The effects of wild blueberry on the cognition and mood of 7-10 year old children

Submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Wild blueberry (WBB) intervention has previously been demonstrated to have beneficial acute cognitive effects in typically developing (TD) children, however it is unclear whether effects persist chronically. This thesis examined the acute (2 h) and chronic (4 week) effects of WBB (253mg anthocyanins) on the cognition and mood of 7-10 year old children, and aimed to extend behavioural data to elucidate the mechanisms by which effects might occur, using event-related potentials (ERPs) and 24 h urinary metabolite analyses.

Initially, acute effects were examined in a TD sample (n=54), where cognition and mood were assessed at baseline and 2 h post-consumption. WBB attenuated forgetting, quickened reaction time (RT) on fast trials, and improved positive affect.

Following this, TD children (n=14) completed cognitive and mood measures 2 h following treatment, concurrent with ERP, using a crossover design. Improved accuracy and RT were seen on cognitively demanding incongruent trials following WBB. Remarkably, these effects occurred simultaneously with higher electrophysiological activation in frontal brain areas associated with increased inhibitory function. Mood effects were not replicated.

Acute and chronic effects were then investigated in TD children (n=23), and in a sub-sample of children with ADHD (n=10) at baseline, 2 h, 2 weeks and 4 weeks. WBB-related acute improvements included faster RT on fast trials across populations, and attenuated forgetting for children with ADHD. Chronic executive function (EF) benefits persisted on cognitively demanding high load trials in both populations. In a final experiment, chronic WBB-related improvements were seen on cognitively demanding incongruent trials in TD children (n=15). Chronic urinary metabolite analyses indicated specific WBB increases in benzoic, vanillic and ferulic acid derivatives across 4 weeks.

Taken together, findings demonstrate cognitive improvements can be seen across acute and chronic WBB intervention in TD children aged 7-10, and preliminarily for those with ADHD. The research also shows 24 h WBB bioavailability in a child cohort for the first time, highlighting metabolites that may cross the blood-brain-barrier and exert cognitive effects. Results add to the evidence that suggests flavonoids may be sensitive to cognitive demand, and novel ERP data implies effects may be mediated by increases in inhibition-related neuronal activation.

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Papers arising from the thesis

Papers published

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Papers in prep

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Chapter 1: Introduction

1.1 General introduction

Diet has become an increasingly important topic of interest in today's society. A healthy diet is deemed essential for typical growth, development and healthy ageing. Consideration of the risk factors involved in poor physical and mental health are an increasing focus of research activity, as well as examination of the potential for using changes to the diet to reduce these risks, or ameliorate onset of illness and disorder. To date considerable research has focused on exploring the implications of eating certain foods on health and cognition. Exploring dietary influences on health in childhood is vital to ascertain whether certain nutrients can promote healthy development, prevent the onset of, or aid in treatment of, developmental disorders. Diet is also important in adulthood, particularly in ageing populations where cognition naturally begins to decline. Recent findings have shown that diets supplemented with a class of naturally occurring polyphenolic compounds called flavonoids (found in high concentrations in a variety of fruits, vegetables and beverages) can promote cognitive performance throughout the lifespan. In this chapter, I will outline the structure of flavonoids, where they can be found in the diet and how they are metabolised, before moving on to a review of the cognitive effects and the potential mechanisms of action behind such effects.

1.2 Flavonoid structure and sub-classes

Flavonoids are a class of polyphenol-rich nutrients found in high levels in foods such as berry fruits, cocoa, wine and tea. There are six main subclasses of flavonoids comprising the flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanins. Each subclass differs in chemical structure (see Figure 1.1) and metabolic processing within the body. Of these six sub-divisions, anthocyanins are the primary subclass found in berry fruits, such as blueberries, blackberries and grapes, and will be the focus of this thesis.

All flavonoid sub-classes originate from an overarching structure of two aromatic carbon rings – a benzene ring (ring B) and a benzopyran ring (rings A and C; Figure 1.1). Subclasses

differ according to the degree of oxidation in the C ring, the hydroxylation pattern of the ring structure or substitution of the C-3 position (Spencer et al, 2008).

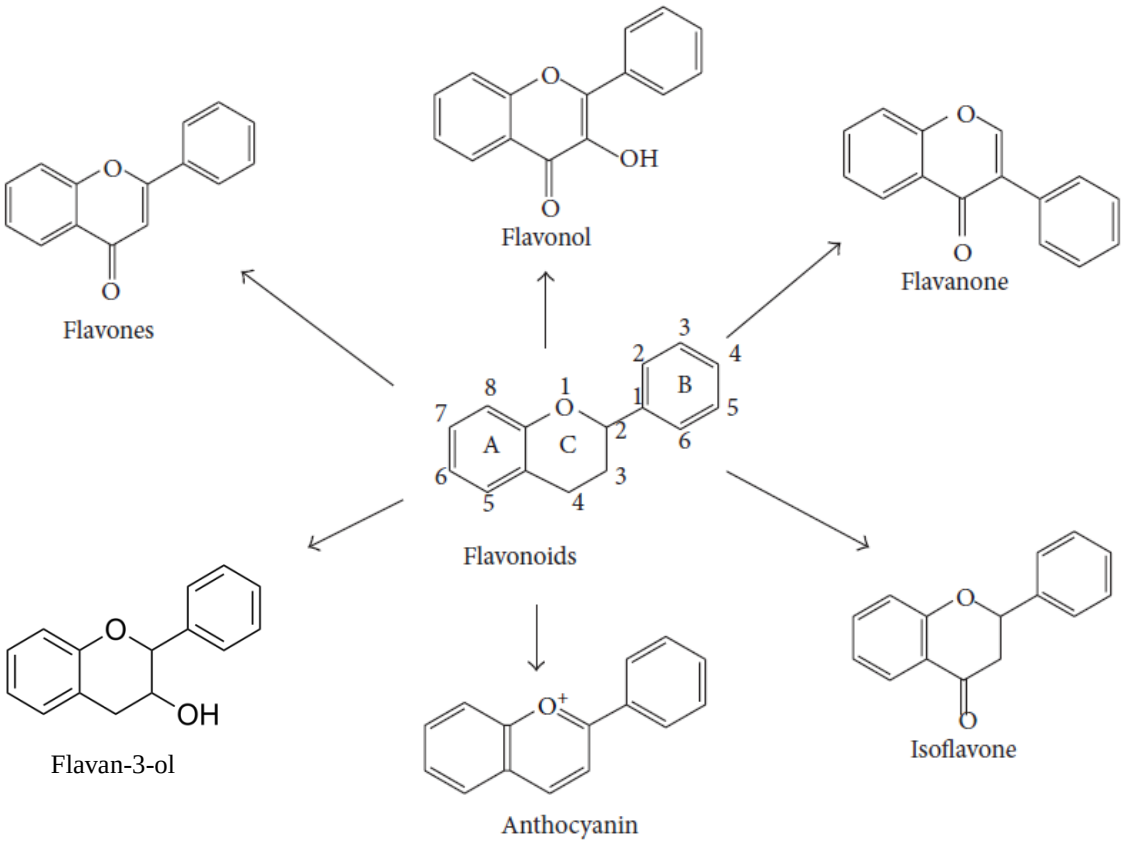


Figure 1.1. The general flavonoid structure, containing a benzene ring (ring B) and a benzopyran ring (rings A and C), is shown in the centre. The six subclasses of flavonoids are also shown, their structure differing based on ring C alterations (adapted from Bitto et al, 2014).

1.3. Flavonoids in the diet

Flavonoids are naturally found in a number of fruits, vegetables and beverages. Anthocyanins are primarily found in berry fruits such as blueberries, blackcurrants or strawberries, as well as in blue and purple vegetables such as red cabbage and aubergine. Flavanols are primarily found in green and black tea, apples and cocoa. Chocolate containing higher proportions of cocoa, such as dark varieties, are therefore more abundant in flavanols compared to milk

varieties. Flavonols occur in many fruits and vegetables such as leeks and onions, and also appear alongside anthocyanin- and flavanol-rich foods such blueberries and tea, respectively. Flavones are present in cereals, celery and herbs such as parsley. Flavanones are the primary subclass found in citrus fruits such as oranges, lemons and grapefruit. Lastly, isoflavones are predominantly found in soy products such as soy beans and soy milk (Beecher, 2003). A varied diet containing a range of fruits and vegetables will therefore be abundant in the different subclasses of flavonoids.

Scientific exploration of the health benefits of blueberry polyphenols has substantially increased across the past decade (Del Rio et al, 2013; Rodriguez-Mateos et al, 2014a), and recent evidence has revealed cognitive benefits and prevention of cognitive decline following acute and chronic consumption, as discussed in section 1.6. Further, there has been a growing necessity to examine the specific bioavailability of polyphenols and the mechanisms of action (MOA) by which they produce such cognitive effects.

1.4 Bioavailability of flavonoids

Most flavonoids are thought to be processed in a similar way within the small intestine, large intestine and liver. Here, they are converted into many metabolic forms, allowing their interaction with a variety of lipid and protein signalling cascades (Zern, et al, 2005; Spencer, Vauzour, Vafeiadou and Rodriguez-Mateos, 2008). Historically, health benefits were considered to occur because of flavonoids' high anti-oxidant capabilities. More recently, work suggests a multitude of potential MOA which will be discussed in sections 1.5 and 1.6.

There is evidence to suggest that whole flavonoids can circulate in the body and cross the blood-brain barrier (BBB), which will be discussed in 1.4.1 and 1.4.2. However, most research has found that larger quantities of circulating polyphenolic conjugates (relative to whole flavonoids) are present in blood and urine after flavonoid consumption (reviewed in section 1.4.3). This suggests that detecting the by-products of flavonoid metabolism may further help to elucidate how flavonoids may affect different areas of the body, including the brain.

1.4.1 Whole flavonoids in the brain

The BBB is a functional property of the microvasculature of the central nervous system (CNS) that regulates transport of cells, molecules and ions between the blood and the CNS (Zlokovic, 2008; Daneman, 2012; Daneman and Prat, 2015). Many nutrients have been found to permeate the BBB (Abbott et al, 2010), including polyphenols (Vauzour, 2012), highlighting the possibility that circulating polyphenols or metabolites from flavonoid interventions could enter the brain (Youdim, Shukitt-Hale, & Joseph, 2004). This has also been confirmed by findings which show localisation of whole flavonoids within animal brain tissues in several cases, most notably after intravenous administration of naringenin (Peng et al, 1998), and oral administration of epigallocatechin gallate (Suganuma et al, 1998), epicatechin (Abd El Mohsen et al, 2002), and anthocyanins (Talavéra et al, 2005; El Mohsen et al, 2006). The latter is of particular interest, as anthocyanins constitute a large proportion of blueberry polyphenols, and could be implicated in the protection against neuronal deficits (Youdim et al, 2000; Sweeney et al, 2002; Joseph et al, 2003; Andres-LaCueva et al, 2005; Williams et al, 2008) or improvements in cognitive function (Rendeiro et al, 2012a; Lamport Dye, Wightman and Lawton, 2012; Bell et al, 2015) that have been observed in animal and human trials, previously. Flavonoid metabolism may be somewhat different depending on the various sub-classes; as the current studies described in this thesis used berries, and anthocyanins are the primary flavonoids in berries, the subsequent discussion of the literature will focus primarily on anthocyanins.

On examining anthocyanins further, Talavéra et al (2005) discovered that rats who received a daily 15g oral dose of an anthocyanin-rich blackberry extract, for 15 days (14.8 mmol anthocyanins/kg/day), showed an increased concentration of anthocyanins in brain tissue. Moreover, a preliminary study, using a single dose of the same intervention by gavage (feeding tube leading to the stomach), showed that this increase in the brain was observable just 30 min after administration.

Passamonti et al (2005) also found presence of anthocyanins in the rat brain only 10 min after intra-gastric administration of grape anthocyanins. This suggests that once anthocyanins reach the stomach they have the potential to be rapidly absorbed into the bloodstream and transported to the brain where they may produce cognitive effects.

1.4.2 Circulation of whole flavonoids in blood plasma and urine

While whole flavonoids, such as anthocyanins, have been detected in animal brains following intervention, their molecular state, and the period of time they are deemed 'bioavailable', has been a potent topic of research amongst nutritional scientists. Prior and Wu (2006) stated that anthocyanins have a half-life of under 2 h in blood plasma, suggesting that degradation and excretion occurs within a short timeframe, post-consumption. Similarly, Kay et al (2004) specified that the maximum concentrations of anthocyanins in urine were also time-limited, appearing up to 5 h after consumption, and reducing by a factor of 1000 at the 24 h time point. The decreases observed following peak concentrations were thought, in part, to be due to degradation. Indeed, once whole anthocyanin concentrations had peaked, the majority of whole, parent anthocyanins ingested were not traceable in plasma and urine afterwards, leaving the mechanisms by which these compounds were expelled, unclear. Wu et al (2005; 2006) remarked that anthocyanin metabolism is an increasingly complex process, and that bioavailability is largely dependent on the differences in the proportion of aglycone and sugar moieties in the intervention, and the stability of the gastrointestinal (GI) tract in the individual. This suggests that measurement of whole, parent anthocyanins may not be the most reliable way to track metabolism of berry polyphenols due to the large variation in stability *in vivo*. Studies have aimed to examine *in vivo* activity by measuring anthocyanins in the brain and in plasma and urine simultaneously. Kalt et al (2008) conducted an animal study which aimed to replicate the increased anthocyanin concentrations found previously in animal brain tissue, and to corroborate these with the presence of anthocyanins in plasma and urine. For this study, pigs were selected due to similarities with human physiology, anatomy and absorption of compounds within the GI tract. Whilst it was evident that anthocyanins were present within brain tissue, their detection in plasma and urine was absent, again questioning assessment of parent anthocyanin bioavailability via plasma and urine. It was proposed that extensive phase I and phase II metabolism may be responsible for such disappearances, with parent anthocyanins being rapidly broken down into several detectable phenolic conjugates - compounds formed through the connection of several chemical phenol groups.

1.4.3. Phenolic conjugates as primary circulatory metabolites

Figure 1.2 shows the metabolic processes that flavonoids undergo in humans.

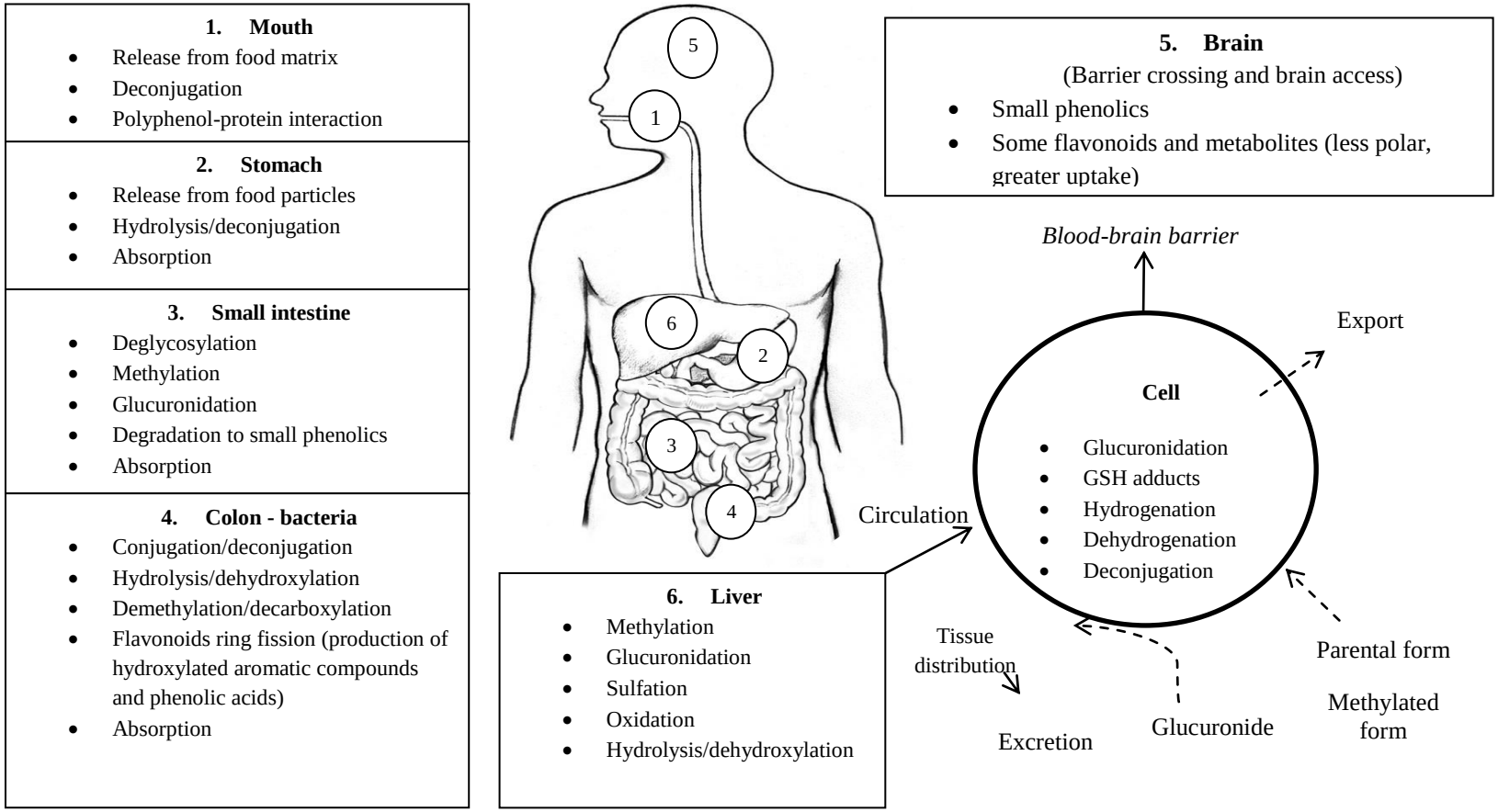


Figure 1.2. Diagram of human flavonoid metabolism (adapted from Karabin, Hudcova, Jelinek, & Dostalek, 2015) showing the processes by which phase I and II metabolism occur.

Since initial studies (Markakis and Jurd, 1974; Hayashi, Ohara and Tsukui, 1996) confirmed that anthocyanins have high levels of instability *in vivo* (for review see Castaneda-Ovando et al, 2009), Kalt et al (2017) revealed that anthocyanin metabolites were 20 times more abundant that parent anthocyanins in urine across a 28 day blueberry juice intervention in healthy adults (age range 24-60 years), suggesting anthocyanin metabolites may be retained in the body over time. However, more importantly, the combined urinary excretion of parent anthocyanins and anthocyanin metabolites in this study accounted for only 1% of the daily anthocyanin dose, suggesting that other compounds might be responsible for anthocyanin-related health effects (Manach et al, 2005; Crozier et al, 2009). Indeed, Kalt et al discovered that aglycone forms accounted for >94% of the total, suggesting anthocyanins break down to form different conjugate compounds.

Collectively, research has suggested that anthocyanins lose substituents of their characteristic flavonoid structure (C6-C3-C6 diphenylpropanoid) during phase I metabolism in vivo, which allows formation of phenolic acids, aldehydes and catabolites (Forester et al, 2009; Kay et al, 2009; Nurmi et al, 2009; Williamson et al, 2010; Czank et al, 2013; Kalt et al, 2017). Moreover, anthocyanins are also thought to undergo conjugation with various groups (glucuronic acid, methyl- or sulfate- groups) via xenobiotic metabolism during phase II metabolism, before or after entering the small intestine (Rodriguez-Mateos et al, 2016; for reviews see Manach et al, 2005; Prior and Wu, 2006; Crozier et al 2009; Marczylo et al, 2009; Czank et al, 2013; Pojer et al, 2013). These conjugates circulate in plasma at concentrations much larger than parent anthocyanins, and have been postulated to be the major compounds circulating in plasma up to 1 h post-flavonoid consumption (Nurmi et al, 2009; Vitaglione et al, 2007; Czank et al, 2013; De Ferrars et al, 2014; Feliciano et al, 2016b). Research has therefore shifted focus from distinguishing anthocyanins, to the detection, distribution and quantification of their phenolic conjugates in blood plasma and urine after ingestion of polyphenol-rich foods. Unlike anthocyanins, many berry polyphenols are not absorbed by the small intestine, but pass through to the colon intact, where they are degraded by gut microbiota (Czank et al, 2013; Feliciano et al, 2016a) and appear in circulation later as phenolic conjugates, at 2 h and 6 h post-consumption (Rodriguez-Mateos et al, 2013). It is therefore clear that polyphenols, anthocyanins and their respective conjugate metabolites are all contributors to the overall pool of berry-derived phenolic metabolites, and should therefore all be quantified in metabolic investigation trials to give a detailed overview of metabolism following flavonoid consumption.

Several studies have focused specifically on the acute bioavailability of blueberry polyphenols within plasma to see how these compounds are metabolised *in vivo* once consumed. Rodriguez-Mateos et al (2013; 2014b; 2016) identified 23 compounds of interest that changed across 0-6 h post-consumption. Increases in plasma levels of polyphenol metabolites at 1, 2 and 6 h post-consumption using varying dosages of wild blueberry (WBB) (310mg, 517mg, 724mg total anthocyanins; 766mg, 1278mg and 1791mg total polyphenols, respectively) were discovered in Rodriguez-Mateos et al's (2013) research. Highest concentrations of metabolites seemed to emerge in a dose- and time-dependent manner. For the lowest dose, the time needed (T_{max}) for each metabolite to reach their maximum concentration in plasma (C_{max}) was 1.5 h post-consumption. For medium and high doses, the T_{max} was 2 h. This is informative for future trials when considering the optimum blueberry dose to administer and the time point to take measurement of health or cognitive parameters. Findings suggest that for blueberry doses between 766mg-1791mg total polyphenols, 1-2 h may be a sufficient time frame to detect peak metabolites in plasma and potentially witness changes in health or cognition parameters.

Further, Rodriguez-Mateos et al (2013) found significant increases in specific metabolite compounds in plasma at 1-2 h and 6 h post-consumption compared to baseline, following the lowest dose (766mg total polyphenols), indicating that concentrations of individual phenolic compounds may be higher following blueberry ingestion. Such increases were evident for 6 compounds at 1-2 h: 2,5-dihydroxybenzoic acid, benzoic acid, vanillic acid, ferulic acid, isoferulic acid and caffeic acid. Interestingly, significant increases were also observed 6 h following the lowest dose for 8 compounds: benzoic acid, vanillic acid, hippuric acid, hydroxyhippuric acid, 3-(3-hydroxy)-phenylpropionic acid, homovanillic acid, 2,5-dihydroxybenzoic acid and dehydroferulic acid, with both 1-2 h and 6 h time points observing increases in vanillic and benzoic acids. No increases were observed at the 3 h time point. These results indicate that 1-2 h and 6 h post-blueberry consumption are feasible and potentially optimum acute postprandial windows to detect blueberry metabolites in plasma at low concentrations. Furthermore, detection of circulating metabolites coincided with increases in flow-mediated dilation (FMD; a measure of endothelial vasodilation) and decreases in neutrophil NADPH oxidase activity. These findings imply that consuming an acute dose of flavonoids ≥766mg total polyphenols may increase metabolite bioavailability to a degree where it could impact vascular function at a time point where cognitive change has

previously been observed. Acute postprandial time points will be discussed further in Chapter 2.

In contrast, little work has investigated the phase II metabolism of blueberry polyphenols in urine. This would be informative to ascertain whether polyphenols are metabolised similarly, or are bioavailable to the same degree, as acute measurement across time. To investigate this, Feliciano et al (2016b) conducted a study where adult males (mean age 33 ± 18) were supplemented daily with 11g freeze-dried WBB powder (302 ± 6mg total (poly)phenols; 150 ± 3 mg anthocyanins) for 30 days. 29 of the 62 phenolic metabolites detected in 24 h urine were at a higher concentration on day 30 compared to day 0 (albeit non-significantly); most notably levels of catechols, benzoic, hippuric, cinnamic and phenylacetic acids had increased, like in the acute data. These findings indicate that continued consumption of WBB across 1 month may lead to increased absorption and excretion of individual phenolic compounds over time. Specifically, hippuric acid was a large contributor (85.5%) to the pool of polyphenol metabolites at day 30; suggesting changes in excretion after chronic supplementation may be driven by specific hippuric acid conjugates. Similar increases in hippuric acid have also been observed after a 12 week daily bilberry intervention (Hanhineva et al, 2015) and an 8 week high-polyphenol diet intervention (Vetrani et al, 2016), as well as after acute WBB intervention (Rodriguez-Mateos et al, 2013), indicating this metabolite may be a potential key marker of berry metabolism acutely and chronically.

Polyphenolic supplementation trials have not always produced consistent metabolic findings. Ferrars et al (2014) did not ascertain any significant differences between urinary metabolites at day 90 compared to day 0 in post-menopausal women supplemented with an elderberry extract (500 mg/day anthocyanins). Likewise, Koli et al (2010) found no significant differences in total urinary metabolites after an 8 week mixed berry intervention (837 mg/day total polyphenols) in middle-aged adults. However, in the latter study, quercetin, p-coumaric acid and 3-hydroxyphenylacetic acid were significantly increased in the mixed berry group compared to the control group, indicating that there may be specific metabolite compounds that increase independently from their conjugate group, as in previous work (Rodriguez-Mateos et al, 2013; 2016; Feliciano et al, 2016b). Increases in individual compounds may therefore be dependent on the type of intervention administered, population recruited, dosage and duration of supplementation, all of which requires further investigation.

1.5 Health benefits and underlying mechanisms

Many health benefits have been observed following flavonoid intervention, such as improved cardiovascular (CV) function (Shrime et al, 2011), and protection from cancer (Bettuzzi et al, 2006) and neurodegenerative disease (Ramassamy, 2006). There has been a growing necessity to explore the biological mechanisms of action (MOA) behind the beneficial effects observed following flavonoid supplementation, to reveal how these dietary constituents can alter health status and physiology *in vivo*. The CV health benefits observed following flavonoid intervention will be discussed in section 1.5.1. Other MOA regarding cognition have been proposed including changes in BDNF regulation and neuronal signalling, which will be discussed alongside the cognitive effects of flavonoid intervention in section 1.6.

1.5.1 Changes in blood flow underlying health benefits

Research has initially assessed the effects of flavonoids on vasoactive properties of the periphery system, often through measurement of flow-mediated dilation (FMD) of the brachial or carotid artery. Increases in vasodilation have been observed after ingestion of flavonoid-rich foods, including 30 minutes (Alexopoulos et al, 2008) and 2 h after cocoa (Schroeter et al, 2006; Monahan et al, 2011), 2 h after green tea (Widlansky et al, 2007), 4 h after cranberry (Dohadwala et al, 2011) and 6 h following orange juice (Morand et al, 2011) consumption. Similarly, peak vasodilatory effects have been observed 1-2 h and 6 h after administration of acai berry (Alqurashi et al, 2016) and blueberry (Rodriguez-Mateos et al, 2013).

Peripheral vasodilatory effects following flavonoid consumption have also been related to other CV benefits such as decreased blood pressure (Taubert Roesen, Lehmann, Jung and Schomig, 2007). Indeed, decreased systolic blood pressure was observed 1-3 h post-cherry juice consumption in Keane, Haskell-Ramsay, Veasey, & Howatson's (2016) study and 3 months after daily WBB extract dosing (Whyte, Cheng, Fromentin & Williams, 2018). Further positive CV outcomes following flavonoid intervention include increased endothelial function (Heiss et al, 2003) and inhibition of platelet build-up (Pearson et al, 2002). As mentioned in 1.4.3., Rodriguez-Mateos et al (2013) found increases in FMD at 1-2 h and 6 h following consumption of 3 separate doses of WBB (766mg, 1278mg, 1791mg total

polyphenols). Moreover, in a second arm of the study, when observing the effects of dose 1 h post-consumption, FMD increased dose-dependently up until 766mg total polyphenols, where it then plateaued. These findings infer that 766mg total polyphenols may be an optimum dose, as doses above this have been shown to decrease blood flow (BF) effects. Indeed, Kay, Hooper, Kroon, Rimm, & Cassidy (2012) revealed FMD effects may occur following an inverted U-shaped curve, where lower and higher doses may produce a less pronounced effect, revealing an intermediate 'sweet spot'. Whilst these results imply flavonoids may elicit BF increases in adults, generalisation to other populations may not be appropriate. Application to individuals with a health diagnosis or to children is limited and requires further investigation of BF changes following flavonoids in these populations.

Acute flavonoid consumption has been posited to alter nitric oxide (NO) activity, which may lead to the peripheral and neural vasodilatory changes observed. Indeed, it is thought that NO release may cause BF effects. NO increases have been observed following 4 days of cocoa-flavanol intervention (821mg flavanols/day), with increased vasodilation following the final acute dose on day 5 (Fisher, Hughes, Gerhard-Herman and Hollenberg, 2003). It was concluded that cocoa-flavanols may be able to induce vasodilation via activation of the nitric oxide system. This suggests a potential mechanism by which high flavonoid foods may moderate BF changes. However, it is unknown whether NO effects persist for other flavonoid subclasses, and further investigations could monitor NO and BF measurement to assess whether such effects are evident after ingestion of other high flavonoid food sources.

It is thought that increases in BF may also be able to occur cerebrally, leading to increased blood flow to regions of the brain. This suggests that flavonoid-related BF effects may impact cognition or mood and this will be discussed in section 1.6.

1.6. Flavonoids and cognition

Over the past decade, there has been an increasing amount of research that has focused on the consumption of flavonoids and cognitive outcomes in animal and human populations. Reviews of the literature have concluded that flavonoids, derived from a wide range of dietary sources, may help to prevent age-related cognitive decline in healthy older adults and may produce cognitive benefits in healthy young and older adults (Macready et al, 2009; Lamport et al, 2012; Bell et al, 2015). Literature reporting cognitive effects in relation to flavonoid

consumption will be discussed here. For the purpose of this review of the cognitive literature, only research recruiting healthy adults will be included. Studies which have recruited participants suffering from mild cognitive impairment (MCI), dementia, Alzheimer's disease (AD) or any other neuropsychological disorder will not be discussed.

1.6.1. Epidemiological evidence

Higher consumption of fruits and vegetables in the diet has been related to increased neuroprotection and positive cognitive outcomes in adult populations (for review see Miller, Thangthaeng, Poulose and Shukitt-Hale, 2017). These effects may be driven by the high dietary flavonoid content found in many fruits and vegetables as suggested by Loef and Walach (2012). Across the past two decades, population, epidemiological and physiological research has suggested that the inclusion of dietary flavonoids may protect healthy ageing adults against cognitive decline and neurodegeneration (for reviews see Macready et al, 2009 and Lamport et al, 2012), which will be further discussed here.

In a longitudinal study examining flavonoid intake and cognition in adults aged >65 (mean age 77, SD 6) at four time points over a 10 year period, those who consumed the highest amount of flavonoids were found to have the most protection from cognitive decline compared to low flavonoid consumers, as assessed by the Mini Mental State Examination (MMSE; Letenneur, Proust-Lima, Le Gouge, Dartigues and Barberger-Gateau, 2007). Similarly, Kesse-Guyot et al (2012) found that higher polyphenol intake was associated with better language and memory performance in older adults (mean age 66, SE 4.6) across 13 years. These epidemiological studies suggest that flavonoids may have the potential to slow the natural cognitive ageing process, and may work dose-dependently; the higher the intake of habitual flavonoids, the better protection from cognitive decline.

Data from the Nurses' Health Study, a cohort of participants completing food frequency questionnaires (FFQs) every 4 years since 1980, discovered that higher intakes of strawberries, blueberries and overall dietary anthocyanidins and total flavonoids were associated with slower rates of cognitive decline in those aged >70 across 6 years (Devore, Hee Kang, Breteler and Grodstein, 2012). These results were found to equate to a delay in cognitive ageing of 2.5 years for high consumers compared to low consumers (calculated using effect size estimates). This indicates that older adults who habitually consumed a diet

rich in flavonoids were protected against the effects of cognitive decline. Similarly, in a cross-sectional study by Crichton , Elias and Alkerwi (2016), significant associations were observed between higher self-reported habitual chocolate consumption and better cognitive performance in adults aged 23-98 (mean age 62), particularly in visuo-spatial memory, working memory (WM), abstract reasoning, scanning and tracking domains, and on the MMSE. Nurk et al (2009) also showed comparable findings when assessing self-reported wine, tea and chocolate consumption in older adults (70-74 years old) using FFQs. Higher consumption of wine was linked to improved object learning, WM and word association (WA); higher consumption of tea was related to improved WM and WA, and higher amounts of chocolate were associated with better WA. Collectively, these studies suggest that habitual high flavonoid consumption, from a variety of food sources, may alleviate cognitive ailments often associated with healthy ageing.

In contrast, Butchart et al (2011) found no associations between intake of flavonoids and fluid intelligence, verbal fluency or memory even after adjusting for individual variation (gender, childhood IQ, socio-economic status, education, smoking and the apoE 14 allele) in adults aged >60 (mean age 70) in a cross-sectional study. Interestingly, positive associations between high intake of flavonoid-rich foods and better cognitive performance were observed before covariate adjustment, and the authors suggest that some of the observed association may be explained by prior IQ. Childhood IQ has been found to account for ~50% of the variance in cognitive performance later in life, indicating that early-life IQ may be a more accurate estimate of adult cognitive ability compared to adult IQ, which may show onset of cognitive decline especially if testing an older adult population. It must also be noted that epidemiological or cross-sectional data using FFQs, especially if completed retrospectively for the previous year, must be interpreted with caution, due to potential bias when estimating flavonoid consumption. Rabassa et al (2015) aimed to address this by investigating the reliability of FFQs in comparison to total urinary polyphenol measurements, and cognition in an older population (>65 years old), at baseline and 3 years. Higher total urinary polyphenols were associated with lower cognitive decline on a WM task (trail making task A; TMT-A) and on MMSE scores, suggesting that those who were better ‘processors’ of polyphenols were the most protected from cognitive decline. Interestingly, total dietary polyphenols, recorded via FFQs, had no relation to cognitive performance. This suggests using a nutritional biomarker, such as urinary analyses, may be a more accurate representation of polyphenol intake compared to self-report food measures in the future.

The vast majority of the epidemiological evidence published has focused on healthy older adults with results suggesting flavonoids may prevent cognitive decline. Although useful in determining a correlation between consumption of a particular food source and cognitive ability, epidemiological studies do not control for the specific dosages, durations and lifestyle restrictions that intervention trials do. Furthermore, the literature includes limited populations, with very little longitudinal evidence from childhood, young adulthood or in clinical populations. Intervention trials are therefore critical in determining the acute and chronic effects of flavonoid supplementation in differing populations.

1.6.2. Evidence from animal models

A number of intervention studies have examined the effects of flavonoids on cognition using animal models, such as rodents. Using rodents in cognitive investigations is advantageous as they share similar visuo-spatial memory skills to humans (Rendeiro et al, 2009). Rodent models also allow for a larger degree of experimental control over confounding factors, such as individual and environmental variability, and can give insight into the underlying neural mechanisms by which flavonoids may exert cognitive effects.

Initial studies (Joseph et al, 1998; Joseph et al, 1999) examining the effects of flavonoids on animal cognition discovered chronic improvements in spatial memory after supplementation with diets rich in spinach, strawberries and blueberries. Joseph et al (1998) supplemented the feed of 6 month old rats with 1-2% (of diet) strawberry extract, spinach extract or vitamin E (an antioxidant-rich, but flavonoid-free control) for 8 months. Prevention of spatial memory retardation was observed for rats on strawberry and spinach extracts compared to those on the vitamin E control, as assessed by an attenuated decline on a Morris Water Maze (MWM), with spinach-supplemented rats showing the largest attenuation. Although flavonoid doses were not specified, strawberry and spinach are both rich sources, indicating the positive effects of chronic dietary flavonoids on cognition. A subsequent study (Joseph et al, 1999) supplementing healthy 19 month old rats with either strawberry (1.48% of diet), spinach (0.91% of diet), blueberry (1.86% of diet) or control extracts for 8 weeks, showed similar benefits. All flavonoid-supplemented animals showed reduced cognitive decline on a MWM compared to controls, and interestingly, blueberry-fed rats showed the greatest improvement in balance and coordination as assessed by an accelerating rotorod and rod walking task – a finding which was not present in strawberry, spinach or control-fed rats. Combined, the

findings from Joseph at al (1998; 1999) indicate a protection from spatial memory deficits associated with healthy ageing after chronic supplementation with dietary flavonoids, specifically foods high in anthocyanins such as spinach, strawberries and blueberries. Antioxidant activity was initially considered to be the MOA behind the cognitive effects observed here. However, measurements of antioxidant activity via oxygen radical absorbance (ORAC) in both studies (Joseph et al, 1998; Joseph et al, 1999) demonstrated there was only a small decrease in oxidative stress, indicating there may be other mechanisms by which cognitive effects persist. Subsequent research (Joseph, Shukitt-Hale & Casadeus, 2005; Milbury & Kalt, 2010; Shukitt-Hale, 2012) also arrived at this conclusion and suggested action on neural signalling pathways may be a more likely MOA for the cognitive effects observed (for short review see Lau, Shukitt-Hale & Joseph, 2005).

Investigations into the action on neuronal signalling pathways and cognition followed. Williams et al (2008) supplemented healthy ageing rats (aged 18 months) with blueberry-enriched feed (2% of diet; 6.68mg/day anthocyanins, 3.85mg/day flavanols) for 12 weeks. Two separate groups consisting of 18 month old and 6 month old rats were employed as control groups, consuming an isocaloric diet that was matched for vitamin C. A cross-maze was used as a measure of spatial memory ability at baseline, 3, 6, 9 and 12 weeks. After only 3 weeks of blueberry-enriched feed, the 18 month old rats showed significantly better performance on accuracy and reaction time (RT) measures compared to 18 month old control-fed rats, which was maintained throughout the remaining 9 weeks of intervention. Improvements for the aged blueberry-fed rats were found to be equivocal to the performance of the younger 6 month old control-fed rats, suggesting blueberry-fed rats underwent a reversal of age-related decline.

Alongside examination into cognitive benefits, Williams et al (2008) also investigated neural mechanisms of interest, namely signalling pathways related to mRNA and protein synthesis, due to their association with long-term memory and synaptic plasticity (Martin, Barad, and Kandel, 2000; Kelleher III, Govindarajan and Tonegawa, 2004). These pathways converge to signal to the transcription factor, cAMP-response element-binding protein (CREB), which binds to genes involved in memory processes (Barco, Bailey and Kandel, 2006). CREB has also been related to BDNF, a key growth factor involved in neuronal survival and functioning, which has been found to regulate memory through the PI3 kinase/Akt/mTOR signalling pathway (Wullschleger, Loewith and Hall, 2006), specifically in regions

susceptible to cell death such as the hippocampus. The hippocampus is key here as it is associated with memory processes, specifically long-term memory.

CREB and BDNF activity was subsequently measured in Williams et al's study to assess activity change after chronic blueberry supplementation. Phosphorylation of CREB in hippocampal regions was significantly increased in aged animals supplemented with blueberry feed, to an equivalent degree to that of young control-fed animals; whereas CREB phosphorylation was significantly reduced in the brains of control-fed aged animals, indicating increased neuronal death in memory-specific brain areas. Increases in extracellular signal-related kinase (ERK1/2), Akt, mTOR and hippocampal Arc/Arg3 levels was also observed in aged blueberry-supplemented animals, suggesting activation of de novo protein synthesis pathways in the hippocampus. This is especially potent as it suggests synthesis of new proteins in memory-related regions. Additionally, hippocampal BDNF levels were significantly increased in aged blueberry-fed rats compared to aged control-fed rats, indicating increased neuronal survival and regulation in memory-specific regions. Indeed, hippocampal BDNF and CREB increases were significantly correlated with the improvements seen in spatial memory performance. Such results signify the ERK-CREB-BDNF signalling pathway to be a potential MOA for flavonoids to exert spatial memory effects in aged rats, a finding which has since been replicated and supported by Rendeiro et al (2013) using a blueberry intervention (2%) in 18 month old rats for 6 weeks, and after 6 months of green tea supplementation in 14 month old mice (Li et al, 2009). Cognitive and motor benefits have also been observed following a 2% blackberry-supplemented diet fed to aged 19 month old rats for 8 weeks (Shukitt-Hale, Cheng and Joseph, 2009). Blackberry-fed animals improved motor performance on an accelerating rotarod, wire suspension and small plank walk, and demonstrated better cognitive performance on the MWM at the end of the intervention. Casadesus et al (2004) also found increases in hippocampal neurogenesis, ERK activation and insulin-like growth factor (IGF-1; a major activator of ERK and modulator of hippocampal neurogenesis, linked with learning and memory processes) in blueberry-supplemented aged animals. Additionally, these increases were found to correlate with improvements in spatial memory.

Similar cognitive results were found when 18 month old mice were supplemented with a daily wild blueberry (WBB) extract or WBB powder for 75 days in Beracochea et al's (2017) study. Contextual memory, measured using a contextual serial discrimination test, was found to significantly improve for rats consuming WBB extract and WBB powder compared to aged

control-fed mice; the latter showing decline across the 75 days. Interestingly, only WBB powder-fed mice showed improvements in spatial and working memory measures, suggesting such domains may be more sensitive to the whole fruit than to flavonoid extracts. Gildawie, Galli, Shukitt-Hale and Carey (2018) discuss this in their review of flavonoids' protective capabilities against cognitive decline, as previous evidence has indicated that ingesting the whole fruit may be more beneficial to neuromodulation than individual anthocyanins (Joseph et al, 2010; Carey et al, 2013). This may be due to the high fibre content of whole fruits in comparison to extracts. This is a hypothesis that should be examined further in relation to the effects of flavonoids on health and cognition by incorporating extract and whole fruit interventions alongside a placebo, and quantifying fibre content.

In addition to studies in aged animals, research has also found cognitive and neuronal changes after flavonoid intervention in younger animals. This would indicate that flavonoids may not only be useful in preventing age-related cognitive decline, but may help to improve cognitive performance in other age groups. Van Praag et al (2007) found improved spatial memory ability in 8-10 week old mice after a single dose of the flavanol (-)epicatechin (2.5mg) compared to control-fed mice. Increased angiogenesis (formation of blood vessels) and neuronal spine density were found in the hippocampal dentate gyri of (-)epicatechin-supplemented mice, suggesting flavonoids may improve the vasculature of specific brain regions which may contribute to memory-related cognitive effects. Furthermore, this study also discovered that genes associated with memory in the hippocampus were up-regulated after consumption of (-)epicatechin, and markers of neurodegeneration were downregulated, supporting previous flavonoid animal research (Joseph et al, 1999) that suggested that regular high consumption of such constituents may prevent cognitive decline in ageing adult rodents. Effects in young animals were also observed in a study by Rendeiro et al (2012b), who found improved performance on a radial arm maze in 2 month old rats supplemented with blueberry (2%) for 7 weeks. ERK-CREB-BDNF modulation of such spatial memory improvements was also observed, again highlighting the possibility for flavonoids to exert positive effects through neuronal signalling pathways.

Research has also begun to investigate neurosignalling after flavonoid intervention in populations where oxidative stress (OS) states have been induced, to ascertain whether flavonoids may help to ameliorate symptoms associated with reduced cognitive functioning. This could be very informative when studying populations who have increased OS (such as older or neurodegenerating adults) or altered response to OS (such as individuals with

developmental disorders like attention-deficit hyperactivity disorder (ADHD; Joseph, Zhang-James, Perl and Faraone, 2015), or clinical symptoms of anxiety and depression (Bouayed, Rammal and Soulimani, 2009). Such populations also display cognitive or behavioural symptoms such as reduced WM ability, lowered attentional capabilities or altered mood states, providing a possibility that flavonoids may be able to lessen symptom intensity by reducing OS through neuronal signalling mechanisms.

Allam et al (2013) examined the effects of grape powder on OS-induced anxiety-like behaviour and memory impairment using 4 groups of rats: healthy control-fed, healthy grape-fed, OS-induced control-fed and OS-induced grape-fed. OS was chemically induced using L-buthionine-(S, R)-sulfoximine (BSO). After 3 weeks of daily supplementation, healthy grape powder-fed (15g/L) rats and healthy control-fed rats made significantly less short- and long-term memory errors on a radial arm maze compared to control-fed rats under chemical induction. The critical finding here was that grape-fed BSO rats displayed significantly less memory errors compared to the BSO control-fed group, suggesting grape intervention may have prevented memory deficits associated with increased oxidative stress. Another interesting finding was the reduction in anxiety-like behaviours observed in grape-fed BSO rats compared to control-fed BSO rats, as assessed through light-dark exploration and open-field tests. Time spent in the unprotected light compartment was significantly reduced in BSO control-fed rats compared to all other rats, indicating an unwillingness to explore and high-anxiety-like behaviour. Similarly, in the open-field test, BSO control-fed rats spent significantly less time in the centre of the open field, displayed fewer rearing behaviours and had an increased count of fecal boli than all other rats, indicating reduced curiosity and increased anxiety.

Critically, grape-fed BSO rats showed significantly less anxiety-like behaviours on all measures compared to BSO control-fed rats. Healthy control- and grape-fed rats also displayed significantly less counts of such behaviours suggesting an absence of oxidative stress may be associated with low levels of anxiety, or that OS results in higher levels of anxiety. Positive changes in ERK-1/2, CREB and BDNF were also observed in grape-fed BSO rats to a similar degree as control-fed and grape-fed rats. Such activation of signalling pathways was not observed in BSO control-fed rats. These findings imply that flavonoids may be able to reduce anxiety and attenuate memory impairment in those with increased OS, potentially through neuronal regulation pathways. Such findings hold implications for the potential for flavonoids to exert effects on mood using the same mechanism as cognitive

change. Future studies assessing mood changes following flavonoid intervention in a human population would be particularly informative to ascertain the link between flavonoids, mood and cognition.

Similar studies have been conducted where young rats were exposed to high-energy and charge particles, which induced oxidative stress and inflammation, and disrupted dopaminergic and cognitive functioning to a level similar to that seen in aged animals. Shukitt-Hale, Carey, Jenkins, Rabin and Joseph (2007) supplemented healthy young rats with 2% strawberry, 2% blueberry or a control diet for 8 weeks, before exposing them to whole-body irradiation with high-energy Fe particles (1.5 Gy of 1GeV/n). As expected, irradiation caused high levels of OS and impaired MWM performance and dopaminergic functioning 1 month after exposure in control-fed animals; however, the flavonoid-rich diets were found to protect against such cognitive and neurological declines. Specifically, strawberry-fed rats showed less spatial deficits in the probe trials of the MWM compared to controls, as they were better able to retain location information (associated with apt hippocampal function). Blueberry-fed rats were found to perform better on the reversal learning aspect of the MWM in comparison to controls, an ability linked to intact functioning of the striatum. This data, amongst others (Casadesus et al, 2005; Rabin, Shukitt-Hale, Joseph and Todd, 2007; Shukitt-Hale et al, 2013; Poulose, Bielinski, Carrihill-Knoll, Rabin, & Shukitt-Hale, 2014), supports the hypothesis that flavonoids may be able to protect against or reverse OS-related cognitive and neurological ailments, such as those seen in ageing populations. Research should continue to investigate clinical animal models and extend interventions to adults to see whether beneficial effects extend to the human brain.

This collection of data suggests that flavonoids may produce beneficial actions on cognitive performance via neuronal signalling pathways, namely those involved in protein synthesis, neuronal communication and growth. A number of studies have investigated the effects of flavonoids on cognition in humans to see if similar mechanisms and outcomes are apparent.

1.6.3. Chronic intervention in adults and effects on cognition

To date, studies using experimental animals suggests that flavonoid effects persist chronically in psychomotor, spatial and WM domains of young and older rodents following a flavonoid-supplemented diet, indicating flavonoids may help to prevent cognitive decline or improve

cognitive functioning across age groups. It is thought that chronic cognitive effects may occur due to changes at the neuronal level over time, for example increased phosphorylation of CREB or upregulation of BDNF. However, the specific MOA underlying chronic consumption is yet to be conclusively determined. Research has begun to investigate the effects of chronic flavonoid intervention in humans, and has discovered that daily supplementation is associated with benefits in cognition (for review see Lamport et al, 2012), especially memory and executive function (Spencer, 2008). Specifically, foods such as cocoa and tea, that are rich in flavanols, and berry fruits, such as blueberries, that are rich in anthocyanins, have been shown to be capable of promoting cognitive improvements when consumed daily over an extended period.

Whyte et al (2018) recruited 65-80 year old healthy adults who subjectively reported memory complaints but were free from psychological diagnoses. Participants either received a 500g daily dose of WBB powder (lowest dose; 35mg total polyphenols), a 1000mg WBB powder daily dose (highest dose; 70mg total polyphenols) or a 100mg/day purified WBB extract (medium dose; 50mg total polyphenols) for 6 months, all containing additional L-cysteine and L-glutathione to promote stability *in vivo*. A polyphenol-free placebo was also employed for comparison. At 3 months, WBB extract was found to improve performance on delayed word recognition of Reys Auditory Verbal Learning Task (RAVLT) and on Corsi Block performance compared to placebo. Systolic blood pressure was also found to reduce following supplementation with the WBB extract across the 6 months. These findings suggest that cognitive decline and high risk CV factors may be ameliorated following a 6 month schedule of a relatively small total polyphenol dose of WBB extract. The authors remark that the additional L-cysteine and L-glutathione may have contributed to increased bioavailability within the body allowing benefits that have emerged using higher dosages in previous studies, to occur in the current study. It is interesting that effects did not persist under the WBB powder, however such effects have persisted before in animal models following extract intervention (see section 1.6.2). Whyte and colleagues comment that although total polyphenol content was highest in the 1000mg (70mg total polyphenols) dose of WBB powder, the proportion of anthocyanins in this dose (2.7mg) was lower than the WBB extract (7mg), indicating anthocyanins may be a critical component of positive cognitive effects.

The cognitive effects observed by Whyte et al are similar to previous findings (Krikorian et al, 2010a; 2010b; Miller, Hamilton, Joseph and Shukitt-Hale, 2018) which have shown memory-related benefits also persist following chronic blueberry supplementation. Miller et

al (2018) recruited older adults (60-75 years old) onto a 90 day intervention of either freeze-dried blueberry (24g/day; ~36mg/g total phenolics; ~19.2mg/g anthocyanins) or a matched placebo, both consumed as a beverage. Participants consuming blueberry were found to have significantly reduced switch cost on a task-switching test, and fewer repetition errors on the California Verbal Learning Test (CVLT) across study visits, compared to placebo. Switch costs are thought to increase with age (Wasylyshyn, Verhaeghen and Sliwinski, 2011), suggesting a reduction in errors may reflect blueberry-related cognitive improvements. The CVLT used in this study was very similar to the RAVLT utilised by Whyte et al (2018), indicating that episodic memory may be particularly sensitive to chronic flavonoid intervention in older populations.

Intervention with other high flavonoid foods in older populations has also shown consistent results, for example chronic supplementation with flavanone-rich orange juice has resulted in beneficial cognitive effects. Kean et al (2015) recruited healthy older adults (mean age 67 years) to consume a daily high-flavanone (305mg) 100% orange juice drink and a low-flavanone (37mg) placebo drink, under an 8 week crossover design, separated by a 4 week washout. Episodic memory (EM) measures were included at baseline and 8 weeks using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD), Verbal Paired Associates (VPA) task, and Spatial Working Memory Test. EF performance was also assessed at each time point using the Go No-Go task, Digit Symbol Substitution Test (DSST), serial 7's, letter fluency and letter memory tests. Mood (measured using the Positive and Negative Affect Scale (PANAS)) and blood pressure was also recorded. Global cognitive function (a composite of EM and EF measures) was found to be significantly better following an 8 week intervention with high-flavanone orange juice compared to the low-flavanone placebo. Interestingly, these effects seemed to be elevated across arms 1 and 2 when the high-flavanone drink was consumed during the first arm, compared to when the low-flavanone drink was consumed during the first arm, indicating a potential flavonoid carryover effect which persisted past the 4 week washout and into the second arm. Results imply that flavonoids may accumulate within the body and continue to benefit cognition after intervention has ceased. Alternatively, these findings suggest that flavonoid intervention during the first phase of learning or encoding may be beneficial for later task performance, and indicates it may be useful to assess the effects of visit order in future trials employing a crossover design to see if carryover effects are replicated. No effects were observed on blood pressure measures. This is unlike Whyte et al's (2018) study which recorded significant

chronic blood pressure decreases at 3 and 6 months relative to baseline following a daily WBB extract. It may be that different flavonoid subclasses exert different effects on vasodilatory function. However, it could also be that 8 weeks is not a long enough time window to initiate long-term CV change in healthy populations. There is limited research that has investigated the effects of orange juice flavanones on CV and cognitive parameters chronically, and the current results indicate that cognitive benefits may persist in healthy older adults. Further investigation into this subclass and its physiological effects under a chronic regimen is therefore warranted.

Not all studies investigating chronic flavonoid effects have produced promising results. Crews et al (2005) recruited older adults (≥60 years old) to consume 32 ounces/day (~909.22ml/day) of a cranberry beverage (containing 27% cranberry juice per volume) or a matched placebo for 6 weeks. No significant effects were observed on a variety of cognitive tests including the Stroop, TMT-A, TMT-B or Digit Symbol-Coding of the Wechsler Adult Intelligence Scale (WAIS). However, polyphenol dose was not stipulated by the authors, meaning we cannot be certain of the amount of polyphenols the intervention contained, making conclusions difficult. Effects may not have emerged due to a potentially low amount of cranberry-derived polyphenols in the drink.

In a further study, Crews, Harrison and Wright (2008) recruited healthy older adults (mean age 69, SD 8) onto a 6 week between-groups, randomised, placebo-controlled proanthocyanin-rich intervention. Participants received one 37g dark chocolate bar (397mg proanthocyanin powder) and one 12g beverage (357mg total proanthocyanins) per day, or a matched placebo. Again, no cognitive effects were observed across the battery employed (which contained the same tasks as Crews et al, 2005). In both of Crews et al's (2005; 2008) studies, the task battery was not particularly cognitively demanding, drawing on simple WM and attentional abilities. This raises the question of task sensitivity in relation to flavonoid intervention; flavonoids may be most beneficial under conditions of increased cognitive demand or when cognitive resources are compromised. As participants were cognitively intact and well educated, it may have been that these samples performed at ceiling, leaving little room for improvement. If more cognitive demand were placed on participants of this calibre, subtle treatment differences may have been revealed – a key detail for future studies to consider when designing flavonoid intervention trials.

At present, there is limited research investigating the physiological or biological effects of chronic flavonoid intervention. Peripheral vasoactive effects have been observed following flavonoid intervention and suggest vasodilation could extend to blood vessels in the brain (see section 1.5.1 for details). Research has begun to focus on allying periphery changes with cerebral changes and finally cognitive measurements, to assess this relationship. Initially, this has been investigated through measurement of cerebral vascular activity using techniques such as arteriol spin labelling (ASL), magnetic resonance imaging (MRI) and functional MRI (fMRI) which will be discussed throughout this section. Such exploration into these parameters could help to reveal the mechanisms through which flavonoids may exert beneficial cognitive benefits over time.

Camfield et al (2012) initiated investigations into chronic effects using a neuroimaging technique called magnetoencephalography (MEG). Middle-aged, healthy participants (40-65 year olds) were administered a daily dose of cocoa flavanols (either 250mg or 500mg) or a placebo for 30 days. Spatial WM and Steady State Probe Topography (SST) measures were taken at baseline and on day 30, to assess neurocognitive changes during completion of the WM task. At the end of the intervention the average amplitude and phase of evoked potentials on the SST varied between groups in centro-frontal and posterior parietal brain regions, indicating increased neural activation following chronic cocoa flavanol consumption. Surprisingly, no effects were observed on the WM task. This may have been due to the participant demographic of Camfield et al's sample; participants consuming all treatments performed at a high level throughout the study, implying subtle changes in cognition may not have been detectable due to ceiling effects. The authors conclude that the higher activation following cocoa flavanol treatment could be interpreted as increased neural efficiency in spatial WM function, in the absence of significant behavioural effects. Similar dissociations between neuroimaging and cognitive findings have been observed by Francis, Head, Morris, and Macdonald (2006) during a functional magnetic resonance imaging (fMRI) study, which will be discussed in section 1.6.5.

Neuroimaging techniques have also been employed in a chronic blueberry (BB) intervention. Bowtell et al (2017) supplemented older adults (mean age 67.5 years) with 30ml blueberry concentrate (387mg anthocyanidins) or a matched placebo every day for 12 weeks. Pre- and post-intervention measures of cognitive function were taken assessing psychomotor function, visual processing, EF, verbal and spatial memory and WM. fMRI testing was completed after the cognitive test battery at both time points, whilst participants completed a numerical Stroop

test. Resting-state perfusion was measured using arteriol spin labelling (ASL) during fMRI, and found increases in parietal and occipital gray matter perfusion for the BB group at 12 weeks compared to baseline. Significant brain activation in task-related areas, including Brodmann areas 4/6/10/21/40/44/45, precuneus, insula/thalamus and the anterior cingulate cortex, an area known to show activation during conditions where errors are likely (Carter et al, 1998), was also observed for BB participants at 12 weeks compared to placebo participants. Taken together these findings suggest that chronic treatment with BB may produce favourable effects on cerebrovascular and cognitive functions. Improvements in WM were also observed for participants consuming BB on the 2-back test, compared to those treated with placebo, supporting previous findings that suggest flavonoids may aid memory-related performance in older populations. However, it is important to note that this test was not completed during fMRI measurement, but took place prior to neuroimaging. No significant effects were observed on the numerical Stroop, which took place during fMRI. Again, dissociation between neuroimaging and behavioural measures are apparent in this study. The increased brain activation detected may reflect increased cognitive effort, or greater increases in task-related blood flow, due to the higher concentration required to perform well on the tasks, without emergence of behavioural effects.

A recent study by Bensalem et al (2018) assessed cognition following chronic supplementation with a daily dose of a polyphenol-rich grape and blueberry extract (PEGB; 600mg/day; 258mg flavonoids) in healthy older adults (aged 60-70 years old) for 6 months. As well as cognition, the researchers also included measures of urinary metabolites; this is the first study of its kind to investigate the relationship between chronic polyphenol metabolism and cognition, and is informative to ascertain whether metabolite bioavailability changes correlate with cognitive improvements over time. PEGB was found to improve free recall performance on the Verbal episodic and Recognition Memory (VRM) test at the end of the intervention. Further, a near-significant ($p=0.058$) association was observed between change in PEGB phenolic metabolite urinary concentration and change in free recall VRM performance across the intervention. Specifically, increased change in native and conjugated epicatechin derivatives were significantly related to increases in VRM free recall, highlighting epicatechin as a potential biomarker of chronic polyphenol consumption. No effects were observed for the other cognitive measure (CANTAB Paired Associate Learning; PAL) employed across participants, which may have been due to small sample size and therefore power to detect an effect in this measure. However, when the cohort was split into a

healthy group and cognitive decliners (a subgroup with advanced cognitive decline based on baseline PAL performance), further benefits emerged. PEGM intervention was associated with better VRM delayed recognition in decliners only, indicating flavonoids aided in protection of age-related cognitive decline, as seen in the previous studies discussed. It is curious that decliners were also found to be the lowest habitual polyphenol consumers at baseline, mirroring epidemiological data that has found significant relationships between lower habitual polyphenol intake and lower cognitive function (see section 1.6.1. for details); however the authors of this study did not examine this association statistically. In decliners, changes in epicatechin metabolites were associated with memory improvements on the free recall and delayed recognition components of the VRM at the end of the intervention. Monomers of flavan-3-ols (epicatechins) and their metabolites may therefore be important to maintain episodic memory integrity, implicating chronic polyphenol consumption as a potential protector from memory-related decline. However, as the current study did not include direct brain or indirect systemic measures, we cannot be certain of the activity underlying metabolite change and cognition. Also, by splitting participants into healthy and declining groups, this would have reduced power further. Future studies should continue to explore cognition and metabolism following chronic polyphenol intervention across varying age groups using sufficient sample sizes, and may want to consider including additional CBF or neuroimaging techniques to try and reveal this relationship.

Clearly, the results arising from the chronic supplementation literature are mixed when considering the effects of different flavonoid subclasses on EF and memory domains. The majority of chronic research has been conducted in older adult populations with results showing that daily inclusion of flavonoids in the diet is beneficial in preventing age-related cognitive decline, particularly in the domain of memory. Inconsistencies in findings have also been apparent but may be explained by the dose of flavonoid administered or the time point at which participants are tested. For example, the duration of the interventions in the literature is varied and may affect the cognitive effects observed. Discrepancies may even be affected by the ratio of flavonoid subclasses within the intervention. There has been no research to date that has investigated total polyphenol-matched interventions which differ in flavonoid subclass ratios. Preliminary work has investigated the metabolism of A-type and B-type procyanidins following cranberry consumption to aid the prevention of urinary tract infections (Gu, 2015), however this work did not include or quantify other flavonoid subclasses. It would be particularly informative to run a total polyphenol-matched intervention study

including treatments with different ratios of flavonoid subclasses to see whether health and cognitive parameters are susceptible to increased concentrations of flavonoid subclasses such as anthocyanins or flavanols, or whether subclasses work synergistically. From the literature (Francis et al, 2006; Crews et al, 2008; Scholey et al, 2010), task sensitivity has also been highlighted as a key component in detecting flavonoid effects; tasks should produce a level of cognitive demand high enough to distinguish between flavonoid-related task performance.

1.6.4. Acute and chronic interventions and effects on mood

Beneficial chronic flavonoid effects have been observed on cognitive outcome measures following a range of flavonoid-rich foods and extracts, as discussed above. Whether these positive effects extend to mood and mental health is a question that researchers are currently investigating. In Kean et al's (2015) crossover study (discussed in section 1.6.3), mood was measured (using the Positive and Negative Affect Scale (PANAS)) at baseline and 8 weeks following a daily dose of high-flavanone (305mg) 100% orange juice and low-flavanone (37mg) placebo in healthy older adults. No significant effects on mood were evident. Although the PANAS has been found to be a relatively stable measure of mood across time (Hughes and Kendall, 2009), it has not been implemented in a chronic flavonoid intervention trial before. Beneficial effects on positive mood using the PANAS in a group of healthy young adults (18-21 years old) and children (7-10 years old) have been observed acutely, 2 h following WBB consumption (253mg anthocyanins; Khalid et al, 2017), indicating mood may be sensitive to BB flavonoids acutely. Pase et al (2013) did find chronic effects on mood in participants following a daily dark chocolate drink (500mg polyphenols) for 30 days, however mood was measured using individual item Bond-Lader Visual Analogue Scales. Participants reported significantly increased calmness and contentedness compared to those under placebo treatment at the end of the 30 day intervention. This suggests that specific mood items, such as calmness and contentedness, may be most sensitive to flavonoid intervention under a chronic regime, whereas overall positive affect may be more able to be detected acutely. Further investigation into the sensitivity of scales to detect mood changes across acute and chronic flavonoid regimens is required.

1.6.5. Acute intervention in adults and effects on cognition

Chronic trials have primarily investigated older adult populations and have shown beneficial effects on cognition. The majority of acute trials have focused on young adult participants where flavonoids have shown potential to enhance cognitive performance over a short time frame (~1-6 h). This has many real-world implications including improvements in short-term mood, workplace efficiency or academic achievement. Intervention with different food sources, comprised of different flavonoid sub-classes has provided varying findings across acute time frames, dosages and populations. This section will aim to summarise the literature and the main findings.

Research conducted in rodents demonstrated significant psychomotor benefits following consumption of a range of high-flavonoid foods (namely blueberries and strawberries; 1.6.2.). Lamport et al (2016a) supplemented healthy young adults with a flavanone-rich orange juice drink (70.5mg total flavonoids; 42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin) or a matched placebo drink, and assessed WM, processing speed, vision, episodic memory and other executive functions prior to and 2 h following consumption. fMRI using ASL was also undertaken at 2 h and 5 h post-consumption to measure CBF. A measure of psychomotor processing speed, the Digit Symbol Substitution Task (DSST), was the only cognitive outcome that showed significant improvements, when compared to placebo and to baseline, indicating the flavonoid psychomotor benefits observed in animals may extend to humans. Absence of other cognitive effects in memory or EF domains may have been due to the fairly low concentration of flavonoids in the orange juice treatment. Previous trials assessing plasma metabolites 1-2 h post-WBB consumption found that a total polyphenol dose of 766mg was potentially optimal to observe elevated levels of circulating metabolites (Rodriguez-Mateos et al, 2013). The dose in the current study may have been sufficient to detect acute psychomotor effects, but may have not been high enough to produce EF and memory effects. Increased CBF using ASL was observed at 2 h for those consuming orange juice, indicating cerebrovascular improvements were observable at the dose administered. It may be possible that such increases in CBF were related to improved psychomotor processing speeds of flavanone participants. CBF changes were not seen at the 5 h time point; however as cognition was not measured 5 h post-ingestion, associations between outcomes at this time point cannot be made.

In a further study by the same research group, Alharbi and colleagues (2016) investigated the acute effects of orange juice at 2 h and 6 h in middle-aged adults (30-65 years old) using a greater total flavonoid dose than previous studies (272mg; 220.46mg hesperidin, 34.54mg narirutin). Participants displayed improvements on a finger tapping task at 2 h and 6 h post-consumption, supporting the psychomotor enhancements seen in animal studies and by Lamport et al (2016a) following acute flavonoids. Attentional benefits were also observed on a continuous performance task (CPT) and maintenance of subjective alertness compared to controls. Such findings suggest that low and higher doses of flavonoids may benefit psychomotor performance in young and middle-aged adults, which may be modulated by changes in CBF 2 h post-consumption. Interestingly, as dose increased from 70.5mg in Lamport et al's to 272mg (total flavonoids) in Alharbi et al's study, the effect size of psychomotor function also increased (reviewed in Bell et al, 2015), and additional cognitive effects appeared. This indicates that lower flavonoid doses may predominantly affect psychomotor domains, and effects on other domains such as attention and alertness may emerge in a dose-dependent manner. This is contrary to animal research conducted by Shukitt-Hale, Carey, Simon, Mark & Joseph (2006) who found that a lower dose of Concord grape juice (10%) resulted in cognitive improvements on a MWM, whereas a higher dose (50%) produced motor improvements. However, it can be argued that the MWM contains a large psychomotor component and cannot be said to measure purely cognition. The MWM improvements observed after 10% grape juice may be more attributable to psychomotor and cognitive performance combined. This would provide additional support to the idea that low and high doses of flavonoids improve psychomotor function. In a second arm to Alharbi et al's (2016) study, regional perfusion was assessed in a different sample of healthy middle-aged adults during fMRI, following the same dose of orange juice (272mg total flavonoids). Acute increases in cerebral activity in inferior and middle frontal gyrus was observed 2 h after consumption of the flavanone-rich drink, suggesting regional, task-related effects persisted across the same time frame as cognitive effects. However, the sample of participants completing fMRI and the sample completing the cognitive battery were different, making it difficult to conclude whether flavonoids had an impact on both CBF and cognition in the same population. This initial research is promising; however it is difficult to ascertain the direct relationship between biological brain mechanisms and cognition, due to the temporal discord of both measurements in this study.

Cocoa flavanol (CF) intervention has also shown acute cognitive benefits in young adult populations. Scholey et al (2009) investigated acute effects during sustained cognitive demand, as induced by 6 repetitions of a 10 minute task battery, in 18-35 year olds. It has been posited that creating a high level of cognitive demand may be critical in detecting cognitive change after flavonoid consumption. Low (520mg CF) and high (994mg CF) doses were administered, as well as a placebo control, in a crossover design separated by 3 day washouts, with testing taking place 1.5 h following consumption. For participants consuming the low dose, self-reported mental fatigue was significantly attenuated at all time points except the third. The low dose of CF was also found to significantly improve WM serial 3 subtractions at all 6 time points compared to placebo. These findings imply that only 520mg CFs are required to elicit a positive effect on cognition, namely prevention from self-rated fatigue and improved WM performance. The high dose resulted in similar enhancements for the first 4 time points of the serial 3's, implying this dose was of benefit, but may not have been effective to a similar extent as the low dose. These findings support Kay et al's (2012) previously discussed theory that flavonoids may act in a U-shaped curve, where effects may be highest at an optimum intermediate dose. Cognition may therefore not be improved by a higher dose; this should be considered in the design of all future flavonoid trials.

It is worthy to note that performance did not improve for either CF treatment on the more difficult serial 7's task, in fact, performance under the high dose was found to worsen. Previous studies have found discrepancies between serial 3 and serial 7 performance following acute doses of a glucose + caffeine energy drink (Kennedy and Scholey, 2004) and *Panax ginseng* (Reay et al, 2005; 2006), and have concluded that the two tasks may draw on different cognitive substrates. Serial 3's relies on a larger psychomotor component, whereas serial 7's is more likely to rely on EF and WM. It has been hypothesised that flavonoids may aid acute performance in psychomotor and WM domains after low and high doses of orange juice flavanones (Lamport et al, 2016a; Alharbi et al, 2016); however the absence of WM improvements in Scholey et al's (2009) study suggests that this may not occur after low or high doses of cocoa-derived flavonoids. Alternatively, although the cognitive task battery should have been sufficiently demanding due to the induction of fatigue, the serial 7's task itself may not have placed enough demand on WM components, in turn, reducing any potential benefits of CF intervention. On the RVIP, reaction time was improved for the high dose of CF during repetitions 3 and 4 only, indicating mild vigilance and psychomotor benefits may occur from a higher dose, around the 2 h time point. However, overall

significant findings following high CF dose were limited compared to those observed following the low dose. From Scholey et al's work, it is clear that acute CF consumption may be of benefit at a lower dose for mental fatigue self-perceptions and for WM that involves primary psychomotor components. Further research is necessary to ascertain the relationship between flavonoid subclass, dose and domain. It seems that a lower dose of CFs and orange juice flavanones may be more beneficial for psychomotor and memory ability, and vigilance may be improved after a higher dose between 1-2 h post-flavonoid intervention.

Field, Williams and Butler (2011) also conducted a cocoa flavanol crossover study in 18-30 year olds whereby participants consumed dark chocolate (720mg cocoa flavanols) and white chocolate 1 week apart. Two hours following each ingestion, visual and cognitive functions were assessed. Improvements in choice reaction time and visual spatial working memory, as well as improved visual contrast sensitivity and time to detect motion direction, were observed following consumption of dark compared to white chocolate. Such findings suggest acute cocoa consumption may provide visual and cognitive benefits to young adults at the 2 h time point, specifically in WM and psychomotor domains. The dose administered in this study falls halfway between the low and high doses used in Scholey et al's (2009) study. This implies that tasks which tap into WM may be capable of improvement following an intermediate, and potentially, 'optimum' dose, as suggested by Kay et al (2012) in relation to FMD increases, and by Rodriguez-Mateos et al (2013) when considering plasma metabolites. Field et al suggest that cognitive changes are likely driven by increases in CBF and may be moderated by improvements in vision. As completion of cognitive tasks requires visual abilities, changes to retinal blood flow may also be a MOA by which cognitive effects persist. Further research is required where visual ability and retinal blood flow changes are incorporated into cognitive and physiological measurements to investigate this mechanism.

Watson et al (2015) investigated cognitive ability in healthy young adults 1 h following consumption of anthocyanin-rich blackcurrants, either as a freeze-dried powder or as a cold pressed juice. A challenging 70 minute cognitive battery (comprising of seven 10 minute sessions) was used to induce cognitive fatigue, and assess the effect of blackcurrant flavonoids under cognitively demanding conditions. Platelet monoamine oxidase (MAO) and blood glucose levels were also monitored. Previously, polyphenols have been shown to have MAO inhibitory effects *in vitro* (Dreiseitel et al, 2009), reducing oxidative stress thought to be associated with impaired cognitive functioning and mood. Attenuation of blood glucose levels have also been demonstrated post-flavonoid consumption by Bell (2017). Declines in

RVIP performance across the 7 sessions were found to be significantly attenuated for those consuming blackcurrant powder extract compared to placebo. Digit vigilance reaction times were also found to slow across all participants; however for those in the blackcurrant juice condition, this was attenuated at repetition 1, 4 and 7. These results suggest that flavonoids may prevent fatigue across tasks which utilise psychomotor and vigilance abilities, 1-2 h post-consumption, as seen previously (Scholey et al, 2009; Alharbi et al, 2016; Lamport et al, 2016a). However, no Stroop, mood or mental fatigue effects were observed. Interestingly, blackcurrant juice inhibited MAO and attenuated blood glucose decline across the 70 minutes, however these effects were not observed for the powder. Bell et al (2015) notes that the differing effects of each blackcurrant treatment on cognition, MAO and blood glucose may have been due to the compositional variances of each. The powder and juice extracts were derived from different blackcurrant cultivars and contained different ratios of flavonoid subclasses. This may have influenced cognitive and physiological outcomes, and highlights that dominance of a particular flavonoid subclass may be associated with improvements in a specific cognitive domain or MOA, which requires further investigation.

Not all flavonoid intervention studies have resulted in cognitive and mood improvements. For example, Hendrickson and Mattes (2008) failed to find any implicit memory or mood effects 1 h following an acute dose of grape juice (600ml; 10ml/kg; 580mg anthocyanins) immediately following a standardised meal, in young adult smokers. However, it is crucial to highlight that implicit memory measurements have not been included in a flavonoid intervention study previously, and results indicate that flavonoids may not exert effects on this domain. As no explicit memory or EF tests were included in this study, it is difficult to ascertain the acute effects of grape juice on domains which have seen prior improvements, such as EF and memory. Bondonno et al (2014) also found an absence of cognitive and mood effects 2.5 h following consumption of fresh whole apple (184mg quercetin; 180mg epicatechin), spinach, or apple + spinach combined. Plasma nitric oxide (NO) increases were observed for all conditions, indicating vasodilatory blood flow increases. However, this MOA may not have affected cognition after apple or spinach supplementation at the 2.5 h time point.

An acute cherry juice intervention (300ml; 55mg anthocyanins) also failed to find cognitive improvements in younger and older adult samples on tests of memory, verbal learning, EF and speed of processing (Caldwell, Charlton, Roodenrys and Jenner, 2015). However, a lack of power was a likely attribute with a total of only 11 participants across healthy young and

old groups. Additionally, the amount of anthocyanins within the cherry juice drink was fairly small compared to studies which have used much larger anthocyanin doses (>200mg) and observed significant cognitive effects. Moreover, a control condition was not employed, making it hard to assess the effects of cherry juice in relation to an energy-matched, low flavonoid comparison. Similarly, no improvements were observed on visual spatial memory, word recognition or word list recall at 1.75 h in young adults (mean age 20, SD 2.9) following acute soy isoflavones (54mg; Vanata and Metzger, 2007). The majority of isoflavone studies conducted are of a chronic nature (for review see Lamport et al, 2012) and report significant improvements in spatial memory and EF domains. However, experimental consistency amongst studies is problematic and findings remain elusive regarding dose, post-consumption time points for testing and cognitive domains studied. It is also thought that isoflavones may be more beneficial for women approaching menopause (Hill and Dye, 2003; Dye, 2008) indicating such interventions in a younger cohort, and in samples including men, may not produce predicted cognitive outcomes at an acute time point. Taken together, these findings may therefore not be indicative of the absence of cognitive effects, but may more likely be a product of insensitive study design, population and treatment dosage choice. Appropriately designed acute interventions, using doses that have shown previous effects, have shown interesting results in young adult populations using neuroimaging techniques such as fMRI.

It is interesting that peripheral vasoactive effects have been seen across the same acute time frame as cognitive effects, and implies vasodilation may extend to blood vessels in the brain (see section 1.5.1 for details). Associating periphery change with cerebral and cognitive change is becoming a focus of the field to assess the potential cognitive MOA at play under acute flavonoid intervention. Measures of cerebral vascular activity have been included within acute flavonoid studies over recent years using techniques such as ASL, MRI and fMRI. Measurement of fMRI blood oxygenation level-dependent (BOLD) signal reflects changes in blood oxygenation in active brain areas. When a BOLD signal is detected, blood flow to a brain region has changed out of proportion to oxygen uptake change (Kim & Ugurbil, 1997). This, in turn, changes the amount of deoxyhaemoglobin in local tissues, which alters the properties of the brain's magnetic field (Raichle, 1998), detectable by fMRI. It has been proposed that such changes occur due to increases in CBF, cerebral blood volume and metabolic rate of cerebral oxygen uptake. It has also been considered that BOLD response is a function of both changes in vasculature and neural activity through neural activity hemodynamic response coupling (alterations in blood volume and CBF; Raichle,

1998; Logothesis et al, 2001). As previously discussed, evidence indicating flavonoids may increase CBF is consistent, and implies flavonoid-induced CBF changes may modulate changes in BOLD at rest and during a cognitive task. Such exploration into fMRI BOLD signals, CBF and cognition has been conducted.

Francis et al (2006) measured BOLD signalling during a switch paradigm task following acute x chronic cocoa flavanol intervention (150mg cocoa flavanols/day) in young adults (aged 18-30). Increases in BOLD signal intensity were observed in the dorsolateral prefrontal cortex (related to response inhibition, EF and WM), right parietal cortex (associated with visual and spatial attention) and the anterior cingulate cortex (ACC; thought to be involved in error preparation; Carter et al, 1998) after 5 days of daily flavanol consumption. The authors conclude that these increases may be a result of altered neuronal activity, a change in vascular response, or both. No cognitive change was observed in the task switching paradigm over the course of the study, indicating BOLD signal may be more predictive of cerebral CV processes than cognition, or highlighting dissociation between neurological and cognitive observations, as has been previously discussed. It is important to note that the final dose of cocoa flavanol on day 5 occurred 1.5 h prior to fMRI measurement, suggesting such an effect may more heavily rely on acute mechanisms, rather than chronic. The researchers did examine this in a second arm to the study, which took a subset of 4 participants and measured CBF via ASL fMRI across the 1-6 h post- consumption period. As expected, increased CBF was observed after acute consumption, peaking at 2 h post-consumption and dropping back off to typical levels at 6 h. Such findings indicate increases in CBF and, by proxy, oxygen, may have been driving regional activation in task-related domains, which approached its peak 2 h following intervention.

Dodd (2012) and Dodd, Moutsiana, Spencer and Butler (2016) continued to test the acute effects of flavonoids on cognitive and neuroimaging parameters in young and older populations. A double blind, crossover trial was performed where young adults (18-25 years old) were administered freeze-dried whole blueberries (BB; 200g fresh equivalent; 631mg anthocyanidins) and an energy matched control, separated by a one week washout. Participants completed a cognitive task battery, which included tests of executive function, memory and mood, at baseline, 2 h and 5 h post-consumption. No cognitive or mood effects emerged at 2 h, however significantly improved accuracy was observed on a WM letter memory task 5 h following BB intervention, suggesting working memory may be susceptible to blueberry flavonoids 5 h postprandially. In healthy older adults (62-73 years old),

performance was found to decline in both treatment groups across time, however maintenance of immediate word recognition performance was observed in participants consuming BB at 2 h and 5 h post-consumption, compared to placebo, indicating a protection from cognitive decline over the course of a day. As in young adults, no improvements in mood or executive function were evident. It seems from these findings that memory performance may be particularly sensitive to acute blueberry ingestion across healthy young and older adults. In a second study, assessing the possible mechanisms of BB function, Dodd (2012) monitored participants' plasma BDNF levels 1 h post-BB consumption (631mg anthocyanidins). BDNF is thought to be active in encoding, consolidation and retrieval of memory (Bekinschtein, Cammarota, Izquierdo and Medina, 2008) through convergence on ERK-AKt-CREB pathways as shown in animal studies (see section 1.6.2), so may be informative in the explanation of flavonoid-related memory effects. Interestingly in young adults, maintenance of BDNF levels was observed 1 h post-blueberry, as opposed to a decrease in BDNF for those consuming placebo, implying BB may ameliorate BDNF decline. In older adults, BDNF alterations were also evident at 1 h; both treatments' BDNF levels declined across the study, however this effect was more pronounced for placebo participants, again indicating BB maintenance in a cognitively ageing population. BDNF activity may therefore be associated with the working memory improvements observed at 2 h and 5 h in Dodd's (2012) previous study. In a third mechanistic study, Dodd (2012) used ASL during fMRI to assess CBF in young adults (18-25 years old) at the 1 h time point. Acute increases in CBF to frontal (particularly precentral and middle-frontal gyrus) and parietal areas (angular gyrus) were observed 2 and 5 h after consumption of the BB intervention (631mg anthocyanidins), suggesting increased blood flow to attention and EF areas. Although conducted separately, these three studies demonstrate the potential for flavonoids to upregulate BDNF, an important factor related to memory function, increase CBF to task-related brain regions and improve cognitive performance across young and older adult populations.

Further BDNF effects have been recently observed. Neshatdoust et al (2016) administered high flavonoid (>15mg/100g) or low flavonoid (<5mg/100g) fruits and vegetables to healthy adults (26-70 years old) who were habitually consuming 3 portions a day, over 18 weeks, increasing portion intake by 2 every 6 weeks. At baseline, serum BDNF levels were related to higher global cognition scores, and after an 18 week intervention, participants consuming high flavonoid fruits and vegetables had significantly increased serum BDNF and cognitive performance compared to low flavonoid and control groups. An additional trial was

conducted by the authors where older adults (62-75 years old) were supplemented daily for 12 weeks with either a high- (494mg total flavanols) or a low-flavanol (23mg total flavanol) cocoa drink. Again, significant increases in serum BDNF were detected at the end of the intervention, correlating with global cognitive performance improvements. Taken together, these findings indicate that high flavonoid fruit, vegetable and cocoa consumption may improve cognition via serum BDNF changes. Indeed, lower BDNF levels are thought to mediate atrophy in hippocampal regions and memory impairment associated with the ageing process (Erickson, Miller and Roecklein, 2012). Increases in BDNF may therefore be indicative of neurodegeneration prevention and maintenance of cognitive health.

Lamport et al (2015) used similar ASL fMRI neuroimaging techniques to Dodd (2012) to assess the acute effects of flavonoids in a healthy sample of older adults (aged 50-65 years). Increased perfusion to parietal brain regions during conscious rest 2 h following a single dose of cocoa flavanols (494mg) was observed compared to a matched placebo (23mg cocoa flavanols). Specifically, increases were observed in the anterior cingulate cortex (ACC) and central opercular cortex of the parietal lobe. The ACC has been associated with environmental and task monitoring, typically in directing attention and learning (see Bush et al, 2000 for review), suggesting such perfusion increases in this region may be connected to cognitive abilities. These findings further support the global CBF increases observed by Francis et al (2006) following a 516mg cocoa flavanol intervention in young adults, and regional increases in frontal and parietal areas observed in Dodd's (2012) work. Taken together, Dodd's (2012), Lamport et al's (2015; 2016a) and Francis et al's findings indicate increased cerebral vasodilatory activity is observable following acute administration of flavonoids, 1-2 h post-consumption, and potentially in brain areas responsible for attention and learning.

Recent adult research conducted by Brickman et al (2014) discovered increased memory performance and CBF volume within the dentate gyrus following 3 months of cocoa supplementation (900mg cocoa flavanols; 138mg epicatechin) compared to a low-flavonoid control mix (10mg cocoa flavanols; 2mg epicatechin). The dentate gyrus is a promising area to find such an effect as it is this hippocampal region which is thought to be affected in age-related memory decline. However, no effects were observed on a delayed-retention task. These results suggest that increased CBF to the dentate gyrus may aid in prevention of neuronal loss often associated with healthy ageing, which may be unrelated to cognitive performance. Similarly, in a study by Haskell-Ramsey et al (2015), CBF increased across 1 h

following an acute dose of cocoa flavanols (500mg), assessed via near-infrared spectroscopy (NIRS), in healthy young adults. Elevated levels of CBF were detected during cognitive task completion, however no effects on cognition or mental fatigue were observed, and no correlation was found between the two. The authors suggest that behavioural effects may only appear when neural resources are depleted, following prolonged task completion. However, it is important to consider that increases in periphery or cerebral vasoactivity may be related to cognitive processes that do not manifest behaviourally, such as strategy formation, cognitive control, effort or inhibition. Such implicit measures have not been included in research to date. On the other hand, unpublished research by Pereira, Vieira, Pavlovskyy and Conde (2018) discovered decreases in brachial and systolic blood pressure, flow-mediated slowing and coronary artery dilation, alongside improvements in speed and accuracy on a test of memory, after an acute dose of high cocoa chocolate (20g;~90%; 18.19±2.64mg epicatechin equivalent/g). Such memory improvements were additionally found to be related to increased pre-frontal cortex perfusion. These results suggest peripheral CV effects and peripheral and cerebral measures may have an impact on processing abilities of memory domains in particular.

Acute consumption of flavonoids, specifically of flavanone, flavanol and anthocyanin subclasses, has shown encouraging findings in relation to cognitive benefits between 1-6 h post-consumption. Taken together with physiological evidence, these benefits may be due to changes in cerebrovascular activity that extend to regional brain areas. Assumptions that increased vaso- and neural activity may influence cognitive change following flavonoid intervention is inconclusive at present, and further work aiming to measure implicit as well as explicit cognitive measures would be informative. Two hours is a post-consumption time point that has been shown to be a somewhat optimal point for flavonoids to have noticeable effects on periphery CV and cognitive systems, potentially working synergistically with flavonoid dosages ≥766mg total polyphenols (Rodriguez-Mateos et al, 2013). Research employing periphery, cerebral and cognitive techniques could give outstanding insight into the health and cognitive effects of flavonoids, including an MOA by which these effects may persist. It is plausible that vasodilatory effects are a contributor to the acute cognitive effects previously observed. Indeed, direct and indirect measures of CBF, including fMRI BOLD, ASL and NIRS have also shown cerebrovascular change following acute flavonoid intervention. Assessment of neuronal activity via EEG, and fMRI at the same time as cognitive assessment, 1-6 h after flavonoid consumption, would also help to inform the field

further of an acute MOA. Initial findings suggest peaks in cerebral vasodilation may coincide with peripheral vasodilation and cognitive peak windows, and so further research examining CBF activity 30 minutes to 6 h post-flavonoid consumption is warranted.

An additional observation can be made when reviewing the acute and chronic flavonoid literature as a whole. Flavonoids appear to impact different cognitive domains within different populations. Lamport et al (2012) note that flavonoid effects are predominantly seen in declarative memory domains. Furthermore, Bell et al (2017) demonstrated that the most reliable episodic memory improvements were found after acute flavonoid intervention in older adults, whereas executive function improvements were most prominent in young adult populations. This suggests there may be an interaction between flavonoid intervention and lifespan stage. Indeed, aging is often associated with memory decline, implying this domain may be particularly sensitive to flavonoid intervention in older adults. This holds promising implications for studies investigating the effects of flavonoids in child populations where EF and memory abilities are still developing. Sensitivity in either or both of these domains in children could highlight a potentially sensitive window where cognitive performance may improve, positively impacting healthy neuropsychological development.

1.6.6. Intervention in children

Recent work from our laboratory has started to address the question of whether flavonoids are beneficial to typically developing child populations. Results have been promising, revealing acute improvements in the cognitive performance of 7-10 year old children supplemented with flavonoid-rich blueberry. Whyte and Williams (2015) initiated the investigations by supplementing 8-10 year old children with a BB drink (200g fresh blueberries; 8g sucrose; 143mg anthocyanins) or a matched placebo, both made with 100ml semi-skimmed milk, using a crossover design, separated by a 1 week washout. A cognitive task battery consisting of a Go No-Go task, Stroop test, RAVLT, Object Location Task and Visual N-back was used before, and 2 h after intervention. Significant BB effects were observed on the delayed recall measure of the RAVLT compared to placebo, indicating acute cognitive benefits persist in school-aged children. Interestingly, proactive interference was also shown to increase for BB participants; previously learnt words had a greater negative impact on the encoding of a new word set. This suggests that children consuming BB may have had greater encoding abilities of the original word list compared to placebos. Similar findings were observed chronically in

Kean et al's (2015) crossover study of middle-aged adults; participants consuming high-flavanone orange juice performed significantly better on the first arm of the study compared to placebo. These effects were maintained in the second arm, 4 weeks later, when consuming placebo, potentially indicating an increase in their encoding ability during the original exposure of the task. Exploration into encoding and retrieval mechanisms following flavonoid intervention would be practical to ascertain the specific components of memory that such an intervention may tap into. Whyte et al found no effects on the other cognitive tests, suggesting flavonoid effects may be most prominent in the domain of memory rather than attention, inhibition or visuospatial memory. However, it is important to note that research has since revealed milk to be a potential inhibitor of polyphenol uptake (Yildirim-Elikoglu & Erdem, 2017). Effects may have therefore not been apparent in other cognitive domains due to the reduced absorption of blueberry polyphenols, resulting in a dose which was not high enough to detect additional cognitive effects. Memory benefits have been observed at low doses in previous adult research (see section 1.6.3 for details), highlighting a potential dose-dependent relationship between flavonoids and functioning across cognitive domains. However, this link is tenuous and deserves additional exploration before assuming a relationship between dose and cognitive domains.

Subsequently, Whyte, Schafer and Williams (2016) aimed to investigate the effects of dose and postprandial time point following doses of WBB mixed with water and low flavonoid squash. Children (aged 7-10) were recruited to consume a 15g WBB drink (127mg anthocyanins; containing a similar amount of anthocyanins to the previous study), a 30g WBB drink (253mg anthocyanins) and a matched placebo drink on 3 occasions, separated by 1 week washouts. Participants were tested on an Auditory Verbal Learning Task (AVLT), a modified flanker task (MFT), a Go No-Go, and a Picture Matching Task (PMT) at baseline, and 1.15, 3 and 6 h post-intervention. WBB-related improvements were again observed on memory measures, with benefits seen on the AVLT measure of delayed recall. All treatments showed significant decreases at each post-intervention time point on this measure, apart from participants consuming the 15g WBB treatment, whose decreases were attenuated. This supports Whyte et al's (2015) previous findings and indicates that a lower dose of BB may improve children's memory performance in the 1-6 h period following ingestion. In Whyte et al (2016), effects were also observed on AVLT final immediate word recall. The 30g WBB treatment was found to significantly improve performance at 1.15 h, whereas performance was found to be worst following the placebo. These results suggest potential dose-dependency

for cognitive effects where a higher flavonoid dose may produce greater improvements. Dose-dependency has been evident in previous studies examining FMD and plasma metabolite increases following acute flavonoid consumption (Kay et al, 2012; Rodriguez-Mateos et al, 2013). These studies found that with increased dose, effects improved up to an 'optimal' dose, after which effects lessened, following a U-shaped curve. Whyte et al's (2016) results suggest that 30g of WBB (253mg anthocyanins) may be an optimal dose to detect memory effects in a child population, which warrants further investigation into doses ≥253mg anthocyanins. Decreases in delayed word recognition performance were also attenuated for 15g and 30g WBB doses in a dose-dependent manner. At 6 h, placebo treatment produced the largest decrease from baseline, whereas participants consuming the 30g treatment showed the least amount of change, with 15g producing a change between the two. This again highlights protection from a drop-off in performance following 15g and 30g WBB treatments following a dose-response pattern. Further, accuracy performance was shown to increase on the MFT for WBB consumers, specifically on cognitively demanding incongruent trials. Specifically, at 3 h post-consumption, dips in accuracy were noted on incongruent trials for those consuming placebo, whereas significant improvements were observed for those consuming 30g WBB. Again, this appeared in a dose-dependent fashion where placebo participants performed the least accurately and 30g WBB participants performed the most accurately, with 15g WBB participants' performance between the two. The BB benefits evident on demanding incongruent trials suggest that flavonoid effects may be particularly sensitive to the cognitive demand of the task, as previously suggested (Francis et al, 2006; Crews et al, 2008; Scholey et al, 2009). Tasks that successfully manipulate cognitive demand, such as the MFT, would be particularly informative to include within future task batteries so that conclusions regarding flavonoid sensitivity under conditions of greater difficulty can be determined.

Finally, Whyte, Schafer and Williams (2017) aimed to explore the effects of flavonoids on the differing levels of cognitive demand using an adapted version of the MFT, the Modified Attention Network Task (MANT). As previously discussed, it is important for cognitive trials to include tasks that are cognitively demanding enough to increase the detectability of flavonoid-related effects. The MANT was created to vary cognitive demand across various levels and trials of the task so that flavonoid sensitivity to EF abilities could be assessed in more detail. WBB powder (30g; 253mg anthocyanins) and matched placebo interventions were administered to typical 7-10 year old children using a crossover design, separated by a 1 week washout. Testing on the MANT took place prior to intervention and 3 h post-

intervention on each occasion, where effects had been observed previously using this dose (Whyte et al, 2016). WBB-treated participants showed significantly faster performance than placebos on the cognitively demanding incongruent and high load trials when presented on-screen for 500ms. This supports the hypothesis that flavonoids may be of most benefit under conditions of increased cognitive load when the task is more challenging. It also supports the notion that tasks used in intervention studies should produce cognitive demand high enough to detect effects. Additionally, trials where a visual cue was used to alert the participants to the upcoming target showed better performance for those consuming WBB. This was found to facilitate performance regardless of task difficulty suggesting that cueing elements may be more beneficial to those who have consumed flavonoids on both easy and difficult task conditions.

1.6.7. Summary of the effects of flavonoids on cognition

Overall, flavonoids have been observed to have beneficial effects on acute and chronic cognitive performance, particularly in domains of psychomotor ability, visuo-spatial processing, episodic memory and working memory. Executive function improvements have also been observed, specifically on tasks linked with attention and processing abilities. Using tasks sensitive enough to detect cognitive change following flavonoid intervention is a critical factor when assessing cognition, and must be considered in future trials. Research to date has concentrated on examining chronic flavonoid effects in animal and healthy older adult (Lamport et al, 2012) populations, whereas acute intervention has mostly been undertaken in healthy young adults (Bell et al, 2017). A small number of studies have investigated the effects of acute flavonoids in school-aged children, a population undergoing biological change in the brain, where flavonoids may be expected to have long-term, developmental impact. Results of acute child studies have been promising, especially in relation to memory and EF, and further exploration into these and other domains are warranted, under acute and chronic regimens. An increasing amount of research has focused on the physiological effects that may underpin cognitive outcomes after flavonoid intervention. To date, relatively consistent findings have been observed in relation to increased CBF, BDNF and regional cerebrovascular perfusion. However, in studies that have attempted to incorporate cognitive and physiological measures, there is often dissociation, where physiological parameters change and cognition remains unchanged. It is unclear whether this is a consistent

phenomenon due to the differences in flavonoid type, dosage and intervention period across studies, rendering results incomparable. Further research is therefore required that aims to corroborate cognitive and physiological outcomes to better ascertain a potential MOA behind the acute and chronic cognitive effects observed.

Chapter 2: Aims of the thesis, specific hypotheses, and methodological considerations

2.1. Thesis aims and research questions

2.1.1. Aims

The primary research aim of this thesis was to investigate whether flavonoid-rich wild blueberries (WBB) exert positive effects on cognition in children. Specifically, domains of executive function (EF), attention and memory were targeted, as these domains of cognitive performance have all been shown to be sensitive to flavonoid-rich intervention in previous animal, adult and child human research. The current thesis aimed to determine to what degree these effects on cognition could be observed under both acute and chronic interventions in a child population. For the purposes of this thesis, acute consumption will be defined as an assessment of outcome measures 0 - 6 h following WBB ingestion. Definition of what accounts as chronic consumption is mixed within the literature, however in this thesis, chronic supplementation will focus on a supplementation period of 4 weeks. The effects of WBB on mood will also be investigated in the thesis. The evidence investigating the specific mechanisms of action that underlie these cognitive effects is limited at present, and no studies have measured cognitive performance alongside physiological measurement in a child population. Therefore, an additional objective was to investigate the acute and chronic physiological responses following WBB, using ERP techniques and measurement of urinary metabolite excretion, to further elucidate potential mechanisms of action by which blueberry flavonoids exert beneficial effects.

2.1.2. Research questions

The research questions explored in the current thesis are:

1) **Will typically developing (TD) 7-10 year old children show improvements in cognition and mood following acute intervention with WBB (reported in Chapters 3, 4 and 5 respectively)?**

Rationale and hypotheses: As shown in previous adult studies, WBB intervention has been shown to improve cognitive function, acutely and chronically. From reviews of adult

literature (Lamport et al, 2012; Bell et al, 2015) it is apparent that these improvements have been observed in the domains of memory and attention 2 h, 3 h and 6 h post-prandially (see Chapter 1 for full review). Similar to adult intervention studies, acute supplementation with a WBB intervention has been found to improve children's executive function and memory performance 1.15 h, 3 h and 6 h post-consumption in 7-10 year old TD children (Whyte and Williams, 2015; Whyte et al 2016; 2017). It is therefore predicted that replication of such findings will be achieved in further samples of typically developing children of the same age. Unlike Whyte's studies (Whyte and Williams, 2015; Whyte et al 2016; 2017), this study will administer blueberry intervention in the afternoon, immediately following lunch, where children may feel cognitively fatigued. Beneficial effects in this immediate post-prandial period have positive implications for the maintenance of concentration and cognitive ability throughout the school day which may improve children's learning capabilities and academic outcomes. Assessment of mood will also be included in the current study and throughout the thesis. Recent epidemiological research has shown that higher flavonoid intake across 10 years has been associated with a reduced risk of depression (Chang et al, 2016), with similar research associating high fruit and vegetable intake with significantly lower incidences of mood disorders across the lifespan (Lai et al, 2014; Mihrshahi et al, 2015). This suggests that there may be an interaction between flavonoid intake and mood, which at present remains largely unexplored.

It was therefore hypothesised that WBB intervention would improve executive function (EF), memory and mood using an acute design.

2) Following acute WBB intervention, will TD 7-10 year olds show improvements in cognition or mood and, if so, is this linked to electrophysiological changes in the brain (reported in Chapter 4)?

Rationale and hypotheses: Evidence has suggested that cognitive and mood benefits may be observed following acute WBB consumption, however the mechanism of action (MOA) by which these effects occur remains to be elucidated. Peak vasodilatory effects measured via flow-mediated dilation (FMD) have been observed 1-2 h and 6 h after WBB consumption, alongside flavonoid metabolite increases in blood plasma (Rodriguez-Mateos et al, 2013). This suggests flavonoid metabolites appear in circulation from 1 h following consumption and may be associated with the vasodilatory FMD changes observed at this same time point.

Improvements in FMD response is indicative of increased blood flow, suggesting flavonoids may have the ability to indirectly increase the delivery of oxygenated blood to brain regions via blood vessel dilation. As Whyte et al (2015; 2016; 2017) showed cognitive benefits occurring within this same time window (1.15 h, 3 h and 6 h) it is plausible to consider increased CBF as a potential MOA for acute supplementation of WBB.

CBF changes have also been linked to changes in activation at a neuronal level. For example, ischemia of cerebral blood vessels has resulted in reduced electrical activity in the CNS (Branston, et al, 1974) and neurons, astrocytes and neurotransmitter-mediated signalling appear to contribute towards CBF regulation (for review see Attwell et al, 2010). Traditionally, energy use was believed to be the primary agent regulating activity-induced blood flow (BF), however there has been a conceptual shift over the last decade towards neurotransmitters inducing vasodilatory BF changes. It is clear from the evidence discussed in Chapter 1 that neurotransmitter and CBF activity are interlinked, and neuroimaging techniques may illuminate such changes following an intervention that has shown changes in brain blood flow and neurosignalling pathways following consumption previously. Neuro-electrical activity may therefore provide an insight into the vasodilatory effects or neuronal activation that may be occurring following acute WBB within the 1-6 h post-consumption window. Therefore, Experiment 2 measured acute neuronal activity via electroencephalogram (EEG) event-related potentials (ERPs) concurrent with cognitive assessment at 2 h post-WBB intervention, to determine the relationship between neuronal activity and cognition in a child population. Measurement of ERPs involves identifying peaks in brain wave amplitude following presentation of a stimulus. Different peaks have been associated with different cognitive abilities. A negative peak occurring ~200ms following a stimulus and a positive peak occurring ~300ms after a stimulus have been related to inhibition abilities. This was relevant to the current investigation as inhibition is a cognitive ability that has been associated with executive function (Diamond, 2002; Ridderinkhof and van der Molen, 1997), where flavonoids have demonstrated positive effects in children previously (Whyte et al, 2016; 2017). It was hypothesised that children would show improvements in cognition or mood, and that ERP activity would show increased N2 and P3 activation in frontal and fronto-central, EF-related regions following acute WBB.

3) Do cognitive and mood effects persist under a chronic design in TD 7-10 year old children, and in a population diagnosed with ADHD (reported in Chapter 5)?

Rational and hypotheses: Although research into flavonoids and their effect on the brain is expanding, little research has systematically investigated (using consistent durations or doses) the effects of long-term consumption on cognition or mood in humans, especially in a child population. Reviews from Lamport et al (2012) and Bell et al (2015) show that chronic effects on working memory, psychomotor ability and executive function are apparent in neuro-typical adults and cognitively impaired older adults; however, little is known about whether these effects are also observable in children. Due to similarities in working memory improvements and mental fatigue reductions observed between young and older adult populations following acute interventions, it is possible that chronic effects may also be observable in a TD child population. It is therefore hypothesised that chronic daily intervention with WBB will improve the cognitive performance and mood of TD 7-10 year olds over a 4 week intervention period.

Due to previous research indicating improvements in attentional abilities in a child cohort (Whyte et al, 2016; 2017), it is possible that children who are diagnosed with attentional deficits (such as those with ADHD) could benefit from WBB supplementation by potentially improving performance on tasks recruiting attentional resources. To date, the majority of research into ADHD nutritional interventions has involved omega-3 supplements (fish oil, flaxseed oil or DHA), but suppositions on outcomes are inconclusive, as will be discussed in Chapter 5. A major shortfall apparent in studies implementing chronic nutrient interventions in this population is the reliance on parent or teacher ratings to determine a change in cognition or behaviour. Such ratings are susceptible to subjectivity and bias, especially if parents or teachers are aware of the treatment the child is receiving. Also, empirical measures of cognitive ability cannot be obtained via this method and require objective measurement via a validated cognitive task or assessment to warrant interpretation of true cognitive change. At present, only a single study has implemented a chronic flavonoid intervention in children diagnosed with ADHD. Trebaticka et al (2006) administered 1mg/kg/day of French maritime pine bark (FMPB), a polyphenol-rich extract, to 6-14 year old ADHD children for one month. At the end of the intervention, children showed reduced hyperactivity, and increased attention and visual-motor control as assessed by teacher and experimenter-reported rating scales.

Clearly, objective cognitive measures are required to assess the impact flavonoids may have on cognition during acute and chronic intervention periods. Experiment 3 will consist of an acute and chronic arm. Typical and ADHD children will be recruited to take part in an acute WBB intervention, testing mood and cognition at baseline and 2 h following WBB to align with the acute cognitive and physiological results previously found across 1-6 h (Whyte et al, 2015; 2016; 2017; Rodriguez-Mateos et al, 2013). Children will then continue onto a chronic arm, with measurements of cognition, mood and parent-rated behaviour being taken after 2 and 4 weeks of daily WBB intervention. It is hypothesised that typical children and children diagnosed with ADHD will show improvements in measures of cognition and mood acutely, and after following a 4 week daily intervention schedule with WBB.

4) **Does chronic WBB consumption alter the excretion of polyphenolic metabolites in 7-10 year old TD children after 4 weeks daily dosing and, if so, are increases in certain metabolites associated with cognitive or mood enhancements (reported in Chapter 6)?**

Rationale and hypotheses: Some researchers believe that anthocyanins cannot accumulate within the body (Kay et al, 2004), however the bioavailability of other polyphenols and their associated metabolite conjugates after acute flavonoid intake needs to be investigated further to ascertain whether polyphenol metabolites remain in circulation for an extended period after an acute dose. Rodriguez-Mateos et al's (2013) work suggests increases in CBF, as measured via FMD change, are related to circulating polyphenol-derived metabolites 1-2 h and 6 h following consumption of WBB. Analysis of metabolites post-WBB ingestion may therefore indicate which metabolites are circulating in the body in the time period where cerebrovascular and cognitive effects have been observed previously. Similar mechanisms may occur chronically, where repeated acute dosing may improve vasodilatory function across time (Rodriguez-Mateos et al, 2016b). Previous research has quantified WBB metabolites in plasma and urine across a 4 week intervention in healthy adult males, where 29 of 62 phenolic metabolites non-significantly increased at day 30 compared to day 0, specifically catechols, benzoic, hippuric, cinnamic and phenylacetic acids (Feliciano et al, 2016b). However, linking cognition to metabolite change has not been attempted previously and, importantly, there has been no research to date which has quantified WBB metabolites excreted in children.

Experiment 4 will involve a 4 week WBB intervention, with measurements of cognition, mood and urinary metabolites at baseline, 2 weeks and 4 weeks to assess chronic cognitive and physiological change. It is hypothesised that repeated consumption of WBB over a 4 week period will alter the metabolism of 7-10 year old TD children similarly to adults (with increases in catechols, benzoic, hippuric, cinnamic and phenylacetic acids specifically), and that changes in cognition may also be observable.

2.2. Methodological considerations

Four experiments were designed to answer these research questions:

- Experiment 1: The acute effects of WBB on the cognition and mood of TD 7-10 year old children (Chapter 3)
- Experiment 2: The acute effects of WBB on the cognition, mood and event-related potentials (ERPs) of TD 7-10 year old children (Chapter 4)
- Experiment 3: The chronic effects of WBB on the cognition and mood of 7-10 year old children with and without ADHD (Chapter 5)
- Experiment 4: The chronic effects of WBB on the cognition, mood and urinary metabolites of TD 7-10 year old children (Chapter 6)

2.2.1. Design

A between-groups design was implemented for Experiments 1, 3 and 4. This design was chosen for Experiment 1 so that children were only required to leave the classroom setting once for baseline testing, minimising disruption to participants' education and to the teacher/School. This design was also chosen for Experiments 3 and 4 to minimise boredom and dropout from repeating the entire experimental procedure twice. Such repetition would have been particularly cumbersome for participants in chronic trials who underwent testing across 0-4 weeks, and may have negatively affected results. There are some disadvantages of using a between-groups design. Individual variation is likely to be higher between groups of participants, decreasing control of confounding factors, such as intelligence level and personality. However, demographic measures assessing the background characteristics of participants (gender, age, intelligence and attentional ability) have been utilised across studies

to ensure all participants have a similar profile. Furthermore, randomisation procedures have been used to reduce the effect of bias occurring in one particular group.

Across all experiments, participants were randomly allocated to their treatment condition to address individual differences that may have affected results. Randomisation was performed by a confederate to ensure the experimenter remained blind and unbiased. The experimenter was unblinded to treatment only once the experiment had ceased and data processing was complete, prior to statistical analyses.

A crossover design was applied in Experiment 2, where participants took part in each experimental condition of the study. This enabled participants to be their own control, minimising the effects of individual variation and improving statistical power. Again, randomisation was performed across treatments and visits, by a confederate. Using this crossover design, it is crucial that a long enough washout period is implemented to avoid carryover effects in subsequent test sessions. This is a major downfall in studies implementing nutritional interventions which use a relatively small washout period. A period of 1 week has been used in Experiment 2 due to previous child blueberry intervention trials that have employed this time frame (Whyte et al, 2015; 2016; 2017). It is also crucial that participants undergo a practice of cognitive task batteries before commencing a trial to eradicate performance increases from practice alone (see section 2.2.2).

2.2.2. Practice effects

Repeated cognitive testing in human participants can often be problematic due to the emergence of practice effects. In crossover or between-groups nutritional intervention designs, participants may undergo cognitive testing multiple times over the course of a day, or across weeks or months. This poses issues as participants become more familiar with the task battery and its' constituents over time, which may lead to increased learning, better strategies to complete the task and enhanced performance. Nutritional intervention effects may therefore be more difficult to detect if cognitive change is related to practice, and type 1 or 2 errors may occur where practice or intervention effects are under- or over-estimated. Additionally, participants may reach ceiling levels of performance, reducing the detection of any cognitive change. It is therefore important for nutritional intervention studies to try and combat practice effects so that intervention interpretations are not impacted.

Bell, Lamport, Field, Butler and Williams (2018) investigated the effect of practice on a selection of cognitive tasks that have commonly been used in nutritional intervention studies (Stroop, Sternberg, serial subtractions, immediate and delayed recall) via 6 repetitions of a task battery. Practice effects emerged for all tasks where performance improved at each subsequent test session, indicating increased familiarity and learning. The effects of practice were attenuated at later test sessions compared with earlier visits; indeed, the strongest practice effects were observed between visits one and two. Bell et al conclude that provision of a familiarisation session on a separate day prior to data collection may help to reduce practice effects on intervention trial outcomes in the future. They also recommend including alternative task forms at each repetition to reduce the familiarity of the task and prevent ceiling effects.

A practice session was therefore included in all experiments on a separate day prior to data collection. Including alternative forms of the MANT, Go No-Go task and PANAS-C was not appropriate due to the necessity to compare responses across standardised variables, however, alternative forms were used across repetitions for the AVLT.

2.2.3. Treatment

Freeze-dried wild blueberry powder from the *Vaccinium angustifolium* cultivar was used as the active treatment in all experiments in this thesis. Fresh blueberries vary in their composition depending on growing conditions and how they are subsequently stored. Using freeze-dried WBB powder, obtained from a large batch harvest, therefore enabled control of polyphenol and anthocyanin content across studies. Powder was stored at -20°C. The cultivar used also has a high total polyphenol content and is rich in anthocyanins (Rodriguez-Mateos et al, 2012). An additional benefit is the publication of bioavailability data for this cultivar (Rodriguez-Mateos et al, 2013; 2016a).

The powder used was provided by the Wild Blueberry Association of North America (WBANA) free of charge. A single dose contained 253mg anthocyanins, equating to approximately 240g or 1 ½ cups of fresh blueberries. This dose was kept constant across the studies, and aligned with previously published data (Rodriguez-Mateos et al, 2013; Whyte et al, 2016; 2017). Two different batches of freeze-dried powder were used in this thesis. As each batch had been grown under different conditions, anthocyanin content varied between

the two. For example, it is likely the second batch of WBB acquired more sunlight during growth, increasing the anthocyanin content found in the skin of the fruit. The second batch was matched to the first batch based on anthocyanin content so results remained comparable between studies. See Table 2.1 for a composition breakdown for each WBB batch.

Inclusion of an appropriate placebo drink was included throughout the thesis to act as a comparable control for those under WBB intervention. This was matched for sugars and vitamin C found in each WBB batch. Details of the placebo drink are outlined in each experimental chapter.

Compound	**Batch 1 (Experiments 1 and 3)**	**Batch 2 (Experiments 2 and 4)**
Total polyphenols (mg/100g)	2239	2900
Anthocyanins (mg/100g)	905	1900
Procyanidins (mg/100g)	400	Not quantified
Total sugars	50	70
Glucose (g/100g)	24	34
Fructose (g/100g)	26	36
Vitamin C (mg/100g)	12	335
Fibre (g/100g)	Not quantified	16

Table 2.1 Composition of wild blueberry powder in batch 1 and batch 2. Batch 1 underwent polyphenol, sugar and vitamin analyses in the Food & Nutritional Sciences Department, University of Reading, UK. Batch 2 was analysed for polyphenols by FutureCeuticals, Illinois, USA, and for sugar, vitamin and fibre by RSSL, Reading, UK.

It is worthy to note that although batches of WBB powder were matched for anthocyanin content, each batch differed in the total amount of total sugars and vitamin C. This was unavoidable due to the composition of each batch being dependent on growing conditions. Differences were successfully controlled for within each experiment by matching the placebo intervention to sugars and vitamin C found in the respective WBB batch, however there were compositional differences across experiments for all constituents except anthocyanins.

2.2.4. Intervention duration

2.2.4.1. Acute

In the case of this doctoral research, an acute postprandial time point of 2 h was selected based on the results of previous psychological and physiological data. Psychological data has shown cognitive improvements at 1 h (Watson et al, 2015), 1.15 h (Whyte et al, 2015; 2016), 1.5 h (Scholey et al, 2009), 2 h (Field et al, 2011; Dodd, 2012; Lamport et al, 2015; Alharbi et al, 2016) and 3 h (Whyte et al, 2017), and mood improvements at 2 h (Khalid et al, 2017) post-flavonoid consumption in humans. This indicates a sensitive time window between 1.15 – 3 h where cognitive change is possible. Physiological data has also found increases in plasma metabolites and FMD at 1-2 h and 6 h post-WBB consumption (Rodriguez-Mateos et al, 2013; 2016a), indicating increased bioavailability of circulating metabolites in this time frame, which may be related to flavonoid uptake in the brain via BBB mechanisms. Elevated BDNF levels have also been observed in humans 2 h post-WBB (Dodd, 2012), and increased CBF has been seen at 2 h post-orange juice (Lamport et al, 2015; Alharbi et al, 2016). This indicates that flavonoids have the ability to affect physiological processes within this time window, and these may be related to the cognitive effects previously observed.

2.2.4.2. Chronic

Chronic intervention will be defined as the assessment of outcome measures after daily supplementation with WBB for an extended period of time. A chronic consumption period of 4 weeks was selected. This period was chosen based on prior research investigating chronic flavonoid supplementation in adult populations, which has shown cognitive effects ranging from 5 days – 6 months (see Chapter 1). Four weeks was deemed an acceptable amount of

time for children to be supplemented with the drinks without adding strain or feelings of over-commitment in families. This was a critical factor to take into account to prevent parents withdrawing their child(ren) prematurely due to commitment issues. Lucas (1994) also provides evidence for significant and potentially permanent positive effects on cognitive function after only a brief period of dietary manipulation (4 weeks on average) in infants. This suggests that 4 weeks is an acceptable and manageable length of time to supplement children for, as well as allowing for potential accumulative physiological and cognitive changes to occur.

2.2.5 Participants

2.2.5.1. Recruitment

All participants recruited in the current thesis were aged between 7-10 years old (inclusive). The brain is believed to undergo many of its biological developments during the first 18 years of life, in particular between the ages of 7-11 years where Piaget (1970) believed children enter a stage of concrete operations, in which they deal with the complexities of representations, events and analogies. Case (1985) and Bjorklund (1985; 1987) believe cognitive development may relate to increases in processing ability, however some have rendered inhibitory control as the potent skill that leads to behavioural and maturational change (Brainerd & Reyna, 1993). For example, the less inhibitory control a child possesses, the more influenced they are by interfering stimuli and the less they can override immature attentional or behavioural reactions (Dempster, 1993). As previous flavonoid literature has revealed speed of processing and EF benefits in adult populations, this may extend to the consolidation of processing and inhibitory skills during the concrete operational stage of child development. An age group of 7-10 years was therefore chosen as it is thought this may be a sensitive period of development where nutrition could influence cognitive change (Smart, 1993; Lucas, 1998; Ruel and Alderman, 2013).

Nutritional intervention within sensitive periods in childhood has been labelled ‘nutritional programming’; it proposes that nutrients act as critical signals affecting receptors in sensitive tissues either directly or indirectly through coupling mechanisms (Lucas, 1994) and have the ability to impact lifelong health (Gabory, Attig and Junien, 2011; Vaiserman. 2014). Further, there are spurts in the growth of the frontal lobes throughout childhood; between birth and 5

years, 7-9 years and 11-13 years (Anderson, 2002; Spencer-Smith and Anderson, 2009). EF abilities also develop alongside these spurts. For example, attentional control develops between 0-5 years, cognitive flexibility, information processing and goal setting between 7-9 years, with full maturation of executive control emerging between 11-13 years. This demonstrates a minimum age of 5 where children may begin to understand cognitive tasks. It is highly likely that the majority of children above 7 years of age will possess the skills to perform cognitive tasks and understand the rules of each.

It is particularly interesting that previous adult and child flavonoid literature has demonstrated beneficial effects on working memory, speed of processing and executive function (e.g. Scholey et al, 2009; Field et al, 2011; Whyte and Williams, 2015; Alharbi et al, 2016; Whyte et al, 2016; 2017), which are domains that are encompassed within the cognitive flexibility, information processing and goal setting skills that develop between ages 7-9. This indicates that flavonoid intervention may help to consolidate these skills in this age range. Data showing cognitive effects following acute intervention with WBB have also been published for this age group (Whyte et al, 2015; 2016; 2017), suggesting the effects observed in adults are extended to developing children.

All participants recruited in the current thesis had attended primary school since Reception grade (aged 4-5). This was important to ensure children had been exposed to the same curriculum, and possessed the basic skills necessary to read and comprehend the cognitive tasks employed. Participants did not possess any developmental or psychological diagnoses, except in Experiment 3 (Chapter 5), where a subset of children with a formal diagnosis of ADHD was recruited. Similarly, participants did not have any health problems (such as diabetes, organ disease) or treatment-related allergies (for example, to fruit or fruit juice). Participants were not on regular medication, except for some participants with an ADHD diagnosis, as detailed in Chapter 5.

Participants were recruited via local primary schools, postings to group email listings, social media pages, community notice boards, the PCLS Child Development Database and through word of mouth. Parent information sheets for each experiment can be seen in Appendix A.

To predict the required sample size for each experiment, a power of 0.9 was determined as optimal for all power analyses across the thesis. However, in some cases this was not achievable due to participant recruitment restraints. In cases where recruitment was challenging, the power threshold was lowered until sample size reached a feasible N for the

study in question. Details of the power calculated and achieved for each study are reported in each experimental chapter.

2.2.5.2 Low flavonoid diet

Participants were required to follow a low flavonoid diet for 24 h before each scheduled test session. This is standard for the field and allows assessment of outcome measures free from the influence of prior dietary flavonoid consumption. A copy of low flavonoid restrictions and a 24 h food log can be found in Appendix B and are detailed in each experimental chapter.

2.2.6 Experimental variables to consider

2.2.6.1 Intelligence Quotient (IQ)

When assessing cognitive ability, it is important to also include an adequate measure of, or proxy for, IQ. Many IQ tests incorporate tasks which involve problem solving, decision making, spatial and verbal ability, and working memory to assess an individual's overall 'general ability' (GA). Tasks that tap into the same domains mentioned are often found in assessment of cognitive abilities, suggesting there is a likely link between general (g) intelligence factors and specific (s) cognitive factors (Spearman, 1927). Indeed, many psychologists have found such links using factor analysis techniques (Cattell, 1963; Gardner, 1999). Measuring general intelligence in experiments assessing cognition is therefore highly informative to check for normality in the sample and assess whether participants are likely to perform adequately on cognitive tasks.

For the current thesis, the British Ability Scale 3 (BAS 3) was chosen as an appropriate measure of GA. The BAS 3 is a test battery that assesses strengths and weaknesses within the domains of an individual's cognitive repertoire, as well as providing an overall measure of GA. BAS 3 contains standardisation data from 2010 and is the most up-to-date version of the battery.

BAS 3 incorporates the original cornerstones of intelligence theories that believe intelligence is comprised of specific cognitive abilities which combine to make an overall GA score (Spearman, 1927). BAS 3 also remains practical to use in a research, school or clinical

setting. The BAS 3 can also help to identify if a child has a learning difficulty, and the nature of that difficulty if present. A support system is also in place should an experimenter need to provide advice to parents or teachers about a child's performance. No participants in the current thesis were identified as having a learning difficulty from BAS 3 results.

The School Age Battery (SAB; ages 6:00 to 17:11) of the BAS 3 was implemented in all experiments of the current thesis. For those scoring less than 3 correct on the first decision point of each School Age subscale, administration of the Early Years Battery (ages 3:00 to 5:11) ensued as per BAS 3 guidelines. Testing continued into the SAB section for these participants up until the point they were no longer able to continue. Each battery contains 6 overlapping core subscales that make up 3 overall clusters (see Table 2.2). The clusters are Verbal Ability (VA), Non-verbal Reasoning Ability (NVRA) and Spatial Ability (SA), from which a General Conceptual Ability (GCA) score is contrived.

Three core subscales (pattern construction, matrices, verbal similarities), one from each cluster (SA, NVRA, VA), were included in this thesis due to time restraints, as detailed in Chapter 3. These particular subscales were chosen due to their successful use in previous child studies at the University of Reading, which demonstrated their feasibility and compliance. These three were also quite different from each other, for example, pattern construction required motor coordination of blocks, matrices required a 'fill in the gap' puzzle paradigm, and verbal similarities involved listening and speaking associative words out loud. It was thought that this combination of subscales would prevent boredom in participants and sustain their engagement.

2.2.6.2 Attention

A measure of baseline attentional ability was considered important in the current scheme of work to assess whether participants were homogenous across each sample on measures of sustained attention and inhibition. Furthermore, it was critical that this measure was objective rather than being recorded via subjective scales where potential bias may have confounded results. A Continuous Performance Task (CPT) was deemed appropriate for use in TD 7-10 year olds and those with a diagnosis of ADHD. Conner's CPT is the most widely used computerised version, measuring attentional abilities in those aged 8 and above. This version is also used to aid diagnosis of ADHD and other attention-related neurodevelopment

Core Scales	Cluster	Battery	
		Early Years	School Age
Verbal Comprehension	VA	GCA	
Picture Similarities	NVRA		
Naming Vocabulary	VA		
Copying	SA		
Pattern Construction	SA		GCA
Matrices	NVRA		
Recognition of Designs	SA		
Word Definitions	VA		
Verbal Similarities	VA		
Quantitative Reasoning	NVRA		

Table 2.2. Core scales and clusters of each BAS 3 battery which contribute towards each age range's respective GCA score. The School Age Battery GCA has been highlighted to indicate the core scales and clusters that contribute towards the GCA for this age group.

disorders and is reported to have appropriate sensitivity of attention deficits between healthy and ADHD child populations (on and off the medication drug methylphenidate; Conners and Sitarenios, 2011). Due to limited resources, the use of Conner's was not possible for this thesis; however, a CPT created at the University of Reading (UoR), which included similar stimuli, was available. In this task, letters of the English alphabet are presented to participants individually on a screen. Participants are instructed to press 'space' for each letter presentation, except for the letter 'X'. When the letter 'X' is presented, participants must press nothing. By using letter stimuli in this task, linguistic abilities are utilised. This includes a sound understanding of the English language, correct grapheme-phoneme interpretation, no visual impairments or visual agnosia, and an adequate level of education or

schooling. This was seen to limit those who may have any linguistic or visual contraindications and may provide an inaccurate measure of attention alone. The task was therefore modified for use in this thesis to display circles of differing colours, rather than letters. By using a familiar shape, linguistic confounds are eliminated from the measurement of the main test components. The modified CPT therefore no longer relies on individual variability in language knowledge or skill, and may have increased sensitivity to detect differences in attentional scores between children with different linguistic abilities. Furthermore, as children who are diagnosed with ADHD often have co-morbid dyslexia, this change would also counteract the task incorrectly measuring a language deficit rather than an attentional deficit. The effect of such modifications has not been studied previously, and sensitivity to detect change to the same degree as the letter CPT remains to be explored in typical and ADHD populations.

It was also believed that using a shape would make the task more achievable for children under 8. The modified task was piloted on a small sample of children (aged between 7-13 years old) and was deemed appropriate and achievable by all that participated. This was on the basis that all participants understood the instructions, and completed the task with >50% accuracy for familiar and novel trials. This level of performance has been implemented previously in cognitive trials (Whyte et al, 2015; 2016; 2017) and is used to infer that children performed above chance levels. Participants also reported increased mental effort on the trials where they had to inhibit a response, demonstrating an apt response to these inhibitory stimuli.

Outcome variables for the CPT were measures of omission and commission. Omission was the percentage of 'orange circle' inhibition trials (out of 30) that participants successfully inhibited pressing 'space' on. A high omission rate indicated good inhibitory ability. Commission was calculated based on the percentage of correctly pressed hits (out of 240) for 'other coloured circles'. High commission rates demonstrated good sustained attention skills.

2.2.7 Cognitive test battery

Reviews investigating flavonoids have revealed key areas of cognition that may be sensitive to intervention in adults; primarily these are executive function (EF) abilities such as attention, inhibition and memory (for reviews see Lamport et al, 2012; 2014; de Jager et al,

2014). To effectively measure these abilities, employment of a cognitive test battery sensitive enough to detect changes across time is required.

In particular, de Jager et al (2014) noted that immediate verbal memory was sensitive to flavonoid intervention in adults and older adults with MCI. Since this review was published, Whyte (2015) and Whyte et al (2015; 2016) have shown such sensitivity in a child cohort (aged 7-10) using a modified version of Reys Auditory and Verbal Learning Task (RAVLT), the Auditory Verbal Learning Task (AVLT). RAVLT was found to be the most commonly cited test of verbal memory used in nutritional RCTs and demonstrated reliable effects across a number of study populations (de Jager et al, 2014). It was decided that an AVLT would be implemented in the current thesis to assess the effects of blueberry flavonoids on verbal memory in a child population. Although this task primarily measured verbal memory, it was acknowledged that some AVLT outcome variables might also be linked to executive function, namely proactive and retroactive interference measures. These measures not only rely on previous and new material being held in memory, but also rely on successful inhibition of an interference list. This should therefore be considered when interpreting results of this task. The task was included in Experiments 1, 3 and 4 (Chapters 3, 5 and 6, respectively). Full details of the AVLT are outlined in Chapter 3.

Secondly, it was important to include a measure of executive function that encompassed frontal-related functions such as attention and inhibition. This is of particular interest in children aged 7-10 due to the spurt in brain development that occurs during this period (Anderson, 2002; Spencer-Smith and Anderson, 2009), where the growth of the frontal lobe accelerates alongside EF abilities. These abilities include the development of cognitive flexibility, goal setting and information processing (Anderson, 2002; Spencer-Smith & Anderson, 2009). Examination of the effects of flavonoids on such abilities during their establishment is of peak interest and may further inform researchers on the extent to which this age range is a 'sensitive' period for cognitive development. Furthermore, it would be particularly revealing given these abilities encompass EF and memory abilities, where adult literature has shown flavonoid-related effects previously (for example, Saunders et al, 2011; Scholey et al, 2010). A Modified Attention Network Task (MANT) was used in all experiments of the current thesis to assess EF-related attentional abilities. This test, and other modified versions of flanker tasks, have been used previously in research investigating cognitive performance in relation to response inhibition (Folstein and Van Petten, 2008), as well as in exercise (Hillman et al, 2009) and flavonoid interventions (Whyte et al, 2016;

2017). Detailed descriptions of the versions of the MANT used can be located in Chapters 3 and 4.

Inclusion of a mood measure (the Positive and Negative Affect Scale for Children; PANAS-C) was enforced for all experiments in this thesis. The PANAS-C (see Appendix C) was chosen due to its relatively quick completion time (~3-5 minutes) and ability to successfully capture current positive and negative mood in a child population across multiple time points (Hughes and Kendall, 2009). Full details of the PANAS-C can be found in Chapter 3.

2.2.8. Justification of statistical approach

In this thesis, data will be analysed using Linear Mixed Modelling (LMM). Using a classical model, such as analysis of variance (ANOVA), assumes that an individual's observations are statistically independent of each other and equally distributed from the group's mean. Consequently, ANOVA models have been deemed to be too restrictive at modelling the likely structure of correlations in a data set (Landau and Everitt, 2004). Using a model that treats individual participant data points as uncorrelated values would not accurately reflect the true nature of repeated measure designs used in my thesis and may lead to false positives or false negatives This is due to these designs including repeated data observations for the same participants across time, which are not uncorrelated values.

On the contrary, LMM allows for adequate analysis when there is individual variability within repeated measure datasets. Such an approach works well for datasets of a biological or psychological nature where there is between- and within-group variation as is the case for experiments in this thesis. Demidenko (2013) uses the 'maple leaf shape analysis' as a clear example of what the LMM does. If you were to take nine maple leaves from the same tree, they would all have significant individual shape variability, yet their shape would look similar. This analogy clearly maps onto between- and within-group parameters of a repeated measure design. The similar shape of all maple leaves represents the between-group parameters (i.e. how particular treatment or demographic groups are performing), and the individual shape variation of each maple leaf relates to within-group parameters (i.e. how one participant is performing at several test points). In the LMM, values from the same participant are acknowledged to correlate and so constitute two 'clusters' – within- and between-group.

A benefit of the model is that then draws from both sources of variation in the analysis, rather than one from source (between-group variation) as in an ANOVA.

A further benefit of LMM is that datasets do not need to be fully complete in order to use this analysis technique. Unlike ANOVA analyses, LMM does not exclude data listwise if there is missing data. All participant data is used within the model to aid the acquisition of between- and within-group means, increasing the power of the analysis and restricting data exclusion. There is not a large amount of missing data across this thesis, however occasionally there are instances of random missing data. By using the LMM technique, participant datasets are not excluded and all available data points are included in the analysis, improving power. Also, dependent variables do not need to be normally distributed. Gelman & Hill (2007) stated the distribution of residuals within the regression model does not influence the outcome of the LMM. This highlights the robust nature of the LMM method under different data distributions.

Throughout this thesis, LMMs have been used in a consistent way to allow accurate comparisons to be made across experiments. As all, or most, experiments reported here have employed the same tasks, it was important that a robust method was chosen and applied systematically so that differences in results could not be attributed to a change in statistical technique. Participant has been included as a random factor across all LMMs to control for the non-independent nature of within-subject data (Sweet & Grace-Martin, 2011). In most analyses (Chapters 4, 5 and 6), a repeated factor has been included to control the covariances (how two variables vary together) for each participant. Each participant will have a covariance structure which includes all of their repeated data observations. In half of cases (Chapters 5 and 6), this repeated factor is Time. For chapter 4, the repeated factor is Visit. The structure that these covariances follow can be specified within a LMM if there are some predictions of how variables may vary together. An unstructured matrix has been applied to all models in this thesis due to the assumption that covariances do not have an underlying structure and are unpredictable. This type of matrix has been deemed suitable for all types of data providing it is not used on a small sample of participants who engage in many repeated time points.

Additionally, baseline performance has been used as a fixed factor in the majority of analyses (Chapters 3, 5 and 6). For ease in this discussion, this will be referred to as using baseline as a covariate. This model structure was chosen to better account for baseline performance and

improves precision when estimating treatment effects. Covariates are often baseline characteristics of participants before treatment consumption occurs, for example age, gender or socioeconomic status. It is important to note that these variables do not vary across time and are not measured again throughout a trial. With this is mind, the baseline performance of a dependent variable (i.e task performance) can be included as such a characteristic, due to it being temporally unchanging throughout a study, and occurring before treatment (Senn, 2006). Baseline demographic characteristics have not been used as covariates in analyses in this thesis; the only covariate used is baseline task performance. Baseline variables are almost always likely to correlate with, or have some bearing on, outcome variables. Applying baseline performance of a task as a covariate therefore accounts for variation in post-treatment values that are predicted by baseline performance. This better isolates the effect of treatment and improves power within the model (Dolan, Green and Lin, 2016). It is also believed that in studies with smaller sample sizes, covariate adjustment can improve precision rather than, or in the same way as, increasing N.

It is also important to highlight the differences between including baseline task performance in a within-group variable such as 'time', or as a covariate. In a traditional, integrated approach, where baseline is included within the model as a time point, it would mean that baselines are treated, alongside post-baseline time points, as a response to treatment. Temporally, and logically this is not appropriate (Senn, 1997; Senn, Stevens and Chaturvedi, 2000; Senn, 2006). Thus, there is a danger in assuming that it is necessary, in designs of a repeated nature, to model baselines as if they are outcomes alongside other post-treatment variables. Senn (2006) stipulates that there should be clear focus on the post-baseline outcomes only, as these are the only variables influenced by treatment. To assume that post-treatment outcomes are rigidly linked to baseline, where no treatment was present, has been deemed "unnatural" (Senn, 1997; 2006; Senn et al, 2000). Although these variables are nested within the arbitrarily labelled variable 'time' rather than 'treatment', the main research question most experimenters are interested in is whether there are differences between treatments at post-baseline time points. Indeed, that is why it is standard to ensure the inclusion of a control group, to compare active treatment against inactive treatment effects across the course of an intervention. During this thesis, post-treatment data points have been categorised as within-group 'time' factors. This allows a comparison between treatment groups at each post-baseline time point and across time within each treatment group that would not be possible if looking at effects of treatment alone.

To include baseline within a time variable would be to also assess between-group differences at baseline within the model. This could potentially confound between-group interactions at post-treatment time points, and produce false positives depending on the extent of group differences at baseline. For example, higher performance in group A at baseline may influence significantly higher performance for this group post-treatment compared to group B. We might therefore conclude that there is a significant treatment effect for group A in this instance, however these differences do not take into account the high performance of this group at baseline and subsequently would result in a false positive. Furthermore, differences between baseline group means may occur non-significantly, and this may still influence post-treatment observations significantly. For example, if performance is higher at baseline for Group A, analyses may show change for this group across time where there may not have been one due to the high baseline. Interpretation of within-group treatment x time effects can also be difficult. The increase in variance attributed to the model by including baseline as a time variable may mask both within- and between-group post-treatment effects. Using the above example, false positive between-group effects observed post-treatment may overshadow a within-group effect in Group B, due to an increased amount of comparisons being made within the model, subsequently reducing power. A within-group difference between baseline and post-treatment, when baseline means are different between groups, is therefore, likely to be non-significant – a false negative. Including baseline as a covariate removes the potential for differing group performance at baseline to confound post-baseline interpretations. This allows a more precise estimate of treatment effects free from type 1 and type 2 errors.

It is also imperative to consider whether comparing performance at baseline within the model is necessary or informative. Assessing for differences at baseline is important; however this analysis can be performed independently from an ANOVA or LMM, and prior to the main analysis using a simple independent t test. In most cases, considering baseline differences within an ANOVA or LMM is to inflate variance of the overall model and subsequently alter the ability to detect between- and within-group changes post-treatment. Throughout the thesis, group differences at baseline have been analysed using an independent t test before conducting LMMs, or in the case of Chapter 4, a one way ANOVA with Drink and Visit as fixed factors. This has been done to assess baseline differences independent of the interference from outcome variables and reduce the variance inflation that often occurs when baseline is included in the model. Alternatively, viewing baseline as a variable capable of

improving outcome predictions by modelling pre-treatment individual differences outside of an integrated model may be a better control in assessing post-treatment effects. Using baseline task performance as such a variable within the thesis will aid in controlling for pre-treatment differences with the aim of improving post-treatment predictions.

Relatedly, there has been debate as to whether using 1) baseline as a covariate (BaC) or 2) change from baseline (CFB; subtracting baseline from post-treatment) scores produces the most unbiased effects. Indeed, for the data in my thesis, it would be possible to calculate change from baseline scores for the outcome variables of interest. Senn (2006) discusses many models that have been proposed comparing methods accounting for baseline (Lord, 1967; Laire & Ware, 1982; Laird & Wang, 1990), concluding that the use of a baseline covariate as a superior choice to CFB in RCTs. Differences between groups at baseline, and the influence of this on post-baseline treatment effects, has also been a focal point in discussion. Many have argued (Holland and Rubin, 1983; Wainer, 1991; Wainer and Brown, 2004) that using each method on the same set of data produces different outcomes. The debate between BaC and CFB analysis is heavily based on Lord's (1967) paradox. This considers a scenario where two groups are weighed at baseline, and again 8 months later. Weight was different between the two groups at baseline, however neither group changed over time. As there was no change, CFB analysis produced a non-significant score of zero for both groups. BaC analyses arrived at a significant outcome due to the differences at baseline being the observed difference at outcome. Lord concluded that where baseline differences were assumed to be zero, BaC and CFB would give the same statistical result. However, this does not mean that using BaC is 'incorrect' if baseline means are different. Senn (2006) reviews the paradox with working counter examples and challenges the assumption that baseline means should be equal. He stipulates that it is easy for researchers to design trials where using BaC is valid and CFB is not, and that the reverse is problematic. When running RCTs, BaC can deal with accidental bias in a way that CFB cannot by accounting for across-participant variance in the raw data, as opposed to experimenters manually creating variables from each participant.

Throughout my thesis, although direct comparisons are not made between baseline and post-baseline time points when baseline is included as a covariate, this does not mean that comparisons to baseline are inherently absent within the LMM models. When baseline is a covariate, pre-treatment performance is averaged across groups to create an estimated mean value. It can be said that each group's baseline performance has been made 'the same'. So, if

between-group differences were to arise post-treatment, this can be indicative of a change from baseline performance, as it has been assumed both groups were identical at baseline. This is perhaps a much better way to quantify and simplify issues arising from differences between groups at baseline compared to integrating with outcome variables, as it is clear that both groups begin at the same level and end up in different positions, from a between-group comparison only. Dolan et al (2016) states that where baseline group differences are present, treatment effects are subject to sampling variability, meaning they will contribute more noise (imprecision) to the data and potentially "miss the mark" (identify false positives and negatives). Covariate adjustment has been shown to improve precision if covariates are good predictors of the outcome. Assessing whether covariates are good predictors can easily be checked by seeing if baseline is a significant predictor of the outcome variable in LMM output. Dolan et al (2016) ran 10,000 iterations on a dataset to compare treatment effects with and without covariate adjustment. True treatment effects were revealed for both analyses and precision of the estimated treatment effects were determined using standard error (SE), which found unadjusted effects to produce a SE of 0.121, and covariate adjusted effects to produce 0.093 – a tighter estimate. Including a highly predictive covariate within the analysis explained some of the noise in the data so that a more precise treatment effect was observed. The predictive capability of baseline performance in the thesis is high due to its' temporal stability and occurrence before treatment. This capability will be assessed via significant effects of baseline within LMMs.

Overall, LMM, a robust statistical technique, will be used in this thesis and will include all available data. It will account for changes in within-group variation over time, increasing power. Baseline will be included as a covariate in analyses allowing estimates for post-baseline treatment effects to be more precise, and reducing the chance of type 1 or type 2 errors arising compared to using baseline within an integrative model. Post-hoc pairwise comparisons will be performed on effects and interactions of interest within LMM analyses. Much like after an ANOVA, these comparisons will identify significant between- and within-variable relationships to further inform the main LMM effects. All post-hocs will be Bonferonni-corrected to reduce the risk of type 1 error.

Chapter 3: The acute effects of wild blueberry on the cognition and mood of typically developing 7-10 year old children

Mood data from this chapter has been published as Khalid, S.*, Barfoot, K. L.*, May, G., Lamport, D. J., Reynolds, S. A., & Williams, C. M. (2017). Effects of acute blueberry flavonoids on mood in children and young adults. *Nutrients, 9*(2), 158.

Cognitive data from this chapter has been published as Barfoot, K. L.*, May, G.*, Lamport, D. J., Ricketts, J., Riddell, P. M., & Williams, C. M. (2018). The Effects of Acute Wild Blueberry Supplementation on the Cognition of 7-10 Year Old Schoolchildren. *European Journal of Nutrition*, 1-10.

*joint first authors

The role of other researchers in this chapter: G. May undertook 50% of data collection.

3.1. Introduction

As discussed in Chapter 1, the health benefits of berry fruits are well established, with consumption leading to both vascular and neurocognitive benefits across the lifespan (for reviews see Lamport et al, 2012; Bell et al, 2015). The majority of research conducted so far, in human populations, has investigated the intervention of flavonoids (from a range of food sources) in middle- and older-aged adult populations, using chronic, repeated dosing regimens. For example, supplementation studies have reported reductions in cognitive impairment, and delayed onset of ageing disorders, such as Alzheimer's disease in older adults after a 12 week supplementation of grape juice (Krikorian et al, 2010a), blueberries (Krikorian et al, 2010b), and cherry juice (Kent et al, 2015). Similarly, greater dietary intake of blueberries and strawberries were associated with a reduced rate of cognitive decline over the course of 6 years (Devore et al, 2012). Supplementation with flavonoids for 5 days to 6 months has also been associated with improved visuospatial memory and improved long-term memory in healthy middle-aged and older adults after supplementation with berry fruits (Miller and Shukitt-Hale, 2012), orange juice (Kean et al, 2015), grape juice (Lamport et al, 2016b) and blueberries (Dodd, 2012; Bowtell et al, 2017; Whyte et al, 2018). Although these chronic studies suggest cognitive benefits from flavonoid interventions administered for several weeks or months in this age range, investigations into cognitive function in the

immediate hours after consumption of flavonoids remains to be elucidated. Few studies have explored the effects of acute supplementation in older adults; and where such trials have been conducted, they have recruited healthy adult populations with results showing improvements in attention, inhibition, visuospatial memory, and executive function (EF) between 1–6 h post-consumption (Macready et al, 2009; Watson et al, 2015; Alharbi et al, 2016). In particular, Scholey et al (2009) discovered improvements on Rapid Visual Information Processing (RVIP) and serial 3s tasks following acute cocoa flavanol intervention, as did Field et al (2011) on a spatial working memory task. This suggests that tasks which tap into EF and memory abilities may be particularly susceptible to acute flavonoid intervention.

Although research to date has examined berry flavonoid supplementation in populations aged 30 and above, many fewer papers have been published investigating the impact of berry flavonoids on cognition in children. In the first study of its kind in this population, Whyte and Williams (2015) assessed the cognitive abilities of 8-10 year olds before and 2 h after consumption of a 200g fresh blueberry drink (143mg anthocyanins) using a Go No-Go task, Stroop, Auditory Verbal Learning Task (AVLT), Visual N-Back and Object Location Task. Significant improvements were observed following blueberry intervention in word recall performance for both short and long delays at 2 h post-consumption, suggesting children's verbal memory may be sensitive to blueberry flavonoid effects at the 2 h postprandial time point. In a similar study (Whyte et al, 2016), the cognitive performance of 7-10yr olds was examined at baseline, 1.15, 3 and 6 h post-consumption of two wild blueberry (WBB) interventions: a 30g WBB drink (253mg anthocyanins) and a 15g WBB drink (127mg anthocyanins), compared to a sugar-matched placebo drink (Whyte et al, 2016). Cognitive performance was measured using the AVLT, Modified Flanker Task (MFT), a Go No-Go Task and a Picture Matching Task. Similar to what was seen previously with this age group, significant WBB-related improvements were observed on the AVLT, where enhanced word recognition performance was observed at every time point tested post-dosing, and immediate word recall was improved at 1.15 h. Improvements were observed for both doses, however performance improved the most following the highest dose (253mg anthocyanins), and was worst following placebo treatment. Additionally, significant improvements in accuracy on the cognitively-demanding MFT were observed at 3 h post-consumption of the 30g WBB drink. These findings highlight that the effects of WBB could be dose-related; AVLT performance may be sensitive to a lower dose, whereas a higher dose is required to impact MFT performance.

Importantly, Rodriguez-Mateos et al (2013) found increases in plasma polyphenolic metabolites and flow-mediated dilation (FMD) at similar time points to the cognitive effects described above, in healthy men. Increases in plasma metabolites and FMD were observed at 1-2 h and 6 h following an acute blueberry dose (766mg total polyphenols; 310mg anthocyanins; ~240 g fresh blueberries). This was a similar dose to that employed in Whyte et al's (2016; 2017) research where a 30g dose equated to ~650-700mg total polyphenols (253mg anthocyanins). Further, a positive correlation was observed between FMD effects and concentration of plasma metabolites. This suggests that the metabolism of polyphenolic compounds may cause changes in FMD, a feasible acute mechanism of action (MOA) for the observed cognitive effects found at 2 and 6 h post-consumption. Interestingly, at 1 h post-consumption, FMD was found to increase dose dependently to 766mg total polyphenols, after which it plateaued. Taken together, these studies suggest a higher dose of WBB may elicit increased cognition and FMD response up to a ceiling dose of 766mg, after which additional WBB may not have additional benefit.

Further findings by Whyte, et al (2017) revealed that a one-off dose of WBB (30g; 253mg anthocyanins), in 7-10yr olds, produced the most benefit on cognitively demanding, incongruent and high load MFT trials, when compared to placebo, 3 h post-consumption. This suggests that blueberry flavonoids may enhance performance when cognitive resources are compromised at certain time points post-consumption.

These studies confirm the findings that flavonoids can positively affect EF and memory abilities after acute supplementation, and clearly show the efficacy of flavonoid interventions in a child population, specifically in those aged 7-10 years old. It is also noteworthy that WBB effects may be amplified under conditions of high cognitive demand on executive function tasks. Interestingly, it is well-documented that children of this age experience a spurt in frontal lobe growth (Anderson, 2002), thought to coincide with enhanced executive functions and the progression of cognitive abilities. It is plausible that this window of accelerated cognitive development is where WBB supplementation may be particularly beneficial.

The effects observed in these trials (Whyte and Williams, 2015; Whyte et al, 2016; 2017) also suggest that the dosage of the intervention, the time point at which participants are tested, and the tasks used influence sensitivity to cognitive effects. Whyte et al (2016) noted that cognitive performance was consistent with a dose-response model, where performance

improved the most following the highest dose (253mg anthocyanins), and was worst following placebo treatment. The anthocyanin content in the treatments used was altered between Whyte and Williams (2015) and Whyte et al's (2016; 2017) studies, due to a switch from using fresh blueberries with milk (143mg anthocyanins) to wild blueberries with water (253mg anthocyanins). Milk has been suggested to impede the absorption of polyphenols, potentially dampening the effects of polyphenol dose (Yildirim-Elikoglu & Erdem, 2017). This change in treatment may have therefore affected polyphenol absorption in Whyte and Williams' (2015) study and reduced the sensitivity to detect cognitive effects. Whyte et al (2016) recommended that future studies coincide with a WBB dose high enough to detect significant cognitive change at 1.15, 2, 3 or 6 h post-consumption (253mg anthocyanins). Despite the different cognitive effects across dose (127mg vs 253mg anthocyanins) in these studies, lower dose participants still showed significant attenuation of decline for delayed recall and word recognition performance. This implies that stable effects can emerge at a lower dose, regardless of post-consumption time point (1.15 h, 2 h, 6 h), and an increased dose may simply reveal different effects on memory and EF domains. It is therefore possible to suggest that sensitivity to detect cognitive changes in blueberry flavonoid intervention studies may be dependent on the dosage, in combination with the time point. On the basis of this previous research, cognitive assessment with a sensitive time window for dose-related effects between ~1-3 hours, which has been shown to correlate with FMD changes, is warranted.

In the present study, we aim to replicate and extend the findings from Whyte and Williams (2015) and Whyte et al (2016; 2017) on verbal memory and executive function by administering an Auditory Verbal Learning Task (AVLT) and Modified Attention Network Task (MANT) before, and 2 h after, consumption of a WBB or placebo drink. These tasks were chosen as they have previously been shown to be sensitive to acute cognitive changes in a child population (Whyte et al, 2015; 2016; 2017). The current study aims to test performance at 2 h post-consumption as this is when flavonoid metabolites have been shown to circulate in plasma, and could potentially cross the blood-brain barrier. Indeed, increased FMD was also observed 1-2 h following acute WBB (766mg total polyphenols) intervention, implying improved circulatory function (Rodriguez-Mateos et al, 2013). We aim to extend the current literature by testing participants in the afternoon, to see if cognitive effects persist in the school environment at a time of day where fatigue is most likely. It is predicted that participants consuming WBB will show improved or maintained performance on memory and

EF measures at 2 h post-consumption, whereas placebo participants will either show no change or a decline. In particular, WBB effects may be more prominent on the more cognitively demanding trials of the MANT (incongruent or high load) as observed in previous research (Whyte et al, 2017). Mood will also be assessed using the children's version of the Positive and Negative Affect Schedule (PANAS-C) before, and 2 h after, WBB or placebo intervention. The sensitivity to detect changes in children's mood following flavonoid intervention is currently unknown; the present study aims to elucidate any changes in positive or negative affect to uncover whether there is potential for WBB to prevent low mood or sustain positive mood across a day.

3.2 Methods

3.2.1 Participants

An a priori power analysis (using G Power 3.1.) based on previous significant findings from our laboratory (Whyte et al, 2016) using an effect size (F) of 0.22, was conducted using F test, repeated measures, within-between interaction parameters. The optimal power sought was 0.9; this produced a required sample size of 58 (n=29 per condition) when completing two repetitions (baseline, post-consumption) ($F(1,56)=4.01$). A total of 54 healthy participants (25 male: 29 female) aged 7-10yrs (M=8.24, SD=0.97) of any ethnicity were recruited from two primary schools local to the University of Reading. This sample size resulted in adequate power of 0.89 at an effect size (F) of 0.22 ($F(1,52)=4.03$). All parents or legal guardians gave written consent for their child to take part and confirmed that the children spoke English as a first language, had not been diagnosed with ADHD, dyslexia or reading impairments, and had no known fruit or fruit juice intolerances. Each child gave written and verbal assent before any research began. All participants followed a low-flavonoid diet 24 h prior to the main test day. The school canteen provided a low-flavonoid lunch to children who had school meals on the day of testing. Parents were asked to provide a low-flavonoid packed lunch to those who did not have school meals.

3.2.2 Treatments

An acute, single-blind, randomised, parallel-groups design was applied with participants randomly allocated to receive either 30g freeze-dried wild blueberries (WBB) or a sugar-matched placebo. The 30g WBB treatment (equivalent to 240g fresh wild blueberries or 1 ½ cups) contained ~650-700mg total polyphenols (253mg anthocyanins). The placebo contained fructose (8.9g), glucose (7.99g) and vitamin C (4ml) to match the concentrations found in the 30g WBB treatment. To aid consumption and palatability, 170ml of cold tap water and 30ml of a low-flavonoid fruit squash (Rocks, UK) were added to both treatments, producing a 200ml drink. Treatments were prepared immediately before being administered to participants in an opaque drinking flask and were consumed through an opaque straw to ensure the participants remained blind to the treatment. Participants were given 5 mins to consume the drink.

3.2.3 Cognitive Tasks

1. *Auditory Verbal Learning Task (AVLT)* – This task measured short-term verbal memory through word list learning, a sensitive format relatively free from associative context (Lezak, 2004). Participants heard an auditory recording of 15 nouns (list A), read at the rate of 1 per second. Each presentation was followed by a free recall of this list (Recalls A1 to A5). A new list of 15 nouns (list B) was introduced as an interference list and was recalled once only (Recall B). Participants then recalled list A after a short delay (2mins) and a long delay (15mins) (Free Recalls A6 and A7). After the final recall, participants were visually presented with 50 nouns containing: words from lists A and B, and 20 additional nouns. They were asked to circle words from list A only (Recognition). Word lists were created for each test session and conformed to an age of acquisition (AOA) rating of 400 or less; this was equivalent to acquiring the words at age 7 or below. All words contained 1 or 2 syllables. Each primary word list (list A) had 7 phonetic and 8 semantic ‘match’ words which were included on the recognition sheet; 2 phonetic and 3 semantic ‘match’ words were also included from secondary word lists (B), providing a total of 20 additional matched nouns for the recognition outcome measure. All lists were

matched for familiarity and concreteness, and word list versions were counterbalanced across test sessions. Word lists can be seen in Appendix D.

For each test session, a series of outcome measures were calculated according to Lezak (2004): immediate word span (Recall A1); words learnt (Recall A5 – Recall A1); final acquisition (Recall A5); proactive interference (Recall A1-Recall B); retroactive interference (Recall A5 – Recall A6); word recognition (the number of words correctly circled). A number of additional outcome measures were also calculated: total acquisition (sum A1 through A5). total recall A (sum A1 through A7 - B) and total delayed recall (A6 + A7) to explore the effects on total word recall for each treatment condition.

2. *Modified Attention Network Task (MANT)* – This task combined a cue-target and flanker test to measure vigilance, selective attention and attention under conditions of cue conflict. In accordance with Whyte et al (2017), participants initially completed four blocks of the MANT each consisting of 80 trials. The blocks consisted of one 120ms target block and one 500ms target block, each presented in silence, or with playground noise, increasing the cognitive load of these trials. A practice block of 35 trials preceded this where target duration decreased from 1000ms to 120ms over 16 trials to allow familiarisation with the speed of response required.

This task was designed to measure response interference. For the main target blocks, a fixation slide was first shown with a cross displayed centrally. This was followed by no cue, a central cue, a double cue or a spatial cue for 120ms with location of the cue (above or below the central fixation cross) randomised between trials. The target stimulus, a single arrow pointing "<" or ">", was then displayed either above or below the fixation cross for either 120ms (two target blocks) or 500ms (two target blocks); stimulus location was not always consistent with prior cue location. The target stimulus appeared individually or surrounded by pairs of flanker arrows, and could be congruent (i.e. <<<<< or >>>>>) or incongruent (i.e. <<><< or >><>>) with the surrounding arrows. Individual arrows exhibited low load whilst congruent or incongruent trials either exhibited medium load (one row of 5 arrows) or high load (two rows of 5 arrows; see Figure 3.1).

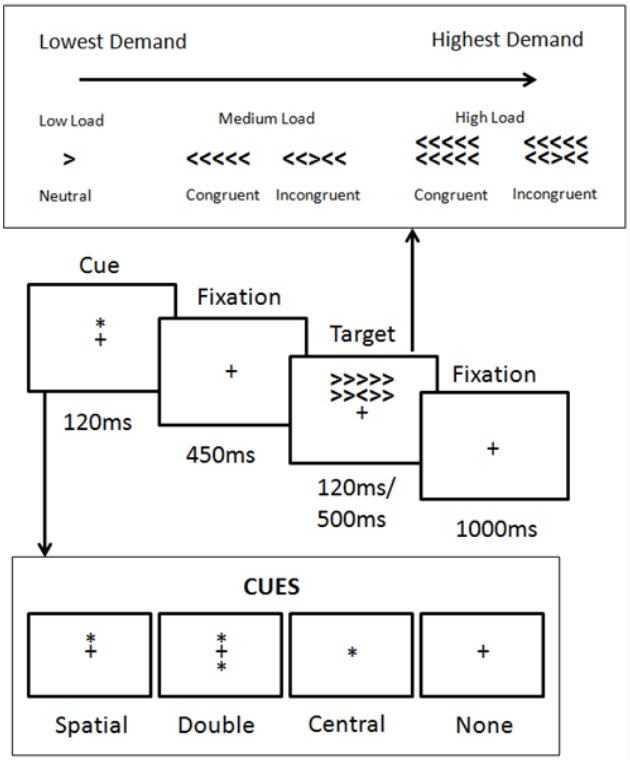


Figure 3.1. Schematic of the Modified Attention Network Task (Whyte et al, 2017)

The stimulus position, congruence, cue and load were randomised between trials, displayed in equal probability. Participants were instructed to press the left or right arrow key on the keyboard according to the direction of the target stimulus arrow for each trial. Although manipulations of cue were included in the MANT across the thesis, cue was not included in cognitive analyses. This was due to unpromising findings from Whyte (2015) and Whyte et al (2016) that showed that the position of the cue did not provide additional benefit to participants. Cues were not removed from the task to maintain task sensitivity and allow for comparisons across experiments in the thesis and published literature.

The outcome measures for the MANT were accuracy (number of correct hits) and reaction time for correct targets (speed of response to target; with reaction times <200ms removed).

3. *Positive and Negative Affect Schedule for Children (PANAS-C)* - The PANAS-C was used to assess current mood (see Appendix C). The PANAS-C is a validated children's version of the Positive and Negative Affect Schedule-NOW (PANAS-NOW) used in adult populations. The PANAS-C is a valid and reliable 30 item (15 positive and 15 negative)

self-report measure of Positive Affect (PA) and Negative Affect (NA), which can be used on multiple test occasions (Hughes and Kendall, 2009). The PANAS-C includes the original 20 items from PANAS-NOW and 10 additional child-friendly synonym items derived from the PANAS-X (Expanded Form; Watson et al, 1988; Crawford and Henry, 2004). Participants were asked to rate the degree to which they were currently experiencing each item, on a five-point Likert scale. The ratings of positive and negative items were summed to calculate an overall positive and overall negative affect score, ranging from 10–50 (lower scores indicating lower levels of positive or negative affect).

3.2.4 Procedure

To accommodate school hours, all sessions took place during the afternoon on school premises. This involved taking participants out of one lesson for ~40 minutes, and after school was finished.

Screening session: Participants took part in a screening session 1-2 days prior to the test day where they completed demographic measures. A computerised version of the children's Raven's Coloured Progressive Matrices (RCPM), a well validated measure assessing nonverbal and reasoning abilities (Raven, 2000), was administered at screening to measure fluid intelligence. This measure was included to ensure all participants were of healthy cognitive functioning for their age. A modified Continuous Performance Task was also employed to measure sustained attention and inhibition abilities. Data for these are shown in Table 3.1. Participants also completed a practice of the main test battery (AVLT, MANT and PANAS-C, see 3.2.3). Finally, all participants were reminded to comply with the 24 h low-flavonoid diet. Parents were telephoned after their child's screening session to give them the date of their child's main test day and to remind them about the dietary restrictions. School canteen staff members were also asked to monitor children's meals on the day of testing and to remind the children taking part in the study not to eat high-flavonoid foods for lunch that day. Compliance and adherence to the restrictions were orally checked with children, parents and canteen staff on the test days.

Test session: A between-groups design was used to minimise disruption to the school and limit the demands on participants. Testing took place during the afternoon, after lunch,

individually in a quiet space at their schools. Children were not required to fast prior to testing, although parents were asked to make sure that their child consumed a low-flavonoid diet for 24 h before the baseline session. Participants consumed either a low-flavonoid packed lunch provided by parents or a low-flavonoid cooked lunch provided by the school canteen on the day of testing. The specific content of the lunch was not standardised or recorded. Lunch was consumed in the 1.15 h period prior to baseline testing.

The main test day consisted of a baseline session and a post-consumption session 2 h later. The initial baseline session commenced at 1200 h, 1245 h or 1330 h immediately before consuming the intervention or placebo drink. After consumption, all children returned to class for a 2 h period during which time they were only allowed to consume water and abstained from exercise. Cognitive performance was assessed at 2 h post-consumption for consistency with Whyte et al (2016); this is where anthocyanin metabolites from WBB are known to peak (Rodriguez-Mateos et al, 2013) leading to their optimum absorption and metabolism within the body. Therefore, the second test session, post drink consumption, took place at 1440 h, 1525 h or 1610 (dependent on time of consumption of the intervention). Here, participants completed a different version of the same test battery to assess any changes in performance. During each test session, participants completed the tasks in the following order: AVLT recalls 1-6, MANT, AVLT recall 7 and word recognition. Test versions of equivalent difficulty were counterbalanced across visits and conditions. Each session lasted approximately 40 mins. Upon completion of the main test session, participants were given a written debrief about the study aims and were given similar debrief information to take home to their parents.

3.2.5 Statistical Analysis

Baseline data comparing each treatment group were analysed using t-tests. All other data were analysed using linear mixed models (LMMs) with baseline performance included as a covariate. Separate LMMs were performed for each dependent variable (DV), and Bonferroni corrected pairwise comparisons were performed on all emergent Fixed Factor effects and interactions.

Drink (placebo, WBB) was included as a Fixed Factor in all LMMs to compare the effects of treatment. For the MANT, Congruency (congruent, incongruent), Load (high load, medium load) and Target Time (120ms, 500ms) were also included as Fixed Factors in the model to detect changes in relation to cognitive load. For the MANT, a post-hoc correlation was also performed between overall accuracy and overall RT with the data from placebo- and WBB-treated participants to assess whether a speed-accuracy trade-off was present. Overall scores for the MANT were derived by averaging placebo and WBB participants' performance across baseline and 2 h for accuracy and RT, respectively.

Data were analysed using SPSS (Version 21.0).

3.3. Results

3.3.1 Demographic and baseline analyses

Demographic data can be seen in Table 3.1. Forty-nine participants were included in baseline analysis of RCPM due to 5 missing data files. There were no significant differences at baseline between groups for RCPM ($t(47) = -0.16$, $p = 0.88$). Forty-eight participants were included in baseline analysis of CPT measures due to 6 missing data files. There were no significant differences between groups for CPT Commissions ($t(46)=-0.19$, $p=0.85$) or Omissions ($t(46)=-0.13$, $p=0.90$). No significant differences were observed between groups for age ($t(52)=-0.034$, $p=0.97$).

Fifty-two participants were included in baseline mood analyses due to 2 missing data files at this session. All participant data was present at the post-consumption time point and were subsequently included in LMM analysis. There were no significant differences between groups at baseline for PA (WBB, $M = 49.00$, $SD = 11.96$; placebo, $M = 49.21$, $SD = 10.04$; $t(50) = 0.07$, $p = 0.95$) or NA (WBB, $M = 20.79$, $SD = 7.43$; placebo, $M = 19.25$, $SD = 3.86$; $t(50) = -0.91$, $p = 0.37$; see table 3.2).

	Placebo (n=25)		WBB (n=29)		
	Mean	**SD**	**Mean**	**SD**	**P statistic**
Age	8.23	1.05	8.24	0.88	0.97
Gender (m:f)	13:12	--	12:17	--	--
School Year (3:4:5)	8:7:8	--	8:7:10	--	--
CPT	(n=22)		(n=28)		
Omissions (%)	10.74	7.14	11.21	16.03	0.90
Commissions (%)	88.48	8.21	89.17	15.62	0.85
RCPM	(n=22)		(n=27)		
	26.55	6.10	26.78	4.40	0.88

Table 3.1. Demographic data for placebo and WBB participants.

Fifty-four participants were included in the baseline analysis of the MANT. There were no significant differences between groups at baseline for MANT accuracy (WBB, M=0.76, SD= 0.14; placebo, M=0.75, SD=0.16; t(52)=-0.156, p=0.88) or MANT RT (WBB, M=557.16, SD= 0.132.84; placebo, M=585.58, SD=102.32; t(52)=0.87, p=0.39; see table 3.2).

Fifty-one participants were included in baseline AVLT analyses due to 3 corrupt data files. There were no significant differences between groups for any AVLT list recalls at baseline (see table 3.2).

Dependent variables	Baseline data		
	Placebo (n=25)	**WBB (n=29)**	**Between-groups p statistic**
	Mean (SD)	**Mean (SD)**	
PA	49.21 (10.04)	49.00 (11.96)	0.95
NA	19.25 (3.86)	20.79 (7.43)	0.37
AVLT – raw data	**(n=24)**	**(n=27)**	
List A			
Recall 1	4.38 (1.47)	4 (1.78)	0.82
Recall 2	6.33 (2.2)	6.15 (2.11)	0.66
Recall 3	8.21 (2.6)	7.41 (1.74)	0.21
Recall 4	8.79 (2.52)	8.52 (2.29)	0.50
Recall 5	9.46 (2.64)	9.12 (2.6)	0.72
List B			
Recall 1	4.54 (1.56)	5 (1.59)	0.19
List A			
Short Delay Recall 6	7.55 (2.22)	7.69 (2.38)	0.47
Long Delay Recall 7	7.04 (2.29)	6.74 (2.1)	0.72
Word Recognition	11.09 (2.66)	10.73 (2.46)	0.51
MANT			
	(n=25)	**(n=28)**	
Accuracy (%)	74.89 (0.16)	75.52 (0.14)	0.88
Reaction Time (ms)	585.578 (102.31)	557.16 (132.84)	0.39

Table 3.2. Dependent variable baseline raw data for placebo and WBB participants.

3.3.2 Mood

Figure 3.2 shows PA and NA scores in both treatment groups 2 h after the intervention.

Positive Affect (PA)

Baseline PA significantly predicted post-consumption PA, regardless of treatment drink ($F(1,54)=18.69$, $p < 0.01$); for every 0.38 increase in baseline PA, post-consumption PA increased by 1. Drink was found to be a trending predictor of post-consumption PA, such that WBB participants ($M = 52.82$, $SE = 1.68$) had higher post-consumption PA than placebo participants ($M = 48.17$, $SE = 1.68$; $F(1,54) = 3.52$, $p = 0.066$; Figure 3.2a).

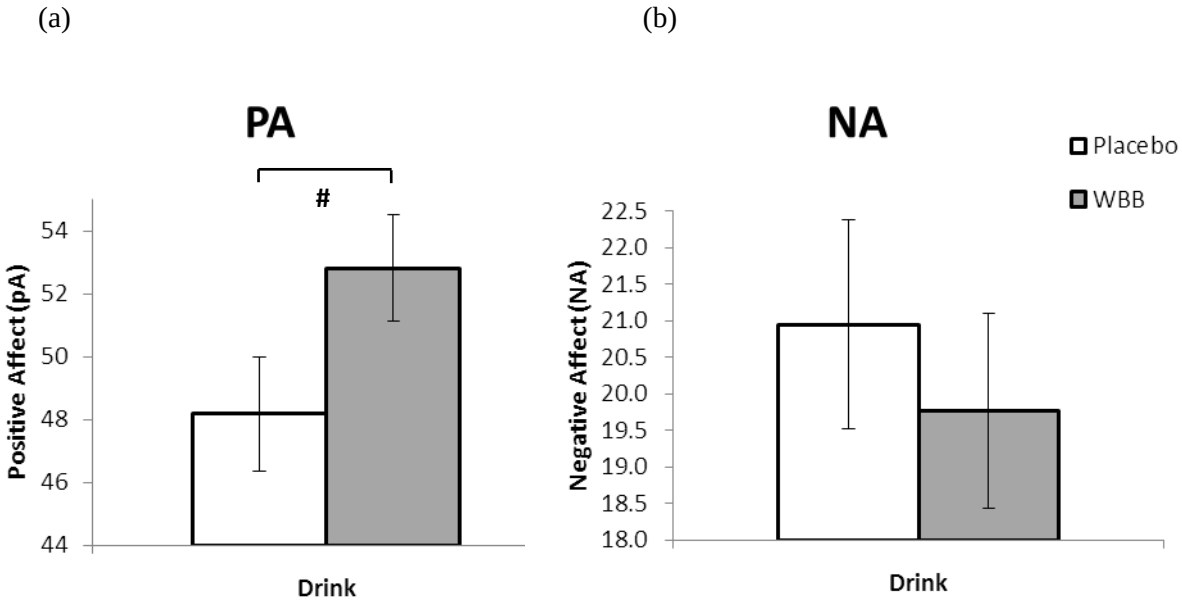


Figure 3.2. Mean PANAS-C mood scores in children aged 7–10 years: (a) Mean PA scores post-consumption of placebo and WBB treatment drinks; (b) Mean NA scores post-consumption of placebo and WBB treatment drinks. # = $0.1 < p > 0.05$

Negative Affect (NA)

Baseline NA ($F(1,54)=2.48$, $p = 0.12$) and Drink ($F(1,54) = 0.36$, $p = 0.55$; Figure 3.2b) did not predict post-consumption NA for either WBB participants ($M = 19.77$, $SE = 1.33$) or placebo participants ($M = 20.95$, $SE = 1.43$).

3.3.3 Modified Attention Network Task (MANT)

Speed-accuracy trade-off

A correlation was performed using placebo and WBB participants' overall accuracy and overall RT performance to determine whether there was a speed-accuracy trade-off in each group. Analyses revealed that as accuracy increased, RT also significantly increased for placebo ($r=0.54$, $p<0.01$) and WBB participants ($r=0.74$, $p<0.01$), implying that there was a trade-off in this study.

Accuracy (proportion correct, 0-1)

Baseline accuracy on the MANT significantly predicted post-consumption accuracy performance, regardless of treatment ($F(1,430.24) = 64.34$, $p<0.01$), whereby for every 1% increase in baseline performance, post-consumption performance increased by 32%.

As expected (Whyte et al, 2017), congruency was a significant predictor of performance, such that participants were more accurate on congruent trials ($M = 0.85$, $SE = 0.017$) than incongruent trials ($M = 0.79$, $SE = 0.017$), regardless of treatment ($F(1,404.22)=26.70$, $p<0.01$). Similarly, load was a trending predictor of performance, such that accuracy on medium load trials ($M = 0.83$, $SE = 0.016$) was better than high load trials ($M = 0.81$, $SE = 0.016$), regardless of treatment ($F(1,371.98)=3.55$, $p= 0.06$). Target time was a significant predictor of performance, such that accuracy on the more cognitively demanding 500ms trials ($M = 0.84$, $SE = 0.017$) was better than the less demanding 120ms trials ($M = 0.80$, $SE = 0.017$), regardless of treatment ($F(1,377.92)=15.49$, $p<0.01$). In contrast, Drink was not a significant predictor of accuracy performance ($F(1,48.52) =0.17$, $p=0.68$). There were no significant fixed effect interactions.

Reaction Time (RT)

Baseline RT significantly predicted post-consumption RT, regardless of treatment ($F(1, 409.03) =74.14$, $p<0.01$), whereby participants' post-consumption RT slowed by 0.33ms for every 1ms speed reduction in baseline RT. As expected, congruency was a significant predictor of performance, such that participants were significantly quicker on congruent trials

(M = 539.32, SE = 10.58) than incongruent trials (M = 587.82, SE = 10.58), regardless of treatment (F(1,398.34)=77.34, p<0.01). Similarly, load was a significant predictor of performance, such that RT on medium load trials (M = 558.48, SE = 10.51) was significantly quicker than on high load trials (M =568.66, SE = 10.51), regardless of treatment (F(1,369.72)=4.19, p= 0.04). Target time (F(1,396.13)=0.56, p=0.45) and Drink (F(45.57)=1.63, p = 0.21) did not independently predict RT performance, however there was a significant fixed effect interaction whereby Drink x Target time predicted post-consumption RT (F(367.82)=5.63, p=0.018).

Pairwise comparisons revealed that WBB participants were significantly quicker on 120ms trials (M =542.59, SE =14.39) than 500ms trials (M =558.43, SE =14.30; p= 0.02; Figure 3.3). However, placebo participants did not show this expected difference in RT between 120ms (M =580.45, SE =15.42) and 500ms (M= 572.81, SE= 15.52) trials. Pairwise comparisons confirmed this and revealed a trend whereby WBB participants (M =542.59, SE =14.39) performed quicker than placebo participants (M =580.45, SE =15.42) on 120ms trials (p=0.078; Figure 3.3). This suggests WBB consumption increased mental alertness following acute WBB for 120ms trials, without change to 500ms trials.

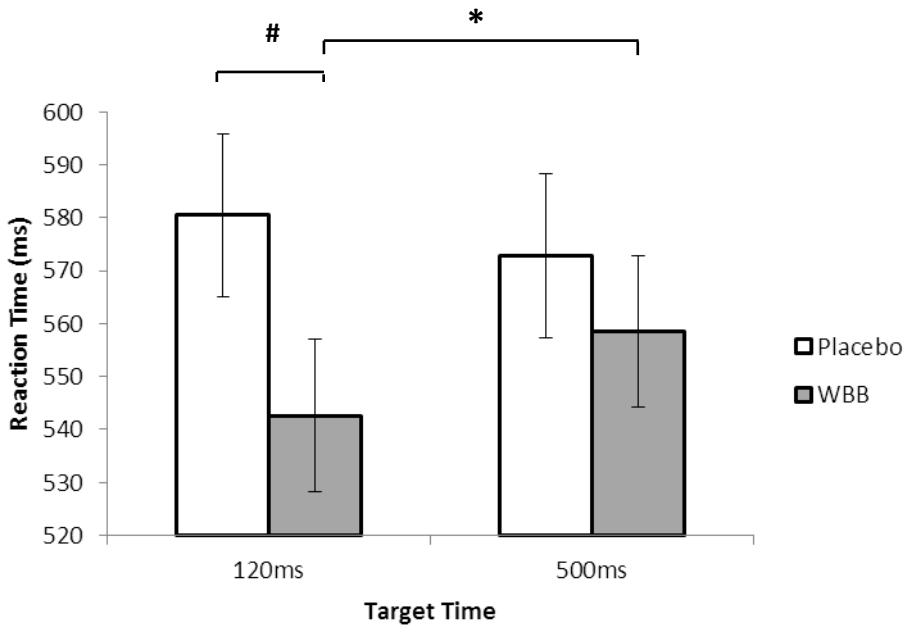


Figure 3.3. Mean post-consumption MANT RT scores of placebo and WBB treatment drinks on 120ms and 500ms trials. *Significant at p<0.05; # Trend at 0.1< p >0.05.

3.3.4. Auditory Verbal Learning Task (AVLT)

AVLT variables	Baseline			Post-consumption (2hrs)		
	Placebo (n=24)	WBB (n=27)	LMM	Placebo (n=24)	WBB (n=27)	LMM
	Mean (SD)	Mean (SD)	Baseline covariate fixed effects statistics	Mean (SD)	Mean (SD)	Drink fixed effects statistics
Word Span	4.40 (1.44)	4.31 (1.38)	F(1,50)=13.60, p<0.01*	4.13 (1.23)	4.04 (1.17)	F(1,50)=0.01, p=0.94
Words Learnt	5.25 (1.70)	4.86 (3.55)	F(1,51)=12.39, p<0.01*	3.79 (1.41)	4.21 (2.08)	F(1,51)=1.52, p=0.22
Final Acquisition	9.44 (2.58)	9.19 (2.57)	F(1,51)=27.94, p<0.01*	7.92 (1.93)	8.25 (2.27)	F(1,51)=1.01, p=0.32
Total Acquisition	36.92 (9.70)	35.11 (8.54)	F(1,52)=43.00, p<0.01*	30.75 (7.76)	33.25 (9.37)	F(1,52=4.69, p=0.035*
PI	0.00 (1.73)	-1.00 (1.96)	F(1,53)=0.69, p=0.41	0.40 (1.58)	0.31 (1.20)	F(1,53)<0.01, p=0.99
RI	2.52 (2.86)	1.36 (2.33)	F(1,53)=1.11, p=0.30	1.76 (2.03)	1.59 (1.64)	F(1,53)<0.01, p=1.00
Total List A Recall 1-7	50.88 (13.68)	49.43 (12.51)	F(1,52)=62.54, p<0.01*	42.08 (11.14)	45.00 (10.92)	F(1,52)=3.82, p=0.056#
Short Delay A6 Recall	7.38 (1.94)	7.02 (1.71)	F(1,52)=57.93, p<0.01*	6.15 (1.55)	6.49 (1.48)	F(1,52)=4.26, p=0.04*
Long Delay A7 Recall	7.04 (2.24)	6.82 (2.11)	F(1,52)=23.48, p<0.01*	5.25 (2.69)	5.93 (2.46)	F(1,52)=1.99, p=0.164
Delayed Recall A6+A7	13.96 (4.74)	14.32 (4.68)	F(1,52)=35.55, p<0.01*	11.33 (4.26)	12.54 (4.33)	F(1,52)=1.21, p=0.28
Word Recognition	11.04 (2.61)	10.56 (2.58)	F(1,51)=23.52, p<0.01*	9.20 (2.74)	9.21 (2.33)	F(1,51)=0.48, p=0.49

Table 3.3. Baseline and post-consumption performance for AVLT outcome measures. LMM fixed effects statistics for Baseline covariate and Drink predictor variables are shown. *significant at p<0.05; # trend at 0.1< p >0.05.

Data for AVLT outcome variables at baseline and 2 h post-consumption can be seen in Table 3.3. LMM analyses revealed that baseline performance significantly predicted post-consumption performance for 10 out of 11 AVLT outcome measures (Table 3.3; word span (β=0.39), final acquisition (β=0.49), total acquisition (β=0.64), words learnt (β=0.28), short delay (β=0.60), long delay (β=0.65), delayed recall (β=0.58), recognition (β=0.56) and total recall 1-7 (β=0.62)). Baseline performance did not significantly predict post-consumption performance for measures of proactive or retroactive interference ($p>0.05$).

Recall performance for each word list at baseline and 2 h post-consumption is presented in Figure 3.4. Performance decreased at 2 h compared to baseline (Figure 3.4), as previously observed by Whyte et al (2016).

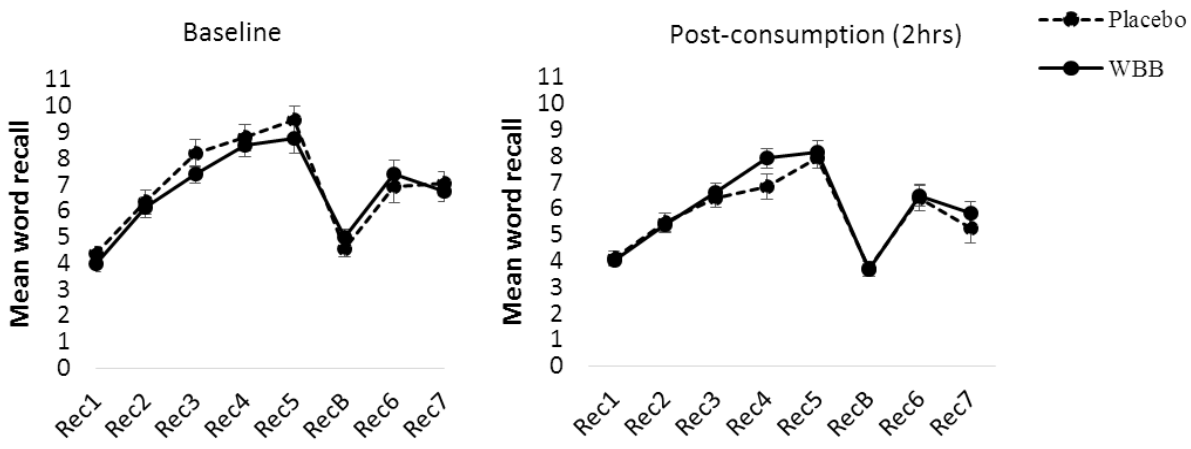


Figure 3.4. AVLT word recall (Rec) performance for each word list (A1-5, B, A6, A7) at baseline and 2 h post-consumption. Mean recall (+-SEM) for each recall by WBB and placebo treatment group is shown

3.3.4.1. AVLT Drink effects

Drink was found to be a significant predictor of post-consumption total acquisition performance (total list A recall across recalls 1-5). LMM analysis revealed that placebo participants had significantly lower total acquisition than WBB participants at the post-consumption time point (Table 3.3; Figure 3.5), suggesting a maintenance of performance in the WBB–treated group. Similarly, short delay trial (recall A6) performance was significantly predicted by Drink, such that WBB participants had better short delay recall than placebo

participants (Table 3.3; Figure 3.6). Again, this is indicative of maintenance of performance in the WBB group alongside task fatigue for the placebo group.

Drink was also found to be a trending predictor for Total 1-7 (total list A recall across recalls 1-7, excluding B) performance. WBB participants had better recall performance than placebo participants, post-consumption (Table 3.3; Figure 3.5). No drink effects were observed in LMM analyses of other AVLT dependent variables (Table 3.3).

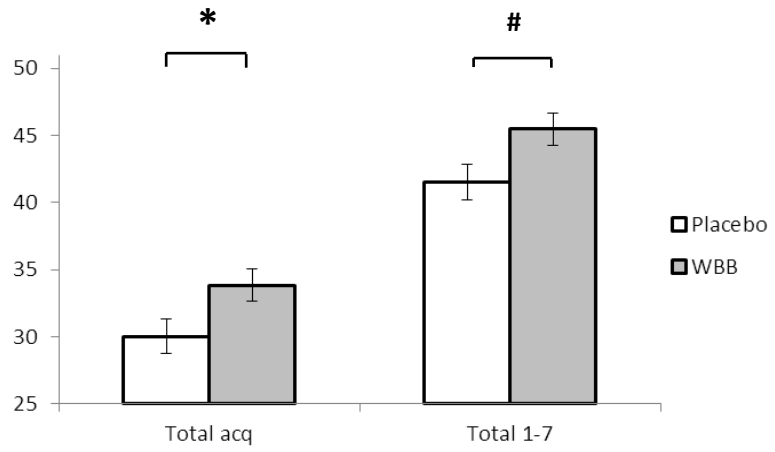


Figure 3.5. Mean Total acquisition (sum of list A1 through A5) and Total 1-7 word recall (sum of list A1 through A5, A6 + A7) of placebo and WBB treatment drinks at the post-consumption time point. *significant at p<0.05; # trend at 0.1< p >0.05.

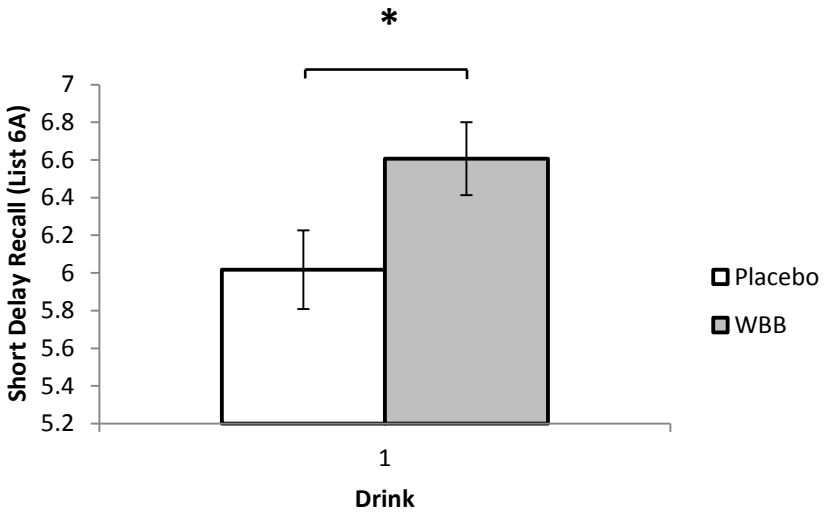


Figure 3.6. Mean short delay word recall of placebo and WBB treatment drinks at the post-consumption time point. *significant at p<0.05; # trend at 0.1< p >0.05.

3.4. Discussion

The current study administered a one-off dose of anthocyanin-rich wild blueberry to typically developing 7-10 year old children following a randomised, placebo-controlled, between-groups design, to investigate the effects of acute consumption on mood and cognition. Here, the blueberry drink tended to improve positive affect, with no changes in negative affect detected. For the MANT, treatment drink did not affect accuracy performance, however significantly quicker RTs were observed for WBB participants when compared to placebo participants on 120ms trials. Similarly, WBB participants showed enhanced performance on the AVLT, recalling more words than placebo participants on measures of total acquisition, short delay and total 1-7 recalls.

The mood effects observed in this study have positive implications for promoting healthy mood in 7-10 year old children across 2 h of the school day. This has several potential benefits; the foremost being that the positive effects that arise from a conscious feeling of happiness could increase optimism and allow children to positively frame future situational encounters (Seligman, 2006). Furthermore, an enhancement in conscious mood could allow children to further enjoy the school environment and increase their motivation for learning. In turn, this could improve attentional abilities, retention of information and therefore, academic achievement (Wong, Wiest and Cusick, 2002). This could have been a possibility in the current study, as mood was measured at the start of the test battery. This may have raised awareness of positive feelings in the children, in turn increasing their motivation to complete the subsequent cognitive tasks. It is uncertain whether positive affect or cognitive performance would alter if the PANAS-C was placed at the end of the test battery. To further explore the effects of WBB on mood and its relationship to motivation and cognition, a battery performed in the reverse order would be informative to ascertain this relationship.

Similar mood-boosting effects of berry interventions have been seen in a young adult population (mean age: 20.14 years) at the same 2 h post-dosing time point (Khalid et al, 2017) using the same WBB intervention (30g) as the current study, and the adult short-form measure of mood (PANAS-NOW). In this study, a within-subjects design was used, and participants were tested in the morning. It was found that young adults reported increased positive affect following WBB intervention, whereas no such effect was reported following

placebo. Thus, the positive effect of blueberry flavonoids on positive affect appears to be robust to variations in experimental design between the ages of 7-10 and 18-21.

The distinctive effect of flavonoids on PA, but not NA, is notable. PA and NA reflect orthogonal facets of mood. A low PA is more highly linked to depression, and high NA is more closely related to anxiety (Oddy et al, 2009; Renzaho et al, 2011). Thus, this data suggests that the effect of flavonoid consumption on mood may be specific to depressive disorders, rather than pervasive across different mood states. It is important to note that diagnosis of mental health disorders or consumption of medication were not specific exclusion criteria in this study, and the data showed normal levels of positive and negative affect (Oddy et al, 2009), indicating a healthy sample.

Mood is by definition a short-term experience. In non-clinical populations mood is usually labile. However, sustained periods of low mood (dysphoria) are a strong predictor of the emergence of major depressive disorder. Therefore, if acute flavonoid consumption improves positive affect, sustained consumption of flavonoids may help prevent dysphoria and thus major depression. Although preliminary, these results are intriguing and warrant focused investigation of the relationship between flavonoids and mood, as well as with mental health more generally.

Beneficial effects of blueberry flavonoids were also observed on the MANT, where WBB participants exhibited quicker RT, without change to accuracy. As expected across both treatment groups, a reduction in accuracy and a slowing of RT was observed for the more cognitively demanding trials (incongruent, high load,) of the MANT, when compared to less cognitively demanding trials (congruent, medium load, respectively). This reflects high internal validity and confirms that manipulation of cognitive demand in congruency and load variables was successful, as participants performed worse on the more difficult trials.

Participants were also found to be significantly more accurate on 500ms trials when compared to 120ms trials, regardless of treatment. This was an unexpected finding and leads to questioning of cognitive load assumptions. An *a priori* assumption of the MANT was that participants would perform less accurately and more slowly on 500ms trials; the premise being that these trials remained on the screen for longer and provided higher visual load, warranting increased cognitive effort from the participant to attend to the stimulus to which they had to respond (Whyte et al, 2017). It was proposed that this longer 500ms exposure also

allows participants more time to be exposed to the dissonance of congruency and load variables (i.e. incongruent and high load trials) on the trial they are observing, increasing cognitive load, and decreasing performance. This assumption was never confirmed for accuracy performance in Whyte et al's (2017) study as no main effect of target time was observed. However, the current study revealed a main effect and, in turn, an opposition to the assumption; participants performed less accurately on 120ms trials, and more accurately on 500ms trials. A likely reason may be that participants succumbed to a 'pacing effect', where they were aware that they had less time to respond to 120ms trials (due to the task instructions outlining 'fast' trials on 2 of 4 blocks), and so responded to the faster pace of visual stimuli alone at a reduced accuracy, due to partial processing of the target stimulus in each trial. On the contrary, for 500ms trials, participants were aware they were being exposed to 'slow' trials (on 2 of 4 blocks) and so had longer to make a correct decision. Furthermore, although the inter-stimulus response window was the same length across both target time variables (1000ms), the time to make a cognitive decision and respond would have differed between trials – 1120ms for 120ms trials, and 1500ms for 500ms trials. This was because the length of time the stimulus was displayed on screen (120ms or 500ms) was also included in the response window, giving more time for participants to respond on 500ms trials. Although this extra time would have likely been unnoticeable to participants, it may have implicitly contributed alongside the explicit knowledge of differences in 'pace' between trials, to reduced accuracy on 120ms trials, and increased accuracy on 500ms trials. Although Whyte et al (2017) used the same instructions and inter-stimulus timings as the current study, it is worthy to note that the post-consumption test time points were different; 3 h versus 2 h, respectively. It is therefore feasible that this extra time allowance on 500ms trials, sensitivity to pace, and subsequent increase in accuracy, were detectable at 2 h post-consumption in the current study, where flavonoid metabolites could be crossing the blood-brain barrier and increasing cerebral blood flow (Rodriguez-Mateos et al, 2013).

In light of the accuracy findings and pacing effects described above, a faster reaction time may not indeed be akin to better performance; participants may be more likely to take longer to make a more accurate response. Correlations between overall accuracy and RT scores revealed that this was the case; as accuracy increased, RT significantly lengthened. From these findings, it can be deduced that slower RTs cannot be used to define increased task difficulty in the current sample; a slower RT is not equivalent to poorer performance, as it allows for a correct response. Whyte et al (2017) did not report an accuracy-RT trade-off in his sample; however he calculated a combined accuracy-RT score and compared performance

between groups, regardless of time point. In the current study, accuracy and RT were directly correlated within each treatment group, regardless of time point to assess the relationship between both variables directly. There was an accuracy-RT trade-off in the current sample for both treatment groups implying all participants responded slower and subsequently performed more accurately. In LMM analyses, if RT effects persisted, accuracy effects were then explored to ascertain if effects were at a cost to accuracy to further explore a potential trade-off.

Regardless of treatment, a difference in RT was not observed between the two target time variables (120ms, 500ms), suggesting participants were not advantaged or disadvantaged by the 380ms response window difference, and did not succumb to the proposed pacing effect on the RT measure. This absence in target time variable differences was also observed in Whyte (2017), where no significant main effect of target time was seen in the measure of RT.

In the current study, when considering treatment effects, significantly quicker RTs were observed on 120ms trials for WBB participants compared to 500ms trials, with no such difference in RT for placebo participants. This is a finding that is also supported by the aforementioned pace effect. Performance could have been slower on 500ms trials, due to a longer response window and subsequent match of pace, and faster for 120ms trials. The fact that only WBB participants showed this difference in RT suggests that they benefitted from these pace cues, and maintained this difference at the post-consumption time point, without detriment to accuracy. The absence of RT differences in the placebo group implies that responses to 120ms trials were slower than expected. This was confirmed in the analysis of 120ms trials, which showed that the placebo group was slower compared to the WBB group. This may have been due to cognitive fatigue and this can be expected in a school environment over the course of an afternoon. The critical finding here was that consumption of WBB significantly attenuated the slowing of RTs, in the presence of a fast-paced cue. The slower RTs observed in the placebo group could be interpreted as a resistance to the 'pacing effects' described above, where they did not use the cue resources available to them ('fast' and 'slow' block instructions) to adapt their response speed to the trial speed. Again, this could be indicative of cognitive fatigue; this group may not have attended to or acknowledged the resources available to them (block instructions) to maximize their success in the task, which subsequently slowed their RT and decreased performance.

An interaction with treatment drink did emerge in RT in Whyte et al's (2017) study; however analyses discovered this effect within 500ms trials, rather than 120ms trials. WBB

participants performed faster than placebo participants on 500ms high load and incongruent trials, at no cost to accuracy. This was seen to support the cognitive load hypothesis, with WBB seemingly overcoming the most cognitively demanding trial combination (incongruent, high, 500ms).

In both the current study and Whyte et al's (2017) study, WBB improvements were shown to persist in the improvement and maintenance of speed of response across 120ms and 500ms trials. Whyte et al (2017) did witness a benefit to WBB participants under conditions of increased difficulty (incongruent, high load), however the inclusion of 500ms as a high load, 'difficult' variable would have had to have been indicated by a significant difference between 120ms and 500ms trials, therefore caution should be taken when interpreting task difficulty. Firstly, the relationship between accuracy and RT in the study sample needs to be determined to define 'good performance'. Secondly, in the case of RT, if a main effect of target time does not persist, caution is to be taken when interpreting 120ms and 500ms trials as either 'easy' or 'difficult' in any subsequent interactions.

Beneficial WBB effects were also observed on the AVLT in the current experiment. As expected across both treatment groups, a reduction in verbal memory 2 h post-consumption was observed compared to baseline for a variety of facets of memory (word span, final acquisition, total acquisition, words learnt, short delay, long delay, delayed recall, recognition and total recall 1-7), indicating increased forgetting at the 2 h time point. In relation to this, the critical finding here was that consumption of WBB significantly attenuated forgetting for total acquisition (total list A recall across recalls 1-5) and short delay recall (recall 6A); as indicated by maintenance of post-consumption word recall following WBB, in comparison to the significant decline seen following placebo for these measures.

This has interesting implications regarding the number of items that can be held in short-term memory (STM). The current theory stipulates that 7 + or – 2 items is a reliable estimate for the capacity of adult STM (Miller, 1956) and this capacity is likely to increase after rehearsal of the material (Atkinson and Shiffrin, 1971). Children have shown an increased memory deficit when compared to adults; however research that has investigated children's STM capacity has been inconclusive (Chi, 1976). Rather, the evidence suggests an absence of successful memory strategies in children that are already developed in adults. The current study supports this deficit in STM capacity, as both placebo and WBB children recalled a mean of 4 words on the first presentation of word list A (Table 3.3; Figure 3.4). Furthermore,

true to rehearsal theory, both groups increased the number of correctly recalled items with each subsequent list A presentation (Figure 3.4); this is often called the 'learning effect'.

Results therefore suggest that WBB participants may be more susceptible to the benefits of rehearsal, and retained more words in their STM, as indicated by significantly increased correct word recalls across lists 1-5 compared to placebo. As Drink effects were not observed at the post-consumption timepoint for word span (recall 1A), results cannot be attributed to poorer memory capacity for the placebo group. These findings indicate that memory strategy use (rehearsal) may have been enhanced for those consuming the WBB intervention, and may have prevented a decline in word recollection. Similarly, a trend in total recall 1-7 also revealed a maintenance of word recall following WBB intervention at the post-consumption timepoint, with a significant decline in performance evident following placebo intervention. This finding is an extension of those observed in total acquisition performance, where WBB attenuated cognitive fatigue.

Significant decline in short delay recall following placebo, and maintenance of performance following WBB further support these findings. This is of particular importance as performance on short delay recall was expected to decline due to preceding interference from list B. The effects of WBB to maintain performance, through enhanced susceptibility to rehearsal, can therefore be shown to pervade across interfering material, rather than detriment performance. These beneficial effects on memory are consistent with Whyte et al (2016), who reported maintenance of delayed and acquisition measures in children at 1.15 h, 3 h and 6 h post-WBB consumption, using an identical 30g WBB treatment and placebo as used in the current study, whilst children consuming placebo showed a decline in performance. This further highlights the effect of WBB treatment in reducing the fatigue effect, specifically in the domain of memory.

In addition to cognitive fatigue, between-session interference on the learning and recalling of information could also help to account for the general decline in memory performance. Words heard at the baseline session may have interfered with encoding and recollection of new words at post-intervention. This would have made less of an impact on the current study which only employed two sessions, compared with Whyte et al's (2016) which included four sessions across the day. Interestingly, the present data provided no evidence of retroactive interference within session; new learning did not appear to alter recollection of previously learned words in either treatment group. This effect was also observed by Whyte et al (2016) and implies a potentially different mechanism between stages of encoding and recollection in

children. It could also infer that RI may be recruiting from a different cognitive domain such as executive function, as participants were able to correctly inhibit new material in order to recall previous word lists. Such interference parameters in a child population are yet to be determined and need further exploration.

The current study demonstrated beneficial affective and cognitive effects of acute WBB consumption on mood, memory and attention in healthy 7-10 year old children. Such findings add to the growing body of evidence that flavonoids are beneficial for healthy brain function, and demonstrate potential for benefits of flavonoid treatment during critical development periods. Several animal and human studies have observed an increase in cognitive ability following acute blueberry interventions with adults (Lamport et al, 2012; Bell et al, 2015), however only three previous studies have explored the effects of berry fruits in children (Whyte & Williams, 2015; Whyte et al, 2016; Whyte et al, 2017). Replications of such findings, alongside biological mechanisms of action, are desirable to further elicit the exact functioning of flavonoids within a child population.

The different mechanisms of action associated with acute and chronic intervention, as well as identification of EF and memory cognitive domain sensitivity at postprandial time points remains to be elucidated. There is evidence to suggest that cognitive improvements are linked to increased cerebral blood flow, however definitive mechanisms are currently unknown, especially in children (Bell et al, 2015). There are a number of plausible mechanisms that may explain the beneficial WBB effects observed in these results. Participants were tested 2 h post-consumption, when flavonoids, or their metabolites, would have been circulating in blood plasma (Rodriguez-Mateos et al, 2013) and blood flow would have been increased in the frontal lobes of the cerebral cortex (Vauzour, 2008). Cognitive processes requiring frontal brain regions, such as EF and processing speed demanded by the MANT, could have experienced this increase in blood flow, and in turn, oxygen, increasing performance capacity. Increased cerebral blood flow to this area may also improve neural regulation in the frontal lobes, where cognitive and emotional control is located. A way in which this could be investigated is through measurement of brain activity whilst performing cognitive tasks after WBB and placebo intervention. This could be explored via electroencephalogram (EEG) with a focus on attention and inhibition-related event-related potentials (ERPs) after the 2 h ingestion window to see whether WBB changes can be recorded in the electrical activity of the frontal lobe (Chapter 4).

Further work to ascertain the chronic cognitive and mood effects of blueberry flavonoids are also warranted, in healthy and clinical populations (Chapter 5). To assess this alongside investigation of bioavailability within children's bodies via non-invasive metabolic examinations would further reveal the physiological effects after chronic blueberry consumption (Chapter 6). This would preferably be conducted alongside cognitive assessment, to converge findings with a robust mechanism of action.

This study demonstrated acute effects of blueberry flavonoid consumption on positive mood, attention and memory in healthy children and young adults. Dietary interventions could play a key role in promoting positive mood and are a possible way to prevent dysphoria and depression. Performance in an academic environment could also benefit from such interventions by increasing cognitive ability through improved attention and recall of information. Given the potential implications of these findings for preventing depression and improving academic ability, it is important to replicate the study and assess the potential to translate these findings to practical, cost-effective and acceptable interventions.

Chapter 4: The effects of acute wild blueberry (WBB) on cognition, mood and event-related potentials (ERPs) in healthy 7-10 year old children

The role of other researchers in this chapter: G. May and A. R. Whyte both undertook data collection (approximately 25% each). C. Cheatham and A. Armer provided valuable training and advice on ERP study design, procedure and analysis. C. Scrivener provided valuable support in software training and ERP analysis.

4.1. Introduction

As discussed in Chapters 1 and 3, there is growing interest in the potential for acute intervention of WBB to improve cognitive and mental health, as shown by short-term improvements in mood, cognition and memory in healthy adults and children. The experiment conducted in Chapter 3 supported these findings; improvements in mood, executive functioning (EF) and short-term verbal memory were observed 2 h after consumption of a 30g freeze-dried WBB drink (253mg anthocyanins) in typically developing 7-10 year olds. This study adds to the current child flavonoid literature which reports similar findings at 1.15, 3 and 6 h post-consumption on similar EF and memory tasks (Whyte and Williams, 2015; Whyte et al, 2016; 2017). However, the potential mechanism(s) of action underlying such effects remains to be elucidated. It is thought that improved acute behavioural effects might result from increases in cerebral blood flow (CBF; Vauzour et al, 2008), particularly to frontal brain regions where areas controlling executive function, attention, inhibition and emotion regulation are situated. Indeed, Rodriguez-Mateos et al (2013; 2016) discovered that increased flavonoid metabolites circulate in blood plasma 2 h after consumption of WBB in young adult men, and this correlated with increases in flow-mediated dilation (FMD). This suggests that circulating flavonoid metabolites could contribute to increased endothelial dilation. Furthermore, Youdim, Dobbie and Kuhnle (2003) and Youdim et al (2004) demonstrated that flavonoids and associated metabolites were able to enter the brain endothelium and traverse the blood-brain barrier (BBB), as shown by flavonoid uptake in *in vitro* mouse and rat cell lines. These findings suggest flavonoids may increase CBF and have the potential to cross the BBB in humans, and exert direct effects on the brain regions involved in cognitive processes, within a 2 h window.

The increased CBF hypothesis (Vauzour et al, 2009) suggests that when performing a cognitive task, there is a demand for more oxygen in task-related brain regions. As

vasodilatory effects have been observed following flavonoid consumption previously, flavonoids may therefore increase the amount of oxygen to these regions under conditions of increased demand. Indeed, studies have demonstrated the efficacy of acute cocoa flavanol consumption through increased CBF in both healthy young (Francis et al, 2006) and older adult (Lamport et al, 2015) populations. Furthermore, Dodd (2012; Dodd et al, 2016) demonstrated increased CBF 1 h post-blueberry consumption, whilst Alharbi et al (2016) demonstrated peak CBF increases at 2 h post-orange juice in healthy adults.

Flavonoid-related vasodilatory effects may, therefore, contribute to cognitive change also seen across acute timeframes (Scholey et al, 2009; Field et al, 2011; Whyte et al, 2015; 2016; 2017; for review see Bell et al, 2015). According to the CBF hypothesis, to successfully complete a cognitive task, frontal regions associated with EF and attention would require higher activation, placing a demand for more oxygen on these brain regions. This demand would likely be aided by the vasodilatory effects of flavonoids delivering more oxygen to frontal areas and could potentially maintain or improve cognitive performance. To verify this hypothesis, it is critical to collect both neurophysiological and behavioural data simultaneously to help understand the potential relationship between increases in CBF in relation to cognitive demand and task performance across the same time frame. This would further inform understanding of whether increases in region-specific brain activity, such as frontal areas, are related to increases in cognitive performance, namely attention and EF, following flavonoid consumption. No study has yet measured real-time neurophysiological and behavioural data after flavonoid supplementation.

A neuroimaging method suitable to assess accurate temporal changes in specific brain regions should be utilised when trying to relate immediate behavioural response measures to real-time brain activity during a placebo-controlled flavonoid intervention. This is so that precise neural activity and cognitive function can be measured during the different demand conditions of a task. Increased cognitive demand should result in less successful performance for placebo participants, yet WBB participants should be protected from this, due to increased vasodilatory effects in task-related regions.

Electroencephalogram (EEG) is a non-invasive, cost-effective measure of instantaneous brain activity, providing the highest level of temporal resolution (0.001-1 second) across the neuroimaging plane (for review see Sejnowski, Churchland and Movshon, 2014). It is therefore a suitable method to assess real-time neurological change. Event-related potentials (ERPs) are recordings of EEGs that are time-locked to either visual or auditory stimuli which

are presented to the participants, giving an accurate simultaneous measure of immediate brain activity, preceding, during or following a behavioural response. Accuracy and reaction time measures from behavioural tasks can indirectly indicate an individual's cognitive ability under different nutritional interventions. However, amplitude and latency changes in the frontal, fronto-central and parietal regions associated with memory, attention, EF and processing can be measured in ERP analysis, giving a more direct measurement of what is happening in task-related brain regions during behavioural responses.

Traditionally, the focus of ERP analysis is on particular positive or negative 'peaks' which occur after the presentation of a particular stimulus. ERPs are named based on the polarity of the peak, which can either be positive (+) or negative (-) (labelled P or N, respectively), and the time to reach peak amplitude after a stimulus has been presented, or a response has occurred (in ms; Figure 4.1). The number within the label of an ERP peak corresponds to the approximate time it takes to reach peak amplitude divided by 100 (as seen in Figure 4.1), and is often labelled as 'latency to peak'.

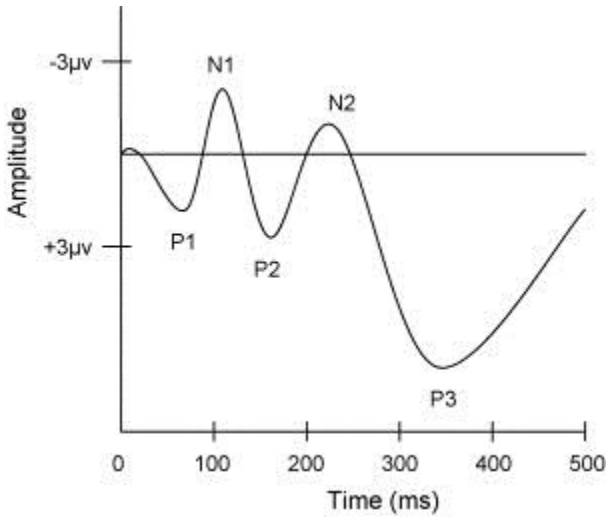


Figure 4.1. Schematic of a standard ERP trace. Peaks are labelled in consecutive order from when they occur after presentation of a stimulus (0ms) and reflect latency to peak, labelled as Time (ms) here. P1, P2 and P3 reflect positive peaks occurring approximately 0-100ms, 100-200ms and 200-500ms after presentation of a stimulus, respectively. N1 and N2 are negative peaks that occur approximately 100ms and 200ms after stimulus presentation, respectively (Dong et al, 2010).

As ERPs are measured across the scalp, particular regions of interest related to the researchers' hypotheses should be stipulated *a priori*. These regions of interest can be specified based on the standardised 10:20 system, an internationally recognised method for choosing electrode positions on the scalp. Certain electrodes are labelled with the lobe or brain region it is recording from, for example Frontal (F), Parietal (P) or Occipital (O). The 10:20 system can be applied to different EEG caps and systems, and allows for standardised comparisons between studies (Klem, Lüders, Jasper & Elger, 1999). In the current experiment, the 10:20 system was applied to the Geodesic cap used which will be described in detail in section 4.2.5.2 and is shown in Appendix E. During analysis, increased ERP amplitude in positive or negative peaks therefore reflects increased activation in the brain regions of interest. Researchers are interested in the differences across individuals in latency to peak or P or N amplitude measures in brain regions of interest to ascertain whether these differences are associated with cognitive processing variances.

As it has been posited that WBB effects occur in attention and EF domains in children (Chapter 3; Whyte et al, 2016; 2017), and that cognitive and vasodilatory effects may be most potent under conditions of increased cognitive demand, tasks that analyse or manipulate cognitive load and conflict should be utilised in a flavonoid intervention that measures ERPs. This would allow researchers to explore the relationship between demand, neural activity and cognitive performance simultaneously, which may uncover specific mechanisms by which flavonoids exert cognitive demand effects. In the current experiment, a Go No-Go task and a modified attention network task (MANT) will be used from which cognitive conflict and demand can be analysed.

ERP studies have employed these tasks previously to assess neural activity under conditions of increased cognitive conflict in brain regions associated with attention, EF and inhibition, namely in frontal and fronto-central areas. This has resulted in higher amplitude of certain ERP peaks in frontal and fronto-central regions being associated with increased inhibitory capabilities on trials of high conflict/demand. For example, increases in both frontal N2 and fronto-central P3 amplitudes have been related to inhibition across several adult studies (Jodo and Kayama, 1992; Falkenstein, Hoormann and Hohnsbein, 1999; Kopp, Matler, Goertz and Rist, 1996a). Particularly, in adults, increased N2 amplitude on successfully inhibited no-go trials (in a Go No-Go task) has been related to response activation processes in frontal areas, whereas increased P3 amplitude on successfully inhibited no-go trials has been associated with better response inhibition in fronto-central areas (Karlin, Martz and Mordko, 1969;

Roberts, Rau, Lutzenberger and Birbaumer, 1994; Bruin et al, 2001), even when no motor response was performed (Pfefferbaum, Ford, Weller and Kopell, 1985).

In a study by Jonkman, Lansbergen and Stauder (2003), N2 activation (150-400ms latency) in adults (aged 19-23) was highest at frontal scalp sites after successful inhibition of no-go trials in a Go No-Go task paradigm. They also found that P3 amplitudes (300-500ms latency) peaked in fronto-central regions in response to successfully inhibited no-go trials. Taken together, these results suggest that successful inhibitory function on high conflict trials may be related to higher N2 and P3 activation at frontal and fronto-central sites. These findings further highlight the suitability of using of a Go No-Go task in the current experiment.

N2 activation has also been associated with response inhibition in a modified flanker task, created by Eriksen and Eriksen (1974; Kopp, Rist and Mattler, 1996b; for review see Folstein and Van Petten, 2008); a task from which the MANT derives. The focus of the analysis in ERP studies are the incongruent flanker trials. This is due to these trials eliciting higher cognitive conflict than congruent trials, in which participants have to inhibit irrelevant stimuli, much like on no-go trials in the Go No-Go task. The same ERP approach can therefore be applied to the MANT, where increased N2 and P3 activation in frontal and fronto-central regions on incongruent trials is associated with higher inhibitory capability. Research has been conducted using flanker tasks, and in comparison to congruent trials, incongruent trials have been found to elicit slower reaction times (RTs), higher fronto-central N2 amplitude and a delayed parietal P3 latency (Bartholow et al, 2005; Coles, Gratton, Bashore, Eriksen, & Donchin, 1985; Gehring et al, 1992; Gratton, Coles, Sirevaag, Eriksen, & Donchin, 1988; Heil, Osman, Wiegelmann, Rolke, and Hennighausen, 2000; Kopp, Rist, & Mattler, 1996b). During the flanker task, modulation of the N2 component is thought to be specifically related to regulation of incorrect response preparation (Folstein and Van Petten, 2008), with increased N2 amplitude associated with increased response conflict (Schmitt et al, 2000). This suggests that a higher level of cognitive demand results in higher N2 amplitude. It also implies that participants may be able to better regulate incorrect responses under high demand compared to low demand conditions. Previous research (Whyte et al, 2016; 2017) has found WBB-related benefits on high demand MANT trials. Taken together, these findings may suggest that N2 activation and cognitive performance may both increase on high demand trials under WBB treatment.

Interestingly, task RT has been found to positively correlate with N2 amplitude; higher N2 amplitudes and slower reaction times were observed on incongruent trials by Yeung et al

(2004). Highest N2 amplitudes have been observed in midline frontal brain areas, suggesting this area may mediate response conflict on incongruent trials, much like it does on no-go trials in the Go No-Go task (for review see Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004). These findings support the notion that high conflict incongruent trials place more demand on cognitive systems neurologically, which can also manifest behaviourally. If WBB can increase N2 amplitude on incongruent trials in frontal or fronto-central regions, this might indicate improved inhibitory function. Quicker RTs may also be achievable due to alleviation of oxygen demand in the brain. Indeed, quicker MANT RTs have been observed previously in child WBB studies (Chapter 3; Whyte et al; 2016).

Significant ERP data has been collected in adult populations; however N2 and P3 ERP activity in children, regardless of intervention, remains to be investigated comprehensively. When comparing adult and child performance, Jonkman et al (2003) discovered that children's (aged 9-10) frontal N2 activation was higher in response to no-go trials than go trials, as in adults, suggesting 9 year olds have mature inhibition processes. Vuiller, Bryce, Szucs and Whitebread (2016) also concluded that by 8 years of age, response inhibition is fully developed. The difference in Jonkman et al's (2003) findings was that children lacked the larger fronto-central P3 activity that adults presented after no-go trials, compared to go trials. This implies that children may show some immaturity in this inhibition-related domain. The authors remark that at present, although it is clear both peaks are related to inhibition, it is unclear whether N2 and P3 represent similar or different inhibition processes, warranting further investigation. Any no-go frontal N2 or fronto-central P3 findings in the current study should therefore be interpreted as non-specific inhibition processes.

Increased activation has also been observed following go trials in a child population. Significantly increased parietal P3 activation was observed in children on go trials compared to no-go trials, which was not seen in adults (Jonkman et al, 2003). Increases in P3 amplitude are thought to be related to greater allocation of attentional resources (Polich, 2007). Increased parietal P3 amplitude may therefore be indicative of increased investment in the target selection process, protecting oneself from non-target interference. Higher go trial parietal P3 amplitudes were found to be related to lower go trial sustained attention scores in children, inferring increased parietal P3 amplitude may have increased resource use to continue attending, but at a cost to performance. Vuiller et al (2016) suggested a similar phenomenon, coined the Superordinate Cognitive Control Network (SCCN) hypothesis, where tasks requiring inhibitory control activate a larger network of frontal and parietal

regions to aid performance in a task. Children may therefore have to recruit more cognitive resources to perform well, but still do not perform as well as adults. This would also infer that decreased parietal P3 activity is indicative of a more efficient target detection and attention process (as observed in adults), as frontal areas are able to efficiently inhibit without recruitment from parietal regions. The SCCN hypothesis also complements the CBF hypothesis. If frontal and parietal regions require more oxygen to complete aspects of a task involving sustained attention, (for example on go and congruent trials), cognitive performance may falter. However, as flavonoids increase vasodilation, these brain regions may be replenished with oxygen under WBB treatment, and this may manifest as maintenance or improvement in behavioural measures.

Adult literature reporting EEG brain activity after acute supplementation with flavonoids has tended to focus on EEG spectral activity rather than ERPs specifically. Spectral activity involves frequency analysis of oscillations (brain waves) in EEG signals, and these have been associated with different states of brain functioning in the past, for example sleep and wakefulness. ERPs, on the other hand, measure the direct electrophysiological response to a time-locked stimulus, as described earlier. Scholey et al (2012) recruited healthy adults (mean age 27.74 years, SD 9.28) to consume Teavigo®, a high flavonoid, caffeine-free *Camelia sinensis* extract from green tea (300mg; 94% epigallocatechin gallate (EGCG); 6% vitamin C; unspecified amount of pine cellulose plant fibre) or a placebo pill (containing flour) in a crossover design. Results showed that 3 h following Teavigo® consumption, EEG spectral increases in resting state alpha (α; associated with relaxation and reduction in cognitive processes), beta (β; associated with behavioural arousal and focused attention) and theta (θ; associated with quiet wakefulness, Cantero et al, 2003) were observed in frontal and central brain regions (specifically frontal and medial frontal gyri) of healthy adults. This study therefore shows that flavonoids have the capability to change electrophysiological measures following acute consumption. In particular, the frontal and medial frontal gyri correspond to Brodmann's area 6, an area primarily labelled as the premotor cortex, whereas the mid frontal gyrus (Brodmann's area 10) is the sensory motor cortex. Both areas are predominantly involved in the planning of movements (Stein, 2017), and the authors conclude that activation in these regions following Teavigo® demonstrated the successful integration of visual, auditory and somatic sensory information. Increased change from baseline for self-ratings of calmness and reduced stress using Bond-Lader scales were also reported following Teavigo® compared to placebo, seemingly supporting the interpretation of α and θ increases. The authors suggest that, taken together, those consuming Teavigo® were in a more relaxed and

attentive resting state, highlighting the ability for flavonoids to exert beneficial effects on neurological and psychological measures.

Watson et al (2018) also demonstrated acute EEG spectral effects 45 minutes after consumption of a single blackcurrant juice serving (500mg polyphenols) in healthy adults (mean age 23 years). Participants consumed a blackcurrant and a matched placebo drink, separated by a 1 week washout, and completed a battery of cognitive tasks (CogTrack™: Simple Reaction Time, Digit Vigilance and Choice Reaction Time), mood visual analogue scales (VAS) and underwent EEG recording immediately prior to and 45 minutes after drink consumption. α spectral power was significantly reduced, indicating higher cognitive processing, and slow wave delta (δ) and θ spectral powers were increased, highlighting anxiolytic effects. Greater alertness and lower fatigue were also implied by increased β and reduced α power. Cognitive performance was expected to improve following ingestion of the blackcurrant drink based on the authors' previous work that showed attenuation of decline on an RVIP task, and quickening of digit vigilance RTs 1 h after blackcurrant extract and blackcurrant juice, respectively (Watson et al, 2015). Interestingly, cognitive performance did not improve in the EEG study; responses were actually found to slow for blackcurrant-treated participants on the choice reaction time task compared to placebo. This is unusual based on the wide array of flavonoid literature that has shown acute cognitive effects (see Chapter 1 for review). The authors interpreted this as an absence of cognitive findings, suggesting the intervention period (45 mins) or small sample size may have affected results. Indeed, only 9 participants were tested indicating the study may have lacked power to detect cognitive effects. No effects on mood were observed in this study, however the VASs used only measured how 'mentally' and 'physically fatigued' participants felt. It is questionable whether such assessments are indicative of general mood, and future studies should aim to include a comprehensive range of mood items to assess different mood states reliably.

As yet, no studies have looked at the real-time changes in electrical activity of adult or children's brains using ERPs after acute supplementation with flavonoids. The aim of the current study is to investigate mood, cognition and ERP changes after acute blueberry flavonoid intervention in healthy children aged 7-10. A placebo-controlled, randomised, double-blind, crossover study was conducted where children received an acute dose of WBB on two occasions, separated by a one week washout. Based on the research previously discussed, ERP measurement in a Go No-Go task will focus on go and no-go trial activation at N2 and P3 frontal, fronto-central regions, and P3 parietal brain regions. For the modified

attention network task (MANT), N2 frontal and fronto-central regions, and P3 fronto-central and parietal regions, on correct congruent and incongruent trials will be the focus.

On the basis of previous studies (Jonkman et al, 2003; Jodo and Kayama, 1992; Falkenstein, et al, 1999; Kopp et al, 1996a; 1996b; Karlin et al, 1969; Roberts et al, 1994; Bruin et al, 2001; Pfefferbaum et al, 1985), it is hypothesised that children will show higher N2 amplitude in frontal regions, and higher P3 fronto-central amplitude when treated with WBB (compared to placebo), after successful inhibition of no-go trials on the Go No-Go task and incongruent flanker trials on the MANT. It is also expected from Falkenstein et al's (1999) findings that this higher amplitude in N2 may correlate with improvements or maintenance of task performance under both treatments, but to a higher degree for those consuming WBB. However, it is not known whether increased fronto-central P3 amplitude will be observed in the current sample due to absence of this in a previous child cohort (Jonkman et al, 2003). A fronto-central P3 hypothesis will therefore be exploratory, and results will provide a clearer account of P3 amplitude in this region after successful inhibition of no-go trials. Decreased activation in P3 parietal areas is also expected after go trials for WBB-treated children, indicating less recruitment from parietal regions and more effective frontal function. Decreased activation is hypothesised to correlate with improved accuracy on go trials for this group.

From previous research (Watson et al, 2018), it is unclear whether faster or slower reaction times are indicative of better task performance. Previous research from Duncan-Johnson (1981) and Verleger (1997) suggests a shorter P3 latency is associated with faster processing speed. Using this information, and based on results from Chapter 3, we hypothesise that decreased parietal P3 amplitude on go trials (on the Go No-Go task), and congruent trials (on the MANT) will correlate with faster and more accurate respective trial performance for those under WBB treatment compared to placebo treatment. For no-go and incongruent trials, higher accuracy rates may be observed alongside higher N2 amplitude and faster RTs (on the MANT) for participants under WBB. For the MANT used in the current study, load and target time have also been employed alongside congruency as manipulations of task difficulty. Significant effects of congruency, load and target time on behavioural accuracy and RT performance will inform the focus of ERP analyses. For example, if congruency is found to be a significant predictor of accuracy or RT performance, analyses of congruent and incongruent trials will be performed in ERP analysis in relation to WBB and placebo

treatments. Thus, analysis will be conducted on trials where successful manipulation of task difficulty (response conflict) has been achieved in the sample tested.

In summary, hypotheses for the Go No-Go task and MANT task are:

- Participants will show higher N2 activation in frontal regions, and higher P3 fronto-central activation (as indicated by greater amplitude of response) when treated with WBB (compared to placebo), after successful inhibition of no-go/incongruent trials
- Higher activation in N2 on no-go/incongruent trials will correlate to improvements or maintenance of task performance for those consuming WBB
- WBB-treated participants will show decreased activation in P3 parietal areas on go/congruent trials
- Decreased P3 parietal activity will correlate with improved accuracy or faster RT on go/congruent trials for WBB participants

4.2 Methods

4.2.1. Participants

Sample size was determined *a priori* using a repeated measures, within factors analysis using G Power 3.1. An effect size (F) of 0.22 was used, based on previous behavioural findings (Whyte and Williams, 2015; Whyte et al 2016; 2017), and a desired power of 0.9. This gave a required sample size of 58 (N=27 in each condition) ($F(1,56)=4.01$). This sample size was deemed unfeasible due to time and resource restraints of the current experiment. A total of 14 healthy participants (4 males: 10 females) aged 7-10yrs (M=8.56, SD=0.97) were recruited from two primary schools in Reading. This was in the same range as the EEG flavonoid intervention study performed previously (9 participants overall; Watson et al, 2018). However, as a result, power for behavioural results was significantly reduced in the current sample. A post-hoc power analysis revealed that 14 participants resulted in a power of 0.33 at an effect size (F) of 0.22 ($F(1,12)=4.75$).

All children spoke English as a first language, had not been diagnosed with ADHD, dyslexia or any other developmental or psychological disorder. No fruit or fruit juice intolerances were present. One participant had an intolerance to cow's milk protein, however this did not affect the intervention treatment.

4.2.2. Treatments

An acute, double-blind, randomised, placebo-controlled, crossover design was employed in the current study. Participants consumed each treatment drink across the course of the intervention at two independent visits, separated by a one week washout period. The order of treatment drink for each participant was randomised by a confederate and kept confidential. Neither participants, parents nor the experimenters were aware of the drink the participant was consuming at each visit. Drinks were also presented in an opaque flask with a black straw to aid blinding procedures.

Due to the supply of a new batch of freeze-dried WBB powder that had an increased amount of anthocyanins per gram compared to the previous batch (see Chapter 2), weight of powder used was adjusted to match the old and new batches on anthocyanin content. Therefore, 13.3g freeze-dried WBB powder (equivalent to 240g fresh wild blueberries) was used which contained 766mg total polyphenols, of which 253mg were anthocyanins (equivalent to that used in Chapter 3). A sugar-matched placebo drink was matched on the amount of fructose (4.79g), glucose (4.58g) and vitamin C (45mg) present in the new batch of WBB treatment. As in the previous experiment (Chapter 3), 170ml of cold tap water and 30ml of Rock's orange squash were added to both treatments to aid palatability. Treatment drinks were prepared by a confederate in the PCLS nutrition lab and were immediately driven to participants either at their school or home by the experimenter or a confederate, 1.5 h before their scheduled test session at the University. This was to allow a 30 minute window at the start of the test session to fit the EEG cap before testing commenced at the 2 h post-drink time point. Participants were asked to consume the drink within 5 minutes, with which all complied.

4.2.3. Cognitive tasks

1. *Go No-Go task* - This task was used to examine response inhibition and was adapted from a task designed by Winter and Sheridan (2014). The concept of the task was to engage participants in a state of sustained attention through repetitive key pressing for certain stimuli (go trials), and initiate inhibition (no key pressing) for particular stimuli (no-go trials). The nature of this task allows for ERP and cognitive analyses to be undertaken on separate go and no-go trials which can be related to sustained attention and inhibitory abilities, respectively.

The task included a main block which comprised of 100 trials containing randomised presentations of 8 cartoon animal stimuli, where 6 animals (cow, goat, horse, pig, rooster and sheep) were regarded as go stimuli and 2 animals (monkey and giraffe) were regarded as no-go stimuli (further details later). Participants were told that they were to press the 'space' button on the computer keyboard to 'catch' the 6 farmyard animal stimuli that were presented on-screen. This engaged participants in a state of sustained attention. Participants were also instructed not to do anything in order to 'not catch' the 2 non-farmyard animals – monkey and giraffe. The main block was preceded by a 'priming' block where all 8 stimuli (cow, goat, horse, pig, rooster, sheep, monkey and giraffe) were regarded as go stimuli. In this block, participants were told to press the 'space' button for every animal they saw in order to 'round it up'. The priming block was included to increase motor prepotency to animals that were regarded as no-go stimuli in the main block (discussed in detail later).

Priming block

Upon loading the task, participants first underwent a priming block where farmyard and non-farmyard animal stimuli (monkey, giraffe, cow, goat, horse, pig, rooster and sheep) were presented one at a time for 600ms in the centre of the computer screen, separated by an inter-stimulus interval of 1150ms. Participants were instructed to press the 'space' key to 'catch' every animal after each one appeared on screen, regardless of farm or non-farm status. If animals were caught within the 600ms response window, participants were shown an image of a cowgirl holding a lasso to indicate the animal had been caught (lasting 500ms). Stimuli presentation order was randomised and each animal was presented to participants 5 times, except for the monkey and the giraffe. Giraffe and monkey stimuli were each displayed 30 times to increase motor prepotency by repeated, speeded go responses to these targets. These targets (monkey and giraffe) required no response (no-go targets) in the main block. According to Winter and Sheridan (2014), no-go trial accuracy should be aided by manipulations that increase no-go stimulus salience, indicating that highlighting no-go trials as important (by increasing trial number) during the go only priming block may allow for better context monitoring and cognitive performance on no-go trials in the main Go No-Go block.

Additionally, on the priming block, participants were told to press the 'space' key especially fast for trials displaying the monkey to win a reward, in order to sensitise them further

towards the monkey stimulus, which required no response (no-go target) in the main Go No-Go block. Upon a successful monkey 'catch' participants were again shown the cowgirl lasso screen as with other successful animal catches (500ms), plus a reward screen of cartoon coins (500ms), followed by an inter-stimulus interval of 150ms.The priming of participants using a reward has previously been found to increase vigilance towards and motivation to respond to the target (Winter and Sheridan, 2014). The inclusion of a reward screen following no-go target stimuli in the priming block would therefore increase salience towards the monkey further and hypothetically improve performance on these trials in the main Go No-Go block. Although priming of the monkey stimulus included a reward for correct trials, and priming of the giraffe stimulus did not include a reward, both trials were matched in the priming block for motor prepotency (each was displayed 30 times). For the purpose of this study, trials that included the monkey and giraffe were grouped and labelled as no-go trials to increase no-go trial N. Manipulation of reward was not considered.

Main block

The main block consisted of 80 trials of go stimuli (14 cow, 12 goat, 12 horse, 14 pig, 14 rooster and 14 sheep) and 20 trials of no-go stimuli (10 monkey and 10 giraffe), presented in a randomised order. This proportion of go to no-go trials was consistent with the Go No-Go task Whyte et al (2016) used in their acute child blueberry intervention and in Winter and Sheridan's (2014) child sample (mean age 8.2, SD 1.97). Participants were instructed to press 'space' to catch each animal that appeared on screen, except for the giraffe and monkey. When the giraffe and monkey appeared, participants were instructed not to press anything. Go and no-go stimuli were presented on screen for 600ms. For go trials, if participants correctly pressed 'space', the cowgirl lasso screen was displayed, lasting 500ms, to show they had caught the animal. This was then followed by an inter-stimulus interval screen displaying a fixation cross for 1150ms, before the next trial. If participants did not press 'space' on go trials, the inter-stimulus interval was shown immediately after without the cowgirl lasso screen (1150ms) to show that the animal had not been caught. For no-go trials, feedback was not provided; correctly and incorrectly inhibited trials were both followed by the inter-stimulus interval (1150ms). The main block was split into two sub-blocks to give participants a small reprieve in between. This reprieve lasted <30 seconds, where participants remained seated and looked at the screen, before the second half of the main block began.

Go trial accuracy (proportion of trials correct, 0-1), go trial reaction time (RT; ms; for correct trials only) and no-go false alarms (proportion of trials incorrect, 0-1) were used as DVs for cognitive performance, in accordance with previous research (Whyte and Williams, 2015; Whyte et al, 2016). A d-prime measure was also calculated by subtracting the false alarm rate z-score from the hit rate z-score for each child (as specified in Stanislaw & Tordorov, 1999). A speed-accuracy trade-off was calculated by converting the false alarm rate and RTs into z-scores (SPSS) and dividing the resulting RT z-scores by the false alarm z-scores (Whyte et al, 2016). Go trial accuracy and no-go trial accuracy were used as DVs in ERP analysis based on prior research (Falkenstein, 1999; Bokura et al, 2001; Jonkman et al, 2003; Ciesielski, Harris and Cofer, 2004; Gajewski and Falkenstein, 2012) and will be detailed later.

2. *Modified Attention Network Task 2 (MANT2)* - This task was a further modified version of the MANT described in Chapter 3. The task was employed to measure vigilance, selective attention and attention under conditions of conflict. Participants completed three blocks of the MANT each consisting of 80 trials. The original MANT (Chapter 3; Whyte et al, 2017) included four blocks of 80 trials; two blocks were presented in silence and two with playground noise. However, in the MANT2, these two noise blocks were removed due to their potential to cause signal interference in ERP measurements. To ensure the task contained enough trials to be sensitive to changes in performance, an additional target time manipulation was included to increase the overall number of trials. Three blocks of 80 trials were therefore presented to participants, consisting of one 120ms target block, one 230ms target block and one 500ms target block to assess participants' behavioural and ERP response to trials of varying visual speed. Specifically, 230ms trials were included to see if an intermediate presentation speed would elicit similar between-treatment differences in performance as those seen on 120ms trials in Chapter 3. This might help to decipher an optimum speed of stimuli presentation where WBB may aid performance. All other elements of the MANT2 (arrow stimuli, congruency, load and cue manipulations) were identical to the MANT as described in Chapter 3. Participants were instructed to press the left or right arrow key on the keyboard according to the direction of the target stimulus arrow for each trial.

A practice block of 35 trials preceded the main block, where target duration decreased from 1000ms to 120ms over the last 16 trials to allow familiarisation with the speed of response required. Participants were required to score 50 or above on this practice block. If this score was not achieved within the first practice block, the practice block was repeated until it was.

The outcome measures for the MANT2 were accuracy (proportion of correct hits, 0-1) and reaction time (RT) for correct hits (speed of response to target; RTs <200ms were removed).

3. *Positive and Negative Affect Scale for Children (PANAS-C)* - The PANAS-C used in the current study was identical to the one used in the previous study (for full details see Chapter 3), and was used to measure current mood. Ratings of positive and negative items were summed to calculate an overall positive affect (PA) and overall negative affect (NA) score which were used as mood DVs for cognitive analysis only. The PANAS-C was not administered during ERP recording. This was due to the measure being administered on paper, meaning ERPs could not be time-locked to participant responses.

4.2.4. Procedure

All sessions took place in the School of Psychology and Clinical Language Sciences (PCLS) at the University of Reading after school hours. Participants were instructed to follow a low-flavonoid diet 24 h before test sessions 1 and 2 to prevent confounding effects of any high-flavonoid foods eaten in the period prior to testing. Parents were given a low-flavonoid diet sheet and an example of a low-flavonoid diet (Appendix B) which they were to implement for their children. If children usually consumed a canteen lunch, parents were asked to pack a low-flavonoid lunch for their child on testing day. Parents and participants were verbally asked at the beginning of each test session whether participants had followed the 24 h dietary restrictions, to which all complied.

Screening session: A screening session took place 6-7 days prior to the first test day at which participants completed demographic measures. First, the participant's head was measured to ensure a suitably sized net was available. Participants with a head measurement above 54.5cm were unable to take part due to the nets available having a maximum diameter of 54 (±0.5)cm. For these children, participation in the trial ceased. For those with a head measurement of 54.5cm and below, participation continued. A modified Continuous Performance Task was used to measure sustained attention and inhibition abilities. Participants also completed three sub-scales of the British Ability Scales 3 (BAS 3; pattern construction, matrices and verbal similarities; Elliott, Murray and Pearson, 1979; Elliott, 1997) to assess general ability (see Chapter 2 for full description). A computerised version of the children's Raven's Coloured Progressive Matrices (RCPM; Raven, 2000) was also administered at this session as an additional measure of fluid intelligence. This measure was included to compare RCPM and

BAS 3 scores, to see if RCPM scores could be used as a proxy for general ability when under time constraints in future studies. These measures of general ability were administered to ensure all participants were of healthy cognitive functioning for their age. Demographic data were not used in analyses and can be seen in Table 4.1.

Participants practiced the main test battery (Go No-Go, MANT2 and PANAS-C, see section 4.2.3). For the MANT2 practice, participants had to score 50 or above to be included. Any child who performed under this score was asked to complete the MANT2 practice block again until they reached this threshold. At the end of the screening, participants were given a blinded sample of both WBB and placebo treatments and were asked to rate how much they liked each drink on a 10-point Likert scale. This was to determine whether one treatment drink was more enjoyable than the other. Participants and parents were then reminded to comply with the 24 h low-flavonoid diet before the next two test sessions. The remaining two test sessions were also arranged at screening, as well as the location of the child on both of these days, to determine where the treatment drink was to be dropped (either at school or home). Parents were telephoned 2 days before each test session to remind them about the 24 h dietary restrictions and to confirm the child's location on the day of testing.

Test session 1: Testing was performed in the child EEG lab in PCLS due to the use of the EGI equipment situated there. The EEG lab is soundproofed to reduce environmental noise interfering with EEG signals. Test sessions during school hours were avoided to minimise disruption to the child's education and to working parents. Participants therefore only attended test sessions after school hours. It was decided that a baseline session at approximately 3.30pm and a subsequent test session at 6.30pm would prove to be highly demanding on participants and their parents, especially as setup and fitting of the EEG cap would take up to 30 minutes and children would have to restrain from food across their usual dinner time. Thus, children in the current study did not take part in a baseline session. A crossover design was employed where children took part in both treatment conditions (placebo and WBB) one week apart in order to act as their own control. Children were not required to fast prior to testing; parents were asked to make sure that their child consumed a low-flavonoid diet for 24 h before the test session, which included breakfast, lunch and snacks on the main test day.

The experimenter (or a confederate) dropped a pre-prepared treatment drink at the participant's home or school 1.5 h before their scheduled test session at PCLS on both main test days. The experimenter remained with the participant whilst they finished the contents of their drink within a 5 minute window. Following consumption, the experimenter left the child's home or school, and reminded children not to exercise or consume any food or drink except water until after their test session at the University.

Participants arrived at the PCLS department 1.5 h after consumption of their treatment drink and the procedure of the session was fully explained to them. Firstly, participants completed the PANAS-C where they were asked to rate how they were feeling on each item 'right now'. The participant was then seated in front of the monitor in the EEG lab and was fitted with a Geodesic Sensor Net (GSN; Electrical Geodesic, Inc.) consisting of a 128-sensor array. For children, the GSN is easy to apply, comfortable, and relatively non-invasive. The GSN is comprised of Ag/AgCl sensors in an elastic-webbed geodesic tension structure. The participant's head was measured to confirm the size of the net to be used, and the experimenter marked the scalp at the vertex for proper placement of the net. The position of the vertex mark was calculated by measuring from the nasion to the inion, and from each pre-auricular point on the side of the head, using the anterior indentations of each ear as a guide, and halving measurements to mark where they crossed at the vertex. Prior to fitting, the GSN was soaked in a potassium chloride and warm water solution for 15 minutes to aid malleability and electro-conductivity. After lifting the net out of the solution, it was gently shaken to remove any excess solution so that it did not drip into the participant's eyes. A towel was placed round the participant's shoulders to avoid them getting wet. The net was then carefully stretched over the participant's head, matching the vertex electrode to the vertex mark, and the ear electrodes with the pre-auricular points. The chin strap and side tension straps were tightened to a comfortable position and all sensors were checked to ensure that they were perpendicular to and in contact with the scalp. Application of the net required around 5-10 minutes and was well tolerated. Fitting of the cap always included at least 2 experimenters to ensure the best fit was achieved.

After the cap was in place, it was plugged into the EGI connector bar to assess impedances. Impedances ≤50kOhm were desirable and appeared green in Netstation. Any sensors that read ≥100kOhm appeared as red in Netstation and sensors that read between 50>100kOhm appeared as yellow. Red and yellow sensors indicated that adjustments were needed before

continuing. Sensors were adjusted by moving any obstructing hair and gently massaging the electrode onto the scalp. If sensors continued to show high impedances, a small amount of electrolyte solution was applied to the sensor's sponge with a pipette, until impedances were within desired limits (≤50 kOhm). Checking of impedances took approximately 15-20 minutes. Whilst this was being done, participants sat without entertainment and were encouraged to 'help' the experimenters by telling them if they saw any red or yellow circles appear on the Netstation computer screen.

The cognitive test battery containing a selection of previously validated and ERP-compatible tasks (see section 4.2.3) was administered to participants. Participants always completed the MANT2 first, followed by the Go No-Go task. A camera, which was time locked to the data being acquired, recorded the participant during presentation of the tasks. The experimenter viewed the participant through this camera from behind a room divider, and manually added markers into the EEG waveform where body movement occurred. The total time watching the screen and recording data did not exceed 40 minutes. On completion of the cognitive test battery, the EEG cap was gently removed from the participant's head, taking approximately 30 seconds.

Participants and their parents were asked if they had any questions and were instructed to return to the department 1 week later as previously planned. Parents were again asked to give their child a low-flavonoid diet for 24 h before the next test session and were contacted 2 days before to remind them of these restrictions.

Test session 2: The same procedure as the previous test session was undertaken, with the experimenter dropping the treatment drink at participants' homes or schools 1.5 h before the test session at the University. Participants consumed the treatment drink that they had not consumed at their first session. At the end of the second test visit, participants and parents were verbally debriefed on the aims of the experiment, and were also given a written de-brief sheet to take home. They were remunerated £30 for their participation.

4.2.5. Statistical analysis

4.2.5.1. Cognitive data analysis

Data was analysed using SPSS (Version 22.0).

Differences between treatments in general ability and attention were assessed by visit using independent samples t tests. The overall BAS 3 t score and RCPM data were z-scored and compared using a paired sample t-test. Taste rating scales comparing ratings of placebo and WBB drinks were also analysed using a paired sample t test. A correlation was performed on overall MANT2 accuracy and overall MANT2 RT with placebo and WBB data to determine whether there was a speed-accuracy trade-off in the current sample. All other data was analysed using linear mixed models (LMMs) with an unstructured covariance matrix. Separate LMMs were performed for each dependent variable (DV), and post-hoc pairwise comparisons were performed on significant or trending interactions, which included Bonferroni corrections.

Drink (placebo, WBB) was included as a Fixed Factor in all LMMs to compare the effects of treatment. For the MANT2, Congruency (congruent, incongruent), Load (high load, medium load) and Target Time (120ms, 230ms, 500ms) were also included as Fixed Factors in the model to assess changes after manipulation of cognitive load.

Following results of these LMMs, Visit (1, 2) was then also included as a repeated Fixed Factor for all tasks to determine any order effects of drinking a treatment first or second. This was based on previous research that has shown that flavonoids may be of most benefit during the initial stage of encoding (Whyte et al, 2015; Kean et al, 2015). As this experiment utilised a crossover design, it was considered that the order of WBB treatment (at initial encoding and 1 week later), may have an effect on cognitive performance. Fixed Factor interactions were also included in the model. For the Go No-Go task, Drink x Visit was included; for the MANT2, Drink was coded in an interaction with each cognitive load variable, as well as with Visit and each cognitive load variable. Participant was input as a random factor in all models to account for individual variation across visits.

MANT2 cognitive analyses were performed before ERP analyses to aid which trial manipulation was to be the focus. As congruency was a significant predictor for accuracy and RT measures, ERPs were segmented into congruent and incongruent trials.

4.2.5.2. ERP data analysis

ERP data was collected using Netstation v4 at a sampling rate of 250Hz. Impedances ≤ 50 kOhm were preferred, however where this was not possible (15% of cases), impedances ≤ 100

kOhm were accepted in the interest of time. Research has stipulated that it is most important that impedances are homogenous across the scalp so that EEG signals are calculated accurately when subtracting from the reference electrode (Ferree, Luu, Russell and Tucker, 2001), so this was the main aim during impedance checking.

Two participant data files (both placebo at visit 2) were not processed for the Go No-Go task. One participant withdrew from the task leaving the session incomplete, and one participant's file contained an error which could not be repaired. Twenty six data files (14 WBB, 12 placebo) were therefore processed for the Go No-Go task. For the MANT2, 5 participant files (1 WBB, 4 placebo) were not processed due to an error regarding markers during recording of data. Twenty three data files (13 WBB, 10 placebo) were therefore carried forward for processing for this task.

Firstly, frame by frame analyses of the video made during stimulus presentation was carried out to identify trials that were unusable due to movement artefacts or eye movements. No trials were removed on this basis. EEG data was then exported to edf format and imported into Brain Vision Analyzer (BVA) to pre-process. Data was re-referenced offline to an average reference through a 70 Hz low band and 0.3 Hz high band filter. Independent components analysis (ICA) was conducted on each participant's data. ICA was deemed the most appropriate analysis to run due to its usefulness as a tool for isolating artefacts and cortical processes from EEG data (for review see Onton, Westerfield, Townsend and Makeig, 2006; Delorme, Sejnowski and Makeig, 2007). During ICA, background interference ('noise') is subtracted from other leads of interest, leaving behind an ERP which is related to the stimulus event. This means that it is possible to look at the electrical potential of certain brain regions of interest in real-time, whilst a participant is completing a task, after being supplemented with an intervention drink.

Following ICA, raw waveforms were then visually assessed for bad channels and artefacts. Individual bad channels were evident across 57.67% of the dataset for the Go No-Go task. The number of bad channels ranged from 1-8 (out of 129) within the individual participant sessions that exhibited bad channels (M=3.8, SD= 2.07). A very small proportion of channels (1.64%) were therefore removed from the overall dataset. Across participants, an average of 65.88 (SD=11.03) go trials (range, 39-79), and 12.85 (SD=3.86) no-go trials (range, 4-19) were included out of the 80 and 20 presented to participants, respectively. Trials were removed due to bad segments which displayed artefacts such as blinking or eye, head, mouth or body movements that were not detectable from video analyses. For MANT2 waveforms,

bad channels were evident across 39.13% of the dataset. Within these participants, the number of bad channels per participant session ranged from 2-6 (M=4.56, SD=1.26); 1.38% of channels were removed from the overall dataset. A mean of 83.83 (SD=11.06) congruent trials (range, 58-96) and 72.91 (SD=22.55) incongruent trials (range, 24-95) were included.

Data was then exported to vhdr format and imported into the MATLAB plug-in, eeglab, as individual trials. The clean data was sectioned into epochs comprised of 200ms pre and 500ms post-stimulus. Windows for analyses were determined based on previous research employing Go No-Go and flanker cognitive tasks (all literature included in 4.1), and from visual inspection of averaged waveforms. Specifically, a mid-latency window of 172-372 ms was chosen around the negative deflection (N2) generally associated with attention and inhibition. Although previous research has shown that N2 may peak later in children (300-400 ms) compared to adults (200-300 ms; Brydges, Anderson, Reid and Fox, 2013), inspection of the current dataset determined N2 windows to span across these ranges, resulting in a chosen window of approximately 200-400ms (see Figure 4.2). This is also consistent with the N2 ERP window observed in children (mean age 9.7 years) in Drollette et al's (2014) exercise intervention study. Non-rounded figures for this window were chosen due to the absence of specific data points at 170 and 370 ms. A long-latency window was chosen at 300-500 ms around the later positive deflection (P3), also generally associated with inhibition. All data were baseline corrected by a baseline of -200 to 0 ms relative to stimulus presentation for both tasks.

Although ERP has good temporal resolution, it is not possible to assess individual trials. The segments of each task condition within each participant's sessions were therefore averaged together. For the Go No-Go task, two conditions were computed: (1) Go correct; (2) No-Go correct. This was so that the ERPs after participants had correctly sustained attention (pressed for Go trials) and inhibited (not pressed for No-Go trials) could be compared across Drink, Visit and Site variables. As the primary focus in this study was inhibitory control, only correct trials were analysed. Go incorrect and No-Go incorrect ERPs were not included in analyses due to cognitive processing on these trials representing different facets of attention and inhibition. Investigating incorrect trials would have focused on the processes involved following inattention (losing focus on continually pressing for Go trials) and unsuccessful inhibition (pressing for No-Go trials) rather than successful sustained attention and inhibition processing capabilities. This approach is consistent with the majority of previous research employing Go No-Go tasks where the focus is on accuracy ERPs rather than inaccuracy ERPs

to assess successful control in attentional and inhibitory domains. However, in the future it would be informative to focus on ERP activity following an incorrect response to stimuli, providing tasks are complex enough to produce a large number of incorrect trials to make reliable comparisons.

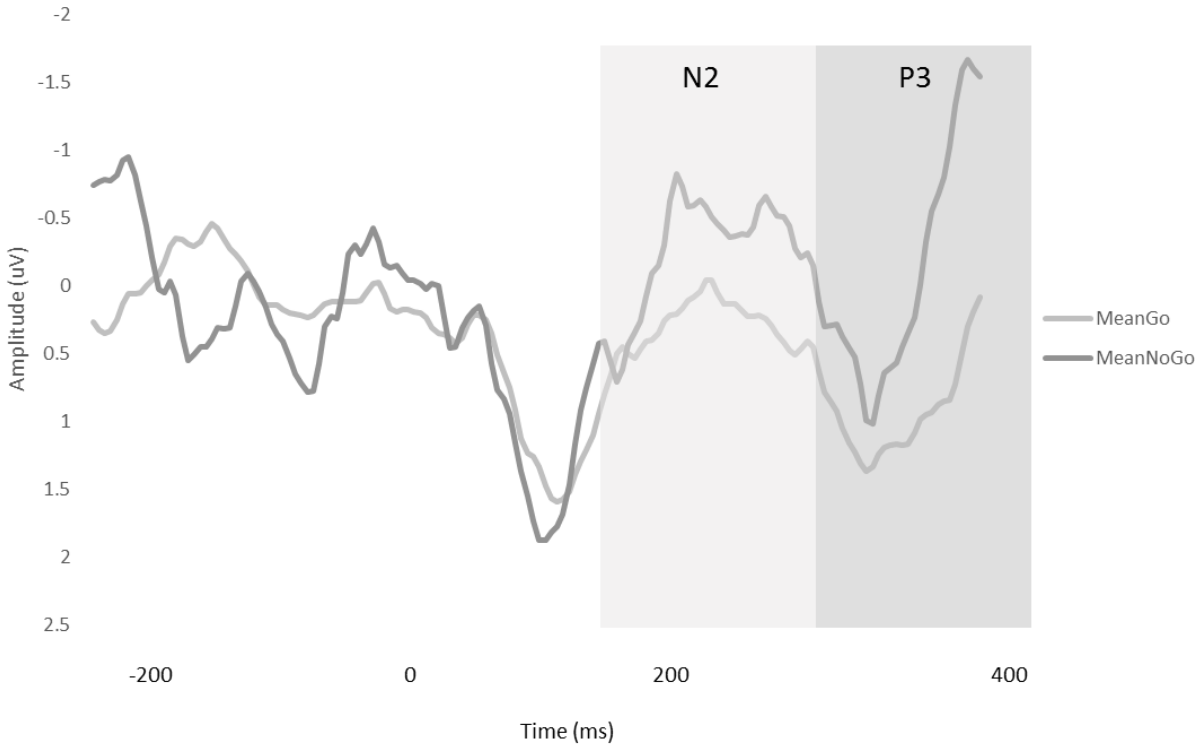


Figure 4.2. ERP grand average trace for Go and No-Go trials on the Go No-Go task. Negativity (-) is plotted upwards. Chosen N2 and P3 peak windows are highlighted respectively.

For the MANT2, two conditions were also computed: (1) Congruent correct; (2) Incongruent correct. Similarly, these two conditions represent correct responses to low and high conflict and were compared across Drink, Visit and Site conditions. The resulting waveforms included information about latency to response and peak amplitude for each participant by treatment drink, task condition and visit for each electrode.

Peak amplitude and latency to peak DVs were calculated from data within N2 and P3 windows by selecting the electrodes of interest relative to brain region locations. The main focus of N2 analyses was on frontal (Fz; electrode 11) fronto-central (FCz; electrode 6) and central (Cz; mean of electrodes 7, 31, 55, 80, 106) components (Vuiller et al, 2016). Differences between P3 Fz (electrode 11), FCz (electrode 6), Cz (mean of electrodes 7, 31,

55, 80, 106) and parietal (Pz; electrode 62) waveforms, established from previous research (Jonkman et al, 2003) as indicative of cognitive control and inhibition, were also explored.

For N2 analysis of the Go No-Go task, data was entered into separate unstructured LMMs for each DV (peak amplitude and latency to peak) with Drink (placebo, WBB), Trial Condition (Go trial correct, No-Go trial correct) and Site (Fz, FCz, Cz) as fixed factors, and Visit (1,2) as a repeated factor. For P3 analysis, the same structure was used except for the inclusion of one extra site (Fz, FCz, Cz, Pz). Participant was also included as a Random factor in all models to accommodate individual variation between participants at each visit.

As previous research has found differences between trial conditions at particular sites, LMM analyses which revealed Drink x Trial x Visit interactions were further split by Site, when Site was a significant predictor. This was done by running Drink x Trial Condition x Visit LMM analyses within each Site. This was necessary to do due to *a priori* predictions involving ERP activity at the first or second visit, under placebo or WBB intervention, during particular trials of a task, at specific regional sites in the brain. Without information from all 4 variables, results would be inconclusive.

For MANT2 analyses, separate unstructured LMMs were also performed on ERP DVs. For N2 analyses, Drink (placebo, WBB), Trial Condition (Congruent correct, Incongruent correct) and site (Fz, FCz, Cz) were used as fixed factors. Again, for P3 analyses an identical structure was employed with the addition of a parietal site (Fz, FCz, Cz, Pz). Participant was included as a random factor and Visit (1,2) as a repeated factor in both LMMs to model the within-subject nature of the study design. Again, in analyses where Drink x Trial x Visit interactions were present alongside Site as a significant predictor, further LMM analyses on each Site ensued.

The most important distinction in the current ERP analyses, across Trial, Visit, Drink and Site variables within amplitude and latency to peak analyses, is the difference in amplitude (N2 or P3) across Trial conditions (i.e. go vs no-go and congruent vs incongruent) and between Drinks (i.e. placebo vs WBB). These will therefore be the focal comparisons of interest in post-hoc analyses. Latency to peak has been included in the analysis due to the exploratory nature of this initial ERP flavonoid trial. Results of the current experiment may allow for more robust hypotheses regarding latency to peak to emerge for future studies.

4.3. Results

4.3.1. Cognitive data

Demographic data is shown in Table 4.1.

	N=14	
	Mean	**SD**
Drink ratio (Placebo: WBB)	14:14	--
Visit 1 (Placebo: WBB)	5:9	--
Visit 2 (Placebo: WBB)	9:5	--
Age	8.56	1.01
Gender (M:F)	4:10	--
CPT		
Omissions (%)	8.80	6.24
Commissions (%)	45.64	19.12
RCPM	28.62	5.33
BAS 3		
Overall t score	50.31	5.54
Matrices t score	47.23	8.28
ADHD IV rating scale (%iles)		
Hyperactivity-Impulsivity	49.67	30.78
Inattention	45.33	33.04
Total	47.58	30.68
Taste rating scale (0-10)		
Placebo	8.92	1.80
WBB	4.23	3.06
Habitual fruit & veg intake at screening (portions per day)	2.67	1.43

Table 4.1. Table of demographic data for all participants

All fourteen participants were included in demographic, PANAS-C and MANT2 analyses. Habitual fruit and vegetable intake was low in the current sample compared to previous cohorts (M=4.9, SD=2.32, Whyte et al, 2016; M=4.67, SD=1.61, Whyte et al, 2017). There were no significant differences between drinks at visit 1 ($t(11)=-0.25$, $p=0.81$) or visit 2 ($t(11)=0.25$, $p=0.81$) for BAS 3 scores. Similarly, no significant differences were evident in

Raven's scores (visit 1, t(11)=-0.53, p=0.61; visit 2, t(11)=0.53, p=0.61), CPT omissions (visit 1, t(11)=-1.19; visit 2, t(11)=1.19) or CPT commissions (visit 1, t(11)=-0.14, p=0.89; visit 2, t(11)=0.14, p=0.89).

Raw data for mood and cognitive DVs can be seen in Table 4.2.

Dependent variables	**Placebo (n=14)**		**WBB (n=14)**	
	Visit 1	**Visit 2**	**Visit 1**	**Visit 2**
PA	58.00 (17.48)	41.33 (14.65)	51.89 (13.93)	50.80 (15.83)
NA	18.20 (2.86)	17.00 (1.87)	17.44 (3.24)	17.60 (2.07)
Go No-Go	**(n=13)**	**(n=14)**		
Go trial accuracy (proportion correct 0-1)	0.83 (0.14)	0.85 (0.14)	0.82 (0.14)	0.81 (0.19)
Go trial RT (ms)	421.35 (50.06)	404.93 (22.98)	413.98 (36.00)	409.01 (45.43)
No Go false alarms (proportion correct 0-1)	0.28 (0.24)	0.36 (0.18)	0.36 (0.21)	0.38 (0.22)
D-prime	0.32 (1.41)	0.09 (1.70)	-0.11 (1.36)	-0.27 (1.04)
Trade-off	-0.60 (1.28)	0.67 (1.56)	0.59 (5.06)	-0.96 (1.05)
MANT	**(n=14)**	**(n=14)**		
Accuracy (proportion correct 0-1)	0.77 (0.28)	0.92 (0.13)	0.89 (0.15)	0.85 (0.20)
Reaction Time (ms)	661.28 (141.37)	585.67 (113.73)	594.18 (109.37)	674.41 (133.82)

Table 4.2. Raw means (SD) for mood and cognitive dependent variables under placebo and WBB treatments at each Visit.

One participant (placebo) did not complete the Go No-Go task during their second visit since they showed signs of distress and asked to withdraw. CPT and RCPM scores were similar to those observed in Chapter 3, suggesting participants had comparable attentional and non-verbal abilities to the previous healthy sample in Experiment 1. A paired samples t test revealed that there were no significant differences between z-transformed BAS 3 t scores and RCPM scores (t(11)=0.22, p=0.83), suggesting that these two measures accurately demonstrated general ability to the same degree. A paired sample correlation performed within this t test confirmed measures were related, showing a significant correlation between BAS 3 t and RCPM scores (r=0.64, p=0.026).

Participants rated the placebo drink as significantly higher in likeability compared to the WBB drink on the taste rating scale (t(12)=6.78, p<0.01).

4.3.1.1 MANT2 data

There was no significant speed-accuracy trade-off in MANT performance when participants consumed placebo (r=0.18, p=0.55) or WBB (r=0.31, p=0.28) treatments.

Accuracy (proportion of trials correct; 0-1)

Initially, LMM analysis including Drink as a fixed factor only, revealed Congruency as a significant predictor; participants were more accurate on congruent trials (M=0.93, SE=0.02) compared to incongruent trials (M=0.82, SE=0.02; F(1,168)=17.19, p<0.01). This was the only significant effect observed, contrasting to prior research where Congruency x Drink effects were observed (Whyte et al, 2016; 2017). It was hypothesised that the order in which participants consumed each treatment drink may have had an effect on results due to prior observations that flavonoids may be of most benefit under stages of initial encoding (section 4.2.5.1). A LMM was therefore performed which included Drink and Visit as fixed factors to assess the effects after consuming WBB and placebo in the first or second trial.

Visit was found to be a significant predictor of MANT2 accuracy such that performance was better at the second visit (M=0.89, SE=0.01) compared to the first (M=0.83, SE=0.01; F (1,322.94)=9.03, p<0.01), regardless of Drink. Congruency was also found to be a significant predictor (F(1,322.94)=49.69, p<0.01). As expected, performance was more accurate on congruent trials (M=0.93, SE=0.01) than incongruent trials (M=0.79, SE=0.01).

Figure 4.3a shows accuracy on incongruent trials whereas Figure 4.4a shows accuracy on congruent trials. Figure 4.3b shows RT performance on incongruent trials whereas Figure 4.4b shows RT performance on congruent trials.

A significant Drink x Visit x Congruency interaction (F(2,302.69)=16.31, p<0.01) was observed for accuracy performance. On incongruent trials at visit 1, those consuming WBB were significantly more accurate than those consuming placebo (p<0.01). At visit 2, these same participants, now treated with placebo, were significantly more accurate than visit 2 WBB-treated participants on incongruent trials (p<0.01). This implies a performance boost for participants consuming WBB first, which was sustained at the second visit under placebo treatment. Indeed, change in accuracy performance between visits 1 and 2 for those consuming WBB first and placebo second was small (6%), indicating this boost was most evident at the first task exposure. This was also supported by significant effects between

visits; performance was significantly more accurate on incongruent trials for those consuming placebo at visit 2 compared to visit 1 ($p<0.01$), and for those consuming WBB at visit 1 compared to visit 2 ($p=0.018$; Figure 4.3a). Although significance was not observed for participants consuming placebo first and WBB second, accuracy improved by 15% across visits for these participants, suggesting WBB intervention at visit 2 did still improve performance.

In addition, visit 1 placebo participants ($p<0.01$), and visit 1 ($p=0.06$) and 2 ($p<0.01$) WBB participants had better accuracy on congruent trials compared to incongruent trials, as expected. However, those under placebo treatment at visit 2 (after consuming WBB first) did not show such differences ($p=0.83$). Performance was similar between trial manipulations for visit 2 placebo participants, suggesting they were able to overcome the cognitive demands of incongruent trials after completing the task under WBB treatment initially. As a between-trial difference (approaching significance) was observed for participants under WBB at visit 1, it could be argued that acute intervention with WBB allowed participants to begin to overcome the cognitive demands required on high cognitive load trials initially, which permitted significantly better performance at visit 2 under placebo treatment. These effects were also observed in a significant Drink x Visit interaction ($F(1,322.94)=27.28$, $p<0.01$).

On congruent trials, accuracy performance was not found to significantly differ between-treatments at visit 1 ($p=0.91$) or visit 2 ($p=0.95$), or between-visits for WBB ($p=0.93$) or placebo ($p=0.88$) treatments (Figure 4.4a).

Neither Drink ($F(1,322.94)=1.88$, $p=0.17$), Load ($F(1,322.94)=0.38$, $p=0.54$) nor Target Time ($F(2,322.94)=0.30$, $p=0.74$) were significant predictors of MANT2 accuracy performance, and there were no further significant interactions to be explored.

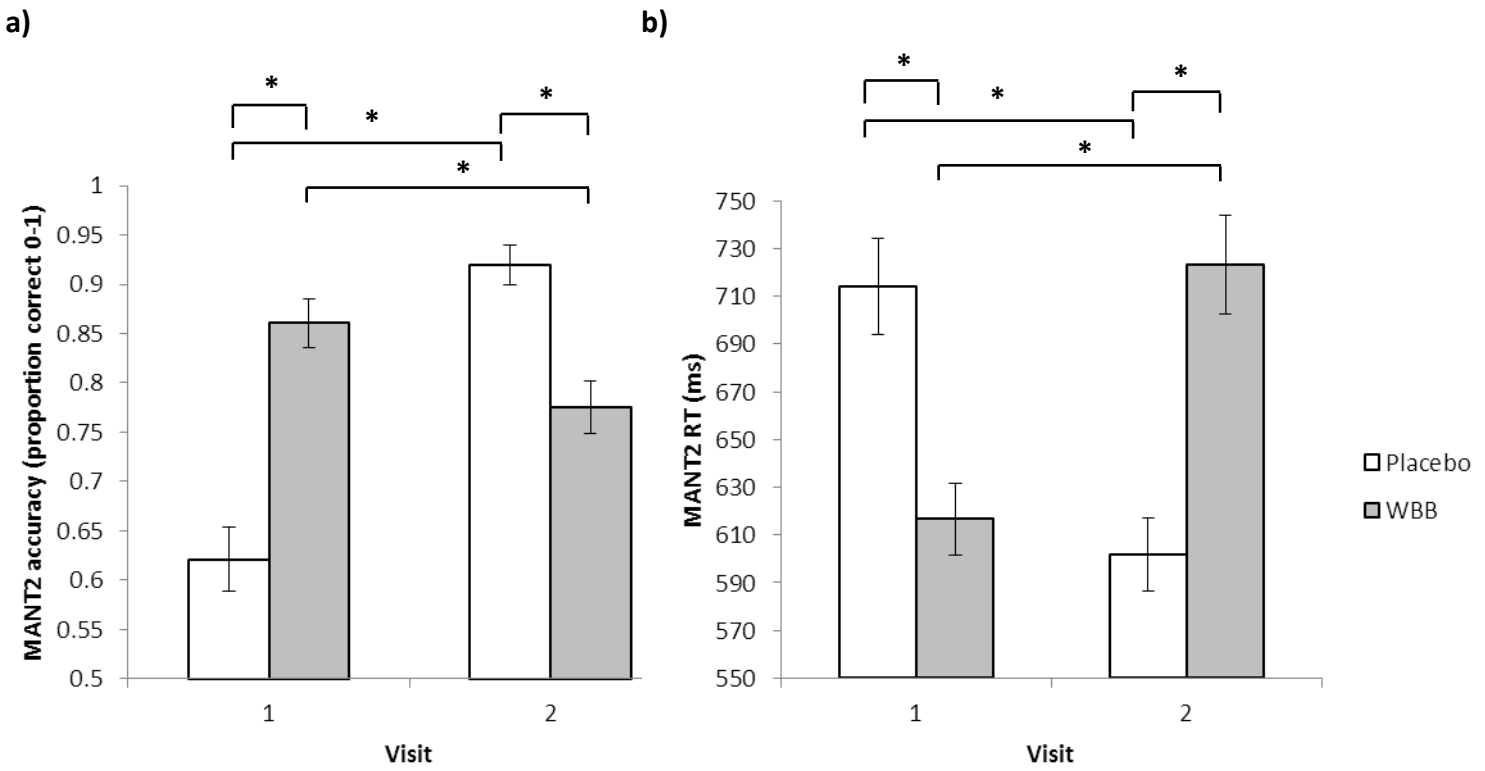


Figure 4.3. Incongruent trial performance on the MANT2 at visits 1 and 2. **(a)** At visit one, WBB-treated participants were significantly more accurate on incongruent trials than placebo-treated participants, and compared to those under WBB treatment at visit two. At visit two, placebo-treated participants were significantly more accurate on incongruent trials than WBB-treated participants and compared to those under placebo treatment at visit one. **(b)** WBB-treated participants were significantly faster on incongruent trials than placebo-treated participants at visit one, and compared to those under WBB treatment at visit two. Placebo-treated participants were significantly faster on incongruent trials than WBB-treated participants at visit two, and compared to those under placebo treatment at visit one. * denotes significance at $p<0.05$.

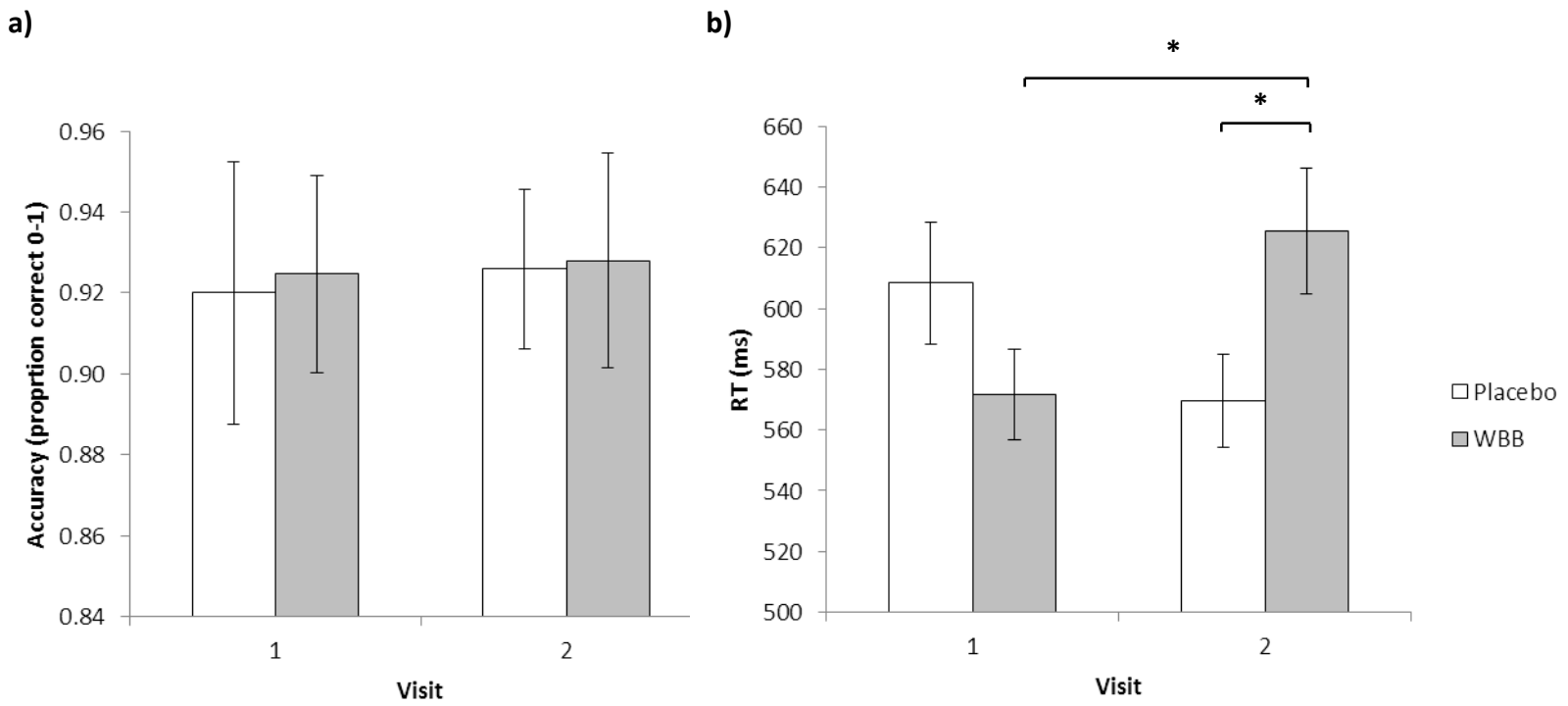


Figure 4.4. Congruent trial performance on the MANT2 at visits 1 and 2. **(a)** No significant differences were observed between-treatments or between-visits for accuracy performance. **(b)** For reaction time performance, participants consuming placebo at visit 2 were significantly faster than participants consuming WBB at visit 2. Participants assigned to WBB at visit 1 were also significantly faster than those assigned to WBB at visit 2. * denotes significance at $p<0.05$.

Reaction time (RT; ms)

As with accuracy performance, a LMM was initially performed using only Drink as a fixed factor. Congruency was found to significantly predict RT performance where participants were faster on congruent (M=587.16, SE=12.46) than incongruent (M=648.34, SE=12.46) trials ($F(1,168)=12.06$, $p<0.01$). A Drink x Target Time interaction was also observed; performance was significantly faster when participants consumed placebo compared to WBB on 120ms (placebo, M=601.92, SE=16.65; WBB, M=628.93, SE=15.83; p=0.017) and 230ms (placebo, M=615.28, SE=16.65; WBB, M=637.43, SE=15.83; p=0.049) trials. Again, to explore the effect of visit order on results, an additional LMM was performed including Visit as a fixed factor.

In LMM analysis which included Visit, participants were significantly faster on congruent trials (M=593.79, SE=9.02) compared to incongruent trials (M=663.98, SE=9.02; $F(1,335.80)=30.30$, $p<0.01$) as expected, regardless of Drink or Visit. Load was also a trending predictor of performance where performance was faster on medium load trials (M=616.97, SE=9.02) compared to high load trials (M=640.80, SE=9.02; $F(1,335.80)=3.50$, $p=0.06$).

A significant Drink x Visit x Congruency interaction occurred ($F(2,310.52)=3.15$, $p=0.04$). As can be seen in Figure 4.3b, WBB-treated participants were significantly faster than placebo participants at visit 1 on incongruent trials only ($p<0.01$). These same participants, consuming placebo at visit 2, were found to be significantly faster than WBB participants at visit 2 on congruent (Figure 4.4b; $p=0.03$) and incongruent trials (Figure 4.3b; $p<0.01$). This suggests that RT performance on both trial manipulations improved when consuming placebo at visit 2 after initial consumption of WBB. This was supported by between-visit interactions with significantly faster performance on incongruent trials for those consuming placebo at visit 2 (after initial WBB) compared to those consuming placebo at visit 1 ($p<0.01$). On incongruent trials, significantly faster RT was evident for WBB-treated participants at visit 1 in comparison to participants receiving WBB at visit 2 (after initial placebo; $p<0.01$). This was also seen on congruent trials: participants were significantly faster when consuming WBB at visit 1 compared to visit 2 (Figure 4.4b; $p=0.036$). As in accuracy analyses, significant differences were observed between trial manipulations for placebo-treated visit 1 ($p<0.01$), and WBB-treated visit 1 ($p=0.036$) and 2 ($p<0.01$) participants, where performance was significantly faster on congruent compared to incongruent trials. This was not true for placebo-treated participants at visit 2 (who consumed WBB first), who showed no such

difference (p=0.14), indicating these participants overcame the more difficult incongruent trials by being able to respond to them at a quicker pace at visit 2, alongside improved accuracy. These effects also occurred in a significant Drink x Visit interaction (F(1,335.80)=37.34, p<0.01).

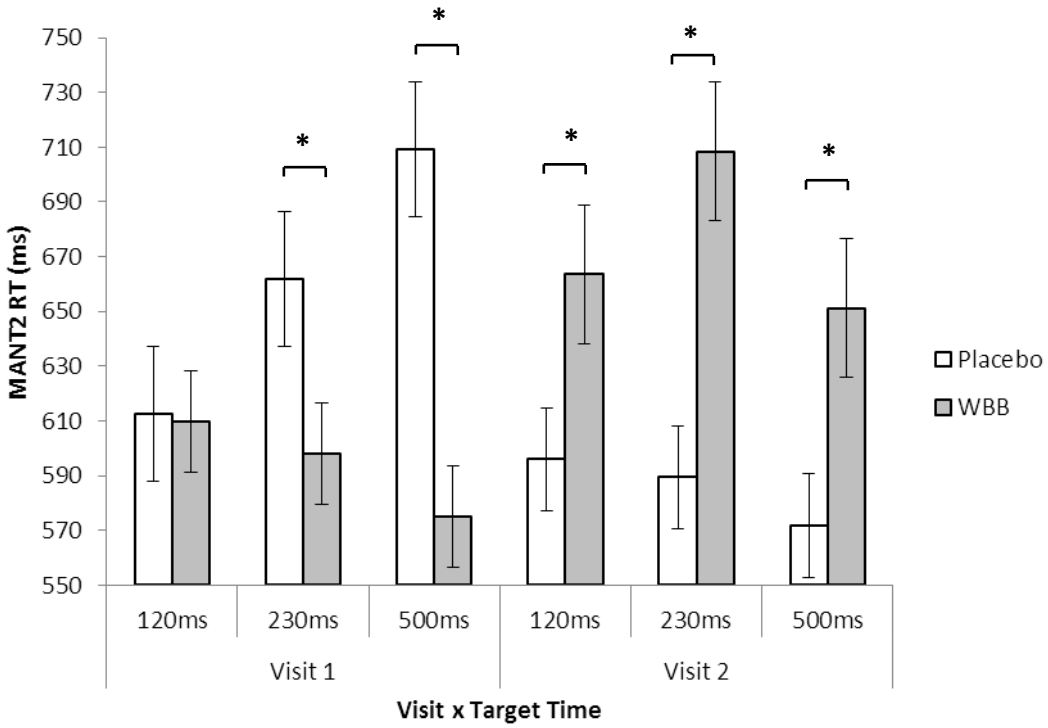


Figure 4.5. Mean (±SEM) MANT2 RT performance for each Target time variable by Visit and Drink. Participants consuming WBB at visit 1 were significantly faster than participants consuming placebo at visit 1, on 230ms and 500ms trials. These same participants, consuming placebo at visit 2, were significantly faster than those consuming WBB at visit 2 for 120ms, 230ms and 500ms trials. * denotes significance at p<0.05.

Figure 4.5 shows a trending Drink x Visit x Target Time interaction that occurred in RT analyses (F(4,310.52)=2.31, p=0.058). Pairwise comparisons revealed that those consuming WBB at visit 1 were significantly faster on medium-paced 230ms trials (p=0.04), and slower-paced 500ms trials (p<0.01) than those consuming placebo. At visit 2, those under placebo treatment (who had consumed WBB initially) were significantly faster on 120ms (p=0.03), 230ms (p<0.01) and 500ms (p=0.01) trials compared to those under WBB at visit 2.

Participants who consumed placebo at visit 1 were the only ones to show between-trial differences, with performance significantly faster on 120ms trials (M=612.60, SE=24.74) compared to 500ms trials (M=709.30, SE=24.74; p=0.019). All other participants did not show such differences, suggesting the consumption of WBB, regardless of visit, eradicated cognitive demand differences under the manipulation of target time.

Neither Drink (F(1,335.80)=0.72, p=0.40), Visit (F(335.80)=0.03, p=0.86) nor Target Time (F(2,335.80)=0.77, p=0.47) were significant predictors of RT performance, nor were there any further significant interactions.

4.3.1.2. Go No-Go data

No significant effects were observed in the analysis of go trial accuracy, go trial RT, no-go false alarms or in the d-prime measure for analyses which included only Drink, or Drink and Visit. There was no speed-accuracy trade-off evident. Statistics can be seen in Appendix F.

4.3.1.3. Mood data

LMM analyses revealed that Drink was not a significant predictor of mood in Drink only analyses (PA (F(1,14)=1.02, p=0.33; NA (F(1,14)=0.006, p=0.94).

For analyses which included Visit, there were also no significant predictors of PA (Drink (F(1,28)=0.09, p=0.76); Visit (F(1,28)=2.58, p=0.12)) nor was there a Drink x Visit interaction (F(1,28)=1.99, p=0.17).

Similarly, neither Drink (1,23.41)=0.007, p=0.94) nor Visit (F(1,23.41)=0.30, p=0.59) significantly predicted NA, and there was no significant Drink x Visit interaction (F(1,23.41)=0.51, p=0.48).

4.3.2. ERP data

4.3.2.1. MANT2 data

Table 4.3 shows the raw data for ERP amplitudes (uV) and latencies to peak (ms) during the MANT2 for each treatment drink at each visit.

Peak	DV	Placebo								WBB							
		Visit 1				Visit 2				Visit 1				Visit 2			
		Fz	FCz	Cz	Pz	Fz	FCz	Cz	Pz	Fz	FCz	Cz	Pz	Fz	FCz	Cz	Pz
N2	Congruent amp	-7.22 (4.04)	-6.06 (2.90)	-2.36 (1.01)	--	-0.69 (3.19)	-1.01 (1.68)	0.10 (1.05)	--	-0.32 (6.51)	-1.52 (5.19)	-0.72 (2.82)	--	-2.79 (2.27)	-2.72 (2.00)	-0.35 (0.91)	--
	Incongruent amp	-4.70 (8.02)	-1.58 (1.91)	-1.30 (0.89)	--	-4.21 (4.07)	-2.44 (1.78)	-1.46 (1.26)	--	-0.53 (5.57)	-1.55 (4.38)	-1.09 (2.11)	--	-1.88 (1.30)	-1.69 (3.18)	0.41 (2.28)	--
	Congruent latency	291.62 (10.39)	293.10 (8.53)	278.17 (6.74)	--	284.18 (6.93)	291.42 (6.45)	278.80 (3.14)	--	282.86 (12.57)	287.16 (12.83)	277.80 (8.83)	--	289.19 (8.35)	291.93 (9.47)	275.81 (7.15)	--
	Incongruent latency	293.16 (12.43)	294.03 (5.09)	276.12 (9.12)	--	282.78 (9.69)	289.75 (9.98)	280.15 (4.70)	--	285.13 (9.55)	28.97 (10.72)	281.38 (6.49)	--	282.79 (15.80)	285.95 (12.58)	278.37 (10.54)	--
P3	Congruent amp	-9.40 (5.57)	-7.71 (4.31)	-3.09 (1.91)	2.24 (4.15)	-2.19 (3.47)	-1.91 (1.86)	-0.33 (1.12)	3.99 (4.86)	-1.71 (7.69)	-2.58 (6.25)	-0.94 (3.43)	4.27 (3.35)	-3.34 (2.57)	-2.71 (1.85)	-0.27 (0.80)	2.46 (3.91)
	Incongruent amp	-8.48 (11.41)	-3.97 (4.58)	-1.08 (1.39)	3.36 (4.90)	-6.63 (6.11)	-4.75 (4.16)	-2.16 (2.38)	1.46 (3.07)	-2.11 (5.68)	-2.51 (4.24)	-1.46 (1.92)	3.76 (4.02)	-3.31 (2.32)	-1.96 (2.83)	0.33 (2.24)	3.03 (2.88)
	Congruent latency	411.34 (4.66)	414.82 (6.23)	406.87 (9.08)	380.40 (7.43)	408.60 (9.11)	408.12 (5.28)	346.90 (153.01)	393.19 (19.39)	406.23 (9.12)	406.39 (8.85)	405.11 (8.12)	398.86 (14.01)	408.40 (12.53)	409.42 (11.92)	404.24 (9.73)	380.51 (21.35)
	Incongruent latency	404.99 (12.27)	406.83 (16.94)	404.70 (8.22)	384.23 (10.74)	404.77 (9.63)	401.33 (8.68)	344.94 (152.37)	394.36 (12.29)	404.83 (4.76)	406.47 (8.31)	406.09 (6.50)	395.23 (7.11)	407.26 (3.75)	412.38 (7.42)	401.95 (6.64)	373.08 (13.15)

Table 4.3. Mean (SD) amplitude (uV) and latency (ms) of MANT2 ERPs (N2, P3) for Fz, FCz and Cz electrodes by treatment drink, visit and trial. P3 ERPs also include Pz electrodes.

4.3.2.1.1. N2 activation

To recap, higher N2 frontal or fronto-central activation was predicted when treated with WBB on incongruent trials, which would indicate increased inhibitory abilities.

Amplitude (uV)

Two significant predictors (Drink and Site) were observed for N2 amplitude on the MANT2. Participants consuming the placebo treatment (M=-2.42, SE=0.57) had significantly higher amplitude than those consuming the WBB treatment (M=-1.30, SE=0.51; $F(1,96.06)=5.36$, $p=0.02$), regardless of Visit, Trial or Site. Activation at the Fz site (M=-2.68, SE=0.59) and FCz site (M=-2.21, SE=0.59) were also found to be significantly higher than at the Cz site (M=-0.69, SE=0.58; all p's <0.05; $F(2,84.81)=6.71$, $p<0.01$), regardless of Drink, Visit or Trial, indicating higher frontal and fronto-central activation during the task, as expected. Visit was also found to be a trending predictor ($F(1,96.06)=3.29$, $p=0.07$), such that N2 activation was higher at visit 1 (M=-2.30, SE=0.60) compared to visit 2 (M=-1.42, SE=0.48), regardless of Drink, Trial or Site, indicating the presence of increased response conflict at the first presentation of this task during ERP recording, which reduces at the second presentation, most likely due to practice effects.

Figure 4.6a and b details a significant Drink x Visit x Trial interaction for N2 amplitude ($F(2,86.06)=5.85$, $p<0.01$). N2 amplitude on congruent and incongruent trials at Visit 1 can be seen in Figure 4.6a. Participants consuming WBB treatment at visit 1 displayed significantly lower N2 amplitude on congruent trials than participants on placebo treatment ($p=0.01$), implying WBB participants experienced less response conflict and used less inhibitory ability on congruent trials. This was predicted, as congruent trials were expected to produce the lowest response conflict out of the two trial manipulations. The fact that WBB participants showed lower conflict suggests they were successfully manipulated by the task demands. On the contrary, participants consuming placebo showed higher N2 amplitude on congruent compared to incongruent trials ($p=0.052$), suggesting these participants found congruent trials the most conflicting. Between-drink differences were not observed on incongruent trials at visit 1 ($p=0.51$), suggesting placebo and WBB participants experienced response conflict and employed inhibition to a similar level on incongruent trials.

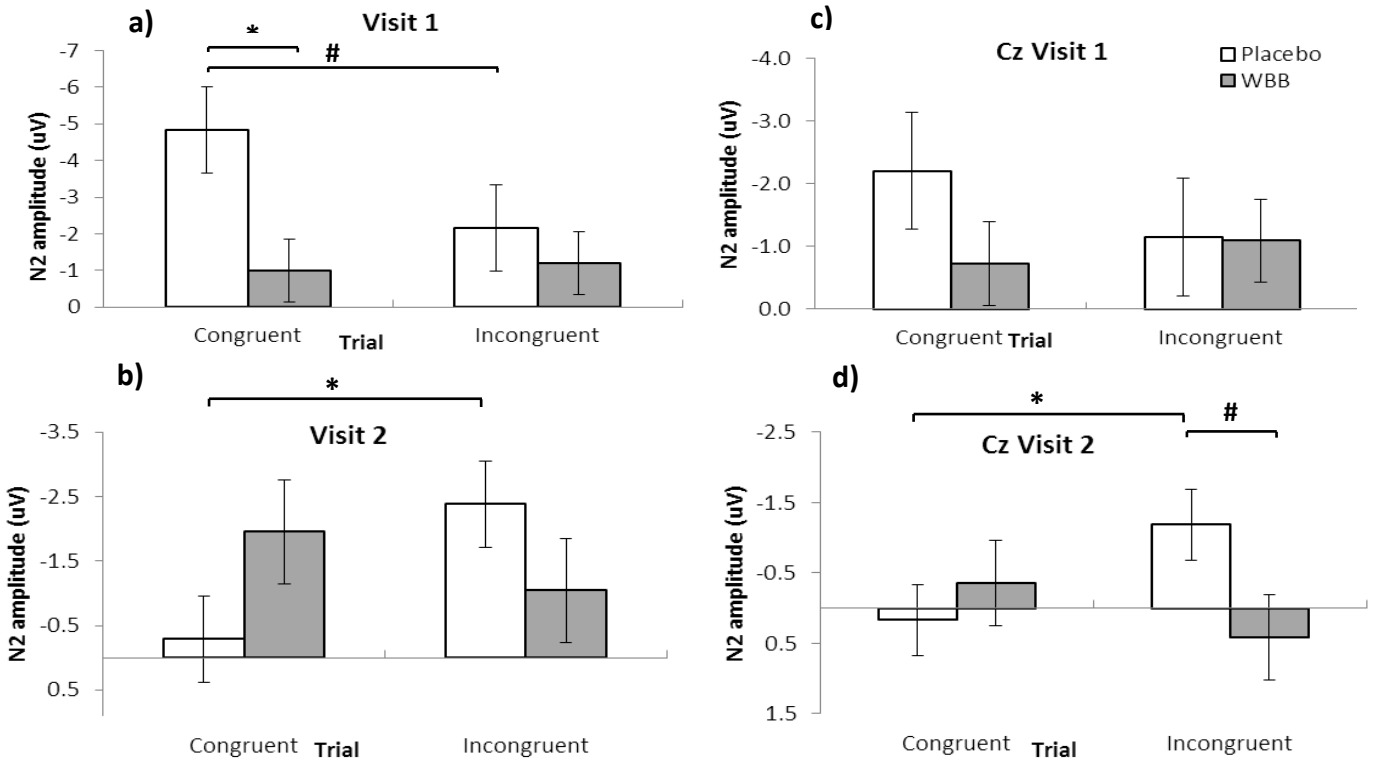


Figure 4.6. Increased negativity (-) = increased N2 amplitude. **a)** Mean (±SEM) N2 amplitude at visit 1, regardless of Site. Lower amplitude was observed on congruent trials for participants consuming WBB at visit 1 compared to placebo, and to incongruent trials. **b)** Mean (±SEM) N2 amplitude at visit 2, regardless of Site. Participants consuming placebo at visit 2 had significantly higher N2 amplitude on incongruent trials compared to congruent trials. **c)** Mean (±SEM) N2 amplitude at Cz site at visit 1. No significant between-trial or between-drink differences were evident. **d)** Mean (±SEM) N2 amplitude at Cz site at visit 2. Placebo consumers had significantly greater N2 amplitude on incongruent trials compared to congruent trials, and when compared to WBB consumers' incongruence amplitude at visit 2. * denotes significance at $p<0.05$; # denotes significance at $0.05 > p < 0.1$.

N2 amplitude on congruent and incongruent trials at Visit 2 can be seen in Figure 4.6b. Participants (who had consumed WBB at visit 1) consuming placebo at visit 2 were found to have significantly higher N2 amplitude on incongruent trials compared to congruent trials ($p<0.01$), implying higher response conflict and inhibitory ability was observed on the more conflicting trials at the second task exposure. This also corroborates with the order effects seen in MANT2 analyses where better performance was observed for placebo participants at visit 2 (who consumed WBB first).

As Site was a significant predictor in the analysis, this interaction was explored within each Site to ascertain the potential brain regions by which Drink x Visit x Trial effects were operating. The interaction was evident at all sites explored: Fz ($F(1,24.26)=3.00$, $p=0.096$), FCz ($F(1,23.94)=4.76$, $p=0.039$) and Cz ($F(1,28.53)=3.86$, $p=0.059$).

Fz and FCz pairwise comparisons mirrored the findings of the main LMM effects reported above (all p's <0.05) with no additional findings. Figures 4.6c and 4.6d show the findings from Cz analyses where a similar pattern of results emerged. N2 amplitude at the Cz site on congruent and incongruent trials at Visit 1 can be seen in Figure 4.6c, where no significant between-trial or between-treatment effects were observed (all p's >0.05). N2 amplitude at the Cz site on congruent and incongruent trials at Visit 2 can be seen in Figure 4.6d. Participants (who had consumed WBB at visit 1) consuming placebo at visit 2 were found to have significantly higher N2 amplitude on incongruent trials compared to congruent trials ($p=0.045$). Additionally, amplitude on incongruent trials at visit 2 was higher for placebo participants (consuming WBB first) compared to WBB participants (consuming placebo first; $p=0.057$; for means see Table 4.3), further indicating that placebo-treated participants were able to overcome incongruent trials at visit 2 after the initial consumption of WBB. Again, this implies a learning effect which was magnified at visit 1 if participants consumed WBB.

Trial ($F(1,84.58)=0.48$, $p=0.49$) was not a significant predictor of MANT2 N2 amplitude, nor did any further significant interactions persist.

Latency to peak (ms)

Significant effects of Site were observed for N2 latency ($F(2,124.23)=43.94$, $p<0.01$). Shorter latency to peak was evident at Cz (M=277.84, SE=1.94) when compared to Fz (M=285.98,

SE=1.94; p<0.01) and to FCz (M=289.80, SE=1.94; p<0.01). Latency was also shorter at Fz compared to FCz (p=0.01).

A significant Drink x Visit x Site interaction was evident (F(4,109.53)=2.53, p=0.045). Upon exploration, a trend was revealed where shorter latency was seen at frontal regions (Fz) for WBB (M=283.34, SE=2.5) compared to placebo (M=290.80, SE=3.50), at visit 1 (p=0.096), indicating a greater ability to process the task when consuming WBB first. This supports the cognitive findings where better performance was observed for those consuming WBB first. As Trial did not persist as a significant predictor, assessing this interaction by Trial was not justified. Assumptions, therefore, cannot be made on whether these differences were present between congruency variables.

4.3.2.1.2. P3 activation

To recap, higher P3 fronto-central activation was predicted when treated with WBB on incongruent trials, indicating increased inhibitory abilities. Lower activation in P3 parietal areas was also hypothesised for WBB participants on congruent trials, conducive of a less effortful recruitment of neural resources to process and attend to the task.

Amplitude (uV)

Drink (F(1,169.19)=10.32, p<0.01) and Site (F(3,151.70)=46.11, p<0.01) emerged as significant predictors of P3 amplitude. Significantly higher amplitude was observed in WBB participants (M=-0.66, SE=0.63) than in placebo participants (M=-2.39, SE=0.68), regardless of Visit, Trial or Site. Amplitude was also highest at the Pz site (M=3.10, SE=0.74) compared to Fz (M=-4.61, SE=0.74), FCz (M=-3.48, SE=0.74) and Cz (M=-1.09, SE=0.74) sites (all p's <0.01), regardless of Drink, Visit or Trial. Visit emerged as a trending predictor, where P3 amplitude was higher at visit 2 (M=-1.07, SE=0.63) compared to visit 1 (M=-1.98, SE=0.68; F(1,169.19)=2.85, p=0.09), irrespective of Drink, Trial or Site.

As shown in Figure 4.7, a trending Drink x Site interaction was evident (F(3,151.70)=2.31, p=0.078). Intervention with WBB significantly increased P3 amplitude by 3.81 uV compared to those receiving placebo intervention at the frontal (Fz) site (p<0.01), regardless of Visit. Similarly, a trend of this nature was also observed at the FCz site (p=0.066), as predicted.

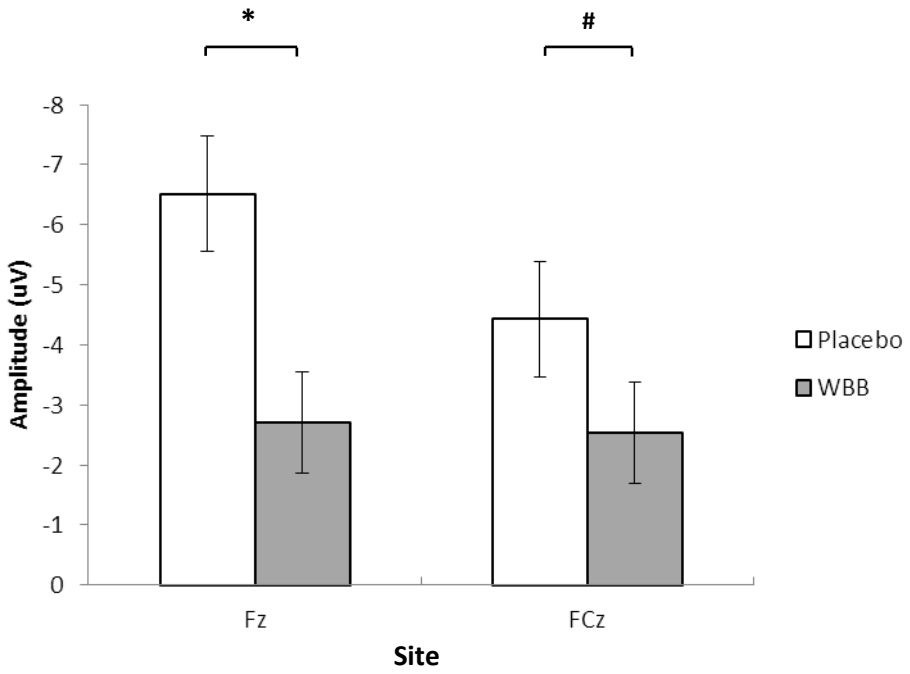


Figure 4.7. Mean (±SEM) P3 amplitude (uV) at Fz and FCz sites. Increased positivity (+) = increased P3 amplitude. Significantly higher amplitude was observed in WBB participants compared to placebo participants at Fz. A trend of this nature was also observed at FCz.

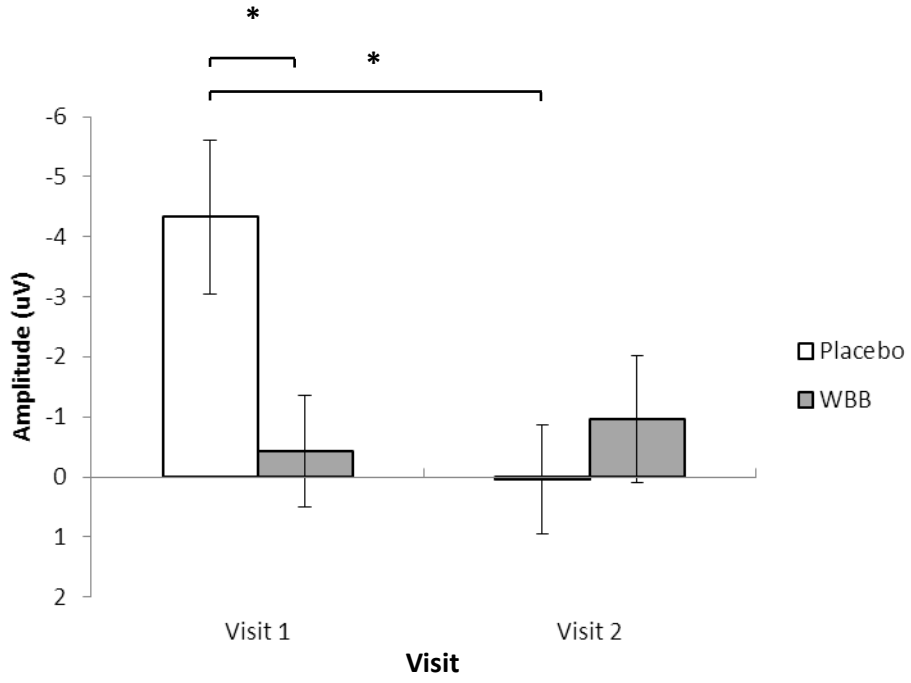


Figure 4.8. Mean (±SEM) P3 amplitude (uV) at visit 1 and visit 2 on congruent trials, regardless of site. Increased positivity (+) = increased P3 amplitude. Significantly higher amplitude was seen for participants consuming WBB first (compared to placebo first) and placebo second (compared to placebo first).

Figure 4.8 shows P3 amplitude on congruent trials. A significant Drink x Visit x Trial interaction was apparent in P3 amplitude analysis ($F(2,148.12)=5.15$, $p<0.01$). At visit 1, significantly higher P3 amplitude was observed on congruent trials for those consuming WBB compared to those consuming placebo ($p=0.018$), suggesting increased allocation of attentional resources in those receiving WBB at the first task exposure. This finding persisted in these participants at visit 2 after receiving placebo, with significantly higher amplitude on congruent trials than incongruent trials ($M=-2.87$, $SE=0.90$; $p<0.01$) and compared to those consuming placebo first ($p<0.01$; Figure 4.8).This suggests that P3 amplitude may increase after initial WBB intervention, and sustain 1 week later after placebo intervention, on congruent trials.

When assessing Drink x Visit x Trial effects by Site, this interaction was evident at all sites (Cz, $F(1,26.80)=4.85$, $p=0.036$; FCz, $F(1,20.61)=3.11$, $p=0.09$; Pz, $F(1,27.56)=3.32$, $p=0.08$) except for Fz ($F(1,23.79)=1.06$, $p=0.31$). Although this interaction trended at the Pz site, post-hoc pairwise comparisons revealed no further Drink, Visit or Trial effects. Unexpectedly, analyses revealed that the WBB effects observed on congruent trials were most evident at the fronto-central site, not the parietal site.

Figure 4.9 displays P3 amplitude at FCz and Cz sites on congruent and incongruent trials at visits 1 and 2 for each treatment. Figure 4.9a details P3 amplitude on congruent trials at FCz. Those consuming WBB at visit 1 showed higher P3 amplitude at FCz compared to those consuming placebo at visit 1 on trials of a congruent nature that approached significance ($p=0.07$). No between-drink differences were observed on congruent trials at visit 2 ($p=0.66$). Figure 4.9b shows P3 amplitude on incongruent trials at the FCz site. No significant differences were observed between treatment drinks at visit 1 ($p=0.62$) or at visit 2 ($p=0.13$) on incongruent trials.

Figure 4.9c shows P3 amplitude on congruent trials at the Cz site. No between-drink differences were seen at visit 1 ($p=0.13$) or visit 2 ($p=0.0.96$). Figure 4.9d shows P3 amplitude at the Cz site on incongruent trials at visits 1 and 2. No significant differences were observed at visit 1 ($p=0.76$). However, significantly higher P3 amplitude was observed for those consuming WBB at visit 2 compared to those consuming placebo at visit 2 on incongruent trials, indicating increased response conflict and inhibition was experienced for those consuming WBB. Trial was not a significant predictor ($F(1,151.70)=0.16$, $p=0.69$) nor were there any further significant interactions.

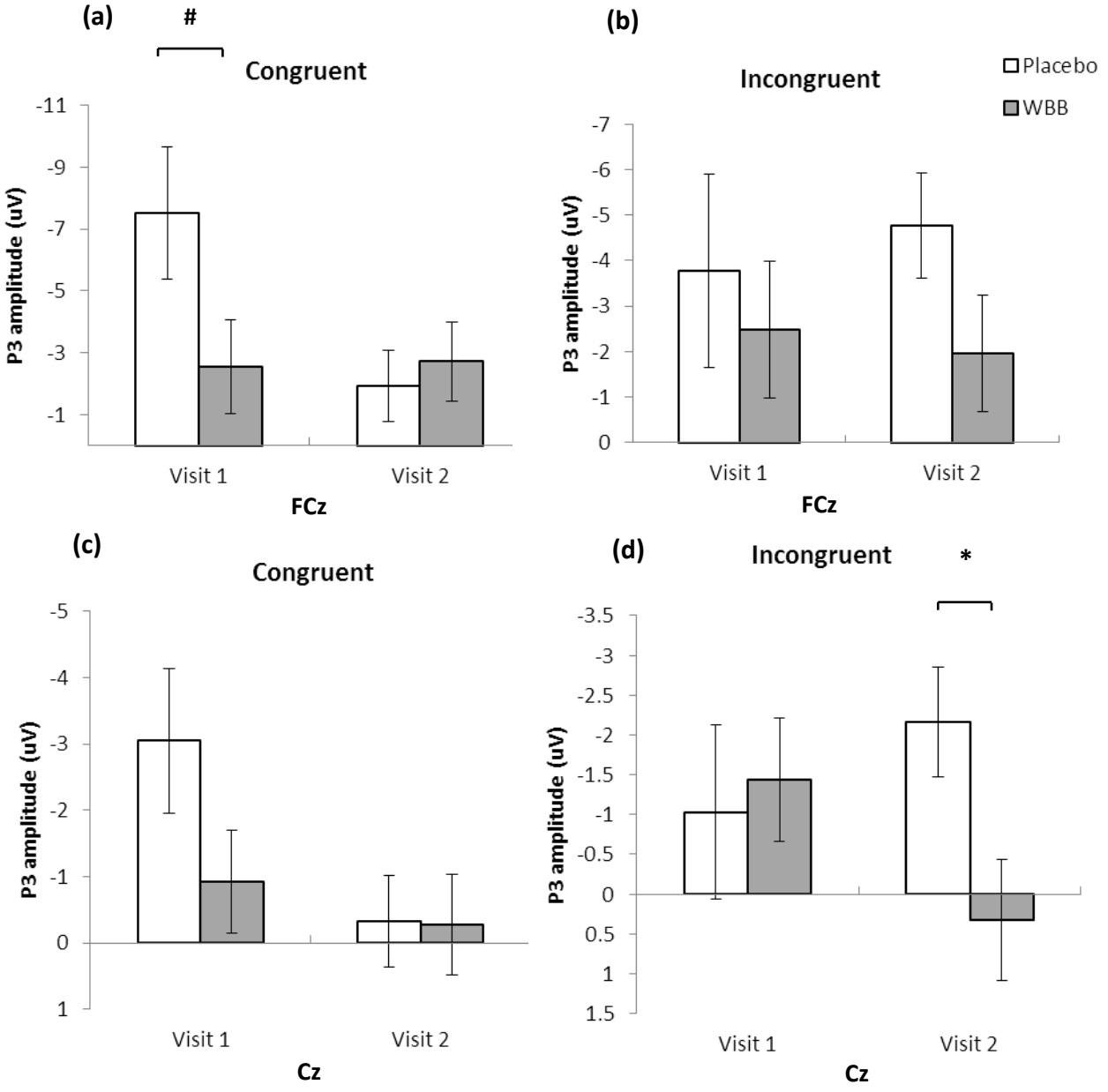


Figure 4.9. Mean (±SEM) P3 amplitude (uV) on visit 1 and visit 2 congruent and incongruent trials at FCz and Cz sites, respectively, for each treatment drink. Increased positivity (+) = increased P3 amplitude**. (a)** At FCz, higher amplitude was observed for visit 1 WBB consumers compared to visit 1 placebo consumers on congruent trials (trend). No effects were seen at visit 2. **(b)** P3 amplitude on incongruent trials at FCz. No between-drink effects were observed at visits 1 or 2. **(c)** P3 amplitude on congruent trials at Cz. No effects were observed at either visits. **(d)** At Cz, no effects were observed at visit 1, however at visit 2 WBB consumers showed significantly higher amplitude compared to visit 2 placebo consumers on incongruent trials. * denotes significance at $p<0.05$; # denotes significance $p=0.05 > 0.1$.

Latency to peak (ms)

Site emerged as a significant predictor of P3 latency ($F(3,92.37)=3.26$, $p=0.025$), however no significant or trending post-hoc effects were revealed.

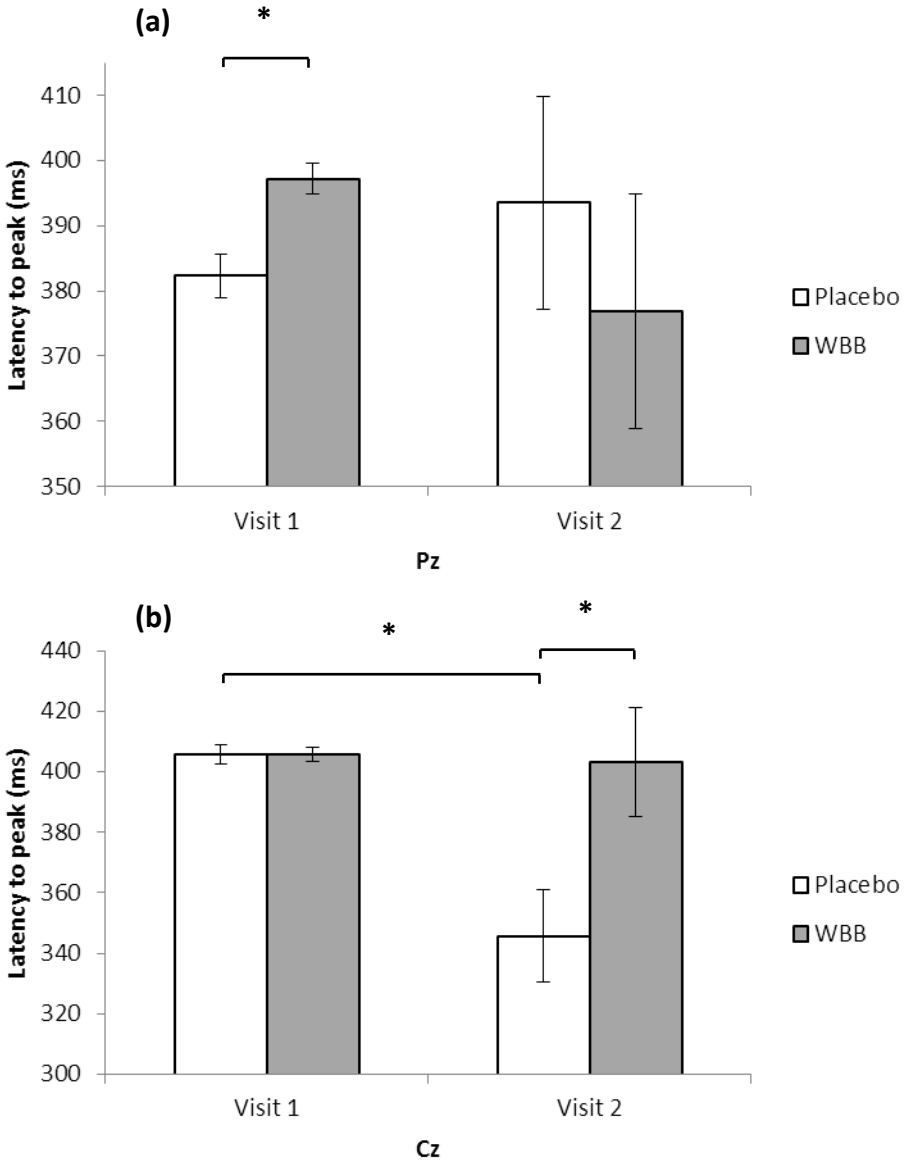


Figure 4.10. Mean (±SEM) P3 latency (ms) at visits 1 and 2 at Pz and Cz sites, for each treatment drink. Longer latency = slower processing**. (a)** Latency at the parietal site. Significantly longer latency was observed for participants consuming WBB at visit 1. No differences were observed at visit 2. **(b)** Latency at the central site. No between-drink differences were evident at visit 1. Significantly shorter latency was seen for those consuming placebo at the second visit in comparison to those consuming WBB at visit 2 and placebo at visit 1.

Drink x Visit x Site appeared as a significant interaction ($F(6,92.49)=2.21$, $p=0.049$). Sites which revealed significance between treatment drinks were Pz and Cz. Figure 4.10a shows P3 latency at Pz for visits 1 and 2. Significantly longer latency was seen for visit 1 WBB participants compared to visit 1 placebo participants at the Pz site ($p<0.01$), suggesting WBB participants did not need to recruit from parietal regions. This also implies that target detection and attentional processing in frontal and fronto-central regions during the first presentation of the task was more efficient, as less recruitment was required from parietal regions. This difference in latency was not seen at visit 2 ($p=0.49$). P3 latency effects were also observed at Cz, which can be seen in Figure 4.10b. No effects were evident at visit 1 ($p=0.98$). However, participants consuming placebo at visit 2 (after initial WBB) demonstrated significantly shorter latencies compared to visit 2 WBB's ($p=0.017$) and compared to visit 1 placebo's ($p<0.01$) at central regions, indicating faster central processing capabilities for those consuming WBB first and placebo second.

No further significant predictors were observed: Drink ($F1,94.25)=1.35$, $p=0.25$), Visit ($F(1,94.25)=2.27$, $p=0.14$), Trial ($F(1,92.35)=0.14$, $p=0.71$), and no further significant interactions occurred.

4.3.2.2. Go No-Go data

DVs	Placebo (n=12)							WBB (n=14)						
	N2			P3				N2			P3			
	Fz	FCz	Cz	Fz	FCz	Cz	Pz	Fz	FCz	Cz	Fz	FCz	Cz	Pz
Go No-Go														
Go amp	-2.73	-2.43	0.21	-2.44	-2.02	0.32	5.74	-1.84	-2.31	-0.11	-1.05	-1.21	0.08	7.51
(uV)	(3.42)	(2.59)	(2.35)	(3.63)	(3.03)	(2.62)	(5.12)	(7.95)	(6.18)	(4.15)	(8.25)	(6.32)	(3.72)	(8.92)
No Go amp	-4.11	-2.89	1.25	-9.86	-5.53	-0.82	10.02	-1.03	-2.37	-0.33	-3.41	-3.88	-0.41	11.25
(uV)	(9.27)	(8.68)	(6.80)	(14.43)	(10.66)	(9.49)	(17.12)	(5.19)	(3.94)	(3.01)	(8.34)	(4.66)	(4.18)	(9.96)
Go latency	278.71	278.44	273.33	403.72	406.06	402.04	381.63	279.5	282.26	275.90	405.14	408.56	401.94	379.16
(ms)	(10.75)	(10.68)	(9.73)	(11.92)	(10.84)	(8.89)	(15.01)	(10.45)	(11.28)	(8.34)	(8.84)	(8.64)	(9.93)	(15.36)
No Go	296.61	297.58	289.74	394.92	398.76	397.96	383.06	283.80	287.96	279.48	402.75	405.54	404.00	381.83
latency (ms)	(41.10)	(37.34)	(37.62)	(30.48)	(25.16)	(15.80)	(20.88)	(19.85)	(16.20)	(11.86)	(16.22)	(15.83)	(12.80)	(25.16)

Table 4.4. Mean (SD) amplitude (uV) and latency (ms) of Go No-Go ERPs (N2, P3) for Fz, FCz and Cz electrodes for each treatment drink. P3 ERPs also include Pz electrodes.

Table 4.4 shows the raw data for ERP amplitudes (uV) and latencies to peak (ms) during the Go No-Go task for each treatment drink.

4.3.2.2.1. N2 activation

Amplitude (uV)

Site was revealed as a significant predictor of N2 amplitude where Fz (M=-2.65, SE=1.17) and FCz (M=-2.54, SE=1.17) activity was higher than Cz activity (M=0.28, SE=1.17; all p's <0.05), regardless of Trial, Drink or Visit ($F(2,97.14)=5.55$, $p<0.01$).

No other significant predictors (Drink $F(1,101.63)=0.60$, $p=0.44$; Visit $F(1,101.63)=0.11$, $p=0.74$; Trial $F(1,97.14)=0.14$, $p=0.71$) or interactions were observed for N2 amplitude.

Latency to peak (ms)

Latency analyses revealed Trial to be a significant predictor; participants had a longer latency to peak on no-go trials (M=289.58, SE=3.30) compared to go trials (M=277.26, SE=3.30), regardless of Drink, Visit or Site ($F(1,79.26)=12.60$, $p<0.01$). This was expected due to no-go trials requiring an increased amount of inhibitory processing, reducing processing speed.

Visit was also a trending latency predictor ($F(1,80.55)=3.49$, $p=0.065$). Latency was shorter at visit 1 (M=280.16, SE=2.47) compared to visit 2 (M=286.68, SE=3.97), regardless of Drink, Trial or Site, indicating faster processing on the first task exposure.

Drink ($F(1,80.55)=0.83$, $p=0.37$) and Site ($F(2,79.26)=1.42$, $p=0.25$) were not significant predictors of N2 latency to peak and no significant interactions were evident.

4.3.2.2.2. P3 activation

Amplitude (uV)

For P3 analyses, Site was also found to be a significant predictor of amplitude ($F(3,136.33)=27.49$, $p<0.01$), regardless of Drink, Visit or Trial. Significantly higher

amplitude was observed at Pz (M=8.44, SE=1.56) than at Fz (M=-4.42, SE=1.56), FCz (M=-3.22, SE=1.56) and Cz (M=-0.23, SE=1.56; all p's <0.01), as expected.

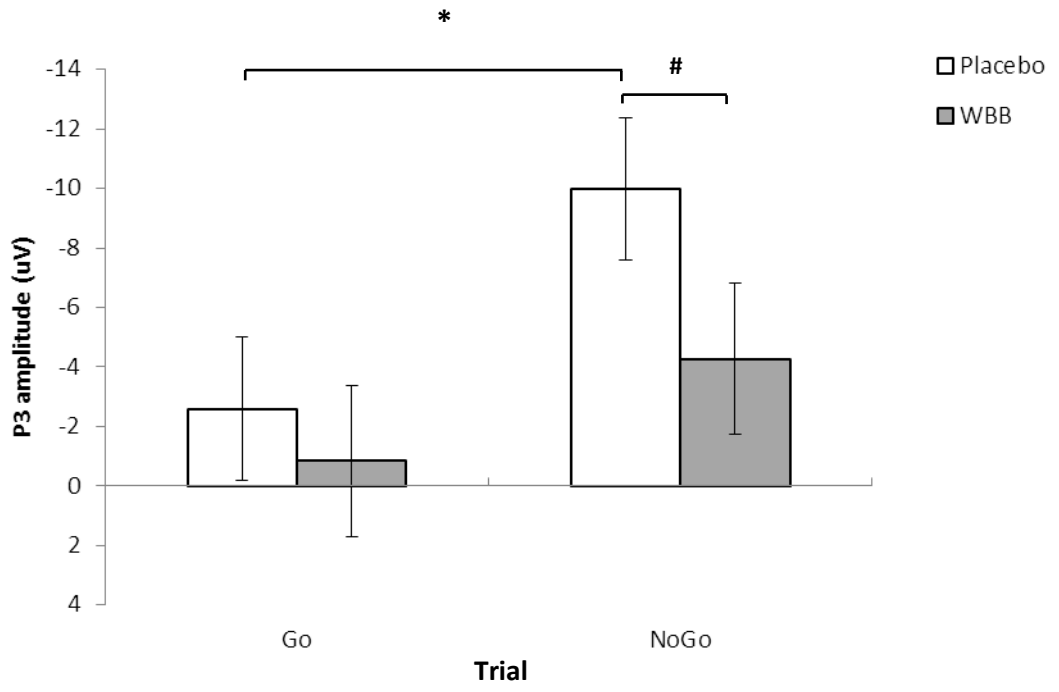


Figure 4.11. Mean (±SEM) P3 amplitude at the frontal (Fz) site. Increased positivity (+) = increased P3 amplitude. Higher amplitude (trend) was observed for participants under WBB compared to placebo on No Go trials. Significantly higher P3 amplitude was evident on Go Trials compared to No Go trials for placebo participants.

Figure 4.11 shows P3 amplitude at Fz on the Go No-Go task, regardless of Visit. A Drink x Site x Trial trending interaction (F(6,135.10)=1.86, p=0.09) was evident. Pairwise comparisons revealed that at the frontal (Fz) site, WBB participants had higher amplitude than placebo participants on no-go trials (p=0.07), suggesting consumption of WBB increased response inhibition capability and cognitive control on the most conflicting trials, regardless of visit. Between-drink differences were not seen on the least conflicting go trials (p=0.58). Also at the frontal site, placebo participants were found to have significantly higher amplitude on go trials than no-go trials (p=0.015), indicating these participants had less inhibitory capabilities on no-go trials. No such difference was seen in WBB participants (p=0.29).

No further significant predictors (Drink F(1,140.12)=2.68, p=0.10; Visit F(1,140.12)=0.89, p=0.35; Trial F(1, 136.33)=1,52, p=0.22) or interactions were observed.

Latency to peak (ms)

Site was a significant predictor of P3 latency, where activation at Pz (M=381.05, SE=2.67) peaked significantly quicker than at Fz (401.94, SE=2.67), FCz (M=405.06, SE=2.67) and Cz (M=401.59, SE=2.67) sites ($F(3,175.56)=24.44$, $P<0.01$; all p's <0.01), regardless of Drink, Visit or Trial, as expected.

Drink ($F(1,178.97)=0.64$, 0.43), Visit (1,178.97)=0.70, p=0.40 and Trial (1,175.56)=0.78, p=0.38) were not significant predictors of P3 latency to peak nor were any interactions evident.

4.3.3. ERP vs. cognitive data analysis

Correlations of interest were derived from significant effects that were revealed in the separate cognitive and ERP data analyses reported above (sections 4.3.1 and 4.3.2). For ease, correlations were placed within two investigatory subsections. The first subsection was labelled 'exploring inhibitory abilities'. Correlations in this section focused on the significant results that were revealed in both cognitive and ERP analyses involving higher N2 and P3 amplitudes and better performance on high conflict trials (incongruent/no-go), which both indicated higher inhibitory abilities for those consuming WBB. These correlations may indicate whether higher amplitudes are associated with higher successful inhibitory abilities on high conflict trials following WBB intervention. The second subsection was labelled 'exploring neural network activation'. This section focused on significant results for P3 activation where significantly longer latency at the Pz site (indicating higher neural efficiency) occurred for WBB participants.

Exploring inhibitory abilities

For the MANT2, correlational analyses were conducted between incongruent N2 amplitude and incongruent trial accuracy performance, at visit 1 and at visit 2. Correlations were performed at both visits as significant WBB-related cognitive and ERP findings were observed at visits 1 and 2. Correlations were then conducted on each treatment drink.

To explore the relationship between the significant cognitive and ERP results observed on P3 latency to peak and cognitive performance, correlations were performed between Cz P3

latency and overall MANT2 accuracy and RT at visit 2. Correlations were only performed on ERP data at the Cz site due to this area revealing significant treatment differences in ERP analyses. Similarly, activity and performance was only correlated at visit 2 as this is where significance had been observed in separate ERP and cognitive analyses. The correlations were then run again split by Drink to ascertain the effect in each treatment group.

For the Go No-Go task, to further examine the significant effects seen for ERP no-go P3 activation and no-go accuracy performance, correlations were performed between no-go P3 amplitude and no-go trial accuracy performance. These were then performed for each treatment drink to see if relationships were more pronounced for WBB or placebo participants. Correlations were not performed by visit here as the significant effects observed on the Go No-Go task and on P3 amplitude occurred regardless of visit.

Exploring neural network activation

Parietal regions were the focus here based on significant P3 ERP findings in parietal regions. MANT2 P3 latency at Pz was correlated with overall performance accuracy and RT at visit 1. P3 data from the Pz site at visit 1 was utilised as this is where significance arose in ERP analyses. These correlations were then performed for each treatment drink.

4.3.3.1. ERPs vs. cognition

4.3.3.1.2. Exploring inhibitory abilities (N2 and P3 frontal and fronto-central regions)

MANT2 data

N2

Correlations were performed between the key significant results from ERP analyses and cognitive measures. As N2 amplitude and MANT2 accuracy were found to significantly increase on incongruent trials in cognitive and ERP analyses, a correlation was performed between these two variables on these trials at visits 1 and 2.

A correlation between N2 amplitude on incongruent trials and incongruent trial accuracy at visit 1 revealed a negative relationship approaching significance (r=-0.54, p=0.068). This suggests that as N2 amplitude decreased, incongruent trial accuracy increased. When split by drink, this finding trended for WBB participants (r=-0.65, p=0.08), but was not significant for placebo participants (r=-0.76, p=0.24; see Figure 4.12).

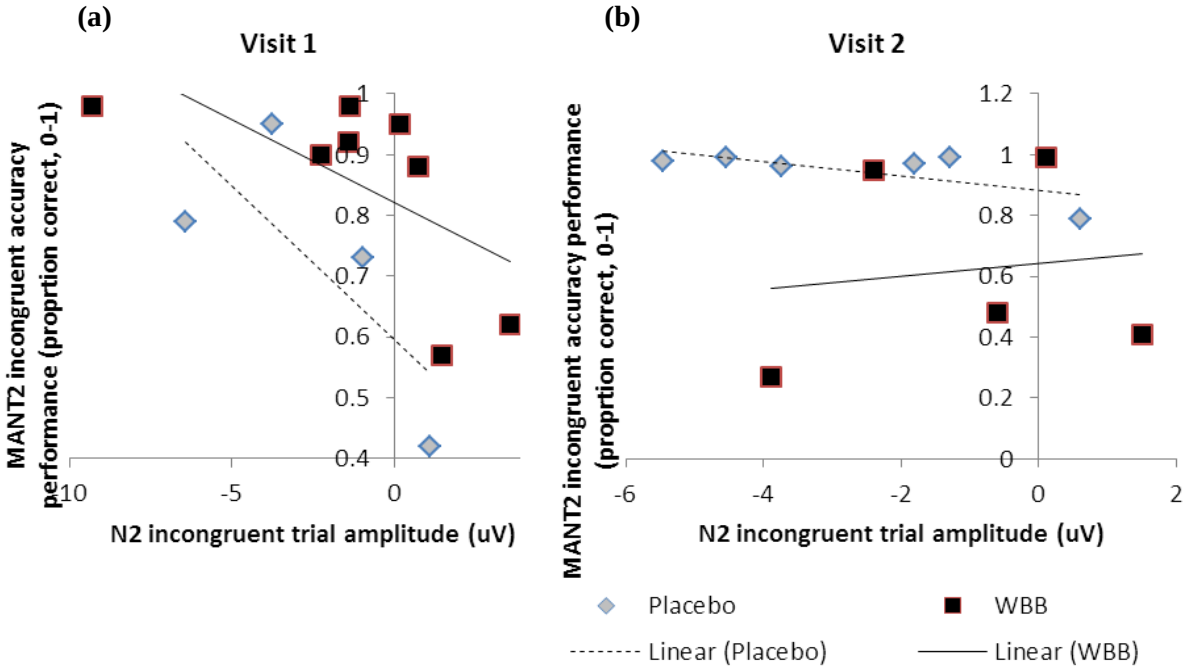


Figure 4.12. Correlational analyses between N2 amplitude (uV) and MANT2 accuracy performance (proportion correct, 0-1) on incongruent trials. **(a)** Visit 1: As N2 amplitude decreased, MANT2 accuracy increased for those consuming WBB (trend). No trend was evident for participants consuming placebo treatment.**(b)** Visit 2: No significant or trending correlations were observed for WBB or placebo participants at visit 2.

The same correlation was performed on visit 2 data. No significant correlation was seen between N2 amplitude on incongruent trials and incongruent trial accuracy at visit 2 (r=-0.28, p=0.41). A significant correlation was also not evident for WBB participants (r=0.13, p=0.83) or placebo participants (r=-0.71, p=0.12) when split by Drink (see Figure 4.12).

It was inappropriate to correlate ERP amplitude with RT performance due to the speed of a participant's response being unrelated to the magnitude of an ERP peak. The latency to peak

ERP measure is more appropriate to correlate with RT performance, however since there were no significant latency N2 effects on the MANT2, these were not performed.

Correlations were not performed between N2 activation and Go No-Go cognitive data as significant N2 activation changes were not observed in Go No-Go ERP analyses.

P3

Significance in P3 amplitude was not observed in MANT2 ERP analyses; hence no correlations were performed for this measure.

A correlation was conducted between P3 Cz latency to peak and overall MANT2 accuracy performance, based on ERP P3 latency findings at the Cz site. This relationship was found to be non-significant (r=-0.19, p=0.57). Significant effects did not occur when split by treatment drink (WBB, r=-0.69, p=0.20; placebo, r=0.36, p=0.49). The same correlation was run on the RT measure to see if speed of processing and speed of response were related. No significant relationship was observed between P3 Cz latency and overall MANT2 RT performance (r=-0.40, p=0.23). This persisted for placebo participants (r=-0.04, p=0.94), however this correlation was significant for WBB participants (r=-0.88, p=0.049), implying that as P3 Cz latency lengthened, RT quickened.

Go No-Go data

N2

No significant effects were observed on N2 peaks for the Go No-Go task in ERP analyses. As a result, no correlations were performed between N2 amplitude, latency to peak and task performance.

P3

A correlation between P3 Fz amplitude on no-go trials and accuracy on no-go trials was performed, based on significant P3 Go No-Go findings at the Fz site, but was found to be non-significant (r=-0.24, p=0.24). When split by drink, no significant correlation was seen for

placebo participants (r=-0.01, p=0.99), however a trending correlation was observed between variables for WBB participants (r=-0.50, p=0.07). This indicated that as P3 Fz amplitude increased, accuracy performance decreased on no-go trials. No latency effects were observed for Go No-Go data; hence no correlations were performed using latency to peak.

4.3.3.1.3. Exploring neural network activation (P3 parietal)

No correlations were performed on amplitude data as no parietal P3 amplitude differences were observed on the MANT2 or Go No-Go tasks in ERP analyses.

Figure 4.13 shows the relationship between P3 Pz latency and MANT2 overall accuracy at visit 1. This correlation was performed based on the significant ERP findings at visit 1 for P3 Pz latency to peak. The relationship was found to be significant (r=0.58, p=0.047) which associated a longer P3 Pz latency with higher accuracy. When split by Drink this effect was not evident for either WBB (r=0.14, p=0.74) or placebo (r=0.34, p=0.66).

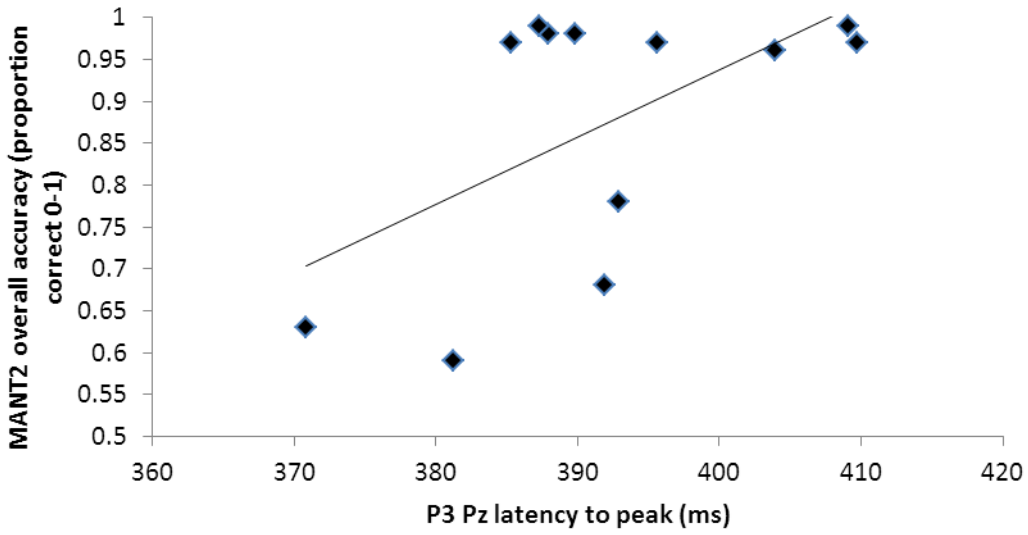


Figure 4.13. Correlational analyses between P3 Pz latency to peak (ms) and MANT2 overall accuracy performance (proportion correct, 0-1) at visit 1. As P3 Pz latency lengthened, MANT2 accuracy significantly increased.

Figure 4.14 shows the correlations between P3 Pz latency and RT measures for placebo and WBB participants at visit 1. This was also conducted based on P3 Pz latency ERP findings at visit 1 to see if processing speed and response speed were associated. Correlational analyses revealed no significant association between P3 Pz latency and MANT2 overall RT at visit 1 (r=-0.22, p=0.50). However, a significant relationship was seen between these measures for visit 1 WBB participants (r=-0.74, p=0.037); as latency lengthened, RT quickened. Placebo analyses also revealed near-significance (r=0.94, p=0.06); for these participants, as latency lengthened, RT also slowed. Correlations were not performed between P3 Pz latency, accuracy and RT measures at visit 2, due to the absence of P3 ERP effects at this visit which can be seen in Figure 4.10a.

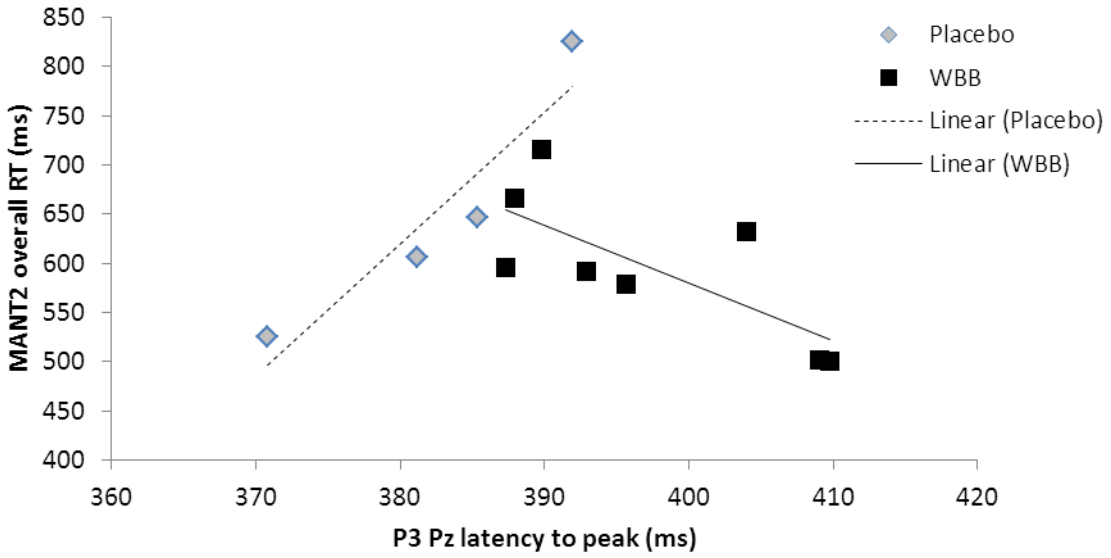


Figure 4.14. Correlational analyses between P3 Pz latency to peak (ms) and MANT2 overall RT performance (ms) at visit 1. For placebo participant, as P3 Pz latency slowed, MANT2 RT slowed (trend). For WBB participants, as P3 Pz latency slowed, MANT2 RT significantly quickened.

4.4. Discussion

The current study recruited healthy 7-10 year old children onto a randomised, placebo-controlled, double-blind crossover trial involving consumption of an acute dose of WBB or placebo, separated by a one week washout. Mood, cognition and EEG ERPs were measured 2 h following treatment consumption to ascertain the cognitive and real-time neurological effects of acute WBB flavonoid consumption.

Previous research has shown promising WBB cognitive effects using a crossover design on a modified flanker task (MFT) and a MANT in child cohorts. Significant acute improvements were observed on a previous MFT, 3 h following WBB (253mg anthocyanins), on incongruent trials (Whyte et al, 2016), suggesting WBB benefitted children on trials of high cognitive demand. Similarly in a later study, Whyte et al (2017) discovered significantly faster performance on incongruent and high load trials for those treated with WBB (253mg anthocyanins) at 3 h compared to placebo, again indicating that WBB may be most beneficial when higher cognitive effort is required. Surprisingly, effects of this nature were not observed in Experiment 1 (Chapter 3). This may have been due to the use of a between-groups design, introducing increased variability between placebo and WBB groups and potentially reducing the ability to detect WBB effects on cognitive load parameters. It was predicted in the current crossover study that WBB effects might emerge on the most cognitively demanding trials (incongruent or high load), as in prior research.

Initially, when assessing the effects of treatment drink across variables, no significant findings were observed. This may have been due to the low number of participants (N=14) included in this experiment due to time and resource restraints. An N of 21 was used in Whyte et al's (2016; 2017) research where load effects were observed, suggesting the current experiment may have been underpowered for cognitive effects. Indeed, a power of 0.33 was reached in the current sample ($F(1,12)=4.75$), indicating sample size was too small to detect behavioural effects. Subsequently, it was considered that the order in which participants had received each treatment drink may have affected performance at each visit. For example, participants consuming WBB first may have been susceptible to carryover effects, or flavonoids may have improved the cognitive mechanisms by which participants were completing the task initially, through better formulation of strategy and increased encoding capabilities. In regards to the latter, in Chapter 3, it was proposed that encoding and retrieval memory systems may be separate entities, and that WBB may improve each to a varying degree under different conditions. This hypothesis was supported by Experiment 1 (Chapter 3) and Whyte et al

(2016) where retroactive interference in the AVLT was absent in both studies, suggesting encoding of new information did not impact recollection of previously learnt material in a child cohort and suggesting these systems may be independent from one another. Additionally, increased encoding of an initial word list was found to interfere with the retrieval of a later word list following WBB in Whyte et al's (2015) study, suggesting a greater 'encoding ability' of the initial list. The effects of WBB at the initial stages of encoding have not been investigated previously. Such exploration could begin by examining the order in which participants receive an intervention or placebo (first vs. second), and the effects this might have on task performance.

In the current experiment, order effects were found to persist in the current sample. At visit 1, participants consuming WBB were significantly more accurate and faster than participants consuming placebo on incongruent trials. This supports the hypothesis that WBB was of most benefit under conditions of increased cognitive load. Moreover, when these WBB participants consumed placebo at visit 2, they were significantly more accurate and faster on incongruent trials than those who consumed WBB at visit 2. This infers that WBB may be most beneficial on cognitively demanding trials at the first task exposure, when participants are encoding the novel aspects of the task and formulating a strategy to perform accurately. This initial benefit may then allow maintenance of greater abilities on the more demanding trials at subsequent time points.

From inspecting the data, it could be argued that participants who consumed WBB first may have already been performing at ceiling (M=0.89, SE=0.15) which may account for their higher performance at visit 2 under placebo treatment (M=0.92, SE=0.13). MANT practice data were examined using an independent t test to see if those assigned to the WBB group first performed at a superior level to placebo in the absence of treatment drink. It must be noted that analysis of practice data is not desirable in a nutritional intervention study due to its highly variable and confounding nature when including it in comparisons across test sessions. Data has been examined here, post-hoc, to elucidate any initial ceiling effects and to reveal potential performance differences between treatment groups in the absence of a baseline session that may impact interpretations. At the practice session, performance was high for WBB participants (M=0.78, SE=0.06) and moderate for placebo participants (M=0.65, SE=0.08), implying that participants assigned to WBB first were naturally better at the task. However, such differences between treatment groups at the practice session were not significant ($t(10)=-1.36$, $p=0.20$), suggesting both groups performed similarly. Significantly

higher performance at visit 1 was therefore unlikely due to higher initial performance prior to treatment for those assigned to WBB. It is interesting to note that performance across each treatment schedule (i.e. participants consuming WBB first and placebo second, and vice versa) improved linearly across practice, visit 1 and visit 2 sessions. This implies that participants' treatment-related improvements may have been in-part due to practice effects. However, such effects should have been reduced due to the inclusion of a practice session, most noticeably across practice and visit 1 sessions as detailed in Bell et al (2015). It is therefore unlikely that practice effects would have accounted for a substantial degree of variability between visits 1 and 2, where treatment effects occurred.

It is also important to note that although visit 2 placebo participants performed better than visit 2 WBB participants, a benefit was also noticeable for those who had consumed WBB at the second visit. For participants consuming WBB first and placebo second, performance between-visits did not show much change, improving accuracy by 6% and reducing RT by 14.84ms, aligning with the idea that encoding was greater at visit 1 and was sustained at visit 2. The small degree of change between visits also implies that these participants did not show large practice effects, supporting a true treatment effect. For participants consuming placebo first and WBB second, accuracy improved more (15%), with RTs decreasing by 9.08ms across the two visits, demonstrating that WBB improved performance at visit 2 compared to the same participants' performance at visit 1 (under placebo). Although these participants showed a larger degree of change between visits, it is unlikely that improvements were due to large practice effects. Both groups completed the task battery three times in total (practice, visit 1, visit 2), suggesting any effects of practice would have equally impacted participants across treatment groups and visits.

Alternative explanations for such WBB order effects do need to be given consideration. Although a 1 week washout was included between visits, and this time frame has been used before in a child population (Whyte et al, 2015; 2016; 2017), higher performance for WBB consumers at visit 1 may have been due to accumulation or carryover effects. Indeed, it is unknown whether a 1 week period is fully suitable from previous research, as Whyte et al (2015; 2016; 2017) did not include drink consumption order as a factor in their analyses. Research by Kalt et al (2014), who administered an acute dose of wild blueberry juice (BJ; 216mg of C3g equivalents) to healthy adults (24-60 years), has suggested that blueberry-related anthocyanins and their metabolites may remain *in vivo* for up to 5 days following a one-off BJ dose on day 0 and an anthocyanin-free diet between day 0-5. Anthocyanin

persistence has also been reported by Wu et al (2011) who suggested that enterohepatic recycling may occur in humans through reabsorption in the intestine or colon, prolonging the half-life of phenolics and flavonoids. Visit 2 placebo effects in the current experiment should therefore be interpreted with caution without sufficient plasma or urinary data to support conclusions. Inclusion of such data would show whether WBB-related metabolites were still present in the body following the 1 week washout and would help to inform order-related results. Future studies should consider conducting a flavonoid crossover study that includes measures of plasma metabolites prior to and following an acute dose, whilst employing a low-flavonoid diet throughout the 1 week washout, to ascertain the extent of acute flavonoid bioavailability.

In the current experiment, participants were found to perform significantly more accurately and quickly on congruent than incongruent trials (as expected) at visit 1 when under placebo and WBB, and at visit 2 under WBB. This was not evident for participants consuming placebo at visit 2, suggesting the consumption of initial WBB at visit 1 may have increased accuracy and reduced RT on congruent or incongruent trials at this visit. Indeed, the means show that placebo participants improved performance on incongruent trials at visit 2 (accuracy, M=0.92, SE=0.02; RT, M=601.83, SE=15.42) compared to their performance under WBB at visit 1 (accuracy, M=0.86, SE=0.02; RT, M=616.67, SE=15.06), accounting for the absence of between-trial differences at visit 2. Incongruent trial improvements were also seen for participants who consumed placebo at visit 1 (accuracy, M=0.62, SE=0.03; RT, M=714.17, SE=20.20) and WBB at visit 2 (accuracy, M=0.78, SE=0.03; RT, M=723.24, SE=20.69), however significant between-trial differences remained at each visit, suggesting incongruent trials remained the most demanding for participants following this treatment schedule. As the difference between congruent and incongruent trials was only a trend for WBB participants at visit 1, this suggests participants may have started to show better accuracy on incongruent trials after initial consumption of WBB at visit 1, which further improved significantly at visit 2 following placebo. Such findings hold promising implications for the role of flavonoids in encoding and retrieval mechanisms. It would be informative to design a study that aims to assess flavonoid intervention at encoding and retrieval separately and simultaneously to further the investigations. This could initially be conducted by utilising order effects, based on the assumption that initial encoding may be higher at visit 1 compared to visit 2. Using an acute crossover, between-groups design, and following a baseline session (where no treatment is consumed), the trial would include consumption of 2 treatments separated by a 1 week washout. Participants would be grouped

into 1 of 4 intervention schedules which would indicate the treatment they would receive first and second: placebo, placebo; placebo, flavonoid; flavonoid, flavonoid; flavonoid, placebo. For example, a participant could be assigned to schedule 1 where they would consume a placebo drink first and a placebo drink 1 week later. Data at each visit could then be compared across schedules, for example, placebo performance could be assessed at visit 2 after WBB or placebo had been consumed at visit 1. Such comparisons would also be performed on WBB data. The schedules would test the impact of consuming placebo and flavonoid at initial and subsequent test points (akin to encoding and retrieval). Although this would not provide a matched comparison between intervention schedules due to different participants taking part in each schedule arm, it would be a good starting indication of whether consumption of flavonoids at first or second exposures are most beneficial in comparison to placebo.

Interestingly, order effects were also present in ERP analyses. It was postulated that frontal N2 and/or fronto-central P3 activity would be higher on incongruent trials for those consuming WBB compared to placebo. This was based on previous literature (Schmitt et al, 2000) that posited response conflict would be higher on trials of an incongruent nature, and would therefore require more inhibitory ability. As WBB interventions have shown effects indicative of increased cognitive control on incongruent trials previously (Whyte et al, 2016; 2017), it was hypothesised that participants under WBB treatment would show superiority in N2 and P3 activation measures. At visit 1, no N2 amplitude differences were observed between treatment drinks on incongruent trials, suggesting all participants experienced the same level of response conflict and utilised similar inhibitory skill. However, at visit 2, placebo participants (who had consumed WBB first) showed increased N2 amplitude on incongruent trials, indicating higher response conflict and improved inhibition on these trials for these participants. This effect occurred at all sites (frontal, fronto-central and central), indicating increased inhibitory ability after initial WBB on the most conflicting trials across frontal and central brain regions. It is important to note that although similar visit 2 effects were apparent in both cognitive and ERP data (improved performance and higher activation in placebo participants who consumed WBB first), there was a dissociation in visit 1 effects. Higher cognitive performance was observed on incongruent trials for those consuming WBB first compared to placebo, however N2 amplitude showed no such treatment differences at visit 1. When exploring correlations between N2 amplitude and MANT2 accuracy on incongruent trials at visit 1 and 2, interesting relationships were revealed. At visit 1, as performance increased, activation was shown to decrease for WBB participants, supporting

the dissociation between cognitive and neurological measures. Although this relationship was a trend, it is promising to see similar significant effects in both cognitive and ERP measures emerge, and correlate. At visit 2, no significant correlations were observed even though amplitude and performance both significantly increased for WBB in the separate analyses.

Interestingly, at visit 1, significantly shorter latency to peak was observed for WBB participants compared to placebo participants at the frontal region. This effect did not occur in an interaction with trial, rendering comparisons within incongruent trials and interpretations involving response conflict and inhibition invalid. However, this result may support the theory of greater 'encoding ability'. Participants may process the task in frontal regions (associated with planning and executive functioning) at a faster speed when exposed to the task under WBB treatment initially, potentially reflecting superior encoding or executive capabilities. This may therefore allow for the higher amplitude and cognitive performance observed in these participants at visit 2. It would be beneficial in the future to conduct a crossover study that includes more time points to assess if cognitive and ERP changes occur and persist in the same way across two visits, and extend beyond the second visit. This may help to elucidate the potential workings under greater 'encoding' or 'executive' ability and may reveal whether neurological or cognitive effects manifest in an order or simultaneously.

P3 activation was also hypothesised to be higher following WBB on incongruent trials at the fronto-central site, reflecting increased inhibitory capabilities based on previous findings (Polich, 2007). Encouragingly, higher P3 amplitude was observed in WBB participants at frontal and fronto-central regions, compared to placebo participants. However, this effect occurred regardless of trial, meaning no inhibition interpretations can be made based on incongruent trial activity. Further, results arose irrespective of visit, not permitting direct comparisons to the N2 activation differences discussed previously. But, it is promising to see that children consuming WBB show frontal and fronto-central P3 increases where they have not been observed before. Jonkman et al (2003) found that children lacked the larger fronto-central P3 activity that adults presented after inhibition trials in their study, implying that children may display some immaturity in inhibitory functions. The current results indicate that brain activation for a child cohort consuming WBB associated with increased processing of inhibitory-related domains, may occur at a similar level to that found in adults. Such findings suggest that the neurological development of children's brains may be able to be influenced by a nutritional WBB intervention. Future studies should investigate whether increased P3 activation at Fz/FCz sites can be observed several weeks later, after an initial

single dose, to see if inhibition effects are temporary or remain after only one treatment. It would also be informative to see whether P3 activation persists under a chronic regimen, where children are supplemented with flavonoids daily, to see whether repeated dosing continues to increase activation.

P3 activation in parietal areas was also a focus in the current study. Jonkman et al (2003) found increased activation in parietal P3 peaks for children on the go trials of a Go No-Go task, which were not observed in adults. The authors hypothesised that, on trials requiring sustained attention like go trials, children may recruit parietal areas to increase allocation of resources, and improve target detection and attentional processing. As adults have more mature processing capabilities, recruitment from brain areas other than frontal, fronto-central and central may not be required. In a child population, P3 parietal activation increases have been linked with a reduction in cognitive performance (Vuiller et al, 2016), suggesting the activation of a larger neural network may be detrimental to performance on a task. Indirectly, this infers that lower P3 parietal activation may be linked to more efficient frontal, fronto-central and central processing (without the need to recruit from parietal areas) and higher performance on cognitive tasks.

MANT2 congruent trials are similar to go trials in that they necessitate sustained attention and elicit reduced cognitive conflict (compared to no-go and incongruent trials in the Go No-Go task and MANT2 respectively), thus the same hypothesis was employed. It was predicted that WBB participants would display lower P3 activation and higher cognitive performance on MANT2 congruent trials. Interestingly, higher P3 amplitude was observed on congruent trials for those consuming WBB at visit 1 and for those consuming placebo at visit 2. However, this effect occurred regardless of Site, meaning conclusions cannot be made regarding the recruitment of parietal areas. Indeed, when analyses were performed by Site, no significant effects were observed in parietal brain regions. This suggests that neither WBB nor placebo participants activated larger neural networks to aid in the allocation of attentional resources, refuting Jonkman et al's (2003) previous research and suggesting the current sample did not engage in recruitment from parietal areas.

Surprisingly, the increased P3 activation observed for WBB participants at visit 1 and placebo participants at visit 2 on congruent trials was most prominent at the fronto-central site. This effect was not expected; higher activation in P3 at FCz was anticipated for incongruent trials due to these trials exhibiting higher response conflict and inhibition processes. These findings could be due to the presence of other variables in the MANT2 that are not included in Go No-

Go tasks. For example, go trials usually contain one stimulus on screen; in the current Go No-Go task, this was an image of a farm animal which required categorical target processing related to whether the image was a monkey, a giraffe or another animal. However, on the current MANT2, congruent trials also contained manipulations of load. This would have meant that on congruent trials, participants would have seen a screen containing either 1 arrow (low load), 1 row of 5 congruent arrows (medium load) or 2 rows of 5 congruent arrows (high load). Under conditions of congruency and high load (2 rows of 5 congruent arrows), response conflict may have been higher compared to conditions of congruency and low load, even though all arrows remained congruent, This may have been due to the higher degree of visual load on screen and may have resulted in the effects observed here; higher response conflict, and therefore inhibition, may have been most prominent in participants consuming WBB first and placebo second on congruent trials containing variations of load. ERPs were not segmented by load in the current sample due to cognitive analyses revealing congruency as a significant predictor and therefore the variable that showed successful manipulation of cognitive demand in this sample. Furthermore, the majority of previous literature (for review see Folstein and Van Petten, 2008) has focused on the effects of congruency in a flanker task under ERP analysis, providing a research base to compare our preliminary effects to. Most of the literature including flanker tasks has found increased N2 and P3 amplitude on incongruent trials, implying congruency as a suitable manipulation in ERP tasks. However, in future, ERP studies should consider segmenting MANT2 trials into levels of difficulty, for example the most difficult trials as incongruent high load arrows and the least difficult as low load single arrows.

P3 latency effects were also apparent in the current dataset. As mentioned, higher parietal activation is thought to be linked with increased allocation of resources which may negatively affect cognitive performance. It was inferred that higher activation would be associated with a shorter latency to peak and, therefore, poorer performance. Longer P3 parietal activation may therefore be linked to more efficient frontal, fronto-central and central processing and higher performance on cognitive tasks, and it was predicted that WBB participants would display longer P3 latency to peak. At visit 1, a significantly longer latency was observed for WBB participants compared to placebo in parietal regions. This implies that participants consuming WBB first were more efficient at frontal and fronto-central processing so did not need to recruit from parietal regions. As this is an effect often seen in adults, not children, it could be implied that WBB aided children to process the task at a higher inhibitory level than those consuming placebo. When exploring correlations between latency and performance measures

at visit 1, longer P3 Pz latency was significantly associated with higher accuracy on the MANT2, regardless of drink. As significance did not emerge in placebo or WBB participants specifically, it is difficult to ascertain whether this relationship was more prominent in WBB or placebo participants. However, when exploring correlations between P3 Pz latency and MANT2 RT at visit 1, significance was observed for both treatment drinks. For those consuming WBB, as latency lengthened, RT quickened. This supports the proposition that a longer latency in parietal areas may be indicative of improved efficiency in frontal, fronto-central and central processing and therefore may lead to higher performance on cognitive tasks. Placebo analyses did reveal near-significance for this correlation but as latency lengthened, RT also slowed. This implies that although placebo participants may have had sufficient processing abilities, this was not able to manifest behaviourally and they were unable to reduce their response speed.

Indeed, significantly shorter latency was observed in central regions for participants consuming placebo at visit 2 (who had consumed WBB at visit 1). This indicates that once children had consumed WBB initially, they were able to quicken central processing capabilities on their second exposure to the task under placebo treatment. When correlating P3 Cz latency with cognitive performance at visit 2, a significant relationship was observed for WBB participants, which indicated that a shorter P3 Cz latency was associated with slower MANT2 RTs. This suggests that participants were faster at processing but slower to respond at visit 2, insinuating a dissociation between neurological and cognitive measures. One potential explanation for these findings is an increase in blood flow to central regions, replenishing oxygen depletion caused by cognitive effort, and allowing for a shorter latency to peak. However, the vasodilatory effects may not have been large enough to produce cognitive change, hence participants' inability to quicken RTs. Again, these results add to the complex relationship between the emergence of amplitude and latency changes when consuming treatment drinks either first or second, and the interpretation of neurological effects in the absence of behavioural outcomes. The effects of order should be considered as a key manipulation in future studies to investigate when and how WBB effects appear and persist in neuroimaging and cognitive domains.

Interestingly, order effects have been noted previously in flavonoid interventions. Lamport et al (2016b) discovered such effects in healthy middle aged adults (40-50 years old) during a 12 week crossover intervention with Concord grape juice (CGJ). Improvements in spatial memory and driving performance were observed following daily CGJ supplementation

relative to placebo. This effect appeared to persist for arm 1 CGJ participants when subsequently receiving placebo at arm 2. Similarly, Kean et al (2015) observed the same endurance effects where improved EF and memory performance was seen 8 weeks after daily flavanone-rich orange juice supplementation in middle-aged adults (mean age 67 years), which was maintained after a 4 week washout. Although these studies were chronic, the carryover effects observed may or may not have been due to accumulation. Kalt et al (2017) found presence of anthocyanin metabolites in the body 5 days after a single dose of blueberry juice (and following a 5 day anthocyanin-free diet). It may therefore be possible that anthocyanins remain in the body longer than initially expected, and the order effects observed in the current experiment, showing higher performance and frontal activation following placebo at the second visit, reflect accumulation from consuming WBB at the first visit. However, the overall number of polyphenols that are metabolised acutely and chronically is unknown, making it difficult to determine whether an acute dose of WBB 2 h prior to testing at visit 2 might produce more or a similar number of metabolites to an acute dose administered 1 week prior. Intuitively, we would predict that an acute dose administered 2 h prior would have a higher number of circulating plasma metabolites than an acute dose administered 1 week prior. If increased bioavailability at 2 h is thought to relate to cognitive effects through increased CBF mechanisms, better cognitive function would be predicted 2 h after consumption of WBB. The current experiment does not show this. Although participants consuming WBB at visit 2 do show EF increases on the MANT2 compared to their performance under placebo at visit 1, they are not as pronounced as participants consuming placebo at visit 2 following WBB at visit 1. This suggests that participants did not benefit from WBB consumption at the second visit to the same degree as the first visit, implying different mechanisms may be at play. Indeed, other researchers have found that flavonoids are expelled from the body within 48 h of consumption, with anthocyanins circulating in the body for up to 5 h only (Kay et al, 2004; Wu, 2005; 2006). This would suggest that flavonoids may not be susceptible to accumulation and may improve attention under initial encoding, subsequently improving performance and maintaining it at future test sessions.

It was also predicted that WBB effects may persist under different manipulations of target time on the MANT2. In Chapter 3, WBB participants were found to be significantly faster on 120ms trials compared to placebo participants and to 500ms trials, indicating increased mental alertness on fast trials. In the current experiment, an additional target time duration (230ms) was introduced alongside 120ms and 500ms trials to increase the total number of trials and to see if effects persisted at an intermediate presentation speed. Performance was

expected to follow a linear pattern, with fastest RTs on 120ms and slowest RTs on 500ms trials following WBB intervention. Significant cognitive MANT2 RT effects were apparent in the current sample. At visit 1, WBB participants were significantly faster than placebo on 230ms and 500ms trials. This contradicts previous findings and shows that WBB intervention benefitted performance on both intermediate and slow trials rather than fast trials. However, at visit 2, those consuming placebo (who had consumed WBB initially) were significantly faster than WBB on 120ms, 230ms and 500ms trials. Taken together, these findings indicate that WBB improved response speed on 2/3s of trial target times at the first exposure, which was further improved on all trial target times at visit 2 when under placebo treatment. Consuming WBB first and placebo second may have enabled participants to overcome the demand of 120ms trials and quicken their RT at visit 2. It is interesting that performance did not initially improve on 120ms trials at visit 1, as previously observed (Chapter 3). This may have been due to the inclusion of an additional level of presentation speed (230ms) which may have reduced sensitivity to the task by decreasing the magnitude of perceived difference between 120ms and 500ms presentation speeds, reducing cognitive demand. Furthermore, the experiment in Chapter 3 contained 4 blocks of MANT trials – 2 of the blocks were presented at 120ms and 2 at 500ms. In the current experiment, only 1 block of 120ms and 1 block of 500ms were presented. This may have reduced the power to detect an immediate acute effect at visit 1 due to participants needing more exposures of each presentation speed to 'master' the task. This mastery can be seen to have been achieved at visit 2 by those who had consumed WBB first and placebo second, indicating WBB enabled better performance on trials of a fast nature to a higher degree than those who consumed placebo first and WBB second.

Whyte et al (2017) did find that the WBB benefits observed for incongruent and high load trials occurred on 500ms trials only, which supports the visit 1 findings that on trials of a slower presentation speed, ability to overcome high cognitive demand may be more achievable for WBB participants. However, a drink x congruency x load x target time interaction was not evident in the current study, making findings incomparable. Further, in Whyte et al's (2017) work, a significant drink x target time interaction did not persist which did not warrant examining target time effects regardless of congruency and load variables, as in Chapter 3. Additionally, in Whyte et al's drink x congruency x load x target time interaction, 120ms trials were never directly compared to 500ms trials as they were in Chapter 3. It is therefore unclear whether such differences occurred in the published data. The interaction between target time variables, cognitive load manipulations and treatment drinks is

unclear at present, and future flavonoid intervention studies should consider persisting with a limited amount of stimuli presentation speeds (i.e. two) to better elucidate interactions from previous and future trials.

It was hypothesised that inhibition, as measured by the Go No-Go task, might show inconclusive behavioural results over the course of the intervention. This task has been used before in an acute WBB trial using the same crossover design, flavonoid dose (253mg anthocyanins) and testing at the 2 h time point (Whyte et al, 2015), but did not find any significant cognitive effects. It was also implemented in an acute crossover trial which saw significant go trial RT improvements at 1.15 h post-consumption following placebo treatment compared to a 15g WBB dose (127mg anthocyanins; Whyte et al, 2016). Although a lack of prior behavioural effects have been observed following WBB, it was important to include the Go No-Go task in the current task battery for measurement of ERPs. This task has been implemented in prior ERP studies and has shown consistent results with increased frontal N2 and fronto-central P3 activation on correctly inhibited no-go trials, in adult and child populations (Overtoom et al, 2002; Jonkman et al, 2003; Jodo and Kayama, 1992; Falkenstein, et al, 1999; Kopp et al, 1996a; 1996b; Karlin et al, 1969; Roberts et al, 1994; Bruin et al, 2001; Pfefferbaum et al, 1985). Indeed, previous flavonoid research has found a dissociation between behavioural and neurological data (Francis et al, 2006; Camfield et al, 2012; Brickman et al, 2014; Bowtell et al, 2017; for details see Chapter 1) suggesting that WBB may affect ERP data in the absence of cognitive findings. It was predicted that N2 and/or P3 activation would be highest on no-go trials at frontal and fronto-central sites for those consuming WBB. In the current study, participants consuming WBB exhibited a trend where higher P3 amplitude was observed on no-go trials compared to those consuming placebo at the frontal site. As higher frontal region P3 amplitudes have been associated with increased response inhibition, this finding suggests that consumption of WBB increased inhibitory capability and cognitive control. As discussed for the MANT2, children have previously been found to lack the larger fronto-central P3 activity that adults presented after no-go trials (Jonkman et al, 2003). However, the current study showed that children consuming WBB displayed higher P3 activity, indicating a boost to inhibition-related domains that was not seen for placebo participants. This may have been due to increased vasodilation to the frontal region which aided replenishment of oxygen to areas involving inhibition.

Although WBB P3 ERP activation was seen, no significant cognitive effects persisted, as expected. When considering a correlation between P3 frontal activation and no-go accuracy performance, higher P3 Fz amplitude was associated with lower accuracy performance. This adds to the body of evidence that demonstrates a dissociation between neuroimaging and cognitive effects. From this data we can conclude that WBB may increase activity to inhibition-related brain regions to allow participants to perform the task accurately, however this increase does not manifest as behavioural improvement. Further, it could be said that the Go No-Go task was not sensitive enough to detect behavioural effects following WBB intervention as was concluded by Whyte (2015) and Whyte et al (2015; 2016). It has been highlighted previously (Scholey et al, 2009; Chapter 1) that tasks need to enforce enough cognitive demand on participants (either through sustained task batteries or manipulation of loading variables) to identify potential flavonoid benefits. Indeed, high performance was observed for both WBB and placebo participants on go trial accuracy (range: 0.81-0.85 proportion correct), and relatively low no-go false alarm rates (range: 0.28-0.38 proportion correct) were seen across visits. Participants may have therefore been performing at ceiling on this task leaving little room for improvement. Future trials should perhaps consider the implementation of a task measuring inhibition that is more sensitive to WBB effects under ERP measurement whilst retaining low visual load (as discussed for the MANT2). This would better allow for behavioural and neuroimaging findings to be detected and help elucidate whether a true dissociation is present.

Absence of significant mood findings in the current experiment was noted for analyses which did and did not include Visit, suggesting the order of treatment did not have an impact on results. Previous mood effects have been detected in a child cohort using a between-groups design (N=54; Chapter 3; Khalid et al, 2017). The lack of mood effects in the current crossover study was therefore not surprising due to the small sample used (N=14), suggesting a lack of power. It is also important to note that the current study did not include a baseline session (where no treatment was consumed), whereas the previous experiment and published research have found beneficial effects when including mood (and cognition) parameters at baseline and acutely. As previously discussed (section 4.2.4), it was not practical to include a baseline session in the current experiment due to timing constraints on children and their parents (notably adherence to out-of-school hours schedule, length of time to setup EEG equipment and length of food abstinence). This limited the ability to assess treatment effects when controlling for baseline in the same participants. However, as participants took part in both treatment arms, and the placebo was suitably matched for sugar and vitamin content, this

provided a comparator for performance following WBB. Future trials should consider testing children earlier in the day whilst on school holidays, so that both baseline and acute test sessions are viable. Time of day effects are yet to be elucidated, and significant WBB benefits have been observed in the morning (Whyte et al, 2015; 2016; 2017) and in the afternoon (Chapter 3) at present, suggesting differences in timings are unlikely to impact results.

It was interesting to note that taste ratings were significantly higher for placebo participants compared to WBB participants. This suggests the current placebo may not have been best matched for taste, and future studies should continue to explore new ways of achieving high treatment palatably. However, as children consumed all drinks, and as drinks were administered 1 week apart, it is unlikely this difference made an impact on results.

Overall, findings from this dataset are encouraging and suggest that benefits of WBB may occur behaviourally and neurologically in typically developing children following acute supplementation. The relationship between neuroimaging data and cognitive performance remains uncertain. The current study seems to support previous data that has shown brain activation in the absence of behavioural effects, and further investigations should focus on the degree at which this might occur over several visits, taking visit order into account. There is a demand for research to examine the chronic effects of WBB on mood and cognition in a child population, as no studies of this nature have been published to date. This would reveal whether repeated consumption of WBB over several weeks may improve cognition and mood as seen in acute trials. Exploration into the effects of WBB on typical children is informative to aid understanding of nutritional interventions through a sensitive period of child development, however it is equally as important to assess such effects in clinical populations such as those with ADHD. Future research should aim to measure mood and cognition in typical child populations and in children with developmental diagnoses to assess the potential therapeutic effects of WBB (Chapter 5). Such findings would help inform the research field of clinical implications and may show promise in the reduction of symptoms or side effects from medication for children diagnosed with a developmental disorder.

Chapter 5: The chronic effects of wild blueberry on the cognition and mood of 7-10 year old children with and without attention-deficit hyperactivity disorder (ADHD)

The role of other researchers in this chapter: D. J. Lamport provided input for the initial stages of this study including the concept, design and procedure.

5.1. Introduction

Findings from both animal and human studies have shown that diets supplemented with flavonoids can lead to improvements in cognitive functioning. Indeed, recent studies have found that acute blueberry flavonoid interventions benefit cognitive performance in children (Whyte et al, 2015; 2016; 2017), which were supported by the experimental results reported in Chapters 3 and 4. There is still a need for investigation into the chronic effects of WBB on mood and cognition in children, as, at present, no research has examined this. Such data would reveal whether daily WBB consumption over a prolonged period may improve cognition and mood as observed in prior acute trials.

Interestingly, Whyte, et al (2017) revealed an acute dose of WBB (30g freeze-dried powder; 253mg anthocyanins) produced significant benefits to 7-10 year olds on the cognitively demanding, incongruent and high load trials of the MANT at 3 h post-consumption. Similarly, data outlined in Chapter 4 also revealed improved performance on incongruent trials of the MANT 2 h post-consumption for children consuming WBB compared to placebo. Taken together, these results suggest that blueberry flavonoids may enhance performance when cognitive resources are compromised i.e when trials are particularly cognitively demanding. WBB-related improvements on trials requiring increased cognitive effort also raise the question of whether blueberry flavonoids could improve performance in a cognitively compromised population. Such research has been conducted in older adults where chronic blueberry intervention has been shown to slow age-related decline and prevent onset of Alzheimer's disease (Krikorian et al, 2010a; 2010b; Lamport et al, 2012; Bell et al, 2015).

Relatedly, an interesting study was also conducted in a sample of urban children (mean age 10.55 years), who already displayed subtle cognitive deficits and early physiological markers of oxidative stress, due to long-term exposure to air pollution (Calderón-Garcidueñas et al,

2013). Results found that supplementation with a cocoa flavanol treatment (680mg/day) for 10.11 ± 3.4 days (minimum 9, maximum 24 days) significantly attenuated expression of the primary inflammatory risk marker, plasma endothelial-1 (ET-1), and improved short term memory, as assessed by letter and object span tasks. Although these results are promising, no research has explored the potential for blueberry flavonoids to alleviate cognitive complaints in children with a clinical diagnosis. Children with attention-deficit hyperactivity disorder (ADHD) often present with cognitive as well as behavioural complaints, such as deficits in working memory and processing speed. Given the aforementioned benefits for attention and memory, ascertaining whether the benefits of flavonoid intervention extends to a sample of participants who have ADHD is of clear practical and theoretical importance, particularly within an educational context. ADHD has been found to negatively affect sustained attention, working memory, executive functioning, processing speed and academic performance, as will be discussed later. These skills are key to successful learning, and so for children with such a deficit, their full academic potential may not be achieved. An intervention that may prevent, alleviate or improve some of these symptoms would be of great benefit to an ADHD population, in which symptoms may be reduced within the school environment, allowing children to reach their full academic potential.

ADHD is a neurodevelopmental disorder that typically presents in childhood (Ford, Goodman, Meltzer, 2003). The disorder encompasses two core symptom categories: inattention and hyperactivity/impulsivity. Children are diagnosed with ADHD if they present with six or more symptoms, across either or both of the categories, which have persisted for at least 6 months. Symptoms also must be negatively impacting academic and social functioning and be inconsistent with developmental level, with symptoms present in two or more settings (e.g. home, school, with relatives; American Psychiatric Association, 2013). Individuals may be diagnosed with one of three presentations of ADHD: combined (≥6 symptoms of inattention and ≥6 symptoms of hyperactivity/impulsivity for ≥6 months), predominantly inattentive (≥6 symptoms of inattention but ≤6 symptoms of hyperactivity-impulsivity for ≥6 months) or predominantly hyperactive/impulsive (≥6 symptoms of hyperactivity/impulsivity but ≤6 symptoms of inattention for ≥6 months). There are many chronic behavioural and cognitive deficits associated with the disorder including atypical executive functioning (Harpin, 2005), poor academic performance (Jensen, Martin, & Cantwell, 1997) poor social functioning (Bagwell, Molina, Pelham, Hoza, 2001; Van der Oord et al, 2005), reduced processing speed and working memory function, dysregulation of affect-motivation-arousal pathways and reduced ability to analyse and synthesise one's own behaviour (Barkley, 1997;

Weigard and Huang-Pollock, 2017). ADHD has an estimated prevalence rate of 5% to 10% in UK school-aged populations and has been deemed a significant public health concern (Scahill and Schwab-Stone, 2000). Healthcare costs of those with ADHD have been found to exceed those without ADHD by more than four times (Holden et al, 2013), and 30-50% of children diagnosed in childhood, continue to display core symptoms into adulthood (Balint et al, 2009). There is, therefore, a pressing need for exploration into cost-effective ways to treat children with ADHD, to reduce the economic and psychological burden of the disorder.

The current primary treatment option for ADHD is short-acting drug therapy, in particular stimulants such as methylphenidate (commonly known as Ritalin), dexamfetamine and atomoxetine (McCarthy et al, 2012). The benefits of short-term stimulant medication have been well documented, showing improvements in parent and teacher hyperactivity ratings after daily dosing of methylphenidate for 4 weeks (Schachter, Pham, King, Langford, & Moher, 2001), and improvements in self-esteem, social and family functioning across several months (Spencer et al., 1996). However, stimulant medication is only thought to be efficacious for 70-80% of ADHD patients (Elia, Ambrosini and Rapoport, 1999), and does not provide relief for all ADHD symptoms. In a review, Swanson (1993) found stimulants to be more than twice as effective in reducing behavioural symptoms of ADHD than improving IQ and achievement measures, highlighting a need for treatments which better target those cognitive symptoms and, in turn, lead to higher academic achievement. Furthermore, several adverse side effects of ADHD medication have been reported including reduced appetite (Schachter et al, 2001), insomnia, stomach aches and headaches (Barkley, McMurray, Edelbrock and Robbins, 1990). This has resulted in increasing concerns regarding administration of stimulant medications to children, in particular from parents of those affected. Again, this emphasises the necessity for novel treatments to relieve ADHD symptoms or negate side effects from existing medication regimens.

Drug-free psychosocial treatments are currently offered, such as cognitive-behavioural therapy (CBT), however these address autonomy, self-management, thoughts, feelings and self-control rather than cognitive ability, directly (Braswell and Bloomquist, 1991). Although psychosocial therapies have been shown to improve the daily functioning of those with ADHD, small effects have been observed in comparison to methylphenidate treatment (Vaan der Oord, Prins, Oosterlaan and Emmelkamp, 2008). This suggests altering neurochemistry may be the most effective method to alleviate symptoms of the disorder.

Although the exact etiology of ADHD remains unclear, it is believed that oxidative stress may play an important role in its development (Dvořáková et al, 2007). A recent meta-analysis concluded that antioxidant production in those with ADHD is typical; however response to oxidative stress is inadequate, resulting in oxidative damage (Joseph, Zhang-James, Perl and Faraone, 2015). Additionally, symptoms of ADHD are also thought to be related to abnormalities in dopaminergic functioning (Sagvolden et al, 2005). Indeed, stimulant medication prescribed to treat ADHD, such as methylphenidate and amphetamines, enhances neural dopamine levels in the striatum and nucleus accumbens by blocking dopamine transporters, leading to reduced behavioural symptoms (Spencer et al, 2005). Furthermore, ADHD has been associated with reduced blood flow to the prefrontal cortex (PFC) which has been found to predict the severity of attentional impairment (Spalletta et al, 2001). Methylphenidate treatment has been found to improve resting regional cerebral blood flow, specifically in the PFC and somatosensory areas (Lee et al, 2005). Such changes are thought to be associated with controlling inappropriate attentional and motor responses to environmental stimuli. Alternative treatments, such as flavonoid-based products, that reduce oxidative stress (Shukitt-Hale, 2012), act on the dopaminergic system by preventing dopamine reuptake (Mercer, Kelly, Horne and Beart, 2005; Yoshitake, Yoshitake and Kehr, 2010), or enhance cerebral blood flow (Dodd, 2012; Rodriguez-Mateos et al, 2013; Lamport et al, 2015) may, therefore, produce similar relief of symptoms.

At present, alternative treatment options remain limited, and there is a pressing need for safe, cost-effective treatments that reduce ADHD symptoms or medication side effects in school-aged children. Most recently, research has focused on nutrition, and whether supplementation with diet-derived nutrients could alleviate cognitive and behavioural symptoms. In the field of ADHD research, such nutritional interventions have been coined Complementary or Alternative Medicines (CAMs). Approximately 50% of parents administer one or more CAMs regularly to their children, such as vitamins, either on their own or alongside conventional drug treatment (Sarris et al, 2011). This implies nutritional options are well tolerated by parents as a treatment for ADHD, most likely due to their plant-based or diet-derived origins, and predicts high acceptability to a future flavonoid CAMs intervention. CAMs are usually administered daily for a chronic period, however it would be interesting to also examine the effect of CAMs acutely. ADHD drug treatment, such as methylphenidate, has an acute benefit on symptoms of behaviour and inattention; it is beneficial for a limited time and ‘wears off’ throughout the day. The potential for a CAMs treatment to act similarly would be particularly useful in situations that require a high level of attention or focus, such

as in exams, or in social situations where noise and movement must be limited, with a return to the 'natural state' of the individual at the end of the day. Indeed, parents often find the decision to medicate their children difficult due to the potential 'dampening' of personality and emotional affect whilst on the medication. Short-acting drugs or CAMs may therefore be preferable for parents of children with ADHD as opposed to a chronic treatment schedule, and both options warrant further research.

The majority of ADHD CAMs research conducted in child populations (6-13 year olds) has involved supplementation with free fatty acids. Small but significant improvements in parent-rated behaviour has been observed following placebo-controlled trials of omega-3 (Belanger et al, 2009; Gustafsson et al, 2010; Stevens et al, 2003; Voigt et al, 2001; Herbaut, 2006), omega-6 (Aman, Mitchell and Turbott, 1987; Arnold et al, 1989) and omega-3 and -6 combined (Hirayama, Hamazaki and Terasawa, 2004; Manor et al, 2012; Raz, Carasso and Yehuda, 2009; Sinn and Bryan, 2007). However, research examining fatty acid intervention has been criticised on the basis of the confounding use of nutritionally unmatched placebos, such as olive oil and vitamin C, and for the subjective nature of parent-rated behaviour reports, which can be subject to bias.

It is common to use behavioural scales completed by a parent or teacher in studies that assess ADHD treatment outcomes in order to measure the treatment's efficacy over time. Although this is a useful measure in evaluating observable symptoms, such scales are likely to be biased, especially if the parent or teacher is aware that the child is taking part in a nutritional trial. Furthermore, parents are more likely to notice a change in behaviour rather than a change in cognition, as the latter is often more subtle. This may be why studies often conclude that stimulant medication is more effective than psychosocial treatment; the medication may be acting on behavioural components, which are more noticeable to parents, teachers and practitioners. Subtle improvements in cognition or mood may not be as easily identified by parents and recorded on rating scales, and may require a more objective assessment measure of these domains. In future trials, it is therefore crucial that objective measures of cognitive performance are included, in addition to rating scales, to provide a better-informed representation of cognitive and behavioural changes in ADHD populations.

Initial exploration of flavonoid supplementation in ADHD populations is promising. Previous research using supplementation with the flavonoid-rich plant, Skullcap (*Scutellaria baicalensis*) has revealed one of its major flavanones, oroxylin A, improves ADHD-like behaviours in spontaneously hypertensive rats (SHR; Kim et al, 2006; 2007; 2008). SHR

animals naturally display similar behaviours to ADHD children, such as hyperactivity, decreased sustained attention and increased cognitive and motor impulsivity: all of which were improved following acute doses of oroxylin A (2 and 10mg/kg; Yoon et al, 2008). Yoon et al (2013) showed such behavioural improvements occur via enhancement of dopamine neurotransmission, much like methylphenidate. Meireles et al (2016) also indicated the potential for flavonoids to be dopaminergic neuromodulators after observing 50% decrease in cation uptake in human neuronal dopaminergic cells *in vitro* following flavonoid exposure. Uptake was found to be altered in the presence of flavonoid derivatives, in particular 4'-methyl-catechin, epicatechin and its related methylated metabolites. These results imply flavonoids may act on dopaminergic neurotransmission and alleviate core symptoms in a similar way as the ADHD drug, methylphenidate.

In a further study, dela Pena et al (2013) administered a one off dose of either an oroxylin A derivative, compound 7-7 (5,7-dihydroxy-6-methoxy-4'-phenoxyflavone; 5 or 10mg/kg), methylphenidate (2 or 3mg/kg) or placebo (saline) to SHR to investigate its potentiality as an ADHD drug. Administration of the flavonoid-derived compound 7-7 resulted in significantly reduced inattention, impulsivity and hyperactivity in SHR for both doses (5 and 10mg/kg). Interestingly, only 2mg/kg methylphenidate alleviated inattention and impulsivity in SHR, with neither the 2 nor 3mg/kg dose ameliorating hyperactivity. These results show promising therapeutic value of flavonoid-derived compounds as an ADHD treatment.

To date, one study has implemented a dietary polyphenolic intervention in children diagnosed with ADHD. Trebaticka et al (2006) supplemented 6-14 year old ADHD children (mean age 9.5) with 1mg/kg/day of Pycnogenol® (Pyc), a polyphenol extract found in French maritime pink bark (FMPB) composed of procyanidins (65-75%, monomers were catechin or epicatechin units), phenolic acids (derivatives of benzoic acid: *p*-hydroxybenzoic, protocatechic, vanillic and gallic acids, or of cinnamon acid – *p*-cumaric, caffeic and ferulic acids; Rohdewald, 2005), calcium (900ug/g), potassium (500ug/g) and iron (65ug/g), as well as traces of manganese (4ug/g), zinc (4ug/g) and copper (<3ug/g; Packer, Rimbach and Virgili, 1999). Daily supplementation continued for one month following a randomised, double-blind, placebo-controlled design. A significant reduction in hyperactivity and inattention was reported, as assessed by the Child Attention Problems (CAP) teacher rating scale. Significant improvements in attention, concentration and visual-motor coordination after 4 week Pyc intervention was also noted, as assessed by weighted scores of five performance subtests from the Prague Wechsler Intelligence Scale for Children (PDW), and

results were found to revert back to baseline levels following a 4 week washout. This suggests that daily, chronic supplementation with flavonoids may improve observable behavioural and cognitive symptoms of ADHD in school-aged children.

In a further study, Dvořáková et al (2007) found urinary catecholamine concentrations were normalised and dopamine, adrenaline and noradrenaline levels were decreased in ADHD children receiving a daily dose of Pyc (1mg/kg/day) for 4 weeks. This also led to less hyperactivity and reduced oxidative stress. These results were found to be specific to a child population, with no such replication observed in a similar study conducted in those with adult ADHD (24-53 years old; Tenenbaum, Paull, Sparrow, Dodd and Green, 2002).

Researchers have also begun to investigate the physiological mechanisms by which antioxidants may reduce oxidative stress in ADHD populations via measurement of glutathione (a major antioxidant that protects the brain from oxidative stress (OS)). The ratio between glutathione's reduced and oxidised forms is a key indicator of cellular health (Owen and Butterfield, 2010). A decreased amount of reduced glutathione (GSH) relative to oxidised glutathione (GSSG) indicates increased OS, whereas an increased ratio of GSH:GSSG demonstrates healthy cell function. A previous RCT (Dvořáková et al, 2006) conducted in children (aged 6-14) with ADHD discovered improvements (via blood samples) in the ratio of GSH to oxidised glutathione GSSG following one month's supplementation of a polyphenolic-rich pine bark extract (Pycnogenol; 1mg/kg body weight/day). As a decreased ratio indicates increased OS, this indicates that in the current study, the extract was metabolised successfully in children's bodies, and may have contributed towards an improved GSH:GSSG ratio. These findings were replicated in a further study (Dvořáková, 2007). Additionally, here, concentrations of urinary catecholamines were seen to be normalised in ADHD children, compared to their characteristically high levels at baseline. Such results demonstrate the feasibility of a chronic 4 week flavonoid trial in ADHD child populations, and demonstrates the potential for behavioural and biochemical changes to occur within a short time frame.

Finally, preliminary findings from the Nutrition and Cognition lab at the University of Reading have indicated improved attention on a modified flanker task (MFT) in 29 typical children and 10 children diagnosed with ADHD (all aged 7-12 years) 3 h following a one-off dose of freeze-dried WBB (30g; 253mg anthocyanins) compared to placebo, under an acute, crossover design. This suggests that blueberry flavonoids may improve cognition in a sample of ADHD children in a similar way to children without ADHD, which holds promising

implications for the learning environment. Most research concerning flavonoid intervention in children has followed an acute design which is effective when assessing the short-term effects on cognitive performance. This is especially informative in a child population as acute improvements may aid learning and retention of information in a school environment. However, it is also vital to investigate whether daily flavonoid intervention benefits children chronically, when supplemented across several weeks. Chronic flavonoid supplementation from a variety of fruit sources has already been shown to improve executive function and working memory in young and aged adults (for discussion see Chapters 1 and 3; Macready et al, 2009; Krikorian et al, 2010a; 2010b; Kent et al, 2015; Devore et al, 2012; Kean et al, 2015; Lamport et al, 2016a; 2016b; Dodd, 2012; Bowtell et al, 2017; Miller et al, 2018). However, no research to date has examined the effect of repeated administration of blueberry flavonoids on cognition or mood in typically-developing healthy children or in children diagnosed with ADHD.

The aim of the proposed study is therefore to investigate the cognitive and behavioural effects of acute and chronic blueberry flavonoid supplementation in 7-10 year old typical children and a subset of children diagnosed with ADHD. This age group has been chosen as it is within a stage of development where there is a spurt of growth in the frontal lobes of the brain (Anderson, 2002; Spencer-Smith and Anderson, 2009). This spurt coincides with a period in which domains which contribute to executive function, such as cognitive flexibility, goal setting and information processing are known to develop (Anderson, 2002), as discussed in Chapter 2. Further, it is most common for children displaying symptoms of ADHD to be diagnosed within this age range; indeed, several inattentive or hyperactive symptoms must be present for at least 6 months prior to age 12 for a diagnosis to be given (American Psychiatric Association, 2013). This therefore highlights a potential age range of interest for the delivery of an appropriate treatment to alleviate emerging symptoms.

It was also deemed important to determine whether any benefits arising from WBB supplementation were specific to those with an attention deficit or may also be beneficial for age-matched and attention-matched peers. In order to assess this, a second control group comprising typically developing children between the ages of 5 years, 0 months and 6 years, 11 months old were recruited into an ‘attention-matched’ group. These children were free from any psychological diagnoses and represented children who had lower attentional abilities than typical 7-10 year old children due to their younger maturational age. Similarities in performance between ‘attention-matched’ and ADHD children at baseline would suggest

that ADHD children were performing at a similar attentional level to 5 or 6 year olds. WBB-related improvements in both of these groups would then suggest that flavonoids act on the attentional capabilities of individuals with underdeveloped or compromised attentional abilities. Improvements in the ADHD group only would suggest that benefits may only be found when there is a cognitive deficit related to ADHD. On the contrary, baseline similarities in performance between typical and ADHD children would suggest that ADHD children hold similar cognitive capabilities to their age-matched peers. Any improvements following WBB in both of these groups would imply flavonoids are beneficial in improving abilities in typical and attention-deficit groups in this age range. If improvements are only observed for typical children, it could be deduced that WBB is beneficial for those with adequate attentional capabilities, but may not extend to ADHD populations.

Additional measures of behaviour and mood will also give insight into whether flavonoid supplementation is able to affect behavioural and psychological components in children. As flavonoids have been linked to decreased incidence of depression (see Chapters 1 and 3 for discussion) and acute positive mood change (Chapter 3; Khalid et al, 2017), inclusion of acute and chronic mood measurements in the current study will help inform researchers of the nature of current mood following flavonoid intervention. Inclusion of chronic, parent-rated behavioural measures will also allow us to observe flavonoid effects that may extend to observable behaviour change, and potentially positively influence symptom management at home. This may lead to future investigations into flavonoids and human behaviour, mood, or a treatment method for developmental disorders, and could even be a viable option for behavioural control in this age group.

An acute x chronic experiment was conducted to investigate the effect of short- and long-term consumption of a WBB drink compared to a placebo drink on a sample of participants who had ADHD, an age-matched control group and an attention-matched control group. It was predicted that typically developing (TD) WBB participants would show acute improvements in verbal memory and EF domains at the 2 h post-consumption time point, as previously found (Chapter 3; Chapter 4; Whyte et al, 2015; 2016), especially on MANT trials of high cognitive demand as seen in Chapter 4 and by Whyte et al (2017). Beneficial acute effects of WBB were also hypothesised for participants' positive mood, as an identical acute design and procedure employed in Experiment 1 (Chapter 3) revealed increased positive affect at the 2 h time point in those consuming WBB. No previous flavonoid studies recruiting children have explored effects following a chronic regimen. It is hypothesised that daily supplementation

with WBB may show similar results to acute studies in the domains of memory, EF and mood, potentially through increased CBF or BDNF up-regulation mechanisms that have been found to occur acutely between 1-2 h (Dodd, 2012; Rodriguez-Mateos et al, 2013) and at 6 h. Alternatively, effects may emerge due to changes in synaptogenesis as observed in chronic animal trials (Williams et al, 2008; Rendeiro et al, 2009). Only one study to date has implemented a flavonoid intervention in children diagnosed with ADHD (Trebaticka et al, 2006), revealing chronic benefits on cognitive components and on parent and teaching behaviour scales after 4 weeks of Pyc. It is therefore predicted that participants with ADHD will show improved cognition and parent-rated behaviour over the course of the 4 week WBB intervention, based on these results.

5.2. Methods

5.2.1. Participants

An *a priori* power calculation was performed to ascertain the required sample size for the current study using a repeated measures, within-between interaction analysis, in GPower 3.0. Forty eight participants (N= 16 per condition) were required to detect a medium effect (F=0.22) at a power of 0.9 across three groups (typical, ADHD, attention-matched) and four repetitions (baseline, 2 h, 2 weeks, 4 weeks) (F(6,135)=2.17). Only 7 participants were recruited into the attention-matched group, and randomisation resulted in an unequal treatment split (2 WBB, 5 placebo). Performance was highly variable across and between participants; 50% of the participants required adjustments to the MANT (i.e. increased rest between blocks, presentation of only 500ms trials) due to the task being too difficult for this age group. Therefore, this group has not been included in any subsequent analyses.

Thirty three participants (6 female) were recruited from the Berkshire and Wiltshire areas, UK. This sample size resulted in an adequate power of 0.85 using an effect size (F) of 0.22 across the 2 demographic groups (typical, ADHD) and 4 repetitions (baseline, 2 h, 2 weeks, 4 weeks) (F(3,93)=2.70). Participants were assigned to 1 of 2 demographic groups: 23 participants (4 female) aged 7-10 years old (M = 7.93, SD = 0.83) were recruited into the 'typical' group and were free from any psychological diagnoses. Post-hoc power analysis performed on this group revealed a power of 0.67 at an effect size (F) of 0.22 across the 2 treatment groups and 4 repetitions (F(3,63)=2.75). Ten participants (2 female) aged 7-10

years old (M= 9.16, SD = 1.07) were assigned to the 'ADHD' group; each had a formal diagnosis of ADHD, given by an educational psychologist in the last 2-4 years. This information was given freely by parents rather than by a professional or clinician. Post-hoc power analysis on this group revealed that at an effect size (F) of 0.22, power was 0.30 across the 2 treatment group and 4 repetitions (F(3,24)=3.01). Three ADHD participants (0 female) had comorbidities which had been diagnosed by a qualified psychologist; these included a child with conduct disorder, a child with autism, and a child with mild autism, dyslexia and anxiety. Two participants (1 female) were taking regular medication for ADHD symptom control. These included daily doses of extended release forms of methylphenidate treatment, Concerta (27mg) and Equasym XL (dose unknown). Parents of both participants were encouraged to continue their child's medication schedule as normal throughout the intervention period.

No participant had any food-related allergies or health conditions e.g. diabetes, high blood pressure, obesity or organ disease which would have excluded them from the study. Demographic data for both demographic groups is shown in Table 5.1.

	Typical				**ADHD**			
	Placebo (n=10)		**WBB (n=13)**		**Placebo (n=5)**		**WBB (n=5)**	
	M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Age	8.07	0.80	7.81	0.86	8.86	1.28	9.45	0.85
Gender (M:F)	9:1	--	10:3	--	5:0	--	3:2	--
CPT	**(n=10)**		**(n=12)**		**(n=3)**		**(n=3)**	
Omissions (%)	7.40	1.67	9.41	1.48	6.94	1.84	8.47	2.18
Commissions (%)	81.33	4.85	79.72	4.27	76.67	3.85	75.56	1.11
BAS 3	56.88	2.23	45.91	2.52	47.80	2.50	47.60	2.62
Taste ratings	8.00	0.47	6.50	0.73	8.00	1.38	7.40	1.12

Table 5.1. Demographic data for placebo and WBB participants in typical and ADHD groups

5.2.2. Design

The study employed a single-blind, between-subjects, placebo-controlled, acute x chronic design where each participant was randomly allocated to either placebo or WBB treatment conditions for the duration of the intervention (see Table 5.1; Figure 5.1). Screening, practice and baseline session data were shared across acute and chronic experimental arms. Participants completed 6 test sessions. A screening session was completed 1 week prior to the baseline test session, which included a measure of parent-rated behaviour. Participants also attended a practice session 1-2 days prior to baseline data collection. The baseline session contained a mood measure and cognitive task battery, followed by consumption of a treatment drink. The acute session took place 2 h after consumption of the treatment, and involved completion of the mood measure and a different, matched version of the cognitive task battery. Participants then continued supplementation with their assigned treatment once a day for 4 weeks, with further data collection of mood, cognition and parent-rated behaviour at 2 and 4 weeks.

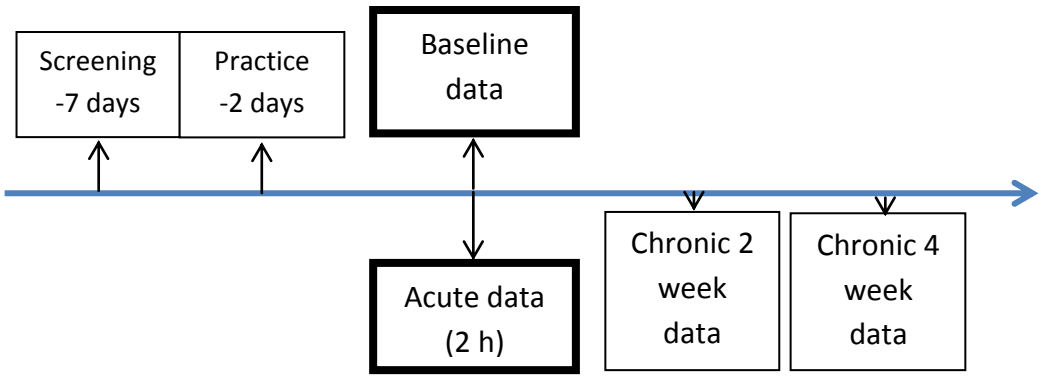


Figure 5.1 Acute x chronic study design comprising mood and cognitive data collection at baseline, 2 h post-consumption, 2 weeks and 4 weeks. Parent-rated behaviour data was collected at screening, 2 weeks and 4 weeks.

5.2.3. Treatment

The WBB drink contained 30g freeze-dried wild blueberry powder (252 mg anthocyanins) and was mixed with 170 ml water and 30 ml Rock's Orange Squash. The dose of WBB was

chosen to match that used in previous paediatric WBB studies (Whyte et al. 2015; 2016; 2017; Khalid et al, 2017; Chapter 3; Chapter 4). 170 ml water and 30 ml of low-flavonoid Rock's Orange Squash was used to aid palatability and flavour. The placebo drink contained 170 ml water, 30 ml Rock's Orange Squash and volumes of glucose (7.99 g), fructose (8.90 g) and vitamin C (4 mg) that were matched to the WBB drink.

One participant's WBB treatment was derived from a different batch of WBB powder and contained 13.3g freeze-dried powder to match the dose of the current study (252 mg anthocyanins). This was due to late recruitment to the study for this participant. A placebo treatment containing matching amounts of glucose (4.52 g), fructose (4.79 g) and vitamin C (45 mg) was also assigned to one participant in order to pair match to the participant on the new batch of WBB. Both treatments were mixed with the same amount of water (170 ml) and Rock's Orange Squash (30 ml) as all other treatment drinks.

All baseline treatment drinks were prepared by a researcher in a food-hygiene approved kitchen at the University of Reading. Drinks were presented in an opaque cup and consumed through a black straw to maintain blinding procedures. Chronic treatments were initially weighed and packaged into sachets by the researcher in the University of Reading kitchen, and stored at -18°C. These were then distributed to participants as 2 x 14 daily sachets alongside an opaque flask, a 500ml bottle of Rock's Orange Squash, a 30 ml measurer, straws and drink making instructions. Parents/guardians were asked to store treatment sachets in their home freezers throughout the intervention period, and ensure their child consumed each treatment drink within 20 minutes of preparation. To our knowledge, all parents adhered to these instructions. Parents were also encouraged to prepare the drink without their child seeing the contents to maintain blinding procedures. It is likely that parents of those in the WBB treatment condition had an idea of the fruit their child was consuming, due to their role in preparation and subsequent exposure to the fruit's purple colour. However, as the study was advertised as a 'fruit drink' study, and participants were only ever exposed to one treatment, it is unlikely the appearance of the drinks would have altered parents' behaviour towards their children.

5.2.4. Measures

Screening

Participants and parents visited the School of Psychology and Clinical Language Sciences (PCLS) at the University of Reading for a screening session to familiarise participants with the researcher and format of the sessions. Here, parents were informed about the study, and fully informed consent from both the participant and their parent was obtained. Participants completed a computerised modified Continuous Performance Task (CPT; for details see Chapter 2) to measure sustained attention and inhibition abilities. This was not used as a diagnostic tool, but as an assessment of homogeneity and normality of attention in each demographic group. CPT data was not used in the analysis of the current study.

Three sub-tests of The British Ability Scale 3 (BAS 3; Elliott, Murray and Pearson, 1979; Elliott, 1997) were administered at screening to measure general ability. The BAS-3 is a well validated and normed measure for schoolchildren aged between 6 years - 17 years, 11 months (Gordon and Elliott, 2001). Three subtests (pattern construction, matrices and verbal similarities), covering both verbal and nonverbal ability clusters, were administered to participants, as detailed in Chapter 2. This measure was included to ensure all participants were of normal cognitive functioning for their age.

During screening, parents were asked to complete a short standardized measure of their child's overt attention and behaviour in the home environment (ADHD Rating Scale IV: Home version). ADHD IV contains 18 statements, 9 relating to instances of hyperactivity/impulsivity, and 9 to inattentiveness. These were summed respectively to calculate a hyperactivity-impulsivity subscale and an inattention subscale, and were totalled to provide an overall behaviour score. These three outcome measures were used in analyses to determine whether overt behaviour had changed over the course of the intervention in each group.

At the end of screening, the participant blind-tasted a sample of their assigned treatment drink, and rated its pleasantness on a Likert scale of '1 – It was horrible' to '10 – I loved it'. This was to ensure that they liked the drink enough to comply throughout the study. Participants who rated their drink as below a '4' were invited to participate in the acute arm of the study only to try to limit potential compliance issues if they were required to complete the 4 week trial. Only one participant rated their drink below a '4' (ADHD placebo); this

participant rated the placebo drink as a '3' and consequently completed the baseline and acute arm of the study only.

Parents were also given a list of high-flavonoid and caffeinated foods that their child was to avoid consuming 24 h before each subsequent session. This included abstinence from high-flavonoid fruits and vegetables, chocolate, fruit juice, tea, coffee, fizzy drinks, energy drinks and pain relievers (e.g. paracetamol, aspirin). This also included instructions to avoid vigorous exercise 24 h prior to each session; 2 x 10 minute mild-moderate-paced walks were deemed acceptable. A 3 day food diary was also administered to parents to record their child's typical diet over the next 3 days. It was deemed impractical for participants to follow a low-flavonoid diet for the duration of the study (4 weeks). If such restrictions were employed, dropout would have likely been high and compliance for those remaining on the trial would have been low. It is also useful to observe the effects of a chronic WBB intervention without flavonoid restrictions to see if effects are observed in dietary conditions akin to real life.

Practice

The practice session took place 5-6 days following screening. The completed 3 day food diary was collected back from parents. The participant then completed a practice battery of the PANAS-C and cognitive tests that would be encountered at each future test session. This was to ensure understanding of the tasks and to reduce practice effects.

Test measures

The main test sessions comprised of the Positive and Negative Affect Scale for Children (PANAS-C; Hughes et al, 2009) and a 40 minute cognitive battery containing the Auditory Verbal Learning Task (AVLT; Lezak et al, 2004) and a Modified Attention Network Task (MANT; Hillman et al, 2009; Whyte et al, 2017). These tests have previously been used with participants aged 7-10 (Whyte et al, 2015; 2016; 2017) and were deemed sensitive to flavonoid effects. See Chapters 2 and 3 for complete descriptions of the tasks used. For chronic sessions (2 and 4 weeks), ADHD IV rating scales were also distributed to parents to complete an assessment of their child's behaviour over the previous 2 weeks.

Home measures

Three-day food diaries were completed by parents at home in between screening, baseline, 2 week and 4 week sessions. These were completed on days when participants were not required to consume a low-flavonoid diet and gave a measure of typical habitual fruit and vegetable consumption. Initially, parents were free to choose the three days to complete the diary, and were then encouraged to keep the recorded days the same throughout the intervention period for consistency.

5.2.5. Test day procedure

Upon arrival at PCLS, participants and their parent/guardian gave written consent to take part in the experiment. Participant demographic data was collected including date of birth, gender, formal psychological diagnoses and any current medications. Parents confirmed that no child had any fruit or fruit juice intolerances or allergies. All test sessions were scheduled during the afternoon to minimise disruption to school hours, and either took place at the University (typical, n= 20; ADHD, n=6) or in the participant's home (typical, n=3; ADHD, n=4).

Participants visiting the University underwent individual testing in a quiet cubicle in the University of Reading's Nutrition and Cognition lab on 6 separate occasions. For those who completed home sessions, the participants were tested individually in a quite space in the home on 6 occasions. Participants who began the study either at home or in the lab, continued with this initial location for the remainder of the trial.

Visit one: Screening

At visit one, participants completed the BAS 3, CPT and taste rating scale, whilst parents completed the ADHD IV. Parents were given an information sheet through which participants were instructed to follow a low flavonoid diet and avoid vigorous exercise for 24 h before each main test session. A three day food diary was also disseminated for the parents to complete for their child over the next week.

Visit two: Practice

At visit two, participants attended the University, or a researcher visited their home, to complete a practice session. Participants completed a practice of the PANAS-C and cognitive task battery, lasting approximately 25 minutes. Both parents and children were reminded of the 24 h flavonoid restrictions imposed for each subsequent test session.

An email was sent to parents 5 days prior to each subsequent test session to remind them to complete the 3 day food diary and follow the 24 h dietary restrictions. Parents were again contacted the day before visits 5 and 6 to remind them to not give their child their treatment drink on the day of their next test session visit, to avoid testing acute effects (see Figure 5.2).

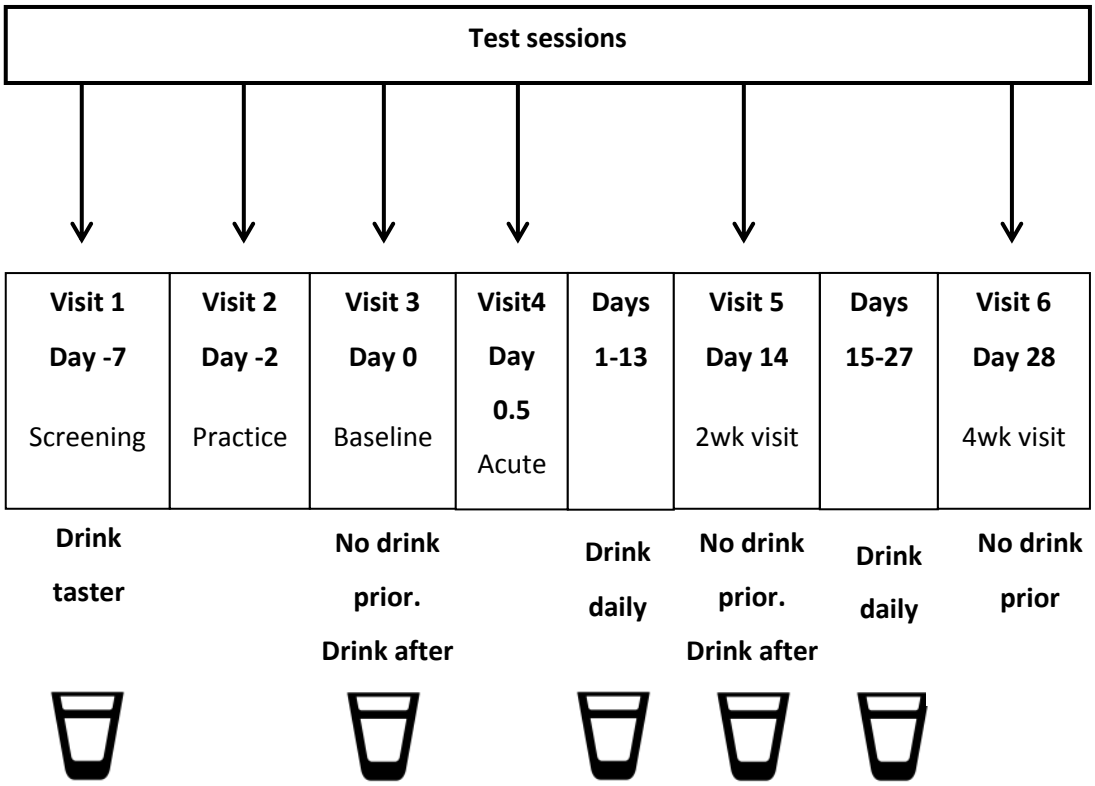


Figure 5.2 Timeline of study indicating when treatment drinks were consumed. A drink taster was administered at Screening, 7 days prior to the start of the intervention. Treatment drinks were consumed daily from day 0 – day 28. On day 0, day 14 and day 28, treatments were not consumed prior to test sessions. On these days, treatments were administered immediately after test sessions. The last treatment drink was consumed on day 28, where intervention ceased.

Visit three: Baseline

At visit three, upon arriving at PCLS or being visited by the researcher, participants completed the PANAS-C and cognitive task battery (lasting approximately 40 minutes), before consuming either a placebo or WBB treatment drink. Participants were then instructed to abstain from food, drink and vigorous exercise over the next 2 h.

Visit four: Acute

The acute session took place 2 h after consumption of the treatment drink. The PANAS-C and a different, matched version of the cognitive test battery were administered to participants, as before. This timeframe coincides with the hypothesised 2 h window of flavonoid absorption and bioavailability (Rodriguez-Mateos et al, 2013) and previous effects of positive mood and cognitive benefits observed 2 h post-WBB consumption (Whyte et al, 2015; Khalid et al, 2017; Chapter 3; Chapter 4).

At the end of the acute session, the drinks-making procedure was thoroughly explained to parents and children verbally, and via a video tutorial created by the experimenter. All necessary equipment was distributed (14 x sachets of treatment, 1 x bottle of Rock's, 1 x opaque flask and straws), in preparation for chronic supplementation. A drink consumption log was also provided to parents to record successful consumption, non-compliance and a space for additional notes. The time of daily consumption over the supplementation period was not stipulated due to the differing home schedules of children and their families. However, once parents found a consumption time that suited, they were encouraged to administer their child's drink at the same time each day, to aid consistency. Typically, participants consumed the treatment drink at ~4-5pm. Parents continued supplementing their children at home (one treatment sachet per day) for 2 weeks. Participants were encouraged to finish all contents of their drink each day. In the event that the drink could not be consumed at the agreed time of consumption, parents were encouraged to try and administer another (fresh) drink before the end of the day. If the drink was not consumed, it was recorded on the drink consumption log as a 'missed day' with an associated reason e.g. ill health. The threshold of total 'missed days' over the 4 weeks was 5 days per participant (82% compliance). The maximum tolerable number of 'missed days' was set to 6 days (78.5% compliance). These numbers are based on the nearest number of days to achieve the desired outcome of 80% compliance (22.4 days) over the total 4 week (28 day) study. If compliance

dropped below 78.5%, those participants' data would be excluded in chronic analyses. Fortunately, no participant dropped below this cut-off in the current study; participants missed an average of 4 days, demonstrating 85.71% compliance. At the end of visit four, parents were given another three day food diary to complete for their child over the next 2 weeks.

Visit five: Week 2

Participants and their parents either visited PCLS, or were visited in the family home 2 weeks into their fruit drink supplementation. Participants abstained from high-flavonoid foods for 24 h before the test session and parents were instructed not to give their child a drink before the session that day, to avoid an acute effect (see Figure 5.2).

Upon arrival, participants completed the PANAS-C and a different, matched version of the cognitive test battery, taking approximately 40 minutes. Parents were asked to complete the ADHD Rating Scale IV based on their child's behaviour over the past 2 weeks, and 3 day food diaries were collected. Participants were then administered their assigned treatment drink by the experimenter, to continue their schedule of supplementation.

At the end of visit five, parents were supplied with more equipment (14 x sachets of treatment, 1 x bottle of Rock's, straws and drink consumption log) in order to prepare and administer their child's treatment drinks for a further 2 weeks, in addition to a 3 day food diary.

Visit six: Week 4

Visit six took place 2 weeks after visit five and marked the end of the intervention. The procedure of this session was identical to visit five, except participants did not receive a treatment drink or additional equipment after testing. Any leftover drinks formulas were collected back from parents at this visit.

5.2.6 Statistical Analysis

Data was analysed using SPSS (Version 22.0). A summary of statistical analyses can be seen in Table 5.2.

Data	Analysis performed	Placebo (N)		WBB (N)	
		Typical (N)	ADHD (N)	Typical (N)	ADHD (N)
Demographics					
BAS		10	3	11	3
Age	Two-way ANOVAs	10	5	13	5
Attention					
Omissions		10	3	12	3
Commissions		10	3	12	3
Taste rating scale		10	3	12	3
Baseline					
PANAS-C	Two-way ANOVAs	10	3	13	4
MANT		10	5	13	5
AVLT		10	5	13	5
ADHD IV		10	5	13	5
Acute					
PANAS-C	LMMs	10	2	13	4
MANT		10	3	13	5
AVLT		10	3	13	5
Chronic					
2 weeks					
PANAS-C		9	2	11	2
MANT		9	3	11	3
AVLT		9	3	11	3
ADHD IV	LMMs	9	3	11	3
4 weeks					
PANAS-C		9	2	11	2
MANT		9	3	11	4
AVLT		9	3	11	4
ADHD IV		9	3	11	4

Table 5.2. A summary of the statistical analyses performed on demographic, baseline, acute and chronic data, including the number of participants who completed each measure at each time point for each demographic and treatment group.

5.2.6.1. Demographic and baseline analyses

Two typically developing participants did not complete the BAS and instead completed the Raven's Coloured Progressive Matrices (RCPM) due to a more restricted period of time to test these children. These scores are not included in demographic data due to lack of comparability, although both participants did show typical, age-appropriate RCPM ability.

Baseline differences in age, attention (CPT omissions; CPT commissions), general ability (BAS 3), taste rating scales and DVs were examined using separate two-way analysis of variances (ANOVAs) with Drink (placebo and WBB) and Group (typical and ADHD) as IVs. ANOVA was deemed the most appropriate model as there was no missing data present at baseline, and analyses did not include varying participant-specific comparisons across time points.

For MANT analyses, to check for a baseline trade-off between speed and accuracy, a correlation was performed between accuracy and reaction time scores.

All 33 participants (23 typical (10 placebo, 13 WBB); 10 ADHD (5 placebo, 5 WBB)) took part in the AVLT and MANT at baseline. Thirty participants (23 typical (10 placebo, 13 WBB); 7 ADHD (3 placebo, 4 WBB)) took part in the baseline measure of mood. Three ADHD participants (2 placebo, 1 WBB) did not take part in the baseline mood measure due to an incomplete understanding of their own mood at the practice session. This was determined by the child expressing a lack of understanding of what mood items meant following adequate explanation from the experimenter. Discrete discussions with the parent confirmed whether children were usually able to decipher how they were feeling. All three participants' parents confirmed that their children typically lacked emotional understanding. It was therefore decided that mood measures would not be taken for these participants for the remainder of the intervention.

5.2.6.2. Acute analyses

Thirty one participants (23 typical (10 placebo, 13 WBB); 8 ADHD (3 placebo, 5 WBB) took part in cognitive tasks (AVLT, MANT) at the acute time point. Two ADHD participants (both placebo) did not take part in the acute session due to attentional difficulties and emotional distress.

Twenty nine participants (23 typical (10 placebo, 13 WBB); 6 ADHD (2 placebo, 4 WBB) took part in the acute mood measure. Two ADHD participants (2 placebo) did not complete the PANAS-C at the acute session due to an insufficient understanding of their own mood and feelings. These participants also did not complete the PANAS-C at any subsequent test sessions due to this reason.

Mood (PANAS-C) and cognitive (AVLT, MANT) data were analysed using linear mixed models (LMMs). DVs for the PANAS-C (PA, NA), MANT (accuracy, reaction time) and AVLT outcome measures were run independently. Baseline performance, Drink (placebo, WBB), Group (Typical, ADHD) and Drink x Group were included within each LMM as fixed factors to compare the effects of treatment within each group whilst accounting for baseline differences. In MANT analyses, additional fixed factors were included to assess the effects of cognitive load; these were Congruency (congruent, incongruent), Load (high load, medium load), Target Time (120ms, 500ms) and Noise (no noise, noise). These variables were also included in interactions with Drink and Group to assess cognitive load differences between treatment and demographic groups. Participant was included as a random factor to acknowledge the dependency of individual data. Post-hoc pairwise comparisons were included within each LMM and were Bonferroni-adjusted for type 1 errors.

5.2.6.3. Chronic analyses

DV baseline data (n=33) was also used in the analysis of the chronic arm.

Twenty six participants (20 typical (9 placebo, 11 WBB); 6 ADHD (3 placebo, 3 WBB) took part in cognitive measures (AVLT, MANT) at the 2 week time point. One ADHD (WBB) and three typical (1 placebo, 2 WBB) participants did not complete the chronic arm of the experiment due to time commitment constraints. One ADHD (placebo) did not complete the chronic arm due to scoring below a '4' on the taste rating scale. In addition, 1 ADHD (placebo) participant withdrew from the chronic arm due to a parental report of increased hyperactivity and disruptive behaviour in the school environment after following their assigned treatment for 3 days. Also, one ADHD (WBB) participant did not take part in the 2 week session due to family illness and unavailability, however supplementation did continue for the full 4 weeks and this participant completed the 4 week session. Twenty seven participants (20 typical (9 placebo, 11 WBB); 7 ADHD (3 placebo, 4 WBB) therefore took part in cognitive measures (AVLT, MANT) at the 4 week time point.

Cognitive (AVLT, MANT) and behavioural (ADHD IV) data were entered into LMMs using an unstructured covariance matrix to model repeat measures. DVs for the MANT (accuracy, reaction time), AVLT outcome measures and ADHD IV percentile data (inattention (IA), hyperactivity-impulsivity (HI), total ADHD behaviour) were run separately. To account for baseline differences, baseline performance was included in each LMM as a fixed factor. Drink (placebo, WBB), Group (Typical, ADHD), Time (2 weeks, 4 weeks), Drink x Group, Drink x Time, Time x Group and Drink x Group x Time were also included within each LMM as fixed factors to compare the effects of treatment within each demographic group. MANT cognitive load fixed factors were entered into the model in the same way as in acute analyses and were included in interactions with Drink, Group and Time to assess cognitive load differences between treatment and demographic groups across the course of the intervention. Again, Participant was included as a random factor to account for individual data dependency, and all post-hoc pairwise comparisons were corrected for type 1 error using Bonferroni adjustment.

Noise was included as a MANT variable in the current study due to the theory of stochastic resonance (SR; Söderlund et al, 2007) which proposes that children with ADHD are able to increase concentration (and therefore performance) when listening to background noise alongside a task. It was predicted that ADHD participants on placebo treatment would improve performance on noisy trials, if the noise provided an aid to concentration. However, it was predicted that ADHD participants under WBB treatment would improve performance on no noise and noise trials. Performance was not expected to change across noise conditions for typical participants.

Twenty four participants (20 typical (9 placebo, 11 WBB); 4 ADHD (2 placebo, 2 WBB) took part in mood measures at 2 weeks and 4 weeks. Three typical participants (1 placebo, 2 WBB) and 2 ADHD participants (1 placebo, 1 WBB) did not take part in the chronic arm of the experiment due to time commitment constraints. Four ADHD participants (2 placebo, 2 WBB) did not complete the PANAS-C at 2 and 4 week sessions due to an insufficient understanding of their own mood and feelings. Due to a reduction in sample size to 4 for this demographic group and to prevent underpowered results for the ADHD group, Group was removed as a fixed factor for baseline mood and chronic mood LMM analysis, and analyses were only performed on typical participants. PANAS-C DVs (PA, NA) were therefore entered into an unstructured LMM with baseline performance, Drink (placebo, WBB), Time (2 weeks, 4 weeks) and Drink x Time as fixed factors.

5.3. Results

5.3.1. Demographic data

Demographic mean data is shown in Table 5.1.

No significant baseline differences emerged between treatment drinks ($F(1,28)=0.60$, $p=0.45$), demographic groups ($F(1,28)=0.09$, $p=0.76$) or in a drink x group interaction ($F(1,28)=0.01$, $p=0.92$) for sustained attention (CPT omissions), or inhibition (CPT commissions; drink ($F(1,28)=0.05$, $p=0.83$), group ($F(1,28)=0.48$, $p=0.50$), drink x group ($F(1,28)=0.00$, $p=0.97$). Taste ratings were also found to be non-significant between drinks ($F(1,31)=1.33$, $p=0.26$), groups ($F(1,31)=0.25$, $p=0.63$) and in a drink x group interaction ($F(1,31)=0.25$, $p=0.63$).

However, significant differences at baseline were seen between demographic groups for age ($F(1, 33)=11.07$, $p<0.01$) with ADHD participants ($M=9.16$, $SD=1.07$) being significantly older than typical participants ($M=7.99$, $SD=0.84$), however no significant differences were observed between treatments ($F(1,33)=0.36$, $p=0.56$) or in a drink x group interaction ($F(1,33)=1.14$, $p=0.30$; Table 5.1).

General ability (BAS 3) was also observed to be significantly different at baseline with placebo participants ($M=53.78$, $SD=7.17$) showing higher general ability than WBB participants ($M=47.18$, $SD=7.88$; $F(1,29)=4.09$, $p=0.05$). This was also a trend in a Group x Drink interaction ($F(1,29)=3.80$, $p=0.06$) and post-hoc t tests revealed typical placebo participants ($M=56.88$, $SD=6.31$) outperformed typical WBB ($M=45.91$, $SD=8.35$; $t(17)=3.12$, $p<0.01$), ADHD placebo ($M=47.80$, $SD=5.59$; $t(11)=2.63$, $p=0.02$) and ADHD WBB participants ($M=47.60$, $SD=5.86$; $t(11)=2.65$, $p=0.02$). No significant main effect of group was observed ($F(1,29)=1.79$, $p=0.19$; Table 5.1).

5.3.2. Baseline data

Mood

For mood, baseline differences were observed between drinks, with participants consuming WBB ($M=53.81$, $SE=3.54$) scoring higher in positive affect (PA) than those consuming placebo ($M=41.12$, $SE=4.07$; $F(1,30)=5.54$, $p=0.026$), regardless of Group. A Group x Drink interaction was also observed for PA ($F(1,30)=4.93$, $p=0.035$) with ADHD WBB participants ($M=59.00$, $SE=6.04$) scoring higher on PA than ADHD placebo participants ($M=34.33$,

SE=2.19; p=0.04). No significant differences were apparent in baseline PA for Group (F(1,30)=0.09, p=0.77), nor were there any significant differences between drinks (F(1,30)=1.35, p=0.26), groups (F(1,30)=1.06, p=0.31) or in a Drink x Group interaction (F(1,30)=0.27, p=0.61) for negative affect (NA).

Modified Attention Network Task (MANT)

Baseline differences were not apparent between drinks (accuracy, F(1,31)=2.34, p=0.14; RT, F(1,31)=1.82, p=0.19) groups (accuracy, F(1,31)=2.41, p=0.13; RT, F(1,31)=0.10, 0.76) or in a Drink x Group interaction (accuracy, F(1,31)=0.76, p=0.39; RT, F(1,31)=0.09, p=0.77) for MANT accuracy or RT scores.

Auditory Verbal Learning Task (AVLT)

AVLT measure at baseline	Placebo	WBB	Drink F statistics
Word Span	4.42 (0.47)	3.17 (0.51)	F(1,32)=3.27, p=0.08 #
Final Acquisition	9.87 (0.73)	6.83 (0.78)	F(1,32)=8.20, p<0.01 *
Total Acquisition	36.83 (2.53)	28.25 (2.71)	F(1,32=5.34, p=0.028 *
Short delay recall	7.42 (0.49)	5.65 (0.53)	F(1,32)=6.03, p=0.02 *
Words learnt	5.46 (0.68)	3.67 (0.72)	F(1,32)=3.26, p=0.08 #
Proactive Interference	-0.28 (0.57)	-0.50 (0.61)	F(1,32)=0.07, p=0.80
Retroactive Interference	1.27 (0.53)	3.08 (0.57)	F(1,32)=5.43, p=0.027 *
Long delay recall	6.56 (0.71)	3.96 (0.76)	F(1,32)=6.18, p=0.019 *
Total 1-7 recall	51.98 (4.02)	34.67 (4.26)	F(1,32)=8.75, p<0.01 *
Delayed recall	6.99 (0.55)	4.80 (0.59)	F(1,32)=7.37, p=0.01) *
Recognition	9.86 (0.98)	8.52 (0.94)	(F(1,32)=0.97, p=0.33

Table 5.3. Baseline means (SD) and LMM effects of Drink on AVLT baseline measures. Placebo participants recalled more words than WBB participants at baseline for measures of word span, final acquisition, total acquisition, short delay recall, words learnt, retroactive interference, long delay recall, total 1-7 recall and delayed recall. * denotes significance at p<0.05. # denotes a trend at 0.05 > p < 0.1.

Table 5.3 shows baseline data and LMM Drink effects for each treatment group on AVLT outcome measures.

A significant main effect of Drink was observed at baseline for 7 AVLT measures: final acquisition, total acquisition, short delay, retroactive interference, long delay recall, total 1-7 recall and delayed recall. A trending main effect of Drink was evident for word span and words learnt. For all Drink main effects, placebo participants recalled more words than WBB participants (Table 5.3).

A trending main effect of Group was also evident for long delay recall; typical participants (M=6.16, SE=0.55) recalled more words than ADHD participants (M=4.35, SE=0.89; F(1,32)=3.01, p=0.09), as expected. No other significant effects of Group, or Drink x Group interactions were present.

5.3.3. Acute data

Raw data for acute dependent variables are presented in Table 5.4.

5.3.3.1. Mood

Positive Affect (PA)

Baseline PA was a trending predictor of post-consumption PA (F(1,29)=3.83, p=0.06). Neither Drink (F(1,29)=0.54, p=0.47), Group (F(1,29)=0.17, p=0.68) nor a Drink x Group interaction (F(1,29)=0.06, p=0.81) was evident.

Negative Affect (NA)

No significant predictors were evident for post-consumption NA (Baseline NA, F(1,29)=2.51, p=0.12; Drink, F(1,29)=1.76, p=0.20; Group, F(1,29)=0.08, p=0.78; Drink x Group, F(1,29)=2.63, p=0.12.)

DVs		Baseline				2 hours			
		Placebo		WBB		Placebo		WBB	
		Typical (n=10)	ADHD (n=3)	Typical (n=13)	ADHD (n=4)	Typical (n=10)	ADHD (n=2)	Typical (n=13)	ADHD (n=4)
Mood	PA	47.90 (14.07)	36.50 (0.71)	48.62 (12.00)	59.00 (12.08)	45.90 (14.69)	45.50 (13.44)	53.23 (17.84)	58.75 (11.18)
	NA	19.60 (7.82)	19.50 (3.54)	17.92 (4.11)	19.25 (4.79)	17.50 (2.12)	21.00 (8.49)	17.69 (5.38)	15.50 (1.00)
			(n=5)		(n=5)		(n=3)		(n=5)
MANT	Acc (0-1)	0.81 (0.12)	0.69 (0.11)	0.68 (0.17)	0.64 (0.11)	0.87 (0.07)	0.72 (0.06)	0.72 (0.16)	0.70 (0.13)
	RT (ms)	550.24 (142.79)	499.51 (63.01)	604.07 (108.32)	603.23 (162.15)	565.96 (148.28)	481.95 (13.53)	599.50 (106.27)	604.12 (83.76)
AVLT									
List A	Recall 1	4.64 (2.06)	4.20 (2.17)	2.83 (1.40)	3.50 (1.00)	4.09 (1.45)	4.33 (1.53)	3.08 (1.08)	4.60 (0.89)
	Recall 2	7.00 (1.26)	6.60 (2.61)	4.08 (1.62)	6.25 (1.50)	6.45 (1.92)	5.33 (1.15)	4.92 (1.08)	5.00 (2.00)
	Recall 3	7.45 (2.77)	7.80 (3.35)	5.50 (1.78)	7.00 (2.31)	6.73 (1.85)	6.67 (2.31)	5.58 (1.44)	6.20 (1.64)
	Recall 4	9.70 (2.16)	7.40 (3.78)	6.42 (2.43)	7.25 (1.50)	7.67 (1.94)	8.00 (1.73)	5.92 (2.11)	6.40 (2.07)
	Recall 5	10.55 (1.44)	9.20 (4.15)	6.67 (3.11)	7.00 (1.41)	7.73 (2.41)	7.67 (2.31)	6.45 (2.25)	7.40 (2.07)
List B	Recall 1	5.00 (1.18)	4.40 (3.05)	3.83 (1.40)	3.50 (1.73)	3.91 (0.83)	4.33 (0.58)	3.25 (1.48)	3.80 (0.45)
List A	Recall 6	9.00 (2.19)	8.20 (4.02)	5.25 (3.45)	5.33 (1.53)	6.90 (2.73)	6.33 (2.52)	3.80 (1.81)	5.20 (2.28)
	Recall 7	7.91 (1.70)	5.20 (3.49)	4.42 (3.20)	3.50 (1.29)	5.10 (2.47)	4.50 (6.36)	2.55 (2.16)	4.25 (1.71)
Word Recognition		11.11 (3.22)	8.60 (4.04)	8.83 (3.76)	9.80 (1.92)	9.56 (3.50)	9.50 (0.58)	7.00 (2.92)	8.67 (2.52)
ADHD IV	HI	50.67 (31.97)	73.50 (33.23)	63.33 (28.67)	94.33 (6.35)	--	--	--	--
	IA	55.22 (32.44)	98.00 (1.00)	65.73 (24.74)	96.25 (2.22)	--	--	--	--
	Total	52.00 (33.41)	94.00 (5.66)	60.27 (26.62)	97.00 (1.41)	--	--	--	--

Table 5.4. Raw mean data (SD) for PANAS-C, MANT and AVLT tasks for placebo and WBB, typical and ADHD participants, at baseline and 2 h post-consumption.

5.3.3.2. MANT

Correlational analyses revealed there was no significant accuracy-reaction time (RT) trade-off present at baseline (r=0.24, p=0.20). Higher accuracy was not associated with a quicker or slower RT.

Accuracy (proportion correct, 0-1)

Congruency was found to be a significant predictor of post-consumption performance; as predicted, performance was more accurate on congruent (M=0.78, SE=0.02) than incongruent (M=0.73, SE=0.02) trials, (F(1,456.37)=10.00, p<0.01). Similarly, Noise significantly predicted post consumption performance; performance was more accurate on trials with noise (M=0.77, SE=0.02) than on trials without (M=0.74, SE=0.02; F(1,430.88)=5.42, p=0.02). Baseline performance also predicted post-consumption performance (F(1,435.80)=44.78, p<0.001).

A Group x Drink x Congruency interaction was revealed (F(2,431.16)=5.59, p<0.01) with placebo typical participants more accurate than WBB typical (p=0.02) and placebo ADHD (p=0.05) participants on congruent trials. Placebo typical were also more accurate than WBB typical on incongruent (p<0.01) trials. This suggests placebo typical participants were of a higher ability than all other groups except ADHD WBB. Both placebo (p<0.01) and WBB (p<0.001) typical participants were found to be more accurate on congruent trials compared to incongruent trials, as expected. These differences between trials were not observed in placebo (p=0.15) and WBB (p=0.62) ADHD participants, suggesting these groups performed similarly on both manipulations of congruency.

Group (F(1,25.71)=2.82, p=0.11), Drink (F(1,25.83)=2.59, p=0.12), Load (F(1,430.88),0.61, p=0.44) and Target Time (F(1,433.29)=1.45, p=0.23) were not found to significantly predict post-consumption performance accuracy, nor were there any other significant interactions.

Reaction time (RT; ms)

Congruency was found to be the only significant predictor of post-consumption RT, showing quicker performance on congruent trials (M=533.33, SE=23.95) compared to incongruent trials (M=593.97, SE=23.90), (F(1,433.12)=45.18, p<0.01).

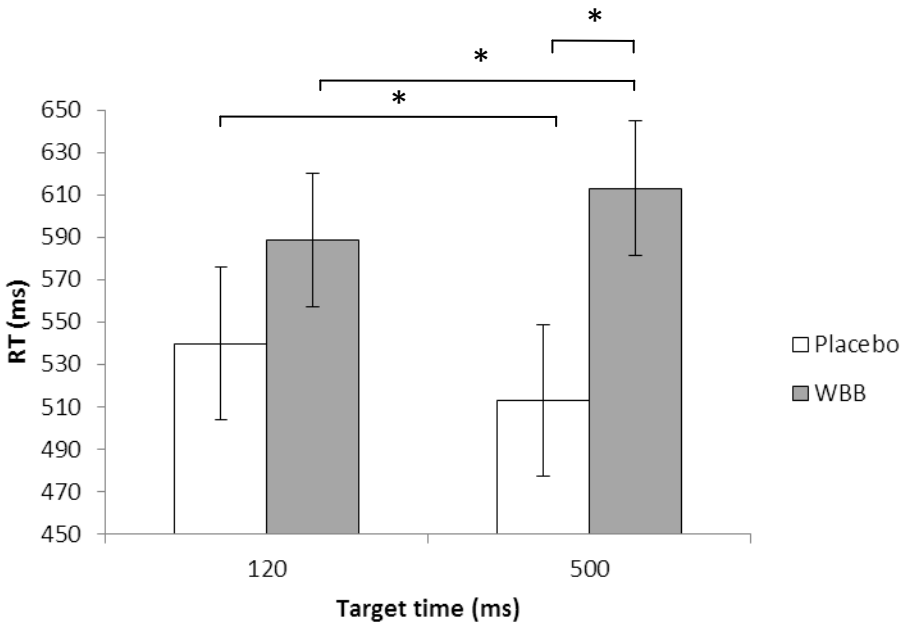


Figure 5.3. Mean (±SEM) 2 h post-consumption MANT RT scores on 120ms and 500ms trials for placebo and WBB participants. Placebo participants show significantly quicker RTs on 500ms trials, whereas WBB participants show significantly quicker RTs on 120ms trials. * denotes significance at $p<0.05$.

A significant Drink x Target Time interaction was observed ($F(1,427.60)=8.86$, $p<0.01$), as shown in Figure 5.3, with WBB participants significantly faster on 120ms trials compared to 500ms trials ($p=0.03$). Placebo participants showed the opposite pattern and were faster on 500ms trials compared to 120ms trials ($p=0.04$), and faster than WBB participants on 500ms trials ($p=0.045$). No significant interaction or pairwise comparisons were observed for performance accuracy, suggesting faster speeds occurred without any cost to accuracy.

No other factor: Group ($F(1,26.71)=0.67$, $p=0.42$), Drink ($F(1,26.95)=2.50$, $p=0.13$), Load ($F(1,427.71)=1.00$, $p=0.32$), Target Time ($F(1,430.71)=0.02$, $p=0.89$), Noise ($F(1,427.61)=0.42$, $p=0.52$) or Baseline performance ($F(1,458.60)=0.14$, $p=0.71$) predicted post-consumption RT, and there were no other significant interactions.

5.3.3.3. AVLT

	Baseline				Post-consumption (2 h)			
	Typical		ADHD		Typical		ADHD	
AVLT measure	Placebo	WBB	Placebo	WBB	Placebo	WBB	Placebo	WBB
Word Span	4.44 (2.24)	2.82 (1.47)	4.20 (2.18)	3.50 (1.47)	4.00 (1.41)	3.18 (1.08)	3.25 (2.50)	3.58 (2.74)
Final Acquisition	10.22 (1.30)	6.18 (2.75)	9.20 (4.15)	7.00 (1.41)	7.78 (2.68)	5.45 (2.46)	5.75 (4.27)	7.50 (2.12)
Total Acquisition	36.89 (8.57)	24.64 (7.99)	35.20 (14.79)	31.00 (7.07)	30.67 (8.22)	24.45 (5.65)	24.00 (17.51)	30.50 (9.19)
Short delay recall	7.50 (1.50)	4.93 (1.60)	7.04 (2.96)	6.20 (1.41)	6.33 (1.32)	4.96 (1.03)	4.80 (3.50)	6.10 (1.84)
Words learnt	5.78 (1.99)	3.36 (2.62)	5.00 (3.32)	3.50 (0.58)	3.78 (2.33)	2.27 (2.41)	2.50 (2.08)	3.50 (2.12)
Proactive Interference	-0.44 (2.70)	-1.00 (1.73)	-0.20 (2.49)	0.00 (1.83)	0.22 (1.30)	0.00 (1.55)	0.00 (1.63)	0.01 (1.53)
Retroactive Interference	1.67 (1.73)	3.36 (1.91)	1.00 (1.22)	3.00 (3.37)	1.44 (2.92)	2.64 (1.86)	1.00 (2.71)	1.50 (2.12)
Long delay recall	7.56 (1.67)	3.91 (2.81)	5.20 (3.49)	3.50 (1.29)	4.44 (3.00)	1.91 (1.70)	2.25 (4.50)	5.50 (0.71)
Total 1-7 recall	53.00 (11.64)	31.36 (12.76)	48.60 (20.67)	38.50 (6.95)	41.44 (12.91)	29.18 (7.48)	31.00 (23.64)	42.00 (9.90)
Delayed recall	3.97 (1.51)	4.42 (2.07)	6.12 (2.89)	4.85 (0.50)	5.39 (2.00)	3.44 (0.94)	3.53 (3.51)	5.90 (1.27)
Recognition	11.11 (3.22)	8.36 (3.56)	8.60 (4.04)	9.25 (1.71)	9.56 (3.50)	6.73 (2.90)	9.50 (0.58)	10.00 (1.41)

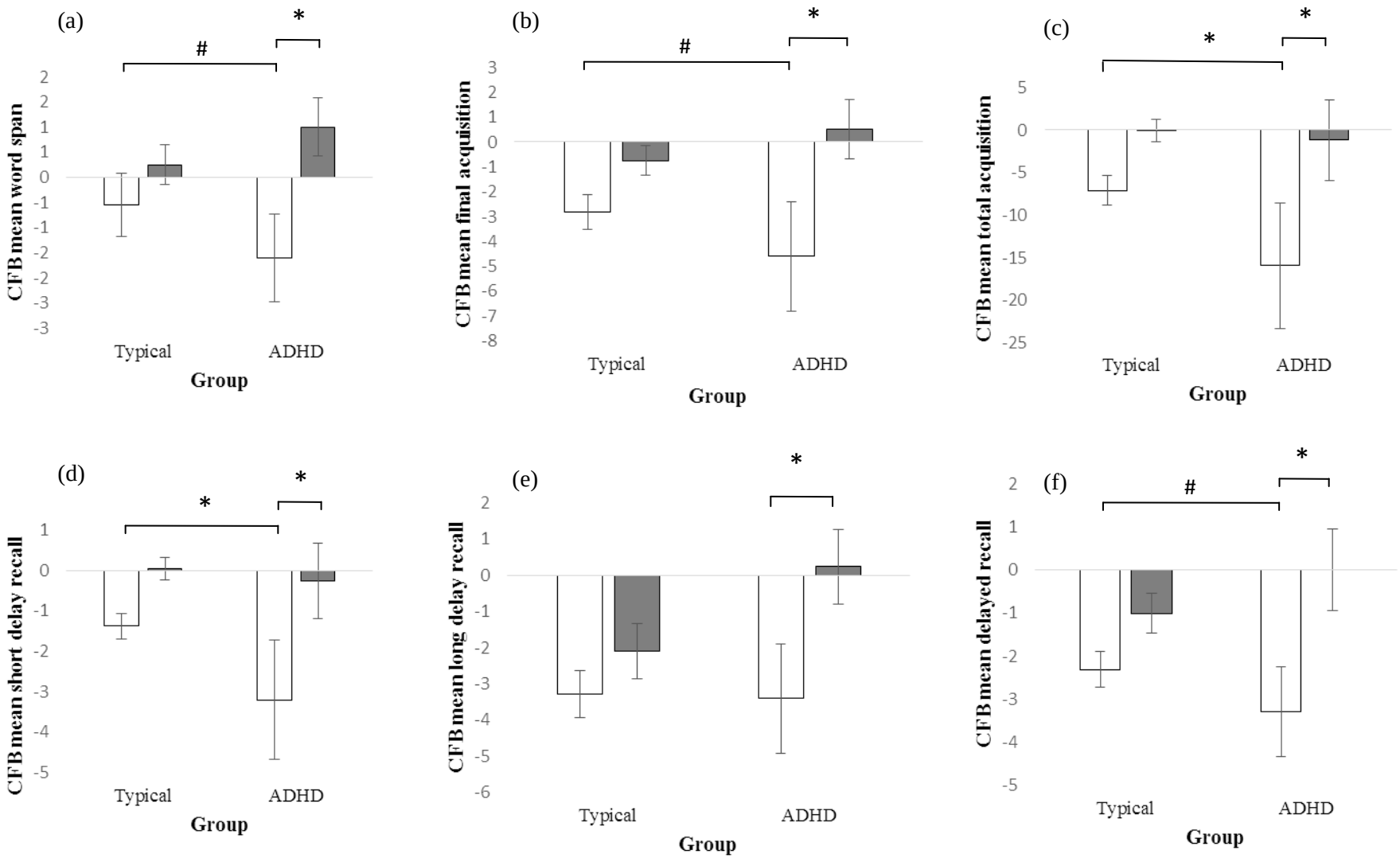
Table 5.5. Means (SD) of the 11 AVLT outcome measures by treatment and demographic group at baseline and 2 h post-consumption.

Table 5.5 shows acute AVLT outcome measure data. LMM analyses revealed Drink x Group interactions for 7 out of the 11 AVLT outcome measures: word span ($F(1,32)=6.05$, $p=0.019$), final acquisition ($F(1,32)=3.95$, $p=0.056$), total acquisition ($F(1,32)=3.54$, $p=0.069$), short delay ($F(1,32)=4.08$, $p=0.05$), long delay ($F(1,32)=3.44$, $p=0.07$), delayed recall ($F(1,32)=5.10$, $p=0.03$) and total 1-7 recall ($F(1,33)=5.26$, $p=0.028$). As predicted, ADHD placebo participants were worse on these measures when compared to ADHD WBB participants (see Figure 5.4). This implies acute consumption of WBB attenuated decline in memory performance for those with ADHD.

ADHD placebo participants also performed worse than typical placebo participants on 6 measures (word span, $p=0.06$; final acquisition, $p=0.078$; total acquisition, $p=0.01$; short delay, $p<0.01$; delayed recall, $p=0.06$); total 1-7, $p=0.02$). No significant group difference between ADHD and typical placebo participants were observed on long delay recall ($p=0.33$), or between typical and ADHD groups for WBB treatment (see Figure 5.4).

Drink was also found to predict post-consumption performance for word span ($F(1,32)=3.20$, $p=0.08$), final acquisition ($F(1,32)=4.71$, $p=0.037$), total acquisition ($F(1,32)=6.67$, $p=0.015$), short delay ($F(1,32)=6.06$, $p=0.019$), delayed recall ($F(1,32)=5.73$, $p=0.02$) and total 1-7 recall ($F(1,33)=7.99$, $p<0.01$), with WBB participants recalling more words than placebo participants in each measure, regardless of group membership.

Group predicted one measure; ADHD participants ($M=10.04$, $SE=0.81$) recognised significantly more words than typical participants ($M=8.07$, $SE=0.47$) in the visual recognition task ($F(1,28)=4.35$, $p=0.04$).


(a)
#
*
CFB mean word span
2
2
1
1
0
-1
-1
-2
-2
-3
-3
Typical
ADHD
Group
(b)
#
*
CFB mean final acquisition
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
Typical
ADHD
Group
(c)
*
*
CFB mean total acquisition
5
0
-5
-10
-15
-20
-25
Typical
ADHD
Group
(d)
*
*
CFB mean short delay recall
1
0
-1
-2
-3
-4
-5
Typical
ADHD
Group
(e)
*
CFB mean long delay recall
2
1
0
-1
-2
-3
-4
-5
-6
Typical
ADHD
Group
(f)
#
*
CFB mean delayed recall
2
1
0
-1
-2
-3
-4
-5
Typical
ADHD
Group

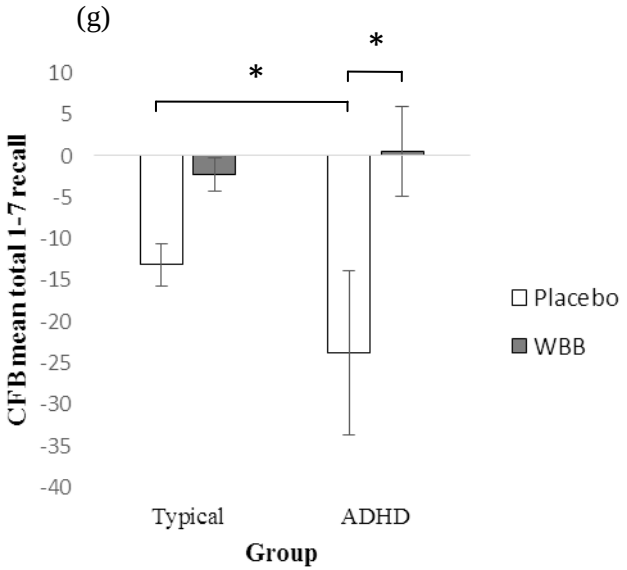


Figure 5.4. Mean (±SEM) acute change from baseline (CFB) scores for 7 AVLT performance measures in placebo and WBB typical and ADHD participants. ADHD placebo participants were significantly worse than ADHD WBB participants on measures of (a) word span (p=0.015), (b) final acquisition (p=0.015), (c) total acquisition (p<0.01), (d) short delay recall (p<0.01), (e) long delay recall (p=0.05), (f) delayed recall (p<0.01) and (g) total 1-7 recall (p<0.01). ADHD placebo participants performed worse than typical placebo participants on all measures (word span, p=0.06; final acquisition, p=0.078; total acquisition, p=0.01; short delay, p<0.01; delayed recall, p=0.06; total 1-7, p=0.02), except long delay recall (p=0.33). No such difference was observed between groups for WBB participants. * denotes significance at p <0.05; # denotes a trend at 0.05 > p < 0.1.

5.3.4. Chronic data

DVs		2 weeks				4 weeks			
		Placebo		WBB		Placebo		WBB	
		Typical (n=9)	ADHD (n=2)	Typical (n=11)	ADHD (n=3)	Typical (n=9)	ADHD (n=2)	Typical (n=11)	ADHD (n=3)
Mood	PA	44.56 (14.54)	--	46.08 (17.92)	49.00 (14.93)	42.56 (15.35)	--	41.58 (20.26)	46.67 (12.66)
	NA	18.33 (6.54)	--	18.38 (5.30)	16.33 (2.31)	17.22 (4.99)	--	17.33 (2.77)	15.67 (1.15)
			(n=3)				(n=3)		(n=4)
MANT	Acc (0-1)	0.94 (0.03)	0.80 (0.16)	0.79 (0.13)	0.88 (0.10)	0.94 (0.02)	0.73 (0.16)	0.87 (0.04)	0.80 (0.15)
	RT (ms)	573.05 (95.34)	515.33 (121.85)	565.93 (98.10)	559.73 (64.51)	560.59 (82.07)	529.71 (205.85)	578.22 (102.26)	547.69 (58.64)
AVLT List A	Recall 1	5.11 (1.62)	3.33 (3.22)	4.27 (1.68)	5.50 (2.12)	4.75 (1.75)	5.67 (2.08)	3.75 (1.58)	3.00 (2.00)
	Recall 2	5.67 (1.32)	6.33 (3.22)	5.09 (2.26)	4.00 (2.83)	5.88 (2.30)	7.67 (4.73)	4.50 (2.88)	4.00 (2.00)
	Recall 3	6.44 (2.30)	8.33 (3.22)	6.09 (2.30)	6.00 (5.56)	7.00 (2.62)	7.67 (4.04)	4.63 (3.20)	6.67 (2.08)
	Recall 4	8.33 (1.50)	5.00 (3.61)	6.18 (3.06)	7.00 (4.24)	7.13 (4.22)	7.33 (3.79)	5.63 (2.20)	6.67 (1.53)
	Recall 5	7.89 (3.33)	6.67 (3.79)	6.82 (2.68)	6.50 (4.95)	8.88 (3.00)	7.00 (4.00)	5.50 (3.96)	8.67 (3.79)
List B	Recall 1	3.00 (1.00)	2.67 (1.16)	3.00 (1.73)	3.00 (1.40)	3.75 (1.98)	3.67 (1.53)	2.75 (1.49)	1.67 (0.58)
List A	Recall 6	7.33 (3.39)	6.00 (3.39)	4.36 (3.33)	5.00 (4.24)	5.88 (2.95)	3.00 (4.36)	3.88 (4.22)	4.67 (5.03)
	Recall 7	6.33 (2.24)	6.67 (3.79)	4.18 (3.57)	3.00 (2.83)	5.75 (4.37)	4.33 (5.86)	3.75 (3.62)	4.33 (4.04)
	Word Recognition	10.11 (3.37)	10.33 (1.53)	8.85 (2.70)	8.00 (2.31)	8.13 (3.00)	10.67 (2.08)	8.50 (3.53)	9.80 (4.60)
ADHD IV	HI	43.00 (30.60)	54.00 (62.23)	50.69 (28.18)	80.25 (20.53)	42.50 (29.76)	67.67 (49.94)	34.55 (26.78)	82.00 (19.51)
	IA	52.13 (30.36)	96.33 (2.89)	56.85 (30.04)	81.00 (21.02)	47.38 (34.05)	95.33 (2.52)	54.64 (26.43)	72.75 (15.82)
	Total	45.50 (29.48)	68.65 (30.64)	54.85 (26.89)	88.75 (9.74)	43.25 (30.78)	73.00 (32.53)	44.27 (25.45)	73.00 (17.30)

Table 5.6. Raw mean chronic data (SD) for PANAS-C, MANT and AVLT tasks for placebo and WBB, typical and ADHD participants, at 2 weeks and 4 weeks into the intervention.

Raw data for the chronic trial can be seen in Table 5.6.

5.3.4.1. Mood

Positive affect (PA)

Baseline PA significantly predicted post-consumption PA, regardless of time point ($F(1,22.01)=6.65$, $p=0.017$). Drink ($F(1,21.87)=0.01$, $p=0.92$) and Time ($F(1,21.02)=0.99$, $p=0.33$) did not significantly predict PA independently or in a Drink x Time interaction.

Negative Affect (NA)

Similarly, baseline NA predicted negative affect at later time points ($F(1,21.74)=21.62$, $p<0.01$). No significant interactions were observed, nor were there any significant predictors of NA (Drink ($F(1,21.46)=0.48$, $p=0.50$); Time ($F(1,22.19)=1.84$, $p=0.19$).

5.3.4.2. Modified Attention Network Task (MANT)

Accuracy (proportion correct, 0-1)

LMM analysis revealed Group ($F(1,24.66)=6.03$, $p=0.02$) as a significant predictor of MANT accuracy performance with typical participants ($M=0.88$, $SE=0.02$) more accurate than ADHD participants ($M=0.77$, $SE=0.04$), regardless of drink or time, supporting the hypothesis that ADHD children have poorer executive function compared to typical children. As expected, Congruency ($F(1,220.30)=30.30$, $p<0.01$), Load ($F(1,212.65)=14.59$, $p<0.01$) and Target Time ($F(1,201.51)=8.46$, $p<0.01$) significantly predicted accuracy: participants were more accurate on congruent trials ($M=0.86$, $SE=0.02$) than incongruent trials ($M=0.78$, $SE=0.02$), on medium load trials ($M=0.85$, $SE=0.02$) compared to high load trials ($M=0.80$, $SE=0.02$), and on 500ms trials ($M=0.84$, $SE=0.02$) in comparison to 120ms trials ($M=0.80$, $SE=0.02$). Unexpectedly, Noise ($F(1,236.12)=0.60$, $p=0.44$) did not significantly predict performance accuracy, and neither did Time ($F(1,177.02)=0.76$, $p=0.39$).

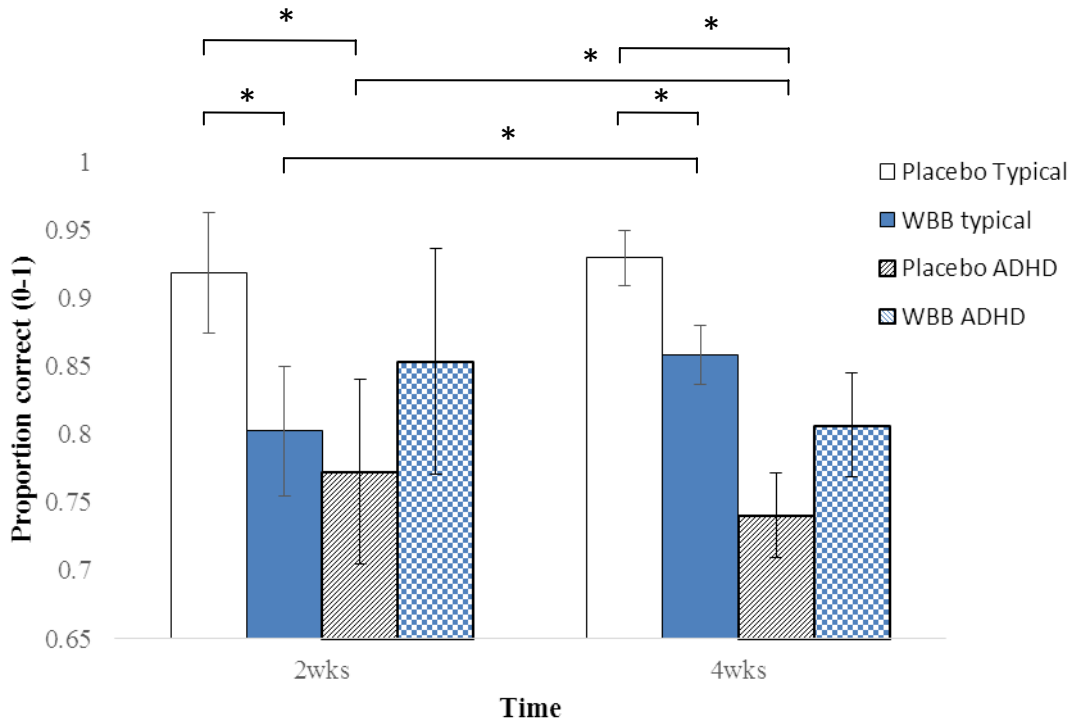


Figure 5.5. Accuracy performance (proportion correct) for placebo and WBB typical and ADHD participants at 2 and 4 weeks on high load trials. Typical WBB participants show a significant increase from 2 to 4 weeks, whereas ADHD placebo show worse performance at 4 weeks compared to 2 weeks on high load trials only. Typical placebo participants are significantly more accurate than typical blueberry and ADHD placebo participants at 2 and 4 weeks on high load trials. * denotes significance at $p<0.05$

Figure 5.5 shows group accuracy performance across the 4 week intervention for high load trials. Interestingly, there was a trend towards a significant Drink x Group x Time x Load interaction ($F(6,247.21)=2.01$, $p=0.065$): ADHD placebo participants were significantly worse at 4 weeks compared to 2 weeks on high load trials only ($p=0.026$); no such effect was observed on medium load trials ($p=0.68$). Typical WBB improvements at 4 weeks compared to 2 weeks were also detected on high load trials ($p<0.01$), with no such change for typical WBB on medium load trials ($p=0.19$). This suggests protection from decline in ADHD children, and enhancements in performance in typical children, may be most potent on trials of high cognitive demand.

Children in the typical placebo group showed significantly increased performance compared to typical WBBs on high ($p<0.01$) and medium load ($p<0.01$) trials at 2 weeks, as well as on

high (p=0.05) and medium load (p=0.01) trials at 4 weeks. Enhanced performance was also seen for typical placebo compared to ADHD placebo participants on 2 week high load (p=0.028), 2 week medium load (p=0.03), 4 week high load (p<0.01) and 4 week medium load (p=0.01) trials. Interestingly, ADHD participants were the only group who showed differences between high and medium load trials, displaying increased accuracy on medium trials for placebo ADHD (p<0.01) and WBB ADHD (p<0.01) at the 4 week time point only.

This effect was also observed as a significant Drink x Time x Group interaction (F(2,176.22)=4.63, p=0.01), and as a trend towards a Drink x Group interaction (F(1,24.72)=3.73, p=0.065).

Reaction time (RT; ms)

Congruency (F(1,212.41)=54.29, p<0.01), Noise (F(1,230.99)=4.44, p=0.036) and baseline performance (F(1,256.75)=17.96, p<0.01) significantly predicted MANT reaction time performance during the intervention. Participants were found to be faster on congruent (M=550.28, SE=19.20) than incongruent trials (M=590.84, SE=19.17), and faster on trials without noise (M=564.59, SE=19.09) compared to trials with noise (M=576.53, SE=19.31).

Similar to accuracy performance, a significant Drink x Group x Time x Load interaction (F(6,247.93)=2.09, p=0.055) showing RT changes in each group across time were most noticeable on trials of differing load, which can be seen in Figure 5.6. For typical participants, those consuming WBB were slower at 4 weeks compared to 2 weeks on high load trials (p=0.025), with no such change on medium load trials (p=0.64). This suggests a slowing of pace for the most difficult trials, in order to make a more accurate response. However, ADHD participants on WBB treatment were faster at 4 weeks compared to 2 weeks on medium load trials (p<0.01), not high load trials (p=0.40), implying these participants were able to enhance their response speed on the easier trials, without cost to accuracy.

Again, ADHD participants were the only group that showed between-load differences. Those consuming the placebo intervention tended to be faster (but less accurate) on high load trials compared to medium load trials at the 4 week time point only (p=0.08). However, ADHD WBB-treated participants were significantly faster (and more accurate) on medium load trials compared to high load trials at 4 weeks (p=0.01).

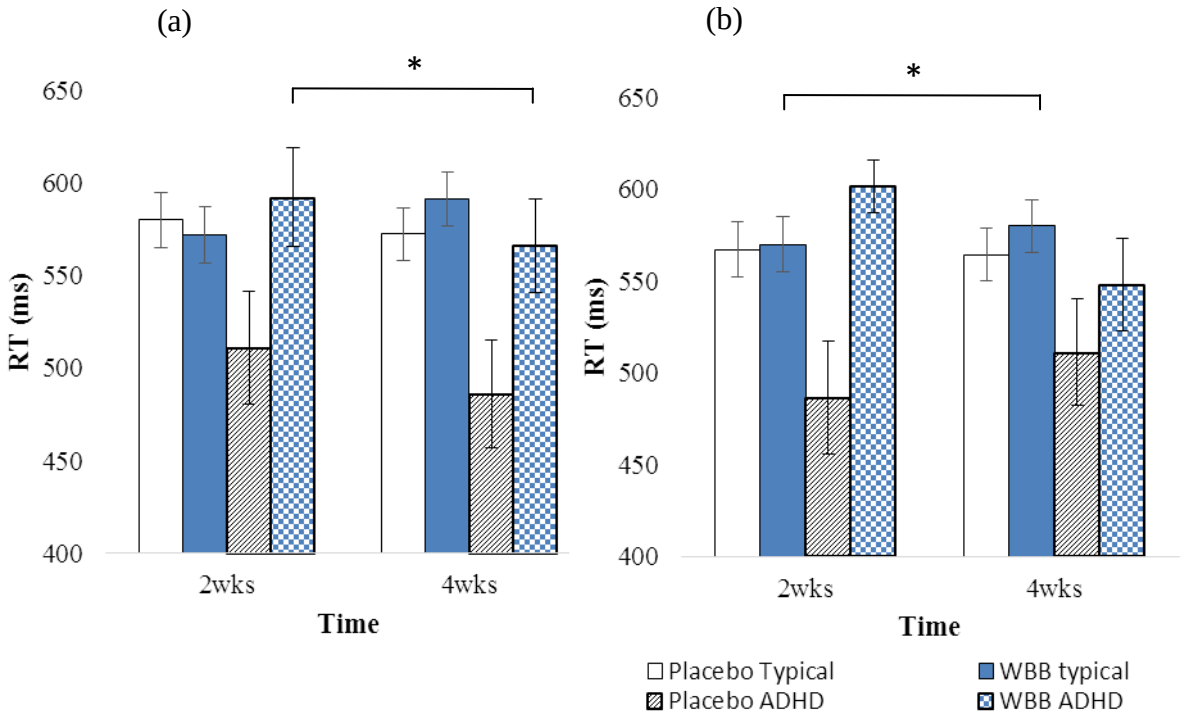


Figure 5.6. (a) MANT RT performance for placebo and WBB typical and ADHD participants on medium load trials. Significantly faster RT was evident for ADHD WBB participants at 4 weeks compared to 2 weeks **(b)** Performance for MANT RT on high load trials for placebo and WBB typical and ADHD participants. Significantly slower RT was observed in typical WBB participants at 4 weeks compared to 2 weeks. * denotes significance at $p<0.05$

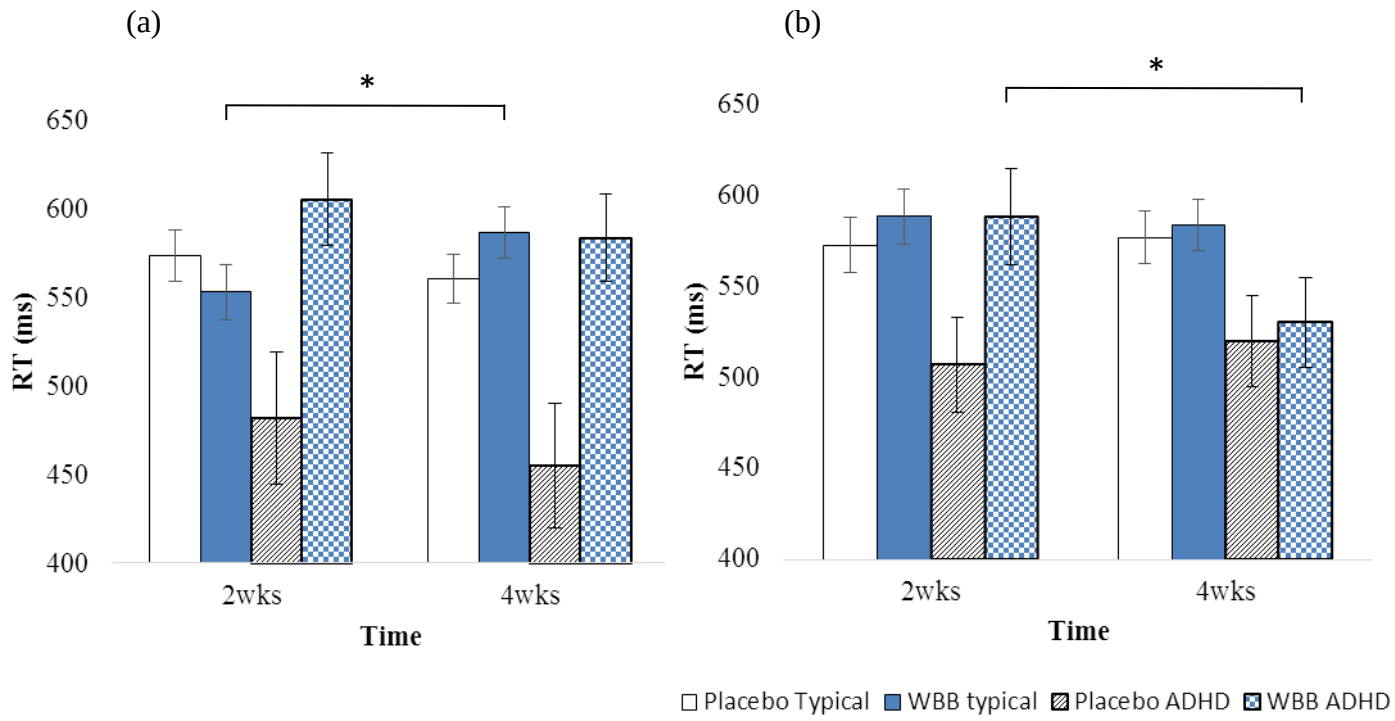


Figure 5.7. (a) MANT RT performance on 120ms trials. Typical WBB participants are slower on 120ms trials at 4 weeks compared to 2 weeks. **(b)** MANT RT performance on 500ms trials. ADHD WBB participants are faster on 500ms trials at 4 weeks compared to 2 weeks. * denotes significance at $p<0.05$.

Lastly, Figure 5.7 shows performance on 120ms and 500ms trials, where a significant Drink x Time x Group x Target Time interaction emerged ($F(6,258.07)=6.27$, $p<0.01$). Post-hoc tests showed that typical WBB participants were significantly slower at 4 weeks compared to 2 weeks on 120ms trials ($p<0.01$). This pattern was not seen for this group on 500ms trials ($p=0.85$). ADHD WBB participants, however, were significantly faster on 500ms trials at 4 weeks compared to 2 weeks ($p<0.01$), with no such trend on 120ms trials ($p=0.33$). This implies increased mental alertness for WBB participants at the end of the intervention, whereby typical participants were slower on 'fast' trials, and ADHD participants were faster on 'slow' trials.

This finding also persisted as a significant Drink x Group x Time interaction ($F(2,200.86)=5.71$, $p<0.01$).

5.3.4.3. Auditory Verbal Learning Task (AVLT)

Figures 5.8a-c show typical participants' performance on each AVLT list recall. Performance follows the expected pattern for this task; participants increase their recall ability across the first 5 presentations of list A, decrease recall at the first presentation of list B, and regain moderate recall of list A at presentations 6 and 7. It is clear that placebo participants performed at a higher recall rate to WBB participants throughout the 4 week intervention, as identified in baseline analyses (see 5.4.2).

ADHD participants' list recall performance is shown in Figures 5.8d-f, and is highly variable at each time point. At baseline (Figure 5.8d), participants display the expected pattern of performance, similar to that of typical participants (Figure 5.8a). However, at 2 (Figure 5.8e) and 4 (Figure 5.8f) weeks, there is substantial variance within treatment groups, and an atypical pattern of performance across list recalls. This might be a result of participant drop out at later time points: all 10 participants completed the baseline session, but only 6 and 7 participants completed 2 and 4 week sessions respectively.

Table 5.7 shows AVLT outcome measures at chronic time points (2 and 4 weeks).

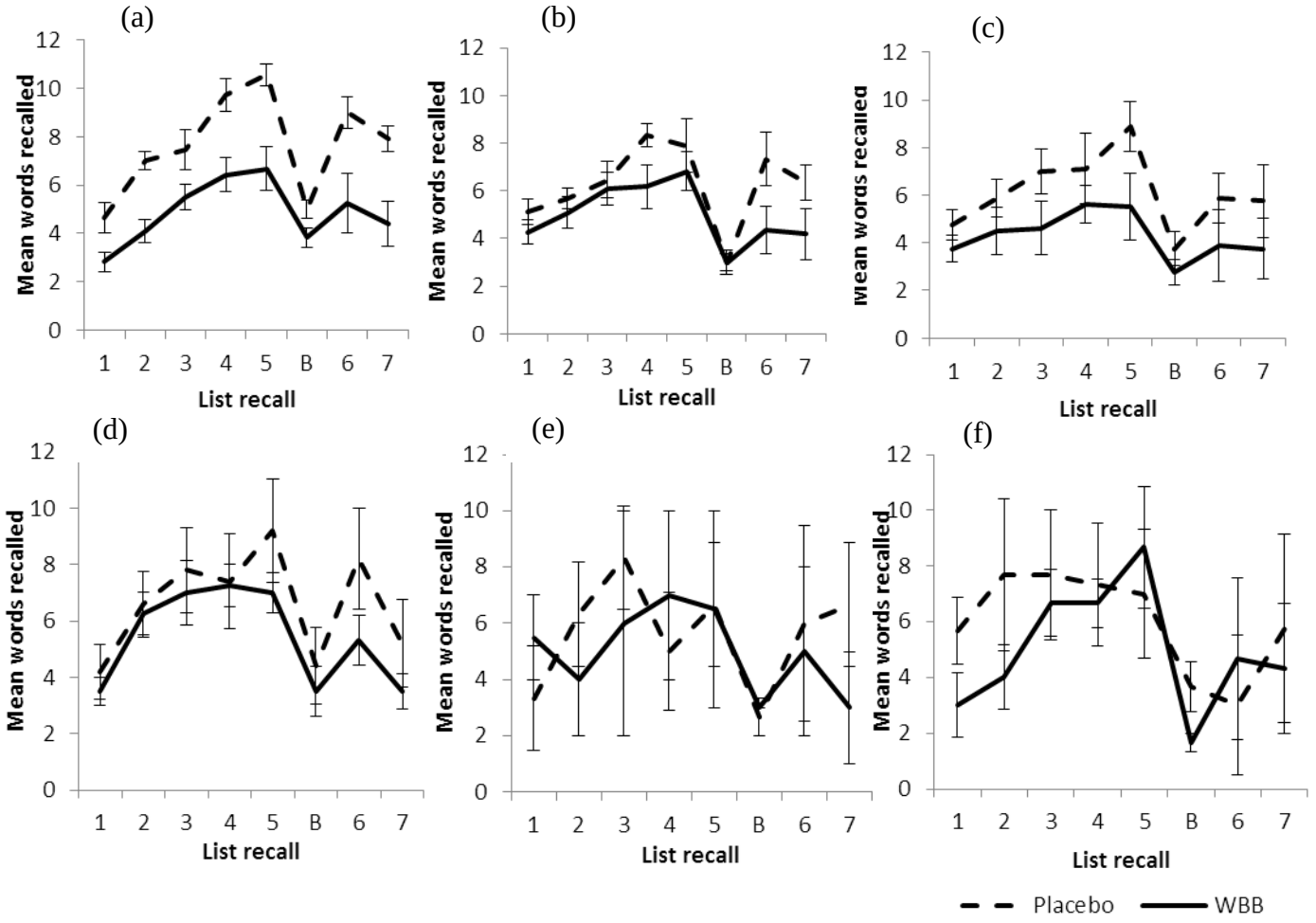


Figure 5.8. Mean (±SEM) AVLT word recall performance for typical participants at (a) baseline (b) 2 weeks and (c) 4 weeks. Performance for ADHD participants at (d) baseline (e) 2 weeks and (f) 4 weeks.

	2 weeks				4 weeks			
	Typical		ADHD		Typical		ADHD	
AVLT measure	Placebo	WBB	Placebo	WBB	Placebo	WBB	Placebo	WBB
Word Span	5.11 (1.62)	4.27 (1.68)	3.33 (3.21)	5.50 (2.12)	4.75 (1.75)	3.75 (1.58)	5.67 (2.08)	4.00 (1.41)
Final Acquisition	7.89 (3.33)	6.82 (2.68)	6.67 (3.79)	6.50 (4.95)	8.88 (3.00)	5.50 (3.96)	7.00 (4.00)	9.50 (4.95)
Total Acquisition	33.44 (7.32)	28.45 (11.01)	29.67 (16.86)	29.00 (19.80)	33.63 (12.84)	24.00 (10.98)	35.33 (18.04)	31.50 (13.44)
Short delay recall	6.69 (1.46)	5.69 (2.20)	5.93 (3.37)	5.80 (3.96)	6.73 (2.57)	4.80 (2.20)	7.07 (3.61)	6.30 (2.69)
Words learnt	2.78 (4.00)	2.54 (1.75)	3.33 (1.15)	1.00 (2.83)	4.13 (1.89)	1.75 (3.77)	1.33 (2.08)	5.50 (3.54)
Proactive Interference	2.11 (2.20)	1.27 (2.57)	0.67 (2.08)	2.50 (2.12)	1.00 (3.21)	1.00 (1.07)	2.00 (1.73)	2.00 (1.41)
Retroactive Interference	0.56 (4.56)	2.45 (3.14)	0.67 (2.89)	1.50 (0.71)	3.00 (1.51)	1.63 (2.67)	4.00 (1.73)	4.50 (2.12)
Long delay recall	6.33 (2.24)	4.18 (3.57)	6.67 (3.79)	3.00 (2.83)	5.75 (4.37)	3.75 (3.62)	4.33 (5.86)	5.50 (4.95)
Total 1-7 recall	47.11 (10.64)	37.00 (16.02)	42.33 (25.74)	37.00 (26.87)	45.25 (18.70)	31.63 (18.28)	42.67 (27.32)	42.00 (25.46)
Delayed recall	6.51 (1.63)	4.94 (2.77)	6.30 (3.55)	4.40 (3.39)	6.24 (3.19)	4.28 (2.79)	5.70 (4.51)	5.90 (3.82)
Recognition	10.11 (3.37)	8.85 (2.70)	10.33 (1.53)	8.67 (2.31)	8.13 (3.00)	8.50 (3.53)	10.67 (2.08)	11.00 (4.58)

Table 5.7. Mean data (SD) for 11 AVLT outcome measures for placebo and WBB, typical and ADHD participants, at 2 weeks and 4 weeks into the intervention.

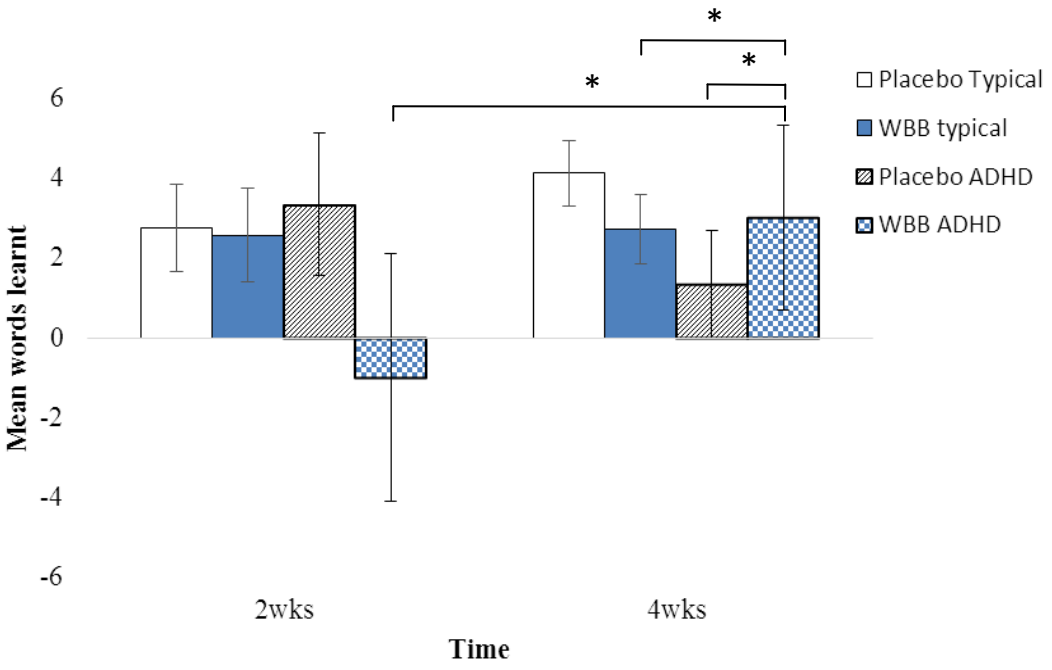


Figure 5.9. Mean (±SEM) words learnt for typical and ADHD placebo and WBB participants at 2 and 4 week time points. ADHD WBB participants were found to learn significantly more words than ADHD placebo and typical WBB participants at 4 weeks. Performance also improved across time for ADHD WBB participants, showing a trending difference between 2 and 4 week learning ability.

Figure 5.9 shows the mean number of words learnt at 2 and 4 weeks sessions for each demographic group and treatment drink. Words learnt was the only AVLT outcome measure to reveal a significant result of interest chronically; a significant Group x Drink x Time interaction was observed ($F(1,28.04)=6.27$, $p=0.018$). ADHD WBB participants learnt significantly more words than ADHD placebo participants at the 4 week time point ($p=0.01$). Interestingly, ADHD participants under WBB treatment were also found to learn significantly more words than typical WBB participants at 4 weeks ($p=0.035$). There was a trend towards ADHD WBBs' performance increasing across time, with this group learning more words at 4 weeks compared to 2 weeks ($p=0.068$). This comparison is, however, likely to be driven by a decrease in performance at the 2 week time point compared to 4 weeks (see Figure 5.9).

No significant predictors were evident for words learnt (Drink ($F(1,26.52)=0.45$, $p=0.51$); Group ($F(1,26.71)=0.17$, $p=0.68$); Time ($F(1,28.04)=1.20$, $p=0.28$)) nor were there any further significant interactions.

Time was found to be a trending predictor of RI; participants suffered increased retroactive interference at 4 weeks (M=3.36, SE=0.47) in comparison to 2 weeks (M=1.35, SE=0.87; F(1,26.32)=4.19, p=0.051), regardless of drink or group. This is indicative of a higher number of words recalled at recall 5 than recall 6, suggesting that by the end of the intervention, participants were much more susceptible to interference from List B.

No significant predictors or interactions were observed for measures of final acquisition, total acquisition, PI, short delay, long delay, delayed recall, recognition or total 1-7 recall.

5.3.4.4. ADHD IV rating scale

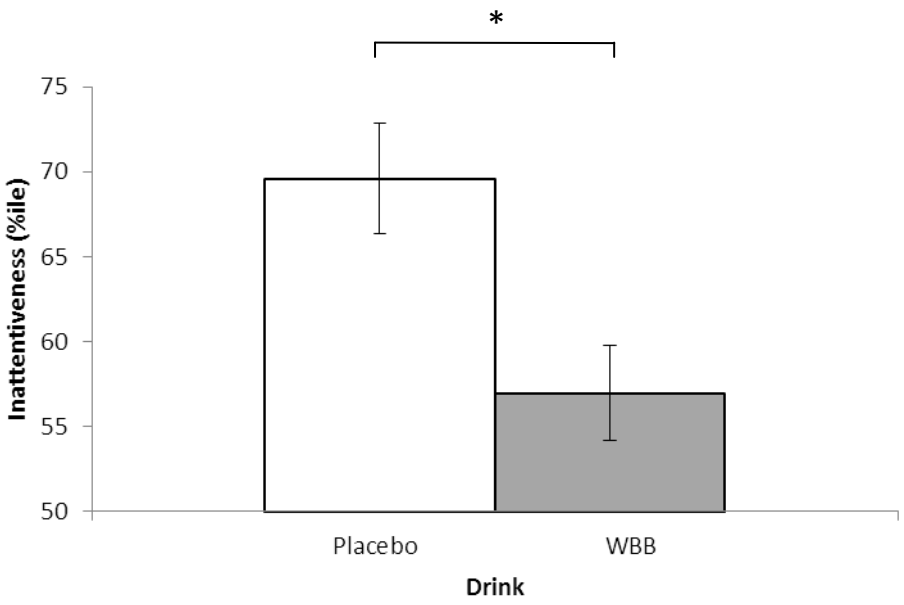


Figure 5.10. Mean (±SEM) chronic ADHD IV inattention scores for placebo and WBB participants, regardless of demographic group or time point. Parents report significantly less inattentiveness for WBB participants compared to placebo participants over the course of the intervention. *denotes significance at p<0.05.

As Figure 5.10 shows, Drink was a significant predictor of inattention (IA). Placebo participants were significantly more inattentive than WBB participants, regardless of demographic group or time point (F(1,51)=8.94, p<0.01). LMM analyses also revealed baseline IA to significantly predict later measures of IA (F(1,51)=145.45, p<0.01). Group

(F(1,51)=0.66, p=0.42) and Time (F(1,51)=0.10, p=0.76) were non-significant and no interactions were evident.

Baseline Hyperactivity-Impulsivity (HI) was found to significantly predict later HI ratings (F(1,27.13)=32.63, p<0.01). No other significant predictors were observed (Drink (F(1,27.17)=0.44, p=0.51); Group (F(1,27.17)=0.09, p=0.76); Time (F(1,24.40)=1.74, p=0.20) nor were there any significant interactions.

Baseline Total Behaviour predicted subsequent Total behaviour ratings (F(1,24.62)=104.41, p<0.01). However, Drink (F(1,23.10)=0.71, p=0.41), Group (F(1,24.58)=0.12, p=0.74) and Time (F(1,22.76)= 1.50, p=0.23) did not significantly predict Total Behaviour, nor were there any significant interactions.

5.4. Discussion

The current study administered a one-off dose of wild blueberry drink to 7-10 year old children with and without a diagnosis of ADHD following a single-blind, randomised, placebo-controlled, between-groups design, to examine the acute mood and cognitive effects of flavonoid treatment in populations of typical and reduced cognitive ability. Participants then continued treatment with a daily dose of their assigned drink for 4 weeks, to investigate the effects of chronic consumption on mood, cognition and behaviour in both populations.

It was predicted that attention and executive function (EF), as assessed by the MANT, would improve following acute WBB. This was based on findings in Chapters 3 and 4 which suggested significant Drink effects persisted 2 h postprandially. Specifically, in Chapter 3, performance for typically developing WBB participants was significantly quicker on 120ms trials compared to placebo and to 500ms trials, indicating WBB improved mental alertness on trials of a fast presentation speed. It was therefore predicted that typical WBB participants may show improvements on faster 120ms trials in comparison to typical placebo participants, or to 500ms trials. Target time effects were observed in the current sample, regardless of demographic group, with WBB participants performing significantly faster on 120ms trials compared to 500ms trials, as predicted. This infers that children under WBB treatment may be able to respond quicker to trials of a faster pace, regardless of other congruency and load variables. Although this finding shows a reduction in speed on faster trials for those consuming WBB, performance on 120ms trials was not significantly different to placebo

participants' 120ms trial performance, as observed previously (Chapter 3). This indicates the two treatment drinks produced comparable speeds in response to fast trials. Placebo participants, on the other hand, performed significantly quicker on 500ms trials compared to 120ms trials, and compared to WBB participants on 500ms trials. This suggests that placebo participants may have been unable to reduce their speed for 'fast' 120ms trials, potentially due to ceiling effects. RTs for these participants were particularly fast at the post-consumption time point (M=539.85, SE=36.10) compared to the RTs observed for placebo (M=580.45, SE=15.42) and WBB (M=542.59, SE=14.39) participants in Experiment 1, and placebo (M=601.92, SE=16.65) and WBB (M=628.93, SE=15.83) participants in Experiment 2, indicating placebo participants in the current experiment may have already reached their fastest speed of response. On the other hand, placebo participants may have been able to quicken their RT when the trial pace was slowed on the 500ms trials, when time to process the stimuli was lengthened. As no cost to accuracy was observed for either treatment drink, slower performance on 500ms trials for WBB participants cannot be attributed to a more deliberate decision to make a more accurate response. It is also important to note that this effect was observed regardless of demographic group. Participants with and without ADHD were therefore included, potentially increasing the variability of results. It would be informative, in future trials, to analyse typical participants' performance without inclusion of other demographic groups, to ascertain whether similar effects still emerge for this group alone. An exploratory LMM analysis was performed on the current dataset for the typical group only. Significant target time x drink effects endured, with significantly quicker performance observed on 120ms trials (M=581.71, SE=35.81) than 500ms trials (M=617.24, SE=35.83) for WBB participants. No significant effects were seen between trials for placebo participants, or between groups ($p>0.05$), indicating previous placebo effects may have indeed been influenced by the inclusion of ADHD participants.

Previous findings have also suggested that acute WBB may benefit children on the most cognitively demanding trials of the MANT, namely incongruent or high load trials. WBB effects persisted in Chapter 4 with improved accuracy and faster reaction times observed when WBB was consumed first, on trials of an incongruent nature, compared to placebo. Similar findings were detected by Whyte et al (2016), who found that performance was significantly better on incongruent trials and by Whyte et al (2017) who discovered higher performance on incongruent high load trials when consuming WBB compared to placebo. A prediction was therefore made that typical WBB participants would perform more accurately

and/or faster than typical placebo participants, specifically on cognitively demanding incongruent or high load trials, in the current experiment.

Unexpectedly, no WBB acute effects emerged within manipulations of congruency or load for typical participants. On the contrary, accuracy performance was found to be higher for placebo participants compared to WBB participants on congruent and incongruent trials. This is reflective of the placebo group's significantly higher general ability compared to the WBB group, as seen in analysis of BAS 3 scores. Although significant baseline differences were not revealed between drinks on the MANT, p values for both accuracy and RT were close to 0.1, and mean values indicated 13% higher performance for typical placebo participants compared to typical WBB participants (see Table 5.4). Importantly, when examining the mean data, little change was observed between baseline and 2 h for each respective treatment group. Placebo participants improved by 0.06 (proportion correct, 0-1) and WBB participants improved by 0.04 at 2 h compared to baseline, indicating a similar pattern for both treatment drinks, mostly likely due to small residual practice effects. Similarly, for RT, those consuming placebo lengthened their response by 15.72ms, whereas participants under WBB quickened by 4.57ms, again demonstrating very small, non-significant changes across time. Although using baseline as a covariate in LMM allows for a more precise estimate of treatment effects at the post-consumption time point (as discussed in Chapter 2), it is paramount for future studies to also consider the change, or lack of it, across time points when interpreting between-group differences, so as not to conflate findings.

An additional hypothesis for the MANT was included in the current study; performance was predicted to be similar across noise and no noise trials for typical participants. This was due to research proposing that typical children may not benefit from noise to the same degree as children with ADHD when completing cognitive tasks. Interestingly, background playground noise was found to increase participants' accuracy on acute MANT trials compared to trials without noise, regardless of drink or group. This rejects the hypothesis that typical children are not able to increase concentration (and therefore performance) when listening to background noise alongside a task (Söderlund, Sikström and Smart, 2007). It is particularly interesting that this effect prevailed across both ADHD and typical groups. According to the phenomenon of stochastic resonance (SR), children with ADHD should regain higher levels of concentration from noise than typical children when completing cognitive tasks. Sikstrom and Soderlund (2007) use the Moderate Brain Arousal (MBA) model to explain this finding. This is a neurocomputational model of ADHD cognition, which proposes that individuals

with low dopamine levels (as often presented in those with ADHD) may require more noise for optimal performance on a task. Task-irrelevant, environmental noise is thought to be translated into internal noise, and it is this internal noise which compensates for the reduced background neural activity and dopaminergic functioning (Solanto, 2002) in children with ADHD. However, the MBA model also predicts that noise should reduce performance for children without ADHD, due to their typically functioning neural and dopamine systems. The current results indicated that noise benefits both typical and ADHD children on an executive function task. It is unlikely that typical children would have hypo-functioning dopamine or neural systems due to the absence of psychological or developmental diagnoses in this group. It can be seen from CPT results that sustained attention and inhibition performance was similar between typical and ADHD participants, suggesting that the typical children in this sample were of a similarly low attentional capability as children with ADHD. This lowered attention may have made the typical children more susceptible to distraction (a key component of the MBA model), allowing them to also benefit from the presence of task-irrelevant noise.

Noise was not found to predict RT acutely, suggesting participants performed more accurately post-consumption at a similar speed of response. Due to these interesting results, future studies should consider including trials which vary in noise intensity to assess if there is an 'optimal' noise level to aid performance in typical and ADHD participants, respectively. Such findings could implicate a useful tool for children with and without ADHD to improve concentration during tasks which recruit executive functions.

Acute consumption of WBB was predicted to improve memory performance in typical children. This was based on prior findings from Chapter 3 and Whyte et al (2015; 2016) that showed acute WBB significantly attenuated forgetting, compared to a placebo group. In the current study, no acute effects were observed in typical participants on memory measures. Participants had comparable scores of general ability and attention, and were of similar mean age to participants in Experiment 1 and previous research, suggesting this was an appropriately matched demographic sample. Results may have therefore been due to statistical power differences between this experiment and previous research. The experiment reported in Chapter 3 found beneficial memory effects in a between-groups sample of 54. Whyte et al (2015; 2016) found significant memory effects in samples of 14 and 21, respectively, using a crossover design, implicating higher statistical power. A sample of 23, using a between-groups design in the current experiment, may therefore not have been

sufficient to detect memory effects significantly. It is reassuring however, that results are in the same direction as prior findings for typical children (see Figure 5.4) and suggest an attenuation of forgetting in the WBB group, on the majority of measures, when compared to placebo. Future trials should continue to explore memory performance in typical children following acute WBB, ensuring samples are sufficiently powered respective of between- and within-group designs.

A post-hoc repeated measures, within-between power calculation based on the current dataset (using G Power 3.0) showed that a power of 0.52 was achieved for typical participants completing each memory measure acutely, (2 repetitions: baseline, post-consumption) across the 2 treatment groups (placebo, WBB), at an effect size (F) of 0.22. To attain a statistical power of 0.9 acutely, 58 participants would have been required, supporting the notion that memory effects were indeed underpowered. A potential step to combat this would be to bootstrap data to obtain an estimate of effects with a larger sampling distribution. However, caution must be taken to not add additional variance to a between-groups study of this nature which could result in false negatives.

Results from Chapter 3 (n=54, between-groups design) have also indicated that WBB may improve positive mood in typical 7-10 year olds. The experiment in Chapter 4 did not support these findings, most likely due to the small sample size employed (n=14, crossover design). Khalid et al (2017) found significant positive mood increases 2 h postprandially in 21 young adults (18-21 years old) under an acute WBB crossover design, suggesting that effects may persist in smaller samples acutely. It was hypothesised that changes in positive mood may be observed across the same timeframe in the current experiment. Typical participants showed no significant change in positive mood at the acute postprandial time point in the current experiment. The study may have been underpowered (n=23, between-groups design), however it is worth noting that acute data did follow the expected pattern of results. At 2 h post-consumption, WBB participants increased positive affect scores by 4.61 compared to baseline. On the other hand, placebo participants decreased in positive affect by a score of 2, compared to baseline. Positive mood effects may therefore persist acutely, providing the study is sufficiently powered to detect significance. Adolescence is acknowledged as a time frame susceptible to the onset of anxiety and depression. Implication of positive mood effects would be highly meaningful for pre-adolescent children, to promote healthy emotional regulation before and during a developmental stage of risk.

It is also worth acknowledging the different activities participants were engaged in in the 2 h post-consumption period in the acute arm of this experiment and Experiment 1 (Chapter 3). In Experiment 1, participants were at school and continued academic activities following drink consumption. This may have increased the level of cognitive demand placed on children in this experiment, leading to a potential reduction in oxygen to brain regions involved in cognitive function. This may have, in turn, increased the potential for WBB to replenish oxygen levels through CBF mechanisms and improve cognition and mood. In the acute arm of Experiment 3, participants were tested at the University and were allowed to either go home or gently walk around the local area. The additional cognitive demand of school work in the post-prandial period of Experiment 1 may have increased sensitivity to detect WBB-related mood and memory effects that were subsequently not seen in the acute arm of the Experiment 3 due to a lack of cognitive engagement. Participant activity in post-consumption time periods needs careful consideration in future studies to ensure participants are exposed to the same level of cognitive demand, and could be used as an additional manipulation of load or fatigue in acute testing paradigms.

No prior research has investigated the effects of chronic WBB in a child population, and the mechanisms by which flavonoids may work chronically are still to be determined. It was predicted as in acute analyses that typical WBB participants would perform better than typical placebo participants chronically. On the MANT, this was expected to be evident on trials that were incongruent or high load based on prior findings (Chapter 4; Whyte et al, 2017). WBB effects were observed in the current chronic dataset; accuracy improvements were evident at 4 weeks compared to 2 weeks on high load trials. RT changes were also observed on high load trials, where RT slowed at 4 weeks compared to 2 weeks. No such effects were seen on medium load trials in either measure. This suggests enhancements in performance in typical children do persist chronically over a 4 week time frame, and may be most potent on trials that require higher cognitive demand, as also observed acutely by Whyte et al (2017) and in Chapter 4. A slowing of pace on the most difficult trials may have allowed participants to implement a strategy where they took longer to make a more accurate response.

Target time hypotheses were also extended to chronic data which predicted performance to be quicker on 120ms trials for typical WBB participants, based on data from Chapter 3. This hypothesis was rejected in the current chronic dataset with participants responding slower on 120ms trials at 4 weeks compared to 2 weeks under a WBB regimen. This implies decreased mental alertness for typical WBB participants at the end of the intervention on the faster trials,

without cost to accuracy, and shows that target time changes occur in a different way chronically. This implies that different mechanisms may be facilitating acute and chronic improvements. Acutely, it is believed that cognitive effects may occur due to increases in CBF, which have been found to persist at 1 and 2 h post-WBB consumption. At present a chronic MOA is undetermined, however it is possible that chronic effects may persist due to repetitive acute ingestion increasing vasodilatory response. Feliciano et al (2016b) investigated urinary metabolites before and after chronic WBB supplementation in healthy adults, and detected increases in polyphenolic metabolites on day 30 of the intervention compared to day 0, suggesting WBB-related compounds may be able to alter metabolism, cross the BBB, and initiate cerebrovascular change across 4 weeks. Such investigation in a child population has been carried out in Experiment 4 (Chapter 6). Cognitive performance has also been found to improve across acute (Whyte et al, 2015; 2016; 2017; for review see Bell et al, 2015) and chronic (for review see Lamport et al, 2012) timeframes. Future work employing an acute and chronic supplementation schedule alongside a standardised physiological measurement such as FMD or NIRS would prove useful in determining whether similar physiological and cognitive outcomes persist acutely and chronically, and whether the same cognitive effects could be emerging from similar or different mechanisms.

Noise was not assumed to predict typical participants' performance chronically, as discussed previously. Indeed, in the current experiment, noise did not predict chronic accuracy on the MANT. As noisy trials were found to warrant improved accuracy in acute analyses, this suggests the phenomenon of SR may not be stable across time or may rely on task difficulty. The tasks' complexity may have been greatest at baseline and acute sessions as the task would have still been fairly new and may have required more concentration or induced mild stress at these time points. Noise may therefore have been most effective at the acute session to improve performance under conditions of increased difficulty or stress. Interestingly, noise was found to predict RT chronically with faster performance on no noise trials, regardless of treatment drink, group or time. As this effect did not persist specifically in either typical or ADHD groups, it is difficult to tell whether one group benefitted more. However, the data suggests that performance is quicker when there is no additional environmental noise in a group of children with and without ADHD, perhaps due to less information to process. Further work should aim to investigate the effects of noise in sufficiently powered samples of typical and ADHD populations under trials of increasing difficulty to see whether SR is dependent on cognitive demand.

An interesting observation in chronic MANT data was the occurrence of load differences in ADHD participants, but the absence of these in typical participants. As expected, accuracy was significantly higher on the less demanding medium load trials compared to high load trials for those with ADHD, under both treatment drinks, at the 4 week time point. No between-trial differences occurred in typical participants at 4 weeks, in either treatment group, indicating performance on both manipulations of cognitive load was similar for typical children. This result may have been due to a combination of increased cognitive capability for typical placebo participants throughout the intervention, as discussed previously, and a WBB treatment effect over time, culminating to non-significance, regardless of drink, at the 4 week time point. Firstly, typical placebo participants may have been less affected by high load trials throughout the course of the intervention due to their higher general ability. Worse performance was observed on high load trials compared to medium trials at 2 weeks (difference of 0.035 proportion correct, 0-1), and performance was 0.026 worse for high load compared to medium load trials at 4 weeks, non-significantly. Between-trial differences were therefore insignificantly small in the typical placebo population across the course of the intervention, suggesting children may have been able to overcome cognitive demand effects prior to treatment due to their higher capabilities. WBB participants on the other hand showed accuracy increases from 2 to 4 weeks. At 2 weeks, typical WBB participants performed worse on high load trials compared to medium load trials by 0.04 (proportion correct, 0-1), non-significantly. Whereas at 4 weeks, performance on high load trials significantly increased compared to 2 weeks (as previously discussed), revealing a difference of only 0.007 between high and medium load trials. This allowed between-trial effects to disappear at the 4 week time point in this group, providing additional support for the beneficial effects of chronic WBB on typical children's EF performance.

It was predicted that chronic consumption of WBB might show similar effects on memory to acute interventions (Chapter 3; Whyte et al, 2015; 2016), with attenuation of forgetting or improvements in memory performance across the 4 week intervention. Significant chronic memory effects were not seen across the course of the current intervention. This may have been due to blueberry flavonoids being metabolised differently when consumed every day, as opposed to a one-off dose, as discussed previously. Indeed, CBF changes have only been observed acutely previously; the mechanisms by which flavonoids impact memory may not be driven by CBF changes chronically. Chronic memory effects may be influenced by an alternative MOA. Williams et al (2008) and Rendeiro et al (2012b) have shown chronic memory effects in rodent models following blueberry intervention (as discussed in Chapter

1). They also revealed chronic changes in BDNF, ERT and AKt pathways. This suggests that blueberries may act on memory systems by enhancing synaptogenesis.

It was hypothesised that acute increases in positive mood that have previously been observed (Chapter 3; Khalid et al, 2017), may persist chronically. Typical participants showed no significant changes in PA under a chronic intervention schedule. Expected data trends were not observed chronically, with participants reporting less positive affect at 2 weeks, and even lower PA at 4 weeks for both treatment groups, in comparison to baseline. Lower PA scores across time may be indicative of increased boredom of completing the scales at each test session; indeed, by 4 weeks, participants had completed 5 repetitions of the PANAS-C. Although this measure has been found to be stable in detecting mood changes across multiple time points, participants may not have been providing the true effort required to assess current mood. Anecdotally, participants tended to complete the PANAS-C quicker at each subsequent test session compared to baseline, indicating participants may have been responding with less effort each time they completed the scale. We would not expect this to be an effect of increased familiarity over time, as this would have been significantly reduced by the practice session. Future trials should therefore encourage participants to fully engage with every PANAS-C item to assess how they feel, and use the full 5 minutes to complete the questionnaire.

Predictions for participants with ADHD were similar to those employed for typical participants. This was based on preliminary unpublished findings from our lab that shows WBB may improve cognition to a similar extent in an ADHD population. Additionally, as attentional benefits have been observed in typical children previously, and as children with ADHD lack attentional and working memory abilities, it was hypothesised that WBB may enhance performance on tasks measuring attention and memory, such as the MANT and AVLT, for those with ADHD. No significant acute treatment effects emerged in the ADHD group on MANT measures in the current study. The usual pattern on the MANT is higher accuracy on congruent trials compared to incongruent trials, which was observed after both treatment drinks in typical participants in the current study. However, it was interesting to observe that ADHD participants did not show expected differences between congruent and incongruent trials, for both treatment drinks, suggesting they performed similarly on both manipulations of congruency. On exploring the means, participants with ADHD consuming placebo did follow the expected pattern, showing reduced accuracy on incongruent trials compared to congruent trials. However, those with ADHD consuming WBB displayed the

opposite pattern and were more accurate on incongruent than congruent trials. Although non-significant, and in a small sample, this suggests that children with ADHD may process congruency variables differently to typical participants on the MANT acutely. Future work should continue to test the hypothesis that performance may be better on trials requiring high cognitive demand in typical populations, but should consider how ADHD populations respond to the task without treatment so that correct assumptions can be made regarding cognitive demand.

Memory performance was predicted to improve following acute WBB for children with ADHD due to previous benefits in typical child populations (Chapter 3; Whyte et al, 2016). Memory scores were expected to lower across a 2 h period as children were performing a different version of the same task within a short time frame, which could lead them to feel bored or tired. Indeed, reductions in memory performance have been observed in typical children across a 1.15, 3 and 6 h period (Whyte et al, 2015; 2016), and 2 h period (Chapter 3) previously. Specifically, placebo ADHD participants were expected to forget a larger proportion of words post-consumption, due to increased inattentiveness and distractibility in this population, whereas WBB consumption was predicted to attenuate forgetting by improving attention to learn and recall words. In the current dataset, this hypothesis was accepted; ADHD participants consuming acute placebo were found to be significantly worse on memory measures when compared to ADHD WBB participants. This implies acute consumption of WBB attenuated forgetting for those with ADHD. This holds promising implications for this population and could indicate WBB as a potential acute CAMs that could improve daily tasks involving memory; for example, information learnt at school or key details regarding family or friends, potentially positively impacting academic achievement or social functioning. However, such implications do need to be made with caution due to the small sample of ADHD participants included in this study, especially as sample size reduced from 10 to 8 in the ADHD placebo group at the 2 h time point. Assessment of memory 2 h postprandially in a sufficiently powered sample would provide a more informed interpretation of acute memory effects before recommendations can be made.

As with typical participants, ADHD participants were also predicted to show positive WBB effects on the MANT under a chronic regimen. Beneficial WBB effects were predicted to emerge on 120ms trials on the MANT, as in acute data and findings from Chapter 3. Interestingly, this hypothesis was rejected. ADHD WBB participants were significantly faster on 500ms trials at 4 weeks compared to 2 weeks. No such findings were evident on 120ms

trials, or for ADHD placebo participants. This implies that participants had increased mental alertness at the end of a 4 week WBB intervention on the slower trials. This may have been due to a lengthened time to process information on 500ms trials. Indeed, children with ADHD have been found to have reduced processing capabilities compared to typical children (Barkley, 1997). 120ms may therefore have been too fast for children with ADHD to quicken their response time. It would be informative in the future to collect MANT data in an ADHD population free from treatment, to ascertain whether cognitive demand assumptions are similar to typical populations. As children with ADHD have various cognitive deficits, including reduced attention, executive functioning and processing capabilities, all of which are encompassed within the MANT, performance may be more variable and show differing results under different cognitive load manipulations that researchers are not yet aware of.

MANT improvements were also expected for WBB compared to placebo ADHD participants on trials requiring increased cognitive demand such as incongruent or high load. Chronic effects were seen in the current experiment in this population. ADHD participants consuming chronic placebo were significantly worse at 4 weeks compared to 2 weeks on high load trials. This suggests that performance was maintained for those consuming WBB on trials requiring increased cognitive demand across the intervention, and WBB participants did not succumb to the cognitive decline that placebos did. Additionally, ADHD participants on WBB treatment were significantly faster at 4 weeks compared to 2 weeks on medium load trials. This effect was not observed on high load trials. This implies that WBB participants were able to enhance their response speed on the easier trials, without cost to accuracy. This holds intriguing implications for children with ADHD who exhibit reduced attentional capabilities. A daily dose of WBB may help sustain attention in cognitively demanding situations and improve processing speed in less demanding conditions after only 4 weeks. However, these implications are limited, due to the small sample tested, and require more data to determine whether findings are replicable.

In addition, a trend occurred in load analyses where ADHD participants consuming placebo were faster, and less accurate, on high load trials compared to medium load trials at the 4 week time point, suggesting they responded more impulsively and at a cost to accuracy. However, ADHD WBB-treated participants were faster, and more accurate, on medium load trials compared to high load trials at the end of intervention. As accuracy for both placebo and WBB ADHD groups was higher on medium load trials at 4 weeks, it could be deduced that WBB ADHD participants were able to enhance their speed and accuracy on the easier trials

after 4 weeks of intervention. Placebo ADHD participants were not able to improve both performance measures simultaneously; they were either able to enhance their accuracy on easier medium load trials or their RT on more difficult high load trials.

Chronic memory effects were expected to occur in an ADHD population following 4 weeks of WBB intervention, based on prior acute findings in typical populations (Chapter 3; Whyte et al, 2015; 2016). At the end of chronic intervention, ADHD WBB participants learnt significantly more words than ADHD placebo participants, suggesting an increase in encoding and recollection capabilities. It is important to note that this can only be interpreted as effective for recalling simple material. For example, the stimuli learnt and recalled in the current study were age-appropriate, 1-2 syllable words, independent from the context of a sentence. Therefore, it cannot be assumed that WBB may improve learning and recollection of educational material without further investigation using such stimuli. Results could be translatable to an educational environment where children learn and recall information for end of term tests, for example memory or spelling tests. However, a larger sample of data is required to make such inferences.

Interestingly, at the end of WBB intervention, ADHD participants were also found to have learnt significantly more words than typical WBB participants, suggesting ADHD participants did not reach the same capability as typical participants, but exceeded them following WBB intervention. In the current sample, ADHD participants were, unintentionally, significantly older than typical participants by 1.17 years, however it is unlikely that this age difference would have made a difference to results. All participants were within the stipulated age range (7-10 years) where a spurt in cognitive development has been theorised (Anderson, 2002), and prior research has not indicated specific WBB memory effects occur by small increments in age (Whyte et al, 2015; 2016). Additionally, ADHD participants often show cognitive deficits, specifically in working memory domains, implying that an advance of 1 year in age may be confounded by an overall reduction in memory capabilities compared to their peers. Higher word learning in ADHD participants holds promising implications for chronic WBB supplementation in this population and should be explored further in better powered intervention studies.

Improvements in parent-rated behaviour were predicted for children diagnosed with ADHD. Previous trials employing these measures across ADHD CAMs interventions have shown that observable behavioural improvements can occur following nutritional supplementation, as assessed by both parents and teachers. It was predicted that positive effects of WBB may

manifest behaviourally as well as cognitively. No behavioural effects were observed for ADHD participants specifically, however, regardless of demographic group membership, placebo participants were rated by parents as being significantly more inattentive than WBB participants overall. When consulting the raw means, placebo participants' inattentiveness remained constant across the course of the intervention, whereas WBB participants showed a decline across time. Although these results do not emerge as a significant Drink x Time interaction it can be inferred that placebo participants do not get more inattentive, but rather WBB participants become less inattentive across the 4 week WBB intervention trial. This holds promise for WBB in terms of improving observable attentional capabilities in the home across a short period of time in children with and without ADHD, and indicates potential for WBB to initiate and maintain long-term behaviour change. Such findings imply that WBB could aid children in the school environment, to maintain their attention and subsequently engage further in academic material. To investigate this further, teacher-rated scales could be implemented in future trials to ascertain whether effects do extend to the learning environment.

Although parent-rated behaviour appeared to change across the intervention, expected baseline differences between demographic groups for sustained attention or inhibition using the CPT did not emerge. This is surprising as the CPT has been used in many ADHD populations who have shown higher omission and commission rates compared to typical participants (Conners, 2004). A reason for the lack of differences in the current study could be due to the modifications performed to the task. Stimuli were modified from letters to coloured circles with the aim to remove lexical confounds such as atypical or slower grapheme-phoneme representations in typical or ADHD participants. Results suggest that stimuli manipulation may have reduced the sensitivity of the task to accurately detect failures in commission and accuracy in omission trials. It must also be noted that the sample size for the ADHD group was small, which may have led to the lack of statistical differences between this population and typical children.

Overall, the effects of the current study are promising and demonstrate WBB benefits may occur acutely and chronically in children with and without developmental disorders such as ADHD. There is a pressing need for more research to investigate the chronic effects of WBB on mood, cognition and behaviour in typical children. Although exploration into disordered populations is equally essential, an understanding of the mechanisms and effects underlying chronic WBB supplementation in a typical sample is critical. As it is currently understood,

acute WBB intervention may improve mood and cognition via increases in cerebral blood flow (Vauzour et al, 2008; Spencer et al, 2009). Physiological changes to the way the body metabolises flavonoids may be a potential indicator for any observed chronic benefits (Rodriguez-Mateos et al, 2013; 2016). Although trials in adult populations have been undertaken, no studies to date have examined the effects of WBB on metabolism in a child population. Furthermore, witnessing high levels of flavonoid metabolites, in plasma or urine, within a similar time frame to cognitive or mood benefits, may be an invaluable marker for whether flavonoids are crossing the blood-brain barrier and potentially moderating such effects. Future investigations should therefore focus on measuring mood, cognition and behaviour alongside physiological measures of flavonoid metabolism (e.g. from urine) following a daily dose of WBB or matched placebo in a typical child population.

Chapter 6: The chronic effects of blueberry flavonoids on cognition and urinary metabolites in 7-10 year old children.

The role of other researchers in this chapter: A. Rodriguez-Mateos provided valuable input and advice on study design, procedure and expertise in the metabolite field. G. Istas provided training in metabolite quantification techniques and data support. R. Feliciano processed urinary samples in Dusseldorf, Germany.

6.1. Introduction

It is clear from the literature discussed so far, and the previous chapters of this thesis, that flavonoids are able to produce cognitive effects. Now, it is important to identify the critical metabolites that may circulate in the body once flavonoids are ingested to ascertain bioavailability. This is important as it may show circulating metabolites of interest at the time of previous vasodilatory and cognitive effects, potentially highlighting a window where flavonoids may be able to cross the blood-brain barrier and impact cognition. Recent exploration has focused on the absorption, metabolism and excretion of berry polyphenols, and their bioavailability within blood plasma and urine. The specific breakdown processes that occur after ingestion of berry polyphenols might provide insight into which metabolites become 'bioavailable' and when they are in active circulation for the body to use. Moreover, detection of polyphenolic metabolites in circulation is an indicator of distribution of these metabolites to many different areas of the body via the bloodstream. At present, the literature is inconclusive regarding whether flavonoid metabolites are able to cross the BBB, although research has suggested polyphenols can enter the brain endothelium and are found in brain tissue, as discussed in Chapter 1. The specific mechanisms by which plasma and urinary metabolites may be distributed to areas, such as the brain, requires further exploration.

As discussed in Chapter 1, many studies have primarily focused on the acute bioavailability of blueberry polyphenols within plasma, and have identified 23 compounds of interest (Rodriguez-Mateos et al, 2013; Rodriguez-Mateos et al, 2014b). However, little work has investigated the phase II metabolism of blueberry polyphenols in urine, or if plasma and urinary concentration of metabolites differ after chronic supplementation. In Feliciano et al's (2016b) study on adult males (mean age 33 ± 18), 29 of the 62 phenolic metabolites detected in 24 h urine samples were at a higher concentration, albeit non-significantly, on day 30 compared to day 0 following 30 day supplementation with 11g/day freeze-dried WBB powder

(302 ± 6mg total (poly)phenols; 150 ± 3 mg anthocyanins). Specifically, levels of catechols, benzoic, hippuric, cinnamic and phenylacetic acids had increased relative to baseline. These findings indicate that sustained consumption of WBB across 1 month may lead to increased absorption and excretion of individual phenolic compounds over time. Similar increases in polyphenolic metabolites have also been observed in plasma after a 12 week daily bilberry intervention (Hanhineva et al, 2015) and in urine after an 8 week high-polyphenol diet intervention (Vetrani et al, 2016). From these results, it can be postulated that blueberry polyphenols are metabolised in-part via conjugation, and that such conjugates will be detected in 24 h urine samples after chronic supplementation. It is important to note that an increase in polyphenolic metabolite excretion at the end of an intervention is not necessarily reflective of a change in metabolic excretion across time. For example, if participants start with a higher concentration of metabolites and finish an intervention with similarly high levels, this does not tell us how much the treatment has changed metabolism across the intervention. To ascertain the degree of metabolic change it would be necessary to also assess change in concentrations across time relative to baseline. This would help account for individual differences in metabolite excretion and increase precision in estimates of treatment effects. Furthermore, the research to date has focused on metabolism in adults; little is known about the similarities and differences in polyphenol metabolism between adults and children.

At present, the only RCTs conducted investigating the physiological mechanisms underlying the beneficial effects of polyphenol consumption in children involve assessing glutathione in those diagnosed with ADHD. As mentioned in Chapter 5, glutathione is a major antioxidant that protects the brain from oxidative stress (OS). An increased ratio of reduced glutathione (GSH): oxidised glutathione (GSSG) demonstrates healthy cell function. Dvořáková et al (2006) found that in children diagnosed with ADHD (6-14 years), the ratio of GSH: GSSG was improved following a one month intervention with Pycnogenol (a polyphenolic-rich pine bark extract; 1mg/kg body weight/day), suggesting intervention with polyphenols may have improved the ratio and reduced OS. In a further study, Dvořáková (2007) discovered that concentrations of urinary catecholamines were normalised in children with ADHD, compared to high levels at baseline. Reductions in hyperactivity were also observed on parent and teacher rating scales (results reported in Trebaticka et al, 2006). Both studies highlight the feasibility of collecting metabolic data from children, and also indicate a potential link between consumption of polyphenols, behaviour change, normalisation of catecholamine concentrations and reductions in oxidative stress.

To date, no data has been published investigating a plasma or urinary metabolite profile following berry consumption in children. A study of this kind could provide insight into whether metabolism of berry polyphenols differs across age groups. It could also help to elucidate a mechanism of action by which cognitive and mood improvements have been observed in 7-10 year olds (Whyte et al, 2015; 2016; 2017; Khalid et al, 2017; Barfoot et al, 2018). Better understanding of changes in polyphenol excretion will inform us when metabolites are in circulation, and potentially crossing the BBB, at which point they have the potential to affect cognitive function. Furthermore, little research has examined the bioavailability of nutritional interventions within a child population, with no research to date investigating the bioavailability during WBB intervention. Thus, this study is one of the first of its kind to combine measurements of cognitive performance with metabolite bioavailability.

In the current study, healthy 7-10 year old children received a daily dose of either a 13.3g WBB drink (766mg total polyphenols; 253mg anthocyanins) or a placebo-matched control drink for 4 weeks. Measures of metabolite excretion were taken at baseline, 2 weeks and 4 weeks using 24 h urinary collections. Urinary excretions covering a 24 h period were used as they reflect the pool of circulating plasma metabolites in the body, from the small and large intestine (Spencer et al, 2008; Rodriguez-Mateos et al, 2016). This method is also less invasive than collections of blood plasma in a child population and is thus more acceptable to parents and children. It was predicted that children would show similar WBB-related metabolic activity to adults following chronic intervention, excreting higher levels of benzoic, hippuric, cinnamic, phenylacetic acids and catechols at day 28 compared to day 0, as in the adult literature (Feliciano et al, 2016). Measures of cognition were also recorded at baseline, 2 weeks and 4 weeks to ascertain the relationship between polyphenolic metabolite excretion and cognitive function. It was hypothesised that participants following the WBB schedule would show chronic improvements in EF, as in Experiment 3 (Chapter 5). Specifically, higher performance was expected for WBB participants on the most cognitively demanding high load or incongruent trials. Although measurement of urinary metabolites will give an indication of the bioavailability of polyphenols in a child population within the 24 h period after consuming an intervention, the direct mechanism between bioavailability and cognitive change cannot be posited. This is due to the varying mechanisms in between circulation and presence in the brain that metabolite measurement does not capture. Correlational results are exploratory and should therefore be interpreted with caution.

As mentioned above, it is also important to use a measure of metabolic change as well as raw excretion to detect change in metabolism across time. Therefore, the relationship between changes in raw excretion between day 0 and day 28 will also be considered to help determine the biological mechanisms underpinning changes in metabolism across time.

6.2. Methods

The research was reviewed and given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (UREC 15/10, UREC 15/58) and was conducted in accordance with the Declaration of Helsinki and Human Tissue Act 2004.

6.2.1. Participants

The sample size calculation used previous metabolite research (Rodriguez-Mateos et al, 2016; Feliciano et al, 2016b) which used 18 and 9 participants, respectively, to inform the current experiment. An N within this range (9-18) was deemed appropriate due to the large amount of individual variation across urinary metabolite measurements found both within and between participants. An *a priori* repeated measures, within-between power analysis using GPower 3.0 was also performed to determine the required sample size for cognitive effects. This analysis showed that, using an alpha level of 0.05, an effect size (F) of 0.22 and a power of 0.9, 46 participants (N=23 per condition) would have been required to detect effects across the 2 treatment groups and 3 repetitions (baseline, 2 weeks, 4 weeks) ($F(2,88)=3.10$). Sixteen participants aged 7-10 years old ($M = 8.48$, $SD = 0.96$) were recruited from the Berkshire area, UK, however one participant withdrew from the study making the total recruited number 15 (8 female). On conducting a post-hoc power analysis, this sample size resulted in a power of 0.4 ($F(2,26)=3.37$). No participant had any food-related allergies or other health conditions, e.g., diabetes, obesity, blood pressure, thyroid, kidney and liver diseases, or psychological diagnoses e.g. ASD, ADHD, which would have excluded them from the study. Demographic data is presented in Table 6.1.

	Placebo (n=7)			WBB (n=8)			
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**P values**
Age	8.46	0.98	7.02-9.07	8.30	0.88	7.00-9.08	0.73
Gender (M:F)	4:3	-	-	3:5	-	-	-
CPT							
Omissions (%)	9.88	4.34	4.58-17.08	4.64	2.87	0.42-8.33	0.02*
Commissions (%)	65.71	20.79	40-93.33	52.92	16.76	23.33-73.33	0.21
BAS 3	52.14	3.13	50-59	52	7.46	40-63	0.96
Taste ratings	9.57	1.13	7-10	5.75	2.82	1-10	<0.01*
Habitual fruit & veg intake [a]	3.67	2.22	0.33-6	4.43	1.12	2.67-5.33	0.43

Table 6.1 Demographic data for placebo and WBB participants. * denotes significance at $p<0.05$; [a] Measured as portions per day.

6.2.2. Design

The study adopted a between-groups, single-blind, placebo-controlled, chronic 4 week design (Figure 6.1). A between-groups design was used in keeping with previous research to minimise demands on participants and their families (Khalid et al, 2017). All children took part in a screening and practice session one week before the baseline test session. The baseline session involved completion of mood and cognitive tasks, followed by treatment consumption. Supplementation then continued daily for 4 weeks, with participants taking part in further cognitive test sessions at 2 and 4 weeks. Twenty four hour urine samples were collected at baseline, 2 weeks and 4 weeks.

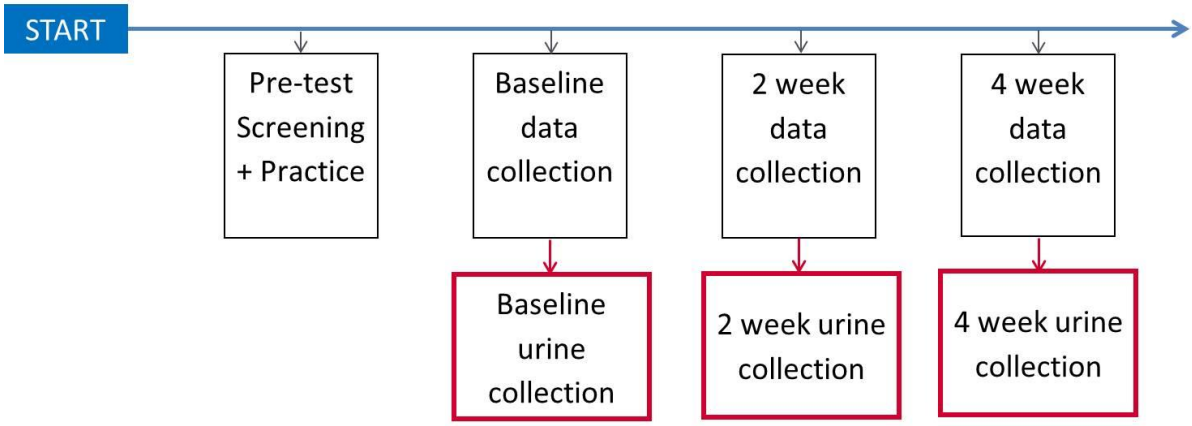


Figure 6.1 Between-groups study design comprising cognitive, mood and urine data collection at baseline, 2 weeks and 4 weeks

6.2.3. Treatment

Prior to screening, participants were randomly allocated to receive either one of two treatment drinks; a wild blueberry (WBB) drink (n = 8; 5 females; mean age = 8.30, SD = 0.88) or a matched placebo drink (n = 7; 3 females; mean age = 8.46, SD = 0.98).

The wild blueberry drink contained 253mg anthocyanins and was prepared by mixing 170ml water, 13.3g of freeze-dried WBB and 30ml of low-flavonoid Rocks Orange Squash. The placebo drink was matched to the WBB drink for sugars (4.79g fructose, 4.52g glucose), vitamin C (45mg), Rocks Orange Squash (30ml) and water (170ml). Drinks were presented in an opaque cup and consumed through a black straw to maintain blinding procedures.

All baseline treatment drinks were prepared in a food hygiene-approved kitchen at the University of Reading immediately prior to consumption. For the chronic 4 week treatment period, placebo and WBB interventions were weighed, packaged and stored at -18°C by the researcher in the University of Reading kitchen, and were distributed to participants as 2 x 14 daily sachets, alongside a 500ml bottle of Rock's Orange Squash, a 30ml measurer, an opaque flask, straws and preparation instructions. Chronic procedures were in compliance with storage recommendations; all participants' parents/guardians confirmed that the treatment sachets were retained in a freezer throughout the intervention period. All chronic interventions were prepared daily at the participants' own homes by their parent(s)/guardian(s), and were consumed within 20 minutes of preparation. It is likely that parents had an idea of what treatment they were preparing due to the differences in colour (orange for placebo and purple

for WBB). However, as this was a between-groups design, parents would not have been exposed to the alternative drink. Additionally, the study was consistently described as a 'fruit drink' study meaning both drinks would have fitted into that description.

6.2.4. Measures

Screening measures

The screening session was carried out to familiarise participants with the experimenter and the format of the sessions. At screening, participants completed a modified Continuous Performance Task (CPT) on a computer which was used to assess, but not diagnose, possible attentional deficits. Differences in CPT performance between-groups were examined, but data was not used in any further analyses of the current study.

Three sub-tests of The British Ability Scale 3 (BAS 3; Elliott, Murray and Pearson, 1979; Elliott, 1997) were administered at screening to measure general cognitive ability (see Chapter 2 for further detail). This measure was included to ensure all participants were of normal cognitive functioning for their age. This was done by observing whether all subscale scores were within the lower and upper ability limits set for the child's age and gender (representing 68% confidence interval for the obtained score). All participants were within these parameters in the current sample.

A practice of the PANAS-C and cognitive task battery was also performed at screening to ensure understanding of the tasks and to reduce practice effects. These practice data were not analysed.

A taste rating scale was distributed to participants at the end of the screening session to assess their likeability of the treatment drinks. This consisted of a 10-point Likert scale ranging from '1 – It was horrible' to '10 – I loved it'. Children were required to taste both treatment drinks in a randomised order. Any child who reported a rating of less than 4 for their assigned drink, as assessed by a confederate, was excluded from the study. No child met this criterion in the current study.

Test measures

For the main test sessions, the Positive and Negative Affect Schedule for Children (PANAS-C; Hughes et al, 2009) was administered to yield measures of both positive affect (PA) and negative affect (NA). The PANAS-C contains 30 items (15 positive and 15 negative emotions) – 20 items from the original PANAS-NOW and an additional 10 child-friendly synonym items sourced from the PANAS-X (expanded form). In addition, a 40 minute cognitive battery was administered alongside the PANAS-C, which consisted of an Auditory Verbal Learning Task (AVLT; Lezak et al, 2004) and a Modified Attention Network Task (MANT; Hillman et al 2009). See Chapter 3 for complete descriptions of the tasks used.

An ADHD Rating Scale-IV: Home version (ADHD IV) was also used in the current study to assess the effect of overt behaviour over the course of the trial. This was due to promising findings from Experiment 3 (Chapter 5) that indicated typical participants' attentional ability may benefit from chronic WBB, as assessed by this scale. Parents/guardians of participants were given an ADHD IV to complete at screening, 2 week and 4 week sessions. ADHD IV contains 18 statements, 9 relating to instances of hyperactivity/impulsivity, and 9 to inattentiveness. These were summed respectively to calculate a hyperactivity-impulsivity subscale and an inattention subscale, and were totalled to provide an overall behaviour score. These three outcome measures were used in analyses to determine whether overt behaviour had changed over the course of the intervention in each group.

Home measures

A three day food diary was disseminated to parents to record their child's typical food consumption across three days (excluding low-flavonoid diet days), between baseline, 2 week and 4 week sessions. This was used to assess habitual flavonoid consumption. The recorded days were kept the same throughout the study, e.g. food diaries were always completed on a Thursday, Friday and Saturday covering two weekdays and 1 weekend day. In addition, a 24 h urine collection log was also distributed to parents to complete where they were asked to log timings of each urination and to list food consumption on the assigned day of the urine collection. This data was used to aid analysis of urinary metabolites, and to check compliance of a low-flavonoid diet during urine collection days.

6.2.5. Urine collection procedure

Three urine containers (1 x 100ml, 2 x 2L) were distributed to parents to collect their child's fasted baseline, 0-12 h and 12-24 h urine, one day prior to test visits two, three and four. The baseline container was required to provide a polyphenol-free, fasted control spot sample for each participant's collection, and did not contain a preservative. Each 2L urine container contained 375mg of L-ascorbic acid to preserve the urine across the collection period.

Instructions were verbally explained to parents and children at screening and test sessions, and parents were given written instructions regarding the urine collection procedure. The first urination of the day was not to be included in any urine collections due to this containing an accumulation of compounds from the previous night. After the first urination of the day, participants were asked to use the 'baseline' container to collect urine before consuming any food. Parents were given a working example to ensure full understanding; "E.g. Your child wakes up and uses the toilet to urinate (1st urine of the day not collected in pot). Your child then does not eat or drink anything (except water) and uses the 'baseline' pot for their next urination. After this urination they can then start eating their low-flavonoid diet for the day." After the 'baseline' sample had been collected, participants were instructed to use the 0-12 h container to collect every subsequent urination. Parents were asked to note down the time the first urination in the 0-12 h pot took place on the urine collection log. Twelve hours after the first 0-12 h urination, participants were asked to use the 12-24 h container to collect subsequent urinations. Again, parents were asked to note down the time the first urination in the 12-24 h container took place on the urine collection log. During the second and third urine collections, the day before visits three and four, the treatment drink was consumed immediately prior to commencement of the 0-12 hour collection. Instructions were amended for these collections, and participants were asked to drink their fruit drink after the 'baseline' collection and before the first 0-12 h urination.

Participants were asked to store the urine containers in a cool bag with ice blocks (both provided) throughout the 24 h collection, and parents were encouraged to replenish with a new ice block every 3-4 hours (excluding overnight). Following all collections, parents dropped their child's samples at the Psychology Department at 9am the following day where they were processed and stored at -80°C (see section 6.2.7 for urinary processing procedure).

6.2.6. Test day procedure

At visit one, on arrival at the Psychology department, participants and their parents/guardians first gave written consent to take part in the study. Parents confirmed that their child had no food intolerances, allergies, developmental diagnoses and were not currently taking medication. In keeping with previous research and to minimise disruption to school hours, children were invited to attend their test sessions at the University after school hours.

Children were individually tested in a quiet cubicle space in the Nutrition and Cognition lab in the University of Reading Psychology Department on three separate visits. At visit one, children completed the CPT, BAS 3, PANAS-C, a practice cognitive test battery and taste rating scale of their assigned drink, whilst parents completed the ADHD IV. Information was then disseminated to parents and children to ensure their child consumed a low-flavonoid diet for the 24 h before each subsequent test session and urine collection. Urine collection procedures were thoroughly explained to parents and children, and the necessary equipment was distributed. Parents were also given a three day food diary to complete for their child over the next 2 weeks.

An email was sent to parents 5 days prior to each test session to remind them to complete the 3 day food diary, follow the 24 h dietary restrictions and abide by the urine collection procedure. Parents were again contacted the day before visits three and four to remind them to not give their child their treatment drink on the day of their next test session visit, to avoid testing acute effects.

At visit two, after children followed a 24 h low-flavonoid diet and urine collection procedure, parents dropped their child's urine collection pots at the University at 9am for processing (see section 6.2.7). Parents returned with their child on the same day to attend the baseline test session during after school hours. The completed three day food diary and urine collection log were collected from parents. Participants then completed the PANAS-C and cognitive task battery before consuming either the placebo or WBB treatment drink. At the end of the baseline test session, the drinks-making procedure was thoroughly explained to parents and children, and the necessary equipment was distributed. Neither parents nor children knew which treatment they were receiving. The time of daily consumption over the supplementation period was not stipulated for ease, due to the differing home schedules of children and their families. Parents were given another three day food diary to complete for their child over the next 2 weeks, as well as clean equipment for the next urine collection.

Parents continued supplementing their children at home with the same treatment that their child had received at the baseline test session.

Two weeks into the treatment supplementation, after following a 24 h low-flavonoid diet and urine collection procedure, parents dropped their child's urine collection pots at the University at 9am for processing (see '6.2.3. Urine collection procedure'). Parents returned with their child on the same day to attend visit three during after school hours. Participants had not received a treatment drink on the day of visit three. The completed three day food diary and urine collection log were collected from parents. Children completed the PANAS-C and a different matched version of the cognitive task battery. Parents were asked to complete an ADHD IV Rating Scale to report their child's behaviour over the previous 2 weeks. Participants were given a further 2 week supply of their assigned treatment, the required urine collection equipment and a three day food diary to complete across the next 2 weeks. Participants were then administered their treatment drink to continue their treatment schedule.

Four weeks into the treatment supplementation, parents again dropped their child's urine collection pots at the University at 9am for processing. Participants attended visit four, after following a 24 h low-flavonoid diet and urine collection procedure for the final time during afterschool hours later that day. A procedure identical to visit three ensued. At the end of visit four, the appropriate equipment was collected, and both the participant and their parent(s) were verbally debriefed on the aims of the study, as well as their assigned condition. A written debrief was also distributed to parents and children, as well as £40 reimbursement, t-shirt and cap for taking part.

6.2.7. Urine processing procedure

Immediately following drop-off at the Psychology department, participant samples were transported to a University of Reading Nutritional Sciences lab to undergo processing.

The total volumes of baseline, 0-12 h and 12-24 h collection samples were recorded, and 100ml of each respective container was retained for further processing after mixing. Samples were centrifuged and measured into 2 x 14 ml falcon tubes, where 1 x 14 ml falcon tube contained approximately 100ul formic acid (FA) to reach a pH of 2.4. 1 ml of urine from each falcon tube was then aliquoted into labelled Eppendorf tubes and stored at -80°C for future analysis. The storage of urine complied with the Human Tissue Act (2004) and all samples

were rendered acellular by centrifugation. All samples that ceased to be used for further analysis were labelled as waste and were destroyed accordingly. When the trial was complete, all 1ml FA urine samples were shipped, on ice, to Dusseldorf, Germany for biochemical analysis.

6.2.8. Urinary analysis procedure

Urine (600 uL) was thawed in an ice bath and centrifuged at 15,000 g for 15 min at 4◦C. Supernatant (350 uL) was diluted (1:1) with phosphoric acid 4% to reduce phenolic-protein interactions and was spiked with the internal standard mix (2-hydroxy-4-methoxybenzoic acid (142 nM), taxifolin (142 nM), morin (142 nM), 7,8-dihydroxycoumarin (143 nM) and phloretin (143 nM)). Each urine sample was loaded (600 uL) on a 96 well u-SPE OASIS HLB plate, washed with 200 uL of water and 200 uL of 0.2% acetic acid and finally eluted with methanol (60 uL), yielding a concentration factor (CF) = 10x. The 96 well collection plates were directly put in the UHPLC autosampler for immediate analysis and sealed with a polyolefin film with a synthetic rubber adhesive to prevent solvent evaporation. The detection of urine (poly)phenol metabolites was performed on a Agilent 6550 iFunnel Accurate-Mass Quadrupole Time-of-Flight Mass Spectrometer (Q-TOF MS) through an electro-spray interface with Jet Stream technology after separation on a 1290 Infinity UHPLC system (Agilent, Waldbronn, Germany). Five uL of each sample were injected in a Zorbax Eclipse Plus RRHDcolumn 2.1 × 50 mm, 1.8 um with a compatible Eclipse Plus guard column 2.1 × 5 mm, 1.8 m (Agilent, Waldbronn, Germany). The mobile phase consisted of 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B) in a 10 min gradient program. The elution profile (flow rate of 0.4 mL/min) started at 1% solvent B and increased to 10% after 5 min, to 25% at 8 min and to 99% at 9.1 min. The percentage of solvent B was held constant for 0.9 min. The gradient was reverted to 1% solvent B for 2 min to equilibrate the column. All samples and standards were analyzed in negative mode with gas temperature 150◦C, gas flow 20 L/min, nebulizer 25 psig, sheath gas temperature 350◦C, sheath gas flow 12 L/min and Vcap 3000 V. Data were analysed and processed using Mass Hunter Workstation Quantitative and Qualitative Analysis software (version B.07.00, Agilent, Waldbronn, Germany). Mass accuracy was verified with a peptide reference solution and the instrument was calibrated daily with a standard mixture to provide mass resolution >20,000 and mass accuracy <1 ppm.

Polyphenol concentrations were calculated using authentic standard curves. These curves were quantified within the Agilent software by calculating the mass of a metabolite compound and the time taken to elute within the gas chamber to produce a urinary concentration value. Recoveries were calculated by normalising urinary concentrations in terms of volume excreted during each collection time point.

6.2.9. Statistical Analysis

All data were analysed using SPSS (Version 22.0).

6.2.9.1. Cognitive analysis

Fifteen participants (7 placebo, 8 WBB) were included in the analyses of demographic, mood and ADHD IV variables. Baseline differences in age, attention (CPT; omissions, commissions), general ability (BAS 3) and taste (1-10) were examined using independent t tests with Drink (placebo and WBB) as the IV.

Mood (PANAS-C) and cognitive (AVLT, MANT) data were analysed by linear mixed models (LMMs) using an unstructured covariance matrix to model repeat measures. Separate LMMs were performed for each dependent variable (DV) in the PANAS-C (PA, NA) and MANT (accuracy, reaction time). Separate LMMs were performed for each outcome measure of the AVLT. Baseline performance was included in all LMMs as a fixed factor. For the AVLT, the number of participants included in analyses varied between list recalls. Missing data in list recalls was due to participants moving past a recording screen, resulting in recall for that list not being captured. Where possible, the experimenter would intervene and verbally ask the participant to say out loud as many words they could remember from the relevant list. This resulted in data still being captured for that particular recall measure. However, due to some participants being tested simultaneously (in individual cubicles), intervention by the researcher was not always possible.

Drink (placebo, WBB), Time (2 weeks, 4 weeks) and Drink x Time were included as Fixed Factors in LMMs to compare the effects of treatment across the intervention period. For the MANT, Congruency (congruent, incongruent), Load (high load, medium load) and Target Time (120ms, 500ms) were also included as Fixed Factors in the model to detect changes in relation to cognitive load. These variables were also contained within interactions with Drink

and Time to assess cognitive load differences between treatment groups across the intervention. Participant was included as a random factor to accommodate the dependency of individual data. Fourteen participants (7 placebo, 7 WBB) were included in the analyses of MANT variables. This was due to corruption of a single participant's MANT data file at the 2 week time point, rendering raw data void for this participant.

All post-hoc pairwise comparisons were corrected for type 1 errors using Bonferroni adjustment within each LMM.

6.2.9.2. Urinary analysis

A total of 71 phenolic metabolites were detected in urine of the current sample. An *a priori* decision was made to quantify key phenolic metabolites that had previously correlated with flow-mediated dilation (FMD; a measure of vascular function) following WBB consumption (Rodriguez-Mateos et al, 2013). This is of particular relevance to the current investigation as cerebral blood flow increases have been suggested as a potential MOA for cognitive effects (Dodd, 2014). Twenty seven key metabolites, as identified through previous research (Rodriguez-Mateos et al, 2016; Feliciano et al, 2016b), were therefore quantified in urine at baseline, 2 weeks and 4 weeks.

12 h and 24 h data were averaged to obtain total excretion levels at each visit, respectively (baseline, 2 weeks and 4 weeks). Time point 0 h was not included in total calculations at each visit, due to this collection only serving as a spot check and having a substantially different volume to 12 and 24 h collections. Individual metabolites and total polyphenols (a mean composite of the 27 key metabolites) were used as DVs in urinary data analyses.

Fifteen participants (7 placebo, 8 WBB) were included in the analyses of urinary metabolites. Differences in total polyphenols at baseline were assessed using an independent samples t-test with Drink (placebo, WBB) as the IV.

An unstructured linear mixed model (LMM) was performed on total polyphenols using Participant as a random factor, and baseline total polyphenol excretion, Drink, Time (2 weeks, 4 weeks) and Drink x Time as fixed factors. Baseline was included as a fixed factor to account for the observable differences between placebo and WBB excretion at baseline, albeit without statistical significance (see Figure 6.8).

Separate linear mixed models (LMMs) were performed for each treatment group on individual metabolites to assess within-group effects across time. Unstructured covariance matrices were used, with Time (baseline, 2 weeks, 4 weeks) as a repeated factor, and Participant as a random factor.

Pairwise comparisons were performed within all LMM analyses and were corrected for type 1 errors using Bonferroni adjustment.

6.2.9.3. Metabolites vs. cognition analysis

6.2.9.3.1. Principle components analysis on metabolite data

Principle components analysis (PCA) was performed on individual metabolites to reduce the data into sets of linear variates (Dunteman, 1989). An exploratory PCA was used to inform future hypotheses on the relationship between metabolites and cognition.

Exploratory PCA was performed on individual metabolite data using raw (baseline, 2 week, 4 week) and change from baseline (CFB; change at 2 week, change at 4 week) scores. This yielded 2 sets of factors; one set representing metabolites with collinear excretion levels, and one representing collinearity in changes of metabolite excretion, respectively. For each PCA, baseline, 2 week and 4 week data were compiled into one column for each corresponding DV to create factor clusters regardless of time point. Factors were retained if eigenvalues ≥ 1, as recommended by Kaiser's (1960) criterion. This was confirmed by consulting the scree plot (a graphical representation of eigenvalues), where factors were not retained to the right of the point of inflexion (Cattell, 1966). The communalities of factors were consulted to confirm factor extraction; these reflect common variance in the data on a scale of 0 to 1, where 0 represents no common variance and 1 indicates total common variance. Therefore, factors with communalities closer to 1 are more reflective of the original data, as there is less random variance (Field, 2009). Inter-factor correlations were expected within each PCA, due to the interlinking nature of the individual metabolites. Oblique rotation was therefore used in both PCA models where correlations between factors were allowed. When there is good reason to suppose that factors could be related theoretically, this method permits variables to load maximally onto distinct factors (Field, 2009). The promax method was applied to the current sample where the delta value (the degree to which factors are permitted to correlate) was set to a default of 0. This value attempts to prevent high correlations between factors and has

been deemed sensible for most analyses (Pedhazur and Schmelkin, 1991; Field, 2009). Both the pattern and structure matrices have been reported by recommendation from Graham, Guthrie and Thompson (2003), and were used in combination to interpret factor loadings. The pattern matrix represents regression coefficients and the structure matrix represents correlation coefficients between each variable and factor (Field, 2009).

Sufficient loadings were determined using Stevens (2002) who stated that for a sample size of 50, 0.722 would be an appropriate loading cut-off. This criterion is based on an alpha level of 0.01 (two-tailed) to control for type 1 errors occurring from multiple tests. Stevens (2002) also recommended only interpreting factor loadings above 0.4; values below this represents variables with little importance to a factor, as they explain the minimum amount of variance allowed in the variable (16%). A cut-off of 0.722 was used for raw metabolite data, which according to Stevens' (2002) guidelines, would explain 52.13% of variance in the sample of 45 (15 participants at 3 time points). A correlation coefficient of 0.3985 was calculated from Stevens' (2002) guidelines, representing a significant factor loading of 0.797. This cut-off was used for the metabolite CFB PCA that had a smaller sample size (n = 24), and would account for 63.52% of variance. The Kaiser-Meyer-Olkin measure of sampling adequacy (KMO) test value was also noted for each PCA to ascertain appropriateness of factor inclusion; factors were therefore retained if they had a significant ($p<0.05$) KMO value ≥ 0.5.

6.2.9.3.2. Combined cognitive data preparation

PCA was deemed inappropriate to use on cognitive data due to the small number, and dependent nature, of the variables. Tests of appropriateness were performed on the data; KMO and Bartlett's test of sphericity were unable to be computed due to the correlation matrix being non-positive definite (NPD). This was confirmed by a determinant value of 0, rendering a singular matrix. Data was defined as NPD because one eigenvalue was a negative number and there were linear dependencies between three eigenvalues, as reflected by values of 0. This was likely due to the collinear nature of AVLT DVs, which made up a large proportion of the cognitive measures. Removal of correlating variables did not improve NPD, so by Wothke's (1993) recommendation, PCA was halted.

Three composite scores were therefore created for cognition at each time point (baseline, 2 weeks and 4 weeks); these were EF (MANT) performance, memory performance and interference. Composites were generated by z-scoring related variables and averaging together

by respective time point. MANT accuracy and RT formed EF MANT performance. Word span, final acquisition, total acquisition, words learnt, short delay, long delay, delayed recall and total 1-7 formed memory performance, and PI and RI formed interference. For CFB analyses, the same procedure ensued for change at 2 weeks and change at 4 weeks.

6.2.9.3.3. Correlational analyses

Metabolite PCA scores were split back into visit time points (baseline, 2 week, 4 week) for correlational analyses. Although multiple regressions were deemed as a preferable statistical technique, the multicollinearity among cognitive DVs in the current sample would not permit unique contribution to a variable. Correlational analyses were therefore performed on metabolite factors and cognitive composites. These were run by respective time point to assess whether metabolite excretion was associated with cognitive performance at each visit. If analyses rendered a significant correlation, the correlation of interest was performed again split by Drink, to assess any treatment relationships.

6.3. Results

6.3.1. Cognitive results

6.3.1.1. Demographic data

Demographic data is presented in Table 6.1. There were no significant differences at baseline between treatment groups for age ($t(13)=0.35$, $p=0.73$), inhibition (CPT commissions; $t(13)=1.32$, $p=0.21$), general ability (BAS 3; $t(13)=0.05$, $p=0.96$) or habitual fruit and vegetable consumption ($t(12)=-0.81$, $p=0.43$). There were significant differences at baseline between groups for sustained attention (CPT omissions; $t(13)=2.80$, $p=0.02$), where placebo participants had a higher rate of failing to respond to non-target stimuli than WBB participants. There were also significant differences between groups for taste ratings ($t(13)=3.35$, $p<0.01$), where drink 'liking' ratings were higher for placebo participants compared to WBB participants.

Raw data for cognitive dependent variables are presented in Table 6.2.

6.3.1.2. Mood

Positive Affect (PA)

Baseline PA significantly predicted post-consumption PA, regardless of treatment drink ($F(1,15)=81.42$, $p<0.01$); for every 0.94 increase in baseline PA, post-consumption PA increased by 1. Time was found to be a trending predictor of post-consumption PA, such that PA was lower at 4 weeks ($M=48.06$, $SE=1.94$) compared to 2 weeks ($M=51.04$, $SE=1.94$; ($F(1,15)=4.27$, $p=0.057$). However, Drink was not a significant predictor of post-consumption PA ($F(1,15)=0.017$, $p=0.90$), nor was there a Drink x Time interaction ($F(1,15)=0.03$, $p=0.86$).

Negative Affect (NA)

Baseline NA significantly predicted post-consumption NA, regardless of treatment drink ($F(1,15)=176.61$, $p<0.01$); for every 0.53 increase in baseline NA, post-consumption NA increased by 1. Neither Drink ($F(1,15)=0.13$, $p=0.72$) nor Time ($F(1,15)=0.30$, $p=0.59$) independently predicted post-consumption NA, nor was there an interaction between Drink and Time ($F(1,15)=1.49$, $p=0.24$).

6.3.1.3. MANT

Correlational analyses revealed that there was no trade-off between accuracy and reaction time (RT) scores at baseline ($r=0.26$, $p=0.35$).

Accuracy (proportion correct 0-1)

Analyses of MANT accuracy scores rendered seven significant and two trending effects. Performance at baseline was found to significantly predict post-baseline performance ($F(1,110.45)=10.53$, $p<0.01$); for every 0.17 increase in baseline accuracy, post-consumption accuracy increased by 1.

Accuracy was found to significantly increase at 4 weeks ($M=0.92$, $SE=0.02$) compared to 2 weeks ($M=0.91$, $SE=0.02$), regardless of treatment ($F(1,119.71)=5.34$, $p=0.02$). As expected, participants were also found to perform significantly more accurately on congruent trials

Dependent variables		Baseline			2 weeks			4 weeks		
		Placebo (n=7)	**WBB (n=8)**	**P**	**Placebo (n=7)**	**WBB (n=8)**	**P**	**Placebo (n=7)**	**WBB (n=8)**	**P**
Mood	**PA**	51.86 (19.91)	54.63 (17.30)	0.78	49.29 (19.42)	52.63 (17.56)	0.73	46.57 (19.58)	49.38 (20.35)	0.79
	NA	20 (12.83)	15.75 (0.89)	0.37	18.29 (6.21)	15.50 (0.53)	0.23	17.86 (7.56)	16.63 (3.29)	0.68
MANT		**(n=7)**	**(n=7)**		**(n=7)**	**(n=7)**		**(n=7)**	**(n=7)**	
	Accuracy (%)	80.75 (11.22)	92.38 (3.73)	0.02	85.43 (16.80)	96.86 (1.63)	0.10	88.52 (12.18)	96.82 (1.18)	0.10
	RT	632.30 (104.28)	644.77 (93.81)	0.82	614.32 (95.05)	621.59 (80.53)	0.88	612.30 (83.86)	609.19 (66.09)	0.94
AVLT		**(n=6)**	**(n=8)**		**(n=7)**	**(n=8)**		**(n=7)**	**(n=8)**	
List A	**Recall 1**	3.50 (0.84)	3.63 (1.51)	0.86	4.14 (1.07)	4.00 (0.76)	0.77	4.00 (1.15)	4.13 (1.13)	0.84
					(n=6)	**(n=8)**				
	Recall 2	5.83 (2.13)	6.38 (2.13)	0.65	6.33 (1.86)	5.75 (1.98)	0.59	5.29 (1.80)	5.75 (1.39)	0.58
					(n=7)	**(n=8)**				
	Recall 3	7.33 (2.50)	7.13 (1.55)	0.85	7.57 (2.15)	7.00 (1.31)	0.54	6.86 (1.95)	6.88 (2.03)	0.99
	Recall 4	7.17 (2.71)	8.38 (2.72)	0.43	7.29 (2.29)	8.00 (2.62)	0.59	7.14 (3.58)	7.13 (1.73)	0.99
	Recall 5	7.00 (2.45)	9.38 (2.50)	0.10	7.57 (2.82)	9.50 (2.51)	0.18	6.71 (3.50)	7.63 (2.07)	0.54
List B	**Recall 1**	4.33 (2.07)	4.50 (1.41)	0.86	3.86 (1.35)	4.50 (1.41)	0.39	3.29 (1.11)	2.88 (1.46)	0.56
List A		**(n=4)**			**(n=6)**	**(n=8)**		**(n=6)**	**(n=8)**	
Short Delay	**Recall 6**	7.50 (1.73)	7.50 (1.31)	1.00	6.00 (1.26)	8.00 (2.73)	0.12	5.33 (2.73)	5.75 (2.76)	0.78
		(n=7)			**(n=7)**	**(n=8)**		**(n=7)**	**(n=8)**	
Long Delay	**Recall 7**	4.86 (2.61)	6.25 (2.71)	0.33	5.14 (2.61)	5.63 (2.33)	0.71	4.71 (1.89)	4.50 (2.27)	0.85
Word Recognition		10.73 (2.36)	11.13 (1.89)	0.46	9.71 (2.50)	10.50 (2.07)	0.52	8.86 (2.80)	10.38 (1.69)	0.22

Table 6.2 Mean (SD) performance for placebo and WBB participants on each dependent variable at baseline, 2 weeks and 4 weeks.

(M=0.93, SE=0.02) than incongruent trials (M=0.91, SE=0.02; F(1,106.44)=13.39, p<0.01), and on 500ms trials (M=0.93, SE=0.02) compared to 120ms trials (M=0.91, SE=0.02; F(1, 106.77)=7.49, p<0.01). Surprisingly, Load was not found to predict performance across the intervention period (F(1,104.14)=0.32, p=0.58). WBB participants' overall accuracy (M=0.96, SE=0.03) was higher than placebo participants' (M=0.88, SE=0.03), although this failed to reach significance (F(1,110.73)=3.12, p=0.098).

A trend was revealed for Drink x Time where WBB-treated participants were more accurate than placebo-treated participants at the 2 week time point (p=0.057). These differences did not persist at the 4 week time point due to a significant increase in placebo participants' accuracy from 2 to 4 weeks (p<0.01; Figure 6.2).

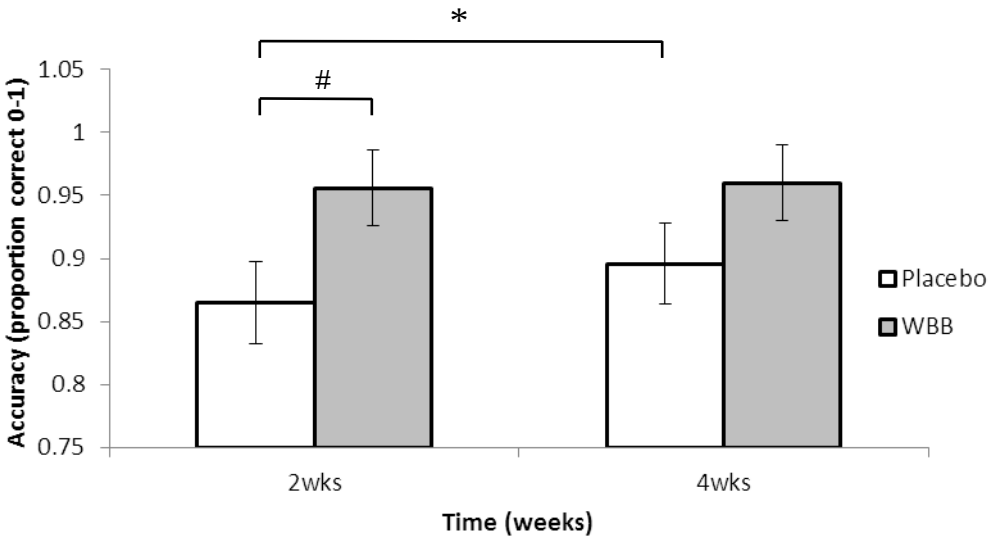


Figure 6.2 Mean (±SEM) MANT accuracy performance at 2 and 4 weeks. A trend towards significantly better performance following WBB at 2 weeks compared to placebo. This effect had disappeared by 4 weeks due to significantly increased performance at 4 weeks compared to 2 weeks for placebo participants only. * significant at p< 0.05; # trend at 0.05 > p < 0.1

Significant interactions were also found for Drink x Congruency (F(1,104.15)=10.74, p<0.01), Drink x Load (F(1,104.38)=4.84, p=0.03) and Drink x Target time (F(1,105.82)=9.39, p<0.01), all showing a similar pattern of results (Figures 6.3-6.5).

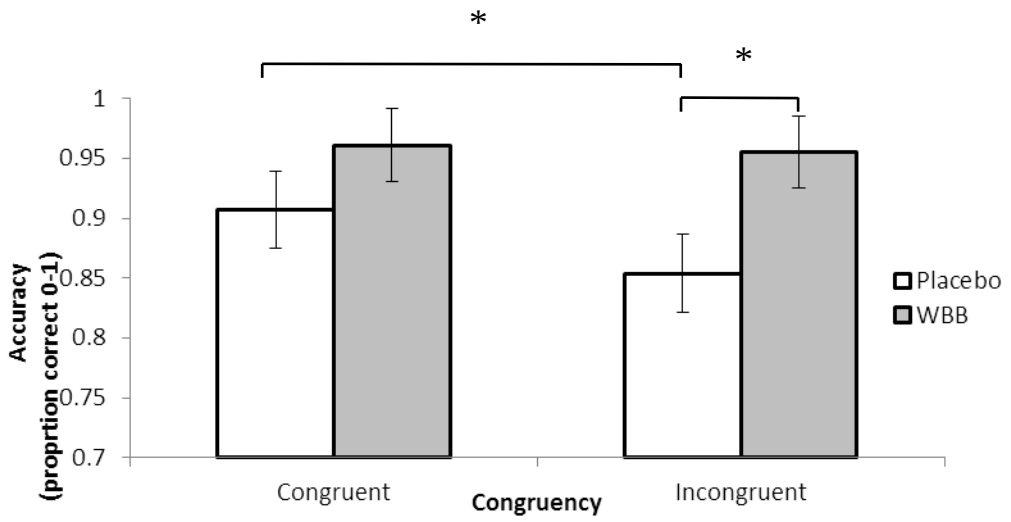


Figure 6.3 Mean (±SEM) post-consumption accuracy scores of placebo and WBB-treated participants on congruent and incongruent MANT trials. * significant at $p < 0.05$.

Accuracy was significantly better on congruent trials than incongruent trials, as expected due to the demands of cognitive load within the task, however this was found to be apparent for placebo participants only ($p<0.01$). Here, placebo participants were performing at 91% accuracy on congruent trials which fell to 85% accuracy on incongruent trials. No such differences in performance were observed between congruent and incongruent trials for WBB participants, with participants performing at 96% accuracy on both incongruent and congruent trials. This suggests that WBB-treated participants were more accurate overall, particularly on incongruent trials. Indeed, WBB participants were found to be significantly more accurate on incongruent trials than placebo participants by 11% ($p=0.038$; Figure 6.3.)

WBB-treated participants were also significantly more accurate than placebo on medium load trials ($p=0.05$; Figure 6.4). A similar pattern was evident for high load trials, though this effect did not reach significance ($p=0.18$). Interestingly, placebo participants were found to be less accurate on medium load trials compared to high load trials at trend level ($p=0.06$), with no such differences observed in the WBB treatment group (Figure 6.4). This suggests poorer performance for placebo participants on medium load trials, an unexpected pattern.

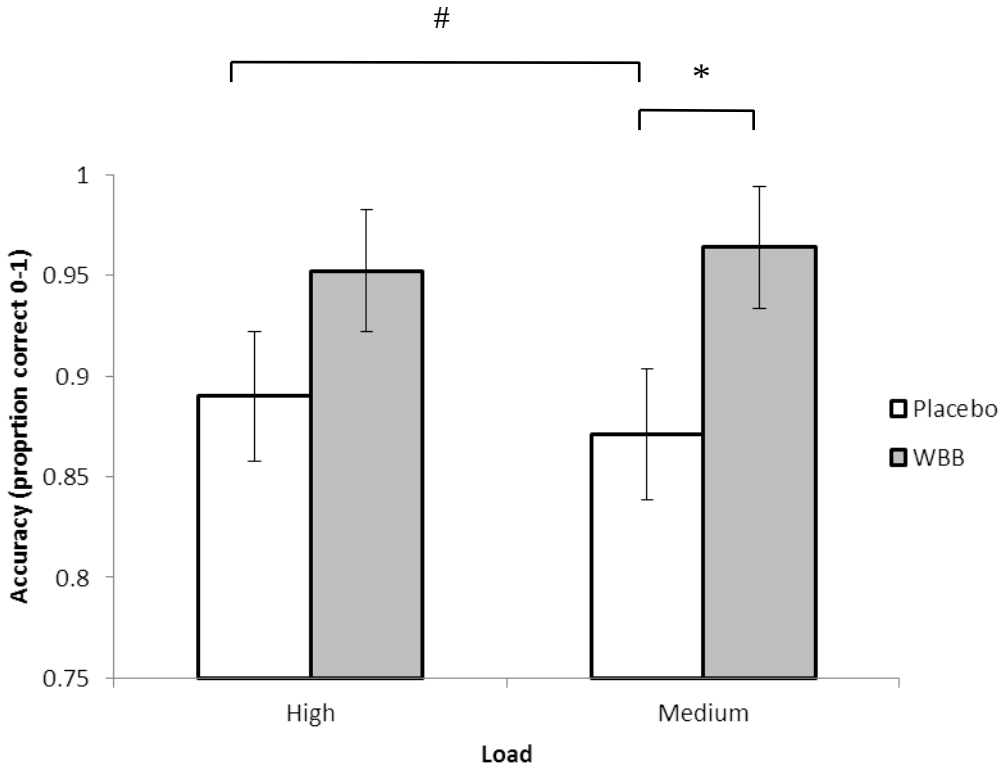


Figure 6.4 Mean (±SEM) post-consumption accuracy scores of placebo and WBB-treated participants on medium load and high load MANT trials. * significant at $p < 0.05$; # trend at $0.05 > p < 0.1$

Significantly increased accuracy was also observed for 500ms trials compared to 120ms trials for placebo participants only ($p<0.01$). The absence of differences in WBB participants ($p=0.87$) suggests a maintenance of performance across different speeds of stimulus presentation within this group. WBB-treated participants showed 96% accuracy compared to 86% accuracy in placebo-treated participants on 120ms trials ($p=0.04$; Figure 6.5), highlighting the benefit of WBB on trials of faster presentation.

Although non-significant, a Drink x Time x Target Time interaction ($F(2, 119.76)=0.44$, $p=0.64$) was explored to examine accuracy performance during significant RT effects. Analyses confirmed that RT improvements observed at 4 weeks compared to 2 weeks for both WBB and placebo participants on 120ms trials occurred without cost to accuracy (WBB, $p=0.048$; placebo, $p=0.12$). In addition, this exploration also revealed improved accuracy for WBB participants compared to placebo on 120ms trials at 2 weeks ($p=0.03$) and 4 weeks ($p=0.06$; Figure 6.6a). Increased accuracy on 500ms trials, compared to 120ms trials, were

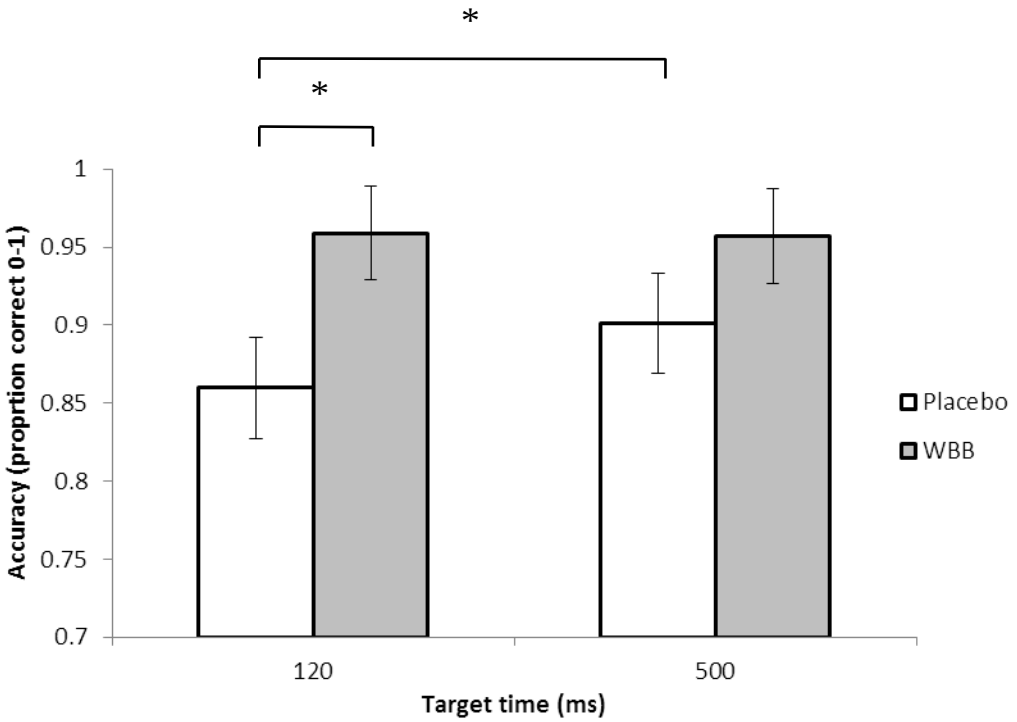


Figure 6.5 Mean (±SEM) post-consumption accuracy scores of placebo and WBB-treated participants on 120ms and 500ms MANT trials. * significant at $p < 0.05$

identified at 2 weeks (500ms, M=0.88, SE=0.03; p=0.02) and 4 weeks (500ms, M=0.92, SE=0.03; p<0.01) in the placebo group only. However, no such differences were observed in the WBB group between 120ms and 500ms trial accuracy at 2 weeks (500ms, M=0.95, SE=0.03; p=0.72) or 4 weeks (500ms, M=0.96, SE=0.03; p=0.54), indicating similar performance between the two target times for the latter group.

Reaction time (RT; ms)

As expected, from performance in previous cognitive studies (Whyte et al 2015; 2016; 2017; Chapters 3, 4 and 5), significantly quicker reaction times were evident for congruent trials (M=607.50, SE=18.05) when compared to incongruent trials (M=634.01, SE=18.06; F(1,112)=19.88, p<0.001). Baseline RT was also a trending predictor of post-baseline performance (F(1,117.76)=3.83, p=0.053); for a speed reduction of 0.11ms at baseline, post-baseline performance reduced by 1ms.

Neither Drink (F(1,14.04)=0.12), Time (F(1,119.77)=1.72, p=0.19), Load (F(1,101.60)=1.44, p=0.23) nor Target Time (F(1,104.31)=1.87, p=0.17) were significant predictors of performance.

There was a significant interaction between Drink x Time x Target Time ($F(2,119.81)=4.17, p=0.02$). Pairwise comparisons revealed that WBB participants were 22.60ms faster on 120ms trials at 4 weeks compared to 2 weeks ($p=0.02$). This was also seen as a trend for placebo participants with performance 20.20ms faster on 120ms trials at 4 compared to 2 weeks ($p=0.058$), suggesting performance improved for both treatments at different degrees of change (Figure 6.6b). This indicates WBB and placebo participants retained accuracy and reduced RT at 4 weeks, indicating practice effects, with the WBB group performing marginally faster on 120ms trials.

Slower RTs were observed on 500ms trials compared to 120ms trials in both placebo ($p=0.02$) and WBB ($p=0.03$) groups, at the 4 week time point only. As placebo participants were found to have increased accuracy on 500ms trials, it could be said that they took longer to make a correct response on these trials. Taken together, these results indicate increased accuracy and quicker RT on 120ms trials for the WBB group.

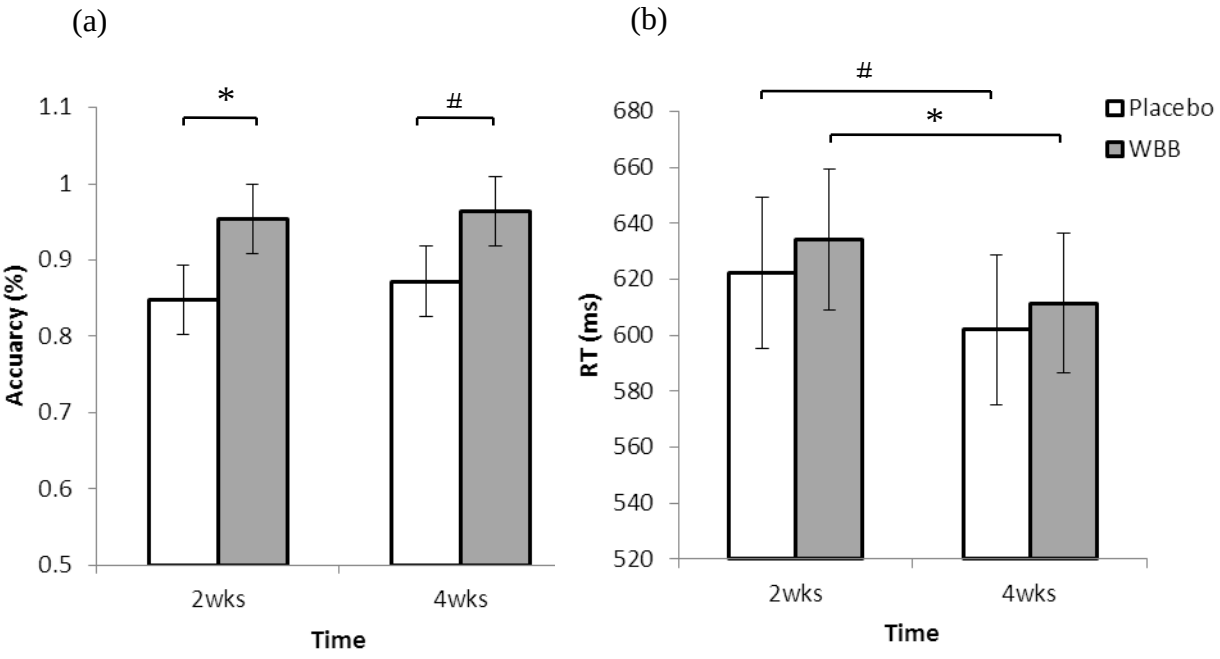


Figure 6.6 (a) Mean accuracy scores and (b) mean RT scores of placebo and WBB participants on 120ms trials at 2 weeks and 4 weeks. *significant at $p< 0.05$; # trend at $0.05 > p < 0.1$

A significant interaction was observed between Drink x Congruence x Load ($F(2,104.30)=4.50$, $p=0.01$), showing significantly quicker RTs for placebo ($p<0.01$) and WBB participants ($p=0.02$) on high load congruent trials (placebo, M=595.91, SE=26.67; WBB, M=618.12, SE=24.99) when compared to high load incongruent trials (placebo M=638.29, SE=26.64; WBB, M=641.94, SE=25.27). This suggests that, as expected, incongruent trials are more difficult. Further analyses also revealed significantly quicker performance on medium load incongruent trials (M=614.52, SE=26.63) when compared to high load incongruent trials (M=638.29, SE=26.64) for placebo participants only ($p=0.01$). This suggests that placebo participants were susceptible to the manipulation of load conditions and performed worse on conditions of high load. No such load differences were evident for WBB participants, suggesting that this group's RTs were potentially not as affected by the manipulation of cognitive load.

6.3.1.4 AVLT

AVLT outcome variable data can be seen in Table 6.3. Decreased performance was observed for both treatment groups across the course of the intervention, most noticeably at the 4 week time point (Table 6.3; Figure 6.7).

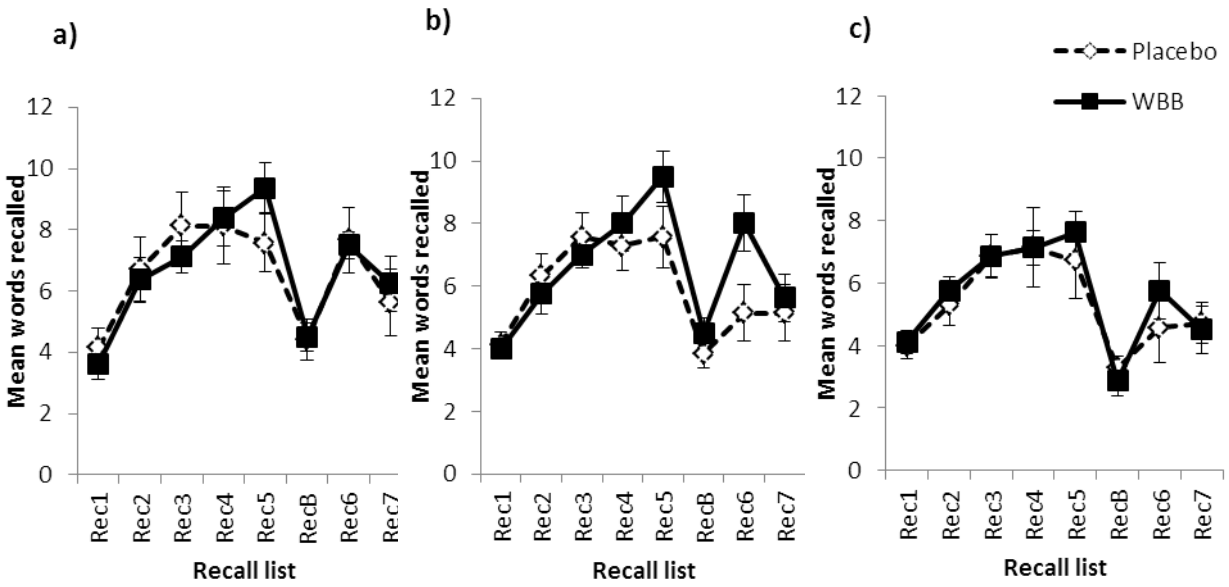


Figure 6.7 AVLT mean word recall (±SEM) by WBB and Placebo treatment groups at a) baseline, b) 2 weeks and c) 4 weeks into the intervention

AVLT calculations	Baseline			2 weeks			4 weeks		
	Placebo	**WBB**	**P**	**Placebo**	**WBB**	**P**	**Placebo**	**WBB**	**P**
	(n=6)	**(n=8)**		**(n=7)**	**(n=8)**		**(n=7)**	**(n-8)**	
Word Span *(A1)*	3.50 (0.84)	3.63 (1.51)	0.86	4.14 (1.07)	4.00 (0.76)	0.77	4.00 (1.15)	4.13 (1.13)	0.84
				(n=6)	**(n=8)**		**(n=6)**	**(n=8)**	
Words Learnt *(A5-A1)*	3.50 (1.87)	5.75 (3.24)	0.16	4.00 (1.67)	5.50 (2.33)	0.21	3.17 (3.66)	3.50 (2.20)	0.84
				(n=7)	**(n=8)**		**(n=7)**	**(n=8)**	
Final Acquisition *(A5)*	7.00 (2.45)	9.38 (2.50)	0.10	7.57 (2.82)	9.50 (2.51)	0.18	6.71 (3.50)	7.63 (2.07)	0.54
Total Acquisition	30.83 (9.85)	34.88 (6.56)	0.37	32.00 (9.00)	34.25 (7.96)	0.62	30.00 (9.88)	31.50 (6.65)	0.73
(sum of list A: 1 to 5)	**(n=7)**	**(n=8)**							
PI *(A1-B1)*	-0.71 (1.25)	-0.88 (2.17)	0.87	0.29 (1.38)	-0.50 (1.20)	0.26	0.71 (0.76)	1.25 (2.12)	0.54
RI *(A5-A6)*	1.14 (1.95)	1.88 (2.47)	0.54	2.43 (3.26)	1.50 (1.20)	0.47	2.14 (2.61)	1.88 (2.53)	0.84
Total List A Recall	36.14 (18.89)	48.63 (8.53)	0.12	42.29 (12.84)	47.88 (11.73)	0.39	39.29 (14.28)	41.75 (9.79)	0.70
(sum list A: 1 to 7)									
Total List Recall	39.86 (21.21)	53.13 (9.49)	0.13	46.14 (13.50)	52.38 (11.90)	0.36	42.57 (14.86)	44.63 (9.36)	0.75
(sum list A: 1 to 7+ B1)									
Recognition	10.29 (2.36)	11.13 (1.89)	0.46	9.71 (2.50)	10.50 (2.07)	0.52	8.86 (2.79)	10.38 (1.69)	0.22
Long Delay Recall *(A7)*	4.86 (2.61)	6.25 (2.71)	0.33	5.14 (2.61)	5.63 (2.33)	0.71	4.71 (1.89)	4.50 (2.27)	0.85
Short Delay Recall *(A6)*	**(n=5)**	**(n=8)**		**(n=6)**	**(n=8)**		**(n=6)**	**(n=8)**	
	6.80 (2.17)	7.50 (1.31)	0.48	6.00 (1.26)	8.00 (2.73)	0.12	5.33 (2.73)	5.75 (2.76)	0.78

Table 6.3 Mean (SD) data for Placebo and WBB participants' performance for each AVLT variable at baseline, 2 weeks and 4 weeks into the intervention.

It is unlikely that fatigue from repeated testing would occur over several weeks in the current study. Chronic decreases in performance may therefore be due to increased boredom leading to lack of engagement with the study. Figure 6.7 shows AVLT list recall performance at baseline, 2 weeks and 4 weeks. Interestingly, placebo participants' performance on the AVLT at baseline did not follow a similar pattern to previous baseline recalls observed in other child samples (Whyte et al, 2015; 2016; 2017; Chapter 3). Previous child samples have demonstrated a linear increase in word recall from recalls 1 –5. However, in the current sample, baseline performance for those consuming placebo was found to increase from recalls 1-3, where it plateaued at recall 4 and decreased at recall 5, highlighting lack of engagement with the study protocol.

LMM analyses revealed that baseline performance significantly predicted post-consumption performance for 5 AVLT outcome measures (final acquisition (β=0.63), total acquisition (β=0.68), recognition (β=0.), total recall 1-7 (β=0.39) and total recall 1-7+B (β=0.40)). There were trends for baseline performance predicting post-baseline performance for 2 outcome measures: words learnt (β=0.34) and RI (β =0.39). Baseline performance did not significantly predict post-consumption performance for measures of word span, short delay, long delay, delayed recall or proactive interference ($p>0.05$).

Interestingly, Time was found to be a significant predictor of delayed recall performance such that participants recalled significantly more words at 2 weeks (M=12.14, SE=0.99) than at 4 weeks (M=7.75, SE=0.99; $F(1,15)=17.08, p<0.01$), regardless of treatment drink. The same finding was observed as a trend in Total 1-7 performance (2 week recall, M=45.24, SE=2.61; 4 week recall, M=40.68, SE=2.61; $F(1,15)=3.10$, $p=0.099$).

Time was also a significant predictor of PI performance ($F(1,15)=9.77$, $p<0.01$); PI was found to be significantly higher at 4 weeks (M=0.98, SE=0.34) compared to 2 weeks (M=-0.11, SE=0.34). A Drink x Time trend was also evident for PI ($F(1,15)=3.59$, $p=0.077$). Pairwise comparisons revealed that PI was significantly increased at 4 weeks compared to 2 weeks, for WBB participants only ($p<0.01$; Figure 6.8).

No significant effects were observed for other AVLT variables.

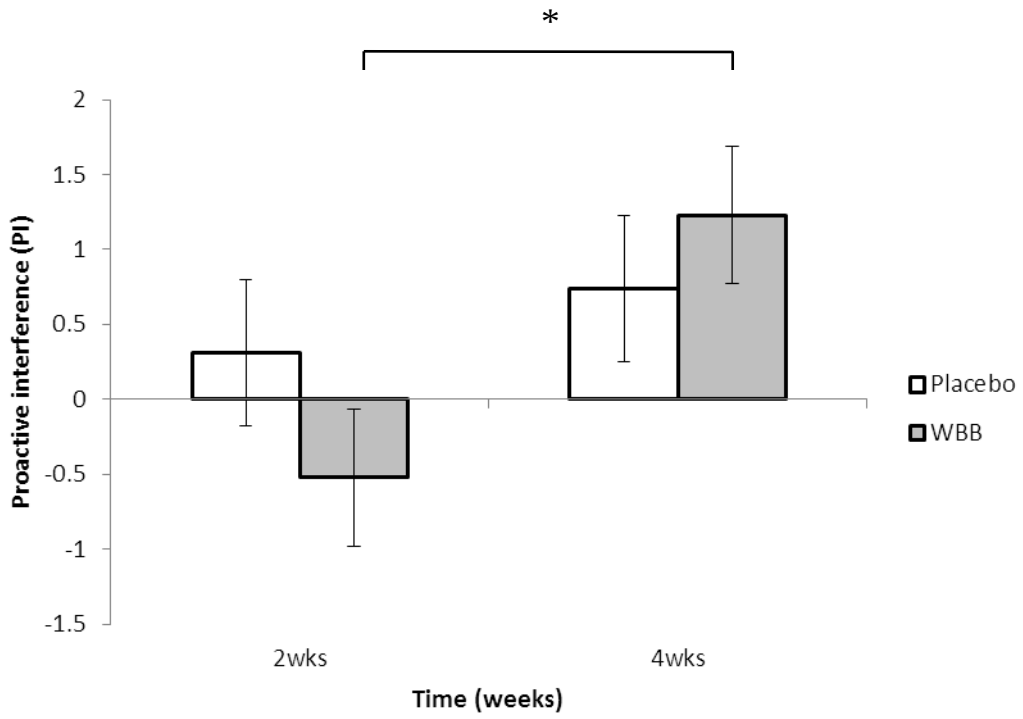


Figure 6.8 Increased proactive interference (PI; mean (±SEM)) for WBB participants at 4 weeks compared to 2 weeks into the intervention. * significant at $p<0.05$

6.3.1.5. ADHD-IV Parent Rating Scale

Baseline inattentiveness (IA) was found to significantly predict IA during the intervention ($F(1,15)=54.41$, $p<0.01$): for every baseline increase of 0.82, inattentiveness increased by 1. Similarly, total ADHD IV scores at baseline were found to significantly predict total ADHD IV scores during the intervention ($F(1,15)=45.45$, $p<0.01$). Total ADHD IV scores were found to increase by 1 for every 1.02 increase at baseline.

Baseline hyperactivity-impulsivity (HI) was a significantly predictor of HI ($F(1,15)=29.73$, $p<0.01$); for every 0.70 increase at baseline, hyperactivity increased by 1. Hyperactivity behaviours were reported less at 4 weeks (M=41.95, SE=4.87), than at 2 weeks (M=48.14, SE=4.87), regardless of treatment drink, however this did not reach statistical significance ($F(1,15)=1.89$, $p=0.19$).

No other effects were observed for behaviour.

6.3.2. Urine results

6.3.2.1. Total polyphenols

There were no significant differences at baseline between treatment groups for total polyphenols excreted ($t(11)=1.39$, $p=0.19$). However, as can be seen in Figure 6.9a placebo participants do seem to be excreting non-significantly higher levels of polyphenols at baseline.

LMM analysis revealed increased excretion of polyphenols for WBB participants over the course of the intervention when compared to baseline, but this was not statistically significant (Time, $F(2,7.57)=3.11$, $p=0.10$). In contrast, placebo participants showed a non-significant decline across the same time period (Figure 6.9a; Time, $F(2,7.23)=1.64$, $p=0.26$). Change in total polyphenol excretion has also been presented in Figure 6.9b.

6.3.2.2. Individual metabolites

Raw individual metabolite data is presented in Table 6.4. A diagram of the change in excretion levels across 4 weeks for all 27 metabolite compounds is presented in Figure 6.10.

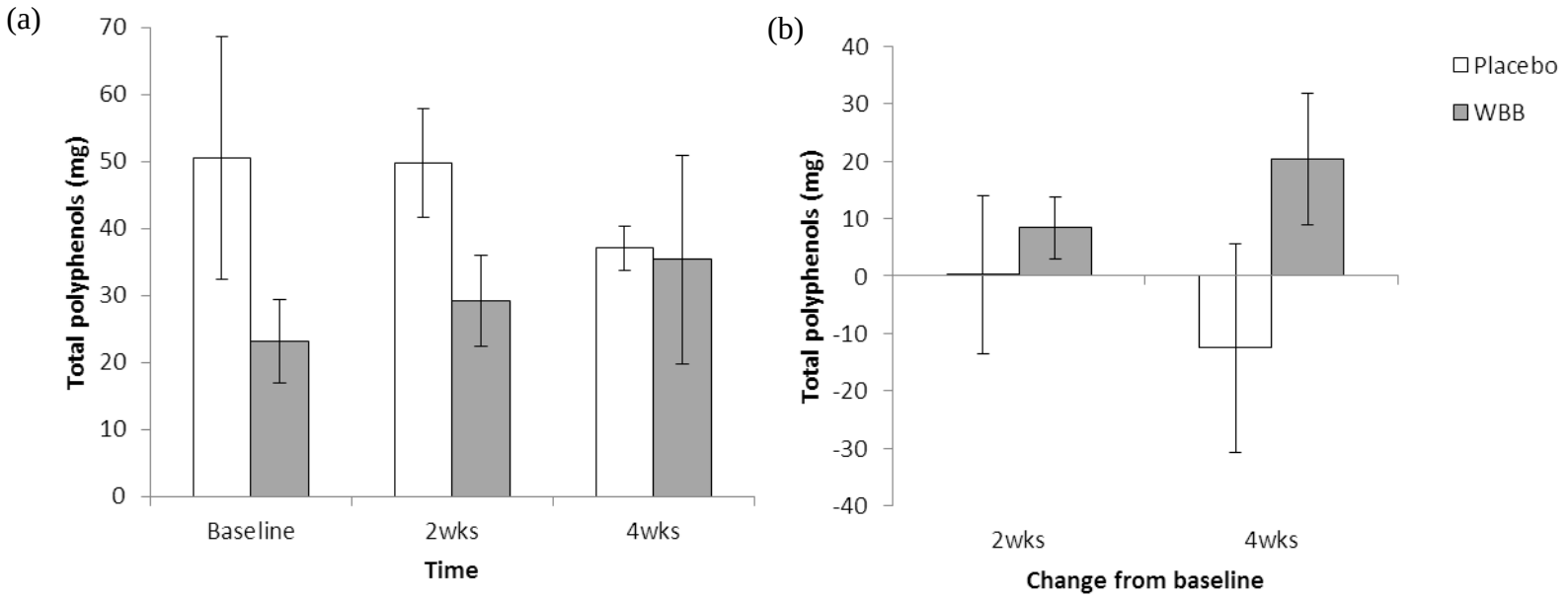


Figure 6.9 (a) Mean (±SEM) raw total polyphenol excretion at baseline, 2 weeks and 4 weeks for placebo and WBB participants, (b) Mean (±SEM) total polyphenol change from baseline at 2 weeks and 4 weeks for placebo and WBB participants.

Twenty seven phenolic metabolites were quantified in urine at week 0, 2 and 4 (as per *a priori* statistical guidelines for both placebo and WBB groups; see 6.3.2; Table 6.4; Figure 6.10).

6.3.2.2.1. Increases in WBB and placebo participants

Increases in excretion were observed in both placebo and WBB groups across the intervention for phenylacetic, valerolactone, benzoic, propionic, and cinnamic acid derivatives.

Five metabolites were found to increase for both WBB and placebo participants at 4 weeks compared to baseline. Increases in 3-hydroxyphenyl acetic acid, 4-hydroxyphenyl acetic acid and benzoic acid were observed for placebo (significantly) and WBB (at trend level) participants. Increases in 3-(4-hydroxyphenyl) propionic acid and (4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate also trended towards significance for both placebo and WBB participants at the 4 week time point (Table 6.4; Figure 6.11; Figure 6.12). Such increases in both groups suggest dietary factors or natural metabolism may have influenced increased excretion of these metabolites, rather than WBB intervention alone.

6.3.2.2.2. WBB only increases

Although not reaching significance, 3 metabolites were unique to WBB consumption and trended towards an increase after chronic 4 weeks consumption of WBB (Table 6.4; Figure 6.11). These were homovanillic acid, syringic acid and dihydro ferulic acid-4-O-B-D-glucuronide.

6.3.2.2.3. Placebo only increases

Seven metabolites unique to placebo consumption were found to increase across the intervention. At 4 weeks, 2 metabolites (ferulic acid 4-O-β-D-glucuronide and dihydro isoferulic acid 3-O-sulfate) significantly increased from baseline. Increases in a further 2 metabolites (protocatechuic acid and 4-methylgallic-3-O-sulfate) trended towards significance for this group. Interestingly, significant effects were also observed at 2 weeks for the placebo

	Urinary Concentration (uG)					
	Placebo (n=7)			WBB (n=8)		
	Week 0 (n=6)	Week 2 (n=7)	Week 4 (n=7)	Week 0 (n=7)	Week 2 (n=8)	Week 4 (n=6)
Pyrogallol derivatives						
Pyrogallol-O-2-sulfate	3172 ± 1949	3043 ± 1745	2126 ± 964	5750 ± 6337	2807 ± 3595	3660 ± 4490
1-Methylpyrogallol-O-sulfate	941 ± 581	902 ± 452	673 ± 428	2424 ± 2457	1055 ± 1102	1599 ± 1635
Phenylacetic acid derivatives						
Homovanillic acid [a]	522 ± 291	1406 ± 1660	626 ± 405	462 ± 310	624 ± 348	431 ± 224
3-hydroxyphenylacetic acid [a b*]	57 ± 36	107 ± 50	126 ± 21	92 ± 68	139 ± 143	161 ± 102
4-hydroxyphenylacetic acid [a b*]	651 ± 88	671 ± 294	778 ± 140	569 ± 250	671 ± 259	778 ± 291
Valerolactone derivatives						
(4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate [a b]	17 ± 38	67 ± 60	7 ± 6	3 ± 5	20 ± 23	25 ± 41
Benzoic acid derivatives						
Benzoic acid [a b*]	53 ± 14	52 ± 9	64 ± 13	48 ± 30	59 ± 18	52 ± 19
Protocatechuic acid [b]	616 ± 208	828 ± 284	662 ± 258	903 ± 592	577 ± 513	799 ± 790
2-hydroxybenzoic acid	0.02 ± 0.02	0.03 ± 0.04	0.03 ± 0.05	0.35 ± 0.40	0.23 ± 0.26	0.27 ± 0.31
4-hydroxybenzoic acid	7 ± 10	18 ± 32	13 ± 16	10 ± 12	12 ± 16	22 ± 26
Vanillic acid	646 ± 312	1152 ± 1420	846 ± 360	413 ± 227	393 ± 285	482 ± 282
4-Methylgallic-3-O-sulfate [b]	670 ± 356	778 ± 705	359 ± 225	994 ± 637	859 ± 1001	1813 ± 2746
2,4-dihydroxybenzoic acid	9 ± 7	8 ± 8	8 ± 5	8 ± 9	8 ± 4	7 ± 4
Syringic acid [a]	265 ± 284	393 ± 377	406 ± 237	87 ± 47	301 ± 270	220 ± 249
Propionic acid derivatives						
3-(4-hydroxyphenyl) propionic acid [a b]	310 ± 133	340 ± 146	435 ± 83	235 ± 265	285 ± 205	175 ± 134
Catechol derivatives						
4-Methylcatechol-O-sulfate	265 ± 156	346 ± 172	203 ± 44	164 ± 73	222 ± 84	171 ± 86
Cinnamic acid derivatives						
t-ferulic acid [b*]	51 ± 22	38 ± 18	44 ± 15	24 ± 13	28 ± 14	37 ± 23
Ferulic Acid 4-O-Sulfate	100 ± 150	439 ± 751	324 ± 544	28 ± 33	83 ±115	71 ± 62
Ferulic acid 4-O-β-D-Glucuronide [b*]	2114 ± 1671	2155 ± 962	3123 ± 2358	713 ± 691	600 ± 382	513 ± 349
Dihydro Ferulic Acid 4-O-β-D-Glucuronide [a]	7194 ± 7401	6879 ± 6718	4095 ± 2589	1214 ± 1001	1911 ± 1122	1310 ± 721
Isoferulic acid	134 ± 118	130 ± 95	168 ± 78	52 ± 49	49 ± 81	29 ± 22
Isoferulic Acid 3-O-β-D-Glucuronide	673 ± 800	459 ± 284	689 ± 546	130 ± 161	126 ±96	68 ± 59
Dihydro Isoferulic acid 3-O-Sulfate [b*]	2445 ± 2407	2281 ± 1462	2925 ± 2305	414 ± 338	513 ± 372	349 ± 411
Dihydro Isoferulic acid 3-O-β-D-Glucuronide [a b*]	3692 ± 4801	7797 ± 4515	6578 ± 4864	917 ± 285	3176 ± 3079	2175 ± 1612
Dihydro Caffeic Acid 3-O-Sulfate	13987 ± 11617	18134 ± 12800	10451 ± 5701	6525 ± 7049	13265 ± 13497	19219 ± 25072
Chlorogenic acid	10685 ± 23737	214 ± 368	217 ± 300	1 ± 1	31 ± 45	58 ± 114
Hippuric acid derivatives						
Hippuric acid	1226 ± 340	1171 ± 443	1158 ± 389	987 ± 394	1347 ± 262	1171 ± 262

Table 6.4 Mean ± SD urinary polyphenol concentrations at week 0, week 2 and week 4 for placebo and WBB participants. [a] denotes metabolites that had higher concentrations after repetitive WBB consumption; [b] denotes metabolites that had higher concentrations after repetitive placebo consumption; * denotes significance at the $p<0.05$ level.

group, with increases in 3-hydroxyphenyl acetic acid and dihydro isoferulic acid 3-O-β-D-glucuronide compared to baseline. Further, t-ferulic acid was found to be significantly higher at baseline compared to 2 weeks for placebo participants.

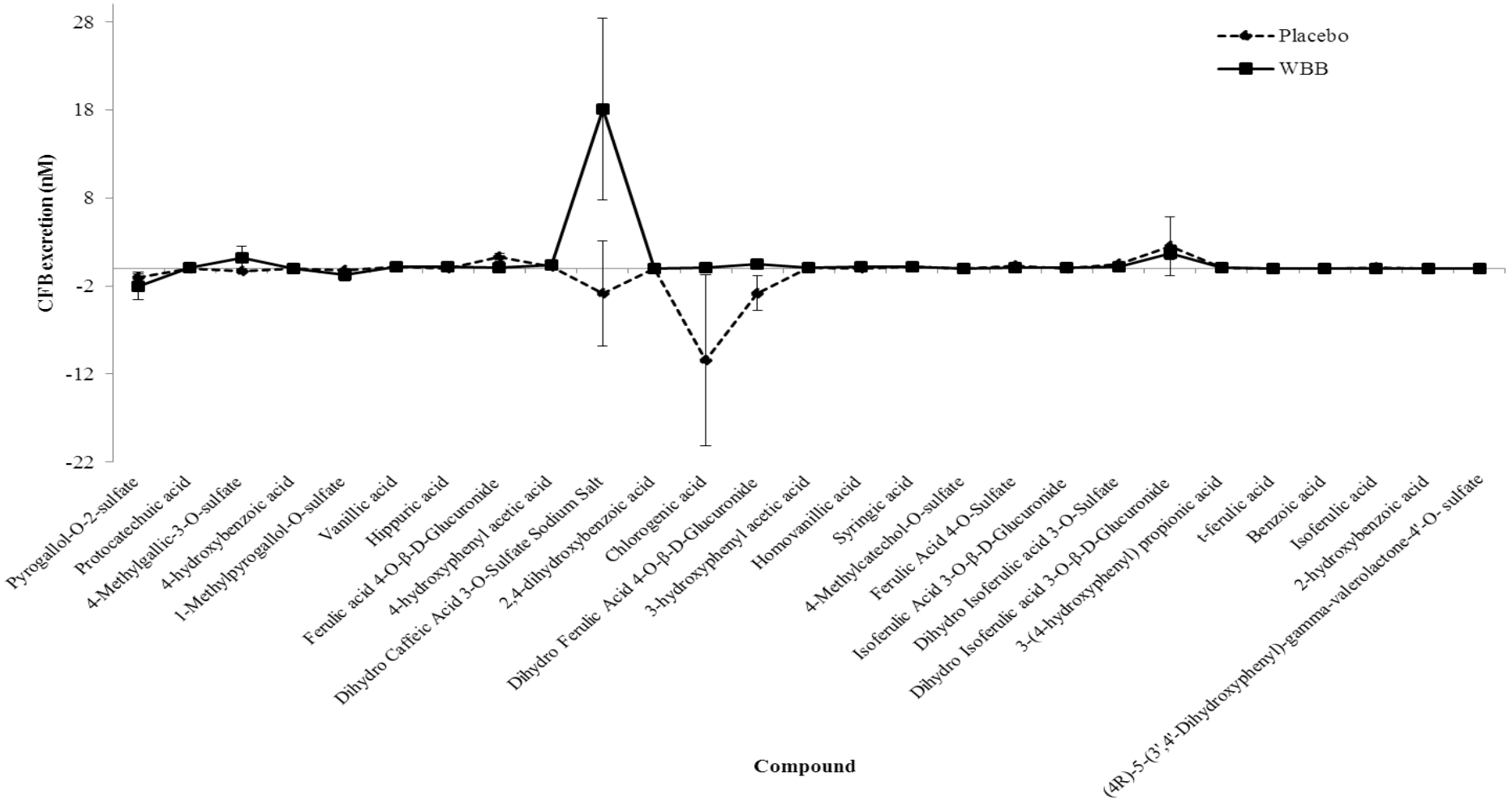


Figure 6.10 Mean ±SEM change from baseline (CFB) data after 4 weeks of daily WBB and placebo supplementation for 27 metabolite compounds

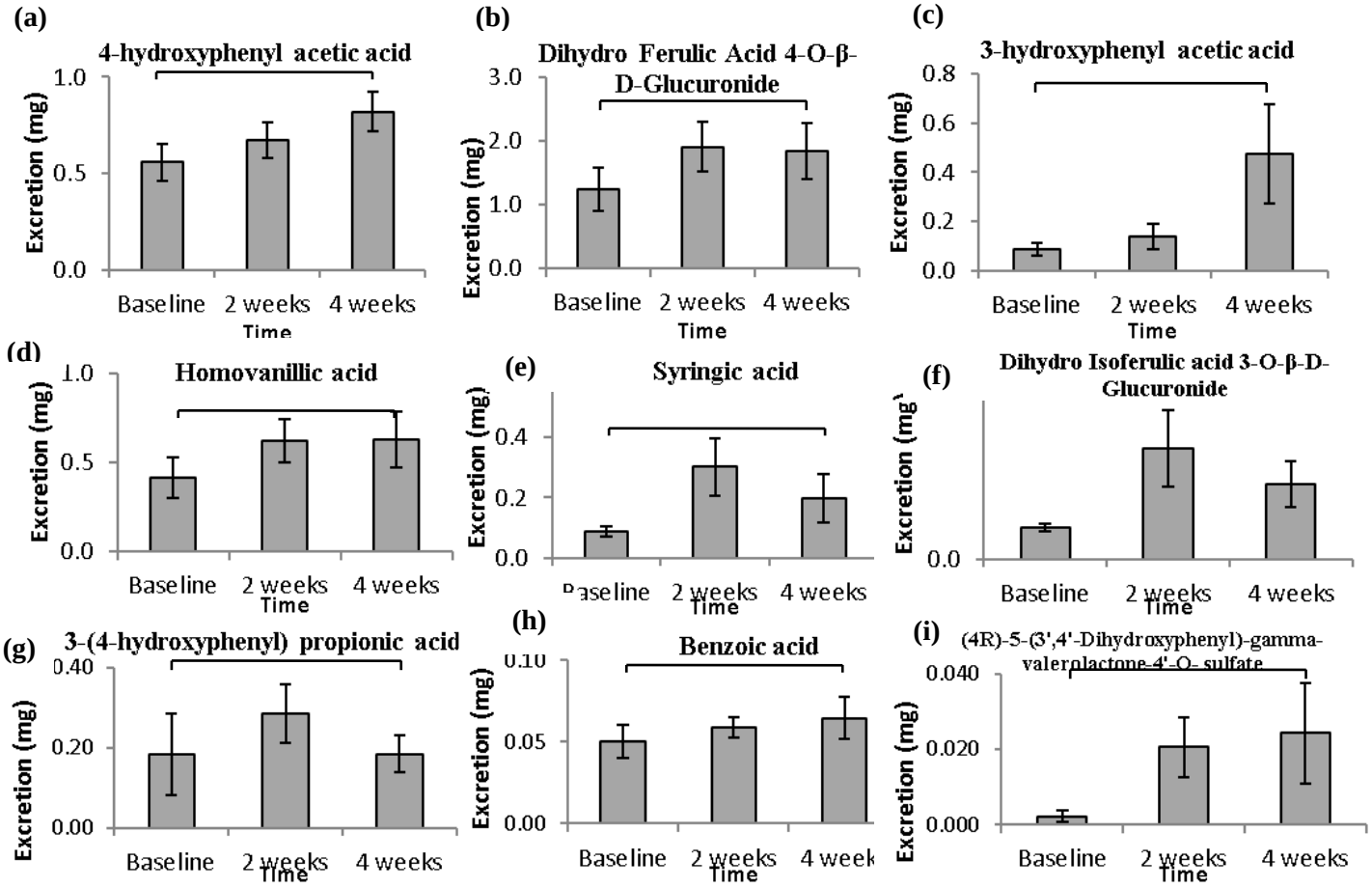


Figure 6.11 Mean ± SEM urine metabolite concentrations of 8 participants supplemented with WBB at baseline, 2 weeks and 4 weeks for (a) 4-hydroxyphenyl acetic acid, (b) dihydro ferulic acid 4-O-β-D-glucuronide, (c) 3-hydroxyphenyl acetic acid, (d) homovanillic acid, (e) syringic acid, (f) dihydro isoferulic acid 3-O-β-D-glucuronide, (g) 3-(4-hydroxyphenyl) propionic acid, (h) benzoic acid, (i) (4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate. Bars in bold represent a significant difference. Bars not in bold represent a trending difference.

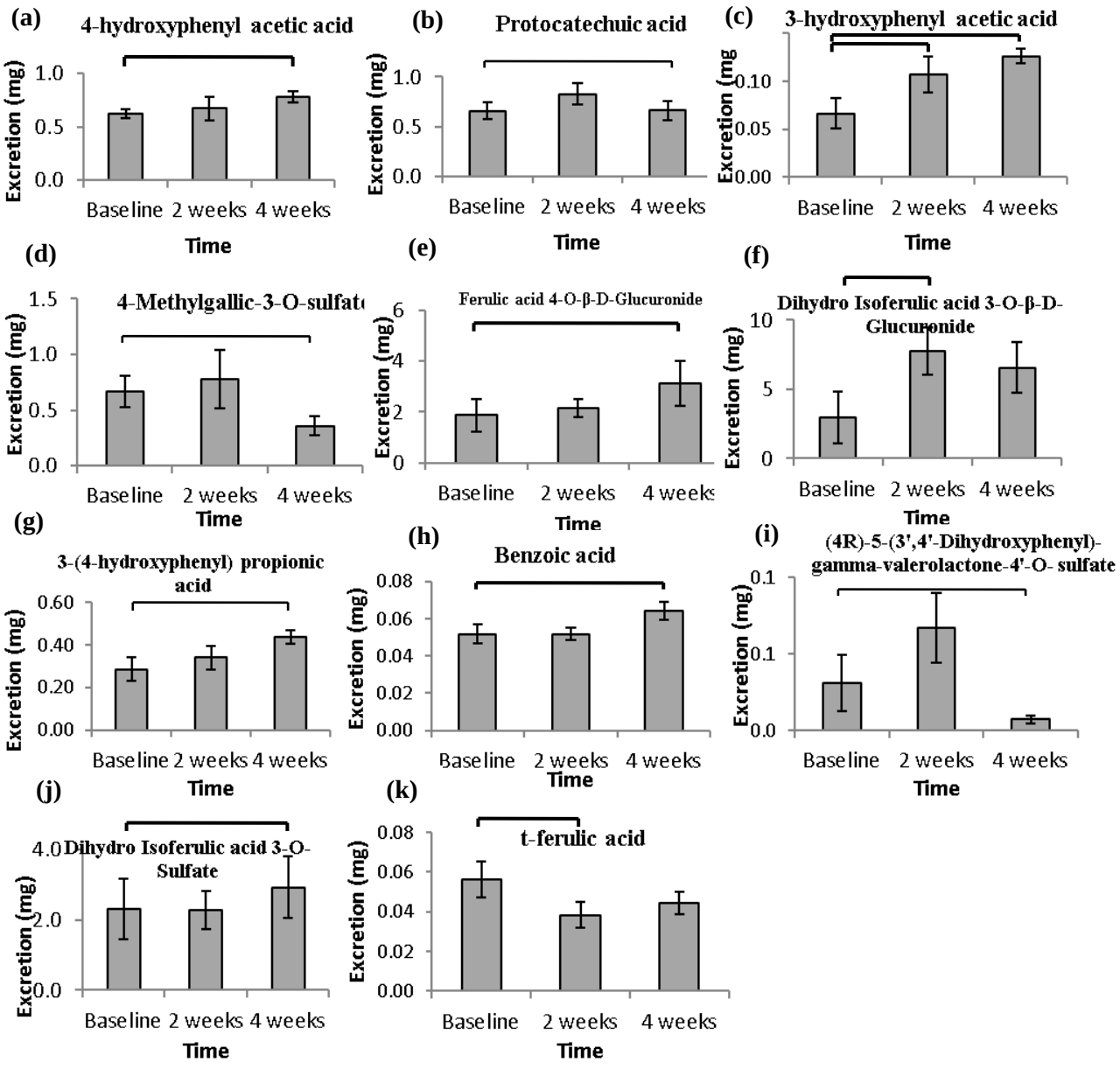


Figure 6.12. Mean (±SEM) urine metabolite concentrations for 7 participants supplemented with placebo at baseline, 2 weeks and 4 weeks for (a) 4-hydroxyphenyl acetic acid, (b) protocatechuic acid, (c) 3-hydroxyphenyl acetic acid, (d) 4-methylgallic-3-O-sulfate, (e) ferulic acid 4-O-B-D-glucuronide, (f) dihydro isoferulic acid 3-O-β-D-glucuronide, (g) 3-(4-hydroxyphenyl) propionic acid, (h) benzoic acid, (i) (4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate, (j) dihydro isoferulic acid 3-0-sulfate, (k) t-ferulic acid. Bars in bold represent a significant difference. Bars not in bold represent a trending difference.

6.3.3. Associations between metabolite excretion and cognitive performance

6.3.3.1. Raw data analysis

6.3.3.1.1. Cognition

Standardised composite scores were created from cognitive variables, producing a measure of EF (MANT performance), memory (AVLT recall performance) and interference (proactive and retroactive) for each respective time point (Table 6.5).

Composite	Time point	Placebo		WBB	
		Mean	SD	Mean	SD
EF	Baseline	-0.40	0.86	0.35	0.44
	2 weeks	-0.30	1.03	0.26	0.39
	4 weeks	-0.28	0.98	0.25	0.44
Memory	Baseline	-0.43	0.86	0.27	0.49
	2 weeks	-0.24	0.84	0.18	0.75
	4 weeks	-0.12	0.94	0.09	0.58
Interference	Baseline	-0.06	0.56	0.06	0.63
	2 weeks	0.27	1.01	-0.23	0.47
	4 weeks	-0.06	0.60	0.05	0.60

Table 6.5. Mean and SDs of standardised composite cognitive scores at each time point (baseline, 2 weeks, 4 weeks) for each treatment group (placebo, WBB).

6.3.3.1.2. Metabolites

The investigation was not restricted to metabolites for which excretion was increased. All metabolites were included in combined data analyses whether they were unchanged, increased or decreased over the intervention period.

A principle components analysis (PCA) was conducted on 27 metabolite variables with oblique rotation (Promax). The KMO (0.48) determined the sampling adequacy for the analysis as "below the bare minimum of 0.5" according to Field (2009). Bartlett's test of

sphericity, χ^2 (351) = 1045.10, p<0.01, indicated that correlations between items were sufficiently large for PCA. Initial analyses of the data obtained 7 components with eigenvalues ≥ 1, conforming to Kaiser's criterion. In combination, these components explained 78.14% of the variance, with the first eigenvalue indicating that component 1 explained 25.56% of the variance. A total of 10 items were eliminated because they failed to meet regression and correlation coefficient loading cut-offs (Stevens, 2002).
For the final stage, a PCA of the remaining 17 metabolite variables using oblique rotation (Promax) was conducted. The KMO verified the sampling adequacy for the analysis, KMO = 0.56 (deemed "mediocre", but acceptable according to Field, 2009). Bartlett's test of sphericity, χ^2 (136) = 581.27, p<0.01, showed between-item correlations to be sufficiently large. Five components with eigenvalues ≥ 1 were obtained, and further met Kaiser's criterion with an unambiguous scree plot. The combination of components explained 75.85% of the variance; this time the eigenvalue for component 1 explained 29.36% of the variance. Oblique rotation provided the best factor structure, and Table 6.6 shows the factor loadings after rotation for pattern and structure matrices. Factor analysis of raw metabolite data suggested component 1 represents ferulic acids, component 2 pyrogallol derivatives, component 3 vanillic acids, component 4 cinnamic-benzoic derivatives, and component 5 chlorogenic acid.

6.3.3.1.3. Combined data

Correlations were performed between cognitive composites (EF, memory, interference) and metabolite factors (ferulic, pyrogallol, vanillic, cinnamic-benzoic and chlorogenic derivatives) at each respective time point. There was a trending relationship between chlorogenic acid excretion and EF performance at baseline (r=.41, p=0.08). When explored, this relationship was apparent within the WBB group only (r=.77, p=0.04). As this was observed prior to treatment consumption, this cannot be attributed to WBB intervention and is likely an effect of consuming a high chlorogenic acid food in the 24 h prior to baseline (such as apples, pears or potatoes; Gonthier, Verny, Besson, Rémésy and Scalbert, 2003). From consulting participant's food logs, no participant appeared to consume particularly high levels of chlorogenic acid-containing fruits, vegetables, tea or coffee on the day of collection, although 5 of the 8 participants assigned to WBB consumed potatoes on the day of baseline collection compared to 6 out of 7 placebo participants. Therefore, it is unlikely that potato consumption had an effect on the WBB group's chlorogenic acid excretion. Instead, it is more plausible that the differences in chlorogenic acid excretion may have been influenced by the foods

Variable	Pattern Component Matrix					Structure Component Matrix				
	1	2	3	4	5	1	2	3	4	5
Ferulic acid 4-O-B-D glucuronide	.967					.928				
Isoferulic Acid 3-O-B-D glucuronide	.966					.931				
Dihydro isoferulic acid 3-O-sulfate	.949					.961				
Dihydro ferulic acid 4-O-B-D glucuronide	.753					.761				.504
Syringic acid	.732					.759				
Isoferulic acid	.538						.664			.536
Pyrogallol-O-2-sulfate		.893					.892			
1-methylpyrogallol-O-sulfate		.871					.862			
4-methylgallic-3-O-sulfate		.853					.848			
Dihydro caffeic acid-3-O-sulfate sodium salt		.794					.814			
Homovanillic acid			.943					.931		
Vanillic acid			.927					.934		
3-hydroxyphenylacetic acid			.475					.472		
Ferulic acid-4-O-sulfate				.925					.921	
4-hydroxybenzoic acid				.747					.793	
2’4’dihydroxybenzoic acid				.594	-.484				.546	-.419
Chlorogenic acid					.890					.883

Table 6.6 Pattern and structure matrices for metabolite raw scores after PCA oblique rotation. Pattern matrix shows regression coefficient factor loadings and structure matrix shows correlation coefficient factor loadings. Values below 0.4 have not been presented according to Stevens’ (2002) guidelines. Factor loadings above the recommended cut-off for statistical significance in a sample of 50 at $p<0.01$ (0.722) have been highlighted.

consumed the day prior to urine collection, for which no food log data was recorded. Excretion of pyrogallol derivatives was significantly related to memory performance at baseline (r=.49, p=0.045). This association was not significant when explored by treatment.

At the 2 week time point, excretion of vanillic acids was found to weakly correlate with a reduction in memory performance (r=-.36, p=0.09), regardless of treatment drink.

There was a weak association between excretion of ferulic acids and reduced interference at baseline (r=-.38, p=0.097). This relationship was also observed at the 2 week time point, but here, ferulic acid excretion correlated with increased interference (r=.43, p=0.056). Neither of these correlations were significant when analysed by separate treatment group.

Vanillic acid excretion was found to be significantly related to reduced interference at baseline (r=-.77, p<0.01), but increased interference at 2 weeks (r=.44, p=0.05; Figure 6.13a). No significant correlations were observed by treatment group at 2 weeks, however at baseline, this reduction in interference was significantly evident in both placebo (r=-.81, p=0.05) and WBB (r=-.76, p=0.047) groups. Lastly, a correlation between excretion of cinnamic-benzoic derivatives and reduced interference at baseline was marginally significant (r=-.46, p=0.058). When explored, this association was significant for WBB participants only (r=-.90, p<0.01).

No significant correlations were observed at the 4 week time point.

6.3.3.2. Change from baseline data

6.3.3.2.1. Cognition

CFB scores were derived from raw standardised composite scores by subtracting baseline performance from 2 week and 4 week performance, respectively. This produced measures of change in EF (MANT performance), memory (AVLT recall performance) and interference (proactive and retroactive) at 2 weeks at 4 weeks.

6.3.3.2.2. Metabolites

A principle components analysis (PCA) was conducted on 27 metabolite variables with oblique rotation (Promax). The KMO and Bartlett's test was not computed due to the correlation matrix being non-positive definite (NPD). This was confirmed by a determinant

value of 0, rendering a singular matrix. Data was defined as NPD because two eigenvalues were negative numbers and there were linear dependencies between another two eigenvalues, as reflected by values of 0. Removal of 21 variables that did not appropriately load onto components, as recommended by Wothke's (1993), abolished incidence of NPD, and tests of appropriateness were computed. KMO (0.39) determined the sampling adequacy for the analysis as "below the bare minimum of 0.5" according to Field (2009). This was likely due to the above data reduction and subsequent diffusion in the pattern of correlations (Field, 2009). However, Bartlett's test of sphericity, χ^2 (15) = 102.61, $p<0.01$, indicated that correlations between items were sufficiently large for PCA. Additionally, several 'rules of thumb' were satisfied, namely Kass and Tinsley's (1979) recommendation of using a minimum of 5 to 10 participants per variable, and Guadagnoli and Velicer's (1988) suggestions that the most important factors in determining reliable factor solutions was the absolute values of factor loadings; when there are four or more loadings larger than 0.6, then the analysis is reliable, regardless of sample size. The data met this criterion. Furthermore, MacCallum, Widaman, Zhang and Hong (1999) suggested that with communalities above 0.6, sample sizes below 100 may be perfectly adequate: the current dataset complies with this criterion. In combination, this indicated that it was appropriate to continue with the current analyses.

For the final stage, a PCA of the remaining 6 metabolite variables using oblique rotation (Promax) was conducted. Three components with eigenvalues ≥ 1 were obtained, and further met Kaiser's criterion with an unambiguous scree plot. The combination of components explained 92.21% of the variance, and the eigenvalue for component 1 explained 36.49% of the variance. Oblique rotation provided the best factor structure, and Table 6.7 shows the factor loadings after rotation for pattern and structure matrices. The variables that cluster on the same components suggest component 1 represents pyrogallol derivatives, component 2 vanillic acids, and component 3 phenylacetic-benzoic derivatives.

Variable	Pattern Component Matrix			Structure Component Matrix		
	1	2	3	1	2	3
Pyrogallol-O-2-sulfate	.977			.977		
1-methylpyrogallol-O-sulfate	.991			.988		
Homovanillic acid		.978			.977	
Vanillic acid		.980			.978	
3-hydroxyphenylacetic acid			-.902			-.888
Protocatechuic acid			.860			.876

Table 6.7 Pattern and structure matrices for metabolite CFB scores after PCA oblique rotation. Pattern matrix shows regression coefficient factor loadings and structure matrix shows correlation coefficient factor loadings. Values below 0.4 have not been presented according to Stevens' (2002) guidelines. Factor loadings above the recommended cut-off for statistical significance in a sample of 25 at $p<0.01$ (0.797) have been highlighted.

6.3.3.2.3. Combined data

Correlational analyses were performed on CFB cognitive composites and CFB metabolite factors. Increased excretion of vanillic acids at 2 weeks was weakly correlated with improvements in EF, compared to baseline (r=.41, p=0.08). No significant correlations were observed when split by treatment group (placebo, r=.41, p=0.42; WBB, r=-.04, p=0.93), or when assessing change in any metabolites at the 4 week time point.

As with raw data analyses, a significant relationship was observed between increased change in vanillic acids and increased interference change at the 2 week time point (r=.48, p=0.047; Figure 6.13b). This did not render any significant associations when split by treatment group (placebo, r=.54, p=0.27; WBB, r=.03, p=0.95), or at 4 weeks of the intervention (r=-.12, p=0.37).

No significant correlations with memory performance were evident.

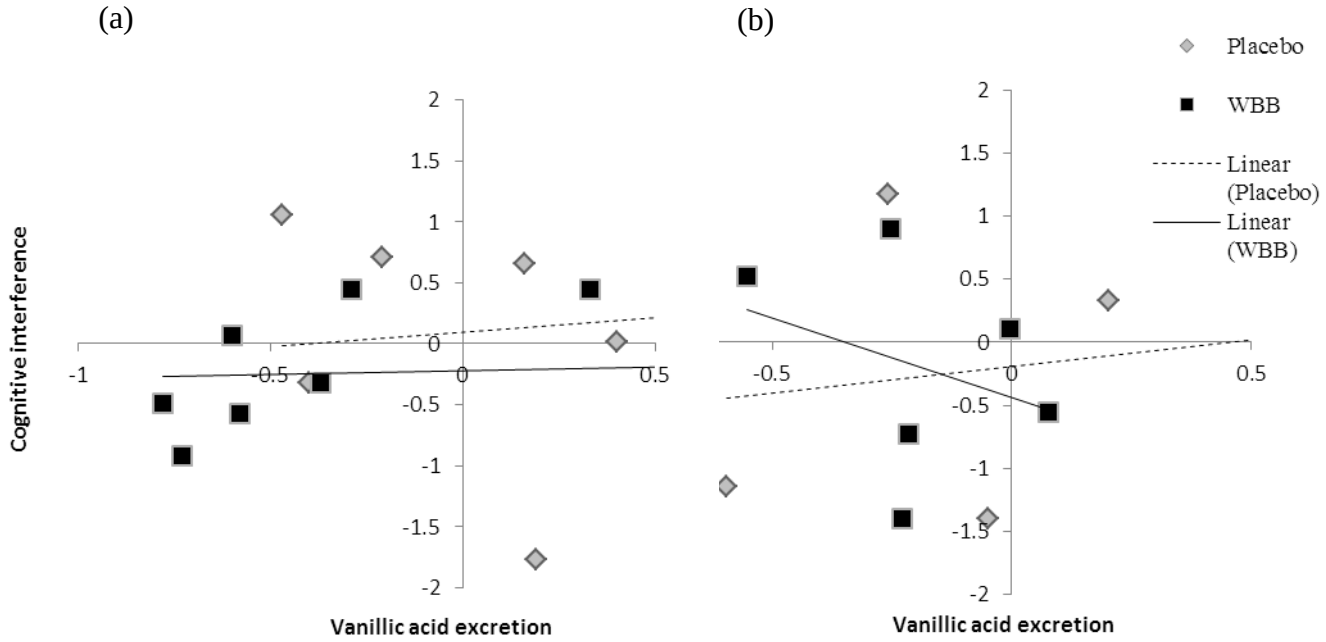


Figure 6.13 Correlational analyses between excretion of vanillic acids and cognitive interference at 2 weeks (a) Significant positive association between excretion of raw vanillic acids and cognitive interference at the 2 week time point, regardless of treatment group. (b) Significant positive association between increased change in excretion of vanillic acids and increased change in cognitive interference at 2 weeks, regardless of treatment group. Significance did not emerge when split by treatment group.

6.4. Discussion

Cognitive findings

The current study administered a daily dose of flavonoid-rich wild blueberry to typically developing 7-10 year old children following a randomised, placebo-controlled, between-groups design, to investigate the effects of chronic consumption on mood, cognition and urinary metabolites.

Accuracy improvements were observed on the MANT following WBB intervention, comprising of increased accuracy on incongruent trials. Additionally, WBB participants did not show decreases on the more difficult incongruent high load trials, whereas placebo participants did. These findings suggest WBB was of most benefit to children under conditions of increased cognitive demand, as also observed by Whyte et al (2017) and in Chapters 4 and 5 of the current thesis. Better accuracy for WBB-treated participants was also observed on medium load trials, with a similar pattern emerging on high load trials, albeit non-significantly. Accuracy and RT were also found to improve on the faster 120ms trials after WBB supplementation indicating improved speed of processing and mental alertness, as in Chapters 3 and 5. One significant memory effect was observed whereby retroactive interference was higher in those consuming WBB treatment, potentially indicating an increased ability to encode words. Interestingly, less hyperactivity was reported by parents at the end of the intervention, regardless of treatment assignment. Although interesting, caution does need to be taken when interpreting typical children's behaviour using a measure designed for those with ADHD. No significant treatment effects were observed in mood or behavioural measures.

On the MANT, increased accuracy was observed for WBB participants when compared to placebo participants at the 2 week time point. This effect did not persist between groups at the 4 week time point, despite sustained performance for WBB participants. This was due to a significant increase in placebo participants' accuracy performance at the 4 week time point compared to 2 weeks. Explanation for this improvement in placebo participants' performance at 4 weeks is ambiguous, but is unlikely to be the result of practice, due to the inclusion of a practice session to diminish such effects (Bell et al, 2017). A likely explanation for the absence of drink effects at 4 weeks is greater variation in the placebo group's accuracy scores. Individual variation in placebo participants' performance at the 2 and 4 week time points was, on average, 1 SD greater than the variation observed in WBB participants at these time points.

This was seen to be primarily driven by lower minimum range scores in the placebo group at 2 weeks (min=0.52, SD=0.17) and 4 weeks (min=0.63, SD=0.12) when comparing to minimum range scores in the WBB group at 2 weeks (min=0.94, SD=0.02) and 4 weeks (min=0.95, SD=0.01). This would have increased the variance in accuracy for the placebo group and likely contributed towards the absence of between-group differences at 4 weeks.

Evidence for beneficial WBB effects on cognitively demanding trials of the MANT were apparent on all levels of task manipulation. WBB participants performed significantly more accurately on incongruent trials than placebo participants, regardless of time point, an effect also observed by Whyte et al (2017) and in Chapters 4 and 5. An interaction between congruency, load and drink was also evident; placebo participants performed significantly slower on high load incongruent trials when compared to medium load incongruent trials. This was an expected effect of the task due to the increased cognitive resource required for high load trials. However, no such difference was observed in WBB participants, suggesting maintenance of performance on the most cognitively demanding conditions of the task for this group. This supports Whyte et al's (2017) research, which suggests supplementation with WBB can overcome the increased demand participants experience in conditions of high cognitive load, and specifically highlights the potential for WBB benefits across conditions of increasing complexity on the MANT, such as high load and incongruence.

Similarly, a load effect was evident in the current study. WBB participants were significantly more accurate on the less cognitively demanding medium load trials compared to placebo participants. This pattern was also observable on the more cognitively demanding high load trials, but did not quite reach statistical significance. Similar to congruency findings, the placebo group's variation in scores was a likely contributor to the absence of significant group differences. Interestingly, WBB participants did not show a significant difference between high and medium load trials, suggesting that WBB participants overcame the demands of high load trials and performed just as accurately as on medium trials.

Accuracy and RT were revealed to be significantly improved for WBB participants compared to placebo participants on 120ms trials at 2 week and 4 week time points. This indicates WBB participants were able to reduce their response speed and still perform as accurately on faster-paced trials after 2 and 4 weeks of daily WBB supplementation. Furthermore, these participants were also significantly quicker on 120ms trials at 4 weeks compared to 2 weeks, with no significant cost to accuracy. Interestingly, accuracy improvements appeared at 2 weeks into the intervention, whereas RT improvements occurred at 4 weeks. This suggests a

potential order effect in the manner in which cognitive ability undergoes change. To be able to perform a cognitive task at a faster speed, participants may initially have to 'master' the task at hand, taking as long as required to make a correct response. This may result in accuracy improving before RT is able to decrease. Once the task is mastered, participants may then be more able to perform the task at a faster pace, maintaining accuracy levels. This is supported by the simple repeating paradigm pattern of the ACT-R Cognitive Architecture model which suggests faster RTs occur after exposure to systematic event sequences, by applying acquired knowledge about the event pattern (Lebiere and Wallach, 2000). Still, the question remains as to whether mastery can be achieved within one exposure of a task, or across several. The current data suggests mastery is achieved by the third exposure, following practice and baseline exposures, at 2 weeks. This is similar to findings in Chapter 5 which also showed overall accuracy improvements by the third exposure at 2 weeks, and at 4 weeks, suggesting mastery occurred with one further exposure in this sample. In the current study, RT was found to quicken for both treatment groups at 4 weeks compared to 2 weeks, indicating mastery of the task. However, a significantly larger RT reduction was observed at 4 weeks compared to 2 weeks for those in the WBB group; the reduction was only seen as a trend in placebo-treated participants, suggesting the WBB group were marginally quicker.

One significant drink effect was observed for the AVLT; WBB participants showed increased proactive interference at 4 weeks compared to 2 weeks. PI can be defined as the interference of previously learnt material on subsequent information recall. In terms of the AVLT, for WBB participants, more words were recalled at list recall A1 compared to list recall B1, reflecting an increase in interference from A1-A5 list presentations. The data suggests that WBB was beneficial at 2 weeks; WBB participants showed reduced interference compared to those under placebo (albeit non-significantly) at this time point. The significant increase in PI for WBB-treated participants at 4 weeks seems to indicate that WBB may detriment performance. Indeed, there may be a 'sweet spot' after 2 weeks of daily consumption by which WBB may be able to positively affect interference parameters, that then decreases at the 4 week time point. Optimal time points and dosages have been posited by Kay et al (2012), Rodriguez-Mateos et al (2013) and have previously been discussed (see Chapter 1), and may be plausible here. Alternatively, such a result could be due to an increased lack of engagement in the task during its fourth presentation within the study; participants may have been less focused on trying to retain lists A and B in memory due to boredom. Recall across lists A1-A7 and B1 was decreased at the 4 week time point compared to 2 weeks for both treatment groups (as shown in Figure 6.7) supporting the notion that all participants' efforts

may have been lower here. As there were no significant between-drink differences at the 4 week time point, it cannot be said that WBB had increased interference compared to placebo. In fact, placebo participants showed increased PI at 2 weeks at 4 weeks, indicating these participants did not improve during the course of the intervention and may have disengaged at 4 weeks.

On the other hand, such results may imply that WBB may aid in encoding information, but not in retrieval, as also observed and interpreted in Whyte et al's (2015) acute study. Surprisingly, PI effects were not observed in the acute experiments reported in Chapters 3, 4 and 5, or in the chronic arm of Chapter 5. Although, between-treatment encoding differences were implied in Chapter 4 where participants who consumed WBB first had better performance than those who consumed placebo first. The absence of PI effects in previous experiments of this thesis indicates that this measure may not be stable following WBB consumption and caution is necessary when interpreting results here. It could also be possible that PI performance recruits from executive function domains as well as memory, indicating WBB participants may have had substantial inhibitory capability at 2 weeks, which declined at 4 weeks, again potentially indicating an optimal daily supplementation duration. Further research should aim to elucidate whether this paradigm is consistent in a child population across acute and chronic time frames to identify the extent to which WBB can aid memory or EF performance.

Interestingly, placebo participants failed to respond to non-target stimuli at a significantly higher rate than WBB participants on the demographic measure of sustained attention. This suggests that those assigned to the placebo group were, by chance, of a lower attentional ability. This was supported by significantly lower accuracy on the MANT for this group at baseline, a task requiring attention. However, accurate response to stimuli in the MANT was consistently high in both placebo (range = 80.75 - 88.52%) and WBB (range = 92.38 – 96.82%) groups across the course of the intervention, suggesting this characteristic may not have been a detriment to placebo participants beyond baseline. Indeed, no significant differences were observed between groups at the 2 or 4 week time point.

Taste ratings were also found to differ; the placebo drink was rated as significantly more likeable compared to the WBB drink. This suggests that the drinks were not fully matched to taste and that differences were detectable to participants, as also observed in Experiment 2 (Chapter 4). This could be due to the texture of the WBB drink, which had a thicker consistency. Future studies should consider including other palatability measures such as

texture and smell to determine aspects of the drinks that participants do not like, to further inform adequate placebo-control matching. The important aspect of the current study is that no participant rated their assigned treatment drink below a 4 (out of 10), indicating their treatment was drinkable and did not cause significant disgust.

The WBB treatment had no effect on PA or NA (PANAS-C), however PA was found to significantly decline at 4 weeks compared to 2 weeks, regardless of treatment, as in Chapter 5. This was to be expected and is likely due to lack of engagement due the tedium and repetitiveness of the study schedule; participants felt less positive on the last test day as the novelty of participating in the experiment had diminished. It is important to note that negative affect did not change over the intervention period, thus participants did not feel more negative at the end of the intervention.

Overall, a lack of treatment-related findings was observed for the PANAS-C, AVLT and ADHD IV. This was unsurprising for the PANAS-C due to the small sample size of the current study. The target sample size (n=46) was partly informed by an *a priori* power analysis, based on cognitive parameters (effect size (F) of 0.22 and power of 0.9; see section 6.2.1). However, sample size to detect a physiological effect was much lower (n= 9-18) based on previous metabolite research that showed high inter-individual variation (Feliciano et al, 2016b; Rodriguez-Mateos et al, 2016). Recruitment of 15 participants in the current experiment suggests the study may be adequately powered for physiological effects, but not for cognitive effects. Significant acute mood findings have been observed previously in a sample of 54 children following WBB intervention (Khalid et al, 2017), suggesting there may not have been enough power to detect a change in the current study. Indeed, post-hoc power analysis resulted in a power of 0.4 at a medium effect size (F=0.22), suggesting mood findings were likely underpowered.

Employment of the PANAS-C, AVLT and ADHD IV in a chronic WBB intervention trial has also not been implemented before. The absence of significant treatment findings on the PANAS-C and AVLT infers that the current sample may not have also been sufficiently powered to detect chronic effects; indeed, previous WBB benefits observed in acute trials (Chapter 3 (n=54, between-groups); Whyte et al, 2016 (n=21, crossover); Khalid et al, 2017 (n=54, between-groups) had a larger number of participants. As for the ADHD-IV, the children recruited in this study were not diagnosed with ADHD and were therefore deemed 'typical'. It may be that the ADHD-IV is not sensitive enough to detect behaviour change in typically developing children; indeed, this measure is usually employed in children with an

ADHD diagnosis. However, reduced inattention over the course of a 4 week WBB intervention was observed in the typical and ADHD sample in Chapter 5. Although the duration of the intervention was the same between studies, the sample size of the current study was noticeably smaller, suggesting there may not have been enough power to detect an effect. Moreover, this measure was not derived directly from the participant, but was a score obtained from parent ratings, again highlighting that a small change in behaviour, or change at school, may not have been noticed and recorded by parents. Interestingly, hyperactive behaviours were reported less at 4 weeks compared to baseline, regardless of treatment group in the current study. However, this finding did not reach significance so conclusions cannot be made regarding overt behaviour change in the current sample.

Metabolite findings

Increased excretion of total polyphenols was observed for WBB participants over the course of the intervention, but this did not reach statistical significance. On the contrary, placebo participants showed a non-significant decline. This implies that repeated daily consumption of a WBB drink across 4 weeks may increase the total concentration of polyphenolic metabolites excreted in 7-10 year old children. However, the degree at which these total changes are driven by individual metabolite concentrations is yet to be elucidated.

Increases in excretion unique to WBB were detected for homovanillic acid, syringic acid and dihydro ferulic acid-4-O-B-D-glucuronide at 4 weeks compared to baseline. Similar increases in homovanillic acid have been observed previously in plasma after daily intake of mixed berries in middle-aged adults for 8 weeks (Koli et al, 2010). However, a previous 4 week WBB trial in young adults (Feliciano et al, 2016b) failed to detect such increases, suggesting homovanillic acids may not exclusively be metabolised from blueberries, but rather from a combination of berries. Indeed, homovanillic acid is metabolised from 4 different flavonoids (Vitaglione et al, 2007), highlighting its potential distinctiveness in blue and red berry metabolism. It is feasible that flavonoids derived from other dietary sources in the 48 h prior to the 4 week urine collection, may have converged with the flavonoids present in WBB, producing increased excretion of homovanillic acid. There is no way of empirically determining this, as only food diaries outlining food consumption during the 24 h urine collection are available. From 3 day food diary data, participants can be said to have average fruit and vegetable consumption when compared to the latest Health Survey for England

(2017), which shows children aged 5-15 consume ~3.1 portions of fruit and vegetables a day. It could, therefore, be possible that children consumed an average amount of fruits and vegetables in the days prior to urine collections, leading to interactions between blueberry and other flavonoids.

Increased concentration of 3-hydroxyphenylacetic acid was evident at 4 weeks following WBB intervention in the current study. These results are also in agreement with Koli et al (2010), who found significantly increased urinary levels of this compound after an 8 week mixed berry intervention. However, higher levels were also observed in the present placebo participants, suggesting increased excretion of this compound may not be specific to blueberries. Indeed, we have seen that this effect exists after mixed berry supplementation (Koli et al, 2010); therefore it is plausible that placebo participants consumed polyphenols from another dietary source in the 24 h prior to the final urinary collection, which was not recorded. It is reassuring to note that although increases were detected in both treatment groups, mean excretion in the WBB group rose by 390ug at 4 weeks compared to baseline, whereas levels rose by only 60ug in the placebo group.

Similar increases were evident at 2 weeks for (4R)-5-(3',4'-dihydroxyphenyl)-gamma-valerolactone-4'-O- sulfate in both treatment groups. The crucial finding here is a 22ug overall increase in this compound for WBB participants at the 4 week time point, compared to baseline. In contrast, a 24ug reduction between baseline and 4 weeks was seen in placebo participants. Although discrete and highly variable, this pattern of results demonstrates the potential that valerolactone derivatives may be indicative of WBB metabolism.

Benzoic acid increases were also detected to similar concentrations after 4 weeks in both treatment groups. Although these compounds can exist within blueberries and other polyphenol-rich foods, Mosele et al (2015) noted that they are also by-products of gut microbiota activity and flavonoid metabolism. They may, therefore, be indicative of other digestive processes or food consumption, unrelated to blueberry polyphenol metabolism specifically, which was not accounted for in the current study. Although there were differences in the activity of specific metabolites between this work and previous research (Feliciano et al, 2016b), urinary concentrations of benzoic, cinnamic and phenylacetic acid groups did increase after repetitive WBB consumption, as previously observed.

It is worthy to note that although total polyphenol excretions between treatment groups appeared to be different at baseline, significance was not observed. Indeed, observable

differences at baseline can occur, as in Feliciano et al's (2016a) quantification of cranberry metabolites; despite participants' compliance with a low polyphenol diet for 72 h before the study commenced, baseline differences in concentrations of phenolic compounds were significant and highly variable. This was noted specifically for levels of hippuric and hydroxyphenylacetic acids, which interestingly, are also endogenous metabolites that are formed from other non-phenolic compounds. Endogenous production of metabolites could also aid in the explanation of a rise in 3- hydroxyphenylacetic acid for both groups as discussed previously.

Increases in hippuric acid were not observed across the current 4 week intervention. This was surprising due to the large contribution of this metabolite to the circulating polyphenols in a previous 4 week WBB trial (Feliciano et al, 2016b), 12 week bilberry trial (Hanhineva et al, 2015) and 8 week high polyphenol diet intervention (Vetrani et al, 2016). Unexpectedly, phase II metabolites such as dihydrocaffeic acid 3-O-sulfate and isoferulic acid 3-O-B-D-glucoronide were also not found to increase in the current 4 week WBB intervention. These findings are not in agreement with Rodriguez-Mateos et al's (2016) previous work, and suggest that blueberry polyphenols may not have changed sulfotransferase and glucuronosyltransferase metabolising capacity in the current sample. Comparisons between excretions of catechol derivatives are futile due to non-quantification of catechol-O-sulfate in the current investigation. However, 4-methylcatechol-O-sulfate was quantified in both studies and shows no change in either, suggesting catechol-O-sulfate may be a better marker to quantify change in catechol derivatives in the future.

It is worthy to note that interactions between foods in the diet and polyphenols can change the efficiency of polyphenol absorption (Spencer et al, 2008). The addition of milk (Serafini et al, 2003), bread, and sugar-containing meals (Schramm et al, 2003) alongside consumption of polyphenol-rich chocolate has previously been found to affect epicatechin absorption. As diets were not restricted for the majority of the current intervention, it is possible that prior consumption of high carbohydrate foods may have impacted polyphenol metabolism during urine collections. This may have been the case for either treatment group; increased carbohydrate consumption for WBB participants on urine collection days (pre- and post-consumption of the WBB drink), and for placebo participants consuming low carbohydrate and high polyphenol foods on non-restriction days.

It is well known that large inter-individual variation exists between individuals across investigations into polyphenol metabolism. Genetic variability in the expression of metabolic

enzymes and transporters is thought to play a part in post-absorptive metabolism, and these metabolic functions are likely to work differently depending on the individual, food source and dose (Vitaglione et al, 2005). High inter-individual variability is therefore expected in investigations of this kind, highlighting the importance of within-participant comparisons. An increase in sample size necessary to detect significant results in a between-groups design is unlikely to control variability; instead, a higher number of measurements across time may better account for within-participant comparisons. However, for crossover designs, a higher number of participants may improve the power to detect metabolite change.

Metabolites vs cognition findings

Higher circulating levels of vanillic acids, and increases in excretion of these metabolites over time, was shown to be indicative of increased cognitive interference. These findings were observed after 2 weeks of participation in the RCT, but were not sustained at 4 weeks. It is important to note that increases in interference associated with both higher levels of vanillic acids during the 2 week urine collection, and the positive change in concentration of vanillic acids at 2 weeks compared to baseline, cannot be attributed solely to placebo or WBB consumption. This was evident in the non-significant correlations observed when results were split by treatment group.

A possible reason for a lack of findings in raw excretion levels could be non-compliance to the low flavonoid diet on the day of, or the day prior to, the 2 week 24 h urine collection. Although food consumption was recorded during the 24 h urinary collection, no measure of compliance was used for the preceding 24 h period. It is possible that participants did not follow dietary restrictions during this unrecorded period. Similar non-significant findings for placebo and WBB participants in regard to excretion change in vanillic acids across the first 2 weeks of the RCT, imply that repetitive consumption of a daily WBB drink did not contribute towards the cognitive effects observed, and was perhaps diluted by other dietary factors. Dietary polyphenol intake across the intervention period was not restricted, and all participants were deemed to have average fruit and vegetable intake, as assessed by their 3 day food diaries, and compared to the 2016 Health Survey for England (2017). Variability was present within the 3 day diaries of each participant, as shown by the range values in Table 6.1. This questions the reliability of using 3 day food diaries as a measure of overall diet, as dietary intake can vary substantially on a daily basis. A lack of a portion size measure also

made it difficult to quantify standard portions of fruit and vegetables, especially within mixed meals.

Basiotis, Welsh, Cronin, Kelsay and Mertz (1987) examined the optimum number of days of food intake required to accurately estimate mean energy and nutrient intake. Energy was found to require 3 days, whereas vitamins were much higher; for example, vitamin A required 41 days. Fruit and vegetables were not included as a component in this study. However, as fruit and vegetable intake is often used to reflect how nutritionally enriched participants' diets are, assessment of vitamins, minerals and polyphenols may be the closest indicator. Basiotis et al's data therefore indicates that a larger number of days may be required to assess accurate mean intake of fruit and vegetables. Therefore, ambiguity in the true intake of fruits and vegetables across the intervention period of the current study is present, and so a moderate to high intake of polyphenols via the diet in placebo and WBB participants across the first 2 weeks of the intervention may have contributed to the above findings without detection in 3 day food diaries. It can therefore only be concluded that vanillic acids derived from a non-specific source are associated with increased cognitive interference.

Higher concentrations of chlorogenic acid present in WBB participants' urine at baseline was also likely due to other factors in the diet, either during or prior to the 24 h urine collection, as no treatment drink was consumed during the baseline collection. The association between chlorogenic acid and EF can also be attributed to non-specific polyphenol consumption rather than WBB consumption per se, and may potentially be linked to the moderate to high intake of chlorogenic-rich foods on the day of baseline collection. A similar finding was observed at baseline whereby higher concentrations of pyrogallol derivatives were associated with higher memory performance, regardless of treatment group. Again, this may be due to polyphenol consumption during or prior to the baseline 24 h urine collection, unrelated to placebo or WBB intervention. Significant associations between excretion of chlorogenic acid, pyrogallol derivates and raw cognition scores, or change in these factors, was not evident at subsequent intervention time points, suggesting these metabolite groups were not associated with cognitive factors once enrolled in daily supplementation. Therefore, it is possible to conclude that these findings hold little meaning in terms of repetitive polyphenol consumption and cognition in the current sample.

Increased levels of ferulic acids were, somewhat tenuously, associated with reduced cognitive interference at baseline, regardless of treatment group membership. In contrast, at the 2 week time point, increased excretion of ferulic acids was marginally related to increased cognitive

interference, regardless of treatment group. It is worthy to note that both of these relationships were weak and non-significant, resulting in ambiguous interpretation. Furthermore, no significant associations were found between changes in levels of ferulic acids and changes in interference. This suggests that as increases in excretion (compared to baseline levels) occur, changes in interference do not occur. Links between ferulic acids and interference should therefore be interpreted with caution.

Research has discovered that ferulic acid may protect against neuronal cell damage and have anti-inflammatory properties (Rukhsana, Agrippinio, Hafiz, Vittorio and Butterfield, 2005). A study examining the effects of daily ferulic acid consumption (0.002% and 0.005% in water) for 28 days in memory-impaired mice, found links between chronic administration and attenuation of memory deficits, as shown by resistance to decline on a spatial working memory task and a passive avoidance performance test at the end of intervention (Kim et al, 2007). This suggests this compound may be useful in preventing cognitive decline associated with dementia. Guohua, Yanhua and Dongzhi (2005) proposed that choline acetyltransferase (ChAT) activity in the cerebral cortex may be dampened in those with Alzheimer's Disease (AD). Kim found declines in ChAT, in memory-injured mice, were attenuated after chronic ferulic acid intervention, posing changes in neurotransmission as a potential MOA behind the effects observed. Similarly, in a mouse model of brain dysfunction indicative of AD, daily treatment with ferulic acid (5mg/kg) for 6 days ameliorated memory deficits, as assessed by a novel object recognition test (Mamiya, Kise and Morikawa, 2008). This research suggests ferulic acid may impact cognitive performance, and may help to explain the link between ferulic acid excretion and interference in the current study. Studies containing a higher number of participants should be conducted to ascertain whether directional interpretations can be replicated to a significant degree, and whether change in ferulic acids excretion is associated with change in interference.

Cinnamic-benzoic derivatives were also found to be strongly and significantly related to a reduction in cognitive interference at baseline, for WBB participants only. This finding cannot be attributed to WBB consumption due to the lack of intervention consumption at the baseline collection. Chance consumption of a higher amount of polyphenol-containing foods in this group is likely the reason for the higher levels of cinnamic-benzoic derivates, and subsequent relationship with interference. Further relationships with cinnamic-benzoic derivates were not observed at any other time point or in CFB analyses. This indicates that non-compliance with the low flavonoid diet could have occurred at baseline for WBB participants, but did not

pertain at 2 week and 4 week collections. Moreover, it is unlikely that the increases observed in cinnamic-benzoic derivates were related to blueberry polyphenols, otherwise results would have been identified at the latter 2 time points.

Overall, this study revealed beneficial effects of chronic WBB consumption on executive function and attention in healthy 7-10 year olds after a 4 week chronic intervention. Alongside cognitive improvements, an overall non-significant increase was observed in total urinary polyphenol excretion for those treated with WBB compared to placebo, similar to in an adult population. Increases in individual metabolites were observed for WBB and placebo groups, namely 3-hydroxyphenyl acetic acid, 4-hydroxyphenyl acetic acid, dihydro isoferulic acid 3-O-β-D-glucuronide, 3-(4-hydroxyphenyl) propionic acid, benzoic acid and (4R)-5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone-4'-O-sulfate. Increases in these metabolites in both treatment groups may have been due to non-compliance to the 24 h food restrictions or via endogenous metabolism. Increased excretion of homovanillic acid, syringic acid and dihydro ferulic acid-4-O-B-D-glucuronide was unique to WBB participants, suggesting these metabolites may be specifically related to blueberry metabolism. Indeed, increases in homovanillic and ferulic acids have been observed previously following berry and ferulic acid intervention trials in adults and mice, respectively, and ferulic acid has previously been linked with the amelioration of cognitive decline; however research investigating the mechanisms between metabolism and brain function are required before making assumptions. Increases in vanillic and ferulic acids were shown to positively correlate with increases in cognitive interference. Such increases in interference may be linked with an improved ability to encode primary information.

It is difficult to determine what proportions of these metabolites were derived from treatment consumption using the current technique, and to measure the consistency of the relationship between metabolites and cognition across time. Caution therefore must be taken when interpreting such data. Future studies employing 24 h urine collections should include 48 h food logs detailing the 24 h periods during and prior to the collection day. This would allow more insight into rates of compliance and the effects of non-treatment polyphenol-rich foods on urinary excretions the following day. Surprisingly, hippuric acid, a major contributor to previous adult urinary pools of metabolites, did not increase across the current intervention. This suggests the metabolism of this compound following repeated WBB intervention may have been different in the current sample, and warrants further investigation comparing adult and child excretion within the same study.

Chapter 7: Final discussion

7.1. Overview of the experiments

This thesis examined the acute and chronic effects of WBB on the cognition (specifically EF, attention, memory) and mood of 7-10 year old children, following on from research that suggested children across this age range may benefit from WBB intervention (Whyte et al, 2015; 2016; 2017). The aims of the thesis were to replicate the acute effects that have been observed previously across a 1-6 h timeframe (Whyte et al; 2015; 2016; 207), and extend the current behavioural data to elucidate the mechanisms by which they might occur, using EEG ERP analysis. Additionally, chronic consumption of WBB in a child sample was investigated here for the first time after 4 weeks of daily dosing. Similarly, the underlying metabolic changes that might occur alongside cognition or mood changes after intervention in a child cohort were explored by assessing urinary metabolites. This data is useful to see whether changes in metabolites and cognition across the same timeframe, are associated.

Cognitive and mood parameters were investigated across four experiments. Experiment 1 used an acute, between-groups design with measurements taken at baseline and 2 h post-consumption to align with known postprandial metabolite and FMD responses (Rodriguez-Mateos et al, 2013) and to utilise a sensitive window where cognitive benefits have emerged in a previous child population (Whyte et al, 2015). This study aimed to replicate these previously observed acute benefits. Cognitive and mood parameters were then tested using an acute crossover design in Experiment 2, where ERP techniques were included to investigate the real-time neuropsychological changes that occurred on cognitive tasks in response to WBB intervention. Such data would help to converge the physiological mechanisms following WBB consumption with cognitive performance, and help to elucidate a potential acute mechanism of action. It was also critical to determine the chronic effects of WBB on cognition and mood in a child sample, to see whether daily supplementation improved outcomes similarly to a one-off dose. Therefore, experiment 3 investigated mood and cognition, using a between-group acute x chronic design, after a 2 h and 4 week time period, respectively, to investigate both acute and chronic effects in a child sample. This study included measures of behaviour chronically and recruited a sub sample of children diagnosed with ADHD to assess the therapeutic effects of WBB. This was due to previous data implying cognitive demand to be important to WBB effects, initiating investigation into these WBB effects in a cognitively compromised population, such as those with ADHD. The last experiment in the thesis tested cognition, mood and behaviour across 4 weeks using a

between-group chronic design, concurrent with urinary metabolite measurement, to assess the bioavailability of blueberry polyphenols following repeated consumption in a child sample. Such data is particularly useful to measure the metabolites that change over time, and see whether such change may be associated with cognitive improvement. Experimental details and outcomes will be discussed further in each section below.

7.1.1. Experiment 1

The initial experiment examined the acute effects of WBB in a typically developing (TD) child population. WBB (253mg anthocyanins) or a matched placebo was administered in the afternoon, after lunch. It was predicted that WBB would maintain or improve concentration and cognitive ability in this post-prandial period. Participants performed a cognitive task battery at baseline and 2 h following treatment, which consisted of the PANAS-C, AVLT and MANT to assess mood, memory and attention/EF, respectively. As previous beneficial memory and EF effects were observed across 1-3 h and 6 h in a child population (Whyte et al, 2015; 2016; 2017), similar results were hypothesised for the current experiment. Many researchers have previously noted that flavonoid effects may be most detectable when tasks require high cognitive demand or induce sustained cognitive fatigue. It was therefore predicted that WBB effects may occur on the MANT, a task that utilises attentional and executive function abilities, specifically on trials of an incongruent or high load nature based on previous research employing a Modified Flanker Task (MFT) and MANT (Whyte et al, 2016; 2017). Congruency or load effects did not emerge in the current experiment, however attentional benefits were observed on trials presented at a fast speed. WBB participants performed significantly quicker on 120ms trials compared to placebo and to 500ms trials, indicating increased mental alertness on trials of a quicker pace. Such findings have not been observed previously and suggest WBB may improve processing and motor speed regardless of congruency or load manipulations. Inability to replicate congruency or load findings may have been due to the blocks of the MANT not being randomised, as they had been in Whyte et al's (2016; 2017) studies. Participants may therefore have become accustomed to the order in which blocks were presented at baseline and 2 h, reducing the cognitive demand and therefore the sensitivity of the task. It was therefore decided that all future inclusions of the MANT in the current thesis would randomise blocks to maintain sensitivity to detect potential WBB effects.

Positive episodic memory effects were also observed in Experiment 1 with participants consuming WBB showing attenuated forgetting for total acquisition, total 1-7 and short delay recall measures on an AVLT. These results aligned with previous research that showed improvements on delayed recall (Whyte et al, 2015; 2016) and final acquisition (Whyte et al, 2016) measures on the same task across a 1-3 h post-consumption window, implying WBB may be beneficial for short-term episodic memory function. Whyte et al (2016) also discovered that WBB prevented decline on word recognition performance, a finding that did not emerge in the current experiment. However, due to the differences in the time of testing between the two experiments (morning vs. afternoon), it may be that recognition effects are more sensitive to WBB intervention in the morning. Children are likely to get fatigued across the school day, with poorer performance occurring in the afternoon compared to the morning. As it has been posited that flavonoids may aid performance under conditions of higher fatigue, it is plausible that WBB effects may be most potent in the afternoon. However, this may not be true across all cognitive domains, and could impact EF, verbal memory and visual recognition memory domains differently. At present it is unclear whether such time of day differences might occur and this warrants further investigation.

Mood had not been examined in a child WBB intervention previously, but was predicted to show marginal improvements over the 2 h period based on previous research that suggested flavonoid intervention increased calmness, contentedness (Pase et al, 2013) and alertness (Alharbi et al, 2016) chronically, and positive mood acutely (Khalid et al, 2017) in healthy adult populations. Analyses revealed a near-significant increase in positive mood for participants consuming WBB, indicating flavonoids may have the potential to impact short-term mood in school-aged children. This is promising as it suggests acute nutritional intervention may help to regulate mood in a pre-pubescent population prior to the high onset risk of mood disorders, such as anxiety and depression, seen in adolescents.

In light of Experiment 1's results, a further acute trial was designed and carried out in Experiment 2 to include physiological acute WBB activity.

7.1.2. Experiment 2

Experiment 2 also aimed to examine the acute effects of WBB on cognition and mood in a TD 7-10 year old child population. Previous metabolite and CBF changes have been observed across 1-2 h and 6 h post-WBB consumption (Rodriguez-Mateos et al, 2013), indicating

WBB may impact vasodilatory blood flow changes. This vasoactivity may extend to the brain via the brain endothelium. Neuroimaging techniques that measure blood flow or electrical activation following flavonoid ingestion whilst partaking in a cognitive task could therefore link real-time neurophysiological activity with cognitive performance and help to reveal a mechanism by which cognitive effects persist. EEG ERP techniques were therefore employed to assess real-time neural activity following both WBB and placebo treatments, using a crossover design. A crossover design was used due to the absence of a baseline session, so participants' outcomes could be compared after consuming WBB and placebo treatments. A baseline session could not be employed in the current experiment due to time constraints including testing out of school hours, duration of EEG setup and length of food abstinence. Changes in ERP activity detected at the same time as changes in cognitive performance could help to elucidate the mechanism of action by which WBB flavonoids exert acute cognitive benefits. A cognitive task battery consisting of a PANAS-C, Go No-Go Task and MANT2 was administered 2 h following each treatment (separated by a 1 week washout) whilst ERP recording was conducted. Measuring episodic memory in an EEG paradigm is difficult at present, and the flavonoid literature to date has not conclusively detected change across an acute design. Additionally, it was necessary to keep environmental noise to a minimum to avoid background ERP signal noise and artefacts. As the AVLT requires participants to talk aloud (creating environmental noise) which also require them to move their tongues and mouths (increasing ERP movement artefacts), the AVLT was not included in this experiment. Instead, a Go No-Go task was incorporated to assess inhibitory abilities.

Target time predictions were carried forward from Experiment 1's findings; quicker performance was expected on fast (120ms) trials for those consuming WBB. It was unclear whether target time effects would persist in Experiment 2 due to the modifications made to the MANT to include 230ms trials as well as 120ms and 500ms trials. 230ms trials were included to increase trial N (and therefore power) of the task when noise blocks had been removed, and provided an intermediate target time duration to see if performance improved linearly across speeds. Faster performance on 120ms trials was not observed in this experiment, although target time differences were apparent. Participants' RT was significantly quicker on 230ms and 500ms trials when consuming WBB first, indicating increased mental alertness for the medium and slow presentation speeds. This did not follow predictions, and may have been due to the inclusion of 230ms trials. These trials may have reduced the perceived speed difference between 120ms and 500ms trials, and therefore decreased task sensitivity.

As randomisation on MANT2 blocks was enforced in this experiment, previous predictions of improved performance on incongruent or high load trials (Whyte et al, 2016; 2017) persisted. Surprisingly, congruency or load effects were not evident in initial analyses. It was considered that the effects of treatment order may have had an influence on results, based on prior findings that have found flavonoid effects to emerge at visit 1 under flavonoid intervention and sustain at visit 2 under placebo (Kean et al, 2015), and so Visit was included in subsequent analyses. Significant accuracy and RT improvements were observed on the more cognitively demanding incongruent trials of the MANT2 for those consuming WBB first. Interestingly, these effects were maintained when these participants consumed placebo second. This suggests WBB may have benefitted participants the most under conditions of initial encoding or strategy formation, and allowed them to maintain a high level of performance at the second visit under placebo treatment. Such results could also imply that WBB may accumulate in the body for longer than initially thought (Wu et al, 2011; Kalt et al, 2014) and requires further investigation. Remarkably, ERP activity mirrored these findings. It was predicted that participants consuming WBB would show higher N2 and P3 activation in frontal or fronto-central regions on trials of an incongruent nature based on previous adult and child ERP studies (discussed in 4.1). Such findings would indicate that participants experienced increased response conflict and an increased ability to inhibit trials of an incongruent nature. N2 effects were observed with shorter latency to peak in the frontal brain region for those consuming WBB at the first visit, implying a quicker ability to process the task, regardless of trial congruency. Significantly higher N2 amplitude was also observed on incongruent trials for those consuming the placebo treatment at the second visit in frontal, fronto-central and central brain regions, mirroring cognitive findings and suggesting successful inhibition was most pronounced at the second visit when WBB had been administered during initial encoding. P3 activation also showed notable findings. Regardless of visit or trial, higher P3 amplitude was seen in frontal and fronto-central brain regions following WBB. This goes some way to indicate increased inhibition, however due to the absence of an interaction with incongruent trials, results are inconclusive. Higher P3 activation was observed on congruent trials for those consuming WBB at visit 1, suggesting such amplitude increases may only be observed under certain conditions; this warrants further investigation.

Taken together, these findings show that cognitive and electrophysiological effects can occur simultaneously 2 h following WBB and can be maintained 2 h following placebo, 1 week later. This data also goes some way to highlighting a potential mechanism of action by which

cognitive WBB benefits emerge. Neurotransmitters have been found to influence CBF changes, and so neuroimaging techniques such as ERP, which assesses neuro-signalling pathways, may provide insight into the neural activation and vasodilatory effects that may occur during the completion of cognitive tasks following acute WBB.

Mood effects were not evident in Experiment 2, however this was likely due to the small sample size (n=14, crossover design). The study may have therefore not been sufficiently powered to detect acute mood effects as previously observed in Experiment 1 (n=54, between-groups design).

From the findings in Experiment 2, it was evident that cognitive demand effects were important when considering the effects of WBB on cognition and mood. WBB seems to aid performance when tasks are difficult and cognitive demand is high. In light of this, it was posited that WBB may be beneficial to populations who are cognitively compromised, such as those with developmental disorders like ADHD. To investigate this, and compare performance under WBB and placebo to a TD population, Experiment 3 was designed to include a group of TD children and a sub-group of children diagnosed with ADHD. It was also important to investigate whether chronic cognitive and mood effects occur after daily supplementation in a child sample, to see whether prolonged consumption elicits the same cognitive benefits as a single dose. An acute x chronic design was therefore employed to merge both aims in Experiment 3.

7.1.3. Experiment 3

Experiment 3 investigated mood and cognition using an acute x chronic between-groups design. For the acute arm, participants were tested on the PANAS-C, AVLT and MANT at baseline and 2 h following either a placebo or WBB intervention. For the chronic arm, participants continued their assigned treatment daily for 4 weeks, undergoing mood (PANAS-C), cognitive (AVLT, MANT) and behavioural assessment (ADHD IV Parent Rating Scale) at baseline, 2 weeks and at the end of the intervention. This study also recruited a sub sample of children diagnosed with ADHD to assess the therapeutic effects of WBB in a cognitively compromised population. Acutely, WBB was found to improve mental alertness on fast 120ms trials of the MANT for children with and without ADHD, as predicted and as was seen in Experiment 1. Attenuation of forgetting was also observed on memory measures (as in Experiment 1) in children with ADHD 2 h following WBB treatment, suggesting maintenance

of auditory-verbal memory abilities in these children where placebo participants dropped off. TD children consuming WBB did show a similar maintenance pattern to children with ADHD consuming WBB hinting towards such attenuation, however differences between treatment groups were non-significant, potentially due to issues with power for these memory measures (typical participants N=23, between-groups); post-hoc power analyses calculated that a sample size of 44 would have been required to achieve a power of 0.8.

Chronically, WBB improved accuracy performance and slowed RTs on cognitively demanding high load trials of the MANT at the 4 week time point for typical children, indicating participants developed a speed-accuracy strategy to better their performance. *A priori* predictions were therefore also met, as accuracy improved on the most demanding trials as seen in Whyte et al's (2016; 2017) research. Interestingly, the target time effects seen previously (Experiment 1 and acute arm of Experiment 3) were not replicated and showed opposite results. RT was found to slow on 120ms trials for typical children at the end of the intervention. This suggests that response to trials of a fast pace might only be able to quicken acutely, and this needs further exploration to assess. Parent-rated behaviour scores also revealed a WBB benefit for children with and without ADHD; WBB maintained inattentiveness across the intervention, whilst placebo participants showed significant increases in inattentive behaviour. This demonstrates that WBB may be able to positively impact overt, attentive behaviour, detectable by parents, in the home environment. Such findings hold promise for the school environment where behavioural management of children with ADHD is a focus. Future studies should additionally include teacher ratings of behaviour to see if WBB benefits extend to the classroom.

Chronic improvements were found for children with ADHD on high and medium load trials of the MANT; WBB participants maintained accuracy performance on high load trials at 4 weeks (where placebos dropped off), as expected, and were able to quicken RTs on medium trials at 4 weeks. The latter finding was not expected and suggests that WBB may benefit children with ADHD on different levels of cognitive demand than TD children. Children with ADHD consuming WBB were also found to be significantly quicker on 500ms trials at the end of the intervention period, which contrasted to the prior predictions that performance would be better on 120ms trials. Taken together, chronic findings on the MANT suggest that WBB may improve performance under cognitively demanding conditions for typical participants, and exert benefits on the less cognitively demanding conditions for children with ADHD. However, a larger amount of data is required before conclusive assumptions can be

made due to the small sample of children with ADHD included. Additional chronic improvements were also seen on the word learning measure of the AVLT, with ADHD WBB children showing increased word learning capability at 4 weeks. Memory effects were not observed chronically for typical children, indicating the memory benefits observed previously in this population (Experiment 1; Whyte et al, 2015; 2016) may not persist chronically. Chronic daily flavonoid interventions have shown memory improvements in healthy adult populations after 3 months (Bowtell et al, 2017; Miller et al, 2018) and 6 months (Bensalem et al, 2018). Whyte et al (2018) also discovered chronic WBB (extract) effects using a different version of the same AVLT used here after 3 months. This suggests that memory effects do persist but are perhaps detectable over a larger time frame in adults. It is unknown whether such effects might emerge for a child population if supplementation extended to daily dosing for 3 months, and future studies should consider a longer intervention period to see if memory parameters are affected by long-term intervention in the same way.

My final experiment aimed to assess cognitive and mood parameters under the same chronic design, whilst also examining the metabolic processes that occur in children following repeated WBB consumption.

7.1.4. Experiment 4

Lastly, Experiment 4 tested cognition, mood and behaviour using a chronic 4 week between-groups design, concurrent with urinary metabolite measurement to assess the bioavailability of blueberry polyphenols following chronic consumption in a child sample. Such data would be particularly useful to witness the metabolites that change over time, and see whether such change is associated with cognitive improvement. A battery of tasks including the PANAS-C, AVLT and MANT were administered at baseline, 2 weeks and 4 weeks, alongside an ADHD IV Parent Rating Scale. Participants provided 24 h urine collections (beginning at 9am on the day prior to cognitive testing) at each of the 3 time points, where a treatment drink was consumed at 0 h for 2 and 4 week collections.

Improved performance was observed on the MANT following chronic WBB, particularly on the cognitively demanding incongruent trials as predicted and as seen previously (Experiment 2; chronic arm of Experiment 3; Whyte et al, 2016; 2017), suggesting WBB was most beneficial under conditions of increased cognitive demand. Interestingly, expected treatment differences on high load trials did not persist; instead, WBB participants were found to

perform more accurately on medium load trials. However, WBB mean data did lean towards higher accuracy on high load trials compared to placebo data, not quite reaching statistical significance ($p=0.18$). It would be useful for future research to ascertain whether incongruent and high load trials are equivocally cognitively demanding, or whether one exerts more demand from cognitive resources. As touched upon by Whyte et al's (2017), it might be more informative to group trials by difficulty rather than congruence and load variables, with incongruent high load trials as the most difficult, and congruent, low load as the least difficult. This may help to elucidate the relationship between WBB and cognitive demand using a difficulty-response paradigm. To reveal the trials that are more or less difficult (for example incongruent medium load vs. congruent high load) it would be beneficial to test how well participants perform under no treatment on the varying trial combinations. This may help to give a normative spread of performance and would hopefully elucidate which trials were of higher demand.

From the chronic results of Experiment 3, where performance on 500ms trials was better than on 120ms trials, it was predicted that WBB effects might occur on 500ms trials here, chronically. Significantly faster RTs were evident on 120ms trials at 4 weeks compared to 2 weeks for WBB participants, refuting findings from the chronic arm of Experiment 3 and suggesting replication of findings from Experiment 1 and the acute arm of Experiment 3. Taken together, results from Experiments 1, 3 and 4 infer that improvements on fast 120ms trials are able to occur acutely and chronically. However, placebo participants also showed this speed reduction as a trend, which has not been observed previously. Results could have therefore been due to residual practice effects, with WBB performing marginally faster. Interestingly, improved performance was observed on fast 120ms trials of the MANT on accuracy scores across the 4 week intervention. This is the first finding that has manifested within accuracy performance, rather than RT, and may reflect a finding specific to chronic supplementation. Due to the non-treatment-specific RT results and emergent accuracy findings in the current experiment, this data may support the notion that RT may not be able to quicken chronically, but accuracy may.

Chronic predictions for memory performance were that WBB participants may show increases across the intervention, based on the improvements observed in the chronic arm of Experiment 3 (for children with ADHD), and the acute effects seen in Experiment 1, the acute arm of Experiment 3 (for children with ADHD), and Whyte et al (2015; 2016). Increased proactive interference on the AVLT was observed following chronic WBB. This indicated

that WBB enabled participants to encode the original list of words to a high degree, which subsequently interfered with the learning of a new word list. This effect was also found by Whyte et al (2015) and fits with the implication that WBB may be most beneficial to children under conditions of encoding, which will be discussed later.

Total urinary polyphenol metabolite excretion was found to increase across the 4 week intervention following WBB, and decrease following placebo, although such changes did not reach statistical significance. This was likely due to the high amounts of variability within and between treatment groups. This was unsurprising as absence of change was observed for total urinary polyphenol excretion in the only other chronic WBB trial published conducted in an adult population (Feliciano et al, 2016b). Specific urinary metabolites were hypothesised to increase over the course of the intervention for those consuming daily WBB, particularly for hippuric and benzoic derivatives, as Feliciano et al (2016b) discovered. Individual polyphenolic metabolites that increased uniquely to WBB across the course of the intervention consisted of homovanillic acid, syringic acid and dihydroferulic acid-4-O-B-D-glucoronide compounds. Although higher excretion of benzoic acid derivatives (syringic acid) did emerge as previously predicted, hippuric increases were not evident. This is surprising as hippuric acid has been a main contributor to the overall pool of polyphenol metabolites following chronic WBB (Feliciano et al, 2016b), bilberry (Hanhineva et al, 2015) and a high polyphenol diet (Vetrani et al, 2016) previously. Specific increases in other metabolites were also observed following placebo, and following both treatments, suggesting metabolic processes unrelated to WBB may have contributed to the overall pool of metabolites detected. Indeed, further work is required to first examine the change in endogenous metabolite production following a strict low polyphenol diet before being able to better ascertain the differing activity under flavonoid treatment. Better control or measurement of habitual diet in the 48 h prior to urine collection would have also been beneficial to reduce the impact of dietary flavonoids on urinary excretions across participants. Interestingly, in Experiment 4, higher vanillic acid concentrations were associated with greater levels of cognitive interference across treatments, and WBB seemed to show protection from such interference, albeit non-significant. However, conclusions about the associations between metabolite excretion and cognition are limited. This is primarily due to the timings of 24 h urine collections and cognitive assessment being offset. Urinary collections took place from 9am (following treatment consumption at weeks 2 and 4) for a 24 h period, ceasing at 9am the following day. Cognitive testing then took place at ~3.30pm on the day urinary collection ceased, leaving a gap of 6.5 h between the end of collection and testing, and 18.5 h between

treatment consumption and testing. The linkage of these two outcomes therefore needs to be cautious with emphasis on causation avoided. Overall, metabolic findings were variable and largely inconclusive, but hold promise for assessing the excretion of berry metabolites in a child cohort in the future.

7.2. Overall summary of experimental findings

Taken together, the experiments within this thesis demonstrate that WBB effects do persist acutely, 2 h following consumption, using crossover and between-group designs. Acute effects were most prominent in memory measures, where WBB seemed to attenuate forgetting for typical children and in a subset of children diagnosed with ADHD. This implies episodic memory as a domain that is particularly sensitive to acute WBB intervention in 7-10 year old children, and holds promise for consumption of blueberries in the promotion of healthy memory functioning and better academic performance. Including flavonoid-rich foods, such as blueberries, in the diets of school-aged children with or without ADHD may aid in benefitting memory performance in the school environment. Specifically, this would be useful in the context of learning academic content, revising for upcoming tests and on days where exams are held, to enhance encoding and recall abilities. Based on the current results, this would also be beneficial for children diagnosed with ADHD. Reduced working memory ability often presents alongside behavioural symptoms of the disorder, which may contribute towards the commonly noted poor academic achievement of these children. Improving memory ability or preventing memory loss, via acute blueberry consumption, may therefore improve working memory, school achievement and quality of life in those with ADHD. Although encouraging, it must be noted that the results from the ADHD cohort in this thesis are preliminary and the effects observed were underpowered, requiring initiation of further investigations using a larger sample size.

It must also be highlighted that although episodic memory effects were observed across the acute experiments that implemented such measures, the specific facets that showed improvements were not entirely consistent across populations. Although the design (between-groups) and intervention duration (2 h) was the same, Experiment 1 showed immediate and total recall effects in TD children (as previously observed by Whyte et al, 2015), whereas Experiment 3 revealed no acute memory effects in TD children, yet benefits on all memory measures apart from word learning, recognition and interference abilities in children with

ADHD. It is thought that acute memory effects may not have emerged for TD children in Experiment 3 due to the inclusion of children with ADHD as a comparator group in analyses. This may have inflated the variance of the sample and reduced power to detect an effect. However, it is promising that the TD WBB group did show a similar pattern to previous results (Experiment 1; Whyte et al, 2015) where memory performance was reduced in placebo participants but this reduction was attenuated for those consuming WBB across several memory measures, albeit non-significantly. Conclusive interpretations cannot be made for those consuming WBB with a diagnosis of ADHD due to an insufficiently powered sample (total baseline N=10, total acute N=8). A post-hoc power analysis revealed that at the acute time point, where only 8 participants were included (3 placebo, 5 WBB), a power of 0.18 was achieved. Future trials should aim to recruit a total sample of 44 to achieve a small acute effect (0.22) at a power of 0.8.

The physiological mechanisms used to explain the cognitive effects of children diagnosed with ADHD in Experiment 3 needs careful consideration. It is likely that CBF increases following flavonoids might also be evident in this population, as had been seen in healthy adults and suggested for typical children. Interestingly, ADHD has been associated with reduced CBF to the prefrontal cortex, and these reductions were also found to predict the severity of attentional impairment in the sample of children tested (Spalletta et al, 2001). In Experiment 3, improvements were observed for children with ADHD consuming WBB on memory, attention and EF measures. As flavonoids have been found to increase CBF to frontal regions, the cognitive enhancements observed may have been due to CBF increases in task-related areas. A study assessing cognitive and neurophysiological measures would help to inform the field whether cerebrovascular change is evident in an ADHD population, and whether this may be a potential MOA for the cognitive effects observed, as posited for typical populations.

Additionally, research has suggested that children with ADHD have an atypical response to oxidative stress (OS) within the body, resulting in higher levels of oxidative damage (Joseph et al, 2015). From the literature discussed in Chapter 1, it is clear that inducing OS in rodents results in decreased cognitive capabilities and maladaptive behaviours – both of which are symptoms of ADHD. Flavonoid supplementation has resulted in improved behavioural and cognitive outcomes for animals with high levels of OS (Shukitt-Hale et al, 2007; Allam et al, 2013), implying flavonoids may reverse the damage caused and subsequently improve OS-induced symptoms. Such reversal is thought to be initiated through changes in neuronal

signalling, such as through ERK-CREB-BDNF pathways (Joseph et al, 2005; Lau et al, 2005; Williams et al, 2008; Milbury & Kalt, 2010; Rendeiro et al, 2012b; Shukitt-Hale, 2012). It would therefore be informative to assess BDNF status in a cohort of children with ADHD following WBB and placebo treatment to ascertain the degree at which neurosignalling improvements may improve ADHD symptoms, namely cognition and overt behaviour.

Memory effects were not observed chronically for TD children, indicating the WBB effects observed previously may only emerge after acute consumption. This implies that different physiological mechanisms may persist for cognitive improvements under acute and chronic WBB regimens. Indeed, chronic memory benefits have been linked with increased BDNF status in the past in animal and human samples (as discussed in Chapter 1), highlighting enhanced neurosignalling as a potential chronic MOA. Investigation into the chronic effects of WBB on memory should continue, perhaps using a longer intervention schedule of ≥3 months, as suggested above. This would help to determine whether improvements in this domain take longer to emerge following daily supplementation of WBB in a child sample as has been found previously in adult populations (Whyte et al, 2018, Miller et al, 2018). The addition of physiological measures concurrent with cognitive assessment, such as BDNF status, would also be informative to try to elucidate the mechanisms underlying acute and chronic effects.

Clear cognitive benefits were also seen on the measure of attention and executive function (MANT) employed across the thesis, particularly where cognitive demand was highest. Experiment 2 revealed results of this nature on incongruent trials, where better performance was observed for those who had consumed WBB 2 h prior. This supports the idea that WBB may enable participants to overcome conditions of increased cognitive load as observed previously by Whyte et al (2016; 2017). Experiment 2 was also the first of its kind to find neuropsychological effects convergent with cognitive effects in a child population, where improved frontal N2 effects were observed for those consuming WBB first and placebo second on trials that were incongruent. Such data supports the proposed CBF mechanism of action by which flavonoids may impact brain function and performance under conditions of increased cognitive demand. If more cognitive demand is placed on participants, this may require a larger draw on neural resources in task-related brain regions. This may, in turn, result in an imbalance where more energy (oxygen) is required to overcome cognitive demands. Flavonoids have been shown to increase CBF and regional perfusion to task-related areas in the brain previously. This implies that flavonoids may be able to replenish the oxygen

required by the brain to sustain or improve attention during tasks or trials which require greater cognitive effort. This would be highly beneficial to children in an academic context when partaking in difficult classroom activities or when sustaining attention throughout a lesson. In these situations, oxygen depletion would likely be high due to the increased cognitive effort required to concentrate. WBB may therefore boost oxygen to the areas required for attention (frontal) and allow for better vigilance and concentration at key points in a lesson where fatigue or distraction would have otherwise been likely.

Indeed, it is interesting to compare the brain areas which have shown activation differences following flavonoid intervention in previous trials to the ERP findings in this thesis. Across the literature, the brain regions that have shown higher perfusion following flavonoids include inferior and middle frontal gyrus (Alharbi et al, 2016), DLPFC (Francis et al, 2006), dentate gyrus (Brickman et al, 2014) and parietal and anterior cingulate cortices (Francis et al; Lamport et al, 2015). All of these areas have been associated with cognitive function. In particular, the DLPFC is a region situated in the frontal lobe that has been related to EF (Baddeley, 2003), WM (Goldman-Rakic, 1988), response inhibition (Ridderinkhof, van den Wildenberg, Segalowitz and Carter, 2004) and emotion regulation (Schore, 2016). The frontal gyrus and anterior cingulate cortex (ACC) are also regions situated in the frontal lobe, associated with attention and error preparation (Carter et al, 1998), respectively. This is particularly interesting in light of the findings from Experiment 2 which showed increased activation in frontal and fronto-central brain regions and increased attention, EF and inhibitory ability after acute WBB intervention across the more demanding MANT2 and Go No-Go trials. Frontal and fronto-central electrodes would have been situated across frontal gyri, ACC and DLPFC regions, indicating increases in neural activation may have occurred alongside vasodilatory effects, and may have mediated improvements in cognition. It is also important to recognise that parietal regions that have previously shown perfusion increases (Francis et al, 2006; Lamport et al, 2015) also showed increased neural activation in Experiment 2, and such activity is thought to be related to increased allocation of attentional resources and visuospatial attention (Robertson, 1998). Again, this corroborates previous flavonoid-related vasodilatory effects with neural changes in an area related to cognitive function. Although we can assume such relationships, it must be acknowledged that Experiment 2 did not measure CBF or regional perfusion; direct parallels can therefore not be made between changes in blood flow and neuronal activity, and future trials should consider implementing measures of both either using EEG-fMRI or EEG-NIRS to examine whether vasodilatory and neuronal increases occur simultaneously during completion of cognitive

tasks following acute flavonoids. Further, Experiment 2 did not include a measure of WM, an additional ability thought be associated with increases in blood flow to the DLPFC following acute flavonoids (Francis et al). It is unclear whether frontal activation or a change in performance would have occurred in memory domains following acute WBB in this experiment, and indicates a potential domain of interest to include in future neuroimaging studies.

Although acute cognitive demand effects were observed in Experiment 2, these were not evident in Experiment 1 and the acute arm of Experiment 3. It is thought that the MANT in Experiments 1 and 3 may not have been cognitively demanding enough as blocks were not randomised, introducing predictability and ease which would not have been present during randomisation of blocks in Experiments 2 and 4. In addition, Experiments 1 and 3 used a between-groups design, whereas prior significant findings (Experiment 2; Whyte et al, 2016; 2017) have been evident when utilising a crossover. This highlights experimental design as a potential influence on the cognitive demand effects observed, and suggests that when individual differences are better controlled for (using a crossover design), there is potentially higher statistical power to detect demand effects. It is problematic to reach a conclusive interpretation on this impact without comparing trials that have used standardised experimental procedures under different designs. The design of a study should be carefully considered in future experiments that wish to test flavonoid effects under different levels of cognitive demand acutely.

Cognitive demand effects were observed chronically. In Experiment 4, higher EF ability was also observed on incongruent trials across the 4 week WBB intervention. This is similar to acute findings from Experiment 2 and Whyte et al (2016), and together suggests cognitive demand mechanisms under conditions of incongruence may persist over short and long-term intervention schedules. Taken together, these findings imply that under conditions of increased mental effort, either a single dose, or daily consumption of a flavonoid-rich food, may provide the necessary boost to maintain or improve cognitive function. Implications of such findings are encouraging, especially in a child cohort, where either of these dietary inclusions would likely be manageable for parents to implement. It is also promising to consider the chronic cognitive demand effects of daily WBB supplementation on school performance. When thinking about the nature of incongruent trials, where one central target arrow is flanked by 5-10 arrows in the opposing direction, it can be interpreted that children consuming WBB may be more able to ignore the flanking distractor arrows and correctly

attend to the target arrow. This ability would be highly beneficial in a learning environment where children are often faced with classroom distractions (such as other children, interruptions etc), but are required to continue attending to the teacher or learning materials. Daily consumption of blueberries, or other flavonoid-rich foods, may therefore prevent distraction and aid focus on the tasks at hand. However, it is unclear how much this effect relies on visual load, and whether results can be translated to other 'distractors' in this way. Although computerised cognitive tasks are informative in assessing particular cognitive domains, it would be interesting to conduct an experiment where cognitive demand was induced by environmental distractors in a classroom setting whilst children completed a difficult, curriculum-based set exercise. This would allow for higher ecological validity and would give some indication of whether the cognitive demand effects observed here and previously on cognitive tasks are translatable to an academic context. Such results would highlight real-life benefits to attention, concentration and learning when consuming a diet rich in flavonoids.

Cognitive demand effects were also observed chronically in Experiment 3, however not on trials of an incongruent nature. TD children's performance was found to improve on high load trials after consuming WBB for 4 weeks. Exclusive effects on high load trials have not been seen previously; they were only revealed when analysed alongside incongruent trials in Whyte et al's (2017) study. An explanation for this inconsistency between WBB effects in congruency and load domains is unknown at present. WBB effects were observed acutely using a crossover design in Experiment 2 and Whyte et al's (2016) study, and chronically using a between-group, acute x chronic design in Experiment 3, insinuating effects prevailed across acute and chronic intervention durations and experimental designs. Experiment 4 utilised the same duration as Experiment 3 (4 weeks), but implemented a chronic design rather than acute x chronic. It may be that the addition of an acute session in Experiment 3 resulted in the emergence of WBB effects on the different, high load trials at the end of chronic intervention. Confidence in this interpretation is, however, low, especially due to the absence of any cognitive demand effects at the acute test point in this study. Manipulation of cognitive load in tasks should continue to help explore the mechanisms behind cognitive demand-related flavonoid effects. Although investigation of these using neuroimaging techniques was undertaken in Experiment 2, full utilisation of congruency and load variables was not achieved, with ERP analyses only focusing on trials of differing congruencies. It is therefore unknown whether ERP load effects occurred. Future studies employing cognitive and neuroimaging parameters should focus on segmenting MANT trials by level of difficulty

rather than by trial type; this would give a better account of flavonoid-related effects on trials of varying cognitive difficulty and their associated neural or vasodilatory action. Demand effects were not observed in Experiment 1, however randomisation of MANT blocks was not utilised, meaning the sensitivity of the task may have been reduced in this sample. This in agreement with previous literature (Scholey et al, 2009; Whyte et al, 2017) that has posited that cognitive task batteries need to place a sufficient demand on or fatigue participants to be able to detect subtle treatment effects, as were observed in Experiments 2, 3 and 4 when MANT blocks were randomised.

As with the acute literature, chronic flavonoid literature has suggested increased CBF and upregulation of BDNF (and its associated pathways) to be the main MOAs behind cognitive effects. Improved CBF has been demonstrated through increased neurocognitive activity in centro-frontal and posterior parietal brain regions following 4 weeks of daily cocoa-flavanol using magnetoencephalography (MEG; Camfield et al, 2012), and by higher perfusion to parietal and occipital gray matter following 12 weeks of daily blueberry using fMRI (Bowtell et al, 2017). These studies suggest flavonoids are able to exert vasodilatory effects on regions in the brain associated with cognitive performance, as seen in acute research. Results are promising, however in both of these chronic studies, no significant cognitive effects were observed during neuroimaging, indicating a dissociation between physiological activity and behavioural outcomes. Similar findings have been observed acutely (for details see Chapter 1) and may be due to a higher degree of physiological activity required to continue at the same performance level, which flavonoids aid. Although preliminary, the chronic CBF research combined with the acute CBF research indicates the same MOA may be present after a one-off dose and after extended daily consumption. It could therefore be that chronic CBF activity is actually an acute effect of the most recent dose. To examine this further, research could measure CBF parameters after an acute dose, after chronic dosing and after chronic x acute dosing within the same study to see the condition where CBF is more pronounced. This would help to reveal whether repeated dosing does elevate CBF to a higher extent than acute, and if so, would suggest chronic vasodilatory effects do occur and are not manifestations of acute effects. Studies should also continue investigating physiological effects in the presence or absence of cognitive effects to highlight the conditions that dissociations may occur in.

Experiment 4 aimed to reveal the specific WBB metabolites that appeared in circulation in the 24 h following consumption, for the first time in a child population. Such data is informative to ascertain the metabolites that may be circulating during cognitive improvements. This

information might vindicate the metabolites that can successfully cross the blood-brain-barrier and act on vasodilatory or neuronal signalling systems. Feliciano et al (2016b) investigated metabolic change after a 4 week chronic WBB intervention in healthy adults and discovered changes in urinary metabolites. Although total polyphenol urinary excretion did not change, certain individual metabolites were near-significantly higher on day 30 compared to day 0 following WBB, namely levels of catechols, benzoic, hippuric, cinnamic and phenylacetic acids. In Experiment 4, only 1 benzoic acid derivative showed WBB-related increases at the end of the intervention. As will be discussed in section 7.3.3, endogenous metabolism and the impact of polyphenol-rich foods consumed in the 48 h prior to urine collections were likely responsible for the lack of replication in this experiment. However, a lack of metabolic change following chronic flavonoid intervention has been observed previously (Ferrars et al, 2014), indicating the nature of repeated flavonoid consumption on metabolic activity is likely to be complex and warrants further investigation across differing populations and timeframes. A correlation was observed between higher levels of vanillic acids and increased cognitive interference, which seemed to be ameliorated for those consuming WBB (albeit non-significant). Bensalem et al (2018) discovered increased urinary concentrations of native and conjugated epicatechin derivatives following a 6 month daily dose of a polyphenol-rich grape and blueberry extract in older adults (60-70 years old) and found this was related to improvements in memory, particularly for those who had advanced cognitive decline. Further data is required to make assumptions about the relationship between metabolic and cognitive parameters, however it is promising that changes in both have been observed across a time period also known to show CBF increases in Experiment 4 and Bensalem et al's work. A study implementing neuroimaging measures concurrent with metabolite excretion and cognitive performance would provide the field with a more detailed view of the mechanisms behind chronic flavonoid effects.

Interestingly, the effect of treatment order was found to be particularly influential in EF and neuropsychological ERP datasets on the high cognitive demand trials. WBB was of most benefit when consumed first, where the first chance for participants to encode and form a strategy for the task was, with high performance persisting at the second visit under placebo treatment. This indicates that WBB may have been critical in the initial encoding process, an assumption that has also been observed in prior research (Kean et al, 2015; Whyte et al, 2015). Participants in Kean et al's (2015) study benefitted most from chronic orange juice consumption when assigned to the intervention arm first and placebo arm second. It is unclear of the exact mechanism behind the order effects observed. It is believed by some researchers

(Kay et al, 2012) that flavonoids do not accumulate within the body, with most having been expelled by 48 h post-consumption, making it unlikely that findings may be driven by physiological accumulation or carryover effects. However, others believe that anthocyanins, phenolics and their metabolites may remain *in vivo* longer than initially anticipated, as discussed in Chapter 4. Kean et al's (2015) research may therefore be seen to support accumulation, as participants consumed a daily high-flavonone treatment for 4 weeks, potentially allowing for a larger 'build up' of metabolites in the body. Higher levels of circulating metabolites following chronic flavonoid supplementation may have allowed for a larger uptake by the brain, inducing cognitive change even once daily dosing had ceased. Although more believable for a chronic design, it is unknown whether accumulation effects might persist following an acute dose. Kalt et al (2014) did find increased concentrations of anthocyanins and their metabolites in the plasma and urine of healthy adults 5 days after an acute dose of wild blueberry juice, whilst following an anthocyanin-free diet. These results imply that blueberry-related metabolites may still be present following a 1 week washout in Experiment 2. However, it is unknown whether such effects persist in a child population and, more importantly, whether prolonged anthocyanin bioavailability is linked to acute cognitive change. Indeed, physiological accumulation may occur, but correlation between higher metabolite levels and improved cognitive function is yet to be determined. Measures of both cognition and metabolite excretion should therefore be implemented across acute and chronic crossover designs to elucidate 1) if WBB metabolites accumulate *in vivo*, and 2) whether accumulation is linked to cognitive effects.

In the absence of sufficient physiological data to confirm or negate accumulation effects, it is possible that encoding processes during initial task completion or strategy formation were sensitive to acute flavonoid intervention and were subsequently improved and maintained over time. Such encoding processes may be mediated by attention. Bentin, Moscovitch and Nihrod (1998) discovered that the higher the attention that is paid to an item at encoding, the higher probability of recall later on. As flavonoid-related improvements have been observed in attention-related cognitive and neuroimaging domains previously, order effects may have arisen due to increased attentional capabilities following WBB during the first task completion. This increase in attention may have enabled participants to master and overcome the demands of the task quicker. Further, WBB was found to benefit performance and ERPs on the first arm (which carried over to the second arm) on the more cognitively demanding incongruent trials of the MANT2. This highlights that WBB-related CBF effects may be most potent on difficult trials and under the most difficult condition (encoding).

Order effects cannot be analysed in this way for other experiments in the thesis since only Experiment 2 used a crossover design. However, effects of WBB on encoding and recall abilities can be examined. The potential benefit of improved attention leading to enhanced encoding and recall was also be seen in the AVLT results of Experiment 4 where increased proactive interference (PI) was observed for those consuming WBB (also observed by Whyte et al, 2015). Attentional capability may have been higher for those consuming WBB when encoding the first word list, which later interfered with the recollection of a new word list. Such interference was not observed in the placebo group indicating these participants may have encoded the initial word list to a lesser extent due to less attentional capacity. However, it is unclear whether these WBB encoding effects arose due to increased attentional or memory capabilities. It may be beneficial in the future to test this hypothesis through adding a higher load on attentional systems, such as dividing participants' attention at the encoding stage. This may help to highlight whether attentional or memory systems are driving encoding effects. PI effects were not observed in Experiments 1, 2 or 3, indicating variability in findings across intervention durations and study designs. An intervention study that specifically aims to examine the effects of flavonoids on encoding and recall abilities would be beneficial in elucidating the cognitive mechanisms behind memory and order effects. This could involve intervention of WBB and placebo at either encoding and recall stages, or at visit 1 or visit 2 in a counterbalanced order. Details of a proposed study were provided in Chapter 4 and will be revisited in section 7.4.

As mentioned above, the DLPFC has been associated with emotional regulation (Schore, 2016), and so mood changes were expected alongside increased frontal activation in Experiment 2. However, no significant change was observed between treatments. As the mood measure was not conducted during ERP recording, the relationship between mood and neuropsychogical measures remains to be elucidated. Caution however should be taken when making assumptions between the regulation of mood and current mood. Regulation insinuates the successful management of emotion over time and may not be related to acute changes in positive or negative affect. The DLPFC is also one of the last brain regions to mature (Miller, 2000), implying successful emotion regulation may not be achieved until adolescence, and may not be appropriate to investigate in a pre-pubescent population. Indeed, the results of Experiment 1 imply that a change in acute mood is achievable through assessment of positive and negative affect in a sufficiently powered child sample consuming WBB. However, acute effects were not observed in Experiments 2 and 3 (likely due to low statistical power), and chronic effects were not evident in Experiments 3 and 4, highlighting the uncertainty of the

durations where changes in mood change might be seen. Studies should continue to focus on current mood rather than mood regulation in child cohorts. It would be informative to investigate the nature of repeated assessment of positive and negative affect in typical children under differing conditions of stress, such as in the lead-up to in-class tests or SATs. It has been noted previously that flavonoid-related cognitive improvements may be more sensitive to conditions of increased difficulty or after induced fatigue (Scholey et al, 2009). This premise may carry over to mood effects; flavonoids may be most sensitive to those with low mood or who are in an acute mood state such as stress. Mood disorders such as depression have been linked with impaired cognitive functioning and this impairment is believed to maintain maladaptive symptoms such as low mood and rumination (Naismith et al, 2003; Tavares et al, 2007; Gohier et al, 2009; Marazziti, Consoli, Picchetti, Carlini and Faravelli, 2010), suggesting that cognitive function is related to mood. Additionally, animal literature has also shown that rats under physiologically- and psychologically-induced stress show a larger degree of positive change in physical and cognitive measures, compared to those who are healthy, following flavonoid intervention (Shukitt-Hale et al, 2007; Rabin et al, 2007; Allam et al, 2013; Shukitt-Hale et al, 2007; Poulose et al, 2014). This indicates flavonoid intervention may be beneficial to a population who are stressed or have low mood. Investigation into mood in a stressed child sample would inform researchers of the nature of mood and stress in school-aged children, and could potentially reveal a time point where flavonoid intervention may be beneficial.

7.3. Limitations of the work

7.3.1. Taste of the treatments

A reoccurring finding in the current scheme of work was that participants rated the placebo treatment higher than the WBB treatment on a scale of likeability. Although sugar, vitamin C and orange squash content were matched, and both treatment drinks were shaken well, the viscosity of the drinks was not similar and this may have contributed to differences in scores. The placebo drink was watery and tasted like a squash mixture, whereas the WBB drink was thicker and had a grainy texture. It would not have been obvious which drink contained WBB due to all experiments being advertised and explained to participants as a fruit drink study, with no reference to any particular fruit. However, participants were informed that they would be randomly assigned to receive an intervention or a control drink. Due to the unusual texture

of the WBB drink, it may have occurred to children that this drink was likely the intervention. This may have affected their performance on the tasks through implicit demand characteristics, and might have jeopardised participant blinding procedures. The differences in taste between treatments would have likely been more noticeable for participants in Experiments 2 and 4 who tasted both treatment drinks at screening and therefore had a comparator. Indeed, ratings were significantly higher for the placebo drink in both these experiments, which could have affected performance when subsequently administered both treatment drinks in Experiment 2 (separated by 1 week) or when administered an assigned drink for 4 weeks in Experiment 4. Interestingly, in Experiment 3, where participants were instructed to only taste their assigned drink at screening, taste differences were not evident. This indicates higher likeability for the placebo drink was more prominent when WBB was administered as a comparator at screening. Participants were not asked at the end of each experiment whether they thought they knew the treatment they had received. Collection of this data would have been informative to see whether the unique taste of the WBB drink was associated with assignment to the WBB intervention.

Another limitation is that participants who were assigned to WBB sometimes struggled to consume the drink and needed encouragement from their parents and the experimenter. Firstly, when testing these children it became immediately obvious to the experimenter that they were receiving WBB treatment, compromising blindness. Another factor that jeopardised blinding was a blue stain which was often observable on the participants' lips or tongue following consumption of the WBB drink. Secondly, the disliked taste and subsequent pressure from adults to consume the drink may have induced an increase in negative mood and formed an undesirable association between the drink and participating in the experiment. Although no effects were observed for negative affect across the thesis, this may have in turn prevented positive affect from increasing in those consuming WBB at subsequent acute and chronic time points in all studies following on from Experiment 1. Indeed, prior research has found that lower palatability can have a negative effect on mood (Benton, 2002; Macht and Mueller, 2007), implying taste may be an important variable to consider and control for in future mood analyses. Dye and Blundell (2002) also found that mood enhancements associated with increased palatability may improve cognitive performance, again highlighting the need for the palatability of treatment drinks to be agreeable with participants so as not to confound cognitive outcome measures. It would be informative in the future to include further measures of palatability rather than taste alone, for example texture, sweetness and tartness, to gauge the specific aspects of the WBB treatment that may require change. This would likely

be easier to implement in an adult population who have had more experience of tastes and textures and would therefore likely give a more informed response. It may also be helpful to experiment with different methods of intervention, for example incorporating WBB into low or no sugar jelly or non-milk based low or no sugar ice cream. The impact of sugar could be avoided by using sweeteners instead to make the treatment more palatable. As children typically associate these foods with high palatability, it may be more achievable to get them to like the treatment and consume more of it than when given as a liquid. However, careful consideration of the other food components incorporated into placebo and WBB treatments for these methods, such as gelatine, sweeteners and milk alternatives, would be required to ensure they did not impact mood or cognitive function before inclusion in a flavonoid intervention study.

7.3.2. Recruitment

Recruitment through schools was initially difficult due to their various commitments. In total, 25 schools were approached which resulted in 2 schools taking part in Experiment 1. Recruitment for Experiments 2, 3 and 4 was increasingly difficult as these studies were not based in the school environment and required participants to visit the University for testing sessions. The same schools were used to distribute information packs for these experiments however this did not yield the same degree of enthusiasm as Experiment 1, most likely due to the additional effort parents would have to make to get their child to the University on several occasions. The demographic of participants successfully recruited through schools for Experiments 2-4 may have also been biased due to the parents having more of an interest in health and nutrition and being more motivated to participate outside of the school location and timetable. Further, children of parents who took an interest in healthy lifestyle choices may have been healthier than the general child population or more educated on nutrition, potentially confounding the sample distribution. Yet, children across experiments fell just below the recommended government guidelines of 5 pieces of fruit and vegetables a day, with no participant reporting extremely high values, indicating the sample was sufficiently varied. Fruit and vegetable consumption in the participants recruited across the thesis was lower than has been previously observed in child samples (Whyte et al, 2015; 2016; 2017). It is therefore likely that parental health or nutrition interests did not impact intake. Habitual fruit and vegetable intake across a participant sample should be considered in the future to see whether

regular consumption of flavonoid-rich foods ameliorates or enhances the effects of flavonoids on cognition or mood.

The majority of recruitment for Experiments 2-4 involved contacting parents from the Child Development Group database (a University-held database comprising of children who were registered from birth), posting on various social network sites and distributing flyers and emails to community organisations. Recruitment was particularly difficult for Experiment 3 which offered no financial remuneration and required children to visit the University 6 times over the course of 4 weeks. Both reasons appeared to be a large factor in parents not signing up their children and so home visits were introduced to try and overcome the burden of travelling. For the sub sample of children diagnosed with ADHD, recruitment was especially difficult and was ongoing for 3.5 years. Families of children with ADHD are often busy and require a larger amount of support to function than typical families. Often these families could not commit to the length of time the study persisted for, even when financial remuneration was introduced to ADHD families in the latter year of recruitment. Participants were mostly recruited via ADHD support social networking pages with testing taking place in the family home. However, the motives behind parents of these children signing up to the study do need to be considered. Individuals are often part of a support group if they require help or are looking for information on treatments. Indeed, many parents enquired as to whether the trial was investigating a supplement pill that they could purchase, or whether the nutritional intervention had shown beneficial effects in children with ADHD before, and often expressed their willingness to 'try anything'. Even though the nature of the research was explained clearly, it may be that the sample of children with ADHD recruited from these groups had parents who were particularly desperate in finding treatment options for their children where medication or alternatives had not worked before. This may have potentially confounded the sample to a medication-resistant or particularly problematic niche group of children with ADHD.

The issues with recruitment across typical and ADHD samples meant that Experiment 3 had low statistical power. On the acute arm, achieved power for significant results in typical participants was 0.52, with power for ADHD participants only 0.23. Chronically, this was 0.60 for typical participant-related effects and 0.26 for participants with ADHD. Therefore over- interpretation of the results in this experiment should be avoided and data should be acknowledged as preliminary. Future studies with sufficiently large sample sizes are required to support conclusions of this study. Experiments 2 and 4 were also underpowered for

cognitive effects, however these studies were powered *a priori* based on physiological parameters.

In retrospect, it may have been better to combine Experiments 3 and 4 to increase power. Indeed, the chronic arm of Experiment 3 was identical to Experiment 4 in design and cognitive procedure. One larger study including TD children and children with ADHD, with a sub-sample of urinary metabolite measures, may have provided larger insight into the chronic effects of WBB on mood and cognition, particularly memory, where TD effects did not persist, potentially due to power. Experiment 3 was an ongoing study, continually recruiting for 3.5 years, whereas Experiment 4 was a time-sensitive study requiring completion in 3 months (due to deadlines for metabolite analysis in Germany). Combination of the two studies was therefore not logistically possible in this case.

7.3.3. Habitual diet

A 24 h low flavonoid diet was implemented before participation in all test sessions to minimise the effects of habitual diet on outcomes measures. Parents were given a low flavonoid diet sheet that informed them of the foods their child should avoid which also contained 2 sample diets should they wish to follow examples for ease. Compliance was only recorded using 24 h food diaries for Experiments 2 and 4. The majority of participants in these trials complied, with only a few deviating from the restrictions. The fact that participants recorded these deviations suggests that they were honest, reducing the likelihood of inaccurate self-reporting. Where restrictions had not been followed for acute experiments (1, 2 and acute arm of 3), participants were asked to return on a different day after successfully following a low flavonoid diet, and were reminded of the foods to avoid. For Experiments 1 and 3, participants and their parents were verbally asked if they had followed the diet to which most affirmed. This was a less reliable way of checking compliance as parents could agree without having to give details, and children may not have been able to remember if they had complied over the previous 24 h period. Future trials should continue to employ 24 h food diaries to hold participants and their parents more accountable to following the flavonoid restrictions.

Although restrictions were in place, a standardised diet including a set breakfast, lunch, dinner and snacks, may have provided better control over the influence of habitual diet. However, levels of commitment were already high for families participating in the experiments, and

preparing a specific diet for one child in the family may have proved difficult to manage, and may have resulted in reduced motivation to participate or an increased likelihood of dropout. Furthermore, such standardisation of diet is not conducive to typical individuals' daily eating habits. Findings from studies that include strict dietary guidelines may therefore not be relevant to the real world.

The influence of habitual diet was highlighted most in Experiment 4 due to the increased excretion of polyphenolic metabolites unrelated to WBB consumption observed. 24 h low flavonoid dietary restrictions were applied the day before and the day of urinary collections. However, 24 h food diaries were only implemented on the day of urine collections, leaving the 24 h period before unmonitored. Although most parents confirmed adherence to the diet for 48 h, some expressed confusion, stating they only thought the diet was to be followed on the day of collection. As the study was resource- and time-limited, participants could not be asked to repeat the restrictions and collections and return for testing on another day on the intervention. All participant data was therefore used, regardless of full compliance. Polyphenolic metabolites have been known to remain in the body for up to 48 h following consumption, meaning foods eaten the day before 24 h urinary collections may have still been in circulation. This may have been noticeable in the increased excretion levels observed in placebo participants, where polyphenol-rich foods eaten in the 24-48 h prior to collection may have been expelled during 24 h collections. Future trials should include food diaries for the 24 h urine collections and the 24 h preceding, to ensure 48 h restrictions have been adhered to. In retrospect, it may have been useful to contact parents the night before the 48 h restrictions began to clarify exactly what was required and to avoid any deviations that would have likely contributed to changes in polyphenol excretion.

7.4. Future work

Future research should build on the initial findings of this thesis by converging cognitive effects with metabolite excretion and a measure of CBF, such as ASL, regional fMRI, or MEG, to better inform the field of a chronic MOA. This would permit measurement of the specific flavonoid metabolites that are circulating in the body when cognitive effects are observed, and would elucidate whether regional CBF effects are also present across the same time frame. This would allow researchers to postulate the specific flavonoid metabolites that

may be able to cross the BBB and exert vasodilatory effects on the brain in the presence or absence of cognitive improvements.

The experiments in this thesis continued the investigations into acute WBB intervention and employed ERP EEG, a neuroimaging technique, for the first time in a flavonoid child intervention. Acute WBB benefits were evident in memory, inhibition, EF and mood domains, and it was discovered that WBB may be most sensitive at the initial stage of encoding. Manipulation of treatment order should be considered in future trials to further assess this. As proposed in Chapter 4, this could be conducted using an acute crossover, between-groups design. Following a baseline session, the trial would include consumption of 2 treatments (placebo, flavonoid), separated by a 1 week washout. Participants would also be grouped into 1 of 4 intervention schedules which would indicate the order of treatments they would receive first and second (placebo, placebo; placebo, flavonoid; flavonoid, flavonoid; flavonoid, placebo). The schedules would test the impact of consuming placebo and flavonoid at initial and subsequent test points (akin to encoding and retrieval). This would be a good indication of whether consumption of flavonoids at first or second exposures is most beneficial in comparison to placebo, and may highlight where effects are more notable in regard to encoding and retrieval mechanisms.

Further, increased activation in task-related frontal and fronto-central brain regions occurred simultaneous with WBB-related inhibitory and EF improvements, demonstrating neural and cognitive activity can occur across the same 2 h time frame where previous CBF and circulating metabolite effects have been seen. Acute findings also posited that WBB may be most beneficial under conditions of increased cognitive demand. Future researchers should consider instigating an acute flavonoid EEG-fMRI or EEG-NIRS study during a cognitively challenging task to examine the relationship between regional perfusion, neuronal activation and cognitive function. Such a study would better elucidate the acute MOA behind acute cognitive flavonoid effects and would also reveal whether oxygen depletion is evident during high demand trials, and whether flavonoids can replete these low oxygen levels. A study of this kind would also expose the relationship between vasodilatory and electrical activity further. This would be beneficial for ascertaining whether flavonoid-related enhancements in CBF are related to the increased neuronal activation observed in this thesis, and if so could imply the use of EEG as a proxy for observing regional CBF changes in the future

Chronic EF findings suggest that WBB may improve performance under cognitively demanding conditions for typical participants, and exert benefits on the less cognitively

demanding conditions for children with ADHD. The specific effects of flavonoid intervention under different levels of cognitive demand requires further investigation in ADHD populations to assess whether flavonoids benefit these children to the same degree as those who are TD. Results could indicate a potential 'sweet spot' for levels of task difficulty where flavonoids might benefit children with developmental disorders.

7.5. Conclusions

Overall, the experiments reported in this thesis are the first to explore the chronic effects and extend the acute findings of flavonoid-rich WBB on the cognition and mood of typically developing school-aged children, and in a sub-sample of children diagnosed with ADHD. It was found that attention, EF and memory did improve following chronic supplementation, especially under conditions of increased cognitive load for both populations. Chronic urinary metabolite assessment was also undertaken for the first time in a typical child sample and indicated specific WBB increases in benzoic, vanillic and ferulic acid derivatives. This helps to reveal the metabolites that may be circulating during cognitive improvements, and indicates the derivatives that may be able to cross the blood-brain-barrier and induce vasodilatory effects on the brain. Acute findings seen in previous child WBB interventions were also successfully replicated in the thesis at the 2 h time point. Effects were prominent in memory measures, where WBB attenuated forgetting in typical children and in a subset of children diagnosed with ADHD. This implies episodic memory as a domain that may be particularly sensitive to acute WBB intervention in 7-10 year old children. Novel mood effects were also observed where WBB improved acute positive affect. This has important implications for maintenance of healthy mood in a pre-pubescent population and prevention against onset of mood disorders in adolescence. EF cognitive load effects also persisted acutely, with improved performance on the more cognitively demanding trials following WBB, as seen in the previous child literature. EEG techniques were employed here for the first time in a child flavonoid intervention. Remarkably, increased electrophysiological activity was observed concurrent with WBB-related EF effects on trials of high demand. Such findings suggest WBB may act on neural activation pathways via increases in CBF in response to regional oxygen depletion, which may be instigated by high cognitive demand.

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Appendices

Appendix A Parent information sheets

A.1 Parent information sheet for Experiment 1

School of Psychology & Clinical Language Sciences

Study: The effect of fruit drink supplementation on the behaviour and cognition of children.

Researchers: Katie Barfoot (Email: [redacted])

Dr Claire Williams (Tel: [redacted]; Email: [redacted])

Fruit Drink Study Information

I hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like your child to participate. However, if you have any further queries or would like to discuss any aspect of the study, please do not hesitate to contact me by email: [redacted]

Background to the study

We are interested in finding out about the effects of a fruit drink on the attention, memory and behaviour of children, particularly children who have attention and/or behaviour difficulties. Some fruits naturally contain high amounts of micronutrients called flavonoids that are found in a number of foods including vegetables, fruits and fruit juices. Recent research with adults has shown that consumption of foods with a high flavonoid content leads to beneficial improvements on such things as attention and memory. A recent study carried out within our department has shown that following consumption of a blueberry drink ,children aged 6-12 years showed improved attention and memory performance. These results have academic and educational implications for children.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading. The researcher on this project has been through the formal Disclosure & Barring Service (DBS) procedure and has been approved by the University of Reading to work with children. The study has been reviewed by the University Research Ethics Committee and has been given favourable ethical approval; which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed. Full agreement from Christ the King to participate in this research has also been granted. The study is funded by the University of Reading and a non-commercial nutritional supplement company.

What does the study involve?

Practice Session

Session 1 will involve your child being taken out of the classroom at a convenient time in the school day to have a 'practice' of all the cognitive tasks that they will encounter at each future test phase. This is so your child can get used to the tasks and ask questions if they are unsure of what to do. This session will last approximately 30 minutes.

Session 1

You will be asked to implement a low-flavonoid diet 24 hours before this Session. Details of what this entails are provided in this pack. If your child usually has school lunches then the researcher will ensure your child receives a low-flavonoid lunch from the cafeteria.

After lunch your child will then take part in the previously practised cognitive task battery taking approximately 40 minutes. Your child will then receive a fruit drink to consume and will then re-join their fellow classmates to continue their school day as normal.

Session 2

This Session will take place 2 hours after Session 1. Your child will be taken out of the classroom and will be asked to perform the same cognitive task battery as before, taking approximately 40 minutes. A small token will be rewarded to your child at the end, e.g. a sticker or small play toy, and they will then be ready to be collected. We will liaise with the school to minimise any disruption to your child's learning where possible. This Session will take place between 3-6pm, so may require you to collect your child later from the school on this day.

About the fruit drinks…

Your child will receive one of two different doses of fruit drinks. The dose received will be randomly allocated. The two drinks will contain organic orange squash and a different dose of flavonoid. One of the drinks will also have a small amount of sugar and vitamin c to match the amount found in the flavonoid drink. All drinks will be prepared hygienically within the university or school, and with the exception of the ingredients already listed, no additives or any other items will be added. A full breakdown of the ingredients can also be made available to you upon request. In order to ensure that it is truly the drinks that are having an effect, we will provide you with a list of foods that are high in flavonoid content, which we will ask your child to avoid eating for 24 hours before each study day.

The sessions are designed to be fun and consist of a few short tasks to do on a computer. One of the tasks will require your child to press arrow keys in accordance with different arrows displayed on-screen. The other task will require your child to listen to a list of words being read out via an audio recording, and then recall as many as possible, in any order.

Why has my child been selected to take part?

We have invited children from local schools aged 6-10 years old to take part in our study. We have targeted children of these ages as they are old enough to understand the tasks. Additionally, around the ages of 7 and 10, children experience a spurt of brain growth in areas related to attention and memory that makes their responses to these tasks of particular interest.

What happens to the data?

All information collected will remain fully confidential and no results from any of the tasks your child performs will be shared with your child's school. All the information you provide us with will be assigned an anonymous number and no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researcher at the top of this sheet will have access. The

data will be used only for research purposes, and in accordance with the Data Protection Act of 1998, they will be destroyed 5 years after the completion of the study.

On completion of this study there is a possibility that that the results will be published in an academic journal. Only the overall results will be referred to in this publication with no direct reference being made regarding your child or the school they attend. Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Does my child have to participate?

No. Participation in this study is entirely voluntary and you are under no obligation to agree to participate. Also, you may withdraw your child at any point during the study without giving any reason.

I'd like my child to participate, what happens next?

Please complete the attached consent forms and return to Christ the King's school office as soon as possible.

I am the researcher on this project, and will be the initial contact for any queries throughout the study, so please do not hesitate to get hold of me via email. However, if by some means you are unable to contact me at any point, then you may also contact Dr Claire Williams (the Principle Investigator and my supervisor; [redacted]) and she will be happy to answer your queries and to pass on any information to me.

Thank you for your help.

A.2 Parent information sheet for Experiment 2

School of Psychology & Clinical Language Sciences

Study: The effects of a fruit drink on the electrical brain activity, cognition and mood in 7-10 year old children

Researchers: Katie Barfoot (Email: [redacted])

Dr Adrian Whyte (Email: [redacted])

Professor Claire Williams (Tel: [redacted]; Email: [redacted])

EEG Fruit Drink Study

We hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like your child to participate. However, if you have any further queries or would like to discuss any aspect of the study, please do not hesitate to contact the researcher by email: [redacted]

Background to the study

We are interested in finding out about the effects of a fruit drink on the attention, memory and mood of children. Some fruits naturally contain high amounts of micronutrients called flavonoids that are found in a number of foods including vegetables, fruits and fruit juices. Recent research with adults has shown that consumption of foods with a high flavonoid content leads to beneficial improvements on such things as attention and memory. A recent study carried out within our department has shown that following consumption of a blueberry drink ,children aged 6-12 years showed improved attention and memory performance, as well as increased positive mood. These results have positive educational implications for children, such as improving memory and attention within the classroom.

This study aims to further investigate these encouraging findings by analysing the electrical activity of the brain, before and after children have been supplemented with either a high or low flavonoid fruit drink. We are hoping to explore the relationship between electrical activity in the brain after fruit drinks, and any subsequent memory, attentional or mood effects within children. We will look at this using a test called an electroencephalogram (EEG), which will record electrical activity in your child's brain. This is a non-invasive test that is regularly used in research and hospitals to monitor brain activity. You may have seen these caps in photos or on science programmes, and may recognise them by their swimming cap appearance. The EEG cap is a stretchy net of sensors and will be soaked in a warm saline solution before being fitted. The sponges on this cap will touch your child's scalp and will enable us to record your child's brain activity, much like a tape recorder, while he/she completes several tasks on the computer. There are no risks involved in participating in this study. Your child will have small marks on his or her head where the sponges were in contact with the scalp. These are merely from the contact (like when you fall asleep on your hand and have marks on your face from your fingers) and will disappear in just a few minutes, generally in less than a half hour.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading. The researchers on this project have been through the formal Disclosure & Barring Service (DBS) procedure and have been approved by the University of Reading to work with children. The study has been reviewed by the University Research Ethics Committee and has been given favourable ethical

approval; which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed. The study is funded by the University of Reading and a non-commercial nutritional supplement company.

What does the study involve?

The study involves 3 sessions in total. The first session will be a screening/practice session and will last approximately 1 hour. The second session will be approximately 1 week later and will last approximately 45 minutes. The third session will be 1 week after the second session, and will last approximately 45 minutes.

For all test sessions, we do not expect you to take your child out of school. Sessions can be arranged in after school hours or at the weekend if preferred. For sessions arranged after school, a researcher will visit your child's school 2.5 hours prior to the scheduled test session to administer a fruit drink.

Screening/Practice session

You and your child will visit the Psychology department and will be briefed on what the study entails. Your child will then be asked to complete several cognitive, behavioural and demographic tasks and will have a 'practice' of all the cognitive tests that they will encounter on the main test sessions. They will also be shown the EEG cap and will learn how it works. This is so your child can get used to their surroundings and the tasks, and can ask any questions about the study. Your child will also be asked to taste a sample of two fruit drinks, and to rate their pleasantness from 1 – 10. You will also be asked to complete a child behaviour rating scale and food questionnaire.

Note: If your child really does not like the drink, shows distress from the taste, or as a result cannot finish the contents, then he/she will not be invited to participate any further.

In order to ensure that it is truly the drinks that are having an effect, we will provide you with a list of foods that are high in flavonoid content, which we will ask your child to avoid eating for 24 hours before the main study day. This diet is well-tolerated by children, and does not restrict your child's food intake to an undesirable degree. If you wish to see this sheet before the screening session, please request from the researcher via the email address provided. Otherwise, full details of this will be made available to you at the screening session.

Test session 1

A low flavonoid diet must be followed for 24 hours prior to the test session start time.

A researcher will visit your child's school 1.5 hours prior to the scheduled test session to administer a fruit drink. Your child must not eat anything in the 1.5 hours following consumption of their fruit drink, but may drink water.

On arrival at the University's Psychology department for the test session, your child will be seated in the EEG lab and will be fitted with the EEG cap. They will then take part in the previously practised cognitive test battery taking approximately 40 minutes.

Test session 2

This session will take place 1 week after test session 1. An identical procedure to this test session will follow, including compliance to the 24 hour low flavonoid diet, a researcher visiting your child's school to administer the drink, and a test session at the Psychology department where the EEG cap will be fitted and a different version of the cognitive test battery administered, lasting approximately 40 minutes.

About the fruit drinks… Your child will receive two different doses of fruit drinks, one on each test session. The dose received will be randomly allocated. The two drinks will contain organic orange squash and a different dose of flavonoid. One of the drinks will also have a small amount of sugar and vitamin C to match the amount found in the flavonoid drink. All drinks will be prepared hygienically within the University, and with the exception of the ingredients already listed above, no additives or any other items will be added. A full breakdown of the ingredients can also be made available to you upon request.

About the tasks... The sessions are designed to be fun and consist of a few short tasks to do on a computer. One of the tasks will require your child to respond to the direction of a target arrow on screen amongst various other arrows. Another task will involve pressing and not pressing certain buttons when particular animals are displayed on a screen. Your child will also be asked to complete a mood questionnaire to see how they are currently feeling at each session.

About EEG… During this study your child will be required to wear a cap which will measure the electrical activity of their brain. This is a painless procedure which will cause them minimal distress. Your child will experience wearing the cap at the screening session. If, during the study, you/your child decide that you/they do not want to continue wearing the cap or perform the tasks, then you/they can withdraw from the experiment without giving a reason. Unusually patterns of EEG activity can be indicative of brain abnormalities such as epilepsy, tumours, and brain injury. We will inform you orally if we observe unusual patterns of EEG activity, and in such cases we recommend that you contact your GP and ask for an official EEG assessment. If you prefer, with your permission, we can contact your GP directly and pass on your child's readings, and aid you in contacting the appropriate organisation.

Why has my child been selected to take part?

We have invited children from the Berkshire area aged 7-10 years old to take part in our study. We have targeted children of these ages as they are old enough to understand the tasks. Additionally, around the ages of 7 and 10, children experience a spurt of brain growth in areas related to attention and memory that makes their responses to these tasks of particular interest. Previous studies have indicated that flavonoids are beneficial for children of this age group; this study aims to explore these findings further.

What happens to the data?

All information collected will remain fully confidential and no results from any of the tasks your child performs will be shared with your child's school. All the information you provide us with will be assigned an anonymous number and no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researcher at the top of this sheet will have access. The data will be used only for research purposes, and in accordance with the Data Protection Act of 1998, they will be destroyed 5 years after the completion of the study.

On completion of this study there is a possibility that that the results will be published in an academic journal. Only the overall results will be referred to in this publication with no direct reference being made regarding your child or the school they attend. Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Does my child have to participate?

No. Participation in this study is entirely voluntary and you are under no obligation to agree to participate. Also, you may withdraw your child at any point during the study without giving any reason.

Will my child be paid to participate?

You and your child will be paid £30 upon completion of the study. Your child will also be given a T-shirt as a token of our appreciation upon completion.

I'd like my child to participate, what happens next?

Please contact the primary researcher, Katie Barfoot **via e-mail** ([redacted]) indicating your interest. A member of the research team will then contact you to arrange the study visits and to answer any further questions you may have.

The primary researcher will be the initial contact for any queries or questions throughout the study, so please do not hesitate to get in touch via email. However, if by some means you are unable to contact the researcher at any point, then you may also contact Professor Claire Williams (the Principle Investigator; [redacted]) and she will be happy to answer your queries and to pass on any information.

Thank you for your help!

A.3 Parent information sheet for Experiment 3

School of Psychology & Clinical Language Sciences

Study: The effect of fruit drink supplementation on the behaviour, cognition and reading performance of children with and without attention-deficit hyperactivity disorder (ADHD).

Researchers: Katie Barfoot (Email: [redacted])
Dr Claire Williams (Tel: [redacted]; Email: [redacted])

Does your child want to take part in a Fruit Drink Study?

We hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like your child to participate. However, if you have any further queries or would like to discuss any aspect of the study, please do not hesitate to contact us by email: [redacted].

Background to the study

We are interested in finding out about the effects of a fruit drink on the attention, memory and behaviour of children, particularly children who have attention and/or behaviour difficulties. Some fruits naturally contain high amounts of micronutrients called flavonoids that are found in a number of foods including vegetables, fruits and fruit juices. Recent research with adults has shown that consumption of foods with a high flavonoid content leads to beneficial improvements on such things as attention and memory. A recent study carried out within our department has shown that following consumption of a blueberry drink ,children aged 6-12 years showed improved attention and memory performance. These results have positive educational implications for children, such as the possibility to benefit reading performance which relies on both memory and attention. This study aims to further investigate these encouraging findings using a sample of children who have Attention Deficit Hyperactivity Disorder (ADHD) and a sample of children who are not diagnosed with ADHD to compare.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading. The researchers on this project has been through the formal Disclosure & Barring Service (DBS) procedure and have been approved by the University of Reading to work with children. The study has been reviewed by the University Research Ethics Committee and has been given favourable ethical approval; which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed. The study is funded by the University of Reading and a non-commercial nutritional supplement company.

What does the study involve?

Session 1

You and your child will visit the Psychology department one week before the study and will be briefed on what the study entails. Your child will be asked to taste a sample of two fruit drinks, and to rate their pleasantness from 1 – 10. Your child will then be asked to complete several cognitive, behavioural and demographic tasks. You will also be asked to complete a child behaviour rating scale, a food questionnaire, and to answer some questions on your child's health.

If your child really does not like the drink, shows distress from the taste, or as a result cannot finish the contents, then he/she will not be invited to participate any further.

Session 2

You will visit the Psychology department one day before the study. Your child will be asked to complete a 'practice' of all the cognitive tests that they will encounter at each future test phase. This is so your child can get used to the tasks and ask questions if they are unsure of what to do.

From the early afternoon of session 2 your child must not consume any of the following:

All fruit and vegetables (except bananas, carrots and sweetcorn)
Chocolate
Fruit juice
Tea and coffee
Fizzy and energy drinks
Pain relievers (e.g. paracetamol, ibuprofen, aspirin)

They must also not partake in vigorous exercise. Two x ten minute mild-moderate-paced walks are acceptable.

You will then be given 2 weeks' worth of the fruit drink formula with instructions on how to prepare it. A video tutorial made by the experimenter showing 'how to make my child's fruit drink' will also be shown to you and a link on where to find the video for future use. You will then be instructed on when you should give your child their drink each day, what to do if anything goes wrong and the experimenter's contact details for any queries during the study. Dates and times for future sessions will also be provisionally booked during this session.

Session 3

This session will take place 1 day after Session 2. On this day you will be required to give your child a standardised breakfast, lunch and snacks until they come into the department. The researcher will inform you on what these foods are well in advance.

You will bring your child to the Psychology department at the University of Reading. They will be seated in a testing room and will take part in the previously practised cognitive test battery taking approximately forty-five minutes. Your child will then receive a fruit drink to consume and you will be asked to return to the Psychology department 2 hours later for further testing. Your child must not eat anything in this 2 hour time period but may drink water.

Session 4

This session will take place on the same day as session 3, 2 hours later. You will bring your child back to the Psychology department to perform the same cognitive test battery as before, taking approximately forty-five minutes.

4 week fruit drink administration

You will then administer your child's drink every day for 4 weeks. Your child must finish all contents each day. In the event that they do not drink it one day, you must try to administer another (fresh) drink that day. If your child still does not comply, you should not administer the drink that day and record it as a 'missed day'. You will also be given a 3 day food diary to complete over the next 2 weeks.

Session 5

This session will take place two weeks after session 4. You and your child will come into the Psychology department. On this day you will be required to give your child a standardised breakfast, lunch and snacks until they come into the department. You will also be asked to not give your child their fruit drink that morning. Your child will then complete the same test battery as before. You will also complete a behaviour rating scale and a food questionnaire. Either you or the experimenter will then give your child their fruit drink for that day.

You will then be given another 2 weeks' worth of the fruit drink formula to continue administration every day, and will be given a 3 day food diary to complete, as before, over the next 2 weeks.

Session 6

This session will take place two weeks after session 5. The procedure of this session will be identical to that session 5, except children will not receive a drink after testing. You will then both be debriefed about the experiment and any questions will be answered. Any remaining fruit drink formula will be collected back from you. You will, again, be given a 3 day food diary to complete over the next 4 weeks.

Session 7

This session will take place four weeks after session 6. In the four week period between session 6 and 7 your child will not be receiving any fruit drinks. You and your child will visit the Psychology department to undergo follow-up testing. The test procedure will be identical to that in sessions 5 and 6.

About the fruit drinks…

Your child will receive one of two different doses of fruit drinks. The dose received will be randomly allocated. The two drinks will contain organic orange squash and a different dose of flavonoid. One of the drinks will also have a small amount of sugar and vitamin c to match the amount found in the flavonoid drink. All drinks will be prepared hygienically within the university or school, and with the exception of the ingredients already listed, no additives or any other items will be added. A full breakdown of the ingredients can also be made available to you upon request. In order to ensure that it is truly the drinks that are having an effect, we will provide you with a list of foods that are high in flavonoid content, which we will ask your child to avoid eating for 24 hours before each study day. A list of optional healthy alternative food will also be provided. Full details of this will be made available to you in advance.

The sessions are designed to be fun and consist of a few short tasks to do on a computer. One of the tasks will require your child to memorise a set of numbers and then be asked to recognise if a particular item belonged to that set or not, by simply replying 'yes' or 'no' accordingly. The other task will require your child to listen to a list of words being read out via an audio recording, and then recall as many as possible, in any order.

Why has my child been selected to take part?

We have invited children from local schools aged 7-10 years old, some with ADHD and others not diagnosed with ADHD, and children aged 5-8 years old without ADHD, to take part in our study. We have

targeted children of these ages as they are old enough to understand the tasks. Additionally, around the ages of 7 and 10, children experience a spurt of brain growth in areas related to attention and memory that makes their responses to these tasks of particular interest.

What happens to the data?

All information collected will remain fully confidential and no results from any of the tasks your child performs will be shared with your child's school. All the information you provide us with will be assigned an anonymous number and no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researcher at the top of this sheet will have access. The data will be used only for research purposes, and in accordance with the Data Protection Act of 1998, they will be destroyed 5 years after the completion of the study.

If your child does not have ADHD and their results indicate they have some characteristics of the disorder, you will be informed by the researcher, and their results explained to you. Please be aware that the researcher is not qualified in diagnosing ADHD and you will need to refer your child to a healthcare professional through your GP for any official diagnosis. We can advise you on the best course of action to take and can put you in touch with those you should seek advice or attention from.

On completion of this study there is a possibility that that the results will be published in an academic journal. Only the overall results will be referred to in this publication with no direct reference being made regarding your child or the school they attend. Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Does my child have to participate?

No. Participation in this study is entirely voluntary and you are under no obligation to agree to participate. Also, you may withdraw your child at any point during the study without giving any reason.

Will my child be paid to participate?

You and your child will not be paid for participation; however your child will be given a T-shirt as a token of our appreciation upon completion of the whole study.

I'd like my child to participate, what happens next?

Please contact me, Katie Barfoot, **via e-mail** [redacted] indicating the information below, **or** tear off the slip below and **send to:** Katie Barfoot, Room 202, School of Psychology and Clinical Language Sciences, Earley Gate, University of Reading, Whiteknights Road, Reading, RG6 6AL. A member of the research team will then contact you to arrange the study visits and to answer any further questions you may have.

I am the researcher on this project, and will be the initial contact for any queries or questions throughout the study, so please do not hesitate to get hold of me via email. However, if by some means you are unable to contact me at any point, then you may also contact Dr Claire Williams (the Principle Investigator and my supervisor; [redacted]) and she will be happy to answer your queries and to pass on any information to me.

Thank you for your help!

A.4 Parent information sheet for Experiment 4

School of Psychology & Clinical Language Sciences

Study: The effect of fruit drink supplementation on the cognition, mood and urine metabolites of children.

Researchers: Katie Barfoot (Email: [redacted])

Professor Claire Williams (Tel: [redacted]; Email: [redacted])

The Fruit Drink Study

We hope to provide all the information you will need about the study in order for you to make an informed decision about whether you would like your child to participate. However, if you have any further queries or would like to discuss any aspect of the study, please do not hesitate to contact me by email: [redacted]

Background to the study

We are interested in finding out about the effects of a fruit drink on the attention, memory and mood of children. Some fruits naturally contain high amounts of micronutrients called flavonoids that are found in a number of foods including vegetables, fruits and fruit juices. Recent research with adults has shown that consumption of foods with a high flavonoid content leads to beneficial improvements on such things as attention and memory. A recent study carried out within our department has shown that following consumption of a blueberry drink ,children aged 6-12 years showed improved attention and memory performance. These results have positive educational implications for children, such as improving memory and attention within the classroom.

This study aims to further investigate these encouraging findings by analysing small molecules called 'metabolites' in urine after children have been supplemented with either a high or low flavonoid fruit drink. Metabolites are natural molecules which are produced within the body as a result of metabolic processes, such as digestion. We are hoping to explore the relationship between the metabolites produced by the body after a fruit drink, and any subsequent memory, attentional or mood effects within children.

Who is running the study?

The study is run by research staff in the School of Psychology & Clinical Language Sciences at the University of Reading. The researchers on this project have been through the formal Disclosure & Barring Service (DBS) procedure and have been approved by the University of Reading to work with children. The study has been reviewed by the University Research Ethics Committee and has been given favourable ethical approval; which means that an independent group did not raise any objections to the study on ethical grounds and have permitted the study to proceed. The study is funded by the University of Reading and a non-commercial nutritional supplement company.

What does the study involve?

Screening session

You and your child will visit the Psychology department, will be briefed on what the study entails and will be provided with all the appropriate equipment for the study. Your child will be asked to taste a sample of two fruit drinks, and to rate their pleasantness from 1 – 10. Your child will then be asked to complete several

cognitive, behavioural and demographic tasks and will have a 'practice' of all the cognitive tests that they will encounter at each future test phase. This is so your child can get used to the tasks and ask questions if they are unsure of what to do. You will also be asked to complete a child behaviour rating scale, a food questionnaire, and will be briefed on the urine collection procedure.

Note: If your child really does not like the drink, shows distress from the taste, or as a result cannot finish the contents, then he/she will not be invited to participate any further.

Urine collection 1

You will be asked to do a 24 hour collection of your child's urine the day before your scheduled visit to the Psychology department. This will involve you supervising your child to urinate into a pot (provided) each time they naturally feel the urge to urinate across 24 hours.

There will be 2 collection pots in total (one for 0-12hrs and one for 12-24hrs) and these will contain a small amount of powdered ascorbic acid (vitamin C) as a preservative. You will be asked to store these pots in a cool bag (provided) between urinations.

Due to the close supervision required on collection days, it is preferable to select a day which you will spend wholly with your child for urine collection days, e.g. a Sunday.

During the 24 hour collection your child must follow a low-flavonoid diet, and so must not consume any of the following:

All fruit and vegetables (except bananas, carrots, mushrooms and sweetcorn)
Chocolate
Fruit juice
Tea and coffee
Fizzy and energy drinks
Pain relievers (e.g. paracetamol, ibuprofen, aspirin)

They must also not partake in vigorous exercise. Two x ten minute mild-moderate-paced walks are acceptable.

Session 1 – Part A

This session will take place the day after your child's first 24 hour urine collection. On this day you will be required to give your child a low-flavonoid breakfast, lunch and snacks until they come into the department, and you will be asked to bring in your child's 24 hour urine pots.

On arrival at the University's Psychology department, your child's urine will be stored for processing. Your child will then be seated in a testing room and will take part in the previously practised cognitive test battery taking approximately thirty minutes. Your child will then receive a fruit drink to consume and you will be asked to return to the Psychology department 2 hours later for further testing. Your child must not eat anything in this 2 hour time period, but may drink water.

Session 1 – Part B

This session will take place on the same day as session 1, 2 hours later. You will bring your child back to the Psychology department to perform the same cognitive test battery as before, lasting approximately thirty minutes.

You will then be given 2 weeks' worth of the fruit drink formula with instructions on how to prepare it. A video tutorial of 'how to make my child's fruit drink' will also be shown to you and a link on where to find

the video for future use. You will then be instructed on when you should give your child their drink each day, what to do if anything goes wrong and the experimenter's contact details for any queries during the study. Dates and times for future sessions will also be provisionally booked during this session.

You will also be provided with 2 empty urine collection pots.

4 week fruit drink administration

You will then administer your child's drink once a day for 4 weeks. Your child must finish all contents each day. In the event that they do not drink it one day, you must try to administer another (fresh) drink that day. If your child still does not comply, you should not administer the drink that day and record it as a 'missed day'. You will also be given a 3 day food diary to complete over the next 2 weeks.

Urine collection 2

You will be asked to do a 24 hour collection of your child's urine (as before) the day before your scheduled visit to the Psychology department.

Session 2

This session will take place two weeks after session 2. You and your child will come into the Psychology department. On this day you will be required to give your child a standardised breakfast, lunch and snacks until they come into the department, and you will be asked to bring in your child's 24 hour urine pots.

On arrival at the University's Psychology department, your child's urine will be stored for processing. Your child will then complete the same test battery as before, lasting approximately 30 minutes.

You will then be given another 2 weeks' worth of the fruit drink formula to continue administration every day, and will be given a 3 day food diary to complete, as before, over the next 2 weeks.

You will again be provided with 2 empty urine collection pots.

Urine collection 3

You will be asked to do a 24 hour collection of your child's urine (as before) the day before your scheduled visit to the Psychology department.

Session 3

This session will take place two weeks after session 2. The procedure of this session will be identical to that of session 2. On this day you will be required to give your child a standardised breakfast, lunch and snacks until they come into the department, and you will be asked to bring in your child's 24 hour urine pots.

On arrival at the University's Psychology department, your child's urine will be stored for processing. Your child will then complete the same test battery as before, lasting approximately 30 minutes.

Your child will now have completed the fruit drink study! You will then both be debriefed about the experiment and any questions will be answered. Any remaining fruit drink formula and/or equipment will be collected back from you.

About the fruit drinks… Your child will receive one of two different doses of fruit drinks. The dose received will be randomly allocated. The two drinks will contain organic orange squash and a different dose of flavonoid. One of the drinks will also have a small amount of sugar and vitamin C to match the amount

found in the flavonoid drink. All drinks will be prepared hygienically within the University, and with the exception of the ingredients already listed above, no additives or any other items will be added. A full breakdown of the ingredients can also be made available to you upon request. In order to ensure that it is truly the drinks that are having an effect, we will provide you with a list of foods that are high in flavonoid content, which we will ask your child to avoid eating for 24 hours before each study day. A list of optional healthy alternative food will also be provided. Full details of this will be made available to you in advance.

About the tasks... The sessions are designed to be fun and consist of a few short tasks to do on a computer. One of the tasks will require your child to respond to the direction of a target arrow on screen amongst various other arrows. The other task will require your child to listen to a list of words being read out via an audio recording, and then recall as many as possible, in any order.

About the urine collection… During this study you will be required to take multiple urine samples from your child which will be used to measure their metabolite concentrations. This is a painless procedure which will cause them minimal distress. If your child would like to experience giving a urine sample before consenting to take part in this research please tell the experimenter. If during the experiment you/your child decide that you/them do not want to take/give a urine sample then you/they can withdraw from the experiment without giving a reason. Unusually low or high metabolite concentrations can be indicative of abnormalities in hormonal regulation. We will inform you orally if we observe unusual metabolite concentrations and in such cases we recommend that you contact your GP and ask for an official metabolic assessment. If you prefer we can contact your GP directly and pass on your readings. This urine collection procedure has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Why has my child been selected to take part?

We have invited children from the Berkshire area aged 7-10 years old to take part in our study. We have targeted children of these ages as they are old enough to understand the tasks and are in control of their own toileting needs. Additionally, around the ages of 7 and 10, children experience a spurt of brain growth in areas related to attention and memory that makes their responses to these tasks of particular interest.

What happens to the data?

All information collected will remain fully confidential and no results from any of the tasks your child performs will be shared with your child's school. All the information you provide us with will be assigned an anonymous number and no name will appear on any of the documents. All data will be kept safely locked at the University of Reading and only the named researcher at the top of this sheet will have access. The data will be used only for research purposes, and in accordance with the Data Protection Act of 1998, they will be destroyed 5 years after the completion of the study.

On completion of this study there is a possibility that that the results will be published in an academic journal. Only the overall results will be referred to in this publication with no direct reference being made regarding your child or the school they attend. Additionally, if you so request, the results of the study will be forwarded to you upon its completion either by post or email.

Does my child have to participate?

No. Participation in this study is entirely voluntary and you are under no obligation to agree to participate. Also, you may withdraw your child at any point during the study without giving any reason.

Will my child be paid to participate?

You and your child will not be paid for participation; however your child will be given a T-shirt and a cap as a token of our appreciation upon completion of the whole study.

I'd like my child to participate, what happens next?

Please contact me, Katie Barfoot **via e-mail** ([redacted]) indicating your interest. A member of the research team will then contact you to arrange the study visits and to answer any further questions you may have.

I am the researcher on this project, and will be the initial contact for any queries or questions throughout the study, so please do not hesitate to get hold of me via email. However, if by some means you are unable to contact me at any point, then you may also contact Professor Claire Williams (the Principle Investigator and my supervisor; [redacted]) and she will be happy to answer your queries and to pass on any information to me.

Thank you for your help!

Appendix B Low flavonoid diet

B.1 Low flavonoid diet sheet

University of Reading

Head of School
Prof Laurie Butler

School of Psychology and Clinical Language Sciences

University of Reading
Earley Gate
Reading RG6 6AL

[redacted]
email [redacted]

Low-flavonoid Diet

Please **avoid** eating foods shown below for **24** hours before **each** visit to the Nutritional Psychology Unit and for the duration of each study day.

- All berries
- Fruit and vegetables (see below for exceptions)
- Fruit juices
- Jams and preserves
- Fruit teas
- Soy products
- Chocolate/cocoa
- Tea (black, green, earl grey etc)
- Coffee
- All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade.
- All dietary supplements

Foods you may eat include those shown below.

- Potatoes
- Rice
- Bread
- Sweetcorn
- Mushrooms
- Carrots
- Bananas
- Pasta
- Meat/fish
- Dairy products
- Nuts
- Snacks – crisps, biscuits, cakes (without chocolate or fruit)

Low-flavonoid meal plan

Breakfast

Toast with a choice of toppings: Butter/spread, marmite, peanut butter, honey and/or banana.

OR

Cereal with milk (no chocolate or fruit cereals).

Lunch

Sandwich with a choice of fillings: Cheese, ham, other meat, egg, fish, peanut butter, butter/spread, marmite.

OR

Croissant, crumpets or other bread-based products with any of the above toppings.

Dinner

Baked white potato with a choice of fillings: Butter/spread, cheese, ham, other meat, egg, fish.

OR

White rice OR pasta with a combination of meat, fish, carrots, sweetcorn, mushrooms, cucumber, lettuce with carbonara/white sauce (no tomato-based sauces).

OR

Fish fingers with chips OR pasta and sweetcorn. (If choosing pasta – plain or with carbonara/white sauce).

Snacks (anytime)

Banana, Carrots, Houmous, Brazil nuts, Peanuts, Rice cakes, White chocolate, Crisps, Crackers, Biscuits (no fruit or chocolate), Cake (no fruit or chocolate), Pastry-based products (no fruit or chocolate), Cereal bars (no fruit or chocolate), Milk, Cheese, Yoghurt (no fruit or chocolate flavours), Meat, Eggs, Custard, Rice pudding.

B.2 24 h food log

Participant ID:
Date of diary:

	Food eaten	Comments
Breakfast		
Snacks		
Lunch		
Snacks		
Dinner		
Snacks		

If you have any problems before or during the 24 hour low flavonoid day please get in touch with the researchers, Katie ([redacted]) or Adrian ([redacted]. Please do not stop abiding by the diet if you think something may have gone wrong.

Appendix C PANAS-C mood questionnaire

PANAS-C MOOD QUESTIONNAIRE

This scale consists of a number of words that describe different feelings and emotions.
Read each item and then tick or cross the appropriate answer next to that word.
Indicate how much you feel this way right now.

	Not much or not at all 1	**A little bit 2**	**Medium 3**	**Quite a bit 4**	**Extremely 5**
Interested					
Sad					
Frightened					
Alert					
Excited					
Ashamed					
Upset					
Happy					
Strong					
Nervous					
Guilty					
Energetic					
Scared					
Calm					
Miserable					
Jittery					
Cheerful					
Active					
Proud					
Afraid					
Joyful					
Lonely					
Mad					
Fearless					

Disgusted					
Delighted					
Blue					
Daring					
Gloomy					
Lively					

Appendix D AVLT word lists

D.1 Practice session

List A	List B
oil	throat
juice	boot
jail	rope
cattle	pedal
camp	pool
jewel	tractor
nail	string
village	pony
rock	desk
berry	ocean
coat	head
chest	skirt
penny	sheet
leaf	plate
gun	mud

D.2 Version 1

List A	List B
egg	arm
ball	glove
drain	pole
palace	gravy
shed	pin
money	mother
coin	wheel
cotton	daisy
card	van
toilet	orange
wood	blood
bin	school
paper	bedroom
clock	rain
salt	face

D.3 Version 2

List A	List B
belt	flame
park	shoe
fox	doll
meadow	ribbon
brush	lamp
building	pillow
tin	knife
breakfast	clothes
tool	bush
painting	honey
star	land
tray	sleeve
garden	button
meat	ring
spoon	tree

D.4 Version 3

List A	List B
band	ship
elbow	lettuce
flag	chin
letter	body
sail	rod
shepherd	marble
knee	box
kitten	soldier
bird	skin
rubber	piano
tail	grape
neck	soup
oven	ticket
nurse	beard
fruit	plant

D.5 Version 4

List A	List B
cage	hedge
sink	grass
forest	pig
woman	coffee
lake	fork
football	river
stew	thread
candy	dentist
crown	stool
teacher	brother
book	girl
hare	foam
insect	ankle
soap	hall
men	room

Appendix E 10:20 standardised system for EEG electrode placement

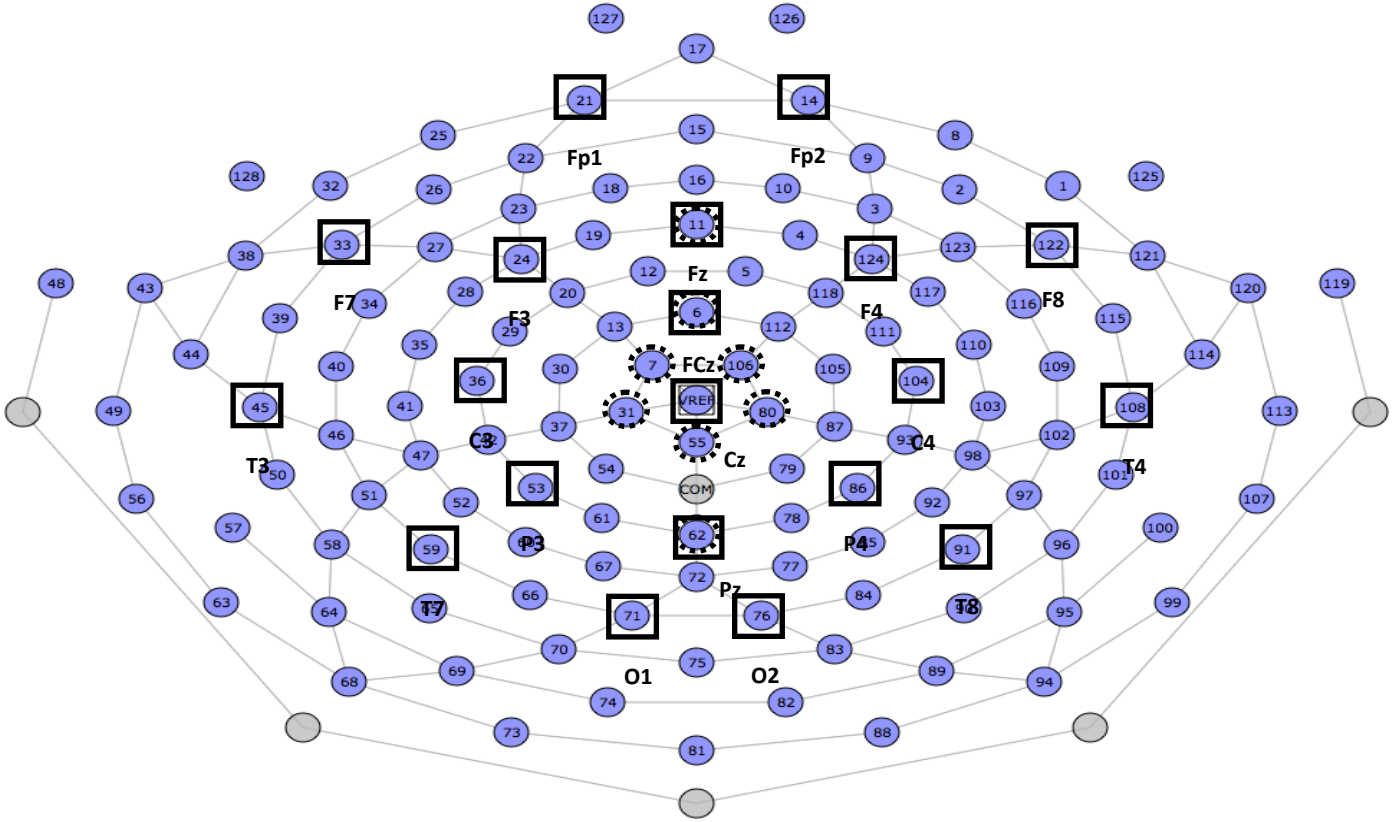

Figure E.1. Electrode locations in the Geodesic 128 cap array. Approximate corresponding positions in the 10:20 system are highlighted using squares. Three single electrodes and one cluster of electrodes were chosen *a priori* as regions of interest for data analyses (shown as dashed circles). These were frontal (Fz; 11), fronto-central (FCz; 6), parietal (Pz; 62) and central (Cz; 7, 31, 5, 80, 106).

Appendix F LMM statistics for Experiment 2 Go No-Go cognitive outcomes

Table F.1. LMM statistics for Experiment 2 (Chapter 4) Go No-Go task

Cognitive task	LMM Model	DV	LMM statistics
Go No-Go	Drink as a Fixed Factor	Go accuracy (hits)	Drink (F(1,12.98)=0.22, p=0.65)
		Go RT	Drink (F(1,13.96)=0.004, p=0.95)
		False alarms	Drink (F(1,13.68)=0.51, p=0.49)
		d-prime	Drink (F(1,13)=0.60, p=0.45)
		Speed-accuracy trade-off	Drink (F(1,14.15)=0.012, p=0.91)
	Drink and Visit as Fixed Factors	Go accuracy (hits)	Drink (F(1,26.20)=0.16, p=0.69) Visit (F(1,26.20)=0.03, p=0.85) Drink x Visit (F(1,26.20)=0.08, p=0.79)
		Go RT	Drink (F(1,26.26)=0.01, p=0.91) Visit (F(1,26.26)=0.61, p=0.44) Drink x Visit (F(1,26.26)=0.17, p=0.68)
		False alarms	Drink (F(1,26.89)=0.37, p=0.55) Visit (F(1,26.89)=0.49, p=0.49 Drink x Visit (F(1,26.89)=0.14, p=0.71)
		d-prime	Drink (F(1,26.53)=0.55, p=0.48) Visit (F(1,26.53)=0.14, p=0.72) Drink x Visit (F(1,26.53)=0.005, p=0.94)
		Speed-accuracy trade-off	Drink (F(1,17.14)=0.04, p=0.85) Visit (F(1,17.14)=0.015, p=0.90) Drink x Visit (F(1,17.14)=1.51, p=0.24)