

# *Nutrition and symbiosis*

Book or Report Section

Accepted Version

Van Emden, H. F. and Douglas, A. E. (2017) Nutrition and symbiosis. In: Van Emden, H. F. and Harrington, R. (eds.) Aphids as Crop Pests, 2nd edition. CABI, Wallingford, pp. 114-131. ISBN 9781780647098 Available at <https://reading-pure-test.eprints-hosting.org/72413/>

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Publisher: CABI

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# 6 Nutrition and Symbiosis

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## Introduction

A single question dominates the study of aphid nutrition: how does plant phloem sap, a nutritionally unbalanced diet of high C:N content, support the remarkably high growth and reproductive rates characteristic of many aphid species? The ‘short answer’ to this question has two elements. First, phloem sap offers a near continuous supply of small organic compounds, principally sugars and amino acids, which require the minimum of digestive processing; consequently, the assimilation efficiency of aphids is exceptionally high. Second, the key nutritional inadequacy of phloem sap, the low essential amino acid content, is met by symbiotic bacteria in the aphid.

The ‘long answer’ to this opening question requires a mechanistic understanding of aphid nutrition and its interaction with the functions of the symbiotic microorganisms, and this is the topic of this chapter. Research on aphid nutrition has been dominated by carbon and nitrogen nutrition, and these topics are addressed in the third and fourth sections, respectively. The fifth section summarizes our current understanding of the mineral and micronutrient requirements of aphids. First, however, the key features of microbial symbiosis in aphids are reviewed.

Two technical advances have been vital in the development of research on aphid nutrition and symbiosis: firstly, chemically defined diets of a composition that is known precisely and can be manipulated readily; and secondly, aphids experimentally deprived of their symbiotic bacteria. Diet studies, often involving bacteria-free aphids, are now used routinely to investigate various aspects of aphid nutrition, e.g. identification of dietary requirements and analysis of nutrient utilization patterns. The value and limitations of such diet-based studies to explore aphid nutrition are assessed in the concluding section of this chapter.

## Microbial Symbiosis in Aphids

### Diversity of microorganisms

Aphids generally bear very few types of microorganisms, usually less than ten taxa. Nearly all aphids of the family Aphididae possess bacteria, known in the traditional literature as 'primary symbionts' and assigned by molecular criteria to a novel genus and species *Buchnera aphidicola* in the  $\gamma$ 3-proteobacteria (*Escherichia coli* is also a member of this group) (Munson *et al.*, 1991). Many aphids additionally bear one to several other bacteria, known as 'secondary symbionts', usually at a 10-fold lower density than *Buchnera*. The secondary symbionts in various aphid species have been studied, and include  $\gamma$ 3-proteobacteria, *Serratia symbiotica*, *Candidatus Hamiltonella defensa*, and *Candidatus Regiella insecticola*, a  $\gamma$ -proteobacterium *Rickettsia* sp. and *Spiroplasma* (Fukatsu *et al.*, 2001; Mran *et al.*, 2005; Sakurai *et al.*, 2005; J. A. Russell *et al.*, 2013). *Buchnera* is coccoid and usually 2–4  $\mu\text{m}$  in diameter, but 'giant' forms exceeding 10  $\mu\text{m}$  occur occasionally. The accessory bacteria are small cocci (<2  $\mu\text{m}$  diam.) or rods of variable length (<2–20  $\mu\text{m}$ ).

Aphids known to lack *Buchnera* are: some members of the subfamily Cerataphidini, which have pyrenomycete yeasts (Suh *et al.*, 2001); the Adelgidae, with both  $\beta$ - and  $\gamma$ -proteobacteria (Toenshoff *et al.*, 2012a; b); and the Phylloxeridae, which apparently have no symbiotic microorganisms (Buchner, 1966).

### Location of symbiotic microorganisms

The *Buchnera* are restricted to insect cells called bacteriocytes (Fig. 6.1a), which are specialized to house and maintain these bacteria, and are located in the haemocoel of the insect. The bacteriocytes are large (up to 100  $\mu\text{m}$  diam.), glistening silver or coloured with green, red, or brown pigment, varying between species, and often aggregated together as a coherent organ known as the bacteriome, lying dorsal to the gut. The *Buchnera* cells are restricted to the cytoplasm (Fig. 6.1b) and occupy c. 60% of the cytoplasmic volume (Whitehead and Douglas, 1993). The tissue distribution of secondary symbionts is generally wider than that of *Buchnera*. Secondary symbionts (Fig. 6.1c) are

very commonly located in ‘sheath cells’ surrounding bacteriocytes, free in the haemolymph and also within bacteriocytes (although apparently never in the same bacteriocyte as *Buchnera* cells (Fukatsu *et al.*, 2000). The incidence of microorganisms in the gut lumen of aphids is variable. The alimentary tract of some aphid individuals is microbially sterile, others have taxa ingested with food and passing directly through the gut, and some bear a more or less resident microbial community, which may include species of the genera *Enterobacter*, *Erwinia*, and *Staphylococcus* (Grenier *et al.*, 1994; Leroy *et al.*, 2011; Sabri *et al.*, 2013; Jing *et al.*, 2014).

### **Acquisition of symbiotic microorganisms**

Aphids acquire their complement of *Buchnera* exclusively from their mother. In other words, transmission of *Buchnera* is obligately vertical. The bacterial cells are released from bacteriocytes abutting the germarium of the aphid ovaries and are transferred directly to the early embryo (blastoderm stage) in parthenogenetic morphs or cytoplasm of the unfertilized egg of oviparae (Fig. 6.1d) (Hinde, 1971; Koga *et al.*, 2012). *Buchnera* probably have been transmitted faithfully from mother to offspring for more than 100 million years and possibly since the origin of the Aphididae, as indicated by the remarkable congruence between the phylogeny of aphids and *Buchnera* and the magnitude of sequence differences between rRNA genes of *Buchnera* in different aphid taxa (Moran *et al.*, 1993).

The phylogenies of aphids and their secondary symbionts are not congruent (J.A. Russell *et al.*, 2003, 2013; Henry *et al.*, 2013). Aphids acquire secondary symbionts from multiple sources: vertically from their mother during sexual reproduction (from male to female), and by feeding. Vertical transmission of most secondary symbionts is regular with an efficiency of >98% (Darby and Douglas, 2003), although lower frequencies have been recorded, e.g. for *Serratia symbiotica* (Peccoud *et al.*, 2014); sexual transmission rates are low and variable (Moran and Dunbar, 2006; Peccoud *et al.*, 2013) and acquisition by feeding is reliable under experimental conditions but of uncertain prevalence in the field (Fukatsu *et al.*, 2000; Darby and Douglas, 2003; Chandler *et al.*, 2008).

### **Significance of symbiotic microorganisms to aphids**

Experiments using antibiotics at a dose that eliminates the bacteria but has no apparent direct effect on aphid metabolism or behaviour have revealed the crucial importance of *Buchnera* to aphids (Wilkinson, 1998). These bacteria-free individuals are called ‘aposymbiotic’ aphids. When adult aphids are treated, reproductive output ceases within 1–2 days, reflecting the vital importance of *Buchnera* for embryo growth (Koga *et al.*, 2007). Aphids treated at birth display normal development (although the final larval stadium may be prolonged) but grow slowly and, on reaching adulthood, are very small and produce no offspring, or a few offspring that are stillborn or die without growing or developing (Table 6.1). Aposymbiotic aphids display no known specific abnormalities, for example in feeding or embryogenesis (Wilkinson, 1998), and the role of *Buchnera* is predominantly, and probably exclusively, nutritional.

The variable incidence of secondary symbionts in aphids indicates that the insect does not require these taxa. Two approaches have been adopted to investigate their significance to aphids: comparisons of the performance of – first, multiple clonal lines naturally bearing different complements of bacteria, and second, single aphid clones with their complement of secondary symbionts manipulated experimentally. The results indicate that the fitness consequences of these bacteria vary with aphid and bacterial genotype, presence of other bacteria, rearing plant and other conditions, especially temperature (Montllor *et al.*, 2002; Oliver *et al.*, 2006; Koga *et al.*, 2007; Chandler *et al.*, 2008). In the pea aphid *Acyrtosiphon pisum*, the secondary symbionts make no evident contribution to nutrition (Douglas *et al.*, 2006a), but genomic data have implicated *Serratia symbiotica* in the nutrition of *Cinara cedri* (Lachninae) (Perez-Brocal *et al.*, 2006; Lamelas *et al.*, 2011). The role of the secondary symbionts in other aspects of the biology of aphids, especially defence, is considered in Chapter 11, this volume.

## Carbon Nutrition

### Sugars

Phloem-mobile sugars are the principal source of carbon for aphid respiration and growth. Virtually all research has been conducted on aphid utilization of ingested sucrose, a disaccharide of glucose and fructose and the sole sugar in the phloem sap of many plants. Aphid utilization of galactose-

based oligosaccharides (raffinose, stachyose, and higher homologues) that occur in the phloem sap of many Cucurbitaceae and Lamiaceae and of sugar alcohols (e.g. sorbitol, dulcitol, mannitol) in the sap of Celastraceae, Prunoideae and Rosaceae has not been studied.

Phloem sucrose poses two physiological ‘problems’ for aphids. First, sucrose is not assimilated directly across animal guts and its nutritional value depends on hydrolysis to its constituent monosaccharides, glucose and fructose. Second, the sucrose concentration in phloem sap is very high, usually between 0.5 and  $>1.0$  M, and its osmotic pressure is considerably higher than that of aphid body fluids (Downing, 1978; Wilkinson *et al.*, 1997). The resultant osmotic gradient would be expected to cause uncontrolled flux of water from the aphid tissues to the gut lumen, and desiccation of the aphid.

Gut enzymes are central to the resolution of both these physiological problems. The aphid midgut has high activity of (i) sucrase,  $\alpha$ -glucosidase that hydrolyses sucrose to glucose and fructose, and (ii) transglycosidase, which synthesizes glucose-based oligosaccharides (3–>15 hexose units), so reducing the osmotic pressure per hexose unit in the gut lumen (Cristofolletti *et al.*, 2003; Price *et al.*, 2007). The monosaccharides are assimilated across the gut wall into the haemolymph via sugar transporters that have been identified (Price *et al.*, 2010), and the oligosaccharides are voided *via* the honeydew (Wilkinson *et al.*, 1997). It is very likely that a single enzyme may mediate both sucrose hydrolysis and oligosaccharide synthesis by inserting a molecule of water and glucose, respectively, at the glucosidic bond (Ashford *et al.*, 2000 Price *et al.*, 2007). The enzyme activity is of aphid (and not microbial) origin as the activity is unaffected by experimental elimination of microorganisms with antibiotics (Wilkinson *et al.*, 1997). Dietary sugars are important, not only as the principal source of carbon but also because they are the single, most important nutritional determinant of aphid feeding rate. The rate at which aphids ingest food varies inversely with the dietary concentration of sucrose (Mittler and Meikle, 1991; Douglas *et al.*, 2006b) (Fig. 6.2). This response generally is interpreted as a compensatory feeding response, i.e. that aphids can compensate behaviourally, at least partially, for low dietary sucrose by feeding at higher rates. Overlaying this feeding response, sucrose acts as a phagostimulant, i.e. sustained feeding by aphids depends on the presence of dietary sucrose. As a result, aphids feed at a progressively increasing rate with declining sucrose concentration to a minimal concentration of 0.05–0.2 M (varying with species and conditions), below which sustained feeding is not exhibited (Pescod *et al.*, 2007).

Research on the behavioural regulation of carbon acquisition by aphids has been conducted almost exclusively with aphids on chemically defined diets. The extent to which these data are relevant to aphids feeding on plants is far from clear. The key issues are:

- The sucrose concentration in plant phloem sap varies both with instantaneous irradiance and over the diurnal cycle (Geigenberger and Stitt, 2000), but the aphid behavioural and physiological responses to fluctuating sucrose concentrations are not fully understood (Cull and van Emden, 1977; Taylor *et al.*, 2012).
- Phloem sucrose concentrations can often exceed 1.0 M (Pescod *et al.*, 2007), considerably higher than the concentrations usually used in diet studies.
- Phloem sap is under positive hydrostatic pressure, and aphids may not be able to regulate feeding rate from sieve elements as precisely as from diets at atmospheric pressure (Turgeon, 2010).

## Lipids

Two lines of evidence indicate that aphid lipids are of endogenous origin: the natural diet of phloem sap contains very little lipid, and the chemically defined diets routinely used for maintaining aphids through multiple generations are lipid-free.

The composition of aphid lipids is most unusual for animals. The dominant lipids are triglycerides with acyl moieties of short chain length. For example in *A. pisum*, myristic acid (C14) and hexanoic acid (C6) make up 90% of the triglyceride fatty acids, and the most abundant triglyceride is 1,3-dimyristoyl-2-hexanoyl glycerol (Rahbe *et al.*, 1994). The triglyceride content and composition of both *A. pisum* and *Macrosiphum euphorbiae* (potato aphid) are not significantly modified by treatment with the antibiotic rifampicin (Rahbe *et al.*, 1994; Walters *et al.*, 1994)(Fig. 6.3a), indicating that these compounds are synthesized by the aphid, and not the symbiotic microorganisms. The principal function of aphid triglycerides is as an energy source when aphids are not feeding (and so do not have access to sugars). The key periods of extended non-feeding are during moulting, flight and, for some species, the adult gynopara and male morphs. It has also been suggested that triglycerides are a crucial energy source for embryos but, to our knowledge, the relative importance of sugars and lipids for energy metabolism of maternal tissues and embryos in virginoparae has not been investigated systematically (Walters *et al.*, 1994). The significance of the unusual fatty acid composition of aphid triglycerides is uncertain. The short acyl chain length ensures that the

triglycerides are above their melting point, and this may be linked to one function of the haemolymph triglycerides as precursors of secretions from the siphunculi. When aphids are agitated, droplets rich in triglycerides of composition similar (but not identical) to the haemolymph triglycerides (Fig. 6.3b) are exuded from the siphuncles, and on release, these siphuncle secretions change into a sticky solid that deters potential predators. The triglycerides containing sorbic acid (C6:2) have also been implicated in defence against microbial antagonists (Brown, 1975) because sorbic acid is a potent fungistatic agent, but this suggestion has not been tested systematically (Brown, 1975).

The principal phospholipids in aphids are phosphatidylethanolamines and phosphatidylcholines; the levels of phosphatidylinositols are very low (Febvay *et al.*, 1992). The fatty acids in aphid phospholipids are generally of longer chain length than in the triglycerides. For example, in the phospholipids of *A. pisum*, the dominant fatty acid is linoleic acid (C18:2). Radiotracer studies have confirmed that, as with the triglyceride fatty acids, the phospholipid fatty acids are synthesized endogenously by the aphid (de Renobles *et al.*, 1986).

Aphids require preformed sterols because, like other insects, they cannot synthesize these compounds *de novo*. The significance of the dietary supply of sterols for aphids is indicated by the improved reproductive output of both *Acyrthosiphon pisum* and *Myzus persicae* (peach–potato aphid) when chemically-defined diets are supplemented with cholesterol or certain phytosterols, i.e. sterols of plant origin bearing an alkyl sidechain (Bouvaine *et al.*, 2012, 2014;). Analyses of the composition of aphid sterols and the performance of aphids on diets with different sterol supplements suggest that aphids may have limited capacity to dealkylate phytosterols (Behmer *et al.*, 2011), although this may vary both across different phytosterols and among aphid species.

## Nitrogen Nutrition

### Amino acids and their sources

The chief nitrogenous compounds utilized by aphids are free amino acids derived from two sources: the phloem sap and the symbiotic bacteria in the aphids.

Amino acids are the dominant nitrogenous compounds in the phloem sap of most plant taxa, and aphids assimilate them very efficiently. Additionally, aphids derive a supplementary supply of

essential amino acids from the symbiotic bacteria in their tissues. The three core lines of evidence are that:

- Aphids do not have a dietary requirement for many essential amino acids e.g. (Douglas *et al.*, 2001; MacDonald *et al.*, 2011).
- The isolated bacteria are capable of essential amino acid synthesis (Sasaki and Ishikawa, 1995; C.W. Russell *et al.*, 2013).
- Amino acids synthesized by the bacteria in aphid tissues are translocated to the aphid partner (Douglas, 1988; C.W. Russell *et al.*, 2013).
- *Buchnera* genome contains genes coding enzymes in the essential amino acid biosynthesis (Shigenobu *et al.*, 2000).

### **The fate of amino acids in aphids**

Aphids mediate considerable amino acid interconversions by transamination and other reactions (Wilson *et al.*, 2010, 2011), and protein synthesis is quantitatively a major fate for amino acids. More than 95% of the amino acid content of aphids is in the protein fraction, and protein accounts for 10–25% of aphid fresh weight.

Some amino acids have important functions other than in protein synthesis. Several amino acids are, or are precursors of, neurotransmitters and neurohormones, e.g. glutamic acid, dopamine (derived from tyrosine) and serotonin (derived from tryptophan). Tyrosine is also a major precursor of cuticle synthesis. One consequence of the link between certain amino acids and cell-signalling interactions is that the biological ramifications of a shortfall in the supply of these compounds might be expected to extend beyond negative effects on protein synthesis to aphid growth and reproductive rate. This issue has not been studied in detail but, as a possible example, 5-hydroxytryptamine (a derivative of dietary tryptophan) has been reported to inhibit the production of alatiform *M. persicae* (Harrewijn, 1978).

Amino acids are also a vital constituent of the haemolymph of aphids. All 20 protein-amino acids are readily detectable in the haemolymph and, as in other insects, the haemolymph amino acids represent both a metabolic store of nitrogen and an osmoprotectant. Haemolymph amino acids play a crucial role in nitrogen nutrition, because the haemolymph is the first destination of both dietary amino acids assimilated across the gut wall and *Buchnera*-derived amino acids released from the

bacteriocytes. Research on the molecular identity, specificity and kinetic properties of the amino acid transporters on the gut and bacteriocyte membranes mediating the flux of amino acids has been initiated. Candidate transporters have been identified from proteomic and transcriptomic analysis of bacteriocytes (Hansen and Moran, 2011; Poliakov *et al.*, 2011; Macdonald *et al.*, 2012). One transporter ApGLNT1 has been localized to the bacteriocyte membrane and has been shown to mediate the uptake of glutamine (Price *et al.*, 2014). The haemolymph amino acid pool has very high turnover. In addition to supporting protein synthesis and other functions (see above), haemolymph amino acids are consumed at a high rate in respiration. For example in plant-reared *Aphis fabae* (black bean aphid), haemolymph glutamic acid is metabolized to carbon dioxide at a rate of 1.2 nmol carbon/mg aphid weight/h (Wilkinson *et al.*, 2001) and, in diet-reared *A. pisum*, respiration accounts for 60% of the total glutamic acid assimilated (Febvay *et al.*, 1995). Perhaps amino acids are important to the energy metabolism of particular cell types, even though, in comparison with sucrose, they are minor respiratory substrates for aphids at the level of the whole organism (Wilkinson *et al.*, 2001).

Ammonia is the principal nitrogenous product of amino acid catabolism. Aphid honeydew contains appreciable concentrations of ammonia, but not the alternative nitrogenous waste compounds, urea or uric acid (Wilkinson and Douglas, 1995). It has been suggested that the symbiotic bacteria contribute to the overall nitrogen nutrition of aphids by recycling nitrogen; i.e. the bacteria assimilate aphid waste ammonia into essential amino acids, which are released back to the aphid. However, genome-scale metabolic modelling of *Buchnera* reveals that this bacterium is a net producer of ammonia (Thomas *et al.*, 2009), and that the principal fate of ammonia in the bacteriocyte is as a substrate for transamination reactions mediating the terminal step in the synthesis of four essential amino acids, isoleucine, leucine, phenylalanine and valine (Macdonald *et al.*, 2012; C.W. Russell *et al.*, 2013).

### **Other dietary sources of nitrogen**

Although research on the nitrogen nutrition of aphids has concentrated on the utilization of dietary amino acids, plant phloem sap does contain other nitrogenous compounds, including proteins and polyamines (Kehr, 2006; Sichhart and Drager, 2013). In general, these compounds are at lower concentrations than are the free amino acids, but they may be biologically significant in certain

plants or in relation to certain amino acids. The phloem sap of cucurbits has been reported to contain high concentrations of protein ( $>1$  mg/ml), but the ‘phloem sap’ exudates obtained from cucurbits are now recognized to be grossly contaminated by the contents of multiple cell types, and the high protein content is almost certainly artefactual (Zhang *et al.*, 2012).

The fate of peptides and proteins in the aphid gut is poorly understood. Evidence that aphids can utilize small peptides comes from the demonstration of aminopeptidase activity in the gut of pea aphids (Rahbe *et al.*, 1995), and the capacity of the dipeptide glycyl-methionine to support the dietary methionine requirements of *A. fabae* (Leckstein and Llewellyn, 1974). The capacity of aphids to utilize dietary proteins is less clear. Multiple cysteine protease genes are expressed in the aphid gut, but it is not established whether they are intracellular or secreted into the gut lumen (Rispe *et al.*, 2008). In the study by Rahbe *et al.* (1995) on pea aphids, various proteins added to the diet passed through the gut to the honeydew without chemical modification; but in contrast experiments by Pyati *et al.* (2011) are indicative of proteolysis in the gut of cereal aphids.

## Minerals and Micronutrients

The study of the mineral requirements of aphids has been dominated by the need for a convenient mineral mixture suitable for maintaining aphids on chemically defined diets, and there has been no systematic analysis of aphid requirements for inorganic ions. All the published diet formulations include relatively high concentrations of potassium and phosphate, reflecting their high concentrations in phloem sap, and of magnesium and sulphate: at micromolar concentrations, iron, copper, manganese, and zinc are included. It is recognized that aphid requirements for other metals may go undetected because these elements are present in the diet as impurities of other dietary constituents. In all probability, the inorganic requirements of aphids are comparable to those of other phytophagous insects.

Early studies indicated that aphids have a dietary requirement for the seven core B vitamins: thiamine (B<sub>1</sub>), riboflavin, nicotinic acid/nicotinamide, pyridoxine (B<sub>6</sub>), pantothenic acid, folic acid, and biotin (B<sub>12</sub>), and these compounds are included routinely in most chemically defined diet formulations (Dadd *et al.*, 1967). Some data, however, suggest that symbiotic bacteria may meet, partially or completely, the riboflavin requirements of aphids. In particular, the *Buchnera* genome

includes the genetic capability for riboflavin synthesis (Shigenobu *et al.*, 2000), and the pea aphid is reportedly independent of dietary riboflavin (Nakabachi and Ishikawa, 1999).

The only other vitamin provided in published aphid diet formulations is ascorbic acid (vitamin C). This reflects its role as both an essential dietary and a chelator of dietary metal ions, especially  $\text{Fe}^{3+}$  (citric acid is also included in diets for the latter purpose) (Mittler, 1976). The lipophilic vitamins A, D, and E are not usually included in diets (see below), suggesting that they are not dietary requirements for aphids.

## Artificial Diet

### History of artificial diets for aphids

A major contribution to the considerable variation in the growth and reproductive performance of individual aphids, even within the progeny of the same mother, is assumed to be variable nutrition. Such variation occurs between apparently identical plants and even between locations on the same leaf. Variance of the mean relative growth rate (MRGR) of *M. persicae* was significantly higher on replicate plants than on an artificial diet (Wojciechowicz-Zytko and van Emden, 1995). The possibility of rearing aphids on a nutritionally uniform substrate has been explored ever since Hamilton (1930) showed that aphids would feed on fluids through a natural membrane (onion epidermis). The real breakthrough came 30 years later, when Mittler and Dadd (1962) published a fully defined artificial diet for *M. persicae*, closely followed by Auclair and Cartier (1963) with a diet for *A. pisum*. Both pairs of workers used stretched Parafilm 'M'™ as the membrane. The diet published by Dadd and Mittler (1966) has proved the most successful and widely used for several species of aphids, yet for many years the diet failed to sustain any species permanently, even for the originators. However, in February 1976, a culture of *M. persicae* was started on the diet at The University of Reading, and this culture persisted without contact with plants before it eventually died out in December 2008. Another successful use has been to introduce antibiotics into aphids to produce aposymbiotic individuals (Douglas, 1988; Prosser and Douglas, 1991).

By comparison, fully-defined diets have been used for many other aphid species, but usually for experiments involving only the few days survival required for testing toxins or behaviour-modifying chemicals (e.g. Corcuera *et al.*, 1985, Hansen, 2006; Fitches *et al.*, 2008; OingNiang *et al.*

2009; Sprawka *et al.*, 2013). However, such short-term experiments usually can be conducted just as well with adult aphids and simple sucrose solutions in the sachets.

Wille and Hartman (2008) tested five diets from different sources with soybean aphid, *Aphis glycines*. The most successful diet was developed from the amino acid profile of young potato plants and sustained the aphid for about 12 days. More host-restricted aphids may need host-related secondary compounds added to the diet to improve phagostimulation (e.g. the addition of lupanine with a pin-feeding population of *M. persicae* (Cardoza *et al.*, 2006). Pike (2010) has claimed that *Pemphigus spyrothecae* should be a good candidate for developing an artificial diet based on the *A. pisum* one. This claim was based on the similarity in amino acid profiles of carcass hydrolysates of the two species, but does not appear to have been tested.

### **Recipe for the diet and practical procedures**

The recipes for the two diets referred to above (for *M. persicae* and *A. pisum*) are given in modified form in Table 6.2. The two have substantial differences in ingredients and their quantities. The glass still with glass-covered elements used at Reading till 2004 appears to have contributed to the success there; nanopure water does not seem as suitable (too pure?) and switching to it may have contributed to the eventual demise of the Reading *M. persicae* culture. It is important that the ingredients are added in exactly the order given in the Table, and that each ingredient is fully dissolved before the next is added. In practice, this involves setting up a magnetic stirrer for much of a day and alternating diet preparation with other work. It appears increasingly difficult to find sources of a pure form of the chelated metals used in the *M. persicae* culture. One alternative could be to use ‘dirty’ chelate mixtures. This may not actually be a bad thing in terms of providing trace elements, but has yet to be tested. A commercial salt mix as in the *A. pisum* recipe may provide another solution.

The diet is not sterilized at this stage, but is divided into convenient 25–30 ml aliquots in tubes (Fig. 6.4a), which can be deep-frozen for up to 6 months until needed. The aliquots are thawed when needed to make large numbers of diet sachets, which can similarly be stored deep-frozen. It is during the preparation of these sachets that sterile procedures are required and the tubes, the thawed diet, and 50 × 35 mm squares of unstretched Parafilm ‘M’™ are placed in a laminar flow unit under a UV lamp for 35 min to sterilize the top surface of the Parafilm squares. Parafilm will deteriorate if exposed to more than 40 min of UV light.

When the UV is switched off, a square of Parafilm is stretched 2-ways from the underside (Fig. 6.4c), to keep the area that covers the opening of the tube sterile, and placed over one end of a diet tube. This forms one surface of the eventual ‘sachet’ of diet. Diet is poured into a syringe fitted with a disposable millipore (0.22 µm) bacterial filter unit and the diet is pipetted into a sterile tube. An automatic pipette set to 0.6 ml dispenses that volume of diet onto the sterile surface of the Parafilm. The liquid is then covered with another stretched sheet of Parafilm, inverted so that the liquid is sandwiched between two sterile surfaces, and the projecting flaps of Parafilm pulled down on to the side of the tube. These flaps can be used to hold two diet tubes together at their open ends (Fig. 6.4d), and this is critical in the way sachets are replaced. The diet deteriorates quickly, especially since aphid stylets are not sterile, and the aphids have to be provided with new sachets every 2–3 days (2 whenever possible).

This replacement involves using the Parafilm flaps to fix the open end of a new diet tube to the open end of the tube where the aphids are feeding. The upper Parafilm of the sachet with the aphids is then gently slit open with the end of a needle, and inverted onto filter paper so that the old diet runs out it and is absorbed. The aphids then rapidly climb the tubes to transfer to the new sachet, with no handling of the insects being necessary.

### **Relation between the diet and plant phloem sap**

Dadd and Mittler’s (1966) diet was based on Mittler’s (1953, 1958) analysis of willow (*Salix acutifolia*) phloem sap obtained from the cut ends of the inserted stylets of *Tuberolachnus salignus* (giant willow aphid). However, developing a successful diet from this basis involved many changes; Dadd and Mittler tested and re-tested different concentrations of individual components against many different backgrounds of a balance of the other constituents. Their 1966 *M. persicae* diet is the diet used at Reading with slight modifications.

The diet is composed of 15% sucrose and 2.4% amino acids. Mittler (1958) reported percentages in willow phloem sap of around 8.3% sucrose and total nitrogen of between 0.03% and 0.13%. Zimmermann and Ziegler’s (1975) compilation of the sucrose concentration in the phloem of 532 plant species in 102 families shows that 49% of the species recorded sucrose in the 10–20% range. An amino acid analysis (H.F. van Emden and M.A. Bashford, unpublished results) is also available for the phloem sap of glasshouse-grown castor oil plant (*Ricinus communis*), a shrub or

small tree that is a host for *M. persicae*. The sap was obtained by the EDTA method (King and Zeevaart, 1974). Figure 6.5 plots the concentration in the artificial diet against the concentration in the castor bean plant phloem sap of those amino acid compounds for which data from both liquids were available. Both Fig. 6.5 and Mittler's (1958) analyses point to the need for higher concentrations of both sucrose and most amino acids in the diet than in plant phloem sap; however, there is no consistent quantitative change in the ratio (contrast especially arginine and glutamic acid in Fig. 6.5).

That concentrations of most ingredients need to be higher in the diet than in phloem sap is not surprising, since uptake from the diet is much reduced compared with that the phloem. Uptake from diet sachets by both *M. persicae* (Mittler, 1970) and *A. pisum* (Auclair, 1965) is 0.3–0.4 µl per aphid per day; only about one-eighth of that of *A. pisum* from pea (*Pisum sativum*) (Auclair and Maltais, 1961). Phloem sap is under considerable pressure (Mittler, 1958), but pressurizing a 10% sucrose solution to 2 kg/cm<sup>2</sup> only extended the longevity of adult *A. fabae* by 20% (van Emden, 1967). One reason limiting the reduced uptake from diet is probably the high osmolality of the aphid haemolymph resulting from the constant high sugar concentration (see earlier under 'carbon nutrition'; also Cull and van Emden, 1977), whereas sugar concentrations in the phloem fluctuate with ambient radiation, with a strong reduction at night. There may also be an element of gustatory 'habituation' to an unchanging food source. T.E. Mittler and H.F. van Emden (unpublished results) found that neonate *A. fabae* attained a mean weight of 147 mg if fed on a cycle of 17 h full diet: 7 h water for three days, but only 127 mg if the full diet was replaced with further full diet for the 7 h period. Moreover, also with *A. fabae*, the same workers found a steady excretion rate of around 0.5 honeydew drops per aphid per hour rose to over 8 drops per hour (Fig. 6.6) for a few hours on full-strength diet after the aphids had been on half-strength diet for just 15 min (van Emden, 1996). Low uptake from diet therefore may well largely have a behavioural component.

### **Aphid performance on the diet**

Even after nearly 30 years on the diet at Reading, *M. persicae* still performed badly compared with the same original genotype when plant-reared (van Emden, 1988; van Emden and Andrews, 1997). Adult weight was lower, generation times extended and fecundity reduced (Fig. 6.7). The main contributor to extended generation time on diet was an adult pre-reproductive period of several days.

Even after 20 years, fecundity was only about a half that on plants. Nutritionally, or through phagostimulation, the diet was clearly sub-optimal. Aphids returned to plants as neonates hardly improved their performance in the first generation (van Emden and Andrews, 1997; Fig. 6.7), but the MRGR of their offspring then rose to match that of individuals maintained on plants throughout. van Emden *et al.* (2014b) used the diet recipe given by Douglas and van Emden (2007) to impose stress on the aphids to demonstrate large variability between *M. persicae* genotypes in their ability to survive on the diet. With six genotypes of *A. pisum*, Vogel and Moran (2005) found that only one required dietary arginine in the diet, and this could be related to a mutation in a *Buchnera* gene involved in amino acid biosynthesis.

Soon after the Reading culture had died out in 2008, we had occasion to measure the titre of alarm pheromone in *M. persicae* on diets with and without a small amount of sodium acetate, added as possibly providing an initial step in the biosynthesis of the pheromone. This proved unnecessary for pheromone production, but unexpectedly gave diet-reared aphids as large as those reared on plants (van Emden *et al.*, 2014a); hence the addition of sodium acetate to our diet recipe (Table 6.1).

## How useful is the diet for studies on aphid nutrition?

What would seem an obvious use of diet – to test cause and effect for apparent correlations between aphid performance and the concentration of nutrients, particularly amino acids, in plants – can in practice prove rather unsatisfactory. There seem to be three main reasons for this:

- It is not possible to increase the concentration of one nutrient without reducing another or changing the balance between compounds. The balances between ingredients seem be as critical to success as the concentration of individual compounds.
- The diet is not strictly a mimic of plant sap (as shown in Fig. 6.5). Beneficial interactions, between for example amino acids and metal availability, which may occur in the homogeneous diet, may not occur in the plant (Mittler, 1972).
- Dadd and Mittler developed their diet to maximize growth and reproduction of *M. persicae*. That any variation in the recipe will lower the performance of the aphid is therefore only to be expected and has indeed proved to be the case (van Emden, 1996), with one exception. For the phagostimulant and essential amino acid methionine alone, doubling the concentration did

significantly increase the growth rate of *M. persicae*, and halving it reduced it.

However, see earlier in connection with aposymbiotic aphids, the diet has contributed substantially to studies on nitrogen metabolism in aphids.

## Conclusions and Future Prospects

The single most perplexing issue in aphid nutrition is the high rate at which the unbalanced diet of phloem sap is transformed into new aphid biomass (see Introduction). As this chapter summarizes, key factors are the speed and efficiency with which aphids assimilate, or otherwise dispose of, ingested nutrients. For example, aphids on many plants ingest a surfeit of sucrose. The sucrose is hydrolysed to completion in the gut lumen and, within 15–20 min of ingestion, the hydrolysis products are assimilated across the gut wall to the haemolymph, or transformed to oligosaccharides that pose no obvious osmotic hazard to the aphid, and voided *via* honeydew. In a comparable fashion, ingested amino acids are rapidly assimilated or voided. This efficiency is compatible with the aphid's absolute nutritional requirement for essential amino acids only because the symbiotic bacteria *Buchnera* provide the aphid with supplementary essential amino acids. The bacteria can be described as 'upgrading' the quality of the diet by converting non-essential amino acids (i.e. amino acids that the aphid can synthesize *de novo*) to essential amino acids (which the aphid cannot synthesize *de novo* and require absolutely). The bacteria may additionally recycle ammonia, the chief nitrogenous waste product of aphid metabolism, but, contrary to some early reports, *Buchnera* has no capacity to promote aphid nitrogen nutrition by fixing atmospheric nitrogen (Shigenobu *et al.*, 2000).

Many issues relating to aphid nutrition and symbiosis remain to be resolved. In particular, we have no physiological information on aphid utilization of phloem sugars other than sucrose, even though many aphid species feed exclusively on plants with galactose-based oligosaccharides as the dominant phloem sugars. Similarly, the significance to aphid nutrition of phloem nitrogenous compounds other than free amino acids is obscure. Compounding these areas of ignorance is the dearth of information on the significance of accessory bacteria to aphids. There are indications in the recent literature that these bacteria may influence aphid host-plant range, thermal tolerance, and propensity to develop into alatiform (dispersal) morphs (Chen *et al.*, 2000; Wilkinson *et al.*, 2001a;

Montllor *et al.*, 2002; Oliver *et al.*, 2003), but the physiological processes underlying these effects are, at present, unknown.

Finally, the main achievements in our understanding of aphid nutrition have come primarily through experiments conducted with aphids on chemically defined diets. However, we have only the most cursory understanding of the nutrition of plant-reared aphids, largely because of the technical difficulties with quantifying or manipulating phloem-mobile nutrients. With the advent of methodologies for routine sampling and analysis of single plant cells (e.g. Tomos and Sharrock, 2001), we are now well placed to address the nutrition of aphids on plants. There is every expectation that aphids display a suite of behavioural and physiological adaptations that optimize nutrient extraction from phloem sap, a diet of far greater biochemical complexity and variability than chemically defined diets.

## Acknowledgements

AED acknowledges the financial support of NIFA grants NYW-2011-04650 and NYW-2014-05217, and the Sarkaria Institute for Insect Physiology and Toxicology.

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Table 6.1. Impact of antibiotics on the performance of apterous *Acyrthosiphon pisum*. Values of mean  $\pm$  s.e. are shown (26 replicates for untreated aphids, 20 replicates for aphids treated with the antibiotic chlortetracycline) (data reproduced from Douglas, 1992).

Aphid	Larval development time (days)	Teneral weight (mg)	Number of offspring
Untreated	$8.2 \pm 0.12$	$3.06 \pm 0.064$	$59 \pm 3.1$
Antibiotic-treated	$10.0 \pm 0.26$	$0.49 \pm 0.017$	0-5

Table 6.2. Composition of artificial diets as mg of dissolved in 100ml nanopure water. It is important that the chemicals are completely dissolved in the order shown, beginning with the left- Chapter\_6MS p.29

hand column (recipe for *Myzus persicae* is modified from Dadd and Mittler, 1966; that for *Acyrthosiphon pisum* is adapted from Auclair, 1965).

	For <i>Myzus persicae</i>	For <i>Acyrthosiphon pisum</i>
sucrose	15000.0 (15g)	35000.0 (35g)
di-potassium hydrogen orthophosphate	750.0	see salt mixture
magnesium sulphate	123.0	see salt mixture
magnesium chloride	0.0	200.0
L-tyrosine	40.0	20.0
L-asparagine hydrate	550.0	300.0
L-aspartic acid	140.0	100.0
L-tryptophan	80.0	100.0
L-alanine	100.0	100.0
gamma-amino butyric acid	0.0	20.0
L-arginine monohydrochloride	270.0	400.0
L-cysteine hydrochloride. hydrate	40.0	6.0
L-glutamic acid	140.0	200.0
L-glutamine	150.0	600.0
L-glycine	80.0	20.0
L-histidine	80.0	200.0
DL-homoserine	0.0	800.0
L-isoleucine (allo free)	80.0	200.0
L-leucine	80.0	200.0
L-lysine monohydrochloride	120.0	200.0
L-methionine	40.0	100.0
L-phenylalanine	40.0	100.0
L-proline	80.0	100.0
L-serine	80.0	100.0
L-threonine	140.0	200.0
L-tryptophan	0.0	100.0
L-tyrosine	0.0	20.0
L-valine	80.0	200.0
ascorbic acid (vitamin C)	100.0	10.0

thiamine HCl (vitamin B <sub>1</sub> )	2.5	2.5
riboflavin	0.5	6.0
nicotinic acid	10.0	10.0
<i>p</i> -aminobenzoic acid	0.0	10.0
folic acid	0.5	1.0
calcium pantothenate (vitamin B <sub>5</sub> )	6.0	6.0
inositol (meso) active	50.0	50.0
choline chloride	50.0	50.0
EDTA Fe(III)-Na chelate pure	1.5	0.0
EDTA Zn-Na <sub>2</sub> chelate pure	0.8	0.0
EDTA Mn-Na <sub>2</sub> chelate pure	0.8	0.0
EDTA Cu-Na <sub>2</sub> chelate pure	0.4	0.0
pyridoxine hydrochloride (vitamin B <sub>6</sub> )	2.5	2.5
D-biotin, crystalline	0.1	0.1
sodium acetate	320.0	0.0
salt mixture *	0.0	6.0

\*Salt mixture U.S.P. XIII (Nutritional Biochemical Corporation, Cleveland, Ohio) used by Auclair (1965) had the following per cent composition:

calcium lactate	32.70
di-potassium hydrogen orthophosphate	23.98
ferric citrate	2.97
magnesium sulphate	13.70
monosodium phosphate	8.72
sodium chloride	4.35
superphosphate	13.58

## Figure Legends



Fig. 6.1. The symbiotic bacteria associated with *Aphis fabae*. a, *Buchnera* are restricted to mycetocytes (m) in haemocoel of aphid; b, *Buchnera* are coccoid cells in cytoplasm of mycetocytes; c, rod-shaped accessory bacteria in aphid tissues; d, *Buchnera* cells are transmitted to the cytoplasm of unfertilized eggs in the ovaries of oviparae. (previously unpublished micrographs).

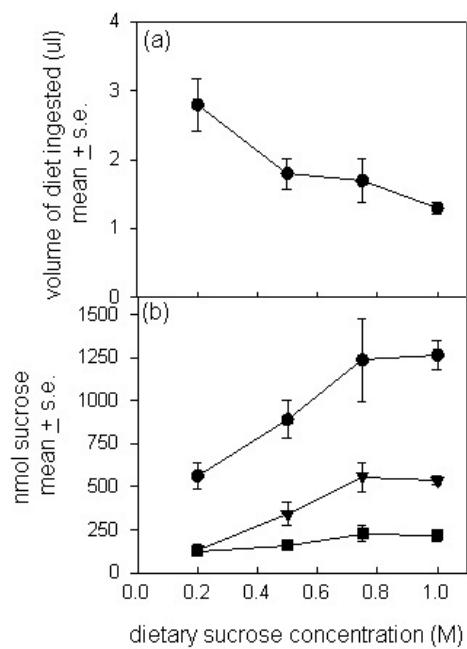


Fig. 6.2. Feeding response of *Acyrthosiphon pisum* to variation in concentration of dietary sucrose. Eight replicate final-instar apterous larvae were caged individually for 48 h to diets containing 0.2-

1.0 M sucrose. (a) Volume of diet ingested. (b) Amount of sucrose ingested (circle) and recovered from aphid tissues (square) and honeydew (triangle) (previously unpublished data of W.A. Smith and A.E. Douglas; methodology as in Ashford *et al.*, 2000)

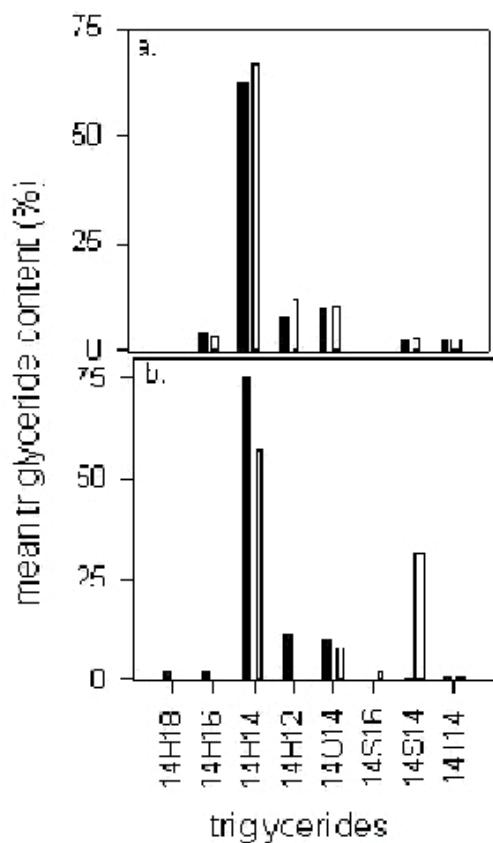


Fig. 6.3. Fatty acid composition of triglycerides in *Acyrthosiphon pisum*. (a) aphids treated with rifampicin to eliminate the symbiotic bacteria (open bars) and untreated aphids (closed bars) reared on chemically-defined diets. (b) Cornicle secretion (open bars) and fat body (closed bars) of plant-reared aphids. 14H18: 1-myristoyl-2-hexanoyl-3-stearoyl glycerol; 14H16: 1-myristoyl-2-hexanoyl-3-palmitoyl glycerol; 14H14 1,3-dimyristoyl-2-hexanoyl glycerol; 14H12: 1-myristoyl-2-hexanoyl-3-lauroyl glycerol; 14O14 1,3-dimyristoyl-2-octanoyl glycerol; 14S16: 1-myristoyl-2-sorboyl-3-palmitoyl glycerol; 14S14: 1,3-dimyristoyl-2-sorboyl glycerol; 14T14: 1,3-dimyristoyl-2-octatrienoyl glycerol (redrawn from Rahbe *et al.*, 1994).



Fig. 6. 4. The artificial diet technique. A, *Myzus persicae* feeding on the diet; B, equipment for dispensing the diet; C, stretching the Parafilm so as to keep one surface sterile; D, new diet tubes with free edges of Parafilm which are used to hold two tubes together, as in E.

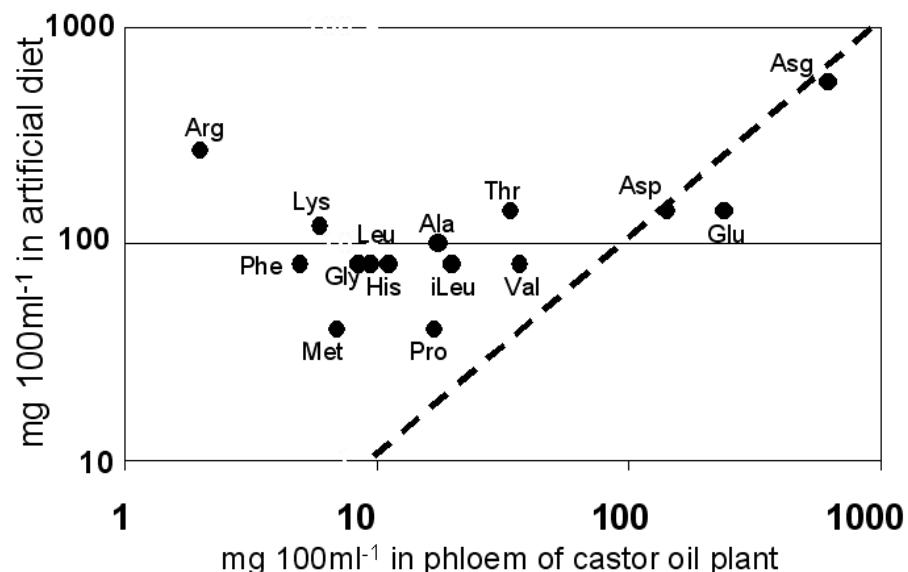


Fig. 6. 5 Relationship between the concentration of individual amino acids in artificial diet and phloem sap of the castor oil plant (*Ricinus communis*); the dotted line is the line of equality in both substrates. Ala, alanine; Asg, asparagine; Asp, aspartic acid; Glu, glutamine; Gly, glycine; Hi, histidine; iLeu, isoleucine, Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, praline; Thr, threonine, Val, Valine.

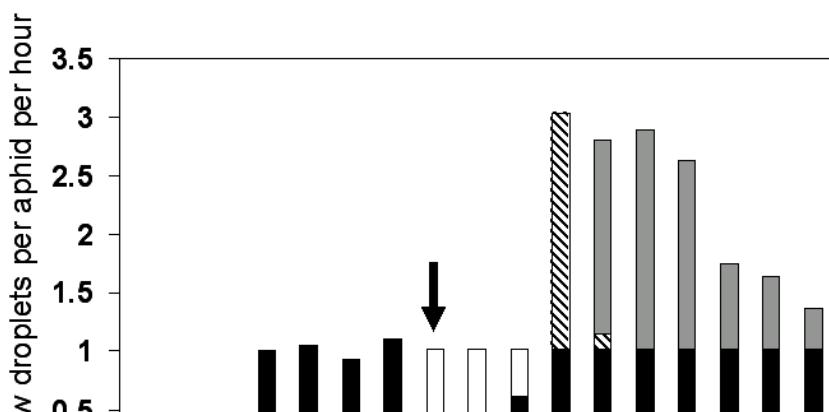


Fig. 6. 6. Mean number of honeydew droplets excreted per hour by *Aphis fabae* on artificial diet before and after a 15 min period (arrowed) on half-strength diet. Black, baseline droplet excretion rate; white, reduction in rate following 1 hour on half strength diet; striped, amount of extra excretion to bring average back to baseline rate; grey, droplet excretion above average baseline rate.

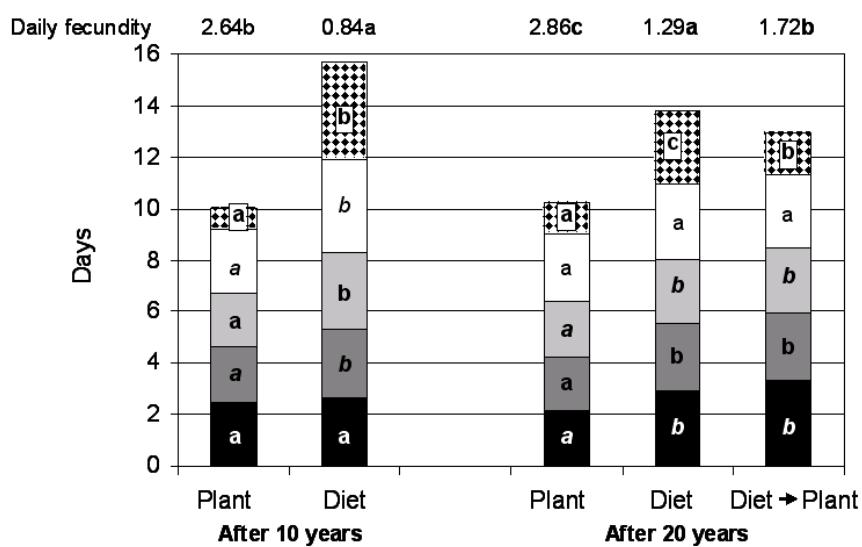


Fig. 6. 7. Development of *Myzus persicae* on plants and artificial diet after ten years on the diet (left) and (right) after twenty years, together with development of aphids born to mothers moved from diet to plants. The divisions of the columns (from bottom to top) are durations (days) of: 1st instar (black), 2nd instar (dark grey), 3rd instar (light grey), 4th instar (white) and the adult pre-reproductive period (chequered).