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1 **Up and away: ontogenetic transference as a pathway for aerial dispersal of**
2 **microplastics**

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28 **Abstract**

29 Microplastics (MPs) are ubiquitous pollutants found in marine, freshwater and terrestrial
30 ecosystems. With so many MPs in aquatic systems it is inevitable that they will be ingested
31 by aquatic organisms, and be transferred up through the food chain. However, to date, no
32 study has considered whether MPs can be transmitted by means of ontogenetic transference i.e.
33 between life stages that utilise different habitats. Here, we determine whether fluorescent
34 polystyrene beads could transfer between *Culex* mosquito life stages and, particularly, could
35 move into the flying adult stage. We show for the first time that MPs can be transferred
36 ontogenically from a feeding (larva) into a non-feeding (pupa) life stage and subsequently
37 into the adult terrestrial life stage. However, transference is dependent on particle size, with
38 smaller 2 μ m MPs transferring readily into pupae and adult stages, whilst 15 μ m MPs
39 transferred at a significantly reduced rate. Microplastics appear to accumulate in the
40 Malpighian tubule renal excretion system. The transfer of MPs to the adults represents a
41 potential aerial pathway to contamination of new environments. Thus, any organism that
42 feeds on terrestrial life phases of freshwater insects could be impacted by MPs found in
43 aquatic ecosystems.

44

45 **Keywords**

46 Food chain; ontology; life stage; Malpighian tubules, microplastics; *Culex pipiens*

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54 **Introduction**

55 Microplastics (MPs) are ubiquitous pollutants found in marine, freshwater and terrestrial
56 ecosystems [1–3]. There is little doubt that plastic and MP pollution is a major
57 environmental concern globally. Despite this, there is relatively little research into the impact
58 of MPs on freshwater ecosystems, with most research concentrating on marine systems and
59 organisms [2]. MPs have been defined as plastic particles smaller than 5mm in size [4,5].
60 However, this simple description covers a wide range of types, including, among others,
61 polypropylene, polyethylene and polystyrene MPs entering the environment in different
62 shapes and sizes, including fibres, pellets and cosmetic beads [6,7]. MPs are categorised
63 based on their origin as primary or secondary types, depending on whether they were
64 released into the environment as MPs (primary) or have degraded to that size in the
65 environment (secondary) [8,9]. Microplastics pass through terrestrial environments in
66 household wastewater [2,10]. Rivers can subsequently deliver MPs into the sea and lakes,
67 where they can be found in high concentrations [11–13].

68

69 Microplastics are ingested by aquatic organisms, and can be transferred through the food
70 chain in both freshwater and marine environments [14–18]. However, to date no study has
71 considered whether MPs can be transmitted by means of ontogenetic transference i.e. between
72 life stages that utilise different habitats. Freshwater environments are inhabited by insects that
73 spend their juvenile stages in water but their adult stages in the terrestrial environment. Such
74 insects include mayflies, dragonflies, midges and mosquitoes, most of which are eaten by
75 terrestrial vertebrates. This raises the potential for MPs to enter terrestrial ecosystems from
76 freshwater habitats aerially *via* transference to adult invertebrate life stages. Here, we thus
77 determine whether 2 and 15 μ m fluorescent polystyrene beads could transfer between insect

78 life stages and, particularly, could move into the flying adult stage. Fluorescent beads were
79 selected to enable MPs to be easily detected in the non-feeding stages and also to allow an
80 investigation of location within the body during metamorphosis. The *Culex pipiens* mosquito
81 complex was selected as a model for this study given their worldwide distribution and broad
82 habitat preference [19]. Mosquitoes develop through four feeding larval instars and a non-
83 feeding pupal stage, and finally emerge into a flying adult.

84

85 **Materials and methods**

86 For additional details of all methods and analyses, see the electronic supplementary material.
87 Two types of MPs were used: a 2 μ m fluorescent yellow-green carboxylate-modified
88 polystyrene (density 1.050g/cm³, excitation 470nm; emission 505nm, Sigma-Aldrich, UK)
89 and a 15.45 \pm 1.1 μ m fluorescent dragon green polystyrene (density 1.06 g/cm³ (5x10⁶
90 particles/ml, excitation 480nm; emission 520nm, Bangs Laboratories Inc., USA). Four
91 treatments were used; a control with no microplastics, a treatment of 8x10⁵ 2 μ m particles/ml,
92 a treatment of 8x10² 15 μ m particles/ml, and a 1:1 mixture of both treatments. Each replicate
93 (five per treatment) contained ten 3rd instar *C. pipiens* larvae in a 50ml glass beaker filled
94 with 50ml of tap water. The control and all treatments contained 100mg of pelleted guinea
95 pig food. Treatments were assigned randomly to a position on the laboratory bench to reduce
96 experimental error.

97 One random individual was removed from each beaker when every mosquito had moulted
98 into the 4th instar, and again when they pupated or emerged as adults. All samples were then
99 placed in separate 1.5ml Eppendorf tubes and stored at -20 °C prior to examination.
100 Microplastics were extracted from mosquitoes by homogenization and filtration. The filter
101 membrane was examined using an epi-fluorescent microscope (Zeiss Axioskop) under a 20x
102 lens to count the number of fluorescent MPs. Adults were further dissected under a binocular

103 stereo microscope (0.7X-4.5X) to extract the gut and quantify the numbers of MPs under the
104 epi-fluorescent microscope [20].

105 All data were analyzed using the statistical software R v3.4.2 [21]. Microplastic counts were
106 analysed using generalized linear models (GLMs) assuming a quasi-Poisson distribution.
107 Uptake of microplastics was examined with respect to 'particle size', 'treatment' and 'life
108 stage'. We performed model simplification via stepwise removal of non-significant effects.
109 Tukey tests were used post hoc for multiple comparisons.

110

111 **Results**

112 No MPs were found in control groups of any mosquito life stage. Densities of MPs were
113 significantly different between life stages ($F_{2, 56}=160.42, P<0.001$), with MP numbers
114 significantly falling as mosquitoes moved between successive ontogenetic levels (all $P<0.001$)
115 (Figure 1, Table S1, S2). Microplastic transference to adults was confirmed by fluorescent
116 microscopy where the beads were detected in the adult abdomen, specifically inside the
117 Malpighian tubules (Figure 2).

118 Significantly more 2 μ m particles were found in mosquito life stages than 15 μ m particles
119 overall ($F_{1, 58}=303.98, P<0.001$). Microplastics uptake was also significantly greater overall
120 in mixed exposure treatments ($F_{1, 55}=6.00, P=0.02$). Although 2 μ m particles were transferred
121 to adults in all instances, we found no transference of 15 μ m particles following single
122 treatment exposures. However, in the mixed MPs treatment, transference to adults of both
123 2 μ m and 15 μ m particles was evidenced (Figure 1).

124

125 **Discussion**

126 Here, we show for the first time that MPs can be transferred ontogenically from a feeding
127 (larval) into a non-feeding (pupal) life stage and subsequently into the flying (adult) life

128 stage. Transference through to adults was found in both MP sizes, although the larger 15 μ m
129 MPs were not ingested as readily as the 2 μ m MPs. Dissection of mosquito adults showed that
130 2 μ m MPs accumulated in the renal excretion system of Malpighian tubules which, unlike the
131 gut, pass from larvae to adult stages without visible reorganization [22]. This has been
132 demonstrated previously to provide a physical transport system between stages during
133 metamorphosis for *Pseudomonas* bacteria and seems to be important for ontogenetic
134 transmission from larvae to adults [23].

135 Few 15 μ m MPs were transferred into adults suggesting that MP size is an important factor in
136 ontogenetic transfer which could be related to the transfer and accumulation of MPs in the
137 Malpighian tubes. Although the translocation mechanism of MPs to the Malpighian tubules
138 is unclear in mosquitoes, analysis of fish, fiddler crab and marine mussels has demonstrated
139 that MPs can be translocated from gastrointestinal tracts into other tissues in a wide range of
140 phyla [24, 25, 26]. Malpighian tubules have an entry point to the gut between the mid- and
141 hindgut of mosquitoes, but the flow of fluid is from the Malpighian tubules to the hindgut
142 [27]. Diptera are known to produce structures called concretions in the Malpighian tubules
143 which have been shown to sequester heavy metals [28]. However, it is unlikely that this
144 pathway would operate with a solid MP.

145 Our results have important implications since any aquatic life stage that is able to consume
146 MPs and transfer them to their terrestrial life stage is a potential vector of MPs onto novel
147 aerial and terrestrial habitats. Ingestion of MP-contaminated organisms by terrestrial
148 organisms is not new [29]. Indeed, the widespread distribution of MPs in marine
149 environments has meant that animals such as fish and shellfish sold for human consumption
150 are contaminated with a range of plastics with a consequent transference of MPs between
151 trophic levels [24]. Unlike MP fibres, which are common in the air and atmosphere, there
152 has been no evidence for MPs being transported into the air [24]. We have demonstrated here

153 that species with aquatic and terrestrial life stages can harbour MPs through their life history.
154 Adults are predated on emergence by many animals including dipteran flies Empididae and
155 Dolichopodidae, whilst resting predominantly by spiders and in flight they are the prey of
156 dragonflies, damselflies, birds (such as swallows and swifts) and bats (31). Where many
157 insects are emerging from a highly contaminated site, the possibility of contamination of
158 these predators could be high. Whilst mosquitoes were used here as a model organism, any
159 freshwater insect that can ingest MPs will likely equally transmit plastics into a terrestrial
160 adult stage. This has implications for organisms that feed on adult mosquitoes with aerial and
161 terrestrial animals accordingly open to MP exposure and transference would appear to occur
162 at a higher rate for smaller MPs.

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252 Figure legends

253 **Figure 1.** Uptake counts of microplastics (MP) across larval (a, b), pupal (c, d) and adult (e, f) *Culex* mosquito stages following single (a, c, e) and mixed (b, d, f) exposures to 2 μ m and 15 μ m beads. Means are \pm SE ($n=5$ per experimental group).

256

257 **Figure 2.** Epi-fluorescent microscope images showing fluorescent microplastic particles
258 within (A) the abdomen of an adult mosquito before dissection, and (B) the abdominal
259 Malpighian tubules following dissection.

260

261 **Ethics**

262 Ethics committee approval was not required.

263 **Data accessibility**

264 Data files are available in online supplementary material.

265 **Author contribution**

266 All authors provided substantial contributions to conception and design, or acquisition of
267 data, or analysis and interpretation of data; were involved in drafting the article or revising it
268 critically for important intellectual content; approved the final version to be published; and
269 agree to be accountable for all aspects of the work in ensuring that questions related to the
270 accuracy or integrity of any part of the work are appropriately investigated and resolved.

271

272 **Competing interests**

273 We declare we have no competing interests.

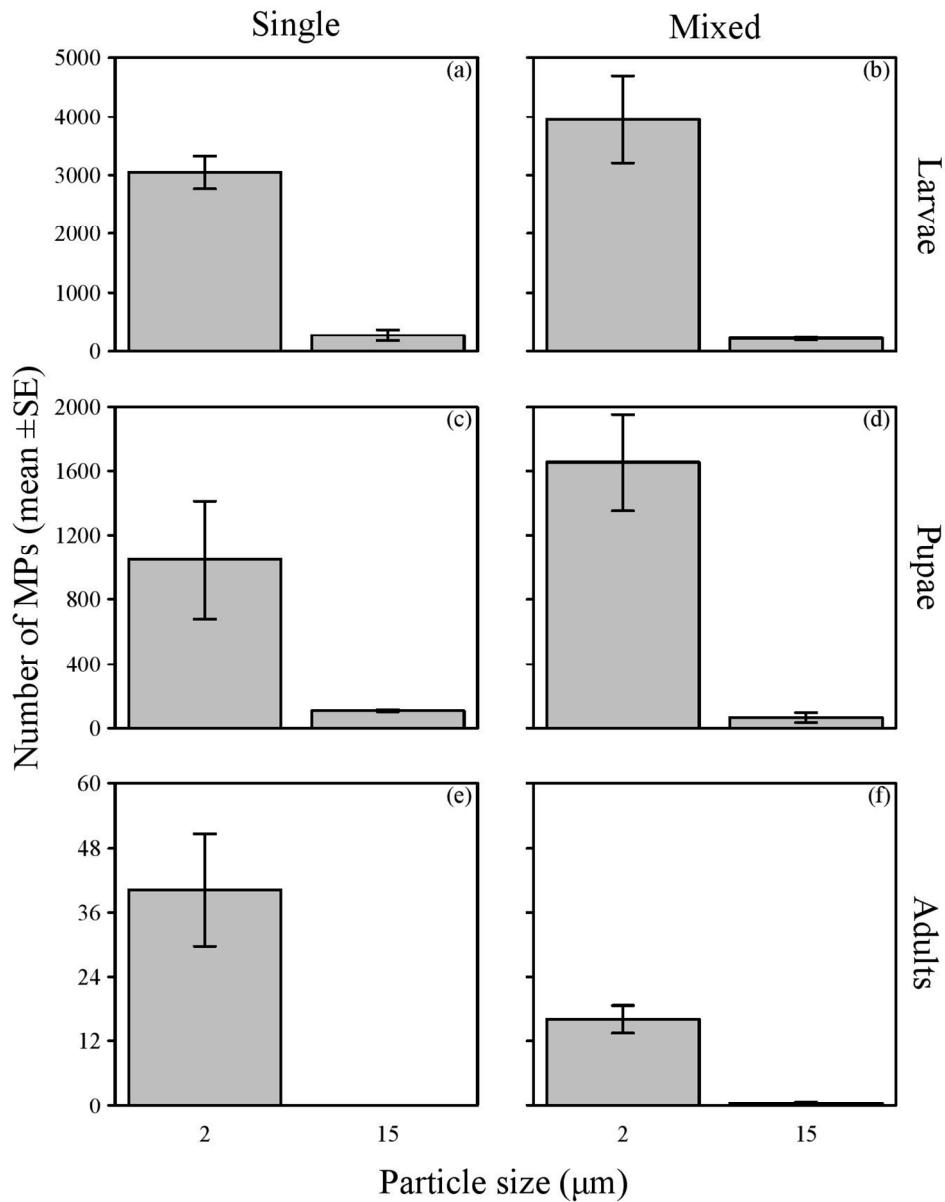
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279



Uptake counts of microplastics (MP) across larval (a, b), pupal (c, d) and adult (e, f) Culex mosquito stages following single (a, c, e) and mixed (b, d, f) exposures to 2 μ m and 15 μ m beads. Means are \pm SE (n=5 per experimental group).

115x144mm (300 x 300 DPI)

