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Examining effects of ontogenic microplastic transference on *Culex* mosquito mortality and adult weight

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Abstract

Microplastics (MPs) continue to proliferate and pollute aquatic and terrestrial environments globally. The impacts of MP pollution on ecosystems and their functioning remain poorly quantified, with most research hitherto focusing on marine ecosystems. There is a paucity of information on the impacts of MPs in freshwater ecosystems, despite the broad range of pathways through which MPs can proliferate and the extensive range of species which actively ingest MPs in these systems. Of particular interest are organisms that bridge aquatic and terrestrial habitats. The present study thus examines the uptake, ontogenic transference and effect of different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 µm) of polystyrene MPs between aquatic and terrestrial life stages of *Culex pipiens* complex mosquitoes. Both 2 and 15 µm MPs transferred from the aquatic larval to terrestrial adult stage of *Culex* mosquitoes, and uptake correlated tightly with initial exposure concentration. However, neither concentration nor size of MPs significantly influenced mortality rates between the aquatic larval and terrestrial adult stage. There was also no impact of MPs on the weight of emerging mosquito adults. We thus demonstrate that MPs can be transferred ontogenically through organisms with complex life histories, presenting a potential pathway for dispersal of MPs into terrestrial environments. We also show that MPs exposure does not affect mortality rates between life stages of freshwater *Culex* populations. This suggests that MPs do not impact nutritional uptakes, with unhampered development to adulthood facilitating subsequent dispersal of MPs aerially and between freshwater and terrestrial habitats.

Keywords: microplastics, mosquitoes, ontogenic transference, habitat transference

1. Introduction

Microplastics (MPs) continue to proliferate in marine, freshwater and terrestrial ecosystems, with biotic impacts frequently unknown (Sighicelli et al., 2018). Microplastic pollution has been detected from the poles to the deep ocean, and more recently in bottled drinking water (Mason et al., 2018; Wagner and Lambert, 2018). Whilst there is little doubt over the enormity of plastic and MP pollution in scale, the vast majority of research has concentrated on marine environments whilst neglecting other ecosystems (Redondo-Hasselerharm et al., 2018; Wagner and Lambert, 2018). To date there is a paucity of information on the impacts of MPs in freshwater ecosystems, despite the broad range of pathways through which MPs can proliferate (Mason et al., 2016), and the extensive range of species which actively ingest MPs in these systems (Canniff and Hoang, 2018; Imhof et al., 2016, 2013; Nel et al., 2018; Qu et al., 2018).

Microplastics have been defined as plastic particles of 5mm or less in size (Imhof et al., 2013; Eriksen et al., 2014). This is a broad definition as MPs manifest in a variety of forms, such as fibres, pellets and cosmetic beads, which all routinely enter the environment (Watts et al., 2014). Microplastics differ in their chemical composition, and can consist of various polymers such as polypropylene, polyethylene and polystyrene (Andrady and Neal, 2009; Rocha-Santos and Duarte, 2014). Furthermore, MPs can either be primary or secondary in origin, with the former released directly into the environment as MPs, and the latter having degraded over time to reach the MP size class (Moor, 2008; Barnes et al., 2009). Despite such inherent variation, there has been little work to compare differential impacts of the varied types of MPs on recipient organisms.

Movement of MPs through terrestrial and aquatic environments has been investigated, and several pathways have been suggested. For example, movement through the air due to wind (Dris et al., 2016) or directly through water courses from wastewater treatment plants into

rivers and eventually the marine environment (Mason et al., 2016; Wagner and Lambert, 2018). Rivers can also deliver MPs into lakes, where they can be found in high concentrations and presumably fall into the sediment (Vaughan et al., 2017). In North America the highest MP concentrations were found in Lake Ontario-Erie, with an average range of 90000 – 6700000 items km^{-2} (Fischer et al., 2016). In Europe, Lake Geneva contained the highest MP concentration with a mean of 220000 (± SD: ± 160000 items km^{-2} (Eriksen et al., 2013), and in Asia, Lake Taihu contained a range of 10000 – 6800000 items km^{-2} (Su et al., 2016).

Given the densities of MPs in freshwater ecosystems, it is likely that they will be ingested by aquatic organisms, and, in turn, probable that they will be transferred up through the food chain (Cole et al., 2013; Sussarellu et al., 2016; Scherer et al., 2017; Redondo-Hasselerharm et al. 2018). Laboratory experiments have demonstrated the uptake of MPs, and it is well established that they are ingested by many invertebrates in both freshwater and marine environments (Imhof et al., 2013; Al-Jaibachi and Callaghan, 2018). However, considerations of whether MPs can be transmitted by means of ontogenic transference, i.e. between life stages within an individual, have remained scarce. Insects comprise an important component of freshwater environments and are often highly abundant (Macadam and Stockan, 2015). Many insects have complex life histories, consisting of successive aquatic and terrestrial stages. Examples of such insects are stoneflies, damselflies, midges and mosquitoes, most of which are eaten by birds in their terrestrial stage. We have recently shown that MPs can be transferred into mosquito adults following ingestion as larvae (Al-Jaibachi et al., 2018). Thus, ontogenic transference of MPs presents a further pathway for MPs to enter new ecosystems from aquatic environments, with the potential to enter organisms that do not feed on the aquatic stages of freshwater or marine organisms.

The present study was undertaken to determine whether MPs which transfer between insect life stages of species with complex life histories could affect survival and adult size, which is

linked to fecundity (Takken et al., 2013). Mosquitoes (Diptera: Culicidae) are ideal for this study since they go through four feeding larval instars, a non-feeding pupal stage and finally emerge into a flying adult that feeds on nectar and/or vertebrate blood depending on the sex and species. Here, we investigate the ingestion of 2 ± 0.2 and 15 ± 1.1 µm fluorescent polystyrene beads, and whether consumption is concentration-dependent. Fluorescent beads were selected to enable MPs to be easily detected in the non-feeding stages and also to allow an investigation of location within the body during metamorphosis. *Culex pipiens* complex mosquitoes were selected for this study because they exhibit a global distribution and colonise a broad range of aquatic habitats, such as stream pools, lake edges, marshes and shallow permanent ponds, alongside both natural (phytotelmata) and artificial containers (Townroe and Callaghan, 2015). The group is also known to be an important food source for birds and other terrestrial organisms in the adult stage (Dow et al., 1994). We hypothesise that: (1) MPs will move ontogenically from larval to pupal stages, and subsequently into adult mosquito stages, and that transference is both MP density- and size-dependent; (2) uptake of MPs will reduce the survivability of larval mosquitoes to the adult stage; (3) exposure to MPs will affect the nutrition and thus development of larval mosquitoes, resulting in smaller-sized adults upon emergence.

2. Materials and methods

2.1. Preparation of microplastics (MPs)

Two types of MPs were used: a 2 ± 0.2 µm fluorescent yellow-green carboxylate-modified polystyrene (density 1.050 g cm^{-3}, excitation 470 nm, emission 505 nm; Sigma-Aldrich, UK) and a 15.45 ± 1.1 µm fluorescent dragon green polystyrene (density 1.06 g cm^{-3}, excitation 480 nm, emission 520 nm; Bangs Laboratories Inc., USA).

The 2 µm MPs were stored as a stock suspension (2.5 mg mL^{-1}) in distilled water and mixed using a vortex (Whirlimixe Cyclon, UK) prior to dilutions. The 15 µm MPs were also stored as stock suspension (1 % solid) polystyrene microspheres. Particles were washed prior to use by adding 1 mL from the stock solution into a 1.5 mL Eppendorf tube and then centrifuging at 9000 rpm for 10 min. The supernatant was discarded and 1 mL of distilled water was added. The solution was then resuspended by using the vortex and centrifuged again at the same speed and duration. This process was repeated two more times.

2.2. Mosquito colonies

Larvae of the *C. pipiens* mosquito complex were obtained from colonies reared at the University of Reading, UK following the methodology of Cuthbert et al. (2018). This colony originated from individuals collected in Cyprus in 2005 by Dr A. Callaghan and have been reared in laboratory conditions since then. Adult *C. pipiens* were fed overnight twice a week with defibrinated horse blood (TCS Biosciences, UK) using artificial membrane feeder (Hemotek, UK). Cotton pads soaked in 10 % sucrose solution were provided for additional sustenance.

2.3. Experimental protocols

We exposed *C. pipiens* larvae to one of two MP sizes (2 and 15 µm) under one of four concentrations (0, 50, 100 and 200 MP mL^{-1}) in a crossed design in the same laboratory where the colonies are maintained (25 ± 2 °C, RH 70 ± 5 %, 16:8 light:dark) for 12 days. In each of five replicates per treatment group, ten third instar *C. pipiens* larvae were placed in a glass beaker (60 × 80 mm) filled with 120 mL of tap water and 100 mg of pelleted guinea pig food for sustenance. Treatments were assigned randomly to a position on the laboratory bench to

reduce experimental error. Microplastic concentrations were quantified at the start and the end of the experiment by taking 5 × 1 mL from different points of each beaker (Table S1).

2.4. Uptake and ontogenic transference

One individual was randomly removed from each beaker once all mosquitoes in the beaker had moulted into the 4th instar, and again when they pupated or emerged as adults. All samples were then washed twice with distilled water to remove MPs from the surface of the mosquito and placed in separate 1.5 mL Eppendorf tubes, before being stored at -20 ºC prior to examination.

Microplastics were extracted from mosquitoes by homogenization and filtration. Mosquitoes were homogenized using a glass pestle in Eppendorf tubes containing 500 µL distilled water. Individuals treated with 2 µm MPs were filtered through a nucleopore track-etched membrane (Whatman, UK) of < 1 µm and 25 mm dia.. Those exposed to 15 µm MPs were filtered through a nucleopore track-etched membrane (Whatman, UK) of < 10 µm and 25 mm dia. using a glass vacuum filter holder connected to a manual air pump. The MPs captured by both filters were quantified under a 20 × epi-fluorescent microscope (Zeiss Axioskop, USA). Adults were further dissected under a binocular stereo microscope (0.7 × – 4.5 ×) to extract the gut and quantify the numbers of MPs under the epi-fluorescent microscope (Coleman et al., 2007).

2.5. Mortality rates

Mortality of successive stages was monitored and recorded daily over the course of the 12 day experimental period. We thus deduced overall proportional survival to the adult mosquito stage in *C. pipiens*.

2.6. Emerging adult weights

Emerging adult mosquitoes from each treatment were weighed using a microbalance (Thermo Cahn, USA), then examined under the epi-fluorescent microscope to ensure that no MPs were attached to the body.

2.7. Statistical methods

All data were analyzed using the statistical software R v3.4.2 (R Development Core Team, 2017). Quantities of MPs in larval, pupal and adult *Culex* mosquito stages were analysed separately using generalised linear models (GLMs) assuming a quasi-Poisson error distribution as counts were found to be overdispersed compared to degrees of freedom. Microplastics were absent from all control groups, and so we excluded this treatment from statistical analyses here. The effects of MPs on proportioned survival of *Culex* from the larval to the adult stage were then analysed separately using GLMs assuming a quasi-binomial error distribution. Then, the effects of MP treatments on adult weights were analysed using ANOVA following log10 transformation to meet normality and homoscedasticity assumptions (Shapiro-Wilk test, $p > 0.05$; Bartlett's test, $p > 0.05$). In all models, we initially incorporated 'concentration' and 'MP size' of MPs as explanatory variables factorially. We then performed backward stepwise deletion of insignificant terms and interactions to facilitate the most parsimonious model fits (Crawley, 2007). We performed *post hoc* Tukey's comparisons where terms significantly affected a response variable at the 95 % confidence level (Lenth, 2016).

3. Results

No MPs were found in control group replicates. Microplastics of both sizes were found in larval, pupal and adult life stages of mosquitoes, however abundances were strongly related to initial exposure concentration and MP size at each ontogenic stage. Abundance of MPs in larval mosquitoes was significantly influenced by initial exposure concentrations ($F_{(2, 27)} = 84.55$, $p < 0.001$), with quantities of MPs significantly higher across all increasing concentration

185 increments (all $p < 0.001$; Figure 1a), and abundances up to a maximum of 255.8 (± SD: ± 8.7)
186 MP larva^{-1}. Significantly greater quantities of 2 µm MPs were found in larval *C. pipiens* than
187 15 µm MPs ($F_{(1, 26)} = 28.53$, $p < 0.001$). Furthermore, there was a significant interaction effect
188 between 'concentration' and 'MP size' ($F_{(2, 24)} = 6.44$, $p = 0.006$), reflecting emergent effects
189 between the variables (Figure 1a). Whilst there were no significant differences in MP quantities
190 in *Culex* larvae at 50 MPs mL^{-1} or 100 MPs mL^{-1} between the two MP size classes (both $p >$
191 0.05), uptake of the smaller 2 µm MPs was significantly higher at concentrations of 200 MPs
192 mL^{-1} than 15 µm MPs at the same concentration ($p < 0.001$).

193 For pupae, exposure concentration also had a significant effect on MP abundance in *C. pipiens*
194 ($F_{(2, 27)} = 4.56$, $p = 0.02$), with abundances up to a maximum of 54.8 (± SD: ± 54.2) MP pupa^{-}
195 ^{1}. Whilst there were similarities in uptake between 50 MPs mL^{-1} *vs* 100 MPs mL^{-1} ($p = 0.31$),
196 and 100 MPs mL^{-1} *vs* 200 MPs mL^{-1} ($p = 0.28$), MP abundances in pupae were significantly
197 greater at 200 MPs mL^{-1} compared to 50 MPs mL^{-1} ($p = 0.01$). Moreover, significantly lower
198 quantities of 15 µm MPs were found in pupae as compared to 2 µm MPs ($F_{(1, 26)} = 133.25$, $p <$
199 0.001; Figure 1b), and this effect was consistent across exposure concentrations as there was
200 no significant 'concentration × MPs size' interaction effect ($F_{(2, 24)} = 0.87$, $p = 0.43$).

201 Microplastics were detected in the adult stage of *C. pipiens* mosquitoes, and MP abundance
202 was significantly greater under increasing initial MP exposure concentrations overall ($F_{(2, 27)} =$
203 14.07, $p < 0.001$) and for 2 µm MP compared to 15 µm MP ($F_{(1, 26)} = 4.71$, $p = 0.04$). However,
204 we found no incidence of MP transfer at 50 MPs mL^{-1} to adults across treatments, whilst MPs
205 were transferred to adults at 2 µm MP concentrations exceeding 100 MPs mL^{-1} , and 15 µm
206 MP concentrations of 200 MPs mL^{-1} (Figure 1c). There were similarities across the
207 'concentration' and 'MPs size' interaction here ($F_{(2, 24)} = 2.09$, $p = 0.15$).

208 Survival to the adult stage was not significantly affected by MP concentration ($F_{(3, 36)} = 0.78$, p
209 = 0.52; Figure 2) or by MP size ($F_{(1, 35)} = 0.31$, $p = 0.58$). There was no significant
210 'concentration × MP size' interaction effect on survival to the adult stage ($F_{(3, 32)} = 2.60$, $p =$
211 0.07).

212 Exposure concentration of MPs did not have a significant effect on the weight of adult *C.*
213 *pipiens* mosquitoes ($F_{(3, 36)} = 1.46$, $p = 0.24$), and weight was not significantly influenced by
214 MP size used during exposure ($F_{(1, 35)} = 0.76$, $p = 0.39$; Figure 3). Similarities were observed
215 for the interaction between 'concentration' and 'MP size' on the weight of adult *C. pipiens*
216 following exposure to MPs ($F_{(3, 32)} = 0.48$, $p = 0.71$).

217 **4. Discussion**

218 Microplastic pollution has increased concurrently in the aquatic environment with human
219 population growth (Rocha-Santos and Duarte, 2014), alongside the high production and
220 consumption of plastic materials (Andrady and Neal, 2009). Investigations have shown that the
221 freshwater environment, including rivers, lakes and streams, contain substantial levels of
222 microplastic pollution, with biotic impacts largely unquantified and pathways for MP dispersal
223 poorly understood (Horton et al., 2017; Wagner and Lambert, 2018). The present study
224 demonstrates the potential for MPs to move ontogenically from feeding larval *C. pipiens*
225 mosquito complex stages, into non-feeding pupal stages, and subsequently into flying adult
226 stages. We also show density- and size-dependence of MP uptake, with greater numbers of
227 particles taken up where MP concentrations were higher, and MP sizes were smaller. Here,
228 MPs did not significantly influence the survival of larval mosquitoes to the adult stage, and the
229 size of adults was not significantly influenced by prior MP exposure.

230 It was no surprise to discover that mosquitoes readily ingested MPs. Larvae of *Aedes aegypti*
231 (Linnaeus), *Anopheles albimanus* Wiedemann, *Anopheles quadrimaculatus* Say and *Culex*

quinquefasciatus Say have all been shown previously to ingest polystyrene latex beads (Dadd, 1971; Aly, 1988). We show that larvae did not ingest as many of the larger 15 μm MPs compared to 2 μm MPs which confirms previous work that suggests that filter feeding mosquitoes ingest particles based on their own size (Merritt et al., 1992), This does not infer selection, but probably reflects physical limitations, for example first instar larvae are unable to ingest latex beads as small as 45 μm in diameter (Dadd, 1971). Mosquito larvae feed using lateral palatal brushes to generate a current that causes water containing food or MPs to approach the mouth (Merritt et al., 1992) and it is possible that either the current or the mouth are not capable of easily dealing with larger fragments. However, MP ingestion at earlier life stages is not relevant for MPs to be passed ontogenically into the adult stage; it is only necessary for the fourth instar to ingest the plastic.

Both 2 and 15 μm MPs were transferred from a feeding (larval) into a non-feeding (pupal) life stage and subsequently into the flying (adult) life stage during metamorphosis. Generally, where MPs were presented at higher concentrations, greater numbers of MPs were taken up by larval mosquitoes, and this differential abundance was sustained throughout their ontogenic development. Although MPs of both sizes were shown to transfer through to the adult stage, this transference only occurred under higher experimental concentrations (100 and 200 MPs mL^{-1}). It is difficult to compare these concentrations to those in the environment since detection is often of a mixed size group of MPs; reporting varies in units used and very small MPs are often not measured (Wagner and Lambert, 2018, de Sá et al., 2018; Hurley et al., 2018).

As with Al-Jaibachi et al. (2018), we found that the MPs accumulated in the Malpighian tubules of adults, however the number of MPs was substantially less than the number of MPs in the two previous life stages. Given the exposure conditions of the present study, it is likely that depuration or excretion reduces MP concentrations over time between mosquito life stages, and particularly immediately post-emergence when adults evacuate their guts (Gillett, 1982).

We suggest that MP size is a very important factor to ontogenic transfer. Small MP sizes can transfer and accumulate faster than large MPs, and in the present study smaller 2 μm MPs were able to transfer to the adult stage at a lower concentration than larger 15 μm MPs.

The extent to which MPs can enter new environments through metamorphosing insects is closely related to plastic toxicity; if MPs have lethal effects on immature stages, then transference and dispersal by adult life stages will not be possible. There was no evidence that MPs had any significant impact on the survival rate of aquatic larval *C. pipiens* through to the terrestrial adult stage. The absence of any negative impact on the weight of emerging adults suggests that the larvae may not have suffered from a lack of nutrition during development. Similar results have been found with studies of MP ingestion in *Daphnia* (Al-Jaibachi and Callaghan, 2018; Canniff and Hoang, 2018). Mosquito larval nutrition determines the extent of metabolic reserves as well as the size of adult mosquitoes upon emergence which, in turn, has a strong impact on fitness (Takken et al., 2013). Although the exposure duration was limited given that third instar larvae were used as the starting point of the experiment, the presence of MPs did not seem to influence the overall nutritional uptake of larvae to the extent where it affected adult growth. Thus, given the sustained survivability and development in the presence of MPs, it is highly likely that mosquitoes which uptake MPs will subsequently disperse MPs aerially into terrestrial food webs from the aquatic environment.

In conclusion, ontogenic transference presents a pathway for MP pollution to disperse from aquatic to terrestrial environments, vectored by mobile organisms with complex life histories. The efficacy of MP transfer is, however, dependent on both MP concentration and MP size, with considerable proportions of MPs lost between larval, pupal and adult *Culex* mosquito stages. Adult weights and mortality were not impacted by MPs, and so the presence of MPs does not seem to influence the viability, development or nutritional uptake of mosquitoes. Future studies should examine the effects of MPs on ontogenic transference in other aquatic

insect larvae, along with a search for evidence of transference into predators. In addition, quantification of MP loss within exuviae would further elucidate the mechanism for MP reductions between ontogenic stages.

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Figure 1. The number of microplastics (MP) across different exposure concentrations (50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 μm) in (a) larval stage, (b) pupal stage and (c) adult stage *Culex pipiens*. Means are ± SE.

Figure 2. Mortality rates between larval and adult stage of *Culex pipiens* exposed to MPs under different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and of different sizes (2 and 15 μm). Means are ± SE.

Figure 3. Weights of emerging *Culex pipiens* adults following exposure to MPs of different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 μm). Means are ± SE.