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Microplastic ingestion rates are phenotype-dependent in juvenile anemonefish[☆]

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ABSTRACT

The potential influence of microplastic debris on marine organisms is an issue of great ecological and socioeconomic concern. Experiments exposing fishes and invertebrates to constant concentrations of microplastics often yield high variation in particle ingestion rates among individuals. Yet, despite an increasing interest in microplastic ingestion in the wild, the potential intrinsic drivers of inter-individual variation have received little attention so far. Here we assessed individual-level ingestion of Polyethylene microspheres by laboratory-reared juvenile anemonefish, *Amphiprion ocellaris*, in relation to (a) ambient particle concentrations and (b) repeatable behavioural traits. We show that microplastic ingestion is highly variable at all tested particle concentrations and that this variation can partially be explained by individual activity levels. Moreover, the relationship between ingestion and behavioural variation increased notably when only the most behaviourally consistent individuals ($n = 40$ out of 60) were considered in the analysis. Our findings indicate that microplastic ingestion rates in juvenile reef fishes may be less dependent on ambient concentrations than expected; instead they are to some degree phenotype-dependent. Care should thus be taken when reporting mean responses to microplastic exposure treatments, because some individuals may not be affected in the same way as others due to differential ingestion behaviour. We also discuss potential ramifications of non-random ingestion variability on population- and community-level responses.

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1. Introduction

Plastic pollution in the marine environment is an issue of rising concern worldwide. Plastic debris has now been detected in every marine ecosystem of the planet, ranging from coral reefs to the Arctic ice sheet and the deep sea (Auta et al., 2017). Global input of plastic waste into the ocean continues to increase (Jambeck et al., 2015) and the weight concentration of plastics in the pelagic environment is predicted to double by 2030 (Isobe et al., 2019). Microplastics are of particular concern because their small size (0.1 μm - 5 mm) makes them available for inadvertent consumption by a wide range of taxa (Wright et al., 2013), including mammals, reptiles, birds, invertebrates and fishes (Egbeocha et al., 2018; Markic et al., 2019).

The ingestion of plastics may cause harm through damage to the

gastrointestinal tract, intestinal blockage, creating a false feeling of satiation or leaching of toxic chemicals (Jovanović, 2017). However, the reported impacts of microplastic exposure on an organismal level vary widely (Foley et al., 2018). For instance, some studies found limited to no impacts on physiology, behaviour or survival of marine fishes (e.g., Mazurais et al., 2015; Tosetto et al., 2017; Jacob et al., 2019), while others detected significant negative effects on feeding rates, growth, body condition, survival, swimming speeds, as well as altered behaviours and differential gene expression (de Sá et al., 2015; Mazurais et al., 2015; Barboza et al., 2018a,b; Choi et al., 2018; Critchell and Hoogenboom, 2018; Naidoo and Glassom, 2019).

The early life history stages of fishes are known to be particularly vulnerable to pollutants due to their small size and less developed immune systems (Weis and Weis, 1989). At the same time, early life stages constitute a critical bottleneck for population replenishment because they naturally experience high rates of mortality (Hixon, 1991; Almany and Webster, 2006). This mortality is not random across a cohort in that faster growing, larger

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individuals have a selective advantage compared to slower growing, smaller conspecifics (Anderson, 1988; Meekan et al., 2006). Individual growth rates in turn are affected by multiple intrinsic (e.g., size at hatching) and extrinsic (e.g., temperature) factors, but are largely driven by food consumption (Boisclair and Sirois, 1993; Wang et al., 1998). It should thus be advantageous for an individual to maximise food intake relative to intraspecific competitors. Where natural planktonic food items are increasingly being replaced by microplastic particles, however, a high consumption capacity may work to an individual's disadvantage.

Food intake rates vary strongly among individuals within groups of fish (McCarthy et al., 1993), probably driven by a complex, reciprocal relationship with group dominance hierarchies, individual metabolic rates and activity levels (Boisclair and Sirois, 1993; Wang et al., 1998; Irwin et al., 2002; Schaefer et al., 2017). Accordingly, direct consumption of microplastics in laboratory exposure studies is often highly variable among individuals, even within the same particle concentration treatments (fishes: Avio et al., 2015; Critchell and Hoogenboom, 2018; McCormick et al. *unpublished results*; sea urchins and ascidians: Messinetti et al., 2018; zooplankton: Cole et al., 2013; Vroom et al., 2017). Rather than representing sampling noise, inter-individual variation in plastic ingestion rates could be related to an individual's past experience, physiology, phenotype, genetic predispositions, or a combination thereof.

Conceivably, an individual's behavioural phenotype may play an important role in its microplastic ingestion rates. Consistent between-individual variation in behaviour ('animal personality') has been receiving extensive interest over the past two decades, and evidence from a wide range of taxa supports the notion that it can be evolutionarily significant (Dall et al., 2004; Wolf et al., 2007). However, not all individuals are equally consistent and there may be significant inter-individual differences in intra-individual variation in behaviour (Stamps et al., 2012). In other words, some individuals are more predictable than others. Between- and within-individual behavioural variation may have far-reaching ecological consequences (Biro and Stamps, 2015) and both may play a role in microplastic ingestion.

Here we explore the relationship of behavioural phenotype with microplastic ingestion rates in a coral reef fish. Specifically, our aims were to assess the relationships of particle ingestion with (1) suspended microplastic concentrations and (2) inter- and intra-individual variation in activity-related behavioural traits in juvenile anemonefish, *Amphiprion ocellaris* (Cuvier 1830). Non-random variation in microplastic consumption could have important individual- and population-level consequences, because some individuals may consistently be more (or less) exposed than others, regardless of ambient concentrations.

2. Materials and methods

2.1. Larval rearing protocol

Anemonefish, *A. ocellaris*, were reared at the National Marine Science Centre, Australia, from breeding pairs collected from the northern Great Barrier Reef (Cairns Marine). Pairs were maintained in separate 90 l tanks with flow-through seawater at 28 °C in a 12/12 h light cycle. Breeding pairs laid egg clutches on the inside of terracotta pots placed in their aquariums for shelter. On the night of hatching (7–9 days post laying), egg clutches were transferred from the parental aquarium to a 60 l black, round larval rearing tank. After hatching, larvae were reared in a semi-closed green-water culture where aquariums had no water exchange during the day and were slowly flushed with filtered, UV-sterilized seawater each night (~1 l min⁻¹). This cycle ensured that larvae could feed *ad*

libitum throughout the day and that any unconsumed food was removed each night. Each morning, diluted algal paste (Nanno 3600) was added to the rearing tank to increase turbidity, thereby reducing larval stress, increasing contrast for larval foraging and adding indirect nutrition (Palmer et al., 2007). Larvae were fed rotifers (*Brachionus* sp.) at approximately 5 individuals ml⁻¹ each morning for the first 3 days, after which *Artemia* nauplii were added at 1 individual ml⁻¹. The ratio of *Artemia* to rotifers was increased each day until larvae were only fed *Artemia* (5 individuals ml⁻¹) from day 8. Larvae were reared to the juvenile stage. To standardize hunger-levels, fish were not fed during the last 48 h before the experiments.

2.2. Individual ingestion rates at different particle concentrations

In the morning of day 24 post-hatching, 50 juveniles of one clutch were randomly assigned to one of five treatments of Polyethylene microspheres (Cospheric UVPMS-BO-1.03, diameter: 180–212 µm, density: 1.03 g cm⁻³, colour: orange): control (0 mg l⁻¹; 0 spheres l⁻¹), low (0.04 mg l⁻¹; 10 spheres l⁻¹), medium (0.2 mg l⁻¹; 50 spheres l⁻¹), high (0.4 mg l⁻¹; 100 spheres l⁻¹), very high (2 mg l⁻¹; 500 spheres l⁻¹). The particles were chosen because they resemble the size range of juvenile anemonefish prey. The treatments represent the lower range of concentrations typically used in exposure experiments (Phuong et al., 2016). The control was included to account for potential cross-contamination or the introduction of particles from external sources.

Ten individuals per treatment were transferred to black 10 l buckets filled with UV-sterilized and 10 µm filtered seawater. After a 1 h acclimation period, the respective number of microspheres were added to each treatment. The spheres were kept in suspension by an air stone in each bucket. Previous experiments showed that juvenile damselfishes exhibit similar levels of ingestion variation in a range of exposure times (10 min–6 h) and start egesting spherical particles rapidly after ingestion (McCormick et al. *unpublished results*). We hence chose an exposure time of 1 h, after which all individuals were euthanized with an overdose of MS-222 (a fish anaesthetic) measured to the nearest tenth of a mm and dissected to quantify the number of ingested spheres.

2.3. Phenotype-dependence of microplastic ingestion

In the morning of day 26 post hatching, a total of 60 individuals were transferred from the rearing tank into individual, numbered 400 ml jars using a small beaker. The individuals originated from three clutches produced by three different parent pairs ($n = 35, 8, 17$, respectively). Holding jars were set in a water bath (~5 cm depth) to maintain temperature at 28 °C and each jar was fitted with an air stone and flow-through seawater (only running overnight). After 5 h of acclimation, each individual was tested in an open field trial to assess routine swimming activity. In this set-up, six Petri dishes (9 cm diameter, 2 cm depth) were placed in 2 × 3 rows on a white PVC plate that was illuminated from below with an LED light source (Fig. S1). For each of 10 trials, six fish were gently scooped from their holding jar and transferred to an individual Petri dish (total $n = 60$). After 3 min of acclimation, their movements were filmed for 2 min from above at 30 frames per second using a GoPro Hero5 camera. After the trial, all fish were placed back into their respective holding jars. The fish were not fed that day and water in the jars was slowly exchanged overnight by a dripping inflow (~0.1 l min⁻¹).

The following day, the open field trials were repeated exactly as the day before, resulting in an overall total of 120 trials (60 individuals × 2 repetitions). Again, all fish were then placed back into their respective jars. After approximately 3 h of re-acclimation,

Polyethylene microbeads (as above) were added to each jar at a concentration of 2.0 mg l^{-1} (approximately 500 beads l^{-1}). After 1 h exposure to the microspheres, fish were euthanized with an overdose of MS-222. Each individual was then measured to the nearest tenth of a mm and dissected to quantify the number of ingested microspheres.

Open field trial videos were analysed using the software Tox-Track (Rodriguez et al., 2018), which extracted a range of movement related variables from the track of each individual (i.e., average speed [mm s^{-1}], average acceleration [mm s^{-2}], mobility rate [% time moving], exploration rate [% of area covered], total distance moved [mm], time frozen [% time motionless]) (Fig. S1).

2.4. Statistical analysis

2.4.1. Particle concentrations

All analyses were conducted in the statistical software R (version 3.5.3). The continuous dependent variable “ingested microspheres” was $\log_{10} + 1$ transformed prior to analysis to improve normality of residuals (Shapiro-test: $W = 0.05$, $p = 0.079$) and homogeneity of variance among treatment groups (Levene’s test: $F_{3,36} = 0.243$, $p = 0.866$). We conducted one-way ANOVA to assess mean differences of particle ingestion among treatments. We then used Ordinary Least Squares (OLS) regression to assess the interactive effect of particle concentration and fish size on particle ingestion. We also ran OLS models by group (within treatments) of fish size vs. particle ingestion. All these analyses excluded the control.

2.4.2. Phenotype-dependence

We estimated adjusted repeatability (r) for all variables across both open field trials while controlling for clutch identity using the package rptR (Stoffel et al., 2017). We generated 95% confidence intervals (CIs) of parameter estimates using 1000 bootstraps. Repeatability was estimated for two data sets, the entire data ($n = 60$) and a subset of the most behaviourally consistent individuals. The latter consisted of the 40 individuals showing the least intra-individual difference in behavioural parameter estimates across both trials. The sample size of $n = 40$ resulted from an arbitrarily chosen threshold of $<10 \text{ mm s}^{-1}$ difference in average speed between the two activity trials (range: $0.4\text{--}116.7 \text{ mm s}^{-1}$).

Due to strong covariation among the repeatable behavioural variables, this data set also included the majority of the least variable individuals for the other variables. Mean particle ingestion did not differ significantly between the two data sets (t -test: $t_{81} = 0.438$, $p = 0.662$; Fig. S3).

We then generated ‘activity levels’ for each individual by extracting the first principal component (PC1) of a correlation-based PCA analyses. Activity levels were generated for both data sets (full data and subset) based on the mean scores of the significantly repeatable behavioural variables across both trials (see results).

Linear mixed effects models were used to assess the effects of phenotypic traits on individual microplastic ingestion. Models were obtained by calling the lmer function in the lme4 package (Bates et al., 2012). An initial model contained two fixed factors, ‘activity level’ (PC1) and ‘fish size’ (mm) as well as their interaction term plus random effects for clutch-id (parent pair) and video-id (six individuals were filmed simultaneously during each behavioural trial). We then used likelihood ratio tests, comparing pairs of alternative models that did and did not contain the effect of interest, to determine whether each effect in the model was significant. We generated marginal pseudo- R^2 values (mR^2 , proportion of variance explained by the fixed factors alone; Nakagawa et al., 2017) for models with significant effects using the function r.squaredGLMM from the package MuMIn (Barton and Barton, 2019).

3. Results

3.1. Particle concentrations

Mean particle ingestion by juvenile *A. ocellaris* increased with increasing particle concentration (Fig. 1a). However, variation in ingestion was high in each treatment (coefficient of variation: low = 0.824, medium = 1.169, high = 0.952, very high = 0.947), resulting in overlap in the number of ingested particles per individual even between the low ($10 \text{ spheres l}^{-1}$) and the very high ($500 \text{ spheres l}^{-1}$) concentrations (Fig. 1a). Significant group differences were only found between the low and the high ($p = 0.014$) and very high ($p = 0.003$) treatment, respectively. There was a significant interaction effect of treatment group and fish size

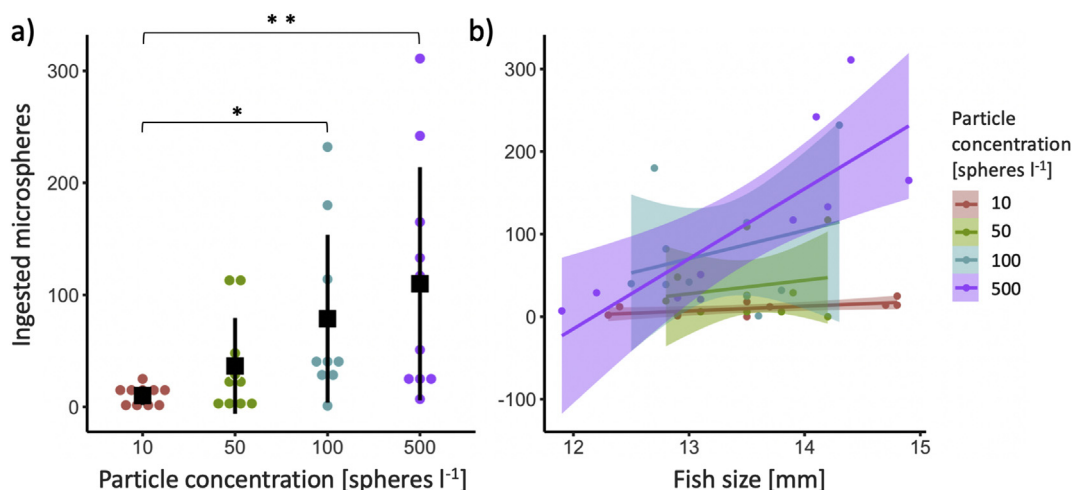


Fig. 1. Particle ingestion of juvenile anemonefish at different microplastic concentrations. (a) Dot-plot of ingested microspheres after 1 h exposure to 10, 50, 100 and 500 PE microspheres l^{-1} ($n = 10$ per group), black squares = mean \pm SD, stars indicate significant group differences after adjusting for multiple tests (** 0.05, **** 0.01); (b) within-group relationships of particle ingestion with fish size; the only significant relationship was within the 500 spheres l^{-1} treatment ($p < 0.001$); for clarity the control group is not shown (ingestion in the control group was zero in all individuals).

($F_{4,35} = 6.471, p < 0.001$); i.e., slopes of particle ingestion vs. fish size increased with increasing particle concentrations (Fig. 1b). However, the relationship was only significant within the very high (500 spheres l^{-1}) treatment (Table S1).

3.2. Phenotype-dependence of microplastic ingestion

Out of the six behavioural variables extracted during movement tracking, four variables were significantly repeatable across both behavioural trials in both the full data set and the most consistent subset (Table S2, Fig. S2). The first principal component of the PCA analysis based on these repeatable variables, the activity level, explained 95.9% and 90.3% of the total variation in the full data and the subset, respectively.

Particle ingestion was highly variable among individuals, ranging from 0 to 161 ingested microspheres (Fig. S3a.) Particle ingestion had a significant positive relationship with activity levels in both the full data set ($\chi^2_1 = 13.75, p < 0.001$) and the subset ($\chi^2_1 = 17.69, p < 0.001$). LMEs including only activity levels as fixed factor explained more than twice the amount of variation in particle ingestion in the most behaviourally consistent data subset ($mR^2 = 0.405$) than in the full dataset model ($mR^2 = 0.19$) (Fig. 2). There was no significant interaction between activity levels and fish size in models of ingested particles based on either the full data set ($\chi^2_1 = 0.199, p = 0.655$) or the subset ($\chi^2_1 = 0.795, p = 0.373$). Fish size alone had a significant relationship with ingested particles in the subset ($\chi^2_1 = 4.766, p = 0.029$), but not in the full data set ($\chi^2_1 = 2.018, p = 0.155$) (Fig. S4, Table S3). Significance in the former resulted from two data points representing particularly small individuals that had not ingested any particles; when these data points were removed, the relationship became non-significant ($\chi^2_1 = 1.355, p = 0.244$). Moreover, fish size was not a significant predictor of activity levels in either the full data set ($\chi^2_1 = 0.786, p = 0.375$) or the subset ($\chi^2_1 = 3.78, p = 0.052$) (Fig. S4).

4. Discussion

Microplastic pollution in the world's oceans presents an increasing environmental issue, particularly for the young, most vulnerable stages of marine organisms. While there is an increasing interest in microplastic ingestion, both in the field (e.g., Egbeocha et al., 2018; Markic et al., 2019) and the laboratory (e.g., Vroom et al., 2017; Procter et al., 2019), the potential intrinsic drivers of

variable ingestion rates have so far been largely overlooked. Our findings indicate that microplastic ingestion by the coral reef fish *A. ocellaris* is not random and that some behavioural phenotypes may be more (less) exposed than others due to inherently higher (lower) consumption levels. Specifically, we found that (1) microplastic ingestion in juvenile anemonefish is highly variable, even among closely related individuals; (2) that this variation may be driven to some degree by inter-individual variation in behavioural traits; and (3) that this relationship is stronger in individuals exhibiting lower levels of intra-individual variation.

4.1. Inter-individual variability in microplastic ingestion

Both ingestion experiments yielded highly variable consumption of microspheres among individuals (Figs. 1 and 2, Fig. S3) that could not be explained by fish size, parentage or rearing history. These findings are in line with other recent laboratory ingestion studies on juvenile coral reef fishes. Critchell and Hoogenboom (2018), for example, reported a range of zero to over 2000 ingested particles in the gastrointestinal tract of juvenile damselfish, *Acanthochromis polyacanthus*, after 1-week exposure to Polyethylene fragments (125–300 μm ; 0.05–0.13 mg l^{-1} ; $n = 23$). Similarly, in another species of juvenile damselfish, *Pomacentrus amboinensis*, individual ingestion ranged between 1 and 33 particles after 1-h exposure to Polystyrene spheres (200–300 μm ; 0.9 mg l^{-1} ; $n = 60$) (McCormick et al. unpublished results). In both cases, the quantity of ingested particles showed only limited relationships with fish size. Likewise, we found that size related to ingestion rates in some instances, but not in others (i.e., only within the highest concentration treatment [Table S1] and in the data subset but not the full data set [Fig. S4, Table S3]).

Critchell and Hoogenboom (2018) further reported that, in another experiment, neither the proportion of fish that had ingested plastics, nor the number of ingested particles per fish were related to the concentration of particles in different treatments (0–0.1 mg l^{-1} , Polyethylene spheres, 1–2 mm, 1.38 g cm^{-3}). While here we did detect a mean increase in ingested spheres with particle concentration, we also found high variation of ingestion at all concentrations (Fig. 1a). When large quantities of particles were available in the water, some individuals filled their entire gastrointestinal tract with plastics (Fig. S1) while others ingested none or only a small number of spheres. This finding entails that microplastics act differently than most other anthropogenic impacts (e.g.,

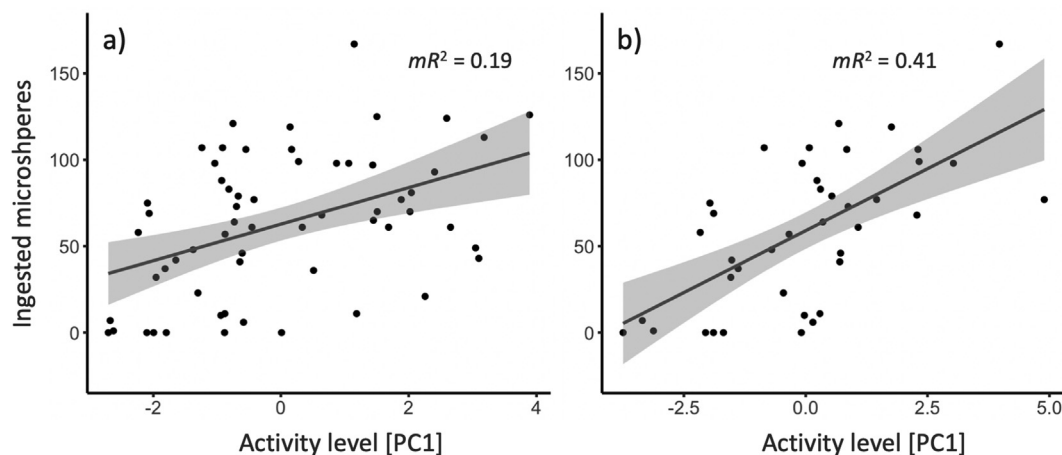


Fig. 2. Regressions of ingestion rates (number of ingested microspheres after 1 h exposure to 500 PE microspheres l^{-1}) with activity level (compound variable of movement related behaviours generated from PCA analysis) based on (a) the full data set ($n = 60$) and (b) a subset of the most behaviourally consistent individuals ($n = 40$) across two repeated behavioural tests. Coefficients of determination represent marginal pseudo- R^2 values estimated from linear mixed effects models (see methods section).

temperature, ocean acidification or chemical pollution), in that exposure to and hence the potential effects of microplastics in laboratory studies will likely not be uniform across individuals. Care should thus be taken when reporting mean responses to microplastic exposure, as some individuals may not be affected in the same way as others due to differential ingestion behaviour.

4.2. Is microplastic ingestion phenotype-dependent?

Consistent inter-individual differences in behaviour have long been recognized as a fundamental aspect of animal ecology (Dall et al., 2004). Among others, behavioural traits have been linked to the acquisition of food resources in that bolder, more aggressive and/or more active individuals typically have higher intake rates (Biro and Stamps, 2008). Our findings indicate that more active behavioural phenotypes within populations (and even within clutches) of juvenile anemonefish may ingest more microplastic particles than less active individuals when exposed to the same particle concentration (Fig. 2). More active phenotypes may hence be subject to higher microplastics exposure than less active individuals, even if they encounter similar ambient particle concentrations in the wild. Whether this observation is a result of inherently higher food intake rates of more active individuals that mistake microplastics for food (e.g., de Sá et al., 2015), or whether more active individuals simply have a higher encounter rate with suspended particles (e.g., Løkkeborg and Fernö, 1999) remains to be investigated.

Phenotype-dependent microplastic ingestion may have far-reaching consequences for marine fish populations and communities. Activity often forms a behavioural syndrome with boldness in that more active individuals are typically bolder and more prone to taking risks (Sih et al., 2012). This syndrome represents a well-known trade-off between higher growth rates (via increased food intake) and an increased probability of predation (via risky behaviour) (Biro et al., 2006). This trade-off is mediated by the bigger-is-better hypothesis, where larger individuals have a selective advantage in terms of predation rates (Meekan et al., 2006). However, if more active (possibly bolder) individuals are also more likely to consume larger quantities of microplastics, the relationship between consumption, growth and survival may be disrupted. Not only may higher plastic intake reduce individual growth rates (e.g., Critchell and Hoogenboom, 2018), but short-term exposure may also increase predation-related mortality in the field (McCormick et al. *unpublished results*). In highly plastic-contaminated areas, more active phenotypes may thus experience elevated vulnerability to predation due to a combination of their inherent behavioural predispositions (i.e., increased risk) and the effects of high microplastic ingestion (i.e., decreased body condition and escape performance). This hypothesis is particularly relevant for early life stages, because pelagic larvae may be more exposed than demersal life stages. Oceanographic features, such as surface slicks and eddies, were shown to aggregate both microplastics and fish larvae, resulting in higher particle exposure within such features than in adjacent waters (Markic et al., 2018; Lestrade et al. *unpublished results*).

In addition to the possible individual- and population-level implications of phenotype-dependent microplastic ingestion, the above-described theory may also affect the wider community via trophic transfer of microplastics. If individuals with higher microplastic ingestion rates are in fact more vulnerable to predation, higher trophic levels may accumulate increased loads of microplastics through secondary ingestion. However, this hypothesis is purely speculative at this point.

4.3. Intra-individual variation in behaviour

The observed relationship between individual activity levels and microplastic ingestion was enhanced notably when only a subset of the 40 (out of 60) most behaviourally consistent individuals were considered (Fig. 2). Behavioural consistency hence appears to be an important factor driving phenotype-dependent microplastic ingestion, providing another indication that varying levels of intra-individual variation are more than random sampling noise (Stamps et al., 2012). Behavioural variability in the less consistent third of individuals stemmed, to the most part, from a disproportionate increase in activity during the second open field trial (Fig. S2). This pattern was unrelated to specific experimental runs or clutch identity. We can thus assume that these individuals have an inherently higher level of intra-individual variation, possibly making them less predictable not only in terms of activity levels, but also regarding plastic ingestion rates.

To get a better understanding of the importance of the consistency of behavioural traits in predicting microplastic ingestion, future studies should increase the number of temporal replicates and include tests of behavioural consistency across different contexts (Carter et al., 2013).

5. Conclusions

Numerous microplastic exposure studies report high inter-individual variation in particle ingestion, yet so far, such observations have remained entirely unexplained. This study indicates that microplastic ingestion rates in juvenile anemonefish, *A. ocellaris*, are not random across individuals, but covary with specific behavioural phenotypes (i.e., more active individuals ingest more particles). We advocate further studies to substantiate the theory of phenotype-dependent microplastic ingestion across a range of taxa. Non-random variation in microplastic ingestion rates could have far-reaching consequences for marine populations and the intrinsic drivers of this variability warrant further investigation.

Data availability

All raw data is available at the Pangaea repository, <https://doi.org/10.1594/PANGAEA.910342>. Routine swimming trial videos are available from the corresponding author upon request.

Ethics statement

This research was conducted according to the guidelines of the Australian Research Council and was approved by the Southern Cross University Animal Care and Ethics Committee under approval number ARA 19/028.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Gerrit B. Nanninga: Conceptualization, Funding acquisition, Investigation, Formal analysis, Writing - original draft. **Anna Scott:** Resources, Supervision, Writing - review & editing. **Andrea Manica:** Formal analysis, Funding acquisition, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2019.113855>.

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