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**Microplastic exposure increases
predictability of predator avoidance strategies in hermit crabs**

Gerrit B. Nanninga^{1,2*}, Cat Horswill^{1,3,4}, Sarah M. Lane⁵, Andrea Manica²,
Mark Briffa⁵

¹Department of Zoology, University of Cambridge, Downing St, Cambridge CB2 3EJ, UK

²School of Life Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

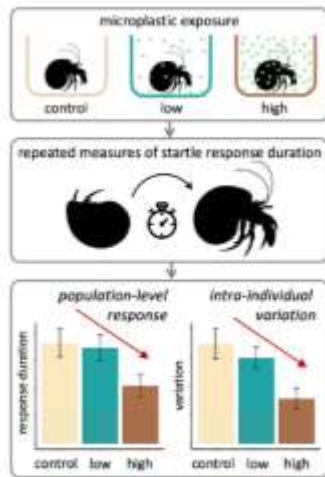
³ZSL Institute of Zoology, Regent's Park, London, NW1 4RY, UK

⁴Centre for Biodiversity and Environmental Research, Department of Genetics, Evolution
and Environment, University College London, Gower Street, London, WC1E 6BT, UK

⁵School of Biological and Marine Sciences, Animal Behaviour Research Group, University
of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

*Corresponding author: gn19687@essex.ac.uk

Graphical abstract



Abstract:

The contamination of natural systems with plastic debris has become one of the most pressing global environmental issues. Microplastics (MPs) are of particular concern because their ubiquity and small size make them available for ingestion by a range of aquatic biota. MP exposure studies are hence proliferating rapidly but are typically limited to the analyses of population-level responses in toxicity endpoints across treatments. Potential contaminant-induced alterations in behavioural patterns, however, could manifest on numerous levels of variation: at the population-level, between individuals and within individuals. Here, we used repeated measures on startle response durations – a risk-avoidance mechanism – in European hermit crabs, *Pagurus bernhardus*, to measure behavioural responses to MP exposure across multiple levels of variation. We found that MP exposure led to a significant decrease of startle duration at the population-level as well as a reduction of intra-individual variation. In other words, crabs became less risk averse on average and their behaviour became more predictable with increasing MP concentrations. Collectively, our findings indicate that MP pollution might increase susceptibility to predation in hermit crabs.

Key words: intra-individual variation, repeatability, anthropogenic contaminants, startle response, predation risk

1. Introduction

The contamination of the world's ecosystems with plastic debris is a key global environmental issue (Ostle et al., 2019). Microplastics (MPs), most recently defined as particles of 1 to < 1000 μm in size (Hartmann et al., 2019), are pervasive in virtually all aquatic ecosystems, from the deep sea to the Antarctic ice sheet (Auta et al., 2017; Rezania et al., 2018). The abundance of MP particles in seawater samples is believed to increase following a power-law function (with a factor of ~ 3) as size decreases (Cózar et al., 2014; Erni-Cassola et al., 2017) and new sampling techniques show that the abundance of small MPs (< 333 μm) might be orders of magnitude higher than previously believed, ranging from 100s to 10 000s of particles per litre of seawater (Brandon et al., 2020). Concentrations of small MPs in the world's oceans are likely to rise even further, because global plastic pollution is still increasing (Geyer et al., 2017), and larger particles already in the system are breaking down into smaller size classes (Thompson and Napper, 2018). Mathematical projections predict a doubling of MP concentrations in the world's oceans over the coming decade (Isobe et al., 2019; Lebreton et al., 2019).

Their small size and ubiquity make MPs available for ingestion by a range of marine taxa, and plastic particles have been found in the gastrointestinal tract of numerous species of mammals, reptiles, birds, invertebrates and fishes (reviewed in Egbeocha et al., 2018; Markic et al., 2019). Exposure studies show that ingestion of MPs may cause harm through damage to the gastrointestinal tract, intestinal blockage, feeding disruption through a false feeling of satiation, leaching of toxic chemicals (inherent and/or absorbed) and translocation into tissues

and organs (reviewed in Anbumani and Kakkar, 2018; Jovanović, 2017). At an individual- and/or population-level, this may entail disturbances in energy metabolism, oxidative stress, reduced growth rates and reproductive success, changes in organ physiology, differential gene expression and lowered survival (reviewed in Egbeocha et al., 2018; Franzellitti et al., 2019; Guzzetti et al., 2018; Wright et al., 2013). However, MP ecotoxicology is an emergent field of research (Bergmann et al., 2015) and scientific evidence for adverse effects is inconsistent (e.g. Foley et al., 2018) and controversial (e.g. Koelmans et al., 2017; Völker et al., 2019). To predict the potential effects of MP exposure at individual- and population-levels we need a mechanistic understanding of toxicity endpoints relevant to fitness, such as behavioural performance (Barboza et al., 2018; Granek et al., 2020).

Exposure to anthropogenic contaminants (e.g. heavy metals, pharmaceuticals, pesticides) has long been known to affect the expression of animal behavioural traits (Sih et al., 2011; Zala and Penn, 2004), and these effects can cascade through population- and community-level processes (Peterson et al., 2017; Wong and Candolin, 2015). The few studies that have so far addressed potential impacts of MP exposure on animal behaviour have yielded inconsistent results (Table S1). Moreover, there is a bias towards studies on fishes and planktonic crustaceans, as well as towards a focus on swimming capacity as the toxicity endpoint. One feature that most existing studies have in common is that they focus exclusively on possible population-level responses across treatments (e.g. different particle concentrations). Yet, behavioural change in response to anthropogenic or environmental factors can occur on a number of levels other than the main treatment- or sample-level (Montiglio and Royauté, 2014; White and Briffa, 2017).

Consistent inter-individual variation in behaviour (aka ‘animal personality’: Sih et al., 2004) has received extensive interest over recent decades and evidence is mounting that it has broad implications for individual fitness, ecosystem function and species evolution (Biro

and Stamps, 2008; Dingemanse and Réale, 2005; Wolf et al., 2007). Behavioural consistency, however, does not imply that individual behaviour is readily predictable. When individuals are observed repeatedly over short periods in the same context, they do not behave in exactly the same way each time (Bell et al., 2009). Behavioural variation observed within individuals is referred to as intra-individual variation (IIV) or predictability (Ram and Gerstorf, 2009; Stamps et al., 2012) and has long been regarded as sampling noise that is homogenous across individuals (Cleasby and Nakagawa, 2011). However, studies across taxa have shown that the degree of IIV can differ between individuals (Stamps et al., 2012), vary systematically across biotic (Briffa, 2013) and abiotic conditions (Briffa et al., 2013), and that it has a direct genetic basis (Henriksen et al., 2019) and potential fitness consequences (Okuyama, 2020; Westneat et al., 2015).

Overall, behavioural responses to anthropogenic (or environmental) factors can be measured on at least five different levels of variation: (1) the population-level behavioural response across treatments/factors; (2) inter-individual variation in behavioural response; (3) inter-individual variation in habituation to exposure (i.e. variation in behavioural reaction norms); (4) IIV (i.e. predictability) across observations within exposure treatments; and (5) treatment-level variation in IIV (Fig. 1). So far, only a small number of studies have simultaneously examined these components of behavioural variation in response to contaminant exposure (Royauté et al., 2015; White and Briffa, 2017) and the possible effects of MP pollution across these different levels of behavioural variation have yet to be assessed.

Hermit crabs represent a popular study system in behavioural research and, accordingly, their responses to anthropogenic disturbances have been studied in great detail (ocean acidification: De la Haye et al., 2011; temperature rise: Briffa et al., 2013; copper contamination: White and Briffa, 2017; anthropogenic noise: Tidau and Briffa, 2019). Their natural habitat renders hermit crabs vulnerable to high MP exposure. Many species inhabit

estuarine ecosystems, which, particularly in urban areas, are prone to high levels of MP contamination from terrestrial run off (Siegfried et al., 2017; Zhang, 2017). Moreover, hermit crabs dwell in the bottom layer of the water column, which often holds comparatively high concentrations of microplastics (Zobkov et al., 2019).

A prominent feature of most species of hermit crabs is their reliance on empty gastropod shells as a portable shelter (Lancaster, 1990). Recent research showed that MP exposure may affect important behavioural capacities in European hermit crabs, *Pagurus bernhardus*, by impairing the process of shell selection (Crump et al., 2020). Plastic-exposed individuals were less likely to contact and enter optimal shells and took longer to do so than control individuals. Accurate shell assessment and selection are highly adaptive, because lower quality shells may reduce growth, fecundity and survival (Lancaster, 1990).

Here we investigate the potential effects of increasing MP exposure on risk avoidance behaviour in *P. bernhardus*. Analogous to measurements of latency to emerge from shelter in other animals (e.g. Beckmann and Biro, 2013), the time taken to re-emerge from their shells (known as a ‘startle response’) is used as a measure of ‘boldness’ in hermit crabs (Bridger et al., 2015; Briffa et al., 2008; Mowles et al., 2012). High levels of IIV in startle response durations are assumed to be a strategy to cope with risk, in that less predictable individuals may incur a selective advantage in predator-prey interactions (Stamps et al., 2012; Briffa, 2013). The aim of this study was to use repeated measures of startle response duration in hermit crabs to examine if and to what degree MP exposure may affect any of the five described levels of behavioural variation. Alterations of startle response behaviour on any of these levels may compromise individual survival and population viability by affecting susceptibility to predation (Briffa et al., 2013).

2. Methods:

2.1. Microplastic particles

Fluorescent green polyethylene microspheres were purchased from Cospheric (Santa Barbara, US, UVPMS-BG-1.100). These particles have a narrow size distribution (10–29 μm), a uniform spherical shape and a density of 1.1 g cm^{-3} . According to the supplier, no solvents are used during the manufacturing processes and all ingredients are inert. Particles consist of pure polyethylene with an encapsulated fluorophore (excitation and emission wavelengths of 515 nm and 414 nm, respectively). These particles were chosen because of their (a) fluorescence, facilitating recognition in the water and inside the exposed crabs, (b) size range, facilitating comparisons to previous studies on MP uptake and toxicological effects in shore crabs, *Carcinus maenas* (Watts et al., 2016, 2014), and (c) near neutral buoyancy, facilitating even and long-lasting distribution of particles in the water column through turbulence created by an air stone.

2.2. Study organisms and behavioural measures

A total of 90 European hermit crabs, *P. bernhardus*, were collected from Hannafore Point, Looe (Cornwall, UK, Grid reference: SX 255523) in August 2019 and transported to the laboratory in Plymouth. Here, crabs were held collectively for three weeks in an 80 l tank of filtered seawater with constant aeration at $15 \text{ }^\circ\text{C}$ on a 12:12 lighting cycle. During this time, crabs were fed *ad libitum* with pieces of fish.

Due to space limitations, experiments were conducted in three consecutive batches of 30 crabs each. Crabs were first isolated in individual food-grade plastic dishes (12 cm diameter) filled with 0.5 l of aerated seawater. Ten individuals each were then randomly assigned to one of three treatment groups: (1) control (0 mg l^{-1}), (2) low (0.1 mg l^{-1} , $\sim 2 \times 10^4 \text{ parts l}^{-1}$), and (3) high (1 mg l^{-1} , $\sim 2 \times 10^5 \text{ parts l}^{-1}$) concentrations of microplastic spheres. These concentrations were in the range of and/or lower than the ones used in most published

exposure studies that typically range from 10^4 to 10^9 (reaching up to 10^{16}) particles per litre (Burns and Boxall, 2018; Phuong et al., 2016). Environmental MP concentrations in marine systems are not well understood, but it is clear that they vary both spatially and temporally (Imhof et al., 2017; Shim et al., 2018), and that they will be increasing substantially over coming decades (Isobe et al., 2019; Lebreton et al., 2019). Moreover, recent research shows that the abundance of small particles ($< 333 \mu\text{m}$) have been critically underestimated with traditional sampling techniques (Brandon et al., 2020; Lindeque et al., 2020). Recent estimates of current environmental concentrations of small MPs in inshore areas reached up to 4×10^4 particles per litre of seawater (Brandon et al., 2020), which is in the range of our ‘low’ treatment. The ‘high’ treatment was instead designed to go beyond environmentally realistic exposure levels of current MP concentrations but approaching exposure levels that many individuals/populations are likely to encounter in coming decades.

Microspheres were added to each dish separately from a stock solution (2.5 g l^{-1} , 5.4×10^8 particles l^{-1}) made with 0.1% Tween-20 surfactant to avoid aggregation of particles. Tween was also added to the control to account for any potential effects it might have on crab behaviour; the final concentration of Tween in the holding dishes was $5 \times 10^{-8} \%$.

During the first 24 h, isolated crabs were left undisturbed to allow for acclimation to the experimental conditions. During the following five days, a startle response was induced once per day by lifting the crab out of the water by hand and inverting it for 5 sec. This caused the crab to withdraw into its gastropod shell. The crab was then placed back into the dish in this inverted position and the time (in seconds) of the startle response was measured; i.e. from the point at which the crab was replaced to the point where it re-emerged from the shell and first contacted the substrate with its walking legs (Briffa et al., 2008). To avoid censored data in our response variable, we did not set an upper limit on startle response duration. The order of observations among crabs was randomized each day. Crabs were not fed during the

experiment. After the fifth behavioural measure, the crabs were carefully removed from their shells using a bench vice. Individuals were then sexed, weighed and examined for parasites and/or damage (e.g. missing limbs).

2.2 Statistical analyses

We used a double-hierarchical generalised linear model (DHGLM) to estimate the behavioural response of hermit crabs to differing levels of plastic contamination. This approach allows explicit modelling of both the population response and residual variance as a function of fixed and random effects (Lee and Nelder, 2006, 1996). The output of the model quantified behavioural response in five ways: (1) the population response to each treatment; (2) inter-individual variation in startle response duration for each MP treatment; (3) inter-individual variation in response to trials for each MP treatment; (4) IIV across trials for each MP treatment; and (5) inter-individual variability in IIV across trials for each MP treatment (refer to SI for full model description).

3. Results

3.1 Population-level variation across MP treatments

At the population-level, startle response duration was significantly shorter in the high MP treatment compared to the control (effect size: -3.07, 95% CRI: 0.56, 5.80) (Table 1, Fig. 2A). By contrast, startle response duration for the low MP treatment did not differ significantly from the control group (effect size: -0.36, 95% CRI: -3.08, 2.19) (Table 1, Fig. 2A). Finally, startle response duration increased by 0.28 seconds (95% CRI: 0.11, 0.54) with each trial (Table 1, Fig. S1).

3.2 Inter-individual variation

Inter-individual variation in startle response durations (Fig. 2B) and inter-individual variation in response to trials (i.e. behavioural reaction norms; Fig. 2C) were not significantly different across treatment conditions based on the 95% credible intervals of the parameters (Table 1).

3.3 Intra-individual variation (IIV)

IIV (i.e. the inverse of predictability) in startle response duration across trials was significantly lower in the high MP treatment compared to the low MP treatment and the control (Table 1, Fig. 2D). Intra-individual variability in IIV across trials was lower in the two MP treatments than in the control. However, this effect was not significant based on the 95% credible intervals of the coefficient of variation for predictability calculated for each treatment (CV_p , Eqn. 6) (Table 1, Fig. 2E).

4. Discussion

The startle response is the primary risk avoidance mechanism in hermit crabs (Vance, 1972). In response to external threat stimuli, individuals retract into their shell and variation in the speed at which they re-emerge can have important consequences for survival (Briffa, 2013). Our findings indicate that microplastic (MP) exposure may affect startle response duration at different levels of variation. At high exposure concentrations, we observed a significant reduction in startle response durations at the population-level (Table 1, Fig. 2), as well as significantly lower levels of IIV across trials (Table 1, Fig. 4A). In other words, exposed crabs emerged faster from shelter on average, and their behaviour became more predictable. Collectively, these findings indicate that MP pollution may increase susceptibility to predation in these crabs.

Re-emergence time following external threat stimuli reflects a trade-off between risk avoidance and the need to perform vital functions, such as foraging (Scarratt and Godin,

1992). Average startle response durations in hermit crabs, *P. bernhardus*, were shown to increase in the presence of predation risk as a mechanism to enhance protection (Briffa, 2013). An indiscriminate reduction in response duration, as observed here in response to MP exposure, is thus likely to increase vulnerability in situations of elevated risk.

IIV in startle behaviour is thought to be adaptive by reducing in individual's predictability by potential predators (Stamps et al., 2012). Conceivably, stochastic re-emergence behaviour is a better anti-predator strategy than predictable re-emergence behaviour. Briffa (2013) showed that IIV increases as a response to predation risk in *P. bernhardus*, demonstrating its relevance as a predator avoidance mechanism. Our findings of reduced IIV (i.e. increased predictability) thus further point towards a decrease in risk avoidance capacity upon MP exposure. Although the effect was marginal, we also found a decreased level of inter-individual variation in IIV (CVp) in the two MP treatments, compared to the control (Fig. 4B). In other words, individuals became more predictable on average and also more similar to one another in terms of IIV. For a predator that sequentially preys upon a range of individuals within a population, a reduction in the prey's startle response CVp would theoretically facilitate higher success rates.

While the underlying mechanisms of the observed MP impacts have not explicitly been investigated here, we can infer potential pathways from existing research. The observed behavioural impacts may in part stem from alterations in metabolic rates. MP particles were taken up by the exposed crabs via ingestion and across the gills (Fig. S2). Similarly, polystyrene spheres of similar dimensions to the ones used in this study were shown to be taken up by shore crabs, *Carcinus maenas*, through oral ingestion, as well as across the gills (Watts et al., 2014). Clogging of the gills by MP particles may in turn lead to higher metabolic demands. Indeed, MP exposure was shown to reduce oxygen consumption in *C. maenas* (Watts et al., 2016). Initial MP exposure could thus lead to positive feedback loops,

where higher ventilation rates due to MP exposure lead to even higher exposure (see Montiglio and Royauté, 2014). Moreover, MP exposure was found to reduce energy reserves in *C. maenas*, by increasing immune functions (Watts et al., 2016), possibly increasing the need to forage for resources. Reduced population-level and IIV in startle response durations may hence represent a response to increased metabolic rates and energy demands induced by MP exposure. Hermit crabs may shorten their startle response to perform elevated respiration rates and/or foraging, thereby exposing themselves to greater predation risk (Biro et al., 2010; White and Briffa, 2017).

Alternatively, or additionally, behavioural impacts may stem from leachates of toxic compounds from the MP particles into the holding water or inside the exposed individuals' gastrointestinal tracts. In line with our findings, chemical leachates from virgin polypropylene particles were shown to decrease vigilance in intertidal gastropods, *Littorina littorea*, thereby increasing their vulnerability to predation (Seuront, 2018). Similar polyethylene particles to the ones used in this study were shown to leach trace amounts of bisphenol, phthalates and UV stabilizers (Chae et al., 2019). When exposed to even low concentrations of phthalates Arctic spider crabs, *Hyas araneus*, exhibited a significantly decreased function of acetylcholine esterase (AChE) activity (Minier et al., 2008). Inhibition of AChE may result in a build-up of acetylcholine, resulting in a continuous and unrestricted stimulation of nerve and muscle fibres, which could explain the premature emergence of exposed crabs observed here. Phthalates and other chemicals may have either leached into the holding water or directly into crab digestive tissues upon ingestion or uptake via gills (Fig. S2).

We found no effect of MP exposure on inter-individual variation in startle response duration or inter-individual variation in behavioural reaction norms (Table 1, Fig. 3). In other words, individuals within MP treatments were equally variable in their startle response and

did not differ in the way their behaviour changed across trials (Fig. 1B & C). Together, these findings indicate that MP exposure had similar effects on all individuals within treatments. This result was somewhat unexpected because individuals typically differ in pre-exposure state and might thus cope differently with contaminant exposure (Montiglio and Royauté, 2014). Here, all of the tested specimens were kept in the same external conditions for weeks before the start of the experiment. This homogenised context may have reduced inter-individual variation in physiological state, thus leading to unrealistically uniform starting conditions.

We observed a significant increase in startle duration across the five repeated trials (Table 1, Fig. S1). A similar trend was identified by White and Briffa (2017) in *P. bernhardus* in response to copper exposure; yet, other studies on the same species found no evidence for either habituation or sensitization across trials (Briffa et al., 2008, 2013; Stamps et al., 2012). Nevertheless, the observed increase in our data was very small (median regression slope = 0.28 [sec]); given our relatively large sample size ($n = 90$ per trial), this pattern was statistically significant, but it is unclear whether such a small change would have important biological implications. Analogous to previous studies in this species, we found no effect of crab weight or sex on startle response durations (Table S2; Briffa et al., 2008, 2013; White and Briffa, 2017) and neither did these two characteristics interact with MP exposure.

To the best of our knowledge, this study presents the most detailed analysis of the effects of MP exposure across different levels of behavioural variation to date. We show that MP exposure significantly reduces startle response durations at the population-level and decreases IIV, i.e. increases predictability in predator avoidance behaviour. Overall, MP contamination may thus leave individuals more susceptible to predation risk, and these effects may cascade through population-level processes.

Ethical note: Crabs were not injured during removal them from their shells and were provided a new shell after measurements were taken. Due to the fact that some crabs had been exposed to plastics, the respective individuals were not immediately returned to the sea following the experiment. Rather, they were held in constantly filtered seawater in the laboratory for a period of three weeks to allow for excretion of accumulated microplastics. Water from the plastic treatments was collected after experiments and particles were filtered out and disposed of appropriately.

Conflict of interest

The authors declare no conflict of interests.

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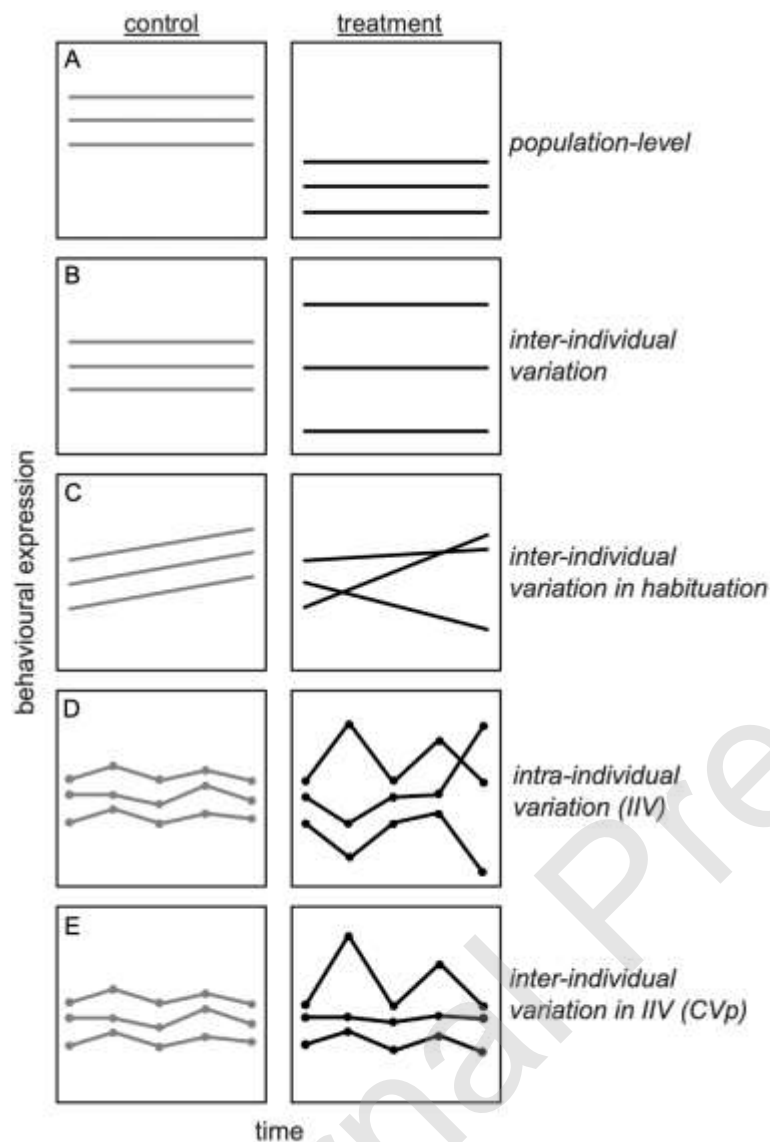


Figure 1: Schematic illustration of behavioural variation in response to a fictional experimental treatment. Each line represents the behaviour of an individual across repeated observations (A-C: regression lines, D-E: actual observations). Differences in response to exposure might be observed at the level of (A) the population response (here, lower expression in the treatment), (B) inter-individual variation (higher variation in treatment), (C) inter-individual variation in habituation across trials (higher in treatment), (D) intra-

individual behavioural variation (i.e. predictability) across trials (higher in treatment), and (E) inter-individual variation in predictability (higher in treatment).

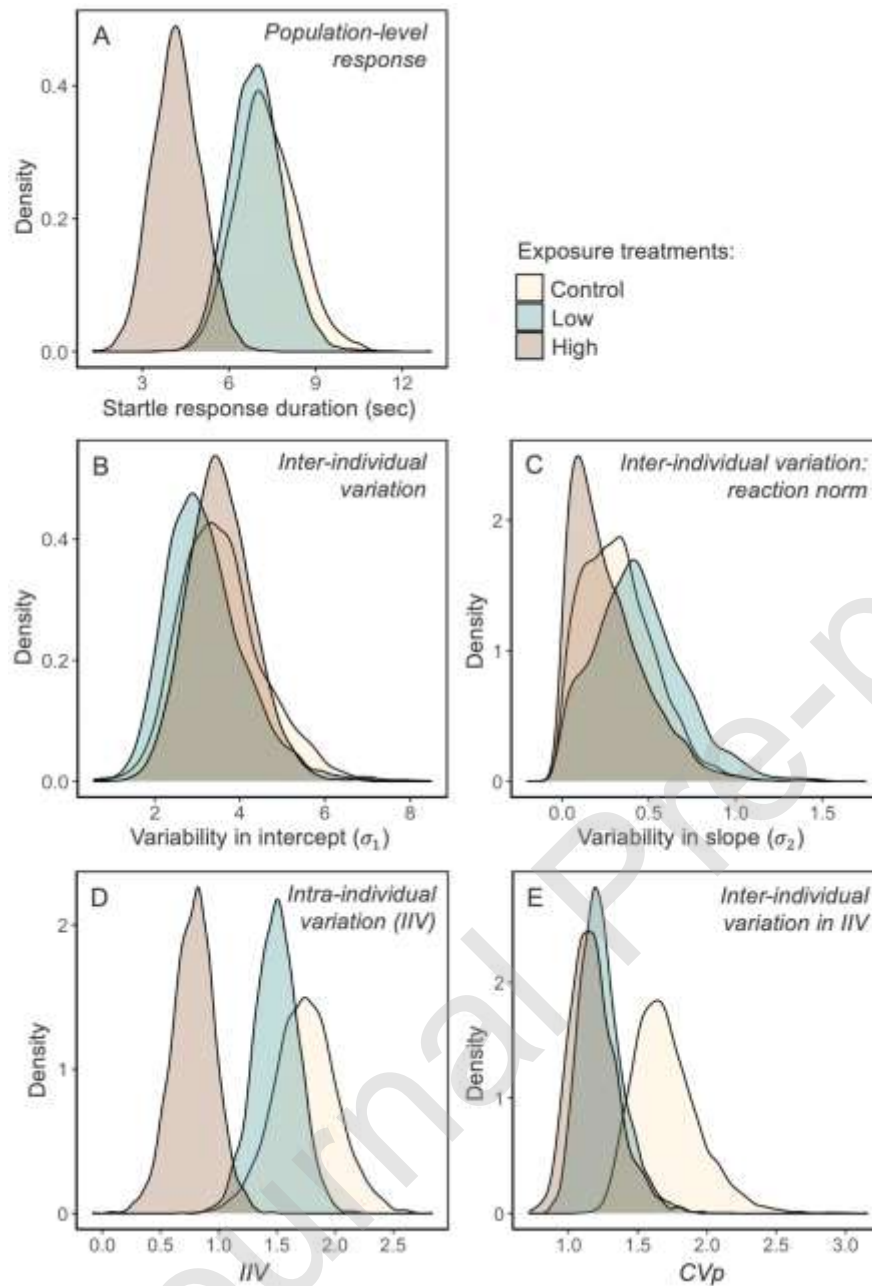


Figure 2: Posterior probability distributions of responses to microplastic exposure treatments at different levels of behavioural variation: (A) population-level response (τ_j , Eqn. 1 [SI]), (B) individual variation in behavioural response ($\sigma_{1,j}$, Eqn. 2) and (C) individual variation in habituation to exposure ($\sigma_{2,j}$, Eqn. 2), (D) intra-individual variation, i.e. predictability (IIV_j ,

Eqn 4) and (E) inter-individual variation in IIV (CVp_j , Eqn. 6). Startle response was significantly shorter in the high plastic treatment than the control and the low plastic treatment (A) based on the 95% credible intervals of the parameters. There was no significant difference in inter-individual variation in either startle response durations (B), or response to trials (variation in behavioural reaction norms) (C). IIV was significantly lower in the high plastic treatment compared to the control and low concentration treatment based on the 95% credible intervals of the parameters (i.e. predictability increased) (D); CVp was marginally higher in the control than in the treatment conditions (E).

Table 1: Credible intervals for parameters in the mean and dispersion parts of the double-hierarchical generalised linear model.

Model part	Parameter type	Parameter	Treatment	Notation	2.5%	Median	97.5%
Mean	Fixed	Intercept		α	5.41	7.24	9.55
		Treatment	Control	τ_1	0.00	0.00	0.00
			Low	τ_2	-3.08	-0.36	2.19
			High	τ_3	-5.80	-3.07	-0.56
	Trial		β	0.11	0.28	0.54	
	Random	Random intercept (between-individual variation in response)	Control	$\sigma_{1,1}$	1.94	3.48	5.81
			Low	$\sigma_{1,2}$	1.72	3.06	5.31
			High	$\sigma_{1,3}$	2.16	3.50	5.14
		Random slope (between-individual variation in response to trials)	Control	$\sigma_{2,1}$	0.02	0.31	0.80
			Low	$\sigma_{2,2}$	0.02	0.42	1.00
High			$\sigma_{2,3}$	0.01	0.21	0.75	
Dispersion	Fixed	Intercept		d_0	1.21	1.74	2.30
		Treatment (IIV)	Control	IIV_1	0.00	0.00	0.00
			Low	IIV_2	-0.90	-0.25	0.38
			High	IIV_3	-1.63	-0.95	-0.32

Random	Random intercept (variation in IIV)	Control	$\sigma_{4,1}$	1.03	1.34	1.81
		Low	$\sigma_{4,2}$	0.68	0.91	1.29
		High	$\sigma_{4,3}$	0.60	0.86	1.25
	CVp	Control	CVp_1	1.35	1.67	2.26
		Low	CVp_2	0.99	1.22	1.62
		High	CVp_3	0.90	1.17	1.58

IIV = intra-individual variation, CVp = coefficient of variation of IIV

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