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Negative effects of microplastic exposure on growth and development of *Crepidula onyx*☆

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ABSTRACT

Microplastics exposure could be detrimental to marine organisms especially under high concentrations. However, few studies have considered the multiphasic nature of marine invertebrates' life history and investigated the impact of experiencing microplastics during early development on post-metamorphic stages (legacy effect). Many planktonic larvae can feed selectively and it is unclear whether such selectivity could modulate the impact of algal food-sized microplastic. In this two-stage experiment, veligers of *Crepidula onyx* were first exposed to additions of algae-sized micro-polystyrene (micro-PS) beads at different concentrations, including ones that were comparable their algal diet. These additions were then either halted or continued after settlement. At environmentally relevant concentration (ten 2-μm microplastic beads ml^{-1}), larval and juvenile *C. onyx* was not affected. At higher concentrations, these micro-PS fed larvae consumed a similar amount of algae compared to those in control but grew relatively slower than those in the control suggesting that ingestion and/or removal of microplastic was/were energetically costly. These larvae also settled earlier at a smaller size compared to the control, which could negatively affect post-settlement success. Juvenile *C. onyx* receiving continuous micro-PS addition had slower growth rates. Individuals only exposed to micro-PS during their larval stage continued to have slower growth rates than those in the control even if micro-PS had been absent in their surroundings for 65 days highlighting a legacy effect of microplastic exposure.

1. Introduction

Microplastics, i.e. plastic pieces that are <5 mm in diameter, are of growing concern worldwide due to their high abundance (Browne et al., 2011; Eriksen et al., 2014) and their ability to absorb other contaminants (Napper et al., 2015). Apart from their physical properties and distributions, the effects of ingesting microplastics on marine fauna have received increasing attention (Van Cauwenberghe et al., 2015). However, the impacts of ingesting microplastics differ between species: some experience adverse effects (e.g., mussel: Browne et al., 2008; lugworm: Besseling et al., 2013; copepods: Cole et al., 2013) while others are little affected by the presence of microplastics (e.g., shore crab: Watts et al., 2014; sea urchin larvae: Kaposi et al., 2014). In addition to interspecific variations in responses to microplastics, ingesting microplastics could have different impacts on different life stages of the same species. Understanding such variations in response between species as well as within a single species are essential for determining the sensitivity of organisms to this particular emerging pollutant.

Experience during one life history stage often affects the performance and development of subsequent stages and is often referred to as legacy or carry-over effect (Phillips, 2002). For example, the adverse impacts on the shell development of larval Olympia oyster (*Ostrea lurida*) reared in low pH persisted for more than a month after larval settlement and placement into normal pH conditions (Hettinger et al., 2012). Such legacy effects were also reported amongst post-metamorphic stages bryozoans (Ng and Keough, 2003), gastropods (Pechenik et al., 2002), and bivalves (Wacker and von Elert, 2002) that were starved or exposed to a sublethal concentration of trace metals as larvae. However, the presence of legacy effect on micro-PS exposure is uncertain, especially for organisms that shift their body plans significantly during metamorphosis. Observations made at one stage might not be generalized to the next, and therefore, studying such legacy effects is crucial for understanding the long-term impacts of microplastics.

Polystyrene (PS) is one of the major plastics produced (Rochman

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et al., 2013) and micro-polystyrene (micro-PS) can either be manufactured directly or degraded from PS products (Lambert and Wagner, 2016). These micro-PS are capable of absorbing other toxicants, such as polyaromatic hydrocarbons (PAHs) (Liu et al., 2016). Absorbed toxins aside, exposure to micro-PS alone can lead to various adverse outcomes, including but not limited to: interruption of subcellular processes (Avio et al., 2015), organ failure (Lu et al., 2016), and reduction of fitness (Sussarellu et al., 2016). In our study site, Hong Kong (22°40′ N, 114°.11′ E), PS accounts for the majority of microplastic pieces found on beaches (Fok and Cheung, 2015) and could pose substantial threats to marine organisms.

The slipper limpet, *Crepidula onyx*, provides a tractable system for studying the impacts of microplastics across life history stages. Veliger of *C. onyx* can be reared to settlement easily in the laboratory (e.g. Chiu et al., 2007; and Chiu et al., 2008). These limpets are protandrous hermaphrodites that brood their young (Hoaglang, 1977) and the size of an individual is positively correlated with the brood chamber sizes, and the numbers of eggs (Strathmann and Strathmann, 1982). Hence, the sizes of reproductive individuals would be crucial for population growth (Newman and Pilson, 1997). Populations with smaller average sizes often have more males (Collin, 1995). Such a skew in sex ratio could imply intensified sperm competition and egg limitation (Parker, 1984). Native to California, *C. onyx* has successfully invaded coastal areas around the South China Sea including Hong Kong (Mukherjee et al., 2013; Huang et al., 1983). Not only does this study organism provides insights on *trans*-life stage effects of microplastics but also the response of invasive species to emerging pollutants.

Preferential feeding behaviors are well-documented in larvae (Okaji et al., 1997; Fernández-Díaz et al., 1994; Checkley, 1982). Larvae of the mud snail *Nassarius obsoletus* showed preference towards specific algal species when offered a mixture of algal cells (Paulson and Scheltema, 1968). Larvae of the oyster *Crassostrea virginica* were able to perform such food selection based on particle sizes and C:N ratios according to their environmental conditions: larvae would normally select small-sized algae over larger ones but they preferred the larger algae during dinoflagellate blooms (Baldwin, 1995). These selection responses based on food quality could be crucial for regulating nutrient and energy intake, and hence, growth and development of the larvae. However, mechanical (size and shape) and chemical cues were often confounded in these studies. The ability of larvae to differentiate algae from microplastics of comparable size could determine its vulnerability to micro-PS exposure.

In the present study, we investigated the effect of microplastic exposure in an environmentally relevant concentration and the role of particle differentiation by exposing larvae to microplastic at size- and concentration-comparable to the algal food present. We took a two-stage approach to first investigate the effects of micro-PS exposure on larval *C. onyx*. We then tracked the performance (growth, survival, feeding, and percentage of males) of juveniles which were continued to be exposed and those that were not to quantify the legacy effect.

2. Materials and methods

2.1. Obtaining larval Crepidula onyx

Crepidula onyx adults were collected from the Tsim Sha Tsui Public Pier, Victoria Harbor, Hong Kong. Newly hatched larvae from dissected egg capsules were used in the first trial and naturally released larvae were used in the subsequent trials. These egg capsules were placed into 500 mL beakers with aerated 0.22 μm filtered seawater (FSW) at a salinity of 30 and at 22 °C until hatching. The hatched/released larvae were divided into 800 mL-beakers with approximately 1000 individuals per beaker and fed with the haptophyte algae *Isochrysis galbana* at a concentration of 2×10^5 cells mL^{-1} (day 0). On the next day, the larvae were randomly allocated to nine 500 mL glass jars at a density of 1 individual in every 4 mL and were starved overnight in FSW at salinity 30 and 22 °C (±0.5 °C).

2.2. Preparation of microplastics solution

The micro-PS stock solution, concentration of 5.0% w/v suspended in deionized water containing sodium azide (0.02%) as a bacteriostatic, was procured from Spherotech Inc (PP-20-10, Illinois, United States). This mirco-PS stock had a nominal bead size of 2.0–2.4 μm and verified size distribution of 2–5 μm that match the size of *I. galbana* (2–3 μm diameter). The Z™ Series Coulter Counter® Cell and Particle Counter (Indiana, USA) was used to enumerate the concentration of beads after 1000 times dilution of the stock with FSW to around 2×10^8 particles mL^{-1}. This solution contained less than 2×10^{-5}% sodium azide and was further diluted 10^7-10^3 times to the desired concentration in each treatment.

2.3. Larval exposure to microplastics

We performed two experimental trials to address the physiological impacts of high and environmentally relevant concentrations of microplastics respectively. In the first trial, larvae were randomly assigned to one of three treatments: 1) the LPR (Low Plastic Ratio), 2) HPR (High Plastic Ratio), and 3) control after starvation (i.e., 2 days post hatching (day 2), with three replicates for each group. The control was only fed with *I. galbana*. Micro-PS were added at 30% and 70% of the algal concentration to the LPR and HPR with final concentration of 6×10^4 particles mL^{-1} and 1.4×10^5 particles mL^{-1} respectively. In the second trial, an additional treatment of the environmental concentration of 10 micro-PS mL^{-1} was added. This concentration was comparable to other studies e.g., Browne et al. (2008) used 2–3 μm microplastics at 42 particles mL^{-1} and Cole et al. (2013) used 20 μm at 75 particles mL^{-1}.

The larvae were incubated at 22 °C (±0.5 °C) and salinity 30 with complete water change every 48 h in a 12:12 light/dark cycle until at least 25% of survived individuals settled spontaneously. All groups were fed every two days with *I. galbana* at a concentration of 2×10^5 cells mL^{-1}, as enumerated with triplicate hemocytometer counts. According to Zhao et al. (2003), larval survivorship to competence is greater than 90% when fed with 1×10^5 *I. galbana* cells mL^{-1} d^{-1} and the survivorship increases with the addition of algae. Larval were raised under still condition as turbulence induced by air pump might reduce larval survivorship (Rehmann et al., 2003; Sawant et al., 2008). Without active mixing, micro-PS used remained mostly suspended between water changes with less than 6% and 13% sinking loss in the LPR and HPRs over 48-hrs (see detailed description in Suppl. Methods and Suppl. Fig. S3). Water was changed completely every two days and the culture jars were replaced with clean ones to avoid any potential particle adhesion.

In the first trial, larvae were enumerated on the first and 14 days post hatching (14 dph); and size measurement was made every other day by micrographing live individuals (5.4 ± 1.1 individuals per replicate, mean ± S.D.). Individuals were immediately returned to their respective culturing jar after micrography. In the second trial, number of larvae were counted every two days and micrographs of live larvae were also taken at this time (6.7 ± 1.4 individuals per replicate, mean ± S.D.). The shell length (Fig. 1A and B) of the larvae were measured with Fiji Image J (Schneider et al., 2012).