Marine Biology (2022) 169:27
https://doi.org/10.1007/s00227-021-04010-x

ORIGINAL PAPER

Effects of microplastics on the feeding rates of larvae of a coastal fish: direct consumption, trophic transfer, and effects on growth and survival

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Received: 27 June 2021 / Accepted: 14 December 2021 / Published online: 18 January 2022

Abstract
Microplastics are now found throughout the world's oceans, and although many organisms ingest microplastics, less is known about how plastics in seawater may affect key processes such as feeding rate, growth, and survival. We used a series of laboratory experiments to test whether microplastics in seawater affected the feeding rates of larvae of the California Grunion, *Leuresthes tenuis*. In addition, we tested whether trophic transfer of microplastics from zooplankton to larval fish can occur and affect growth and survival of fish. We measured feeding rates of grunion larvae at various concentrations of 75–90 µm and 125–250 µm polyethylene microplastics and under both still water and turbulent conditions. In these experiments, exposure to microplastics had modest effects on feeding rates, though responses may be somewhat complex. Low concentrations of microplastics increased feeding rates compared to the control, but at higher concentrations, feeding rates were indistinguishable from those in the control group, though effects were small compared to natural variation in feeding rates among individual fish. Experiments to test for trophic transfer of microplastics revealed that grunion larvae that were fed brine shrimp exposed to high concentrations of microplastics had lower growth rates and elevated mortality rates. Overall, our results suggest that the direct effects of microplastics on feeding rates of California Grunion during the early larval phase are minor, while the trophic transfer of microplastics from zooplankton to larval fish may have significant effects on their growth and survival.

Keywords Bioaccumulation · California Grunion · Fish larvae · Fragments · Ingestion · *Leuresthes tenuis* · Mortality · Plastic debris · Size

Introduction

Since the introduction of plastics to the global market in the 1940s, the amount of plastic waste in the ocean has increased exponentially (Thompson et al. 2009; Ostle et al. 2019; Wilcox et al. 2019), and recent estimates suggest that over 8 million tons of plastics enter the ocean annually (Jambeck et al. 2015; Law 2017; Borelle et al.2020). The same features that allow plastics to be durable and inexpensive make their disposal difficult, and once present in nature, plastics can become a persistent contaminant, especially in marine ecosystems (Rochman et al. 2013a). Despite efforts to reuse and recycle plastics, large quantities of plastic end up in waterways, and eventually wind up in the ocean. Through prolonged exposure to ultraviolet radiation and ocean waves, plastics break down into smaller fragments, and particles < 5 mm in diameter are generally referred to as microplastics (NOAA 2016). In addition, some microplastics are manufactured to be < 5 mm (e.g., microbeads from beauty products, textile fibers, plastic pellets, etc.) and some of these plastics end up in the marine environment because of improper disposal and accidental spillage (Fendall and Sewell 2009; Lestari and Trihadiningrum 2019). Although getting an accurate count of microplastics in the ocean is a challenge—especially for very small particles that are difficult to detect—microplastics are found throughout the world's oceans, and can be very abundant in coastal waters near large cities (e.g., Browne et al. 2011; Sutton et al. 2016; Wiggin and Holland 2019).

Responsible Editor: K.D. Clements.

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The resemblance of microplastic particles to zooplankton, and the co-occurrence of microplastic particles and zooplankton in the ocean (Gove et al. 2019; van Sebille et al. 2020) may cause organisms to mistakenly ingest microplastics as food. For example, Gove et al. (2019) reported that the median density of microplastics in surface slicks—regions that larvae are routinely concentrated in (Shanks 1983; van Sebille et al. 2020)—was 126-fold higher compared to ambient waters. These results suggest that larval fish may often feed in an environment where the concentration of microplastics is locally high. Indeed, microplastics have been found in the guts of many species (bivalves, adult fish) (e.g., Browne et al. 2008; Lusher et al. 2013; Lo and Chan 2018); fish larvae may be particularly susceptible to the presence of microplastics in their environment (Mazurais et al. 2015; Rodrigues et al. 2019; Jacob et al. 2020). During early development, most larval fishes experience a critical period for survival when they exhaust the energy supplied within the yolk sac and must begin feeding on their own (May 1974). Because larval fish need to grow quickly to avoid predation (Bailey and Houde 1989; Johnson et al. 2014), they must consume volumes of food that are large relative to their body sizes. Larvae that have evolved to become voracious and relatively indiscriminate feeders can be at an advantage under natural circumstances when fast growth is favored, but these same characteristics may make fish larvae predisposed to consuming microplastics. It is becoming increasingly clear that consumption of microplastics can have ill effects for the health of individual fish (de Sá et al. 2015; Mazurais et al. 2015; Athey et al. 2020); therefore, exposure to microplastics may pose a significant threat to fish populations as well (Cong et al. 2019; Limonta et al. 2019).

Microplastics in the sea may affect the feeding rates of larval fishes in several ways. First, microplastics may affect the ability of larval fish to detect and consume prey. With floating plastics in the surrounding water, fish may find it difficult to isolate zooplankton and capture them effectively. For instance, if zooplankton and microplastics appear close together, fish may not have a clear approach at a zooplankter. Furthermore, if a fish mistakenly captures a microplastic particle, it may decide to stop feeding for a while and forego opportunities for additional feeding and growth. Conversely, it is also possible that microplastics in seawater actually stimulate feeding. For example, if larval fish actively inspect microplastic particles that are dispersed in seawater, then the effective foraging volume of a larva may increase, thus leading to more encounters with zooplankton and greater feeding rates. Very little is known about whether microplastics affect prey detection and capture, and more research is needed to understand these mechanisms.

Second, direct ingestion of microplastics may interfere with further feeding and digestion. For example, ingested microplastics may cause false satiation and decrease feeding rates (Welden and Cowie 2016). They may also accumulate in the gastrointestinal tract and interfere with digestion of food and assimilation of organic matter (Jovanović 2017). If the ingested plastics are large enough, they may even block the digestive system entirely (Wright et al. 2013; Pedà et al. 2016), and be immediately fatal to the animals (Law 2017; Pannetier et al. 2020). Furthermore, once ingested, microplastics that have chemical additives or have adsorbed chemicals from the environment may leach these chemicals into the tissues and bloodstream of the organism (Teuten et al. 2009; Rochman et al. 2013b; Koelmans 2015; Kumar et al. 2021). In addition to physiological harm, the stress of chemicals in the body may alter feeding behavior and hunting performance (de Sá et al. 2015; Pannetier et al. 2020).

Finally, there may be trophic transfer of microplastics up the food chain, and larval fish may ingest microplastics indirectly by consuming zooplankton that have ingested plastic particles. Accumulation of microplastics by organisms that are near the base of the marine food web may be especially important, because many zooplankton feed by filtering particles from seawater and, therefore, may be less able to discriminate plastic particles from organic particles of similar size (phytoplankton, etc.). The concentration of plastic particles is magnified as plastics are transferred between trophic levels through ingestion. Hasegawa and Nakaoka (2021) reported that fish exposed to plankton pre-exposed to microplastics have ingested 3–11 times more microplastic beads than when fish have direct exposure to microplastics. Zooplankton that feed at lower trophic levels are very abundant relative to larval fish, and a single fish larva can consume many zooplankton. If zooplankton ingest microplastics, then the plastic ingested by hundreds to thousands of zooplankton may accumulate inside the gut of the larval fish. Larval fish that consume microplastics from this pathway may have some deleterious physiological effects. Athey et al. (2020) reported that short-term accumulation of microplastics had impeded growth and slowed the development of larvae of Inland silversides, *Menidia beryllina*. In other marine organisms (e.g., copepod, bivalves, adult fish), microplastic exposure has resulted in reduced growth rates (Besseling et al. 2014; Lo and Chan 2018), decreased reproductive output (Cole et al. 2015), and decreased survival (Mazurais et al. 2015).

Despite the growing interest in the study of microplastics, there is little information regarding the effects of microplastics on feeding rates of larval fishes. In this study, we tested whether microplastics in seawater affected the feeding rates of larvae of California grunion, *Leuresthes tenuis,* a fish commonly found along the coast of Southern California. Our study consisted of several experiments. First, we tested whether exposure to microplastics in seawater had direct effects on feeding activity and food consumption rates.

We also quantified the incidence of microplastic ingestion, and whether ingestion propensity varied with microplastic concentration. Finally, we tested whether trophic transfer of microplastics from zooplankton (brine shrimp nauplii) to larval fish occurred, and whether this indirect pathway had subsequent consequences for growth and survival of larval fish.

Methods

Study species

The California Grunion, *Leuresthes tenuis,* is a marine fish that is common in the coastal waters of Southern California, and ranges from San Francisco Bay, California to Punto Abreojos, Mexico (Clark 1938; Walker 1952; Martin et al. 2013). Although life during the larval phase of grunion is very similar to many other coastal fishes, grunion are known for their unique spawning mechanism of going completely out of water to lay eggs on sandy beaches (Martin and Swiderski 2001). Spawning events occur ~ 14d apart, and once the subsequent tide cycle is at its peak (spring tides that typically occur ~ 14 days later), rising tides and waves stimulate the eggs to hatch, and the larvae return to the sea. Grunion larvae and small juveniles (< 25 mmTL) may be found along the shoreline near spawning beaches (D. Johnson, *personal observation*), and it is likely that they compete much of their larval development in shallow waters (Allen and Horn 1975; Suntsov et al. 2012). The Southern California coast is a highly urbanized environment, and it is likely that most grunion larvae develop in waters with a relatively high concentration of microplastics (Clark et al. 2016; Wiggin and Holland 2019).

To produce grunion larvae for these experiments, adults were collected from Seal Beach, CA immediately prior to spawning, and strip-spawned to collect and fertilize eggs. Embryos from each female were buried in moist beach sand and placed inside 475 mL containers. Embryos were then incubated at room temperature (20–21 °C) in the lab for 2 weeks (Ehrlich and Farris 1972; Smyder and Martin 2002). Following the incubation period, embryos were hatched and larvae were kept in a recirculating seawater system. Larvae were housed in 7-L circular tanks (12 in total) that were fitted with acrylic tops and received an inflow of 10 mL/s (see Tasoff and Johnson 2019 for additional details). Drains were covered with a fine mesh that prevented the escape of larvae and all tanks drained to a larger sump where seawater was filtered with a 10-micron mesh and aerated before recirculating within the system. Grunion larvae were approximately 7 mm SL at hatching, and by the time they were 14 days post-hatch, they were 8.5 mm SL, on average.

Manipulation of microplastics

To create microplastic fragments to be used in our feeding experiments, we grinded low-density polyethylene (LDPE) and high-density polyethylene (HDPE) pellets into smaller pieces using a household blender for 5 min. These plastics were not previously exposed to the environment, and our study of larval feeding thus focused on the physical effects of microplastics, rather than the effects of adsorbed chemicals. The plastics were mixed with fresh water during the blending process and the mix of water and plastic fragments was run through a set of sieves and rinsed to obtain our size of interest, 125–250 µm. Plastics within this size range were then added to seawater to create a stock solution that was stored in a 125 mL, sealed glass bottle. We did not analyze the plastics for chemical content, but it is unlikely that leaching of any chemical additives played a role in these short-term feeding experiments (< 4 h). Pellets were white and odorless, and a small aliquot of the concentrated microplastic fragment solution was introduced to a much larger container of seawater minutes before the feeding experiments.

We also purchased polyethylene microspheres that were 75–90 µm in diameter (Cospheric, Santa Barbara, CA, USA). These were added directly to seawater to create stock solutions, and stock solutions were stored in sealed, 80 mL glass bottles. The size ranges of microplastics were chosen, because similar sized microplastics can be common in coastal waters (Wiggin and Holland 2019), and because many of the zooplankton that larval fish feed on may be within this size range. Microplastics were stored as a concentrated stock solution that was added as needed to the containers of seawater used in the feeding experiments (see below). Stock solution concentration for experiment #1 was 1.245×10^3 particles/mL and for experiment #2 was 1.169×10^3 particles/mL. To make solutions of microplastics for our feeding experiments, the stock solution was mixed vigorously and a sample from the stock solution was pipetted in to a solution of seawater (e.g., 60 µL of stock solution was aliquoted to create our medium concentration in experiment 1). Previous research in our lab suggests that experimental solutions made by this procedure have a coefficient of variation < 3.5% for concentration of microplastics (Chhor 2021).

Measuring feeding rates

We ran two sets of feeding experiments. The overall procedure was similar, but we conducted these feeding experiments under two conditions: one where the water containing larvae was still and one where the water was turbulent. Our feeding experiments began with larvae that were 3 days post-hatch. By this time, grunion larvae have exhausted their yolk sac and rely on external feeding (May 1971). During feeding trials, single larvae were placed in a container with seawater,

a randomly assigned microplastic treatment, and a known number of zooplankton prey (brine shrimp nauplii). For each feeding trial, we would have 3–4 replicates per treatment, for a total of 12–16 per day. Because counts were made by a single observer, and because we relied on collections from the wild, feeding trials were replicated on different days and across different ages of fish. Brine shrimp nauplii, *Artemia salina*, were used as food source and are nutritionally adequate for larval grunion (May 1970). In our feeding experiments, grunion larvae began with a diet of 75 brine shrimp nauplii per individual and this amount was adjusted as the larvae aged. For example, larvae aged 15 days post-hatch were fed 250 brine shrimp nauplii per individual. Feeding levels were chosen to provide larvae with a slight excess of food and were based on age-dependent feeding rates observed in previous laboratory studies (May 1971). To prepare the microplastic concentrations in each container, aliquots of a stock solution of microplastics in seawater were added. The volume added was determined by the target concentration for each experimental treatment (see Table 1). Brine shrimp nauplii were enumerated in plastic counting trays under a dissecting microscope before being decanted into the containers containing the seawater and microplastic treatments. Brine shrimp nauplii were given approximately 5 min to acclimate before a single grunion larvae was added to each container using a strip of Nitex mesh to net the larva. At the end of each feeding trial, grunion larvae were removed and preserved for dissection. To count the number of brine shrimp remaining in each container, seawater was filtered through a fine mesh (20 µm Nitex nylon) that was small enough to catch brine shrimp nauplii by pouring over the contents of the container slowly into the mesh. Containers were rinsed and filtered multiple times. Nauplii retained in the mesh were counted under a dissecting microscope. Before moving over to another container, mesh was rinsed thoroughly to remove any stray artemia. Feeding rates were calculated as the number of nauplii added minus the number of nauplii remaining, divided by the duration of the feeding trial. Procedural control trials with no grunion larvae were conducted for each concentration of microplastics. These trials suggested that nauplii can be counted with > 95% accuracy and that counting accuracy did not differ among microplastic treatments. For each experiment, trials were repeated every few days and feeding rates were measured for larvae of ages that varied from 3 to 14 days post-hatching.

To analyze the effects of microplastic on feeding rates of fish while also accounting for the age of fish, we used an Analysis of Covariance (ANCOVA). Since fish naturally eat more as they grow older and larger, we needed to account for the effects of age. Age was a significant source of variation in all of these analyses (see Results), but because the main focus of the study was the effects of microplastics, in our data displays, we compared how the residual effects differed by microplastic groups. In other words, we corrected for the effects of age when making these comparisons. Note that for all feeding rate experiments, larvae that died during a trial or were obviously stressed (e.g., larvae that remained immobile on the bottom of the container throughout the feeding trials) were excluded from the analysis. Out of a total of 204 trials, 9 were excluded. All statistical analyses in this study were conducted using R (R Development Core Team 2021).

Feeding experiment #1: still water environment (125–250 µm LDPE)

Each larva was placed into a 475 mL plastic cup filled with 300 mL of seawater. Each cup was randomly assigned an experimental treatment (control, low, and medium), all of the same microplastic type, with the corresponding microplastic density found in Table 1. These concentrations include values comparable to observed concentrations in coastal environments (Desforges et al. 2014; Wiggin and Holland 2019). Each feeding trial lasted approximately 4 h and larvae were fasted for 16 h before each feeding trial. In a single day, each treatment combination was replicated three-to-four times, and the entire procedure was replicated on multiple days.

A complimentary version of this experiment focused on measuring activity levels during feeding. The experimental design was the same, except we used slightly different set

Table 1 Concentration of microplastics used in experiments designed to test the effects of microplastics on feeding and activity rates

Treatment	Still water environment (# of particles/300 mL)	Turbulent environment (# of particles/520 mL)	# Of particles/L
Control (C)[a,b,c]	0	0	0
Low (L)[a,b]	25	–	83
Medium (M)[a,b,c]	75	130	250
High (H)[c]	–	546	1050
Very High (VH)[b,c]	500	867	1667

[a]Treatments in feeding experiment #1

[b]Treatments in activity rate experiment

[c]Treatments in feeding experiment #2

of microplastic concentrations (control, low, medium, and very high; Table 1), and microplastic type (HDPE). HDPE fragments were used, because they dispersed more evenly in the water column than LDPE fragments which tended to float. Our original intent was to repeat feeding experiment 1 with HDPE fragments, but equipment failure caused a die-off of the entire cohort of larvae that were to be used in the repeated experiments. Because larvae from these wild-caught fish were in limited supply, we proceeded by testing whether activity was affected by HDPE fragments and we needed to use larvae with a wider range of ages (3–36 days post-hatch; average = 22 d). Activity level was assessed by observing a single fish for 30 s every 15–30 min (individual fish were observed 2–3 times during each replicate run of the experiment). Containers were visually divided into four equally sized quadrants, and during the 30 s of observation, the number of times a larva moved to different quadrants of the container was recorded (e.g., Levell and Travis 2018). A single observer measured all activity values. Activity value was averaged across all observations for each fish and fish within treatment combinations were treated as the unit of replication in our statistical analyses.

Feeding experiment #2: turbulent environment (125–250 µm HDPE and 75–90 µm HDPE)

After experiment #1, it was apparent that not all of the microplastics remained evenly dispersed within the seawater containers. In particular, some of the microplastics stuck together on the surface, and although this decreased the effective concentration of microplastics within the water column, the proportional decrease was likely to be the same across microplastic treatments, because the volume of fragments on the surface appeared proportional to the overall concentration. Even so, the response of fish larvae to microplastics may be affected by water motion, and examining the response of fish larvae in a turbulent environment may be an informative contrast. Nearshore environments are turbulent (Davis 1985; Woodson 2018), and in the wild, larval fish are likely to experience microplastics that are regularly re-suspended and dispersed. To evaluate feeding rates within an environment where microplastics and zooplankton were regularly re-suspended, we conducted feeding trials within an apparatus that slowly rotated the containers and kept the microplastics in suspension.

The apparatus (hereafter referred to as the 'rotisserie') consisted of a 7.62 cm-diameter PVC pipe that was cut in half across the entire length with both ends capped (Fig. 1). The pipe was 57.2 cm long and four containers could be contained within each apparatus. Containers were clear plastic jars that were 520 mL in volume [7.62 cm in diameter and 12.7 cm high]. Each jar was secured to the rotating pipe using cable ties. Attached to the caps of the rotating pipe were axles made of 10 mm-diameter steel tubing. These axles were set within ball bearings that were attached to 90° steel brackets that were elevated on wooden blocks screwed to a plywood base. Axles could rotate freely within the ball bearings and an electric clock motor was attached to one axle and rotated the apparatus slowly during the feeding trials (1–3 rotations per minute). The slow rotating mechanism allowed the re-suspension of microplastics in water without impeding the feeding rates of larvae (see "Results" section). Preliminary trials revealed that HDPE fragments produced a more homogeneous mix of microplastics in seawater than LDPE fragments did and our feeding experiments within the rotisserie apparatus used HDPE plastics throughout. This experiment was conducted once with 125–250 µm fragments and once with 75–90 µm diameter spheres as the source of microplastics.

Each larva was placed in a 520 mL container that was filled with seawater. Each rotisserie held 4 containers and there were 3 identical rotisseries. Thus, 12 feeding trials could be run at a time. One rotisserie contained one replicate of each of the four microplastic treatments, and the placement of replicates within the rotisserie was randomized. Each container was randomly assigned an experimental treatment with their corresponding microplastic density found in Table 1. After adding the microplastics in their respective containers, we slowly stirred the seawater to break the surface tension and ensure that the plastic particles were

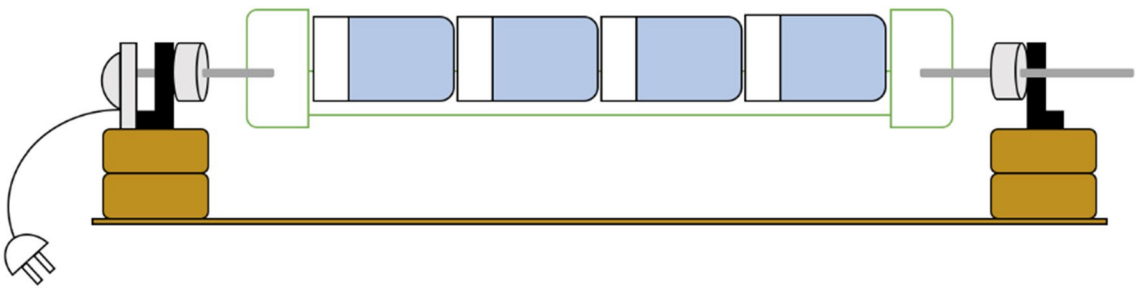

Fig. 1 Rotisserie apparatus designed to measure the effects of microplastics on the feeding rates of larval fish in a slowly rotating, turbulent environment (1–3 RPM)

suspended in water. Then, we carefully closed the lid to eliminate any air in the container. Each feeding trial lasted 2 h, and larvae were fasted for 16 h before each feeding trial. It was not practical to measure activity rates in these experiments. In contrast to the still water experiments where larvae could be viewed against a white background, in the turbulent water experiments, the rotation of the apparatus meant that larvae in the clear containers would be viewed against a background that alternated between light and dark. This precluded visual tracking of larvae, because larvae were rarely visible against a dark background.

We used a General Linear Model to test whether the feeding rates of larval grunion were affected by microplastic concentration, microplastic size, and larval age. Microplastic concentration was treated as a discrete factor to account for any non-linear changes in feeding rate with concentration. Microplastic size was treated as a factor to compare between the two groups (125–250 µm vs. 75–90 µm). Age was included as a continuous covariate, because model residuals in this analysis and others indicated that feeding rate increased linearly with age. We tested all two-way interactions and the three-way interaction among age, microplastic size, and concentration (see Supporting Information for more details).

After the trials from feeding experiments #1 and #2 were complete, all larvae were euthanized using a solution of tricaine methanesulfonate (MS 222; 250 mg/L seawater) and preserved in a 10% formalin solution. Grunion larvae examined under a dissecting microscope and instances of microplastics ingestion were recorded. After visual inspection of the mouth and gills, the intestinal tract was dissected. Using a scalpel, we carefully made a small incision to the intestine. Using a probe, we examined the inside of the intestinal tract for microplastics. All fish examined had partially digested artemia in their stomachs, and some fish had ingested microplastics (see "Results" section). To analyze proportion of ingestion of microplastics of fish, we used a simple Chi-squared test. This test analyzed whether the proportion of ingestion of microplastics of fish was the same for all experimental treatments.

Bioaccumulation experiment

The aims of this experiment were to test whether microplastics can be transferred up the food chain, and to test whether growth and survival of larval grunion were affected by transfer of microplastics from zooplankton prey (brine shrimp nauplii). Zooplankton were incubated with and without microplastics, and these zooplankton were fed to larval fish. We then measured growth and survival of larval fish over a 15-day period.

At 2 days post-hatching, brine shrimp nauplii were cultured within 1 L cones of seawater that were gently aerated and mixed completely by a steady stream of bubbles at the bottom of the cone. Brine shrimp cultures were assigned to either a control treatment (no microplastics), or treatments with microplastics (8×10^6 spheres/L). Microplastics consisted of fluorescent polyethylene spheres that were 1–5 µm in diameter and had a specific gravity of 1.3 (Cospheric, Santa Barbara, CA, USA). Two color forms were available, and we tested both green and blue colored spheres in these experiments. Cultures of brine shrimp nauplii were fed to larval grunion in our main experiment (see details below) and were refreshed every 4–5 days to maintain an adequate food supply. We regularly checked brine shrimp nauplii for presence of microplastics in their guts by examining a subsample of at least 10 nauplii under a dissecting microscope illuminated by a UV light (370 or 450 nm; Fig. S1).

Grunion were hatched in the lab and groups of grunion larvae were randomly assigned to each of the three experimental treatments: a control group fed nauplii exposed to no microplastics; an experimental group fed nauplii that were exposed to green microplastics; and an experimental group fed nauplii that were exposed to blue microplastics. There were three replicate groups per treatment. Grunion larvae were kept in 7-L circular basins, with each container carrying 55 grunion larvae. This experiment was then repeated in the next year (2020) with a slight modification. In this round, newly hatched group of grunion larvae was randomly assigned to only two experimental treatments: a control group fed nauplii exposed to no microplastics and an experimental group fed nauplii that were exposed to green microplastics (prior results indicated that different colored particles did not differ with respect to rates of uptake by nauplii or effects on larvae; see "Results" section). In this trial, there were four replicate groups per treatment. Grunion larvae were kept in 7-L circular basins, with each container carrying at least 20 grunion larvae. Later on, both data were pooled together to represent seven replicates for the control, and ten replicates for the microplastic treatment.

To measure growth of larval fish, digital photographs were taken on days 0, 12, and 15 post-hatching. For each measurement, a sample of ten larvae were euthanized and photographed under a dissecting microscope. Size was measured by placing digital landmarks in standard, easily identifiable locations on the photographs of fish (Fig. S2). The edge of the body was not always discernable in the photographs, but many morphological features were always identifiable (e.g., terminus of the gut, notochord tip, etc.). Size of larvae was described by the area of a standard polygon connecting six landmarks on the body of the fish (Fig. S2). This measure was highly correlated with other measures of size (e.g., standard length), and it incorporated variation in the body depth and condition of the fish. Growth rates of grunion larvae were calculated by taking the difference between the area of the individual fish at the end of the

experiment and the average area of larvae from that same family at hatching, and then dividing by the number of days between measurements. To test whether growth rates differed among the experimental treatments, we used a Linear Mixed Effects Model (LMM). Treatment type and year were included as fixed effects, and because larvae were collected from different families and distributed across treatments, family identity was included as a random effect.

To measure the mortality for each family and treatment, each day, we would check the basins for presence of dead larvae and record them accordingly. Using a pipette, dead grunion were carefully removed and later examined. Daily mortality rates for each group of larvae were calculated as $\frac{-\mathrm{Ln}(P)}{t}$, where P is the proportion of larvae alive at the end of the experiment and t is the duration of the experiment (15 days). Mortality was measured for each group, and we used a linear mixed-effects model to examine whether mortality rates differed among experimental treatments while also accounting for family-to-family variation in overall mortality rates. Experimental treatment (presence/absence of microplastics and year) was treated as a fixed effects, and family was included as a random effect. In this randomized experiment, differences in mortality rates between the control and microplastic treatments would be evidence of a causal effect, even if the proximate mechanism of mortality was not known exactly. To further examine potential associations between microplastics and mortality, we collected the dead fish and examined their gastrointestinal tract for the presence of microplastics, which were detectable under UV light.

Results

Feeding experiment #1: still water environment

Feeding rates were measured for a total of 57 grunion larvae across different ages and different concentrations of microplastics (Table 1). In general, feeding rates increased with age of larvae (Table S1), reflecting the fact that the appetites of fish naturally increased as they developed and grew larger. Examination of model residuals confirmed that a linear model was sufficient to describe the increase in feeding rates with age. After accounting for this variation, the different levels of microplastic significantly influenced feeding rate ($P = 0.0119$; Table S1). In particular, feeding rates were highest when larvae were exposed to low levels of microplastics. Whereas, at medium concentrations of microplastics, feeding rates were slightly lower than the control (Fig. 2A).

In a version of the still water experiment, activity levels (measured by movement within the containers and measured over multiple intervals for each fish) were evaluated for a total of 89 grunion larvae at different ages and different concentrations of microplastics (Table 1). Grunion activity generally increased with age ($P = 2.26 \times 10^{-5}$; Table S2), and even when variation associated with age was accounted for, activity levels were still highly variable from individual to individual. There was no significant variation with microplastic treatment ($P = 0.418$; Table S2), though the results generally mirrored the results for feeding rates in that activity rates were slightly higher for grunion larvae exposed to lower concentrations of microplastics (Fig. 2B).

A total of 147 grunion larvae were examined for presence of microplastic particles in their guts, and after these 4-h feeding and activity trials in still water, we found 7 larvae (4.76%) had collectively 9 particles of microplastics visible within their digestive system. Of those 7, 3 larvae died soon after ingesting the microplastic particles. Rates of ingestion did not vary significantly among microplastic concentrations (Chi-square test, $\chi^2 = 0.992$, $P = 0.803$, df = 3). Fewer fish were examined for the very high group, because it was only used in the experiment that measured activity (Table 2).

Feeding experiment #2: turbulent environment

Feeding rates were measured for a total of 123 grunion larvae across different ages and different concentrations of microplastics (Table 1). 57 grunion larvae were from experiments that used 125–250 µm HDPE, and 66 grunion larvae were from experiments that used 75–90 µm HDPE. Interaction terms were not significant, indicating no complex responses of feeding rate (e.g., effects of microplastic treatments on feeding rate did not differ by microplastic size, nor did the effects of age differ among the experimental treatments; Table S3). Again, feeding rates increased as larvae became older and grew larger ($P = 1.11 \times 10^{-4}$; Table S3) and examination of the residuals indicated that linear model was sufficient to describe the increase in feeding rate with age. There was a significant effect of microplastic size ($P = 5.49 \times 10^{-8}$; Table S3), reflecting the fact that average feeding rates were higher in the treatments with 125–250 µm particles than with 75–90 µm particles (mean feeding rates of 0.145 and 0.064 nauplii per minute, respectively). After accounting for age and particle size, there was no strong evidence of significant variation in feeding rates with microplastic concentration ($P = 0.201$; Table S3), but feeding rates were slightly higher at intermediate concentrations of microplastics (Fig. 3)—a pattern that was similar to feeding rates in the still water experiment. We also note that average feeding rate in the still water experiment (0.098 nauplii per minute) was similar to the average feeding rate in the turbulent environment (0.104 nauplii per minute).

A total of 143 grunion larvae were examined for presence of microplastic particles in their guts, and after these

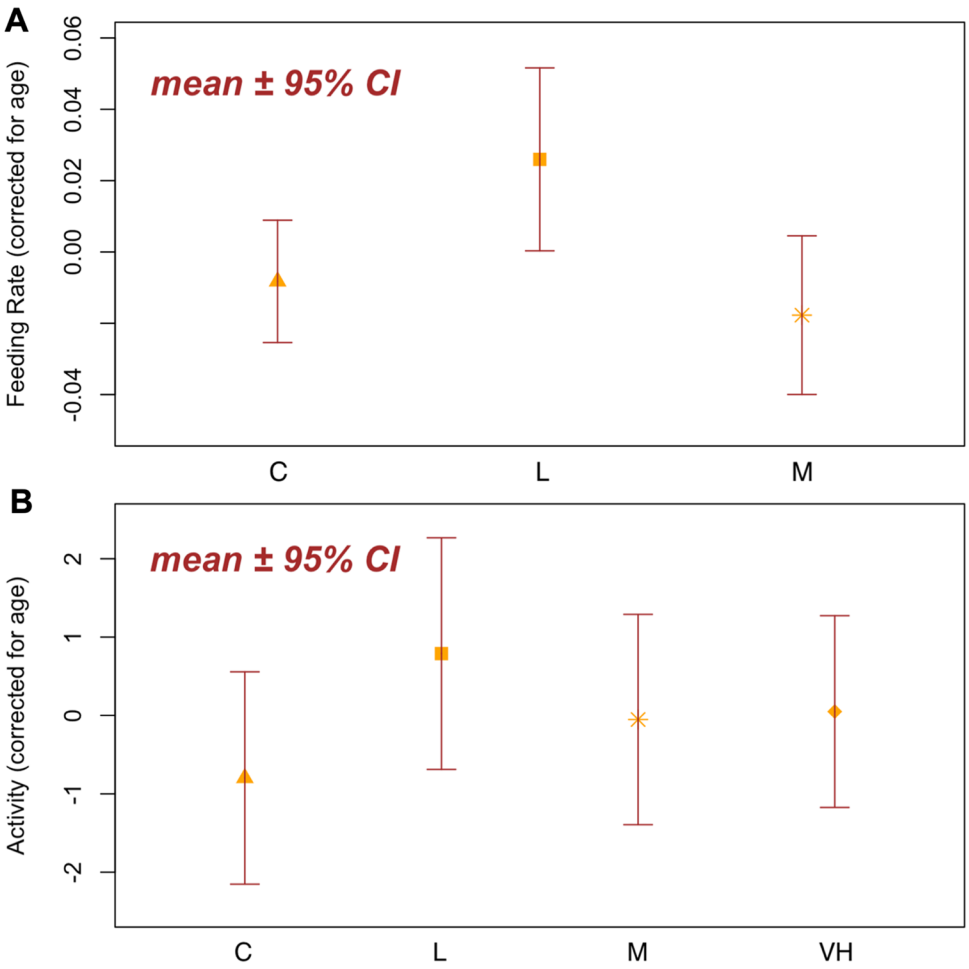

Fig. 2 Effects of microplastic exposure in the still water experiment. (**A**) Relative feeding rates of California Grunion larvae exposed to different concentrations of microplastics (125–250 µm). Positive values indicate feeding rates above the average for a larva of comparable age. Negative values indicate lower than average feeding rates. **(B)** Relative activity levels of California Grunion larvae exposed to different concentrations of microplastics. Activity was quantified as the number of times a focal fish crossed from one quadrant to another for 30 s rounds of observation. Focal fish were observed for 2–3 rounds to get a measure of average activity level. Positive values indicate responses greater than the age-specific average

Table 2 Proportion of grunion larvae sampled that ingested microplastics within feeding experiment #1 (still water environment)

		Microplastic treatment			
		C	L	M	VH
No. of fish w/microplastic	Died upon ingestion	2	0	1	0
	Lived upon ingestion	0	1	2	1
No. of fish inspected		40	40	41	26
%		5.0	2.5	7.3	3.9

Treatment names correspond to the concentrations listed in Table 1

Table 3 Proportion of grunion larvae sampled that ingested microplastics within feeding experiment #2 (turbulent environment)

		Microplastic treatment			
		C	M	H	VH
No. of fish w/microplastic	Died upon ingestion	1	1	0	0
	Lived upon ingestion	1	2	4	0
No. of fish inspected		35	36	36	36
%		5.7	8.3	11.1	0

Treatment names correspond to the concentrations listed in Table 1

2-h feeding trials in turbulent water, we found 9 larvae (6.30%) had collectively 24 particles of microplastics visible within their digestive system (Table 3). Of those 9 larvae, 2 had died soon after ingesting microplastics. Rates of ingestion varied slightly among microplastic concentrations, but this variation was not statistically significant (Chi-square test, $\chi^2 = 3.70$, $P = 0.296$, df = 3).

Bioaccumulation experiment

Our visual assessments confirmed that brine shrimp nauplii exposed to smaller microplastics in their environment regularly ingested microplastics, and revealed no contamination of our control group. Of all the nauplii (340) examined in the microplastic group after pooling data from the different colored microplastics together, 269 (79.1%) had microplastics visible in their guts, while in the control, none of the

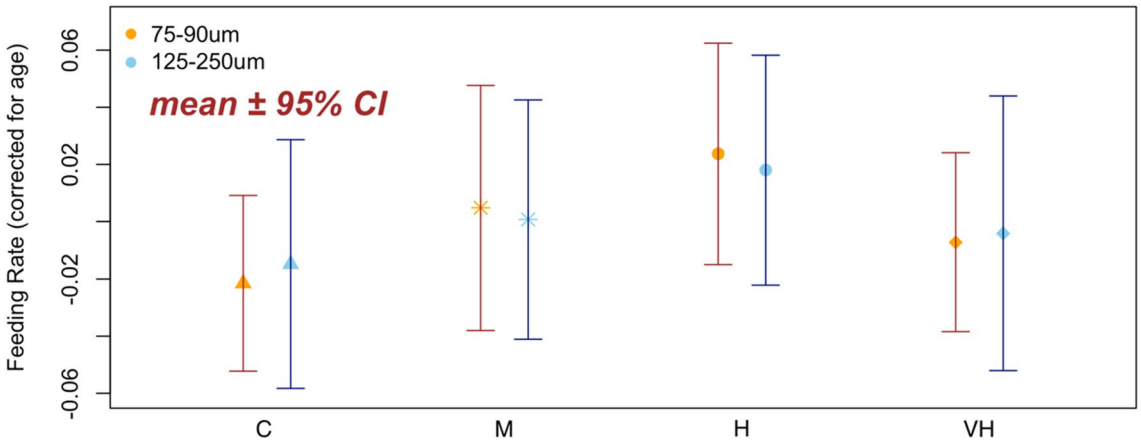

Fig. 3 Relative feeding rates of California Grunion larvae exposed to different concentrations of microplastics (75–90 µm and 125–250 µm HDPE). Positive values indicate responses greater than the age-specific average

170 nauplii sampled (0%) had visible microplastics in their guts. Of 36 fish sampled from the control treatments, 0 had microplastics visible in their guts. Of 76 fish sampled from the microplastic treatments, 41 had microplastics visible in their guts (Table S4).

Growth rates were analyzed twice during the duration of this experiment. For the first time in 2019, growth rates were analyzed by measuring size-at-age of larvae exposed to three different treatments: control, blue microplastics, and green microplastics. Within an age group, larvae that were fed brine shrimp that were exposed to microplastics were smaller on average (12th day = − 16.81%, 15th day = − 12.93%) compared to the control. Thus, growth rates were reduced in both microplastic treatments, and the effects were similar for the two different colors of microplastics (*T* test, $T = 0.996$; df = 117; $P = 0.161$). Because the color of microplastics used in this experiment did not alter the responses of fish growth, data for both treatments with both microplastic colors were pooled. In the following year, growth rates were analyzed by measuring larvae exposed to only two different treatments: control and the microplastics (green-colored). Data collected from both years were compared (Fig. 4A), and growth rates were analyzed by measuring the change in size per day. Growth rates in 2020 were significantly different compared to 2019 ($P = 1.10 \times 10^{-4}$; Table S4). Despite this year-to-year variation, effects of microplastics still resulted in a significant reduction of larval growth ($P = 0.0140$; Table S5).

Grunion larvae that were fed nauplii exposed to microplastics had elevated mortality rates compared to the control (Fig. 4B). However, the precision of the mortality rate estimates was low, in part because mortality is averaged within each family per treatment and there were a relatively low number of replicate groups (10 replicate families per treatments). Mortality rate did not vary significantly among experimental treatments ($P = 0.148$; Table S6) despite the trend toward higher mortality in the microplastic-exposed treatments (Fig. 4B). In addition, examination of dead larvae on days 6–12 of the experiment found that 7 of the 16 fish that died in the microplastic treatments had visible microplastic beads in their gastrointestinal tract (Table S4). Of the 6 dead larvae found from the control treatment, 0 had visible microplastic in their guts (Table S4).

Discussion

We found little evidence that microplastics in seawater interfere directly with feeding rates of grunion. If anything, having a low concentration of microplastics in water seemed to increase feeding rates. However, we found that some grunion ingested microplastics and that indirect consumption and trophic transfer reduced growth and likely the survival of larvae. Based on the results of our laboratory feeding experiments, microplastic particles of the sizes and types we tested did not have an immediate, negative effect on feeding rates of California grunion during the early larval phase. Although it is conceivable that microplastics can interfere with the ability of a larval fish to capture food (e.g., de Sá et al. 2015), we found little effect on feeding rates in the short term. While not statistically significant in all of the single experiments, the pattern of slightly elevated feeding rates at low concentrations of microplastics was observed in multiple feeding experiments and activity rates were slightly elevated in a separate experiment that focused on activity while feeding (cf. Figs. 2A–B and 3). We suspect that the presence of microplastics in seawater stimulated larval fish to feed, possibly because particles in the water stimulate a feeding response, or attract larval fish to areas where planktonic organisms may aggregate. Average activity

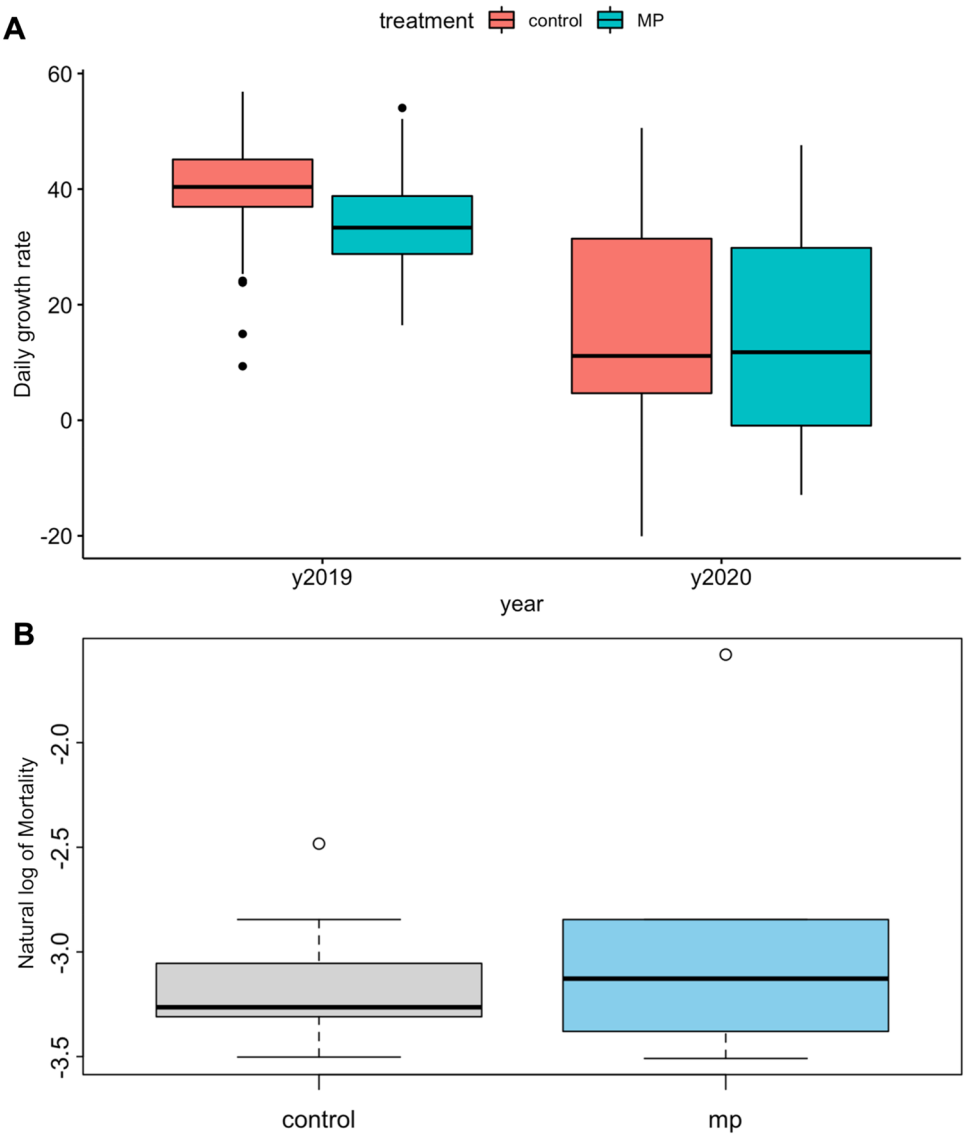

Fig. 4 Effects of trophic transfer of microplastics. (**A**) Growth rates of grunion larvae fed with brine shrimp that were either exposed to microplastics or not. (**B**) Mortality rates of grunion larvae fed with brine shrimp that were either exposed to microplastic or not. The outlier data points are from a single family that had high rates of mortality in both treatments

rates of grunion larvae increased by almost fourfold at low concentration and by at least twofold at very high concentrations of microplastics. If fish were actively inspecting particles (which were dispersed in the containers), this would manifest as greater swimming activity. However, the hump-shaped relationship with microplastic concentration suggests that movement of fish was possibly impeded by high concentrations of microplastics.

Recent studies have demonstrated that odors emitted by plastic debris that has been exposed to the environment can cause for foraging behaviors to be activated in Anchovy, *Engraulis mordax*; however, virgin plastics in this study may not have the same concentration of odors and may not have the same effects (Savoca et al. 2017). Though it is hard to say how much the odor of microplastics could be detected in our study, increased activity when exposed to virgin microplastics has been detected in zebrafish as well (Limonta et al. 2019). Limonta et al. (2019) reported that the cause of the increased level of activity induced by this type of microplastic was not clear; however, up-regulation of the circadian clock gene was observed in their data which suggests possible alteration on the circadian mechanism. These studies and our own suggest interesting links between microplastic exposure and the activity of larval fish, including feeding activity, but more studies will be needed to fully illuminate the behavioral mechanisms involved.

In microplastic research, it is now clear that fish and other marine organisms can ingest microplastics, and that microplastic egestion can occur. Although it was not part of our study, ingested microplastics that stay in the gut may interfere with feeding. Residence times of microplastics in the gastrointestinal tract are not well known, and egestion can occur as soon as several hours after microplastics exposure (Cousin et al. 2020) and may take up to 49 days for total

egestion in some species (e.g., *Seriolella violacea*) depending on the size and type of microplastic (Mazurais et al. 2015; Ory et al. 2018). Mazurais et al. (2015) reported that there was little effect of microplastic ingestion on sea bass larvae because of its high potential for egesting microplastics. However, different species are likely to have different capacities for egestion and we are not aware of any study that have investigated the egestion ability of California Grunion larvae. Since retention of microplastics in the gut of fish may play a pivotal role in the overall effects of microplastics, future studies should test this in more detail.

We note that fasting the fish before each feeding trial may result in elevated feeding rates, but the goal of our experiments was to compare relative feeding rates of larval fish when exposed to various concentrations of microplastics in seawater. Although fasting may elevate feeding rates during brief assays (e.g., a few hours), both the period of fasting and the duration of the feeding trials were the same for all fish in our experiments. Therefore, any effects of fasting would be consistent across our experimental treatments. Furthermore, our 16 h period of fasting included 12 h of darkness—conditions under which grunion larvae do not feed (May 1971).

Although our short-term feeding trials were not designed to evaluate the long-term effects of exposing grunion larvae to microplastics, some of our data suggest that microplastics may have a cumulative, long-term effect on populations. A small, but non-trivial, proportion of fish (5.52% overall) ingested microplastics during the course of these 2–4-h experiments, and 31.25% of those fish died upon ingesting microplastics. The interpretation of these data is, however, complicated by the fact that some of the fish in the control groups were found to have ingested microplastics. There were never any microplastics visible in the control treatments, and we suspect that the ingested microplastics may have come from the housing tanks, or may have been introduced accidentally (e.g., microplastics stuck to the Nitex mesh net that we used to transfer larvae). The sizes of microplastic fragments in the guts of fish were similar between fish from the control and microplastic treatments, suggesting that they came from the same source. The frequency of ingestion in the microplastic treatments should thus be considered relative to the controls, and in that sense, ingestion was not significantly elevated when fish were exposed to higher levels of microplastics. That said, fish still ingested microplastic fragments and a substantial proportion of those larvae that ingested microplastics appear to have died shortly after. Even if ingestion rates are low per microplastic encounter, grunion larvae feed sporadically throughout the day (up to 14 h during spawning season) and in the wild, the cumulative encounters with microplastics may be appreciable, especially considering that plankton, fish larvae, and microplastic debris can all be concentrated together by oceanographic features (Shanks 1983; Gove et al. 2019; van Sebille et al. 2020). Because of the mortality risk associated with ingesting microplastics, it is possible that ingestion of microplastics leads to a chronic, low-level increase in mortality risk (Naidoo and Glassom 2019). Future research should examine such chronic effects in greater detail.

Results of the bioaccumulation experiment demonstrate that microplastics ingested by zooplankton can ultimately impede growth rates of larval fish that feed on the zooplankton. Even within a short-term experiment (15 days), larval fish that ate prey exposed to microplastics had growth rates that were on average lower (14.8% in 2019; 6.5% in 2020) than fish in the control treatment. Growth rates for year 2020 were low compared to year 2019, probably because of the differences in the age and energetic content of brine shrimp. Due to the COVID-19 pandemic, access to our laboratory facilities was restricted in 2020 and we were unable to change the brine shrimp cultures as frequently as in 2019. Nauplii fed to the fish in 2020 were on average older, and older nauplii can have a much lower energy density (Vanhaecke et al. 1983). Despite the overall difference in growth among years, there was a significant decline in growth when larval fish ate prey that were exposed to microplastics. Mortality rates of larval fish over 15 days after were also elevated by 31.7% on average when fish were fed prey that had been exposed to microplastics. Growth did not differ between groups of larvae that were fed different colors of microplastics. This is consistent with high rates of ingestion of both microplastic colors by brine shrimp (see "Results" section) and the idea that brine shrimp nauplii are fairly indiscriminate eaters. Other studies have shown that ingestion of nanoplastics and microplastics by zooplankton is common (Cole et al. 2013; Desforges et al. 2015; Sun et al. 2017; Cousin et al. 2020), and that retention of microplastics is fairly low (Wang et al. 2019), zooplankton as a group may be particularly susceptible to the negative effects of microplastic pollution. It is becoming increasingly clear that microplastics may be transferred up the food web (e.g., Setälä et al. 2014; Cousin et al. 2020; Hasegawa and Nakaoka 2021; Stienbarger et al. 2021), more studies of the ecological effects of trophic transfer are needed. Our study found immediate effects on growth and a trend toward greater mortality when larval fish consume zooplankton that have ingested microplastics.

Our findings of substantial effects in the bioaccumulation experiment but little effect in the feeding experiments may be explained by a combination of factors. First is that the exposure times were much shorter for the feeding experiments (< 4 h) than for the bioaccumulation experiments (~ 2 weeks). With longer exposure, fish may ingest more microplastics (either directly or indirectly through consumption of microplastics in prey) and effects may accumulate (Kelpsiene et al. 2020). In addition, the microplastic size used in the bioaccumulation experiment was smaller than

the size used in the feeding experiments. Small microplastics that are ingested may aggregate within the gastrointestinal tract and cause blockages the same way larger microplastic fragments do (Wright et al. 2013; Pedà et al. 2016; Jovanović 2017). However, smaller particles may have additional effects. Other studies have found that retention of small microplastics in the gut has caused some organisms (e.g., bivalves, fish) to internalize plastics and translocate them into their cells and tissues (von Moos et al. 2012; Zitouni et al. 2020; Sendra et al. 2021). Von Moos et al. (2012) reported that the uptake of microplastics in the digestive system and into the cell via endocytosis in *Mytilus edulis* resulted in a significant increase in the formation of granulocytoma and significant decrease in lysosomal membrane stability. Both of which are indicative of stress, toxicological response, and inflammatory response from microplastic exposure (von Moos et al. 2012).

At the outset of the experiment, it was not clear whether brine shrimp nauplii would ingest microplastics in appreciable amounts, and we started these experiments with a high concentration of microplastics. Their effects on mortality and growth of grunion larvae were strong, but the process of bioaccumulation and trophic transfer will be dependent on the concentrations of microplastics zooplankton encounter in the wild, as well as the residence time of microplastics in the gut of zooplankton. Future studies should examine the effects of microplastics in lower concentrations and we recommend experimental designs that expose larvae to a wide range of concentrations of microplastics. Future studies should also examine the effects of other microplastic types (e.g., fibers). The effects of microplastics are likely to be type- and concentration-dependent, and it would be useful to characterize the steepness of these relationships and to identify any nonlinearities. Such information will be useful for evaluating the sensitivity of fish populations to varying degrees of microplastic pollution.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00227-021-04010-x.

Acknowledgements We would like to thank James Chhor, Callyn Shelly, Brian Stirling, and Darien Satterfield for collecting and providing the grunion eggs used in this study. Mohammad Shobieiri and Brent Scheiwe constructed the rotisserie apparatus. We also thank Bailey McCann and Janelle Paz for helping to care for the grunion and assisting in the feeding experiments. Dr. Bruno Pernet and James Chhor helped with photography.

Author contributions Both authors contributed to study conception and design. CAU collected and analyzed data and wrote the first draft of the manuscript. DWJ analyzed data and contributed to the writing of the manuscript.

Funding This research was supported by California State University Council on Ocean Affairs, Science and Technology (COAST) and CSU-LSAMP (Louis Stokes Alliance for Minority Participation) which is funded through the National Science Foundation (NSF) under Grant #HRD-1826490 and the Chancellor's Office of the California State University.

Data availability The datasets generated during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest All authors declare no conflicts of interest.

Ethical approval Experiments were performed with the approval of California State University's Institutional Animal Care and Use Committee.

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