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ARTICLE

## Effect of Species Assemblage on Juvenile Growth and Condition in Three California Estuarine Fishes

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### Abstract

We investigated the physiological response of the endangered Tidewater Goby *Eucyclogobius newberryi* to the presence of Threespine Sticklebacks *Gasterosteus aculeatus* (native to California) and Rainwater Killifish *Lucania parva* (nonnative). A fully factorial experimental design was used to examine species assemblage effects on juvenile fish over a 28-d period. Growth characteristics (weight, SL, and relative condition factor [ $K_n$ ]) and stress hormone levels (cortisol) were assessed under ample food conditions and at a salinity of 15‰. Weight and SL of Tidewater Goby increased throughout the experiment; growth did not differ in relation to fish assemblage treatment, but significant differences in growth were observed between sampling dates within the experiment. Rainwater Killifish exhibited marginal increases in weight, SL, and  $K_n$ , but these increases were not different among assemblage treatments or over time. For Threespine Sticklebacks, weight and SL increased during the final 2 weeks of the experiment, resulting in significant differences over the entire experimental period; however, growth characteristics of this species did not differ among assemblage treatments. Cortisol levels in all three species were not significantly affected by assemblage treatment. The present results indicate that juvenile Tidewater Goby are not adversely affected by native Threespine Sticklebacks or nonnative Rainwater Killifish under stable abiotic conditions in the absence of food limitation.

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Along the Pacific coast of North America, losses of aquatic species in estuaries and significant shifts in fish assemblages have been caused by a combination of physical habitat modifications, changes in abiotic conditions, and species introductions (Moyle and Light 1996; Marchetti et al. 2001; Leidy 2007). In the San Francisco Bay Estuary (the largest Pacific coast estuary in North America), habitat modification and loss are attributable in part to the filling of wetlands, the placement of hardened structures (e.g., dikes and armored shorelines), and the establishment of levees (Medellín-Azuara et al. 2013). Manipulation of the hydrologic cycle (particularly salinity) by

changing the timing and quantity of freshwater flow through the San Francisco Bay Estuary has resulted in the alteration and homogenization of abiotic conditions (Fleener et al. 2010; Suddeth et al. 2010; Null et al. 2014). Furthermore, within this altered estuarine system, species introductions have occurred at a high rate as the homogenized habitat conditions reduce the advantage of locally adapted species and favor the establishment of nonnative species (Moyle et al. 1986; Marchetti et al. 2001; Matern et al. 2002). The success of an introduced species is believed to be more dependent upon physiological compatibility with the local habitat rather than

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an ability to coexist with the species currently occupying the local habitat. For native species that are already adapted to the local environment, however, the successful introduction of a novel species poses an unknown challenge that can lead to reductions in the native species' distribution and population numbers (Moyle and Light 1996; Matern 2001).

Reintroduction of federal and state regulated and commercially valuable species (e.g., steelhead *Oncorhynchus mykiss* and Chinook Salmon *O. tshawytscha*) has been used as a recovery tool to counter the loss of native fishes in California (Anderson et al. 2014). Many factors must be evaluated prior to a reintroduction attempt, such as current habitat condition, proximity to other populations, species life history requirements, and current habitat occupants' potential role in the success or failure of a targeted reintroduction (USFWS 2005; Anderson et al. 2014). Predatory species can be identified and are often easier to evaluate as a biological constraint than species that utilize the same resources in the habitat, thus potentially imposing competitive pressure (USFWS 2005). Competition between species with similar niches can result in limitation or exclusion of the less-dominant species from key resources, such as food and cover, and this dynamic can vary in intensity depending on the life stage or season (Osenberg et al. 1992; Ward et al. 2006; Grether et al. 2009; Britton et al. 2011; Pegg and Britton 2011). Due to this complexity, species assemblage and interspecific competition present an often poorly understood dynamic when evaluating the potential success of reintroducing a target species.

The Tidewater Goby *Eucyclogobius newberryi* is a small (<55 mm TL), annual species endemic to coastal California lagoons and estuaries but has been extirpated from the San Francisco Bay Estuary, where it was last encountered during the 1960s (Swift et al. 1989; Swenson 1999; USFWS 2005). This species is protected by the U.S. Endangered Species Act and has been the target of reintroduction efforts at other locations in California (Lafferty et al. 2010). In areas where Tidewater Goby are still present, they typically are locally abundant; however, habitat can be limited, as these fish are usually excluded from areas with swift currents or large tidal exchanges (USFWS 2005; Chamberlain 2006). Artificial breaching of coastal lagoon habitat has detrimentally impacted the Tidewater Goby by increasing the magnitude of tidal exchanges, thereby reducing habitat suitability and affecting population stability (USFWS 2005; McCraney et al. 2010). The alteration of coastal estuarine habitat and the introduction of nonnative species have been identified as potential factors jeopardizing the distribution of the Tidewater Goby (Swift et al. 1989; Moyle 2002; USFWS 2005).

The impacts of management practices (e.g., lagoon breaching and development) can be curtailed to prevent further effects on Tidewater Goby; however, the presence of an established nonnative species that has similar habitat requirements is much harder to remedy (Swift et al. 1989; Matern 2001; USFWS 2005; Lafferty et al. 2010). The Rainwater Killifish *Lucania parva*, which is native to saltmarshes and estuaries of

the East Coast and Gulf Coast regions of the United States and Mexico, is thought to be a potential competitor that could be detrimental to the recovery of Tidewater Goby (Swift et al. 1989; Crawford and Balon 1994; USFWS 2005). The introduction of Rainwater Killifish into the San Francisco Bay Estuary during the 1960s corresponded with the last known collections of Tidewater Goby in that habitat; currently, the two species do not co-occur in the wild (USFWS 2005).

Although the impacts of habitat alterations and predatory fishes have been investigated for Tidewater Goby, the potential effects of nonnative species with similar habitat requirements are not well understood (Swift et al. 1989; Swenson 1999; Matern 2001; USFWS 2005; Chamberlain 2006; Lafferty et al. 2010). Knowledge of nonnative species' impacts on Tidewater Goby could be instrumental in (1) understanding the Tidewater Goby's ability to naturally recolonize habitat or (2) facilitating the success of reintroduction efforts. To support assessment of potential reintroduction locations for extirpated native Tidewater Goby, we tested the effects of a novel, nonnative species (a potential competitor, the Threespine Stickleback *Gasterosteus aculeatus*). We selected the Threespine Stickleback as the native species for this experiment because it is documented to co-occur with Tidewater Goby across much of its range and because it occupies habitat from which Tidewater Goby have been extirpated. Threespine Sticklebacks have also been shown to exhibit pre-emptive exploitative competition with other native estuarine species by reducing the availability of invertebrate prey (Spilseth and Simenstad 2010). Use of a native heterospecific fish in our experiment was crucial for untangling the effects of Tidewater Goby interactions with a native species relative to a novel, nonnative species (i.e., Rainwater Killifish). Interactions between species were assessed by examining the survival, growth, and stress hormone levels of juvenile fish under stable estuarine conditions in the laboratory, where resources (food and shelter) were not limited. Our objective was to better inform evaluations of potential Tidewater Goby reintroduction sites in the San Francisco Bay Estuary.

## METHODS

### Fish Collection and Holding

On September 22, 2012, juvenile Tidewater Goby were collected by seining in Salmon Creek Lagoon, Sonoma County, California (38°21'11.42"N, 123°3'57.02"W). At the time of collection, water temperature in Salmon Creek Lagoon was 15.8°C, salinity was 4.7‰, and dissolved oxygen concentration was 9.94 mg/L. On September 23, 2012, juvenile Rainwater Killifish were collected by seining in an unnamed tidal slough that drained into Bothin Marsh Preserve, Marin County, California (37°53'15.26"N, 122°31'31.17"W). Water temperature in the unnamed slough was 18.8°C, salinity was 33.6‰, and dissolved

oxygen concentration was 12.04 mg/L. Juvenile Threespine Sticklebacks were collected on September 23, 2012, via seining in the lower Coyote Creek watershed (37°52'34.09"N, 122°31'35.11"W) and Novato Creek (38°5'58.67"N, 122°34'6.72"W), both located within Marin County. During collection within lower Coyote Creek, water temperature was 18.4°C, salinity was 31.5‰, and dissolved oxygen concentration was 9.12 mg/L. In Novato Creek, water temperature was 20.8°C and salinity was 0.3‰. In the field collections, juvenile Rainwater Killifish and Threespine Sticklebacks were selected based on their size similarity to Tidewater Goby and were transported using aerated coolers to the Romberg Tiburon Center for Environmental Studies at San Francisco State University, Tiburon, California. Fish of each species were maintained separately in indoor, recirculating aquaria under a natural photoperiod until experimentation. Temperature and salinity conditions during the acclimation period are described below.

Using incremental salinity changes achieved through partial water changes, the salinity in each aquarium was gradually adjusted to 15‰ from the salinities measured at field collection sites. The target salinity of 15‰ was the median between conditions recorded during reconnaissance-level surveys conducted in Marin County on June 28 and July 26, 2012, and at Salmon Creek Lagoon on September 8, 2012. The value of 15‰ falls within the salinity range of occupied habitats and physiological tolerances for Tidewater Goby (Chamberlain 2006), Threespine Sticklebacks (Moyle 2002), and Rainwater Killifish (Fuller et al. 2007). Tidewater Goby were initially held at 4.8–7.0‰ for 1 h before being introduced into one of two aquaria at a salinity of 10‰. Tidewater Goby were then exposed to increasing salinity at a rate of 0.6‰ per day for 9 d until 15‰ was reached; the fish were held at 15‰ for 3 d before experimentation began. Rainwater Killifish and Threespine Sticklebacks collected from lower Coyote Creek were held in the transport coolers for 1 h at 26.9‰ before being placed into one of three species-specific aquaria at 23.4‰. Salinity in the three aquaria was decreased at a rate of 0.9‰ per day for 8 d and then was maintained at 15‰ for 5 d before the start of experimentation. Threespine Sticklebacks collected from Novato Creek were held in the transport cooler for 1 h at 7‰; next, they were introduced into an aquarium where salinity was increased at a rate of 0.9‰ per day for 8 d until attaining 15‰, at which the fish were held for 5 d prior to the experiment. Threespine Sticklebacks that were collected from the two field sites were pooled together after the salinity transition to 15‰ but before being introduced into the experimental cells. In all acclimation tanks for the duration of the acclimation period, the temperature was maintained at  $17.7 \pm 1.4^\circ\text{C}$  and the dissolved oxygen concentration was maintained at  $8.1 \pm 0.6$  mg/L.

### Experimental Design

**Tank composition.**—Growth, survival, and cortisol levels in juvenile Tidewater Goby, Rainwater Killifish, and Threespine Sticklebacks were investigated under controlled laboratory

conditions. Aquaria were divided in half with a watertight, black-acrylic divider sealed with silicone, thereby creating two 18.9-L experimental cells. Each cell measured 25.4 cm long  $\times$  27.94 cm wide  $\times$  30.48 cm high. All cells were isolated from the other cells by covering one additional side in black plastic sheeting and using completely separate water sources so that the fish in one cell could not see or sense the fish in any other cell (Schofield 2004). A standardized volume of clean sand and green ribbon (to simulate submerged aquatic vegetation) was uniformly distributed throughout each cell. The experiment cells were designed to provide equal ratios of cover (ribbon) and open water. Artificial vegetation has been used to successfully mimic natural habitat structure for fish in laboratory conditions (Keller and Brown 2008).

All aquaria were held in a temperature-controlled seawater table that maintained a temperature of  $17.5 \pm 1.4^\circ\text{C}$  in each cell over the duration of the experiment. All replicate cells had temperatures that were within a maximum daily range of  $0.5^\circ\text{C}$  of each other. Air stones were used to maintain the dissolved oxygen level at  $8.13 \pm 0.3$  mg/L in each cell. Salinity within each cell was held at  $15.4 \pm 0.3$ ‰. Internal tank circulation and water quality were maintained separately for each cell by using aquarium filters (Whisper Model PF10; Tetra, Blacksburg, Virginia) that were fitted with fine-mesh screens to reduce water velocity. Temperature and oxygen concentration were measured in each cell every other day (Model 85 meter; YSI, Yellow Springs, Ohio); ammonia, nitrate, nitrite, and pH in each cell were measured once per week (Saltwater Master Test Kit; API, Chalfont, Pennsylvania). Water changes equal to 5–15% of cell volume were conducted weekly to maintain water quality.

We conducted a 28-d experiment to test the effects of species assemblage on growth and basal stress hormone levels in juvenile Tidewater Goby, Rainwater Killifish, and Threespine Sticklebacks. The experiment incorporated a fully factorial design with six assemblages (1 assemblage/cell; 2 species/cell) that accounted for both intraspecific competition as well as interspecific competition. In total, 20 fish were introduced into a cell at the same time and were held under one of the six assemblage treatments (3 replicates/treatment): (1) 10 Tidewater Goby and 10 conspecifics; (2) 10 Tidewater Goby and 10 Rainwater Killifish; (3) 10 Tidewater Goby and 10 Threespine Sticklebacks; (4) 10 Rainwater Killifish and 10 Threespine Sticklebacks; (5) 10 Rainwater Killifish and 10 conspecifics; and (6) 10 Threespine Sticklebacks and 10 conspecifics.

**Feeding.**—Fish were fed a diet that primarily consisted of live *Daphnia pulex* cultivated on Phyto-Feast (Reed Mariculture, Campbell, California). Feedings were supplemented with either frozen brine shrimp *Artemia* spp. (San Francisco Bay Brand, Newark, California) or Marine-S pellets (Hikari Sales USA, Hayward, California). Feedings occurred once daily; the contents of a dish were poured randomly around the top of a cell, allowing the *D. pulex* to swim throughout the cell. In pilot studies using this feeding method, the tested fish all had access to food and consumed the food within a 1-min period. During the

experiment, food was apportioned evenly among 18 randomized dishes (1 dish/cell) to ensure that the quantity of food administered was the same for all cells. The food quantity was standardized such that (1) all fish were observed consuming the food and (2) all of the food was consumed within 1 min of application.

### Growth Measurements

Fish SL and weight measurements were recorded every 14 d: (1) on day 1, when fish were moved from acclimation tanks to experimental cells; (2) on day 15, the experimental midpoint; and (3) on day 29, the last day of the experiment. On days 1 and 15, all fish were netted and temporarily held in an aerated container. Fish were then individually removed for measurement and were returned to a separate aerated container to ensure that each individual was counted only once. Weight measurements were taken in accordance with the methods of Anderson and Neumann (1996): live fish were lightly blotted dry; placed in tared, water-filled weighing boats (at 15‰ salinity); and weighed to the nearest 0.001 g (Model XS105 dual-range micro-scale; Mettler-Toledo, LLC, Columbus, Ohio). Standard length was measured to the nearest 0.5 mm by using dial calipers. On day 29, all fish in a given cell were captured and euthanized with an overdose of tricaine methanesulfonate (MS-222 [Finquell]; Argent Chemical Laboratories, Redmond, Washington) within a 5-min period. Fish were measured for SL and weight, placed in individually labeled centrifuge tubes, and flash-frozen in liquid nitrogen or on dry ice. Specimens were then stored at  $-80^{\circ}\text{C}$  until their use in the analysis of cortisol concentration.

Length and weight data were used to calculate the relative condition factor ( $K_n$ ) for comparison between fish assemblages (Anderson and Neumann 1996; Froese 2006). For Tidewater Goby, SL and weight data from 118 individuals measured at Salmon Creek Lagoon were used to create a log-transformed plot from the equation

$$\log_{10}W = \log_{10}a + (b \cdot \log_{10}L),$$

where  $W$  is weight (g);  $L$  is the SL (cm);  $b$  is the slope of the regression line; and  $a$  is the intercept value (see the Supplement available in the online version of this article). For Rainwater Killifish and Threespine Sticklebacks,  $a$ - and  $b$ -values were taken from FishBase (Froese 2006; Froese and Pauly 2014). The  $a$  and  $b$  parameters determined for each species were applied to the weight-length relationship

$$W' = aL^b,$$

where  $W'$  is used to calculate the length-specific standard weight. The  $K_n$  value for an individual was then calculated as

$$K_n = W/W'.$$

### Biochemical Analysis of Stress

*Whole-body homogenization.*—The whole body of each fish ( $n = 45$  fish/species) was homogenized via the protocol used by

Hasenbein et al. (2013). The frozen sample was thawed, weighed, cut into quarters, and then homogenized in 1 mL of ice-cold  $1 \times$  phosphate-buffered saline (PBS; 4.3-mM sodium phosphate, 136.8-mM sodium chloride, 2.7-mM potassium chloride, and 1.47-mM monopotassium phosphate, pH 7.4). The blades of the homogenizer were washed with 1 mL of PBS, which was then combined with the sample and vortexed. The sample was split in half, and one of the halves was immediately used for cortisol analysis.

*Cortisol extraction and analysis.*—Cortisol extraction was performed by using methodology adopted for Zebrafish *Danio rerio* (Alsop and Vijayan 2008; Cachat et al. 2010) and optimized for juvenile Delta Smelt *Hypomesus transpacificus* (Hasenbein et al. 2013); Delta Smelt were of similar size to the juvenile fish used in our study. Tissue homogenate was spiked with 2.0 mL of diethyl ether, vortexed, and centrifuged at  $3,200 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatant was extracted without touching the pellet and was transferred to a new glass test tube. Preliminary analysis determined that over 90% of total-body cortisol was extracted with a single wash of the tissue homogenate. For the experiment, this process was repeated two more times to obtain maximal cortisol extraction from the tissue homogenate with diethyl ether (Cachat et al. 2010), and the supernatant from the three washes was combined. The pellet was then discarded, and the extracted supernatant in the glass test tube was left overnight in a fume hood to ensure that the diethyl ether had fully evaporated (Cachat et al. 2010). The next day, samples were resuspended in 1 mL of PBS, vortexed for 30 s, and stored at  $4^{\circ}\text{C}$  overnight. Cortisol was then measured by using an ELISA-based assay in accordance with the manufacturer's instructions (Neogen, Lansing, Michigan). Cortisol samples were run in duplicate, and mean blank absorbance values were subtracted from each sample value. Cortisol concentrations were calculated with a four-parameter sigmoid standard curve and were then corrected for dilution and standardized by sample weight (50% of the fish weight used for cortisol). This extraction method allowed us to detect unstressed and stressed cortisol levels in Tidewater Goby (D. A. Chase and A. E. Todgham, unpublished data).

### Statistical Analysis

Statistical analyses were conducted in R version 2.15.0 (R Development Core Team 2012) to evaluate the main effect of fish assemblage on response variables (SL, weight,  $K_n$ , and cortisol level) for each fish species. Data were first visually examined for normality and heteroscedasticity based on  $Q-Q$ , density, and residual plots of the linear model to ensure that the assumptions of parametric analysis were met. A mixed-effects linear model was used to evaluate whether it was necessary to include the effect of tank as random error in the model. If significant differences were

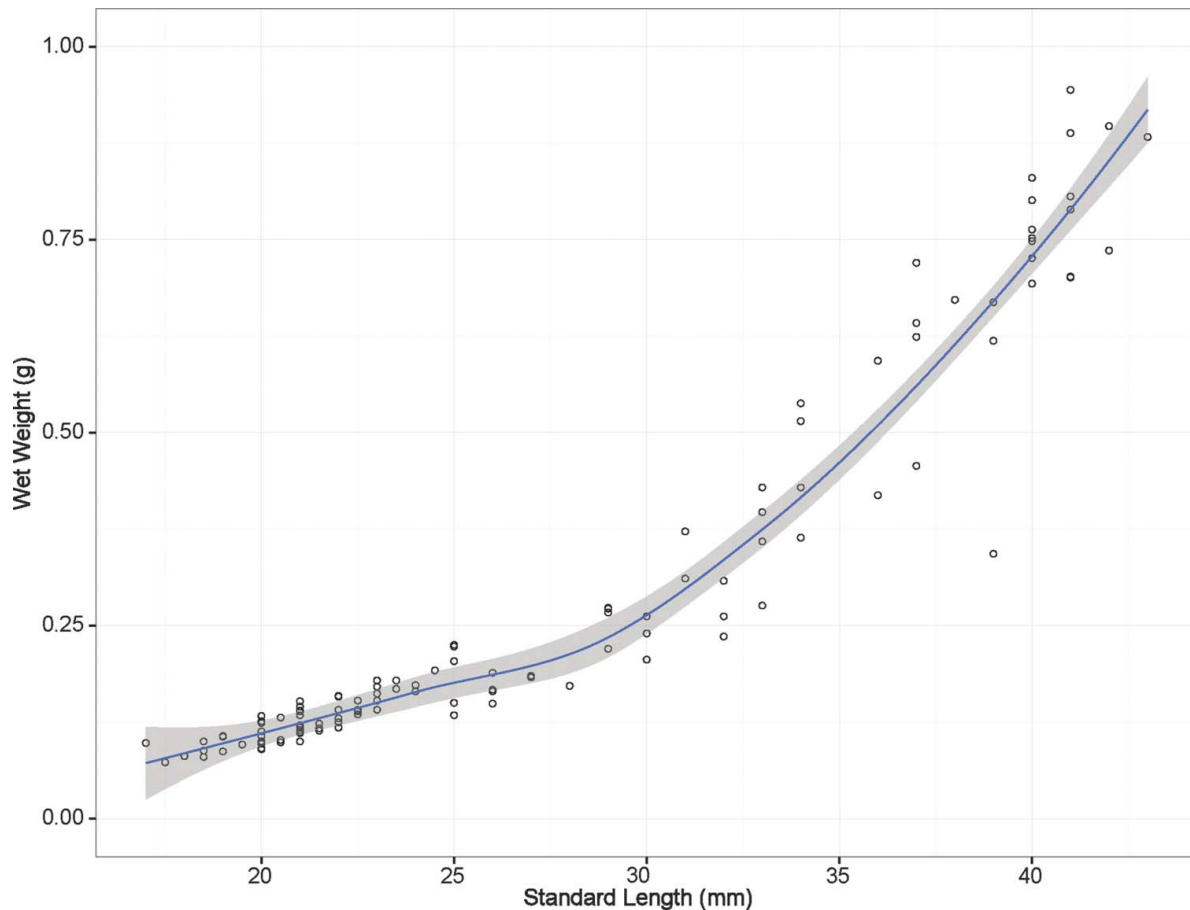


FIGURE 1. Standard length (mm) and wet weight (g) of Tidewater Goby surveyed at Salmon Creek Lagoon, California. The solid line represents the polynomial trend line (shading = 95% confidence interval).

encountered, a Tukey–Kramer multiple comparison test was carried out. Weight and SL measurements were assessed by using a linear model with fish assemblage and day as fixed factors. The tank effect was included as random error in the linear mixed-effects models for the  $K_n$  of Tidewater Goby; the weight and SL of Rainwater Killifish; and the weight, SL, and  $K_n$  of Threespine Sticklebacks. Repeated-measures analyses could not be used with this data set because individual fish were not marked and therefore were not individually tracked during the experiment. For cortisol data from day 29, weighted means were examined in a one-way ANOVA with fish assemblage as the main factor; this was followed by a Tukey–Kramer multiple comparison test to distinguish differences in cortisol for Tidewater Goby and Threespine Sticklebacks. Because cortisol data for Rainwater Killifish did not meet the assumption of normality, a nonparametric Kruskal–Wallis test was applied when comparing differences in cortisol among Rainwater Killifish in different assemblage treatments. The threshold for significance ( $\alpha$ ) was set at 0.05. All data are reported as mean  $\pm$  SE unless otherwise stated.

## RESULTS

### Field Survey of Tidewater Goby Morphometrics

Overall, 118 Tidewater Goby were measured and weighed at the Salmon Creek Lagoon site between June 24 and August 14, 2013. In general, the range of weights at a given length was narrower for juveniles (<27 mm SL) than for reproductive adults (Figure 1). Tidewater Goby exhibited a substantial increase in weight at lengths between 30 and 45 mm SL; this corresponds with the onset of sexual maturity and the increased fecundity with increasing size (Swift et al. 1989; Swenson 1993, 1999). For Tidewater Goby sampled at Salmon Creek Lagoon, the weight–length relationship had an  $a$ -value of 0.01647 and a  $b$ -value of 2.66, and these parameters were used for the calculation of  $K_n$ .

### Growth

Tidewater Goby in all assemblage treatments increased in SL and weight throughout the duration of the experiment (Figure 2). Tidewater Goby weight was not significantly affected by the fish assemblage  $\times$  day interaction (linear

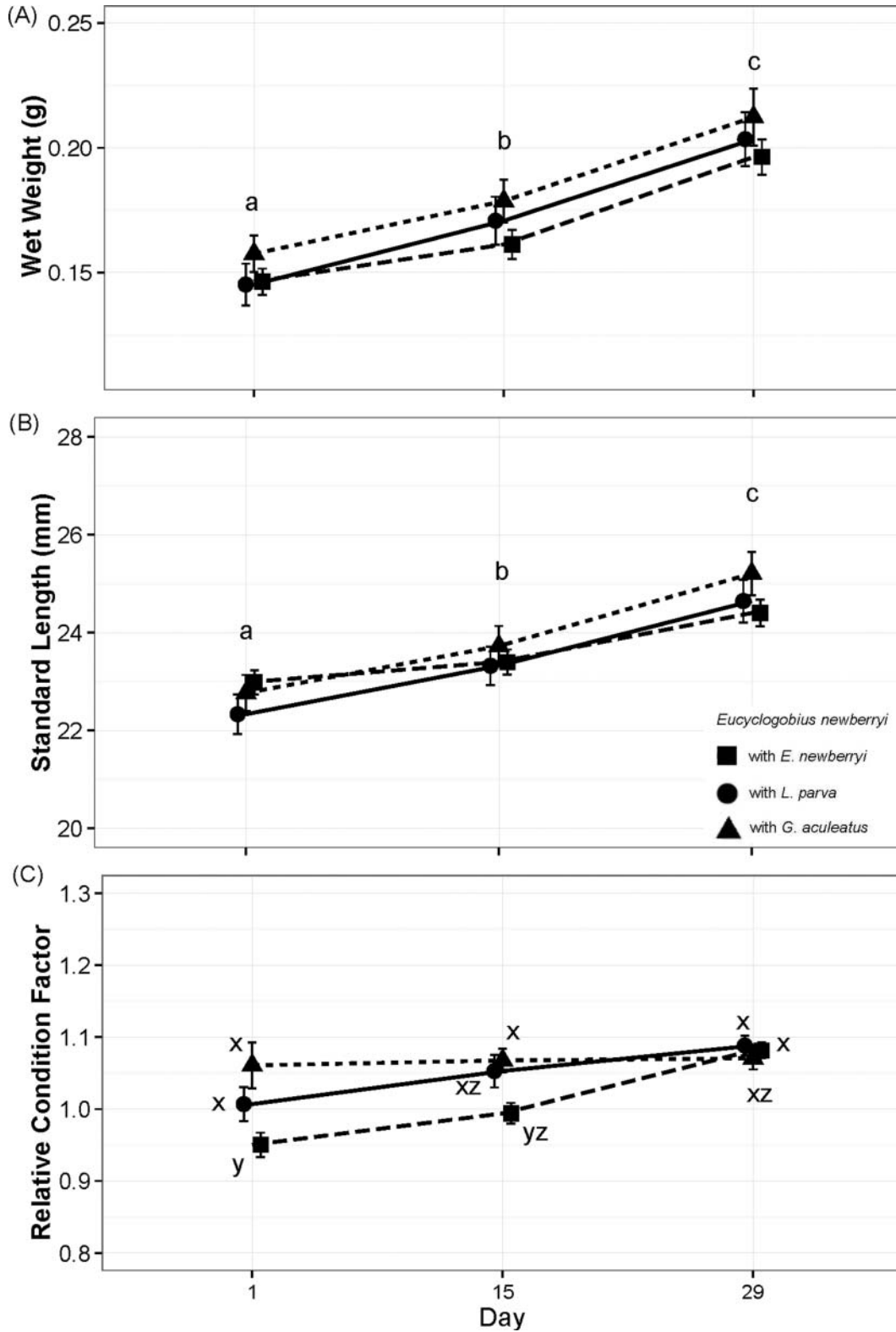


FIGURE 2. (A) Wet weight, (B) SL, and (C) relative condition factor of Tidewater Goby *Eucyclogobius newberryi* on days 1, 15, and 29 in the presence of conspecifics (squares), Rainwater Killifish *Lucania parva* (circles), or Threespine Sticklebacks *Gasterosteus aculeatus* (triangles). Data represent mean  $\pm$  SE for 30–60 fish. In panels A and B, differing letters represent significant differences ( $P < 0.05$ ) between days for all assemblage treatments combined. In panel C, means without a letter in common are significantly different ( $P < 0.05$ ) between species assemblages across all days.

TABLE 1. Survival (%) of Tidewater Goby, Rainwater Killifish, and Threespine Sticklebacks within each experimental fish assemblage, as measured on days 1, 15, and 29.

Species	Versus Tidewater Goby			Versus Rainwater Killifish			Versus Threespine Stickleback		
	Day 1	Day 15	Day 29	Day 1	Day 15	Day 29	Day 1	Day 15	Day 29
Tidewater Goby	100	100	100	100	100	100	100	96.7	96.7
Rainwater Killifish	100	100	100	100	100	100	100	100	96.7
Threespine Stickleback	100	93.3	86.7	100	100	96.7	100	100	96.7

mixed-effects model:  $F_{4, 351} = 0.14$ ,  $P = 0.968$ ) or by fish assemblage ( $F_{2, 355} = 0.58$ ,  $P = 0.077$ ); however, day did have a significant effect on weight ( $F_{2, 355} = 5.78$ ,  $P < 0.001$ ), as weight increased significantly at each sampling time point (Tukey–Kramer multiple comparison test, day 1 versus day 15:  $P = 0.008$ ; day 1 versus day 29:  $P < 0.0001$ ; day 15 versus day 29:  $P < 0.01$ ; Figure 2A). Weight increases on days 15 and 29 were similar in magnitude for Tidewater Goby in all fish assemblage treatments. We found no significant fish assemblage  $\times$  day interaction effect ( $F_{4, 351} = 0.78$ ,  $P = 0.538$ ) or fish assemblage effect ( $F_{2, 355} = 1.01$ ,  $P = 0.363$ ; Figure 2B) on the SL of Tidewater Goby. There was a significant effect of day on SL ( $F_{2, 355} = 24.42$ ,  $P < 0.0001$ ), and SL increases were similar for Tidewater Goby in the Rainwater Killifish and Threespine Stickleback assemblage treatments; however, only a minimal increase in SL between day 1 and day 15 was observed for Tidewater Goby in the conspecific assemblage treatment (day 1 versus day 15:  $P = 0.031$ ; day 1 versus day 29:  $P < 0.0001$ ; day 15 versus day 29:  $P < 0.01$ ). The fish assemblage  $\times$  day interaction effect on  $K_n$  was significant ( $F_{4, 345} = 3.53$ ,  $P < 0.01$ ): Tidewater Goby  $K_n$  exhibited the greatest increase on day 29 in the conspecific assemblage treatment; nearly equal increases on day 15 and day 29 in the Rainwater Killifish assemblage treatment; and little to no change over the study period in the Threespine Stickleback assemblage treatment (Figure 2C). Survival of Tidewater Goby remained high throughout the experiment (Table 1).

The weight of Rainwater Killifish did not change significantly over the course of the experiment, although there were slight increases in weight between day 15 and day 29. Rainwater Killifish weight was not significantly affected by the fish assemblage  $\times$  day interaction (linear mixed-effects model [with the tank effect as random error]:  $F_{4, 344} = 0.11$ ,  $P = 0.980$ ), the fish assemblage ( $F_{2, 6} = 1.39$ ,  $P = 0.320$ ), or the day (linear mixed-effects model:  $F_{2, 348} = 1.92$ ,  $P = 0.149$ ; Figure 3A). Likewise, the fish assemblage  $\times$  day interaction ( $F_{4, 344} = 0.10$ ,  $P = 0.980$ ), fish assemblage ( $F_{2, 6} = 0.905$ ,  $P = 0.453$ ), and day ( $F_{2, 348} = 2.30$ ,  $P = 0.101$ ) did not have significant effects on Rainwater Killifish SL (Figure 3B). Due to small increases in SL and weight throughout the experiment, Rainwater Killifish exhibited no change in  $K_n$  (Figure 3C), and there was no significant fish assemblage  $\times$  day interaction effect ( $F_{4, 344} = 0.14$ ,  $P = 0.967$ ), fish

assemblage effect ( $F_{2, 354} = 0.202$ ,  $P = 0.817$ ), or day effect ( $F_{2, 354} = 0.002$ ,  $P = 0.998$ ) on  $K_n$ . Throughout the experiment, survival of Rainwater Killifish remained high (Table 1).

Threespine Sticklebacks increased in weight during the experiment (Figure 4A). Threespine Stickleback weight was not significantly affected by the fish assemblage  $\times$  day interaction (linear mixed-effects model [with the tank effect as random error]:  $F_{4, 331} = 0.14$ ,  $P = 0.968$ ) or by fish assemblage ( $F_{2, 6} = 0.256$ ,  $P = 0.781$ ), but there was a significant effect of day ( $F_{2, 335} = 13.219$ ,  $P < 0.001$ ). Threespine Sticklebacks in all fish assemblages had an initial nonsignificant weight increase between day 1 and day 15 (Tukey–Kramer multiple comparison test:  $P = 0.653$ ), followed by a significant increase in weight between day 15 and day 29 ( $P = 0.0003$ ; Figure 4A). Changes in SL for Threespine Sticklebacks were similar to the trend observed with weight: the fish assemblage  $\times$  day interaction effect ( $F_{4, 331} = 0.29$ ,  $P = 0.882$ ) and the fish assemblage effect ( $F_{2, 6} = 0.213$ ,  $P = 0.814$ ) were not significant, whereas day had a significant effect on SL ( $F_{2, 335} = 18.363$ ,  $P < 0.001$ ; day 1 versus day 15:  $P = 0.1401$ ; day 1 versus day 29:  $P < 0.0001$ ; day 15 versus day 29:  $P = 0.0002$ ). Threespine Sticklebacks in all fish assemblages exhibited small increases in SL on day 15 and slightly greater increases on day 29 (Figure 4B). For the  $K_n$  of Threespine Sticklebacks (Figure 4C), the fish assemblage  $\times$  day interaction effect ( $F_{4, 331} = 0.42$ ,  $P = 0.792$ ) and the fish assemblage effect ( $F_{2, 6} = 0.061$ ,  $P = 0.942$ ) were not significant, but the effect of day was significant ( $F_{2, 335} = 4.16$ ,  $P < 0.05$ ; day 1 versus day 15:  $P = 0.0498$ ; day 1 versus day 29:  $P = 0.0259$ ; day 15 versus day 29:  $P = 0.9579$ ). Threespine Sticklebacks in all fish assemblages exhibited a decrease in  $K_n$  during the experiment. Threespine Sticklebacks experienced minor levels of mortality in all fish assemblages, but overall survival remained high (Table 1).

### Whole-Body Cortisol Levels

At the end of the 29-d trial, there was no significant effect of fish assemblage on cortisol level in Tidewater Goby (nested ANOVA:  $F_{6, 28} = 1.125$ ,  $P = 0.373$ ; Figure 5A), Rainwater Killifish (Kruskal–Wallis rank-sum test:  $\chi^2 = 2.17$ ,  $df = 2$ ,



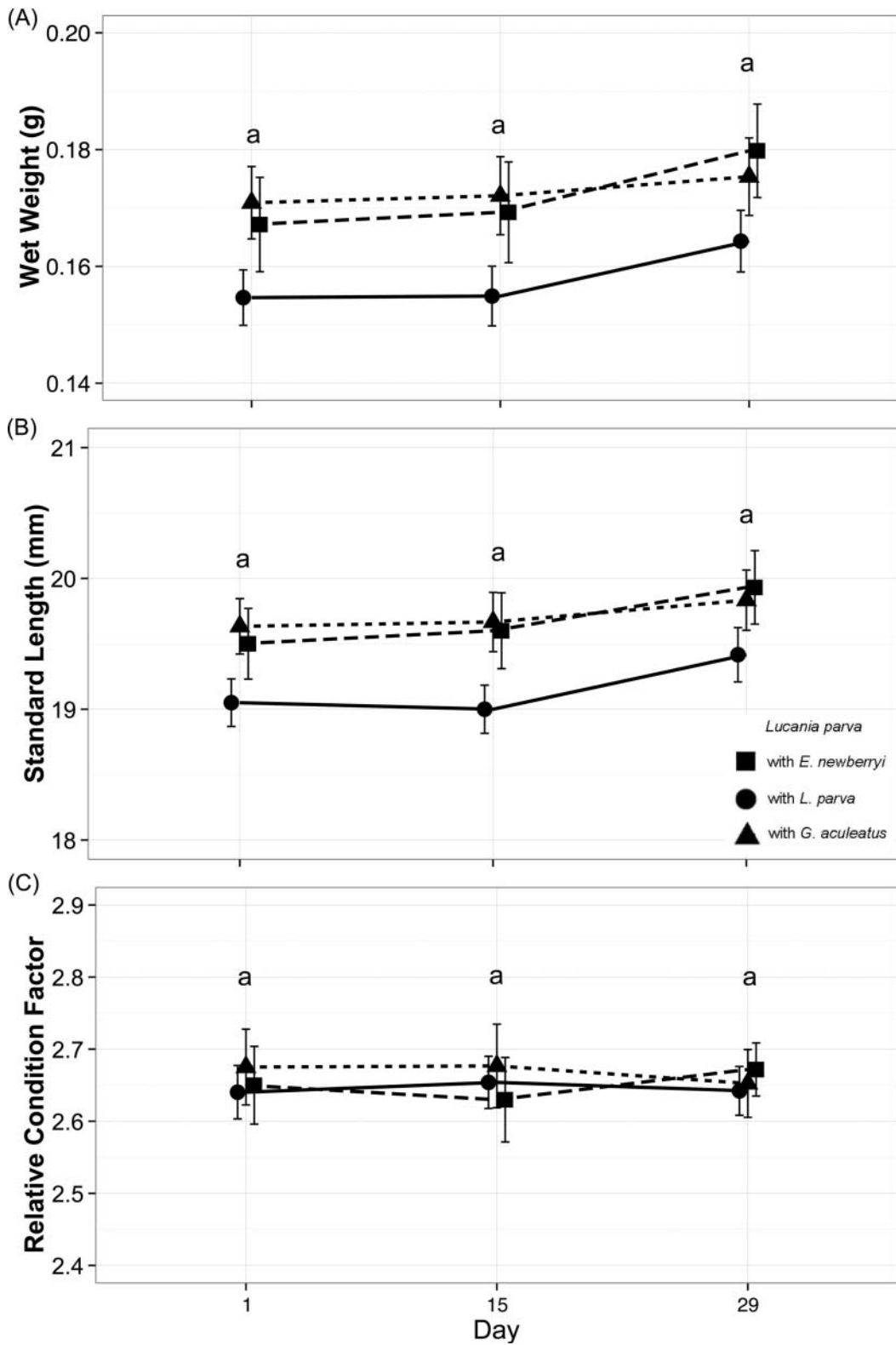


FIGURE 3. (A) Wet weight, (B) SL, and (C) relative condition factor of Rainwater Killifish *Lucania parva* on days 1, 15, and 29 in the presence of conspecifics (circles), Tidewater Goby *Eucyclogobius newberryi* (squares), or Threespine Sticklebacks *Gasterosteus aculeatus* (triangles). Data represent mean  $\pm$  SE for 30–60 fish. Differing letters represent significant differences ( $P < 0.05$ ) between days for all assemblage treatments combined.

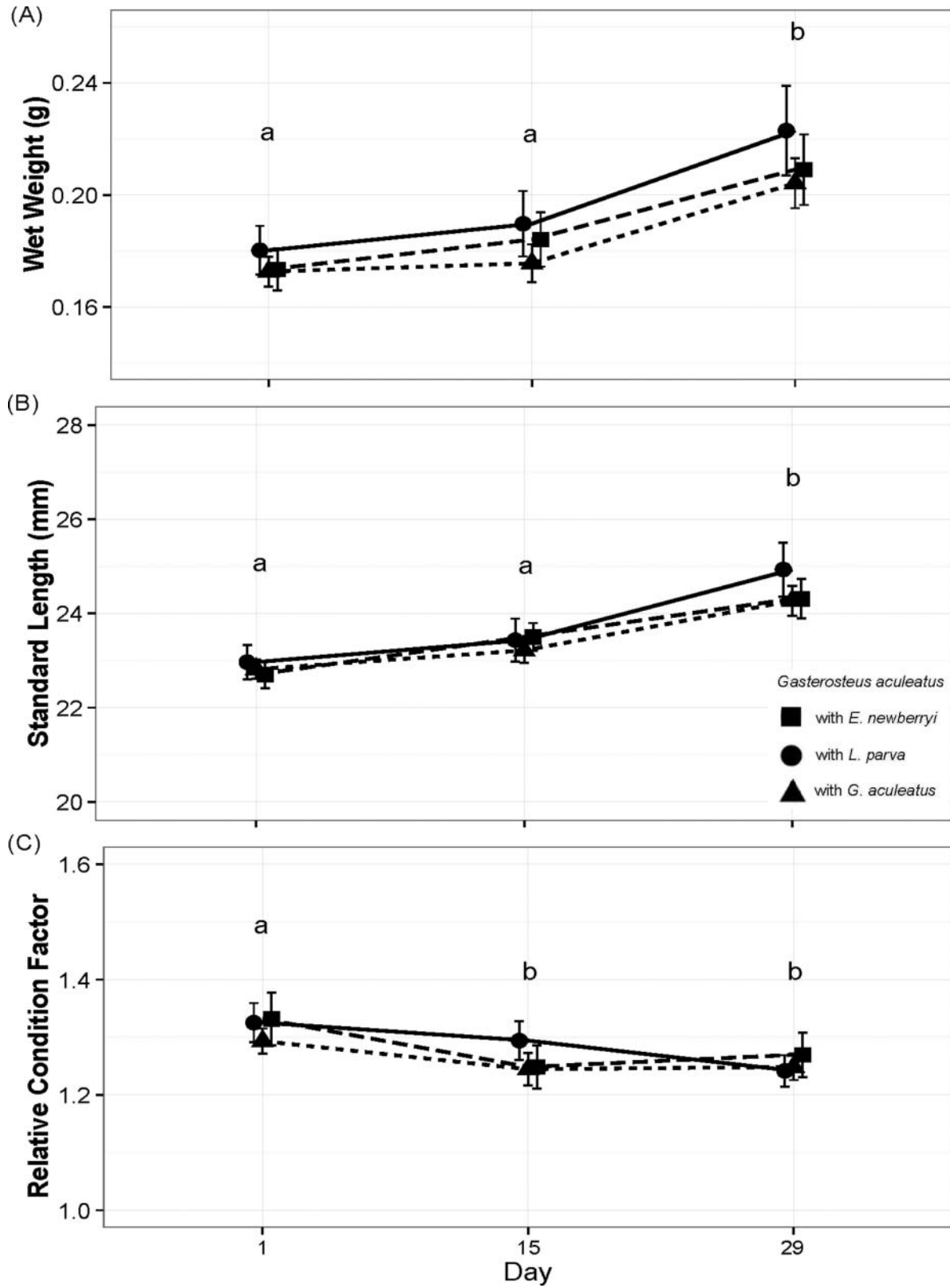


FIGURE 4. (A) Wet weight, (B) SL, and (C) relative condition factor of Threespine Sticklebacks *Gasterosteus aculeatus* on days 1, 15, and 29 in the presence of conspecifics (triangles), Tidewater Goby *Eucyclogobius newberryi* (squares), or Rainwater Killifish *Lucania parva* (circles). Data represent mean  $\pm$  SE for 30–60 fish. Differing letters represent significant differences ( $P < 0.05$ ) between days for all assemblage treatments combined.

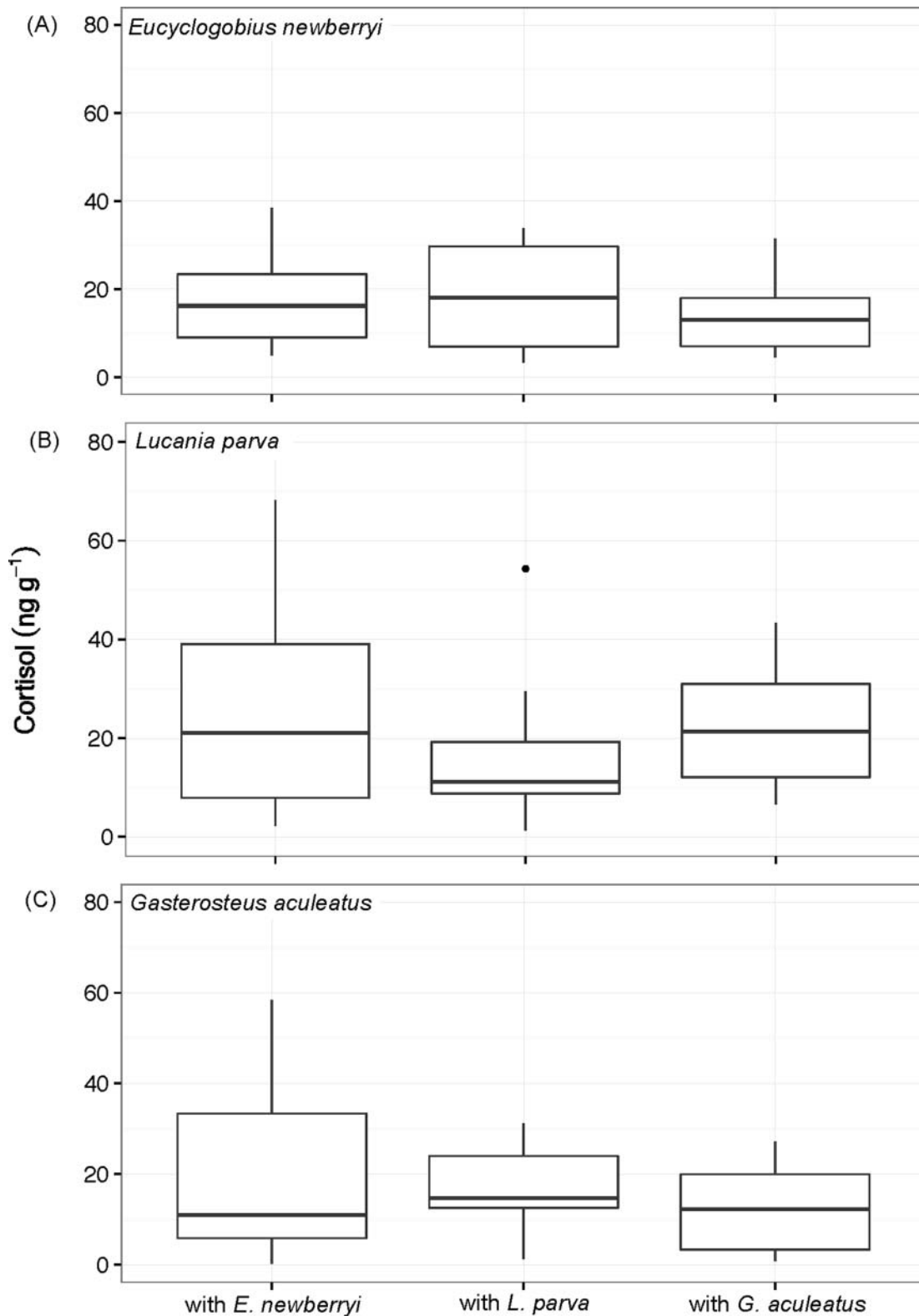


FIGURE 5. Cortisol levels (ng of cortisol/g of fish) measured on day 29 for fish within each assemblage treatment: (A) Tidewater Goby *Eucyclogobius newberryi* when in the presence of conspecifics ( $n = 11$ ), Rainwater Killifish *Lucania parva* ( $n = 14$ ), or Threespine Sticklebacks *Gasterosteus aculeatus* ( $n = 12$ ); (B) Rainwater Killifish when in the presence of conspecifics ( $n = 14$ ), Tidewater Goby ( $n = 18$ ), or Threespine Sticklebacks ( $n = 12$ ); and (C) Threespine Sticklebacks when in the presence of conspecifics ( $n = 9$ ), Tidewater Goby ( $n = 10$ ), or Rainwater Killifish ( $n = 9$ ). The lower and upper boundaries of the box represent the third and first quartiles, respectively, the lines within the box represent the median, black circles represent outliers, and the whiskers represent upper and lower values within  $1.5 \times$  the interquartile range.

$P = 0.337$ ; Figure 5B), or Threespine Sticklebacks (nested ANOVA:  $F_{6, 19} = 0.508$ ,  $P = 0.795$ ; Figure 5C). Higher variability in measured cortisol levels for Rainwater Killifish in the presence of Tidewater Goby was due to the results for one tank, in which six individual Rainwater Killifish exhibited cortisol levels ranging from 35.2 to 66.7 ng/g.

## DISCUSSION

Successful reintroduction of an extirpated species requires not only suitable habitat but also an understanding of the potential effects of negative social or competitive interactions with species that already occupy the location. This may be especially true (1) for interactions with species that were introduced after the species of concern was extirpated; or (2) when the species of concern and the other species are not found in overlapping locations. In the present study, we were interested in understanding the interactions between juvenile Tidewater Goby and two species—one native (Threespine Stickleback) and one nonnative (Rainwater Killifish)—that are currently present at San Francisco Bay Estuary habitats once occupied by Tidewater Goby. The impact of different species assemblages in the absence of food limitations was assessed by determining juvenile survival, growth, and cortisol concentration, with the objective of supporting evaluation processes for potential sites of Tidewater Goby reintroduction.

### Growth

Under ample food conditions, Tidewater Goby did not exhibit negative impacts associated with either the Rainwater Killifish assemblage or the Threespine Stickleback assemblage. A positive growth response and high survival were recorded for Tidewater Goby in each fish assemblage. There was no indication that the presence of Rainwater Killifish or Threespine Sticklebacks affected the ability of juvenile Tidewater Goby to reach sexually mature sizes ( $>27$  mm SL; Swift et al. 1989). Although interactions between Tidewater Goby and nonnative species have not been thoroughly investigated, Striped Bass *Morone saxatilis*, centrarchids, and African clawed frogs *Xenopus laevis* have all been documented as preying upon Tidewater Goby. Under laboratory conditions, Tidewater Goby were aggressively intimidated, outcompeted, and preyed upon by Shimofuri Goby *Tridentiger bifasciatus* (Lafferty and Page 1997; USFWS 2005). Competition with Rainwater Killifish and nonnative Yellowfin Goby *Acanthogobius flavimanus* has also been speculated to affect Tidewater Goby populations (Lafferty and Page 1997; USFWS 2005; Lafferty et al. 2010). However, we demonstrated that when food was not limited, Tidewater Goby had no negative interactions with Rainwater Killifish over a 29-d period. Our observations for Tidewater Goby when held with Rainwater Killifish are contrary to the effects shown for similar nonnative taxa in California. Mosquitofishes *Gambusia* spp., which occupy a

similar ecological role as Rainwater Killifish, have been shown to reduce juvenile survival and growth in native fishes (Jordan 2002; Rincon et al. 2002; Mills et al. 2004). There is concern that a novel, nonnative species would have a different and adverse effect on Tidewater Goby than a co-occurring native species. Our results suggest that in the absence of food limitation, Tidewater Goby do not respond negatively to interactions with a novel, nonpredatory species. Additional research is needed to enhance our understanding of how Tidewater Goby interactions with other species might change under more realistic environmental conditions that reflect estuarine variability and patchy food availability (Cloern and Jassby 2012).

Although the observed growth pattern for a given species was consistent across the different fish assemblages, there were species-specific differences in growth throughout the experiment. The growth trend for Tidewater Goby in all assemblage treatments was consistently positive, whereas only small increases in growth over the experimental period were detected for Rainwater Killifish. In studying Rainwater Killifish growth under laboratory conditions, Dunson and Travis (1991) identified a greater increase in weight over the same experimental duration. The lower growth in the present study may be attributable to the cooler water temperature we used ( $17^{\circ}\text{C}$ ) relative to that used by Dunson and Travis (1991;  $26^{\circ}\text{C}$ ).

Positive growth of Threespine Sticklebacks was found within each assemblage tested; however, growth during the last 2 weeks of the experiment was significantly greater than growth during the first 2 weeks. Threespine Sticklebacks are more aggressive upon first interaction with conspecifics (Utne Palm and Hart 2000), and our results provide evidence that this increased initial aggression extends to interactions with other species as well. Utne Palm and Hart (2000) reported that juvenile Threespine Sticklebacks were less aggressive with conspecifics over time, as aggression levels dropped after 2 weeks and further decreased after 4 weeks. Our findings support a reduction in conspecific aggression as well as interspecific aggression by Threespine Sticklebacks: growth increased greatly for Threespine Sticklebacks in each assemblage treatment after 2 weeks, possibly representing a switch in energy allocation from aggressive behavior to growth.

### Whole-Body Cortisol Levels

Social interactions have been shown to affect cortisol levels, with subordinate fish in a size-matched hierarchy experiencing elevated and chronic stress when in the presence of a dominant fish (Sloman et al. 2001; DiBattista et al. 2006). Assemblage treatment did not appear to manifest negatively as chronic stress for any of the species during our study. The cortisol levels measured for Threespine Sticklebacks in the current experiment (1.3–58.4 ng/g; Figure 5C) were well below the reported range for stressed fish (Bell et al. 2007; Pottinger

et al. 2011). The cortisol levels we report for Tidewater Goby (3.3–33.3 ng/g; Figure 5A) and Rainwater Killifish (1.3–66.7 ng/g; Figure 5B) represent the first published values for these species; hence, there are no previous values with which to compare our measurements and characterize the level of stress. Given that growth was not negatively impacted during acclimation to laboratory conditions or under different species assemblage treatments, our study fish were unlikely to have been experiencing chronic stress (Barton and Iwama 1991). However, previous research on Golden Shiners *Notemigonus crysoleucas* and sturgeons *Scaphirhynchus* spp. has shown that cortisol levels reflecting the basal or unstressed condition in some species are indicative of stress in other species (Barton et al. 2000; Lankford et al. 2005; Sink et al. 2007). Furthermore, higher fish densities in Yellow Perch *Perca flavescens* can attenuate the cortisol response (Haukenes and Barton 2004). Therefore, additional research is needed to characterize cortisol levels that represent the unstressed condition. Due to our sampling regime, we did not measure cortisol until after the 28-d feeding trial was complete, so we were unable to assess potential differences or acute changes in cortisol level due to (1) the holding density experienced by the wild fish in the laboratory or (2) initial interactions between species once the assemblage treatments had begun. Further research into the range of cortisol levels exhibited by Tidewater Goby and Rainwater Killifish in response to changes in environmental and experimental conditions will be needed to better understand their generalized stress responses under laboratory conditions and in nature.

### Species Interactions and Competition

The ample food provided during the experiment may have prevented adverse species interactions or competition, as excess prey availability ensured that all individuals in a tank had access to prey, thus limiting fish interactions during feeding. Although feeding behavior was not quantitatively evaluated, visual observations suggested that interaction between fish during feeding was limited. Tidewater Goby feeding behavior was consistent with descriptions of their midwater feeding during both field and laboratory experiments (Swenson and McCray 1996; Swenson 1999). Threespine Sticklebacks also pursued and consumed prey in the middle to upper portions of the water column, similar to the species' previously documented feeding behavior (Hart and Gill 1992). The primary feeding approach used by Threespine Sticklebacks often led them to feed higher in the water column than Rainwater Killifish, as the latter species primarily preyed upon *Daphnia* that swam to the tank bottom and were closer to the substrate. This spatial partitioning during feeding may have reduced the potential for "scramble" competition (Ward et al. 2006) between Rainwater Killifish and the Tidewater Goby or Threespine Sticklebacks. However, scramble competition between the two native species was apparent: on multiple

occasions, Threespine Sticklebacks were observed to dart in front of Tidewater Goby to consume prey (D. A. Chase, personal observation). The quantity of food administered at each feeding period likely precluded competition for food, as the more dominant individuals were unable to prevent the less-dominant fish from accessing prey (Johnsson et al. 2005). Additional research will be required to determine (1) whether limited food resources would affect growth and survival during interactions between these species and (2) how variability in fish density could factor into the intensity of competition (Mittelbach 1988; Bohlin et al. 2002).

Adverse social interactions, including competition, can manifest at different life stages and can have conservation implications if recruitment, growth, and reproduction are affected (Johnsson et al. 2005; IEP-MAST 2015). Our experimental results indicated that the effects of nonnative and native species interactions on juvenile Tidewater Goby growth did not differ from the effects of conspecific interactions. However, the impacts of Rainwater Killifish on Tidewater Goby may be more pronounced at other life stages. Tidewater Goby have a pelagic larval stage in which newly hatched embryos are only 4–5 mm TL; therefore, early life stages may be susceptible to predation by Rainwater Killifish, which could lead to reduced recruitment (Dunson and Rowe 1996; Swenson 1997). It should be noted that potential predation on the pelagic Tidewater Goby larvae would not be limited to Rainwater Killifish, as Threespine Stickleback adults can consume prey up to 8 mm TL (Hart and Gill 1992). Tidewater Goby larvae would thus be susceptible to predation by both species, with potential effects on recruitment. Furthermore, our laboratory experiments manipulated two-species fish assemblages at one density in the absence of predators and did not represent natural species assemblages in the wild. Habitat use and food selection by juvenile fish have been documented to change when predatory species are detected, so the presence of piscivores might force Tidewater Goby and Rainwater Killifish to compete for cover and available invertebrate prey within submerged aquatic vegetation beds (Mittelbach 1981; Jordan 2002).

### Conclusions

Based on our experimental results obtained under conditions of stable brackish salinity, stable temperature, and ample food availability, the nonnative Rainwater Killifish and the native Threespine Stickleback do not represent a significant risk to Tidewater Goby reintroduction. Tidewater Goby did not demonstrate reduced survival, diminished growth, or elevated cortisol levels when in the presence of either species. We presented the first published cortisol levels for Tidewater Goby and Rainwater Killifish, and this information will supplement future studies evaluating cortisol levels in stressed fish and field populations. Furthermore, our study provided the first weight–length relationship and  $K_n$  values for Tidewater

Goby from a wild population, which can be used for future monitoring of existing Tidewater Goby populations as well as those that are reintroduced to areas of extirpation. Our observations of fish behavior during the experiment support the potential for competitive interactions to occur between these species if food resources become limited. Although estuaries are viewed as productive habitats that are typically conducive for juvenile fish growth, variability in abiotic conditions and pollution from urban runoff can reduce productivity and impact food web dynamics in estuarine systems. Limitations in food availability across seasons and as an indirect result of climate change are likely to occur (Parker et al. 2011). Therefore, competition for food between the Tidewater Goby and other native and nonnative species should be examined in future studies to more accurately assess the potential for negative species dynamics within estuarine habitats considered suitable for Tidewater Goby reintroduction.

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