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Short communication

Establishing optimal broodstock sex ratios for the royal gramma (*Gramma loreto*) in small scale system aquaculture

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A R T I C L E I N F O

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A B S T R A C T

The royal gramma *(Gramma loreto)* is vulnerable to anthropogenic impact in its coral reef habitats, and is deemed an "indicator" species of pollution. It is also heavily fished for the aquarium trade, being among the top 1% of imported marine aquarium fish species into the U.S. The objective of this study was to establish optimal sex ratios of broodstock groups for aquaculture as an alternative to wild capture, while balancing territorial aggression. Three two-month breeding trials were carried out in six 23 L aquaria stocked with one male and one, two, or three females. During the trials, each aquarium was filmed weekly for one hour at first light to record courtship, nest use, and aversive behaviors. Behaviors were quantified with JWatcher software and analyzed in repeated measures ANOVAs. Results suggest that in small aquaria, a sex ratio of one male to two females may be optimal for spawning because courtship and nest use behaviors are optimized while aversive behaviors are moderated. Past efforts to aquaculture the species have typically been conducted at broodstock sex ratios of 1:1. These results offer the potential to increase productivity of commercial aquaculture of the species.

1. Introduction

The royal gramma *(Gramma loreto)*, a popular fish traded in the marine aquarium industry, is representative of coral reef fishes facing multiple threats to the sustainability of its populations. It is a small planktivore found on shallow reefs around Bermuda and the Bahamas, through the Greater and Lesser Antilles to the islands off Venezuela and throughout the Western Caribbean (Böhlke and Chaplin, 1968; Robins et al., 1986). It is especially sensitive to sewage-based pollution. Its decline in abundance near sewage outfalls led Duque et al. (2010) to identify it as an "indicator species." The royal gramma is also preyed upon by the invasive Indo-Pacific lionfish (*Pterois volitans,* Albins and Hixon, 2008), causing further depletion of wild populations. These effects are compounded by heavy fishing pressure for the marine aquarium trade. Out of 1802 fish spp. imported into the U.S. for the industry, it ranks among the top 1% of imports, with ~86,000 individuals coming into the country annually (Rhyne et al., 2015). In Puerto Rico, one of its export countries, this species is the most commonly exported fish (12, 520 fish annually) in the country's marine ornamental collection industry (LeGore et al., 2005).

The royal gramma is thus a candidate to develop for commercial aquaculture as a sustainable alternative to wild harvest. Additional features of its candidacy include its ease of culture and its potential profitability. It provides paternal care to eggs until relatively large and robust larvae are hatched, facilitating larval rearing by culturists (Wittenrich, 2007). Its average U.S. retail price point of $22 ranks the royal gramma as the 8th most valuable among the U.S.'s top 20 imports (P. Anderson, unpublished data).

The royal gramma has been successfully aquacultured (Wittenrich, 2007), yet it is not commonly encountered in commercial aquaculture production. This may be in part due to its lower fecundity relative to other species, rendering the economic viability of an aquaculture venture of this species tenuous. Current breeding recommendations for broodstock are to house them in pairs (Wittenrich, 2007). However, in nature, aggregations of a dozen or more royal grammas occur in association with large coral heads, caves, and ledges, and spawn in social groups consisting of 1–2 males and 3–9 females (Asoh and Shapiro, 1997). Nest-holding males spawn routinely with multiple females, with nests simultaneously containing eggs from multiple females (Asoh, 1996; Asoh and Yoshikawa, 1996). Therefore, an artificially manipulated broodstock social structure of a pair of fish in an aquaculture setting may not take advantage of the full reproductive and parental care

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output of a given male. However, there are difficulties in establishing broodstock groups. Adult males can be aggressive and territorial to conspecifics, especially conspecific males (Wittenrich, 2007). The objective of this study was to establish optimal sex ratios of *G. loreto* broodstock groups by investigating courtship, nest care, and aversive behaviors in small scale system aquaculture, while balancing territorial aggression.

2. Materials and methods

All protocols were approved by Mystic Aquarium's Institutional Animal Care and Use Committee (Protocol 15010).

2.1. Broodstock acquisition and quarantine

Wild-caught royal grammas were obtained through wholesalers and quarantined for a minimum of 35 days in a 590 L sea table divided into 23 × 22 cm ventilated compartments, each containing a 13 cm section of 5 cm diameter PVC pipe, capped at one end, to serve as a shelter. The system was filtered via mechanical sock filter and bio-balls, and thermoregulated at 23.3 °C ± 1.5 °C (mean ± SD, range 18.0 to 26.3 °C) with aquarium heaters. Artificial seawater was mixed using Instant Ocean™ sea salt (Spectrum Brands Holdings, Inc., Madison, WI), mixed with dechlorinated fresh water and kept at a salinity of 30.93 ± 2.07 ppt (range 27.30 to 38.50 ppt). Fish were maintained under the following water quality parameters, measured weekly: pH 8.25 ± 0.08, (range 8.00 to 8.46), NH_3 0.02 ± 0.08 mg/L (range 0.00 to 0.50 mg/L), NO_2 0.00 ± 0.02 mg/L (range 0.00 to 0.16 mg/L), NO_3 8.10 ± 13.41 mg/L (range 0.00 to 40.00 mg/L), and alkalinity 218 ± 8 meq/L (range 180 to 240 meq/L). This system was maintained with ambient fluorescent room lighting on a photoperiod of 12 L:12 D. Husbandry on this system consisted of siphoning debris every other day with water refill, constituting small frequent water changes, and weekly changing of the filter sock.

Over the 35-day quarantine period, fishes were exposed to a quarantine protocol advised by the attending veterinarian consisting of bath treatments: Chloroquine (10 mg/L), metronidazole (10 mg/L), oxolinic acid (10 mg/L), and/or praziquantel (2 mg/L); each treatment was followed by a 50% water change and the addition of 50 g of activated carbon to the system's sump for 24 h. In addition, a diet schedule of medicated gel feeds was supplied; the dosage of each medication was formulated per 1 g feed and 1 mL H_20: Amprolium (1.04 μL of 9.6% solution), fenbendazole (50 μL of 100 mg/mL suspension), metronidazole (13 mg), praziquantel (8 mg), and/or salinomycin (0.8 mg). These were mixed with a low-fat aquatic gel diet (Formula 5ME2, Mazuri®, Richmond, IN). In addition, fish were fed a mix of frozen krill, Mega Marine™, mysis (Hikari Sales USA, Hayward, CA); Formula One™ and Prime Reef™ (Ocean Nutrition, Newark, CA); TDO Chroma Boost™ (Reed Mariculture, Campbell, CA); and/or fish eggs (San Francisco Bay Brand, Newark, CA).

During the quarantine period and prior to experiment, fishes were anesthetized via immersion in 80–200 mg/L of MS-222 in aquarium water buffered 2:1 with sodium bicarbonate, and sexed by standard length measurement (males ≥4.5 cm, Asoh, 1996). Males were tagged with a white visible implant elastomer (VIE) tag using a manual elastomer injection system (Northwest Marine Technology, Inc., Anacortes, WA); VIE tags were applied subcutaneously to the dorsocaudal musculature.

2.2. Experimental system and design

2.2.1. External environment

An XR3 rack system (Pentair Aquatic Habitats®, Minneapolis, MN) comprised the experimental broodstock system. It consisted of a three-tier rack of six 23 L aquaria, 56 cm L x 30 cm W x 20 cm H. The system was maintained inside the Joint Aquaculture Research Laboratory, operated in partnership between the Marine Science Magnet High School and Mystic Aquarium. The system was situated in one corner of the laboratory and enclosed on the front and left side by a partition made of black plastic sheeting to minimize disturbance from laboratory workers. Four viewing flaps were cut into the front sheet to allow for unobtrusive observation with videocamera or the naked eye. Activity inside the compartment was limited to husbandry, feeding, and occasional tours that always occurred after filming for the day.

The laboratory was controlled for temperature and photoperiod. The system was illuminated by ambient fluorescent room lighting supplemented with an aquarium fluorescent hood lighting fixture hung 1.2 m in front of the system. Photoperiod was maintained at 11 L:13 D by a self-adjusting wall switch timer (Intermatic®, Libertyville, IL) on the room lights, and a digital light timer on the hood. A 30-min dawn and dusk interval was added to the photoperiod of trial 3 using the hood.

2.2.2. System parameters

The system was filtered by a pre-filtration sock, submerged bio-balls, 50 μm pleated cartridge filter, and UV disinfection (dose of 110 mJ/cm2 toward the end of lamp life). The biofiltration media was pre-cycled via 30-day bath in a biofiltration seeding system routinely operated by Mystic Aquarium prior to addition of fish in the system. Artificial seawater was mixed using Instant Ocean™ sea salt mixed with dechlorinated fresh water and kept at a salinity of 29.87 ± 0.80 ppt (mean ± SD, range 28.00 to 32.00 ppt); and thermoregulated at 24.8 °C ± 1.4 °C (range 22.1 to 28.0 °C). Fish were maintained under the following water quality parameters, measured weekly: pH 8.23 ± 0.18 (range 7.40 to 8.40), NH_3 0.00 ± 0.00 mg/L (range 0.00 to 0.00 mg/L), NO_2 0.00 ± 0.00 mg/L (range 0.00 to 0.00 mg/L), NO_3 14.81 ± 11.64 mg/L (range 0.00 to 30.00 mg/L), and alkalinity 215 ± 8 meq/L (range 200 to 220 meq/L). Tanks were siphoned of debris every other day and refilled, constituting small frequent water changes; the pre-filtration sock and 50 μm pleated cartridge filter were cleaned weekly, and tanks scrubbed as needed.

2.2.3. Establishing sex ratios

Three trials, two months each in duration, were conducted to determine an optimal sex ratio of harem groups in small (23 L) aquaria. Socio-sexual groups were created with male to female (M:F) sex ratios of 1:1, 1:2, and 1:3. At the start of each trial, two sample groups of each sex ratio were established. Thus, a sample size of 6 groups of each sex ratio were tested over the course of the three trials. On the first day of each trial, 1–3 randomly selected putative females (< 4.5 cm, Asoh, 1996) were pre-stocked into tank one. On day two, tank one was subsequently stocked with one male (≥ 4.5 cm, Asoh, 1996), tagged with a white VIE tag. Tank two was then pre-stocked with 1–3 randomly selected putative females. On day three, a single male was introduced into tank two, and tank three was stocked with 1–3 randomly selected females. This method was repeated for stocking all six tanks for each trial.

2.3. Broodstock management

This experiment was conducted using an agile approach (Project Management Institute, 2017), using observations from previous trials to modify husbandry procedures and broodstock habitats for better egg production and hatching success in subsequent trials, for other studies. The variables described below thus differed for each trial, but were statistically accounted for by modeling for trial effect in the resulting ANOVAs (see section 2.5).

2.3.1. Nutrition and nest design trial #1

Broodstock were fed twice daily to satiation with frozen mysis (Hikari Sales USA, Hayward, CA) and TDO Chroma Boost™ (Reed Mariculture, Campbell, CA). Four nests were added to each broodstock tank; nests consisted of 13 cm lengths of 5 cm diameter PVC, capped at one end (Fig. 1A). Natural *Chaetomorpha* spp. algae was provided in the tanks for fish to use as nesting material. Broodstock tanks were surface

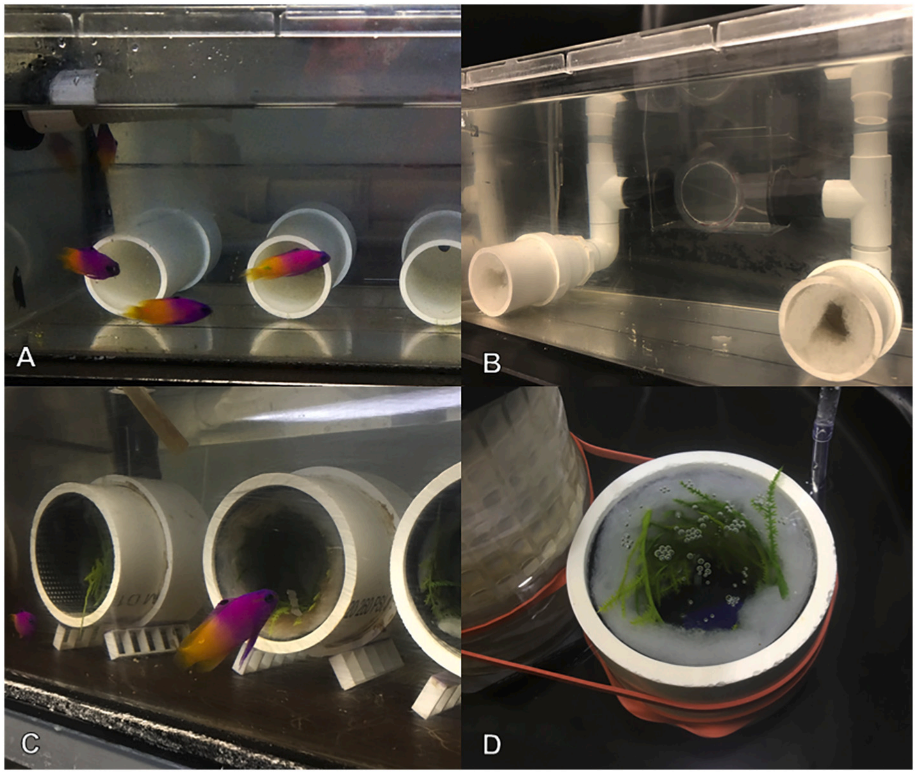

Fig. 1. Nests used in trials. A) Trial #1 PVC nests, B) trial #2 larval collectors, C) trial #3 nests that doubled as D) egg incubators.

skimmed into 1.9 L buckets designed to trap larvae; water was subsequently surface skimmed from buckets via strainers fitted with 200 μm screen to retain larvae in buckets.

2.3.2. Nutrition and nest design trial #2

A varied diet of one single-ingredient and three blended frozen feeds was selected for feeding four times daily to satiation. The blended diets were chosen because multiple ingredients were considered to provide a variety of nutrients necessary for egg production. Diet items chosen included mysis and Mega Marine™ (Hikari Sales USA, Hayward, CA), and Formula One™ and Prime Reef™ (Ocean Nutrition, Newark, CA).

Larval collectors, a type of nest to facilitate capture of larvae, were modified from a published design (Gilles et al., 2008) and installed in broodstock tanks (Fig. 1B). The collectors consisted of two 7 cm lengths of 5 cm diameter PVC fitted with polyester batting and a 5 mm screen at one end to serve as nests; there were two collectors per tank. The nests were then reduced to 1.9 cm PVC, following a 90° elbow up to a tee that deposited water into a specimen container to collect and isolate larvae from adults. A 5.7 cm diameter hole was drilled in the middle of the specimen container and fitted with a 200 μm screen to allow water flow out of the container. Water flow from the nests to the specimen container was driven by the placement of a rigid airline tube into the PVC arms down to the elbow.

2.3.3. Nutrition and nest design trial #3

The trial 3 feeding regime was modified based on feeding response and palatability. One frozen food blend and three marine origin single ingredient frozen foods were selected: Mega Marine™, krill, mysis (Hikari Sales USA, Hayward, CA), and fish eggs (San Francisco Bay Brand, Newark, CA). Fish were fed this diet to the point of satiation.

The design of trial 3 nests facilitated the discovery of egg clutches, and served a duplicate function as egg incubators for manual hatching of eggs in larval rearing tanks (Fig. 1C, D). Four nests per experimental tank were constructed, each out of 7.0 × 7.6 cm diameter PVC pipe, capped at one end. Furniture batting and plastic faux leaf garland was stitched with fishing line to a 22.9 × 6.4 cm semi rigid (#7) plastic mesh with square apertures of 2.8 mm in length, which maintained the cylindrical structure of the batting. Two single column rows of diffraction grating were mounted to the bottom of caps to act as stands. PVC cylinders containing the nests could be disengaged from the caps, rotated 90°, affixed inside larval rearing tanks and aerated by an airstone placed at the bottom of the cylinder to incubate eggs manually.

2.4. Data collection

2.4.1. Filming courtship behavior

Groups were filmed with a Sony Handycam NEX-VG30GH (HD, 60i frame rate) starting on the day following male introduction during the first hour of light (as male courtship occurs at dawn, Asoh, 1996), and then filmed once weekly thereafter for a total of eight weeks. The videographer followed the male and any females he may have been interacting with during recording; males were identified by white VIE tag.

2.4.2. Courtship and spawning behavior

Behaviors were identified and quantified based on an ethogram of behaviors observed in naturally occurring harems of royal grammas (Asoh, 1996; Asoh and Yoshikawa, 1996; Table 1). Behaviors were categorized as aversive, courtship, and nest use. Aversive behaviors were any aggressive or avoidance behaviors displayed in relation to other individuals, such as a mouth attack or to swim away. Courtship behaviors consisted of body displays from males toward females, with females either following or parting ways from males in response. Nest use behaviors included nest entries and maintenance.

2.5. Data analysis

Fish behavior from video review was quantified using JWatcher© software (v 1.0, Blumstein and Daniel, 2007) to quantify aversive, courtship, and nest use behaviors of males and females. Videos were coded by a single, consistent coder for each trial. While the coders

Table 1
Ethogram of aversive, courtship, and nest use behaviors of the royal gramma (*Gramma loreto*), adapted from Asoh (1996) and Asoh and Yoshikawa (1996).

Name	Definition
Aversive behaviors	
Bite	Attacks a fish using its mouth.
Chase	Drives an approaching fish away.
Mouth attack	Opens mouth widely toward an approaching fish.
Swim away	Withdraws from an approaching fish.
Courtship behaviors	
Follow	In response to an approach and courtship display from another fish, attends fish all the way into a nest.
Part way	In response to a fish approach and courtship display, follows fish incompletely, and then returns to feeding site.
Quiver	Faces a fish and shakes its body.
U – turn with stop	Rapidly swims toward a fish with median and paired fins flattened against the body, stops in front of fish, faces fish for a brief moment with median and paired fins erect, and then turns quickly and swims rapidly toward or into a nest with median and paired fins flattened against the body.
Simple U – turn	Rapidly swims toward a fish with median and paired fins flattened against the body, makes a quick turn in front of fish, and then swims rapidly back to or into a nest.
U – turn with quiver	Rapidly swims toward a fish with median and paired fins flattened against the body, stops in front of fish, then the anterior part of body shakes slightly while facing fish with median and paired fins erect. Then makes a quick turn and swims rapidly toward or into a nest with median and paired fins flattened against the body.
Nest use behaviors	
Detritus clear	Enters a nest, remains for a short period (mean ± SD = 19.5 ± 10.4 s and range = 3–41 s), then exits with mouth closed, and then opens mouth widely and spits a cloud of silt into the water column.
Hover	Remains stationary under or in front of nest entrance.
Nest maintenance	Pushes, trims, or adds pieces of algae to the algal mat at a nest entrance with mouth; or carries pieces of algae inside a nest. May first spit the algae out, then retrieve portions of it to carry into a nest. Typically repeats the spitting then retrieving sequence two to three times before finally swimming back to the nest.
Protrude	Remains inside a nest at the nest entrance with head sticking out from the nest.
Simple nest enter	Enters a nest without carrying algae or removing silt. Remains inside for a period of time (mean ± SD = 23 ± 61 s and range = 2–440 s) before exiting.

differed among trials, this effect was also designed to be partitioned out as part of the trial effect in the ANOVAs. Male behavioral measures were corrected for time in sight, and female aversive behaviors were divided by the number of females in the tank to achieve a measure of aversive behaviors performed per female. Measures were subsequently rank transformed and tested in repeated measures ANOVAs, assessing the main fixed effects of the *number of females* and *week*, the block effect of *trial* (to account for the aforementioned lighting, nutrition, nest-design, and coder variables), and *tank* (= subject) effect, nested within the *number of females* and *trial*. The interaction effects of *number of females* * *week, number of females* * *trial*, and *week* * *trial* were also included in the model to partition out additional variability from the dataset. The effect of interest was the *number of females.* Tukey's tests were used to discriminate significant differences among levels of the *number of females* ($n = 1$, 2, or 3 females) when found to be significant. Statistical tests were conducted in Minitab (v. 17, State College, PA).

3. Results

3.1. Courtship behaviors

There was a significant effect of the *number of females* on the number of courtship behaviors performed by males (Repeated Measures ANOVA, $F_{2,78} = 5.94$, $p = 0.004$). Males in tanks with one or two females performed more courtship behaviors per hour (0 < 12 < 41, 1st quartile < median < 3rd quartile, respectively) than did males in tanks with three females (0 < 0 < 1, Tukey's Test, $p \leq 0.010$, Fig. 2A).

Female courtship behavior was a rare occurrence overall, hence the flat lines shown for median measures of this behavior in tanks with one and three females in (Fig. 2B). However, repeated measures ANOVA verified that there was a significant effect of the *number of females* on the number of courtship behaviors performed by females (Repeated Measures ANOVA, $F_{2,86} = 12.60$, $p < 0.001$). Females in tanks with two or three females performed more total courtship behaviors per hour (0 < 0 < 1) than did females in tanks with one female (0 < 0 < 0, Tukey's Test, $p < 0.002$, Fig. 2B).

3.2. Nest use

Males spent time in nests to maintain them, guard them, and mate with females in them. There was a significant effect of the *number of females* on the amount of time males spent using nests (Repeated Measures ANOVA, $F_{2,78} = 10.92$, $p < 0.001$). Males spent more time using nests in tanks with one or two females (10 < 17 < 28 min) than in tanks with three females (4 < 5 < 9 min, Tukey's Test, $p \leq 0.001$, Fig. 3A).

Presumably, females primarily use nests for the purposes of mating with males. There was a significant effect of the *number of females* on the number of times females entered nests (Repeated Measures ANOVA, $F_{2,86} = 12.60$, $p < 0.001$). Nests were entered more often by females in tanks with two or three females (1 < 5 < 13) than in tanks with one female (0 < 0 < 7, Tukey's Test, $p \leq 0.002$, Fig. 3B).

3.3. Aversive behavior

There was a significant effect of the *number of females* on the number of aversive behaviors males displayed toward females (Repeated Measures ANOVA, $F_{2,78} = 6.00$, $p = 0.004$). Males displayed aversive behaviors more often in tanks with three females (12 < 22 < 66) than in tanks with one or two females (2 < 4 < 10; Tukey's Test, $p \leq 0.007$, Fig. 4A).

When the numbers of aversive behaviors performed by females were partitioned to each female, there was a significant effect of the *number of females* on the number of aversive behaviors each female displayed toward each other (Repeated Measures ANOVA, $F_{1,\,42} = 8.59$, $p = 0.005$). Females displayed aversive behaviors toward each other more often in tanks with three females (3 < 6 < 14) than in tanks with two females (1 < 3 < 7, Fig. 4B). Of course, females who were the only females in their tank performed no female-to-female aversive behavior.

4. Discussion

Results from this study suggest that in small (23 L) aquaria, a male kept at a 1 M:2 F sex ratio exhibits more courtship behaviors, spends more time in his nest, and exhibits more nest care and guarding behaviors than males kept at other sex ratios (1 M:1 F and 1 M:3 F). The time that females spent inside the nests, presumably exclusively for mating, were higher among groups of 1 M:1 F and 1 M:2 F sex ratios. Over time, male aggressive behaviors were lowest in the 1 M:1 F sex ratio groups, moderate in the 1 M:2 F ratio groups, and highest in the 1 M:3 F ratio groups, while female aggressive/aversive behaviors (per female) were lowest in a 1 M:2 F sex ratio, and highest in the 1 M:3 F sex ratio. Preliminary results thus suggest that a sex ratio of 1 M:2 F in small aquaria (23 L) is optimal for spawning because courtship and mating behaviors were maximized while aggressive and aversive behaviors were moderated. These results thus offer to improve upon the potential productivity of commercial aquaculture of the species.

Future work might rigorously test the effect of diet and nutrition on fecundity and egg viability. The broodstock nests should also continue to be optimized, so they may be attractive to and used by broodstock, easy

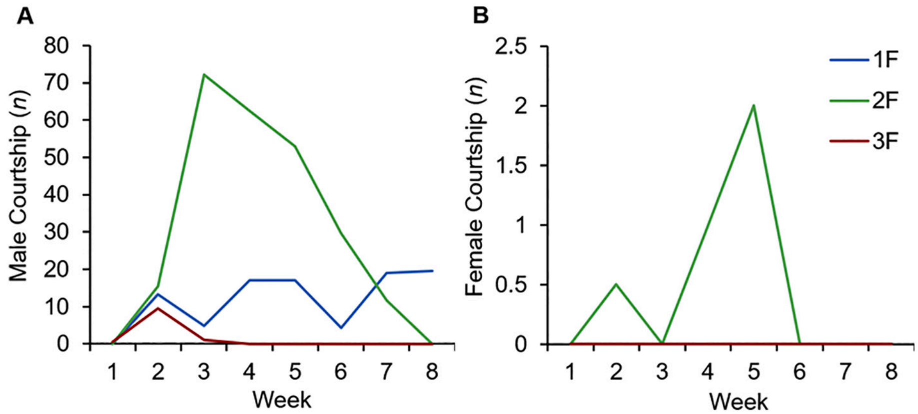

Fig. 2. Line graphs of median counts of the number of courtship behaviors performed by A) males and B) females.

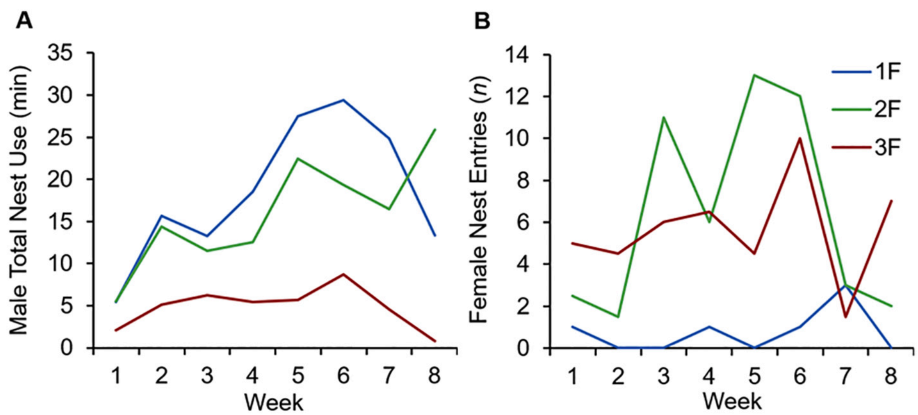

Fig. 3. Line graphs of A) median time males spent using nests, and B) median counts of the number of times females entered nests.

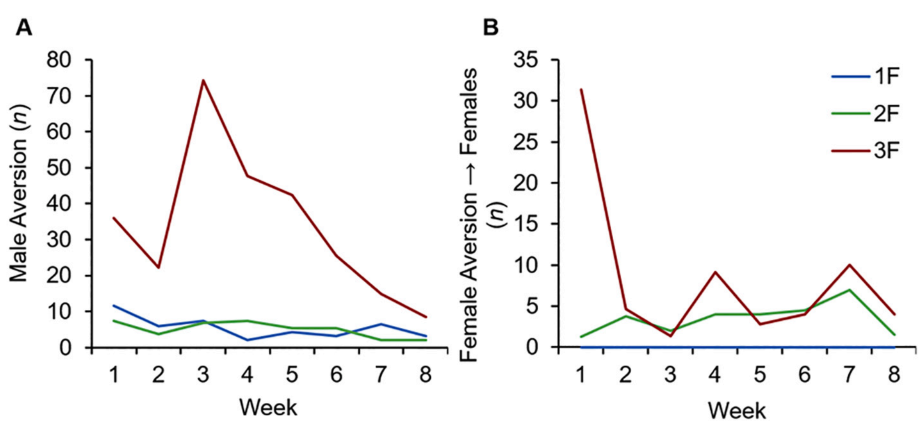

Fig. 4. Line graphs of median counts of the number of aversive behaviors performed by A) males and B) females toward other females.

to manipulate and investigate for the presence of eggs, and serve a dual purpose as effective egg incubators. Increasing aquarium size may allow for the opportunity to test larger nest structures, increase the number of nests per tank, and test different broodstock sex ratios.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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