Invasion frustration: can biotic resistance explain the small geographic range of non-native croaking gourami *Trichopsis vittata* (Cuvier, 1831) in Florida, USA?

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Abstract

Croaking gourami *Trichopsis vittata* is a non-native fish species that has maintained a reproducing population in Florida, USA, since at least the 1970s. However, unlike most other non-native fishes in Florida, *T. vittata* has not spread beyond its very small (ca. 5 km^2) range. We suspected the inability of *T. vittata* to colonize new habitats may be due to biotic resistance by the native eastern mosquitofish *Gambusia holbrooki*. In laboratory experiments, we show that *G. holbrooki* causes physical damage to *T. vittata* and that *T. vittata*'s growth is reduced in the presence of *G. holbrooki*. While the effects of *G. holbrooki* on *T. vittata* were sub-lethal, they were severe enough to hamper its growth and could affect recruitment in the wild. These results support the hypothesis that small non-native fishes may be excluded from establishment or may only establish small ranges due to pressure from *G. holbrooki*. Biotic resistance may reduce invasion success and thus consideration of species interactions is useful for natural resource managers trying to evaluate the potential risk of new invaders.

Key words: invasiveness, *Gambusia*, non-native fishes, sub-lethal effects

Introduction

A central question of invasion biology is: What factors facilitate or impede the geographic spread of an organism once it is introduced into a novel environment? In other words, why are some invasions successful while others fail? Multiple factors affect invasion success, including propagule pressure, abiotic constraints (i.e., environmental characteristics), species traits (life-history variables such as body size) and biotic resistance (e.g., predators, competitors). Successful invaders must overcome successive abiotic and biotic barriers to establish and spread, moving through a series of invasion stages in a step-wise fashion (Blackburn et al. 2011). First, there must be sufficient propagule pressure to overcome Allee effects and establish self-sustaining populations. Second, the newly-introduced species must be able to tolerate the abiotic conditions into which it has been introduced or move to a more suitable habitat. Finally, the potential

invader must be able to withstand the pressures of competition and predation by other species in the new environment to establish a population that persists over time and is capable of spreading across the landscape. This study focuses on the final factor, biotic resistance, specifically with respect to non-native fishes in Florida, USA.

In Florida's freshwater habitats, 128 species of foreign non-native fishes have been documented, and at least 36 have established reproducing populations (Robins et al. 2018; USGS-NAS 2020). The large difference in the number of documented versus established non-native fishes in Florida suggests the presence of barriers or factors that impede establishment. Identifying and understanding factors that facilitate or obstruct establishment and dispersal of non-native fishes may assist natural resource managers in assessing the risk of new invaders.

There are many non-native fish species that have failed to establish reproducing populations in Florida's environments, even though they seem to satisfy the first two requirements (i.e., sufficient propagule pressure and ecophysiological suitability). Small, colorful fishes such as platys/swordtails (*Xiphophorus* spp.), guppies (*Poecilia reticulata* Peters, 1859), tetras (family Characidae), and gouramis (family Osphronemidae) constitute a group of non-native fish species that are common in the aquarium trade, and this popularity may equate to increased propagule pressure as pets are released (Duggan et al. 2006). These fish are also farm-raised in Florida, from where they may escape (Tuckett et al. 2017; Hill and Tuckett 2018). However, despite expectations of significant propagule pressure and the possibility of ecophysiological suitability, these species rarely establish permanent feral populations (Tuckett et al. 2017). Furthermore, when they do establish it is usually only in a small, localized area (Tuckett et al. 2017). Sometimes these localized populations persist for decades, while in other cases they become extirpated after a few years (e.g., *Rocio octofasciata* (Regan, 1903); Lawson et al. 2017).

Biotic resistance has been invoked as a hypothesis that may explain the lack of establishment and spread of this group of small fishes (Hill et al. 2011; Thompson et al. 2012; Hill and Tuckett 2018). Biotic resistance is the ability of communities to limit invasion by non-native species, and several hypotheses have been developed around this central idea, including the dependence of biotic resistance on species richness of donor and recipient regions (Elton 1958; Fridley and Sax 2014), community saturation (Stachowicz and Tilman 2005), and dominant or strongly-interacting species in the introduced range (Wardle 2001; Henriksson et al. 2015). Herein, we evaluate this hypothesis for a non-native fish species that has not successfully spread in Florida, the croaking gourami *Trichopsis vittata* (Cuvier, 1831). We suspect its lack of geographic spread may be due to biotic resistance by the strongly-interacting, native eastern mosquitofish *Gambusia holbrooki* Girard, 1859.

Gambusia holbrooki is an aggressive small-bodied fish that typically reaches 6.0 cm total length (TL; Pyke 2005). It is native to fresh and brackish-water habitats of Atlantic and Gulf slope drainages in the southeastern USA (Page and Burr 2011). Locally abundant and widespread in Florida, it is the most common fish in the state (Robins et al. 2018). Its introduction across the world has been suspected of causing severe negative effects on native fauna (reviewed in Pyke 2008), resulting in its inclusion on the list of the world's worst 100 invaders (Lowe et al. 2000). Thompson et al. (2012) suggested that non-native fishes reaching > 15 cm TL could grow large enough to withstand *G. holbrooki* predation and eventually establish and spread in Florida. However, in comparison, small-bodied fishes (< 15 cm TL) were subjected to aggression, predation and competition by *G. holbrooki* that consequently inhibited their survival and reproduction.

Trichopsis vittata is a small-bodied gourami (Family: Osphronemidae) that reaches 6.5 cm TL (Schofield and Pecora 2013). Its native range includes Java, Borneo, Sumatra, peninsular Malaysia, Thailand, and the Mekong basin in Cambodia, Laos, and Vietnam (Kottelat 1985; Rainboth 1996; Norén et al. 2017). Non-native introductions have been reported from the Philippines (BFAR 2006 in Norén et al. 2017), India (Knight and Balasubramanian 2015), Myanmar (Norén et al. 2017), Bangladesh (Norén et al. 2017; Akash and Hossain 2018) and Florida, USA (Schofield and Pecora 2013). Most reports of non-native introductions note *T. vittata*'s presence in the aquarium trade as the most likely vector for its movement outside the native range. The species was first detected in Florida in the 1970s, and has persisted for more than four decades in a relatively small geographic area (less than 5 km^2) near Arthur R. Marshall (ARM) Loxahatchee Wildlife Refuge (Schofield and Pecora 2013). The species' limited spread in Florida is puzzling because it is more tolerant to ecophysiological variables such as salinity, low temperature, and low-oxygen than many other non-native fishes that have spread across south Florida (Schofield and Schulte 2016). To evaluate the hypothesis that *T. vittata* may be restricted from spreading by the presence of *G. holbrooki*, we studied interactions between the two species in laboratory experiments.

Materials and methods

The study was conducted in two parts to identify whether *G. holbrooki* exhibited aggression towards *T. vittata* (Part 1) and the ultimate outcome of the interaction in longer-term trials (Part 2). In Part 1 (Ruskin, FL) we documented fin damage and aggressive interactions between *T. vittata* and *G. holbrooki* when placed into mesocosm tanks containing artificial vegetation. In Part 2 (Gainesville, FL) *T. vittata* were placed into mesocosm tanks that mimicked a natural environment and contained a complement of native species (shrimp, fishes) and live vegetation. Goals for that experiment

Table 1. Summary of experimental design for mesocosm experiments.

Location	Time period	Trial	Length of experiment	Density of *T. vittata*	Density of *G. holbrooki*	Replicates
Ruskin	Summer 2014	–	15 days	11 m^{-2}	0	2
				11 m^{-2}	21 m^{-2}	5
				11 m^{-2}	86 m^{-2}	5
Gainesville	Summer 2014	1	30 days	11 m^{-2}	0	5
				11 m^{-2}	11 m^{-2}	5
	Spring 2015	2	30 days	11 m^{-2}	0	5
				11 m^{-2}	21 m^{-2}	5
	Spring 2019	3	29 days	11 m^{-2}	0	4
				32 m^{-2}	0	4
				11 m^{-2}	21 m^{-2}	4

were to evaluate effects of *G. holbrooki* on *T. vittata* growth, fin damage, and bubble-nest building. Details of the experiments are given below; a summary of experimental design is provided in Table 1.

Trichopsis vittata used in all experiments were collected at ARM Loxahatchee National Wildlife Refuge with dipnets and transported to the laboratory in aerated coolers. Upon collection, fish were in poor condition with damaged fins. They were held in the laboratory for several weeks before the experiment began, during which time their fins healed and they appeared healthy. Gainesville experiments occurred over three time periods, and fresh *T. vittata* were collected for each. *Gambusia holbrooki* were collected on-site at each facility and had no prior experience with *T. vittata*.

Ruskin

Part 1 of the study was conducted in oval polyethylene tanks in a covered greenhouse at the University of Florida, IFAS Tropical Aquaculture Laboratory in Ruskin, Florida. Treatments consisted of *G. holbrooki* at three densities (0 [control, n = 2], 21 [n = 5], and 86 [n = 5] fish m^{-2}). Densities of *G. holbrooki* were within the range typically found in ditch environments (25–150, Hill and Tuckett 2018), vegetated littoral zones of lakes (8–44, Chick and McIvor 1994), and vegetated marshes (8–98, Freeman et al. 1984; Streever and Crisman 1993; Schaefer et al. 1994) in central and southern Florida and were similar to those used in previous studies of *G. holbrooki* effects on non-native fishes (Hill et al. 2011; Thompson et al. 2012). Bottom surface area of the plastic tank was 1.0 m^2, water depth was maintained at 23 cm, and aerated well water was trickled into the tubs to prevent accumulation of nitrogenous wastes. Water parameters were within acceptable ranges for the experimental fish. Artificial vegetation was added to each tank to provide cover for *T. vittata* and consisted of 2 cm × 40 cm black, buoyant, plastic strips glued to a rectangular plastic grid (61 cm × 91 cm) at a density of 216 strips m^{-2} following Thompson et al. (2012). After adding artificial vegetation and aerated well water, *G. holbrooki* were captured from ponds onsite and acclimated for one week. They were stocked into the experimental tanks on 2 September 2014 and checked for mortality for three days. When *G. holbrooki* mortality was noted, dead fish

were removed and replaced to maintain the target density. Following the three-day period, 10 *T. vittata* were stocked in each tank and the experiment ran for two weeks. Fish were fed once daily (Aquafeed, Cargill®, Minnetonka MN). Experimental tanks were checked for mortality daily over the course of the two-week experiment. When mortality was noted, fish were promptly removed. No fish were replaced after *T. vittata* were stocked.

We performed behavioral observations on each tank for 30 minutes on two occasions, 5 September 2014 (1 hour after stocking *T. vittata*) and 19 September 2014 (immediately before the experiment concluded). These observations followed previously-established focal animal procedures (Martin and Bateson 1993; Thompson et al. 2012) and were designed to examine the number of aggressive interactions *T. vittata* experience at two *G. holbrooki* densities. The observer positioned themselves on an elevated platform over the tank and waited up to 10 minutes for fish to resume normal activities. Individual *T. vittata* were initially randomly selected following a quick scan of the tank, observed for a maximum of five minutes or until the animal was lost from sight, whereupon a new fish was selected from a different part of the tank for observation. We tallied the number of agonistic interactions directed at *T. vittata* by *G. holbrooki*; interactions were recorded when the focal animal experienced nips (or attempted nips) to the caudal region or was rapidly chased with a response by the focal animal (Thompson et al. 2012; Carmona-Catot et al. 2013; Magellan and García-Berthou 2015). Agonistic interactions between *T. vittata* conspecifics and *T. vittata* agonistic interactions (nips and/or chases) directed at *G. holbrooki* were also recorded.

After the two-week experiment, all *T. vittata* were removed, visually inspected, and scored for caudal damage as follows: 0 for no damage, 0.5 for moderate damage (less than 50% of the fin area), and 1.0 for severe (greater than 50%; Figure 1; following Thompson et al. 2012).

Gainesville

Part 2 of the study was conducted outdoors under a clear plastic roof at the U.S. Geological Survey (USGS) in Gainesville, Florida. We used round, polyethylene mesocosm tanks that measured 1.07 m diameter at the base and 1.13 m at the water surface. We used the mean of these values to determine the area of 0.95 m². Tanks were filled with water to a depth of 23 cm; the total volume in each tank was 219 L. Water from an on-site well continuously flowed through the system and tanks were aerated with low-pressure air attached to an airstone. About 1 cm of sterile sand was scattered on the bottom of each tank.

We added live vegetation and small aquatic invertebrates to mimic a marsh-type environment. Eight liters of *Hydrilla verticillata* was added to each tank to provide refuge; it covered roughly 65% of the water surface. The *Hydrilla* was collected from on-site ponds and added to the mesocosms
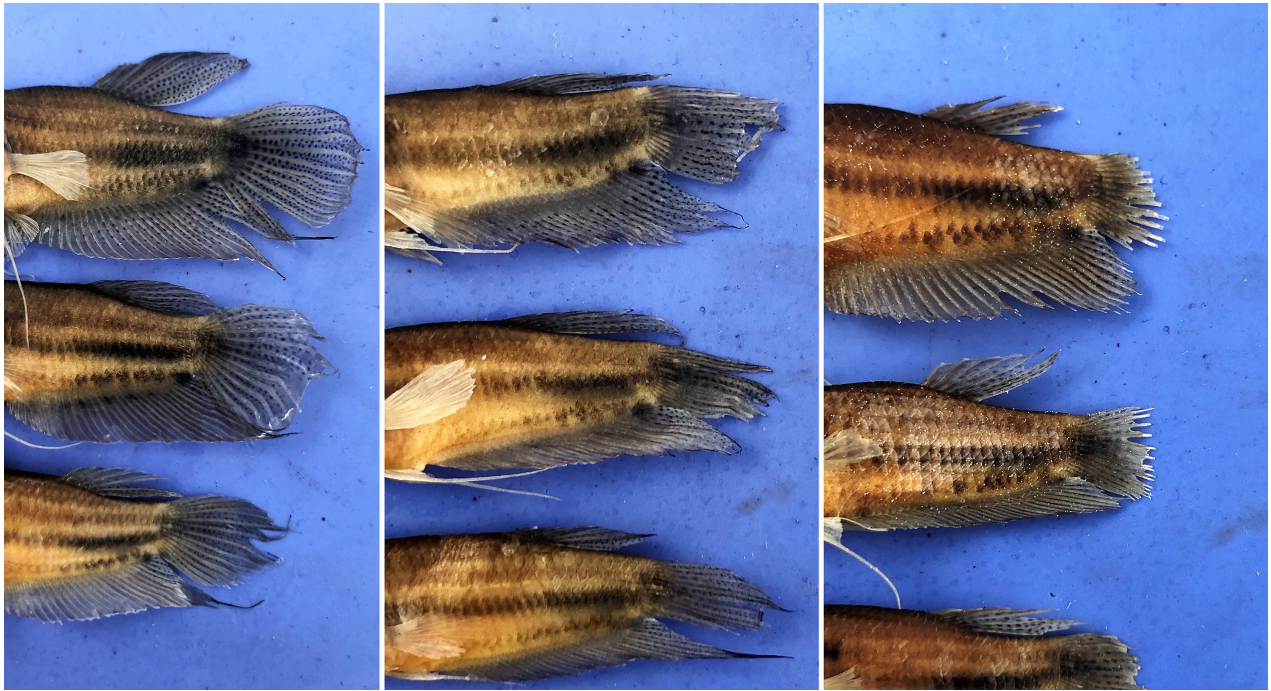
Figure 1. Examples of caudal fin damage scoring for *Trichopsis vittata*. Left = 0 (no damage); center = 0.5 (< 50% damage); Right = 1 (> 50% damage). Photos by Kristen Reaver, U.S. Geological Survey.

without washing or agitating to retain clinging aquatic invertebrates. Additional aquatic invertebrates (including but not limited to: cladocerans, copepods, rotifers, ostracods, and amphipods) were added to serve as a food source for the fishes. Those animals were harvested from on-site ponds using pattern samplers (Brakke 1976) and added to the tanks several weeks before any fish were added. In addition, fish were fed commercially-prepared flake food (Pentair egg yolk, worm and spirulina flake) *ad libitum* five times per week to supplement the live food sources. Fish were stocked at three different densities: n = 10 per tank for density of 11 m^{-2}, n = 20 per tank for density of 21 m^{-2}, or n = 30 per tank for density of 32 m^{-2} (see below and Table 1 for details). *Trichopsis vittata* and *G. holbrooki* were stocked into the tanks on the same day so that neither species would benefit from prior residence.

The experiment was conducted during three different periods (Table 1): In Summer 2014, we compared two treatments: 11 *T. vittata* m^{-2} + 11 *G. holbrooki* m^{-2} compared to an experimental control with 11 *T. vittata* m^{-2} and no *G. holbrooki*. Because we saw minimal effects of *G. holbrooki* on *T. vittata* in that trial, we increased the number of *G. holbrooki* in each tank in subsequent experiments. In Spring 2015 we compared tanks with 11 *T. vittata* m^{-2} + 21 *G. holbrooki* m^{-2} to experimental controls with 11 *T. vittata* m^{-2} and no *G. holbrooki*. In Spring 2019 we ran the third set of trials, which were designed to uncouple two confounded variables from previous experiments (presence of *G. holbrooki* and fish density). Treatments were 1) 11 *T. vittata* m^{-2}; 2) 11 *T. vittata* m^{-2} + 21 additional *T. vittata* m^{-2} (total 32 *T. vittata* m^{-2}); 3) 11 *T. vittata* m^{-2} + 21 *G. holbrooki* m^{-2}.

Table 2. Number of aggressive interactions from *G. holbrooki* against *T. vittata* per 30 min observation period. Estimates and 95% confidence intervals derived from *glmer* model as described in the text. Experiments performed in Ruskin (see text for details).

Fish density (fish m^{-2})			Replicates	Time	Aggressive interactions (No. 30 min^{-1})	95% Confidence Interval	
T. vittata	*G. holbrooki*	Total				Lower	Upper
10	21	31	5	Beginning	0.50	0.16	1.52
				End	0.23	0.07	0.76
10	86	96	5	Beginning	3.44	1.79	6.59
				End	1.56	0.71	3.46

Trials lasted 29 or 30 days from the day *T. vittata* and *G. holbrooki* were introduced to the tanks (Table 1). Environmental parameters were measured at least once per week with a YSI (Yellow Springs Instruments Professional Plus [Pro Plus]; Yellow Springs, CO) multiparameter instrument. When *T. vittata* built bubble nests, we recorded two variables: the number of new nests built per tank and the number of days each particular nest persisted (i.e., nest-days). At the end of the thirty-day experimental periods, tanks were drained and *T. vittata* were collected, batch-weighed (by tank), and preserved. We inspected *T. vittata* for caudal fin damage and scored them in the same way as the Ruskin fish.

Data analyses

All analyses were conducted in the statistical software program R (Version 3.6.0; R Core Team 2019). All functions used are available in R base packages unless otherwise noted. For all analyses below, 95% confidence intervals for each treatment were generated using the function *ggeffect* (Lüdecke 2018).

Ruskin

We tested whether the number of aggressive interactions by *G. holbrooki* toward *T. vittata* varied by treatment (density of *G. holbrooki*) and between the beginning and end of the two-week trial period. This test was performed as a log-link Poisson linear model using *glmer* (Bates et al. 2015) with treatment and time as fixed factors (Table 2). Tank was included as a random effect to control for repeated sampling. The *glmer* function call was:

No. interactions ~ *G. holbrooki* m^{-2} + Time + (1 | Tank),
family = poisson(link = "log")

To test the effect of *G. holbrooki* or *T. vittata* density on fin damage of *T. vittata*, we calculated the mean caudal fin damage score per tank, then analyzed with a logit-link Gaussian linear model using *glm*. Because the location, tank size, fish density and trial duration differed between Ruskin and Gainesville, each location was analyzed separately. For the Ruskin study, number of *G. holbrooki* ($0 < 21 < 86$ m^{-2}) was included as an ordered factor. The *glm* function call was:

logit(mean fin damage) ~ *G. holbrooki* m^{-2}

Table 3. Number of *T. vittata* with each fin damage class (0: Undamaged, 0.5: < 50%, 1: ≥ 50% damage) recovered after the trial period, and number of unrecovered (dead or missing) fish. Number of nests and nest-days observed in Gainesville are also shown.

Location	Fish density (fish m^{-2})			Trial	Replicates	*T. vittata* fin damage (No. fish)				Nests	Nest Days
	T. vittata	*G. holbrooki*	Total			0	0.5	1	Unrecovered		
Ruskin	10	0	10	–	2	19	1	0	0	–	–
	10	21	31	–	5	32	14	2	2	–	–
	10	86	96	–	5	11	27	9	3	–	–
Gainesville	11	0	11	1	5	34	16	0	0	4	9
	11	0	11	2	5	39	11	0	0	2	8
	11	0	11	3	4	38	0	0	2	3	11
	11	11	22	1	5	4	36	9	1	3	9
	11	21	32	2	5	1	25	24	0	0	0
	11	21	32	3	4	10	27	0	3	4	6
	32	0	32	3	4	110	5	0	5	4	5

Gainesville

We tested the effect of *G. holbrooki* or *T. vittata* density on fin damage of *T. vittata* in the Gainesville study, but in addition to density of *G. holbrooki* (0 < 11 < 21 m^{-2}), we also included density of *T. vittata* (11 < 32 m^{-2}) as ordered factors. Trial (Summer 2014, Spring 2015, or Spring 2019) was also included as a fixed effect. The *glm* function call was:

$$logit(\text{mean fin damage}) \sim G.\ holbrooki\ \text{m}^{-2} + T.\ vittata\ \text{m}^{-2} + \text{Trial}$$

Number of new nests were pooled for each mesocosm and analyzed with zero-inflated Poisson regression using *zeroinfl* (package *pscl*; Jackman 2017). Factors were identical to the caudal fin model. Number of nest-days (cumulative number of days nests persisted in each mesocosm) was compared among treatments in the same way. The *zeroinfl* function calls were:

$$\text{Nests} \sim G.\ holbrooki\ \text{m}^{-2} + T.\ vittata\ \text{m}^{-2} + \text{Trial} \mid 1$$
$$\text{Nest days} \sim G.\ holbrooki\ \text{m}^{-2} + T.\ vittata\ \text{m}^{-2} + \text{Trial} \mid 1$$

Finally, we compared treatment differences in *T. vittata* mass change ([final mass – initial mass]/initial mass, pooled for each mesocosm) using *glm* with identical factors as the previous models.

$$\text{Mass change} \sim G.\ holbrooki\ \text{m}^{-2} + T.\ vittata\ \text{m}^{-2} + \text{Trial}$$

Results

Survival of *T. vittata* was high in both Ruskin and Gainesville. There were five unaccounted fish out of 120 in Ruskin from four tanks, or 96% overall survival, while in Gainesville there were 11 unaccounted fish out of 400 from nine tanks for a 97% survival rate (Table 3). Water quality readings were within the normal range for these species in aquaria (Supplementary material Table S1).

Aggression

In Ruskin, there were 32 *G. holbrooki* to *T. vittata* aggressive interactions recorded (Table 2). There were more aggressive interactions per 30-minute

observation period in the 86 m^{-2} *G. holbrooki* treatment compared to the lower-density treatment (21 m^{-2}; P < 0.001), and the number of interactions at the end of the trial were fewer than the number at the beginning (P = 0.039). The number of interactions in the 86 m^{-2} treatment was 6.9 times higher than in the lower-density treatment, while the density of *G. holbrooki* was 4.1 times higher, suggesting a synergistic effect on aggression from higher density. There was only one *T. vittata* to *G. holbrooki* aggressive interaction recorded and three *T. vittata* intraspecific interactions across all treatments.

Fin damage

In Ruskin, there was significantly more damage to the caudal fins of *T. vittata* when they were in tanks with more *G. holbrooki* (P = 0.010; Table 3). While only one out of 20 *T. vittata* (5.0%) in the control treatment (no *G. holbrooki*) had damaged caudal fins after the two-week study period, 16 out of 48 (33.3%) had fin damage in the low-density treatment (21 *G. holbrooki* m^{-2}), and 36 out of 47 (76.6%) in the high-density treatment (86 *G. holbrooki* m^{-2}), with nine of those fish having severely damaged fins versus only two in the low-density treatment.

In Gainesville, caudal fin damage of *T. vittata* was also greater in treatments with more *G. holbrooki* (P < 0.0001; Table 3), but not in the treatment with more *T. vittata* (P = 0.292). The Spring 2019 trial had overall less fin damage across all treatments (P < 0.001). In neither the Ruskin nor Gainesville trials was there a non-linear component detected (P = 0.832, 0.560 respectively), so the higher-density *G. holbrooki* treatments were associated with more caudal fin damage than the lower-density treatments in both locations.

Nesting

No nests were ever observed in Ruskin experiments. In Gainesville, 20 nests were observed over the three trials, for a total of 48 nest-days (Table 3). No treatment effect was found in number of new nests or nest-days (P > 0.9), but the low number of nests overall suggests that the power to detect differences among treatments was low. All treatments showed a relatively equivalent amount of nesting effort (2–4 nests each) except the highest-density *G. holbrooki* treatment (21 *G. holbrooki* m^{-2}) in the Spring 2015 trial. That treatment also showed the highest level of fin damage: 48% of *T. vittata* showed the highest level of fin damage whereas no more than 18% of *T. vittata* showed the highest level in any other treatment.

Mass change

There was no consistent change in the mass of *T. vittata* over all Gainesville experiments combined (P = 0.582); however, increasing density of *G. holbrooki* was associated with a possible loss of *T. vittata* mass (P = 0.066), and in the Spring 2019 trial the *T. vittata* in all treatments showed an overall mass gain of 42% (P < 0.001).

Discussion

Trichopsis vittata was first recorded from Florida in 1978 (Lee et al. 1980) and was presumed extirpated following the lack of reports for many years (Shafland et al. 2008). It was then rediscovered in 2012 near the original sighting location (Schofield and Pecora 2013), suggesting it had persisted in the same small area (less than 5 km^2) since the 1970s. We show here that the aggressive and ubiquitous *G. holbrooki* imposes biotic stress on *T. vittata*, possibly limiting its abundance and geographic spread, and explaining the latter species' enigmatic invasion history in south Florida. Aggressive attacks on *T. vittata* resulted in considerable damage to fins under experimental conditions and may explain the common observation of poor condition of *T. vittata* individuals collected in the field.

Biotic resistance seems more important than abiotic stresses in limiting population expansion for *T. vittata* in Florida. Schofield and Schulte (2016) showed that *T. vittata* was able to handle typical abiotic stressors found in Florida environments (low oxygen, periodic low temperatures, salinity), and in some cases it performed better in environmental challenges than more widespread fish invaders. Thus, abiotic stress alone is unlikely to explain the failure of this species to spread over several decades. However, interactions with other species such as *G. holbrooki* may be hindering the ability of the species to colonize new habitats. In this study, we provide evidence that biotic resistance by *G. holbrooki* could be providing sublethal stress to *T. vittata* that contributes to its inability to spread. Further, we suspect that *G. holbrooki* reduces the reproductive success of nesting *T. vittata* and likely has large mortality effects on larval and juvenile *T. vittata*. We observed qualitative evidence of impacts on nesting *T. vittata* (i.e., fewer nests in tanks with *G. holbrooki*) but our design was not adequate to test this statistically due to the low number of nests observed. Negative effects on larval, neonate, and juvenile fishes have been widely reported for *G. holbrooki* (Meffe 1985; Taylor et al. 2001; Thompson et al. 2012), though our research design did not address this question.

Results from the mesocosm experiment in Ruskin confirmed that *G. holbrooki* exhibited aggressive behaviors (e.g., chasing and fin nipping) directed at *T. vittata*. We interpret these aggressive interactions as the proximate cause of the ultimate consequences of reduced growth and physical damage to the caudal fins of *T. vittata*. Fin damage increased with *G. holbrooki* density in both Ruskin and Gainesville, even though the experimental conditions differed, indicating the effect was robust. Furthermore, these results are consistent with previous research demonstrating the importance of *G. holbrooki* in resisting the invasion of small-bodied fishes in Florida (Hill et al. 2011; Thompson et al. 2012). While survival of *T. vittata* in our experiments was relatively high, sublethal stress via aggression and physical damage could translate into increased
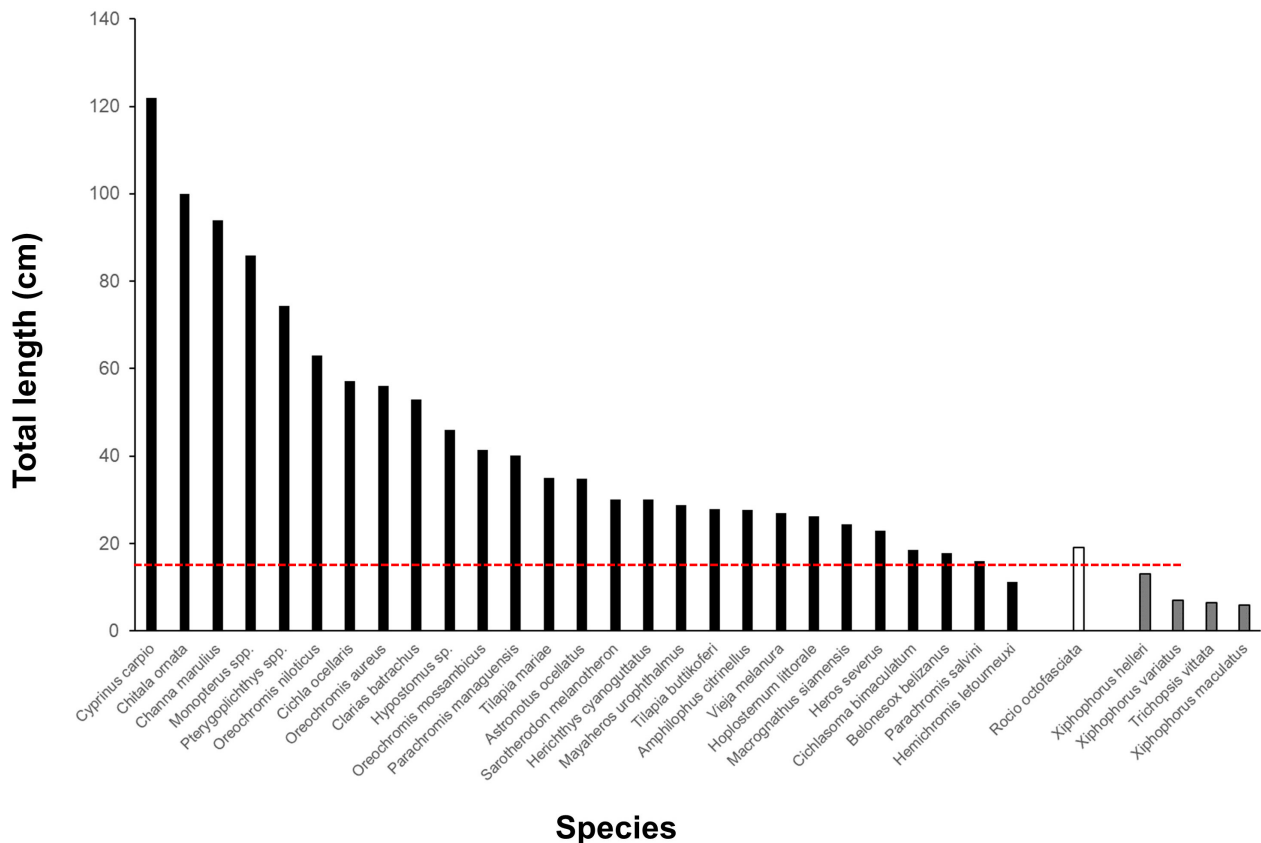
Figure 2. Graphical comparison showing maximum lengths of non-native fishes in Florida. Black bars indicate permanently established fishes, white bar denotes possibly established species and grey bars represent locally established species. The red line indicates 15 cm total length. Data and citations for maximum total length measures are provided in Table S2 and Appendix 1.

mortality over longer time periods than 30 days, lower fitness and an inability to colonize new environments.

Biotic resistance by *G. holbrooki* may be limiting the ability of *T. vittata* and other small non-native fishes to establish and spread in Florida. Amongst other benefits of large body size, species that grow to about 15 cm TL or more seem to be capable of obtaining a large enough size to escape harassment and aggression by *G. holbrooki*. This hypothesis seems to be borne out when examining the size of established non-native fishes in Florida (Figure 2; Table S2). Except for one species (*Hemichromis letourneuxi* Sauvage 1880; see below), all permanently established non-native fishes grow larger than 15 cm TL. Four fishes that have only established local populations (three *Xiphophorus* spp. and *T. vittata*) do not grow larger than 15 cm TL. One species that is possibly established grows to slightly larger than 15 cm TL (*Rocio octofasciata* (Regan 1903)). Obtaining a large body size likely infers additional advantages other than escaping biotic resistance by *G. holbrooki* (e.g., increased reproductive output).

Hemichromis letourneuxi is the one permanently-established invader that is an exception to our hypothesis. It reaches only 11.2 cm TL (Shafland 1996), though more commonly 7.5–10.0 cm TL (Hill 2016), yet *H. letourneuxi* has colonized much of the southern half of the Florida peninsula (USGS-NAS

2020). Its ability to spread may be due to the fact that, unlike other small fishes, it is not vulnerable to aggression by *G. holbrooki* (Hill 2016). Based on our own experiences with this species, we speculate that its success could be due to its extreme aggression. Future research into the factors that facilitate *H. letourneuxi*'s spread in Florida could be useful in risk screening and assessment.

Overall, *T. vittata* in south Florida is established in a small region in ditch, canal and wetlands habitats where it co-occurs with a relatively simple assemblage of native and non-native fishes, suggesting that species diversity *per se* or species saturation are not specific elements of resistance. Piscivorous non-native fishes in these systems include *Mayaheros urophthalmus* (Günther, 1862)*, Clarias batrachus* (Linnaeus, 1758), and *H. letourneuxi,* all of which may prey upon *T. vittata* (Schofield and Pecora 2013). Other strongly-interacting native species besides *G. holbrooki*, such as largemouth bass *Micropterus salmoides* (Lacepède, 1802), may limit further expansion of *T. vittata* into or through deeper or more open habitats (Hill et al. 2011; Hill and Tuckett 2018).

Although not explicitly addressed in this study, habitat suitability and connectivity may also facilitate or impede range expansion of *T. vittata*. In its small range in Florida, the species is found in cypress swamps and alongside the edges of canals, habitats that appear superficially similar to those in its native range (Schofield and Pecora 2013). Future research into habitat requirements of *T. vittata* may shed light on the importance of habitat factors in the range of this species.

While the factors contributing to successful invasions have ostensibly been attributed to one factor or another (such as abiotic versus biotic), often a combination of influences is found (Hill and Tuckett 2018), which will vary across the landscape. For example, even in the absence of biotic resistance, the northern range extent of *T. vittata* would be limited by temperature (Schofield and Schulte 2016). In addition to working in concert to limit invasion success, these two dominant filters likely interact to weaken or strengthen invasion resistance. For example, temperature mediates competitive interactions between invasive *G. holbrooki* and native Iberian toothcarp, *Aphanius iberus* (Valenciennes, 1846) (Carmona-Catot et al. 2013). *Gambusia holbrooki* is less aggressive at lower water temperatures and decreases aggressive fin nips and chases as salinity increases (Alcaraz et al. 2008). Additionally, disturbance by human activity can reduce the ability of native freshwater fish assemblages to resist non-native fish invasions (Baltz and Moyle 1993). However, reduced biotic resistance in human-modified environments could be due to the various human activities in addition to greater disturbance, including increased propagule pressure (Duggan et al. 2006) and the potential masking effects of human activity (Leprieur et al. 2008).

One important question is whether our laboratory experiments adequately characterize the interactions between *G. holbrooki* and *T. vittata* that would take place in more complex environments with varying fish communities and environmental conditions. We provide several lines of evidence suggesting that they do. For example, when *T. vittata* was collected from the ARM Loxahatchee National Wildlife Refuge, the captured fish were in poor shape with damaged fins, presumably due to *G. holbrooki* aggression. Thus, prior to the experiments discussed here, these fish had to be reconditioned before stocking in the mesocosms. Further, we obtained similar results in both the simple, two-week experiments in Ruskin and the four-week experiments with a more complex food-web in Gainesville. Despite varying complexity and temporal scale between the Gainesville and Ruskin experiments, the results were consistent. Instead, it might be possible that we have underestimated the importance of biotic resistance in these environments because of the potential for multiple-predator effects, which can enhance the effects of biotic resistance (Harvey et al. 2004).

We have shown that the invasion of *T. vittata* has likely been restricted by *G. holbrooki* via biotic resistance and suggest that this same mechanism may be at work on other non-native fishes. *Gambusia holbrooki* has been documented to affect persistence of the green swordtail, *Xiphophorus hellerii* Heckel, 1848, variable platy, *Xiphophorus variatus* (Meek, 1904), and zebra danio, *Danio rerio* (Hamilton, 1822) through agonistic interactions similar to our results with *T. vittata* (Hill et al. 2011; Thompson et al. 2012). Given the aggressive behaviors noted by *G. holbrooki* and the resulting reduced growth and caudal fin damage, *G. holbrooki* appears to be affecting the range and population growth of *T. vittata*. Interestingly, *T. vittata* has not been completely eliminated, and seems to have persisted for decades without significant range expansion. Future studies of *T. vittata* could provide a more robust understanding of the mechanisms of persistence.

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Ethics and Permits

The authors have complied with institutional policies governing the humane and ethical treatment of the experimental subjects. All procedures were in compliance with Institutional Animal Care and Use Committee regulations (approved protocol USGS/SESC 2014-14 and UF

#201408492). Data for this study are publicly available (Schofield et al. 2019). Fish were collected under Special Use Permit B14-005 from ARM Loxahatchee Wildlife Refuge.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Water quality data from Gainesville experiments.
Table S2. Maximum total lengths of non-native fishes in Florida.
Appendix 1. List of references for Table S2.

This material is available as part of online article from:

http://www.reabic.net/aquaticinvasions/2021/Supplements/AI_2021_Schofield_etal_SupplementaryMaterial.xlsx