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Copeia

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Sex-Dependent Cold Tolerance at the Northern Invasive Range Limit of *Gambusia affinis* on Cape Cod, Massachusetts

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Many invasive species may be constrained in their ability to invade high latitude or elevation habitats due to limits of cold tolerance. However, isolated populations at the edge of these species' geographic ranges may face a combination of strong selection and lack of maladaptive gene flow that allows them to adapt to the cold and invade further. Here we show that an isolated population of *Gambusia affinis* (on Cape Cod, MA, USA), one of the most invasive fish in the world, can survive temperatures lower than any reported tolerances for other populations, with large, long-lived females showing more tolerance than males. Indeed, male acute cold tolerance appears insufficient, suggesting the population may persist through winters mainly via the ability of mated females to delay offspring production until spring. This combination of female cold tolerance and life history may open the door for further expansion of this invasive species into high latitude freshwater habitats.

SPECIES distributions can be constrained by history, vicariance, and maladaptation to environmental conditions at range margins. In previously glaciated regions, some species may be absent simply due to barriers to dispersal and colonization. In other cases, species might lack the adaptive genetic variation required for more extreme conditions. Traditionally, cold temperatures have often been considered a barrier to the spread of invading organisms to higher latitudes or elevations (Janzen, 1967; MacArthur, 1984; Eme and Bennett, 2008). However, numerous examples of contemporary evolution (Prentis et al., 2008; Moran and Alexander, 2014) suggest that adaptation of cold tolerance can occur on ecologically relevant time scales. Moreover, humans may facilitate this contemporary evolution by removing other constraints on this adaptation.

New populations face several genetic constraints. The ability for marginal populations to adapt to novel environmental conditions can be limited by insufficient genetic variation and inbreeding in small founding populations. However, some anthropogenic species introductions overcome this limitation by introducing large numbers of individuals from a diverse genetic background (Lockwood et al., 2005; Roman and Darling, 2007). At the same time, in moving species beyond their natural dispersal limitations, introduced populations can be freed from genetic load imposed by the influx of locally maladaptive genes from populations adapted to different environments (Haldane and Ford, 1956; Polechová and Barton, 2015). Similarly, habitat fragmentation via anthropogenic land-use change may reduce the flow of maladaptive genes into marginal populations (Fischer and Lindenmayer, 2007; Lindenmayer and Fischer, 2007), especially when patches are sufficiently distant or the between-patch matrix is sufficiently unsuitable (DiBattista, 2008; Hanski, 2011; Fountain et al., 2016). Isolation from maladaptive gene flow can enable local adaptation and range expansion (Urban and Skelly, 2006; Urban et al., 2008), assuming sufficient standing genetic variation exists or is produced within the marginal population to allow adaptation (Bell and Gonzalez, 2009; Carlson et al., 2014). Such human-facilitated local adaptation may be particularly troubling when it enables the spread of invasive species.

Modes of cold adaptation are highly specific to the life history of an organism. Long-lived, less-mobile species face clear selection to tolerate colder temperatures, as they face repeated bouts of low temperatures that they cannot escape in space or time (Lithgow, 1996; Bubliy and Loeschcke, 2005; Abele et al., 2009; Wu and Storey, 2016). However, more-mobile species can escape the cold in space through migration or thermal refugia (Peterson and Rabeni, 1996; Sims et al., 2004; Brodersen et al., 2011). Shorter-lived organisms can also escape the cold in time by exhibiting seasonal adaptation of cold tolerance (Bennett et al., 1997; Bergland et al., 2014; Noh et al., 2017) or by employing resting stages or seed banks that persist through winter (diapause; Denlinger, 1991; Danks, 2006; Sunday et al., 2012; Podrabsky et al., 2015). Typically these tolerant stages are tied to reproduction with mating occurring prior to death of much of the population and regeneration of the population from the tolerant stages come spring. Thus, species' life-history traits are a key factor when considering the potential for cold adaptation and range expansion in invaders. Cold-adaptive life-history traits can also vary between sexes as traits that facilitate cold tolerance may be associated with sex-specific reproductive traits such as body size or energetic demands (Schultz et al., 1998).

Here we investigate the cold tolerance of Western Mosquitofish (*Gambusia affinis*) in the Quashnet River, Mashpee, Massachusetts, USA. This introduced population of mosquitofish is one of the farthest north populations, and the farthest east population, of *Gambusia* in continental North America (USGS, 2016). It has persisted in isolation for more than two decades (>40 generations [Pyke, 2005]) in a set of abandoned lotic cranberry bogs hydrologically connected to the main river channel (Hartel et al., 2002). *Gambusia* spp.—particularly *G. affinis*—exhibit immense adaptive capacity to

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local environments, including temperature (Hagen, 1964; Stearns, 1983a, 1983b; Meffe, 1991; Meffe et al., 1995; Stockwell and Weeks, 1999; Langerhans et al., 2004; Moffett et al., 2018). One of the most widely distributed invasive fish in the world (Fryxell et al., 2015), *Gambusia* spp. have profound ecological impacts on recipient communities (Hurlbert et al., 1972; Hurlbert and Mulla, 1981), often at the expense of native species (Pyke, 2008). Despite their broadly accepted classification as invasive, *Gambusia* spp. are still introduced globally for their presumed utility in controlling mosquito larvae (Haas and Pal, 1984; Blaustein, 1992; Walton, 2007; Chandra et al., 2008).

We examine the cold tolerance of male and female mosquitofish of varying size in the Quashnet River using a pseudo-lethal chilling experiment. We compare the cold tolerance of this population to those in other published studies with similar acclimation methods to determine whether this population has an unusually low cold tolerance. We also compare cold tolerances across sexes, as difference in female and male longevity (years vs. months), size (females are several times larger), and mating strategy (males rush to mate, females store sperm; Pyke, 2005) set the stage for sex-dependent selection on cold tolerance. We evaluate two sets of hypotheses. First, we test whether this population at the extreme north and east of the species range possesses exceptional cold tolerance. Second, we examine whether cold tolerance is sex-dependent after accounting for sex-specific differences in size. If there is sex-dependent selection and sex-linked cold adaptation, we would anticipate females to withstand colder temperatures than males, as they are longer lived, and their reproductive success more tightly associated with repeated broods and thus longevity.

MATERIALS AND METHODS

Mosquitofish collection.—We collected mosquitofish from the Quashnet River bogs (41.636497°N, 70.507118°W) in September 2018 using seine nets and dip nets. We held mosquitofish in the lab at 18°C until January 2019, during which time we fed the fish abundant powdered flake food. For their final week in the lab, we acclimated mosquitofish to a temperature of 13°C. We stopped feeding the mosquitofish 18 hours before experimentation. To confirm that the mosquitofish were *Gambusia affinis* (not Eastern Mosquitofish, *G. holbrooki*), we examined several male gonopodia under a microscope (Rauchenberger, 1989).

Cold tolerance experiment.—We conducted a pseudo-lethal cold tolerance experiment with our mosquitofish, in which we examined the temperature at which mosquitofish ceased responding immediately to outside stimuli, and the temperature at which mosquitofish lost the ability to maintain their vertical orientation—our proxy for mortality.

We placed 61 mosquitofish (43 females, 18 males) in 5 cm wide plastic mesh tubes in a 75-liter pool. Beginning at 13°C, we used a combination of chillers and ice in plastic bags to lower the temperature of the pool 1°C every 90 minutes, with a final temperature of 1.5°C dictated by our equipment. This rate of cooling is comparable with the night-time cooling rate of small ponds and stream eddies inhabited by mosquitofish (Woolway et al., 2016) and thus reflects a realistic winter scenario. We used several small pumps to keep water circulating through the tank and ensure equal cooling of all fish. Forty-five minutes after starting each drop-in temperature, we prodded each mosquitofish with a black plastic rod and counted whether the mosquitofish avoided the rod and maintained its vertical orientation as response to stimulus and survival, respectively. As fish that failed to respond to stimuli but were still able to maintain orientation clearly retained some muscular abilities (and indeed, survived for hours and in much colder water after failing to avoid the stimulus), we did not count failure to respond to stimuli as a pseudolethal endpoint. We assumed that fish that failed to maintain their vertical orientation would eventually perish at the current temperature, as they were unable to complete basic muscular functions, including ram and opercular ventilation. If a fish failed to maintain its vertical orientation, we removed it from the experiment and then slowly warmed the fish back to room temperature. All but one fish that lost vertical orientation recovered successfully after being warmed, though we euthanized fish after experimentation in accordance with our permitting and IACUC protocol. We then sexed and weighed each fish.

We fit the following binomial generalized linear model in R (R Core Team, 2016) to predict mosquitofish response to stimulus and maintenance of orientation:

$$p(M,T) = \frac{1}{1 + \exp\left(\beta_{0_s} + \beta_{1_s} \times M + \beta_2 \times ln(T)\right)} \tag{1}$$

Where p = probability; M = mass; T = temperature; β_{0_s} = sex-specific intercept, β_{1_s} = sex-specific mass slope, and β_2 = general temperature slope. After a mosquitofish was removed from the experiment due to loss of orientation (i.e., presumed to die), we treated all data for that individual at lower temperatures as failures for both response to stimulus and maintenance of orientation. Ln-transforming T decreased AIC by 9.8 in the response to stimulus model and increased AIC by 0.2 in the orientation model. We therefore used ln(T) in both models for consistency, due to the lack of overwhelming evidence for or against ln(T) in the maintenance of orientation model (Burnham and Anderson, 2004), and because of the biological realism of having survival below 0°C unlikely in freshwater.

We calculated the lower stimulus-response temperature for 50% of fish (LRT50) and lower lethal temperature for 50% of fish (LT50) for fish of a given sex using the fit coefficient values from equation (1):

$$T_{50} = \exp\left(-\frac{\beta_{0_s} + \beta_{1_s} \times M}{\beta_2}\right) \tag{2}$$

We calculated standard error intervals for LRT50 and LT50 as the temperature at which the confidence interval from model (1) crossed 0.5 using the *glm.predict* function in R:

$$0.5 = \hat{p}(M,T) \pm \varepsilon_{\hat{p}}(M,T) \tag{3}$$

In the case of LT50, our estimates were extrapolations, as more than half of fish from both sexes survived to 1.5°C. Nonetheless, the error estimates for these LT50s are relatively low (see Results), and the most conservative interpretation of our LT50s is that they are certainly between 1.5°C and 0°C.

Quantitative review.—In order to determine whether our study population had a uniquely low cold tolerance, we conducted a quantitative review of reported thermal toler-

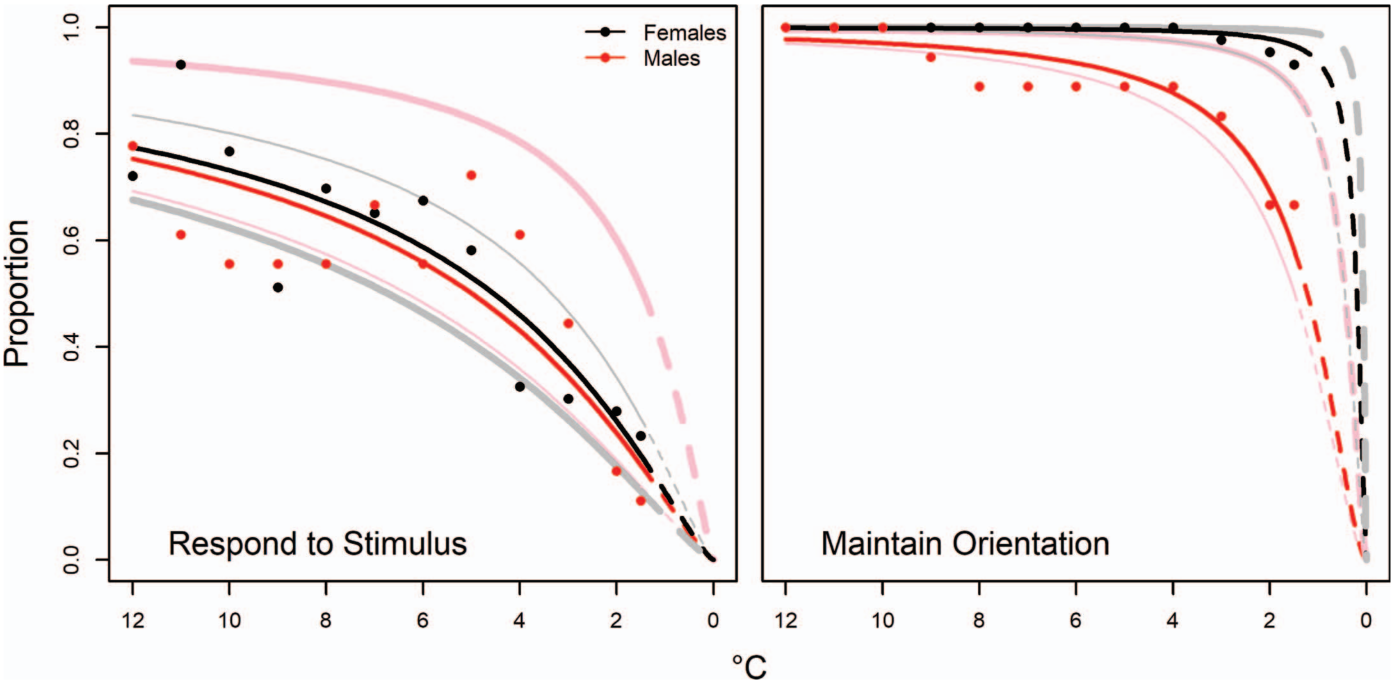

Fig. 1. Proportion of mosquitofish that responded to a stimulus and maintained orientation while experiencing progressively decreasing temperature. Points show average proportions for all females and males. Lines show model predictions: dark lines for fish of average mass; thin, light lines for fish at the 5% lower range of fish mass; and thick, light lines for fish at the 95% range of fish mass (calculated separately for each sex). Dashed lines show model extrapolation between 1.5 and 0°C.

ance values for *Gambusia affinis* and *G. holbrooki* (Table S1; see Data Accessibility). We included results for both species as they have overlapping ranges, hybridize, and are poorly distinguished in earlier literature. We used exhaustive searches of "Gambusia + thermal" in Web of Science to locate papers that reported upper or lower thermal tolerances for *G. affinis* and *G. holbrooki*. For each population within each paper, we calculated maximum and minimum upper and lower thermal tolerances (LT50). As we were most interested in comparing the ranges of reported thermal tolerances to those of our study population, we pooled multiple acclimation temperatures within each reported population. We compared the ranges of reported LT50s for various populations of *Gambusia* to the LT50s of average males and females in our study.

We used published temperature data from three thermistors in the Quashnet River (Briggs et al., 2018) to examine how the cold tolerance of our study population relates to minimum winter temperature. We then compared our estimated LRT50 and LT50 for average males and females to daily maximum and minimum surface water temperature for each thermistor.

RESULTS

Cold tolerance experiment.—While response to stimulus decreased with temperature, nearly all mosquitofish maintained orientation to 1.5°C (Fig. 1). In general, females ceased responding to stimuli at higher temperatures than males, but had greater ability to retain orientation at lower temperatures and thus lower extrapolated LT50s (Figs. 1, 2; Tables 1, 2). Smaller males ceased responding to stimuli at higher temperatures than larger males, while females showed the opposite trend, with larger females failing to respond to stimuli at higher temperatures. However, both males and females showed increased tolerance (ability to maintain orientation) to colder temperatures with increased size.

Quantitative review.—Male mosquitofish from the Quashnet River had cold tolerances (extrapolated LT50 = 1.23°C) at the far lower end of the overall range from published studies (0.6–15.5°C), and females from the Quashnet River had cold tolerances (extrapolated LT50 = 0.20°C) that were below those of other published studies (Fig. 3; Otto, 1973; Johnson, 1976; Al-Habbib and Yacoob, 1993; Meffe et al., 1995; Carveth et al., 2006).

The LT50 for the average female mosquitofish from the Quashnet River fell just below the lowest minimum daily temperature from the four winters reported in Briggs et al. (2018), while the LT50 for the average male was reached roughly once per winter (Fig. 4). The LRT50 for both average males and females roughly corresponded to the average winter temperatures reported in Briggs et al. (2018).

DISCUSSION

Here we found that a population of mosquitofish that has been isolated for more than two decades in a stream that occasionally approaches 0°C had correspondingly low LT50s—among the coldest thermal tolerance of any reported for *G. affinis* or *G. holbrooki* in the literature.

Cold adaptation in isolation.—We hypothesize that adaptation in isolation has contributed to the striking cold tolerance of Quashnet River mosquitofish. Quashnet mosquitofish—particularly females—were more cold tolerant than any other reported population. Similar acclimation methods (see Table S1; see Data Accessibility) in other papers reduce the likelihood that differences in cold tolerance

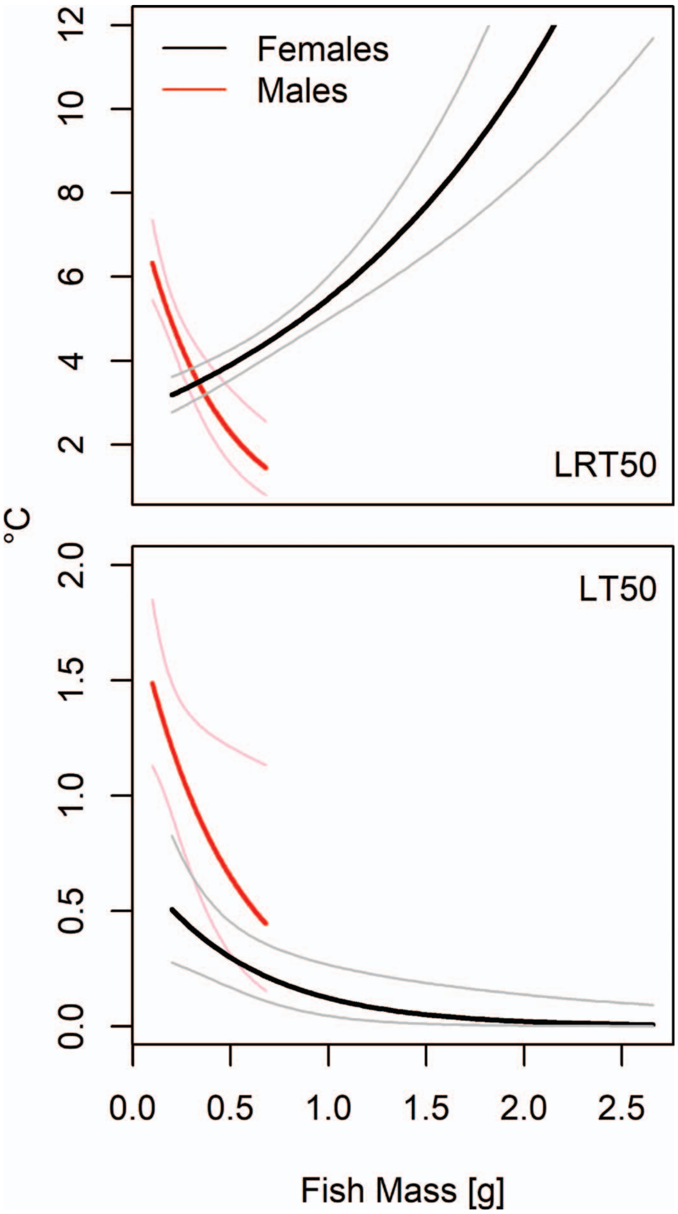

Fig. 2. Estimated lower temperature of 50% loss of response to stimulus (LRT50) and 50% mortality (LT50) for male and female *Gambusia affinis* of various sizes from the Quashnet River, Massachusetts, USA. Thinner, lighter lines represent ± one standard error. Note that estimates below 1.5°C are extrapolations beyond the range of our data.

between Quashnet and other mosquitofish are due only to plasticity associated with experimental acclimation. Therefore, genetic evolution is more likely an explanation for the extreme cold tolerance of Quashnet mosquitofish, potentially including genetic evolution of plastic capacity or epigenetic inheritance of cold tolerance (Bonduriansky et al., 2012; Slotsbo et al., 2016; Scheepens et al., 2018; Karunakar et al., 2019). Furthermore, enhanced cold tolerance in Quashnet mosquitofish is unlikely simply due to size, as even our smallest females had lower extrapolated LT50s than any reported literature value (Figs. 2, 3).

Evolution in the Quashnet River system may be enhanced by isolation. The nearest neighbor population of mosquitofish to the Quashnet River is at least 150 km away (USGS, 2016), making gene flow into the Quashnet population highly unlikely. Thus, more than two decades (>40 generations) of isolation may have facilitated the uniquely low cold tolerance of Quashnet mosquitofish. While the exact details of mosquitofish introduction to the Quashnet River are unknown, we suspect that the introduction was motivated by mosquito control. As such, introductions for mosquito control typically involve huge numbers of fish. The Quashnet River also has numerous groundwater-fed springs (Rosenberry et al., 2016), which may have provided initial winter refugia for mosquitofish (Craig et al., 2019). Therefore, we doubt that this population experienced a significant founder effect and demographic bottleneck during colonization compared to wild populations generated by smaller dispersal events.

Sex-dependent selection.—Significant differences in cold tolerance between sexes suggest sex-dependent selection in Quashnet mosquitofish. Mosquitofish are highly sexually dimorphic, with females being several times larger than males. Even after accounting for their larger mass, females still had higher survival rates than males (Fig. 2). Additionally, females typically live much longer than males, with male life spans being less than a year and females living up to a few years (Pyke, 2005). Because female reproductive success is highly tied to size and longevity effects on fecundity, females may experience stronger selection to survive through the winter. Male *Gambusia* invest a large proportion of energy and resources on mating (Martin, 1975; Hughes, 1985), to the extent that they experience deterministic growth upon maturation. As such, males may face stronger selection to mate during warmer months than to survive through colder months. Such sex-dependent selection would explain why the LT50 for female mosquitofish in the Quashnet River fell below the winter minimum temperature, while the LT50 for males was reached in most winters. Even if some males manage to survive in small springwater refugia in the Quashnet River in the winter, their LT50 indicates that any males outside of refugia would perish, while females could survive anywhere in the river.

The mating system of these fish may be especially important to facilitating this capacity for sexual dimorphism of cold tolerance (Kahn et al., 2013). Females store sperm for

Table 1. Likelihood ratio tests for Quashnet mosquitofish lower thermal tolerance.

	Response to stimulus			Maintenance of orientation		
Dependent variable	χ^2	df	*P*	χ^2	df	*P*
ln(Temperature)	105.2	1	**<0.001**	33.9	1	**<0.001**
Sex	6.0	1	**0.014**	4.3	1	**0.038**
Mass	11.0	1	**<0.001**	5.1	1	**0.024**
Sex × Mass	10.0	1	**0.0016**	0.02	1	0.89

Table 2. Model coefficients for Quashnet mosquitofish lower thermal tolerance.

	Response to stimulus		Maintenance of orientation	
Parameter	Estimate	se	Estimate	se
β_{0_F}	−1.30	0.29	0.53	1.11
β_{0_M}	−2.67	0.39	−1.00	0.68
β_{1_F}	−0.86	0.24	2.94	1.98
β_{1_M}	3.24	1.42	3.44	2.96
β_2	1.66	0.13	1.66	0.31

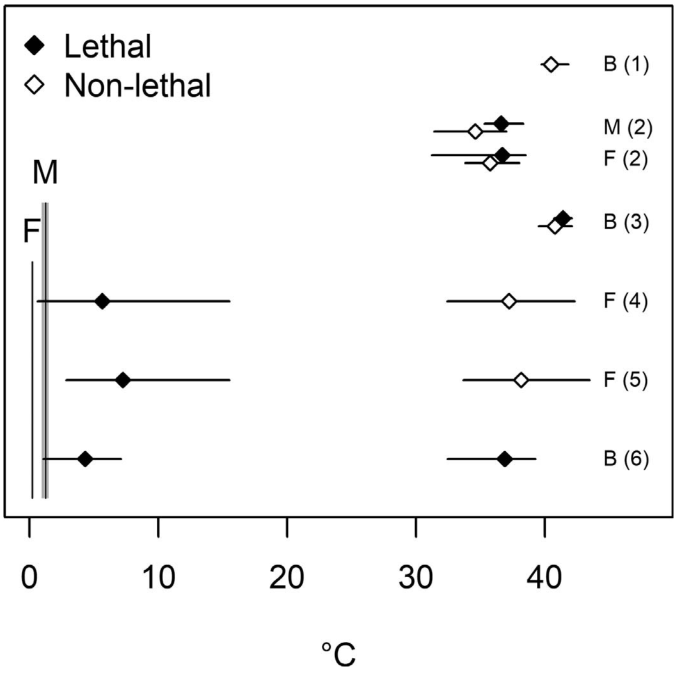

Fig. 3. Lower (left) and upper (right) reported thermal tolerances for *Gambusia affinis* and *Gambusia holbrooki*. Each point and line represent the mean and range, respectively, for a particular population, with lethal and non-lethal assays distinguished. Significant variation in reported values for a given population is typically due to varied acclimation temperatures and separate testing on males and females. Gray lines show extrapolated 50% lower thermal tolerance predictions and standard errors for average males and females from our study. Letters indicate sexes included, and numbers refer to studies in Table S1 (see Data Accessibility).

months, even when not actively reproducing (Hildebrand, 1917; Krumholz, 1948; Haynes, 1993), so it is possible that populations could persist through winter die-offs of essentially all males, with sex ratios restored by new births of males in the spring. Indeed, large winter reductions in population size, followed by quick spring rebounds are common in other populations of *Gambusia* (Pyke, 2005).

Interestingly, females and males showed opposite size-dependent behavioral responses to cooling. Larger females ceased responding to stimuli at higher temperatures than smaller females, while males showed the opposite trend (Crowder and Magnuson, 1983; Stevenson, 1985; Ospina and Mora, 2004). Larger females may face less predation risk than other mosquitofish due to predator gape limitation, and thus could face decreased selection for metabolically difficult activity at cooler temperatures. Other studies of *Gambusia affinis* have also shown that larger females tend to be behaviorally less sensitive to predation risk (Wood et al., 2019). Larger females typically also have greater fat stores, and likely face less need to forage at challenging colder temperatures (Reznick and Braun, 1987).

Invasion risk.—The observed cold tolerance near 0°C in this population of mosquitofish indicates that they have adapted to overcome one of the primary abiotic constraints to expansion into high latitude freshwaters. In particular, the robust cold tolerance demonstrated by female mosquitofish here is troubling, as females can store sperm from multiple males and begin gestation months after insemination (Hildebrand, 1917; Krumholz, 1948; Haynes, 1993). Thus, only a single inseminated cold-hardy female coming from this population could establish a new population at similar higher latitude locations outside the normal species range. Based on our findings, it is possible the only real limit to range expansion of the species may be the extent of ice formation in their preferred shallow-water habitat. Predicted increases in winter water temperature in the region due to climate change (Fernandez et al., 2015) suggest these abiotic conditions will become less challenging for the northward expansion of mosquitofish. The observed cold adaptation of this population combined with this species' known negative impacts on culturally and economically important native fish (Pyke, 2008) paint a worrying picture for the continued spread of this highly invasive fish. However, simply because this population of mosquitofish shows the capacity to tolerate cold conditions does not mean that invasion is inevitable. Two other critical factors will influence the ability of these fish to establish in uninvaded habitats: dispersal to new habitats and the biotic state of potential recipient communities.

The Quashnet River drainage basin is largely isolated from other systems of rivers and streams, thus limiting the potential for direct movement of mosquitofish into other freshwater systems. However, *Gambusia* can, under some conditions, develop tolerance to levels of salinity approaching seawater (Al-Daham and Bhatti, 1977; Chervinski, 1983; Purcell et al., 2008). The Quashnet River drains into the Waquoit Bay estuary, which is connected to several other major freshwater systems and often exhibits significant dips in salinity (D'Avanzo and Kremer, 1994; Tomasky et al., 1999; Abraham et al., 2003). Thus, it is possible that Quashnet mosquitofish could invade other stream networks via Waquoit Bay. There is also potential for humans to facilitate spread of mosquitofish through purposeful or incidental relocation. While regulations are already in place that prohibit the movement of mosquitofish in much of New England, these regulations were also in place when mosquitofish were first introduced to the Quashnet River (Stephen T. Hurley, pers. obs.). Many people in the New England region are likely unfamiliar with mosquitofish, increasing the risk they might be collected and moved as seemingly harmless 'minnows.'

In the event that mosquitofish from the Quashnet population find their way to new, northern habitats, their ability to establish will also depend on the ecological state of the recipient communities. Healthy, stable ecological communities are often thought to be resistant to biological

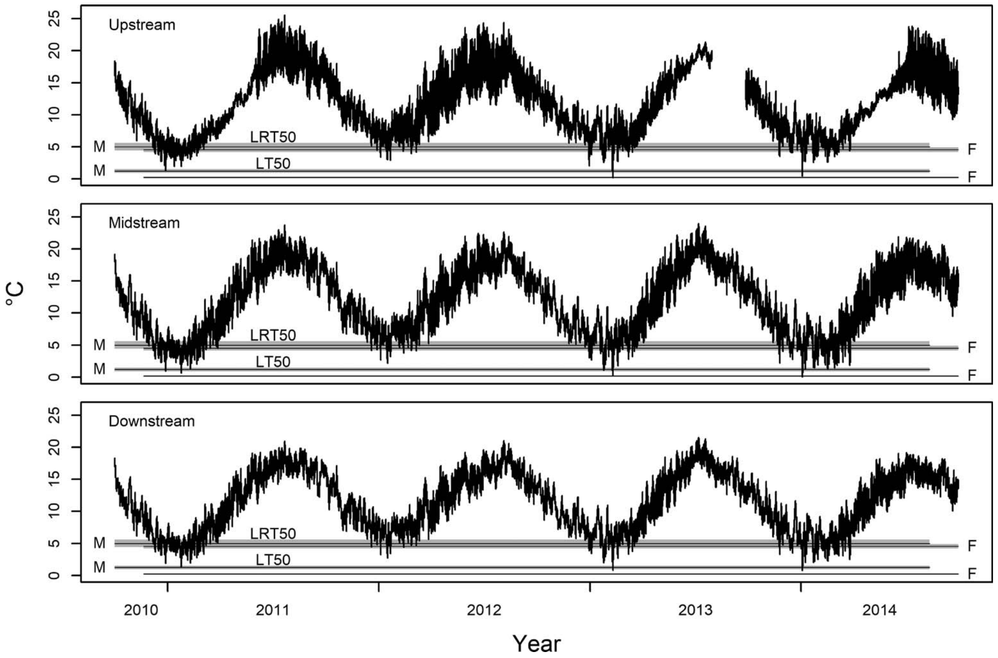

Fig. 4. Daily temperature ranges for Quashnet River surface water from three thermistors over four years. Data from Briggs et al. (2018). Lines and bars show mean ± standard error 50% lower thermal response (LRT50) and lower thermal tolerance (LT50) predictions for average males and females from our study.

invasions (Kneitel and Perrault, 2006; Romanuk et al., 2009), and mosquitofish thrive in marginal habitats for other species, such as drainage ponds and ditches (Lee et al., 2017). In this instance, having systems that support a robust and diverse population of top predators may be critical in lowering the chance that mosquitofish establish in new habitats. The overwintering survival of freshwater fishes in northern latitudes has been shown to be lower in the presence of predators (Hurst, 2007). Indeed, that this and other populations of *Gambusia* (Winkler, 1979) exhibit a decrease in their ability to respond to stimulus at lower temperatures may make them more vulnerable to predation during winter months, particularly if their predators are well adapted to foraging at low temperatures (e.g., salmonids). Therefore, restoring degraded communities and protecting those already in place in areas at risk of invasion may help prevent the continued spread of mosquitofish.

Conclusions.—The population of *Gambusia affinis* in the Quashnet River has an extremely low cold tolerance, with some individuals predicted to survive temperatures below 0.2°C. The combination of extreme temperatures and isolation from other populations has likely contributed to this adaptation aided by the sex-specific life history and enhanced thermal tolerance of female mosquitofish. This life history and tolerance makes female mosquitofish a particular invasion-threat—a single female can establish a new population. Taken broadly, these findings support the conclusion that invasion risk assessments need to incorporate both adaptation in isolation and the ecological effects of sexual dimorphism (Fryxell et al., 2015, 2019). In addition, ongoing monitoring of cold-adapting mosquitofish populations is necessary to ensure their spread is limited.

DATA ACCESSIBILITY

Supplemental material is available at https://www.copeiajournal.org/ce-19-332. All data analyzed in our experiment can be found in Tables S2 and S3. Literature temperature tolerance values for *G. affinis* and *G. holbrooki* can be found in the cited papers or their supplements.

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