Multiple interacting stressors influence development, growth, and morphology of Pacific Chorus Frog (*Pseudacris regilla*) larvae

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Abstract: Freshwater ecosystems are threatened by climate change and invasive species. Amphibians are important members of freshwater communities and are susceptible to negative effects of climate change and invasion. Furthermore, both climate change and invasion can influence density of amphibian larvae at the microhabitat scale because of larval clustering. To understand the effects of climate change and invasion on Pacific Chorus Frog *Pseudacris regilla* (Baird and Girard, 1852) larvae, a $2 \times 2 \times 2$ factorial experiment was carried out with a climate treatment (future/historical; simulated as changes in both temperature and hydroperiod), indirect exposure to the invasive Brook Stickleback *Culaea inconstans* (Kirtland, 1840) fish (chemical cues present/absent), and conspecific density (conspecific present/absent). We measured tadpole survival and development rate along with variables related to body shape, including mass, growth, growth rate, body mass index, tail fin depth, tail muscle depth, tail muscle width, and rear limb length. We assessed the effects of 3 common and important stressors (climate, invasive species, and density) alone and in combination with the other stressors to explore their interactions. Climate change, as simulated by our study, had large effects on Pacific Chorus Frog tadpole development and body shape. Brook Stickleback chemical cues had smaller but important effects on body shape and development rate when combined with additional stressors. Competition because of presence of a conspecific tadpole also reduced survival and had large effects on body shape. Pairwise interactions were largely additive or antagonistic. Three-way stressor interactions were most often antagonistic, resulting in less-than-additive effects of the 3 stressors together. However, 3 synergistic 3-way emergent interactions were detected. When all 3 stressors were present, development rate was faster than expected but rear limb length and tail muscle width were smaller than expected. Our results illustrate that climate change and conspecific density have wide-ranging effects on amphibian growth, development, and morphology—effects which may carry over into the terrestrial stage and reduce fitness. Furthermore, our findings reveal complex stressor interactions that were not predictable from single stressor effects or pairwise stressor effects, illustrating the difficulty in predicting multiple stressor interactions in freshwater systems.
Key words: competition, climate change, invasion, temperature, hydroperiod, density, tadpole, fish

Freshwater ecosystems are vulnerable habitats threatened by the effects of climate change and invasive species (Heino et al. 2009, Carpenter et al. 2011, Angeler et al. 2014, Reid et al. 2019). The physical effects of climate change in freshwater systems include warmer temperatures, increased variability of precipitation, acidification, and altered flow rates (Arias et al. 2021). These altered physical factors can drive population declines alone or may interact with other environmental stressors to cause loss of biodiversity (Reid et al. 2019). Invasive species are increasingly common in freshwater ecosystems and pose an additional threat to native populations (Gallardo et al. 2015). The effects of invasive species include predation, biodiversity loss, and ecosystem degradation (Pyšek and Richardson 2010). Invasion and climate

change threaten freshwater systems, both individually and through complex interactions. Effects of invasive species on these ecosystems can depend on physical conditions in the environment (Loewen et al. 2020); thus, effects of invasion are expected to change under the various physical effects of climate change (Rahel and Olden 2008).

Amphibians are important components of many freshwater communities, and climate change is expected to have large effects, both direct and indirect, on amphibian populations (Blaustein et al. 2010). Climate change can influence amphibian distributions (Lawler et al. 2010), phenology (Todd et al. 2011), immune function (Rollins-Smith 2017), growth and development (Thurman and Garcia 2017, Tasker et al. 2022), and behavior (Jara et al. 2019). For pond-breeding amphibians, warming waters and shorter hydroperiods are the primary physical changes of concern related to climate change. Warmer waters have been shown to accelerate development rates, reduce length, mass, or both at metamorphosis and increase growth rates (Tejedo et al. 2010). Warmer water can also interact with other stressors, such as the presence of predators, to reduce survival (de Mira-Mendes et al. 2019). Shorter hydroperiods accelerate development rates, reduce survival, alter immune function, decrease growth rates, produce smaller metamorphs, and alter morphology (Lane and Mahony 2002, Richter-Boix et al. 2006, Johansson et al. 2010, Tejedo et al. 2010, Edge et al. 2016, Brannelly et al. 2019). Warmer temperatures have been shown to produce both longer (Blouin and Brown 2000) and shorter (Gomez-Mestre and Buchholz 2006) hind legs. Exposure to a reduced hydroperiod also produces conflicting results for leg length (Richter-Boix et al. 2006, Johansson et al. 2010, Bredeweg et al. 2019), resulting in no clear effect of either temperature or hydroperiod on leg length (Tejedo et al. 2010). Tail morphology also changes in response to some environmental conditions. For example, under drying conditions (reduced hydroperiod), larval *Pelodytes punctatus* had narrower tail fins, but no changes in tail muscle were detected (Richter-Boix et al. 2006). Habitat alterations due to climate change can also influence how species respond to other stressors. For example, hydroperiod can influence the response of amphibian larvae to predators (Lent and Babbitt 2020). Thus, physical changes associated with climate change can have wide-ranging effects on amphibians.

Invasive species have been identified as the cause of ⅓ of amphibian extinctions and have a broad range of effects on this taxonomic group (Bucciarelli et al. 2014, Nunes et al. 2019, Falaschi et al. 2020). Aquatic-breeding amphibians, including those that breed in ponds, may be especially sensitive to invasive species because of their evolutionary history in habitats without large-bodied predators such as fish. Indeed, fish are one of the most commonly introduced large predators and have had negative effects on amphibian populations across the globe (e.g., Hecnar and M'Closkey 1997, Bucciarelli et al. 2014, Préau et al. 2017). A recent meta-analysis determined that the presence of invasive fish reduced fitness, growth, and activity in amphibians while increasing avoidance activity (Nunes et al. 2019). Both large and small fish are efficient predators on larval amphibians (Remon et al. 2016, Miró et al. 2018, Vannini et al. 2018). Fish and other predators can also induce morphological changes in amphibians (Van Buskirk and McCollum 2000, Van Buskirk 2001, Relyea 2001a, Katzenberger et al. 2014, Florencio et al. 2020). Predators also tend to reduce amphibian mass at metamorphosis, growth rate, and hind limb length while increasing development time (Tejedo et al. 2010, Edge et al. 2016). The effects of predators can be context dependent. For example, Pacific Chorus Frog (PCF) (*Pseudacris regilla* [Baird and Girard, 1852]) tadpoles reduced their activity in the presence of a predator at 20°C but not at 10°C (Jara et al. 2019). Also, naïve populations do not always respond to invasive predators with adaptive changes (Nunes et al. 2019), suggesting that native prey do not always recognize new predator species.

Both climate change and invasive species can influence competition among tadpoles. Reduced hydroperiod due to climate change can reduce water volume, effectively increasing the density of tadpoles in a pond. Warmer water can also alter competitive relationships among individuals and between species (Govindarajulu and Anholt 2006, Liles et al. 2017). Microhabitat use can shift because of predator avoidance, resulting in aggregations of native species away from predatory invasive species. Also, amphibian populations are understood to be regulated by density-dependent factors, particularly in the early life-history stages (Biek et al. 2002, Vonesh and De la Cruz 2002). A recent meta-analysis compiling the effects of stressors on amphibians found that increased density tends to slow development rate, reduce mass of tadpoles at metamorphosis, and decrease growth rate (Edge et al. 2016). Morphological changes in response to competition, typically narrower tail fin and muscle tissue, and shorter leg length, have been observed in several studies (Tejedo et al. 2000, Relyea 2002, Relyea and Hoverman 2003, Relyea and Auld 2005, Van Buskirk 2009). Competition and density at the larval stage can result in carryover effects to the terrestrial stage, which is also observed for the effects of climate change and invasive species (Goater 1994, Van Buskirk and Saxer 2001, Lane and Mahony 2002, Relyea and Hoverman 2003, Johansson et al. 2010, Bredeweg et al. 2019), making understanding the interaction among these 3 stressors important for predicting effects of environmental change on amphibians.

Generally, stressors are assumed to interact in 1 of 3 ways. The additive effects model is most commonly used in ecological studies. In an additive effects model, the null expectation is that the effect of the 2 stressors together is the same as the sum of the 2 stressors individually. In a synergistic interaction, the effect of the 2 stressors together is greater than the null expectation. In an antagonistic interaction, the effect

of the 2 stressors together is less than the null expectation. These expected interactions can be expanded to include any number of interacting stressors. Recent meta-analyses on the effects of multiple stressors in freshwater systems found that antagonistic interactions were the most commonly observed interaction type (Jackson et al. 2016, Burgess et al. 2021). However, the choice of model underpinning the null expectation and the direction of effects influence the classification of stressor interactions (Côté et al. 2016, Schäfer and Piggott 2018, Burgess et al. 2021).

In this experiment, we tested the individual and combined effects of climate change, invasion, and competition (via conspecific density) on a common native amphibian, the PCF. PCFs are found in freshwater habitats across western North America. Previous work suggests PCFs respond plastically to both climate change and invasion. Like other amphibians, PCFs exposed to warmer temperatures, reduced hydroperiod, or both had accelerated metamorphosis, usually at the cost of emerging at a smaller size compared with tadpoles in control treatments (Koprivnikar et al. 2014, O'Regan et al. 2014, Thurman and Garcia 2017, 2019, Tasker et al. 2022). Invasive fishes have been found in bodies of water historically and currently inhabited by PCF larvae, and PCFs demonstrate morphological and behavioral plasticity in response to invasive predators, including when the population is naïve to the predator (Benard 2006, Preston et al. 2017, Wilson et al. 2018, Garcia et al. 2019). Exposure to a species of invasive fish resulted in narrower tail fins (but not reduced muscle) in naïve PCFs, and exposure to a native insect predator resulted in deeper tail fins for PCFs in California, USA (Benard 2006). Larval density also affects PCF survival in combination with other factors. In a field survey, PCFs in low-density populations had increasing survival with increasing water temperature, but PCFs in high-density populations had reduced survival with increasing water temperature (Govindarajulu and Anholt 2006). Density also reduced survival of PCF larvae when coupled with the presence of invasive American Bullfrog (*Lithobates catesbeianus*) tadpoles, where survival was lower in high-density treatments (Adams 2000).

In this study, we sought to understand the individual and combined effects of climate change, invasive species, and competition (via increased conspecific density) on PCF survival, growth, and development. Our primary interest was in the interaction among the stressors, and we hypothesized that H1) the 3 stressors would interact antagonistically (*sensu* Côté et al. 2016, Jackson et al. 2016, Burgess et al. 2021), resulting in a less-than-additive effect when all 3 stressors were present. We also were interested in the individual effects of each stressor on PCFs and developed a priori hypotheses about effects on survival, growth, and development. Regarding survival, a pilot study found that survival of PCFs was lower under warmer temperatures when paired with a conspecific (BAB, unpublished data), so we hypothesized that

H2) increased density would reduce survival under future climate conditions (warmer water, shorter hydroperiod). Regarding growth, we hypothesized that H3) all 3 stressors would result in reduced growth. Finally, climate, predation, and density affect development differently, so we hypothesized that H4a) exposure to future climate conditions (warmer water, shorter hydroperiod) would result in a faster development rate, H4b) exposure to invasive predatory fish chemical cues would result in a slower development rate, and H4c) higher density conditions would result in a slower development rate due to increased competition.

METHODS

To answer our research questions, we conducted a fully factorial experiment in the laboratory. We tested the effects of climate change (modeled as increased temperature and reduced hydroperiod, simultaneously), invasive fish chemical cues (Brook Stickleback, *Culaea inconstans* (Kirtland, 1840), hereafter, sticklebacks), and competition (modeled through increased conspecific density) on PCF larvae. Sticklebacks are currently invading ponds in the region where this study was conducted, including ponds historically occupied by PCFs. Their effects on PCFs are not well understood, though they act as predators on native amphibians (BAB, unpublished data). We measured survival, growth, growth rate, mass at metamorphosis, body mass index, tail fin depth, tail muscle depth, tail muscle width, hind limb length, and development rate. We used 2 approaches to understand the effects of the stressors individually and in combination. First, we used traditional linear models to analyze the outcomes related to each stressor and stressor combination. Second, because sample size determines, in part, the power to detect interactions via traditional statistical models, and traditional models may be biased against detecting synergistic interactions even when present (Burgess et al. 2021), we also used the method of Tekin et al. (2020), including normalization via rescaling, to calculate interaction effects.

Animal collection

We collected PCF larvae from a fishless pond in the Dishman Hills Conservation Area, Spokane County, Washington, USA, on 29 May 2019. Tadpoles were collected from the entire pond to avoid collecting from a single kin group. We collected sticklebacks from Turnbull National Wildlife Refuge, Spokane County, Washington, on 28 June 2019. We transported the experimental organisms to the laboratory on ice to reduce transport stress. In the lab, we transferred the animals to 33 × 19 × 21-cm plastic holding tanks filled with tap water treated with NovAqua® Plus™ and AmQuel® Plus™ (Kordon®, Hayward, California) in a temperature-controlled room set to 14°C. Prior to the experiment, we kept PCFs in holding tanks in groups of 8 to 10 ind. and sticklebacks in groups of 15 to 30 ind. In stickleback tanks we used

aquarium bubblers to maintain oxygen levels. We fed tadpoles a mixture of rabbit chow and fish flakes (3:1 by volume) ad libitum and sticklebacks a mixture of frozen prey (rotifers, brine shrimp, bloodworms, cyclops; San Francisco Bay Brand, Newark, California) ad libitum. We removed waste daily and conducted partial water changes as needed to maintain water quality. We held tadpoles in the 14°C lab for 30 d. From d 30 to 39, we moved tadpoles to a 22°C room during daylight hours and then returned them to the 14°C room overnight for re-acclimatization to diurnal temperature fluctuations. The experiment began 40 d after the tadpoles were collected from the field.

Experimental design

The treatments consisted of climate conditions (historical temperatures/hydroperiod and projected future temperatures/hydroperiod), invasive stickleback chemical cues (present and absent), and conspecific density (conspecific present and absent) in a fully factorial design ($2 \times 2 \times 2$) replicated $8\times$ ($n = 8$, total tanks = 64). Although this design limited our ability to detect nonlinear responses, adding more treatment levels to each stressor would have necessitated a corresponding reduction in replication. We opted to emphasize replication because variation was expected to be high based on previous work with this species. We arranged glass tanks (9.5 L; $31 \times 16 \times 20$ cm), filled to a depth of 15 cm at the start of the experiment, on wire shelving in a temperature-controlled room set to 14°C with a 14:10-h photoperiod. Treatments were randomly assigned to tanks, and PCF tadpoles were randomly assigned to treatments. Tanks were arranged such that each shelf (1.2×0.9 m) held 9 tanks. Tanks were spaced so that the glass walls did not touch to avoid heat conduction between warmer or cooler tanks.

Our study combined all 3 stressors in a fully factorial design to understand how the stressors may interact. Climate conditions that increase temperatures and reduce hydroperiod, invasion by nonnative species, and competition with conspecifics are likely to co-occur at the levels modeled in this study. Temperatures and hydroperiods for climate treatments were based on historical conditions and future projections for the study area, sticklebacks are found at very high densities in the invaded areas (BAB, unpublished data), and conspecific density effects are well known for PCFs and other frog species.

Climate treatment The 2 climate treatments were designed to reflect historical climate patterns and the predicted future climate. Warmer temperatures and reduced hydroperiod are expected to covary due in part to increased evaporation rates (Arias et al. 2021), so we simulated climate conditions by manipulating temperature and hydroperiod as a single treatment. We designed scenarios, described in Tasker

et al. (2022) and briefly described here, to mimic conditions in seasonal ponds similar to the pond where the PCFs were collected. We estimated historical and projected future water temperatures using historical temperature data and future air temperature projections from the Downscaled Coupled Model Intercomparison Project 3 (CMIP3) and CMIP5 Climate and Hydrology Projections archive (http://gdo-dcp .ucllnl.org/) for the Spokane River tributary area for the months May through September. For historical data, we downloaded data for May through September from the y 1976 through 1999. We then randomly extracted a daily minimum and maximum temperature for each date across all years, resulting in a single daily minimum and maximum for each date. For future projections, we downloaded the BCCAv2 CMIP5 data from all available climate models and used a simple ensemble mean of all models to obtain daily minimum and maximum air temperature values. All air temperature data were converted to water temperatures by using an observed relationship between water and air temperature in 3 ponds near the population used in the current study (supplement 1 in Tasker et al. 2022). Because the future projections were the result of an ensemble mean, the daily variation in minimum and maximum temperature was much higher in the historical data. To remove this extra variation and smooth the historical data, we calculated the mean difference between daily minimum and maximum temperatures between the projected future and historical temperatures for the month of June. Then, we estimated the daily historical minimum and maximum for each date by subtracting the mean difference (minimum: 1.45°C, maximum: 2.24 °C) from the daily future projections. For both treatments, we simulated a diurnal cycle of temperature with the lowest temperature at 08:00 and a continual warming until the peak at 18:00. Daily minimum and maximum temperature varied by day, based on future daily minimum and maximum temperatures, with an overall increasing trend in both treatments over time (Fig. S1). A distributed network of microprocessors (Arduino® Nano; Arduino, Somerville, Massachusetts) and small computers (Raspberry Pi®, Cambridge, United Kingdom) controlled each tank's temperature and recorded the actual temperature of each tank every 5 min. A single waterproof temperature probe (model #DS18B20; Maxim Integrated™, San Jose, California) monitored individual tank temperatures, and a power-modulated 50W ViaAqua® aquarium heater (Commodity Axis®, Camarillo, California) heated each tank (https://github.com/scott-whitworth/bancroft_tempera ture_control; Tasker et al. 2022).

In addition to temperature treatments, we manipulated hydroperiod to reflect the evaporation rate of the simulated seasonal ponds as described in Tasker et al. (2022, supplement 1). Briefly, we extracted data on hydroperiod from a published paper on montane environments (Lee et al. 2015) and combined it with projected open-water potential evapotranspiration data from the Downscaled CMIP3

and CMIP5 Climate and Hydrology Projections archive. To imitate evaporation of ponds over time, we removed a small amount of water from the tanks each week. Because water loss is greater in warmer, drier climates, we removed more water from the future treatments than the historical treatments. In the historical climate treatment, we withdrew 1.2 cm of water from each tank each week. In the future climate treatment, we withdrew 2.2 cm of water each week. In some weeks, higher water temperatures resulted in high rates of evaporation, reducing the tank water level below the set weekly depth. We replenished water as needed to maintain consistent hydroperiod levels within treatment groups. All tanks started with 15 cm of water. After 4 wk, the historical tanks were 10.2 cm deep and future tanks were 7.1 cm deep, at which point water levels were kept static to prevent exposure of the aquarium heaters in the future treatments.

Chemical cues from invasive sticklebacks   Previous work suggests sticklebacks quickly consume amphibian larvae when confined in the same tank (BAB, unpublished data). Therefore, we used chemical cues rather than direct interaction to understand the nonlethal effects of invasive stickleback. We filled plastic tanks (33 × 19 × 21 cm) with 6.8 L of treated tap water and used standard aquarium bubblers to aerate them. We kept sticklebacks in groups of ~30/tank. To minimize the effect of feces (which can be food for tadpoles) or additional cues from stickleback food, we designed a tank rotation. Because we added cues daily (including sham cues from tanks without fish), 14 sets of tanks were designated for cues (7 with fish, 7 without). Fish were held in a feed tank for 5 d where waste was removed daily. Stickleback food was prepared by adding 3 blocks of frozen prey to 400 mL of treated water. Sticklebacks were fed 100 mL of stickleback food/tank each feed day. On d 6, fish were individually netted and transferred to a clean tank (designated as the fish cue tank) and held for 24 h without food before removing the fish back to the feed tank. We removed waste products, and the water in the cue tank was then used as the fish chemical cues for the day's treatment. Control cue tanks were paired with fish cue tanks and were treated the same except for the presence of fish. Every day, 400 mL of water was removed from each experimental tank and replaced with 400 mL of either stickleback cue water or control cue water, depending on the treatment group.

Conspecific density   To test the effects of conspecific presence, treatments were randomly assigned such that ½ the tanks had 1 PCF larva and the other ½ had 2 PCF larvae. Tadpoles were visually size matched to prevent asymmetric competition related to size effects. Tadpoles were fed ad libitum to control for resource access between conspecific absent and present treatments. Because we fed the tadpoles ad libitum, competition effects were more likely due to interference competition rather than exploitative competition.

Data collection and analysis

We staged all tadpoles (mean: stage 33, range: 30–37; Gosner 1960) and photographed each tadpole in water from the top and side prior to the start of the experiment. We removed tadpoles from the experiment when they reached Gosner stage 42 (forelimb emergence; Gosner 1960), signaling metamorphosis to the terrestrial life stage, or if a tadpole died. To measure morphological differences and growth during the experiment, we weighed tadpoles and photographed them from the top and side at the end of the experiment. In the conspecific present treatments, we weighed and measured the 1st tadpole that reached stage 42. We euthanized the tadpoles with an overdose of buffered MS-222 (tricaine methanesulfonate; Millipore Sigma, Burlington, Massachusetts) after taking photographs and mass and preserved them in 70% ethanol. Three tadpoles were not weighed until 3 d post euthanasia. We subsequently weighed 10 tadpoles immediately after emergence and again 3 d post euthanasia to create a standard curve from which to estimate the emergence mass of the 3 tadpoles (Fig. S2). We detected a clear relationship ($r^2 = 0.97$, $p < 0.0001$) with which we estimated the emergence mass of the 3 tadpoles. We analyzed mass with and without these 3 tadpoles, but analyses are presented with the estimated masses. One tadpole progressed from stage 41 to stage 45 over the course of 24 h, which we excluded from all analyses except survival and development because it had developed past stage 42 and was not comparable morphologically.

To measure linear growth and morphological characteristics, we used ImageJ (Schneider et al. 2012) with the beginning and end photographs. From these photographs we measured snout vent length (SVL) and calculated growth over the course of the experiment as the proportion of the initial size gained during the experiment ([final SVL − initial SVL] / initial SVL) (Annibale et al. 2018, Tasker et al. 2022). We calculated growth rate as the growth (final SVL − initial SVL) divided by the number of d each tadpole was in the experiment before reaching Gosner stage 42. We calculated scaled indices for all morphological variables (body mass, tail muscle depth, tail muscle width, tail fin depth, leg length) to account for inherent allometric relationships between aspects of size and morphology (Peig and Green 2009). These scaled indices calculate the value for each morphological variable (e.g., mass) at a fixed body size. To calculate the scaled indices, we first checked for outliers (none were identified) and then used standard major axis regression in the *smatr* package (version 3.4–8; Warton et al. 2018) in R (version 4.1.1; R Project for Statistical Computing, Vienna, Austria) to identify the variable with the best correlation with the measurement to be scaled. We used total body length (including tail) to scale body mass and tail muscle depth (vertical linear measurement across the deepest part of the tail muscle from a lateral view), and

we used mass at stage 42 to scale tail fin depth (the vertical linear measurement across the deepest part of the tail minus the muscle depth from a lateral view), tail muscle width (at the widest part of the tail from a dorsal view), and leg length (distance from body to base of tibia of left rear limb). We then used the slope coefficient to scale body measurements by using the Thorpe–Lleonart method as modified by Peig and Green (2009). Because of correlation among variables, we used principal component analysis to summarize all growth and morphology measurements into fewer uncorrelated variables. We centered and scaled all variables before generating principal components with the prcomp function in R. We retained the first 2 principal components for further analyses, representing 87% of variation in the data (Table 1).

We measured development rate by calculating the number of stages passed/d. In conspecific treatments we assumed that the tadpole emerging 1st was the tadpole with the more advanced stage at the start of the experiment.

Our analysis objectives were 2-fold. First, we analyzed the outcome of our experiment using traditional statistical models. We analyzed tadpole survival with a binomial generalized linear model (GLM) with a logit link. Because of the scarcity of mortality events, we used Firth-adjusted estimates (Walker and Smith 2019) implemented in the *brglm2* package (version 0.8.2; Kosmidis et al. 2021) in R. Because we had fewer mortality events than treatment groups, we did not include interactions in our model (e.g., only main effects were included). Growth and morphology were represented by principal components 1 and 2, which we analyzed with 3-way analysis of variance with the aov function in base R statistics. We analyzed development rate with a gaussian GLM with a log link. For body shape (PC 1 and PC 2) and development, we used fully factorial models, including all 2- and 3-way experimental factor (climate, conspecific density, fish cue) interactions.

Table 1. Principal component analysis (PCA) of growth and morphological variables for Pacific Chorus Frog (*Pseudacris regilla*) tadpoles (Gosner stage 42). SVL = snout vent length.

PCA results	PC 1	PC 2
% of variance	70	18
Eigenvalue	5.56	1.66
Factor loading		
Mass at stage 42	0.413	0.090
Proportional growth ([final SVL − initial SVL] / initial SVL)	0.359	−0.310
Growth rate	0.301	−0.463
Scaled mass index	0.036	0.765
Tail muscle depth	0.375	0.230
Tail muscle width	0.411	0.075
Tail fin depth (excluding tail muscle depth)	0.363	0.195
Leg length	0.409	−0.004

For all GLM models, we used analysis of deviance based on likelihood ratio χ^2 tests to assess the significance of terms using the Anova function in the *car* package (version 3.0–11; Fox et al. 2021) in R. We used likelihood ratios (rather than Wald tests) because of small sample sizes (Agresti 2007). Prior to analysis, we assessed model assumptions via residual plots, and we conducted post hoc analyses with the *emmeans* package (version 1.7.0; Lenth et al. 2021) in R.

We also used the method of Tekin et al. (2020), including normalization via rescaling, to calculate interaction effects because traditional linear models may be biased against detecting interactions (Burgess et al. 2021). This method uses rescaling to define interactions relative to baselines of synergy or antagonism, which reveals the magnitude of interactions and results in multimodal distributions (Tekin et al. 2020). Interaction effects are expressed as deviation from additivity (DA) values, where 0 = perfectly additive effects (e.g., no interaction). We categorized interaction effects under this framework as synergistic if DA < −0.5, additive between −0.5 and 0.5, antagonistic buffering if between 0.5 and 1.3, and antagonistic suppression (extreme antagonism) if DA > 1.3. These calculations allowed us to explore the relative influence of each stressor and stressor combination and to characterize interactions as additive, antagonistic, or synergistic regardless of the strength of the interaction. Furthermore, we were able to separate contributions of all individual and pairwise effects from the emergent and net interaction effects of 3 stressors using the methods of Beppler et al. (2016), including rescaling based on the minimum value of the single stressor effect when the unscaled metric was positive. The emergent interaction represents the effect when all 3 stressors are present (beyond the multiple pairwise effects from the combinations of 3 stressors interacting in pairs); thus, the emergent interaction is the effect beyond what would be predicted from the pairwise effects. The net interaction is the effect of the 3 stressors beyond the individual effects of each stressor, which identifies the presence of a 3-way interaction that is larger than would be predicted based on the individual effects alone (Beppler et al. 2016, Tekin et al. 2018). These interaction methods were developed and tested in the drug-interaction literature using small sample sizes and potentially low statistical power. For example, Beppler et al. (2016) had sample sizes as low as 2 and as large as 9 for their triple drug interaction studies. We used R statistical software for all analyses and graphing, and we created graphs with the packages *ggplot2* (version 3.3.5; Wickham et al. 2020) and *patchwork* (version 1.1.1; Pedersen 2020).

RESULTS
Linear models
Survival All PCFs metamorphosed or died by d 41, at which point the experiment was terminated. In partial

support of H2, survival was not affected by climate treatment or presence of fish chemical cues, but presence of a conspecific reduced the odds of survival by 61% (Table S1). Seven tadpoles died, 6 of which were in the conspecific treatment groups. Of the 6 tadpoles that died in the conspecific treatment group, the corresponding paired tadpole survived to metamorphosis in all cases (i.e., all tanks with 2 tadpoles had at least 1 surviving tadpole).

Growth and morphology   Principal component 1 (PC 1) loaded strongly and positively for all morphological variables except scaled mass index and accounted for most (69%) of the variance in the data (Table 1). Larger positive values of PC 1 indicate a larger overall body size, faster growth rate, greater linear growth, greater muscle mass in the tail, larger tail fin, and longer legs. Principal component 2 (PC 2) loaded positively for scaled mass index and negatively for growth rate and linear growth (proportion of original size obtained during the experiment; Table 1). In support of H3, conspecific density affected body size (PC 1; $p < 0.0001$; Table S2), where tadpoles raised with a conspecific entered metamorphosis with smaller bodies, including lower mass (by 37%), and reduced leg length (by 48%), and had 45% lower linear growth compared with tadpoles alone (Fig. 1A). Climate treatment also affected body size (PC 1; $p = 0.002$; Table S2) in support of H3, where tadpoles in future climate treatments had lower PC 1 scores (Fig. 1B). For example, mass was 28% lower and both linear growth and leg length were 37% lower in tadpoles exposed to warmer

temperatures and decreased hydroperiod than those in the historical climate treatment. Furthermore, an antagonistic interaction was observed between climate and fish cues ($p = 0.02$; Table S2), such that under historical climate conditions, fish chemical cue presence reduced body size ($p = 0.01$), but under future climate conditions, no effect of fish chemical cue was observed beyond the effect of climate alone ($p = 0.44$; Fig. 1B). No effects of any treatment were observed for PC 2 (Table S2).

Development   A 3-way interaction was detected for development rate (Table S3). In support of H4a, development rate was 23% faster under future climate conditions overall (Fig. 2). In support of H4b, under future climate conditions and in the absence of fish chemical cues, the presence of a conspecific reduced development rate by 23% relative to when all 3 stressors were present ($p = 0.02$). In contrast to the expectations of H4b, presence of fish chemical cues interacted antagonistically with conspecific presence to restore rapid development under future climate conditions (Fig. 2). Results from all pairwise comparisons are shown in Table S4.

Multiple stressor effects

Interactions between pairs of stressors were all classified as either additive or antagonistic. Out of 27 pairwise interactions, >½ (16; 59%) were classified as additive (Table 2). After additive pairwise interactions, antagonistic pairwise interactions were the only other observed pairwise interaction type,
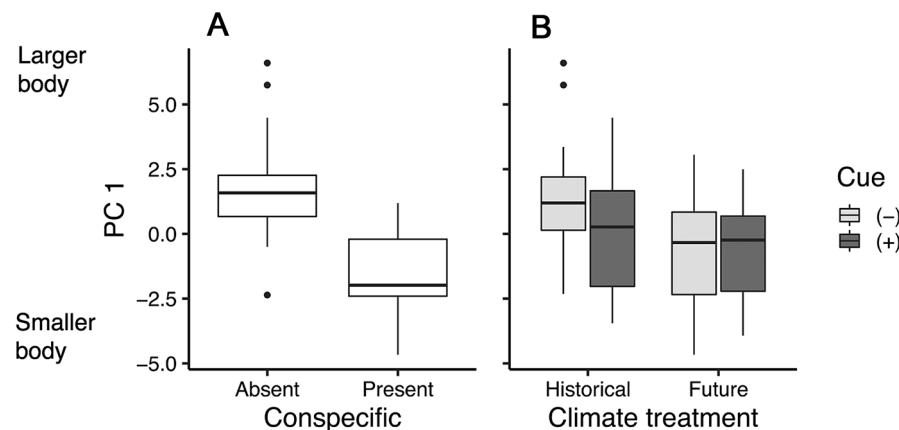

Figure 1. Relative body shape at Gosner stage 42 (Gosner 1960) in Pacific Chorus Frogs (*Psuedacris regilla*), represented by the 1st axis (PC 1) of a principal component analysis of tadpole morphological and growth measures. Larger positive values of PC 1 indicate greater mass, higher growth rate, greater linear growth, increased tail muscle mass, increased tail fin depth (excluding muscle depth), and longer leg length. Tadpoles were smaller overall when raised with a single conspecific tadpole compared with tadpoles raised alone ($p < 0.0001$) (A). Tadpoles were smaller under future climate conditions (elevated temperature and reduced hydroperiod) compared with tadpoles under historical climate conditions ($p = 0.002$) (B). Fish chemical cues reduced body size under historical conditions ($p = 0.01$, indicated by asterisk on plot), but did not reduce body size under future climate conditions ($p = 0.47$) (B). Boxplots show median (horizontal lines) and 1st and 3rd quartiles + 1.5× interquartile range (whiskers). Dots represent data beyond the 1.5× interquartile range. Data were analyzed using analysis of variance. Post hoc test for fish cue and climate interaction done via estimated marginal means.
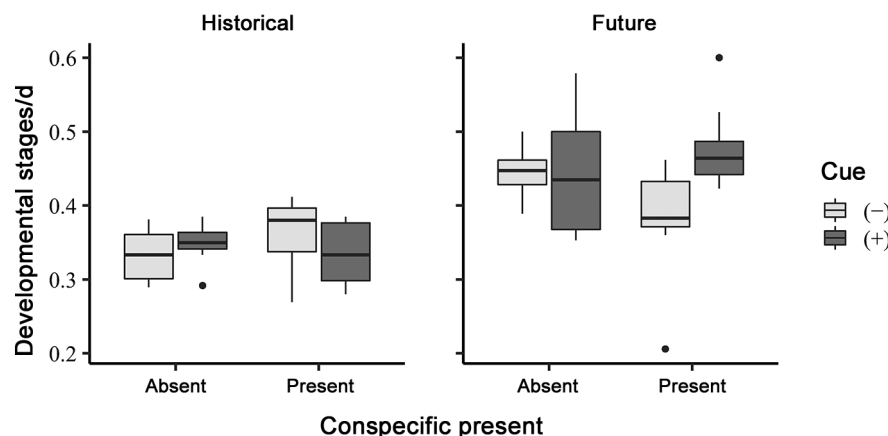
Historical / Future

Figure 2. Development rate of Pacific Chorus Frogs (*Pseudacris regilla*) as Gosner (1960) stages/d. Each tadpole was staged prior to the experiment. All tadpoles were removed at stage 42. Exposure to warmer temperatures with reduced hydroperiod (future) climate treatment resulted in overall faster rates of development (Likelihood ratio = 38.97, df = 1, $p < 0.0001$; Table S3). In the absence of fish chemical cues, presence of a conspecific reduced development rate under future climates. When fish chemical cues were added, rapid development rate was restored, even in the presence of conspecifics (3-way interaction term: Likelihood ratio = 6.59, df = 1, $p = 0.01$). Boxplots show median (horizontal lines) and 1st and 3rd quartiles + 1.5× interquartile range (whiskers). Dots represent data beyond the 1.5× interquartile range. Data were analyzed with a Gaussian generalized linear model with a log link.

representing 11/27 interactions. Of these, 6 were the more extreme antagonistic suppression classification (Table 2). Notably, all pairwise interactions between climate and conspecific presence were classified as additive. Most pairwise interactions between conspecific presence and fish cues were also additive, with the exceptions of mass, scaled mass index, and tail muscle width. Pairwise interactions between climate and cue had smaller antagonistic effects (antagonistic buffering) on mass and leg length and larger antagonistic effects (antagonistic suppression) on other outcomes except for fin depth (Table 2).

Generally, the effect of 3 stressors was not predictable from the effects of individual stressors or the effects of the pairwise combinations. Specifically, additive interactions were less frequent when all 3 stressors were present. Each outcome variable had 2 interaction effects calculated for the 3-way interaction, a net interaction (effect of 3 stressors relative to individual stressor effects), and an emergent interaction (effect of 3 stressors relative to pairwise effects of stressor combinations; Beppler et al. 2016). For net and emergent effects, only 1 outcome variable each had 3-way interactions classified as additive (tail muscle depth and scaled body mass index, respectively; Table 2). In contrast to H1, not all 3-way interactions were antagonistic. Synergistic interactions were more frequent for 3-way net effects (6/9) than for 3-way emergent effects (3/9). Specifically, when all 3 stressors were present, development rate was faster than expected but scaled leg length and tail muscle width were smaller than expected relative to the additive assumption (Table 2, Fig. 3). Antagonistic interactions were less frequent for net effects (only scaled body mass index and growth rate) but were the most frequent emergent interaction (Table 2,

Fig. 3). Overall, effect sizes revealed that scaled body mass index, tail muscle depth, and tail fin depth were less affected relative to other outcome variables, whereas leg length and linear growth were most affected by the stressors (Fig. 3). For example, when all 3 effects were present, leg length was 69% lower than the control and linear growth was 54% lower.

DISCUSSION

Our goal was to quantify the effects of climate change (modeled as increased temperature and reduced hydroperiod simultaneously), invasive fish chemical cues, and conspecific density on PCF larvae, both as individual effects and as interacting stressors. Our study highlights the wide-ranging effects of 3 important stressors on a generalist amphibian species and illustrates the complexity of predicting multiple stressor interactions from single or pairwise effects. Intraspecific competition (density) had the largest relative impacts on all measured outcomes except development rate (Fig. 2), followed by the effects of simulated future climate conditions (warmer water temperature, rapid drying). Brook Stickleback chemical cues had relatively smaller effects, particularly under future climate conditions, but were often important in the presence of the other stressors, evidenced by the increased incidence of antagonistic suppressive emergent interactions when all 3 stressors were present. The preponderance of antagonistic suppressive interactions suggests that in most cases, overall effects did not increase proportionally to the number and individual effects of stressors. However, when all 3 stressors were present, leg length and tail muscle width were reduced more than expected and development rate was faster than expected (synergistic 3-way net and emergent

Table 2. Multiple stressor classification of all interactions using the stressor classification from Tekin et al. (2020) to assign deviation from additivity (DA) values for 3 environmental stressors on growth, morphology, and development of Pacific Chorus Frog (*Pseudacris regilla*) tadpoles. This framework classifies interactions into synergistic (<-0.5), additive ($-0.5-0.5$), antagonistic buffering (AB; 0.5–1.3), or antagonistic suppressing (AS; >1.3), which is a more extreme form of antagonism than AB. Net interactions are the effect of 3 stressors beyond expected based on the effect of each stressor alone. Emergent interactions are the effect of 3 stressors beyond expected based on the pairwise effects. Three-way interactions have both net and emergent classifications, whereas 2-way interactions do not. All 2-way interaction DA values are rescaled as described in Tekin et al. (2020), and all 3-way DA values are rescaled as described in Beppler et al. (2016).

Measured outcome	3-way interaction: Climate × conspecific × cue			2-way interaction: Climate × conspecific		2-way interaction: Climate × cue		2-way interaction: Conspecific × cue	
	Interaction type	DA	Effect classification	DA	Effect classification	DA	Effect classification	DA	Effect classification
Mass (g)	Net	−1.04	Synergistic	0.09	Additive	0.85	AB	0.85	AB
	Emergent	2.53	AS						
Growth (cm)	Net	−0.62	Synergistic	−0.16	Additive	1.44	AS	−0.27	Additive
	Emergent	5.52	AS						
Growth rate (cm/d)	Net	1.15	AS	−0.10	Additive	2.55	AS	−0.22	Additive
	Emergent	66.23	AS						
Scaled body mass index	Net	3.75	AS	−0.04	Additive	5.47	AS	−8.81	AS
	Emergent	0.22	Additive						
Tail muscle depth	Net	−0.04	Additive	−0.15	Additive	7.11	AS	−0.05	Additive
	Emergent	5.27	AS						
Tail muscle width	Net	−0.84	Synergistic	0.17	Additive	1.35	AS	0.85	AB
	Emergent	−0.80	Synergistic						
Tail fin depth	Net	−2.73	Synergistic	−0.07	Additive	0.06	Additive	−0.01	Additive
	Emergent	1.88	AS						
Leg index	Net	−0.55	Synergistic	0.16	Additive	1.29	AS	−0.20	Additive
	Emergent	−0.80	Synergistic						
Stages/d[a]	Net	−6.46	Synergistic	0.27	Additive	1.77	AS	0.13	Additive
	Emergent	−1.21	Synergistic						

[a] Stages/d were all higher than the control, so we followed the method of Tekin et al. (2020) and calculated the effect sizes using control/experimental instead of experimental/control.

interactions). Thus, in some cases, overall effects increased more than proportionally to the number of stressors.

Survival response to competition

As hypothesized, tadpoles raised with a conspecific had a higher mortality rate than tadpoles raised alone, though no interaction with climate treatment was observed. The potential for density to reduce survival has been documented in several species, including PCFs (Brockelman 1969, Semlitsch and Caldwell 1982, Loman 2004, Govindarajulu and Anholt 2006). The density used in our experiment was much lower than typical densities used in laboratory experiments. The range in our experiment was 0.2 tadpoles/L at the beginning of the experiment to 0.4 tadpoles/L at the end of the experiment in future climate tanks compared with a mean ±SE of 43.8 ± 19.8 tadpoles/L from 73 intraspecific laboratory experiments (Skelly and Kiesecker 2001). However, the density range in our experiment was within the range of observed PCF tadpole densities in the field (Kupferberg 1998, Govin-

darajulu and Anholt 2006, Bancroft et al. 2008). Estimated larval survival for many species is low, in part because of density dependence, so the mortality observed in our study is unlikely to be important at the population level (Biek et al. 2002, Vonesh and De la Cruz 2002), particularly as it was not enhanced by other stressors.

Growth response to stressors

Changes in body size and morphology at metamorphosis can influence aspects of the terrestrial phase. Tadpoles in our study entered metamorphosis at both a lighter mass and shorter SVL after exposure to future climate conditions and because of competition with a single conspecific. Lower mass at metamorphosis can result in lower survival in the terrestrial stage (Goater 1994, Lane and Mahony 2002). In 1 study, individuals that were lighter at metamorphosis were at higher risk of death during the overwintering period if additional mass was lost (e.g., during poor conditions) (Garner et al. 2011). In that study, individuals that were heavier at metamorphosis
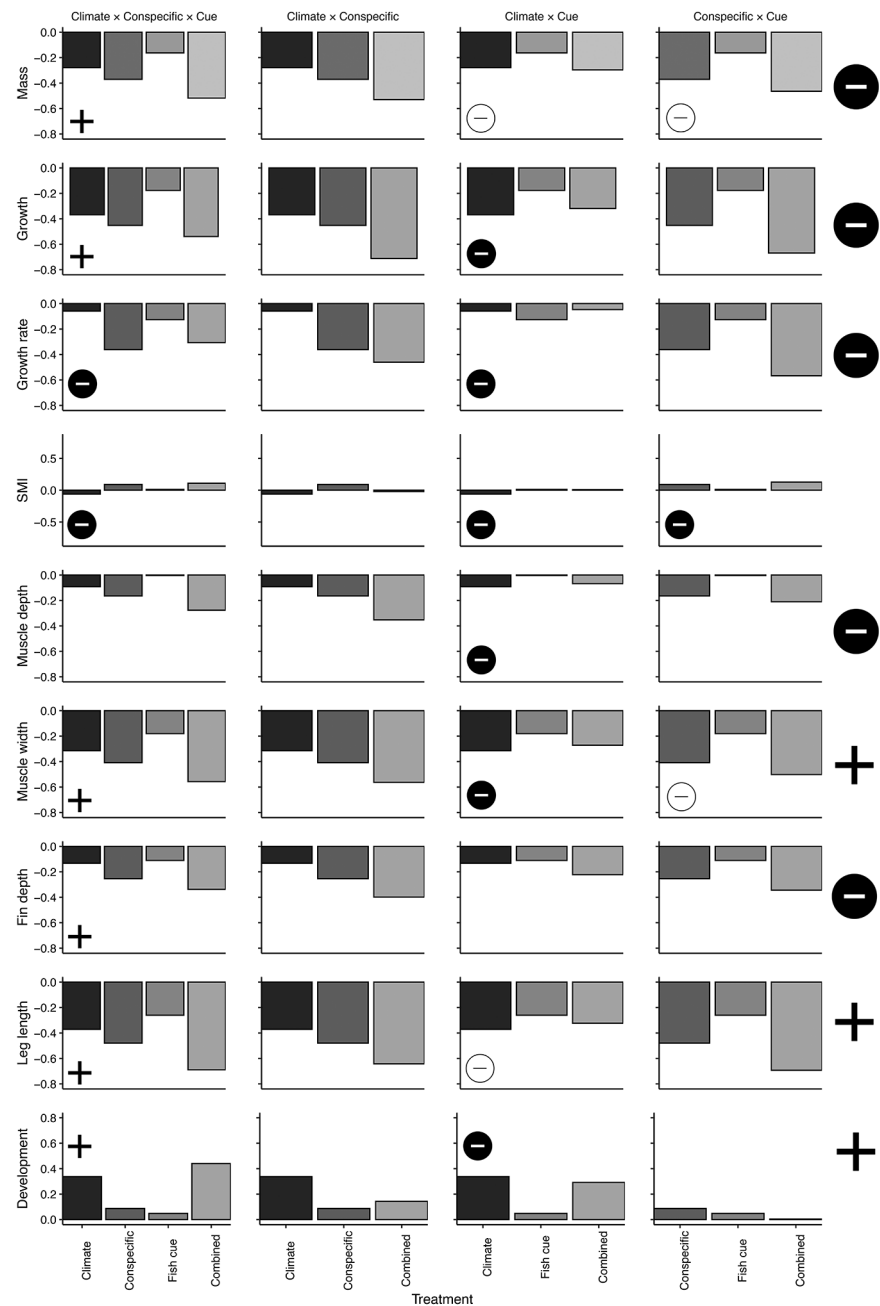
Figure 3. Effect sizes and interaction classification for all 3-way and pairwise stressor combinations for all measured outcomes. Bars represent the change relative to the control condition (1 − [treatment mean / control mean]). Note that the values for development are calculated as ([treatment mean / control mean] − 1) because of the higher rates of development in all experimental groups relative to controls. Negative values indicate that treatments reduced the outcome, and positive values indicate that treatments increased the outcome relative to the control. Symbols indicate stressor classification (plus sign = synergism, filled circle with minus sign = antagonistic suppression, open circle with minus sign = antagonistic buffering, no symbol = additive). For 3-way interactions, the symbol inside the plot indicates the classification of the net interaction, and the symbol at the far right of each row indicates the overall emergent interaction. SMI = scaled body mass index.

were not at higher risk, even if additional mass was lost during the overwintering phase. These results, coupled with our results, suggest that high-density larval populations under future climate conditions may enter the overwintering period at a higher risk of mortality. Mass at metamorphosis is also

correlated with aspects of performance, including jump distance, sprint speed, and endurance (Beck and Congdon 2000, Tejedo et al. 2000, Watkins 2001).

Smaller body size (length) is correlated with lower metamorphic survival, and body size at metamorphosis is correlated

with adult body size (Berven 1990). Size at metamorphosis has been linked with multiple aspects of fitness in amphibians (Earl and Whiteman 2015). Leg length also has important implications for terrestrial stages. Along with SVL, leg length is important in predicting maximal jump distance for anurans, including PCFs (Johansson et al. 2010, Bredeweg et al. 2019). Maximal jump distance is assumed to be important for predator escape and thus survival in the terrestrial stage. Emerson (1986) estimated that differences in leg length would need to exceed 10% before measurable performance differences would be observed, which was exceeded by the tadpoles in our study exposed to future climate conditions, higher density, and predatory fish cues (37–69% reductions). Moreover, leg length was 1 of 2 outcomes to exhibit negative synergistic net and emergent effects of all 3 stressors, suggesting that the effects of these stressors on leg length was greater than expected based both on each individual stressor effect and the pairwise effects of the stressors. Thus, jump performance was likely reduced more than would be predicted by exposure to these 3 stressors, which may affect survival in the terrestrial stage. Shorter legs could be partially compensated for by increased muscle power in the leg muscles. However, leg muscle may be augmented by material reabsorbed from the tail (Van Buskirk and Saxer 2001), suggesting that tadpoles with reduced tail muscle may have less leg muscle mass as juveniles, compounding the effects of these stressors.

Tail morphology relates to energy availability and tradeoffs in both the aquatic and terrestrial stages. Changes to tail morphology are often attributed to adaptive phenotypic plasticity in response to selection. Competitors induce narrower tail fins and narrower muscle, whereas predators induce deeper tail fins and wider and deeper muscle tissue (Relyea 2001b, 2002, 2004). Deeper tail fins and tail muscle appear to be adaptive because tadpoles with that morphology are better swimmers (Johansson et al. 2010) and survive at higher rates in the presence of predators (Van Buskirk et al. 1997, Van Buskirk and McCollum 2000, Relyea 2001b). However, increased tail fin depth may come at a cost to body size or development (Van Buskirk and Saxer 2001, Florencio et al. 2020). In the presence of fish predators, it may be more adaptive to allocate energy to rapid development rather than increased tail tissue. The weak effects of fish chemical cue on tail morphology we observed may have been due to the lack of alarm cues from conspecific prey in the cue water, habituation to fish chemical cues, the naïve population from which we drew our study population, or a combination of all 3 (Mirza et al. 2006, Gonzalo et al. 2007, Polo-Cavia and Gomez-Mestre 2014, Pueta et al. 2022). The narrow tail morphology we observed in the presence of a conspecific competitor is typical and has been hypothesized to be due to reduced resource availability and the resulting tradeoff between tail tissue and body growth rate (Relyea 2002). The response of tadpoles in our study to simulated future climate conditions mirrored the response to presence of a competitor, suggesting that future climate conditions imposed a similar tradeoff between resource allocation. However, tadpoles in future climate conditions likely invested energy in rapid development, as reflected by their 33% faster development rate relative to controls. These rates are similar to those found by previous studies on this species (Thurman and Garcia 2019, Tasker et al. 2022).

Development rate response to stressors

As expected, development rate was sensitive to environmental conditions, with climate by far the most important predictor. Our finding that development rate increased under future climate conditions was consistent with previous studies (Lane and Mahony 2002, Richter-Boix et al. 2006, Tejedo et al. 2010, Edge et al. 2016, Ruthsatz et al. 2018, Tasker et al. 2022). When both future climate conditions and competition were present, development rate was depressed, though not below the weak effect of competition alone. Interestingly, when competition was combined with both other stressors, the overall effect was a more rapid rate than expected (positive synergistic emergent interaction). The addition of a 3rd stressor in the form of fish chemical cues restored the rapid development rate seen under future climate conditions alone.

The result of no effect of fish cues alone but a synergistic 3-way interaction reflects the variable effects predators have on development. Some studies have found no effect (e.g., Lane and Mahony 2002) or observed an increased development rate in the presence of predators at certain densities (Lent and Babbitt 2020), whereas others have observed a reduced development rate (e.g., Van Buskirk and Saxer 2001). A recent meta-analysis found that, in general, predators tend to slow development (Edge et al. 2016). The lack of typical response to fish chemical cues in our study suggests that the PCFs may not have perceived Brook Sticklebacks as potential predators. Previous work found that development rate of Long-Toed Salamander (*Ambystoma macrodactylum*) larvae was accelerated by exposure to cues from Brook Sticklebacks fed earthworms and depressed by exposure to cues from Brook Sticklebacks fed Long-Toed Salamanders (Reed 2016), suggesting that the diet of the predator matters for prey interpretation of risk. The design of our study did not include feeding sticklebacks with PCF larvae and, therefore, likely underestimates the true effect of predator cues in this system.

Interactions among environmental stressors

Understanding the interactions among stressors is necessary for both basic and applied ecology. Traditional methods of analysis for factorial experimental designs suffer from several issues, making detection of interactions difficult (Tekin et al. 2020). For example, we used GLMs with interaction terms to identify interactions for growth, tail muscle width,

leg length, and development; however, in this traditional analytical approach, the strength of the interaction (in this case, the size of the effect) and sample size drives the ability to detect ecologically important effects. Using an additional method developed for other fields in biology (e.g., genetics, drug interactions) allowed us to identify emergent interactions for all 3-way exposures for all but 1 outcome (scaled body mass index). In addition, our identification of antagonistic pairwise interactions between climate and fish cue for multiple response variables suggest strong suppressive effects of future climate conditions on fish chemical cues. However, these analyses do not include measures of variance and are based on the cutoffs suggested by Tekin et al. (2020). Therefore, these 2 approaches should be seen as complementary. Coupling traditional analysis with a different perspective on interactions allows for a fuller picture of the processes and patterns in the data.

In our analyses, most of the emergent 3-way interactions were antagonistic (5/9), suggesting that more stressors do not necessarily result in proportionally worse outcomes for PCFs. Furthermore, our results highlight that predicting multiple stressor interactions from single stressor or even pairwise interactions is difficult. Relative to single stressor effects, 3-way stressor net interactions tended toward synergisms in our analyses. However, after accounting for both single and pairwise effects, the overall effect of the 3-way stressor exposures tended towards antagonism, similar to patterns in drug interactions (Beppler et al. 2016, Tekin et al. 2016). Using a traditional GLM framework alone would suggest most 3-way interactions in this system are additive, but using the emergent interaction framework allowed us to identify the preponderance of antagonistic interactions. Without understanding the interactions under this framework, we would expect to see larger effects (additive rather than antagonistic) of multiple stressors in this system based on single stressor effects or pairwise interactions.

Phenotypic plasticity in PCFs coupled with the tendency toward antagonistic multiple stressor interactions may enable this species to cope with the rapid changes induced by climate change and other types of environmental change (Urban et al. 2014); however, more research is needed to understand the full extent of the carry over effects of environmental change in the larval stage on adult fitness for native amphibians. Tropical and subtropical amphibian populations are likely at greater risk because of climate change (Duarte et al. 2012), but our results suggest that expected changes in water temperature and hydroperiod have potential long-term effects for even temperate species, particularly when coupled with other stressors.

ACKNOWLEDGEMENTS

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