Behavioral Interactions between a Native and an Invasive Fish Species in a Thermally Heterogeneous Experimental Chamber

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Abstract: Mechanisms of the displacement of native fish by nonnative fish can include agonistic behaviors that push native fish species out of their preferred habitat, including their thermal optima. To examine these interactions, we built an experimental thermal preference chamber to evaluate: (1) the thermal preference of native, glacial relict northern redbelly dace *Chrosomus eos*; (2) if the thermal preference and movement changed in the presence of the invasive western mosquitofish *Gambusia affinis*; and (3) the direction of agonistic interactions. We hypothesized that *G. affinis* would express agonistic behavior toward *C. eos*, because *G. affinis* is widely recognized as an aggressive invader. Given the temperature range of the experimental chamber, i.e., 20–30 °C, *C. eos* selected an average of 24.3 °C as its thermal preference. After *G. affinis'* introduction, the thermal preference of *C. eos* increased by 1.7 °C and the movement, given by distance (cm) travelled, increased by 21%. Contrary to our prediction, more agonistic interactions were observed in *C. eos* toward *G. affinis*. These results indicate that agonistic behavior of *G. affinis* toward native fish species may be species- and condition-specific, and may not always be the primary mechanism of native species' displacement. Biological invasions are a global issue and altered thermal regimes are expected to continue. This study provided the novel approach using of a thermally heterogeneous thermal chamber to examine thermal preferences and aggressive interactions between a native and an invasive species. Future research should examine other life history traits that may be conveying the competitive advantage to *G. affinis*.

Keywords: agonistic behavior; thermal preference; condition-specific competition; *Chrosomus eos*; interspecific competition; *Gambusia affinis*; northern redbelly dace; western mosquitofish

1. Introduction

Nonnative fish are implicated in the demise of native fish globally, but a mechanistic understanding of how displacements take place is often lacking [1–3]. Mechanisms of displacement may include agonistic interactions, where aggressive behaviors by nonnative species push native species out of their preferred habitat, including their thermal optima [4–6]. Western mosquitofish *Gambusia affinis* (Baird & Girard, 1853) is listed as one of the 100 worst invasive species by the International Union for Conservation of Nature and has been implicated in the decline of freshwater fish [7–9], amphibians [10,11], and invertebrates [12–14]. A suite of ecological traits confer competitive advantages to *G. affinis*, including broad physiological tolerances (i.e., eurythermic) and high fecundity [15,16], but their aggressive behaviors are considered a key mechanism in native fish's displacement [17–19]. These agonistic interactions can result in an increased movement, which

is an energetic cost that can require fish to consume more food [20], increase predation risk [21], and reduce growth [22].

The northern redbelly dace *Chrosomus eos* Cope, 1861 = *Phoxinus eos* (Cope, 1861) is declining on the western edge of its distribution, where it is a glacial relict species. Although the species is stable throughout much of its range and has an IUCN status of "least concern", it is listed as "endangered" in Colorado, and "threatened" in Nebraska and South Dakota [23]. Threats to *C. eos* include alterations to its habitat and the presence of invasive species [23]. *C. eos* prefers off-channel lentic habitats, and has a strong preference for spring-fed habitats that are densely vegetated, such as beaver ponds [24,25]. In occupied habitats > 0.3 m deep, Bestgen [24] observed a strong thermal stratification, with surface to bottom temperatures ranging from 27 to 18 °C, respectively. Likewise, *G. affinis* uses the same habitat and has an overlapping thermal range with *C. eos*. *G. affinis* tolerates temperatures up to 39.5 °C [26] and has a preferred temperature, under laboratory conditions, of 31 °C [27]. Because *C. eos* occupies a thermally stratified habitat, its localized displacement by *G. affinis* may result a thermal displacement.

Temperature regulates the physiological and metabolic processes of ectothermic fish and is considered the abiotic ecological master factor [28,29]. As such, temperature influences growth [30], development [31], and behavior [32]. In habitats with micro-spatial thermal variation, ectotherms behaviorally thermo-regulate, fine-tuning trade-offs between thermal and competing resources (i.e., food) to optimize physiological and metabolic processes—ultimately maximizing the fish's fitness [33–35]. The thermal displacement of a native species by a non-native species has the potential to interrupt the former's thermal regulation, therefore interrupting the optimization of its physiological and metabolic processes. Instances where the inter-specific competitive dominance changes as a result of the environment (i.e., temperature) are known as condition-specific competition [36–39].

Studies have investigated temperature condition-specific competition by examining swimming performance among salmonids [38] and aggressive interactions of *G. affinis* [39]. While the foraging success and aggression of *G. affinis* increased significantly at higher temperatures, these fish were subject to thermally constant tanks at only three experimental temperatures [39]. Our current study aimed to examine behavioral interactions between native and nonnative species in a thermally heterogeneous setting simulating a thermally stratified micro-habitat, such as off-channel pools or beaver dams [40]. We observed fish's aggression to test for the presence of inter-specific competition and the frequency of *C. eos*' displacement to a non-preferred habitat. Aggressive behaviors in fish are influenced by a variety of factors including their size, societal status, prior residency, and temperature [41–43]. Elucidating how strongly thermal conditions mediate inter-specific competition is critical for predicting the population persistence of native species, especially for species with a relatively narrow thermal tolerance such as glacial relict species [44].

To inform conservation actions, we hypothesized that the displacement from preferred thermal range occurs due to agonistic behavior of *G. affinis* toward *C. eos*. Trials were conducted in a thermal preference chamber to determine: (1) the thermal preference of *C. eos*, (2) if the thermal preference changed with the addition of the invasive *G. affinis*, and (3) the direction of agonistic interactions.

2. Materials and Methods

2.1. Ethics Statement

The care and use of experimental animals complied with the State of Colorado's animal welfare laws, guidelines, and policies as approved by the State of Colorado Scientific Collection License #05–AQ963.

2.2. Experimental Fish

Adult *C. eos* (average total length (L_T) ± SD = 40 ± 4 mm) were obtained from the Colorado Parks and Wildlife John W. Mumma Native Aquatic Species Restoration Facility in Alamosa, CO, USA. Adult *G. affinis* (31 ± 4 mm) were captured using a two-person

pull seine from the South Platte River, near Greeley, CO, USA. *C. eos* and *G. affinis* were held in ten five-gallon holding tanks at the Colorado Parks and Wildlife Aquatic Research laboratory in Fort Collins, CO, USA. The stocking density of *C. eos* and *G. affinis* was 18–20 and 10–12 fish/tank, respectively. Holding tanks were separated by visual barriers, and supplied with on-site well water, aerated, and heated to 25 °C (±1 °C) using submersible aquarium heaters. Fish's health and holding tank temperatures were monitored daily. Test fish were held in holding tanks for at least 21 days prior to testing, and were fed ad libitum rations of Tetramin tropical flakes two times per day. Additional feeding of *Tubifex* spp. occurred once per day. Fish were fasted 12–14 h prior to the initiation of the experiment and were not fed during testing to limit the influence of their digestion on their temperature selection.
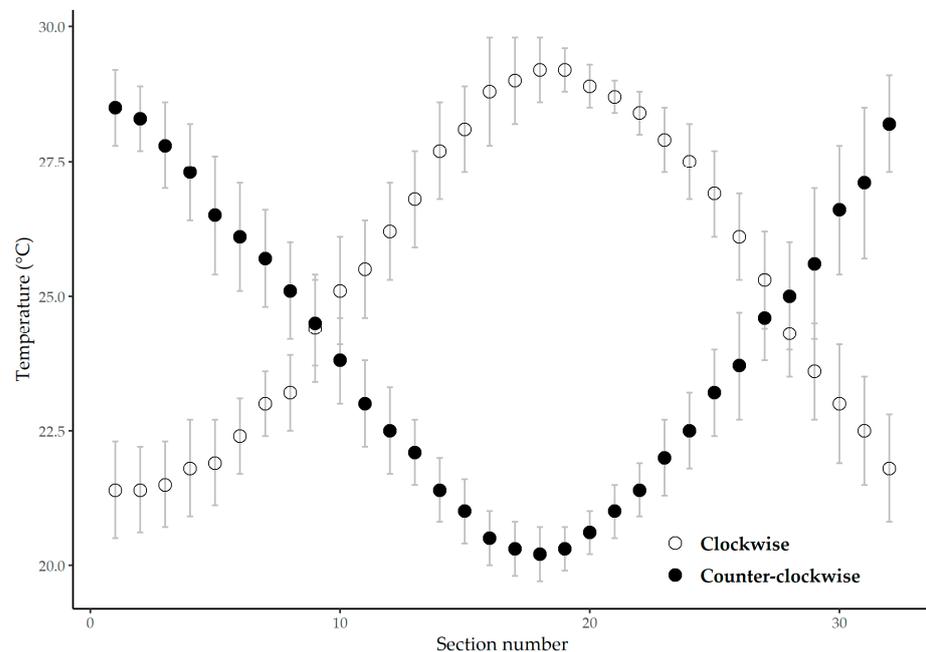
2.3. Experimental Apparatus

We used a modified Myrick-type thermal preference chamber to quantify the thermal and behavioral response of *C. eos* to the introduction of *G. affinis* (Figure 1) [45]. We made several modifications to the original thermal preference chamber design as described by Myrick et al. [45]. Most notably, we decreased the number of constant-headwater distribution reservoirs from four to two and eliminated the distribution lines that carried the source water directly to the mixing chamber. Water was instead piped from a warm (31 ± 0.2 °C) and a cool (19 ± 0.2 °C) constant-headwater distribution reservoir into two stacked mixing rings located above the chamber. The top mixing ring was a complete circle and supplied water for diluting the temperature of the bottom mixing ring. The bottom mixing ring was nearly a complete circle but, unlike the top mixing ring, was capped at both ends. Water from the top mixing ring was funneled into the bottom mixing ring at 20 evenly spaced locations, effectively diluting the concentration of the bottom mixing ring's source water along the ring. The highest concretion of the bottom mixing ring's source water was located at the center point between the two capped ends and decreased as it radiated out from the center point toward each capped end (Figure 1). The dilution of the bottom ring from the top ring created a 10 °C circular thermal gradient (21.2 ± 0.71 to 29.4 ± 0.59 °C). Switching between cool and warm water delivered to the top mixing ring reversed the orientation of the thermal gradient and allowed us to randomize the thermal gradient orientation during trials. When cool water was delivered to the top mixing ring, the water temperature decreased from 30 °C to 19 °C as it traveled from the center point to the two capped ends. The gradient was reversed when warm water was delivered to the top mixing ring.

Water from the bottom mixing ring was funneled into the 20 sections (increased from 8 in the original Myrick-type [45] thermal preference chamber) of the mixing chamber. Water flowed from the mixing chamber into the swimming chamber through 60 evenly spaced holes (3 per section). The swimming chamber's outer wall had a diameter of 101 cm, and the inner wall was 80 cm. Water then flowed through 32 evenly spaced holes, into the 32 sections (increased from 8) of the effluent channel before finally flowing through 32 holes (1 per section) to three drains located in the base of the chamber. The apparatus created a continuous 10 °C circular thermal gradient (mean segment temperature ± 95% CI; 21.2 ± 0.7 to 29.4 ± 0.6 °C) with the capacity to control flow direction (Figure 2). We randomized the thermal gradient orientation between trials to ascertain whether fish were selecting a temperature and not a location in the chamber (Figure 2). The thermal chamber was enclosed with a curtain, and a 122 cm × 122 cm sheet of white Plexiglas was mounted above the chamber to eliminate external disturbances during trials and allow for a uniform lighting of the chamber.
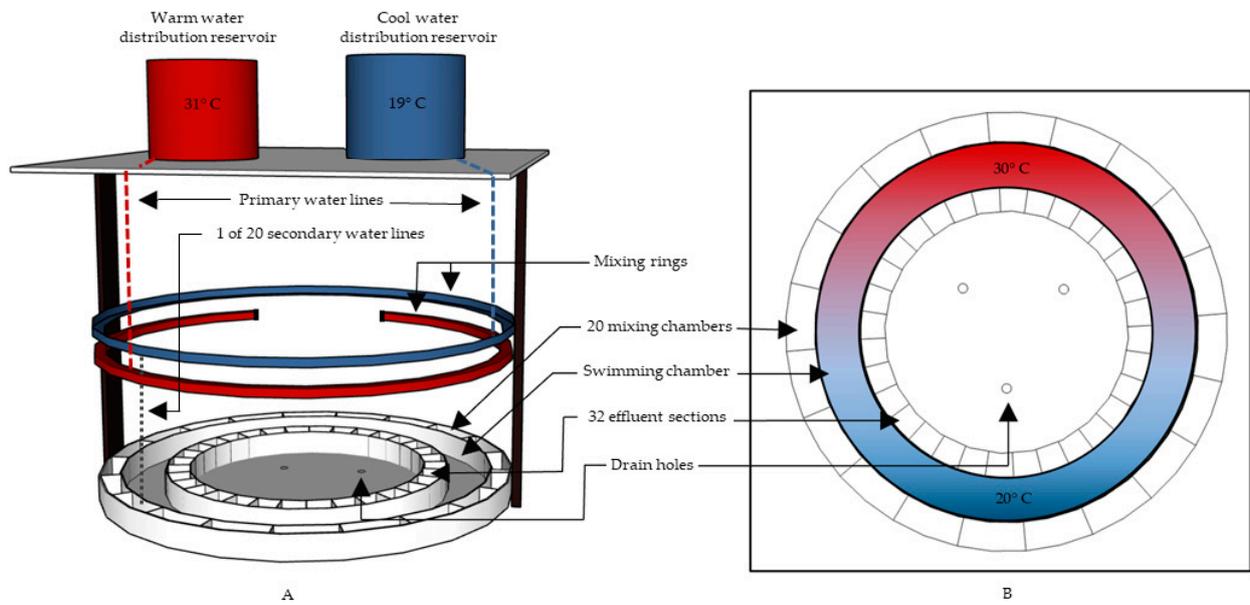
Figure 1. Lateral (**A**) and overhead (**B**) views of the thermal preference chamber that was used to quantify fish's thermal preference and movement (modified from Myrick et al. [45]). One of the 20 secondary water lines is shown to provide an example of the water distribution from the top mixing ring to the bottom mixing ring and finally into the mixing chamber. The remaining 19 secondary water lines were equally spaced around the mixing rings, with their location corresponding to the center of each mixing section.

Figure 2. Mean (±standard deviation) water temperature recorded immediately prior to initiating trials assigned to one of two flow directions, clockwise (open circles) or counter-clockwise (closed circles). The water temperature was measured in the swimming chamber at the center of 32 equidistant visual sections.

2.4. Experimental Fish Trials

We first estimated *C. eos*' thermal preference, and then proceeded to add *G. affinis* (i.e., the treatment) to quantify the thermal and behavioral response of *C. eos* to the introduction of *G. affinis*. A total of 11 trials were completed, and each trial consisted of two 60 min runs. Run 1 represented the reference condition period and was used to establish the thermal

preference and travel distance of an individual *C. eos*. After a 15 min acclimation period, Run 2 began, representing the treatment period, which was used to observe changes in *C. eos'* thermal preference and travel distance due to the introduction of three *G. affinis*.

Prior to each trial, we randomly selected a thermal gradient orientation (clockwise or counter-clockwise) and allowed the preference chamber to equilibrate for 30 min. We then measured the thermal gradient of the annular chamber at 32 equally distanced locations (Figure 2). At the initiation of each trial, we removed one *C. eos* from its holding tank and introduced it into the thermal chamber at the location corresponding to the fish's acclimation temperature of 25 °C. Test fish were randomly selected and measured for L_T prior to their introduction into the thermal chamber. After a 1.5 h acclimation period, we began the reference condition period of the trial, which included only the single *C. eos*. The results of this hour in the thermal chamber established the thermal preference of *C. eos*. Following the reference condition period, we randomly selected and introduced three *G. affinis,* bringing the total number of fish in the chamber to four. The *G. affinis* were introduced to the chamber at the same location as the initial *C. eos*.

During trials we video-recorded fish's movement and behavior with four web-cameras mounted 1.5 m above the surface of the water (Figure 3). Collectively, the four cameras were positioned to capture the entirety of the swimming chamber (Figure 3). Post-trial we used the video files to note the location, and thus temperature, of all fish at the start of every minute during reference condition and treatment periods, for a total of 60 temperatures for each fish. We noted fish's location (nose position) in relation to the 32 effluent sections. To estimate *C. eos'* movement travel, we visually tracked and recorded the number of sections each *C. eos* travelled through in the first 10 s of each minute during the reference condition and treatment periods and multiplied the number of sections traveled by the center width of each section (9.6 cm). Finally, to quantify the species interactions we counted the number of chases that occurred during the first 10 s of each minute in the treatment period.
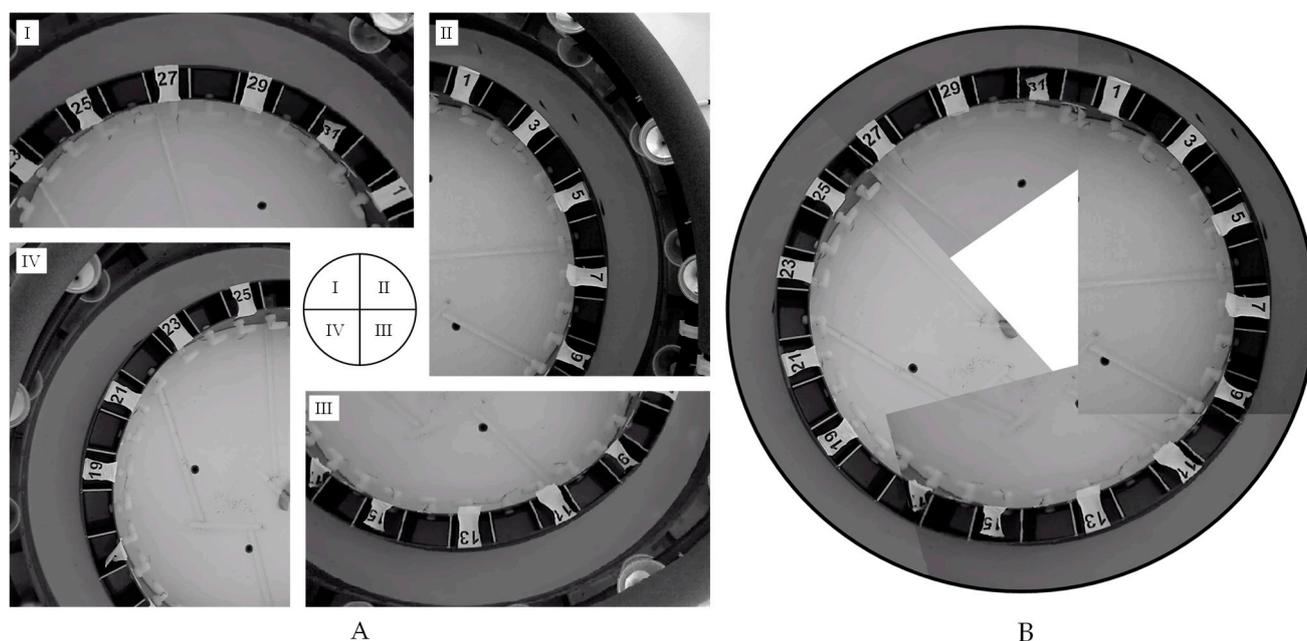
Figure 3. Photographs of the thermal preference chamber. (**A**) Display of the four camera angles. (**B**) Display of when the four images are merged into one. Note: There are four fish in Figure **A** (II) and in the upper right corner of Figure **B**.

2.5. Statistical Methods

We tested whether the temperature selected and distance moved by the *C. eos*, differed pre-treatment and after three *G. affinis* individuals were introduced into the chamber using hierarchical linear models. The response variable was the mean water temperature across sections that the single *C. eos* individual occupied, or the number of sections that the *C.*

eos individual travelled through during a 10 s period at the beginning of each minute for 60 min. The binary explanatory variable, x_i, was the presence of *G. affinis* (0 = pre-*G. affinis'* introduction, 1 = post-treatment). Because the temperature was continuous and the movement was discrete data, the temperature was modeled to follow a normal distribution, and the movement followed a Poisson distribution. Given a vector of temperature ($temp_i$) and movement ($move_i$) data across minutes and trials, the models are written:

$$temp_i \sim Normal\left(\alpha.temp_{j[i]} + \beta.temp_{j[i]}\, x_i,\, \sigma_y^2\right) \tag{1}$$

$$move_i \sim Poisson\left(\lambda_i\right) \tag{2}$$

$$log\left(\lambda_i\right) = \alpha.move_{j[i]} + \beta.move_{j[i]}\, x_i \tag{3}$$

We let the intercepts ($\alpha.temp_j$ and $\alpha.move_j$) and slopes ($\beta.temp_j$ and $\beta.move_j$) vary by trial j to which observation i belong, and σ_y refers to the residual term (i.e., standard deviation). That is, we considered that the initial temperature selection or travel distance by *C. eos*, and its shifts after *G. affinis'* introduction, could differ among trials. To model inter-trial variation and synthesize the overall pattern across trials, we modeled the intercepts and slopes as random effects, where $\mu_{\alpha.temp}$ is the mean overall temperature selected, and $\mu_{\alpha.move}$ is the total number of sections moved by a *C. eos* across 10 s periods prior to *G. affinis'* introduction with standard deviations equaling to $\sigma_{\alpha.temp}$ and $\sigma_{\alpha.move}$ (Equation (4)). Similarly, $\mu_{\beta.temp}$ is the mean overall change in temperature selection, and $\mu_{\beta.move}$ is the mean overall change in the number of sections traveled due to the presence of *G. affinis* with standard deviations equaling to $\sigma_{\beta.temp}$ and $\sigma_{\beta.move}$:

$$\alpha.temp_j \sim Normal\left(\mu_{\alpha.temp},\, \sigma_{\alpha.temp}^2\right);\ \alpha.move_j \sim Normal\left(\mu_{\alpha.move},\, \sigma_{\alpha.move}^2\right) \tag{4}$$

$$\beta.temp_j \sim Normal\left(\mu_{\beta.temp},\, \sigma_{\beta.temp}^2\right);\ \beta.move_j \sim Normal\left(\mu_{\beta.move},\, \sigma_{\beta.move}^2\right) \tag{5}$$

We additionally fit a model that evaluated whether inter-trial variation in intercepts and slopes could be explained by variation in the body length of *C. eos* between trials, because agonistic inter-specific interactions depend frequently on the body lengths of fish [46–48]. Specifically, we fitted linear models on intercepts ($\alpha.temp_j$ and $\alpha.move_j$) and slopes ($\beta.temp_j$ and $\beta.move_j$), where μ_j is the mean-standardized body length of *C. eos* (total length in mm) used in trial j:

$$\begin{aligned} \alpha.temp_j &\sim Normal\left(\gamma_0.temp + \gamma_1.temp * \mu_j,\, \sigma_{\alpha.temp}^2\right)\\ \alpha.move_j &\sim Normal\left(\gamma_0.move + \gamma_1.move * \mu_j,\, \sigma_{\alpha.move}^2\right) \end{aligned} \tag{6}$$

$$\begin{aligned} \beta.temp_j &\sim Normal\left(\delta_0.temp + \delta_1.temp * \mu_j,\, \sigma_{\beta.temp}^2\right)\\ \beta.move_j &\sim Normal\left(\delta_0.move + \delta_1.move * \mu_j,\, \sigma_{\beta.move}^2\right) \end{aligned} \tag{7}$$

C. eos body length did not affect temperature selection or movement; therefore, we primarily report results based on the model described above (Equations (1)–(5)).

Models were analyzed with a Bayesian approach using a Markov chain Monte Carlo (MCMC) method in the program JAGS [49] called from the program R [50] with the jagsUI package [51]. Uninformative priors were used for all parameters. Posterior distributions of parameters were estimated by taking every fifth sample from 10,000 iterations of three chains after a burn-in period of 2000 iterations. The model convergence was checked by visually examining plots of the MCMC chains for good mixture as well as ensuring that the R-hat statistic was less than 1.1 for all model parameters [52]. We considered effects of explanatory covariates (i.e., *G. affinis* introductions and *C. eos* body size) statistically significant when >95% of posterior samples were either positive or negative; we report the proportion of posterior samples (PPS) that were in the same direction (positive or negative) as the posterior mean value. This metric ranged from 0.5 to 1. If covariate effects are zero,

50% of posterior samples are approximately positive or negative; if effects are unequivocal (i.e., posterior samples do not overlap zero), 100% of posteriors samples may be positive or negative.

3. Results

3.1. Thermal Preference

Prior to *G. affinis* introductions, *C. eos* occupied an average temperature of 24.3 °C ($\mu_{\alpha.temp}$) that varied between trials ($\sigma_{\alpha.temp}$: mean = 2.5 °C). After *G. affinis* were introduced, *C. eos* selected significantly (PPS = 0.98) higher temperature ranges (mean = 1.7 °C higher ($\mu_{\beta.temp}$); Figure 4). The magnitude of changes in temperature selection varied by trial, ranging from −1.3 °C to 4.7 °C.
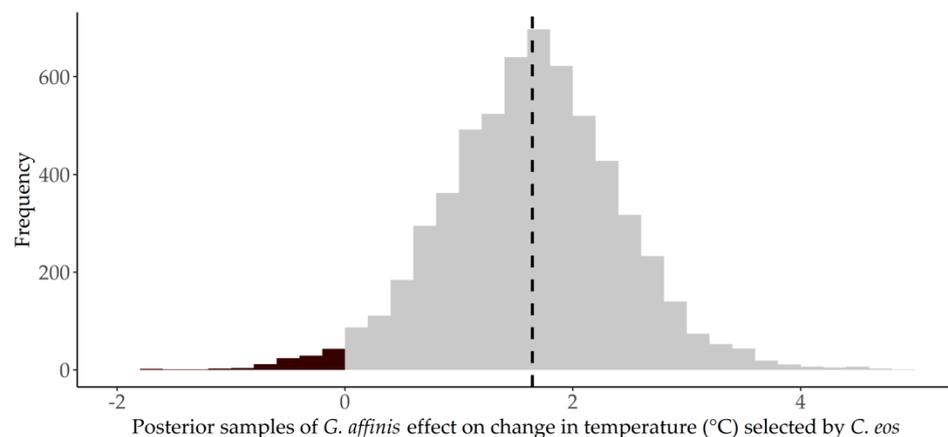
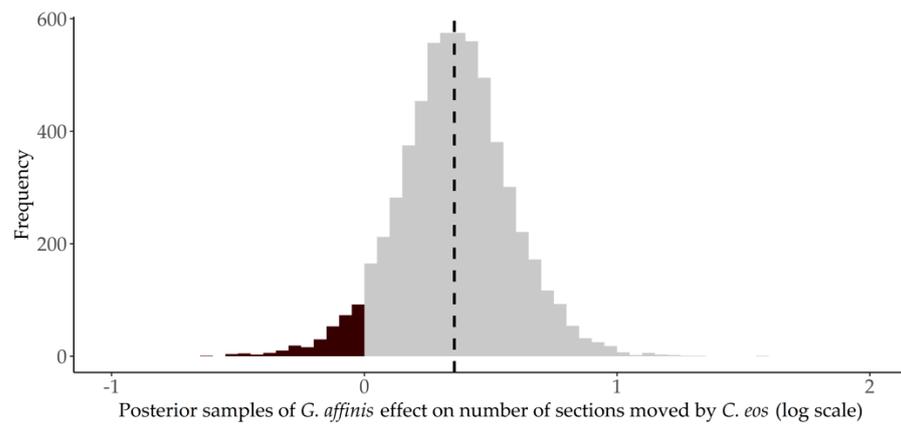

Figure 4. Proportion of posterior samples (PPS) of *Gambusia affinis* introduction effects on the change in temperature selected ($\mu_{\beta.temp}$) in $\beta.temp_j \sim Normal\left(\mu_{\beta.temp}, \sigma^2_{\beta.temp}\right)$ by *Chrosomus eos*. Posterior samples that are positive are shown in gray and those that are negative are shown in black. The vertical black dotted line shows the mean posterior value.

The body length of *C. eos* did not explain the inter-trial variation in its temperature selection ($\gamma_1.temp$: mean = −0.38, PPS = 0.69) or movement distance ($\gamma_1.move$: mean = 0.70, PPS = 0.63) before *G. affinis'* introduction, or changes in its temperature selection ($\delta_1.temp$: mean = −0.09, PPS = 0.53) or movement ($\delta_1.move$: mean = 0.07, PPS = 0.56) in response to *G. affinis* introduction.

3.2. Movement

C. eos' movement distance increased 21% after *G. affinis* were introduced. Without *G. affinis*, *C. eos* moved an average of 10 sections (96 cm) per 10 s across minutes and trials. After *G. affinis* introductions, *C. eos* moved an average of 12 sections (116 cm), and this effect was statistically significant ($\mu_{\beta.move}$: mean = 0.36 (log scale), PPS = 0.96) (Figure 5). Again, the change in the movement distance of *C. eos* varied by trial, ranging from two fewer sections moved to nine more sections of movement after *G. affinis'* introduction.

Figure 5. Proportion of posterior samples (PPS) of *Gambusia affinis'* introduction effects on the distance moved ($\mu_{\beta.move}$) in $\beta.move_j \sim Normal\left(\mu_{\beta.move}, \sigma^2_{\beta.move}\right)$ by *Chrosomus eos*. Posterior samples that are positive are shown in gray and those that are negative are shown in black. The vertical black dotted line shows the mean posterior value.

3.3. Agonistic Interactions

Contrary to our prediction, more agonistic interactions were initiated by *C. eos* toward *G. affinis* than the opposite. We recorded interspecific interactions (i.e., chases) in 2562 out of 6600 total seconds of observations (39%). Of these, 1976 observations (77%) were chases by *C. eos* of *G. affinis*, and 348 observations (14%) were chases by *G. affinis* of *C. eos* (Figure 6). In the remaining 238 observations (9%), *G. affinis* and *C. eos* chased each other, and we could not determine which species initiated the interactions.
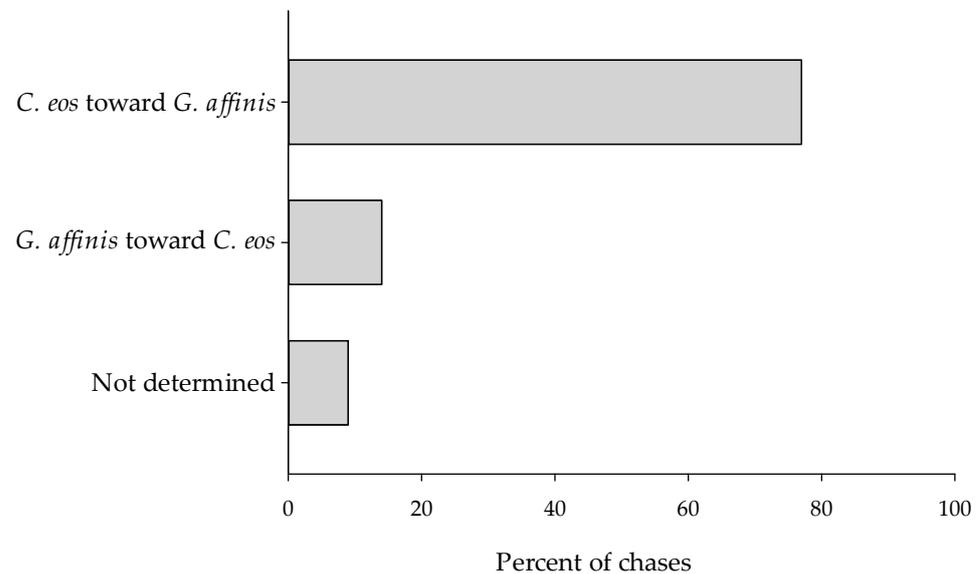
Figure 6. Direction of agonistic interactions between *C. eos* and *G. affinis*.

4. Discussion

Our results indicated a temperature preference of *C. eos* (24.3 °C) slightly lower than the temperature preference of 25.3 °C estimated by Stauffer et al. [53] when integrating temperature preferences of fish acclimated to five different temperatures (6, 18, 24, 30, and 33 °C). However, the preferred temperature of 24 °C-acclimated *C. eos* reported by Stauffer et al. [53] (24.0 °C) was similar to the preferred temperature of the 25 °C-acclimated *C. eos* in this study (24.3 °C). The similarity in the preferred temperatures between these two studies was interesting given the differences between the *C. eos* source populations used in each study. The source population of the hatchery-propagated *C. eos* used in this study was West Plum Creek (a tributary to the South Platte River, CO, USA), which is

at approximately 2,000 m in elevation, whereas the source population of *C. eos* used by Stauffer et al. [53] was Spratt Creek (a tributary of the Thunder Bay system, Lake Huron, Michigan, USA), which is at an approximate elevation of 220 m. As a glacial relict species in Colorado, *C. eos* may have experienced cooler temperatures than other populations in *C. eos'* native range, however our results indicate that *C. eos'* preferred temperature remains stable across geographic regions when acclimated to similar temperatures.

The introduction of *G. affinis* into the thermal preference chamber increased *C. eos'* movement, given by distance (cm) traveled, by 21%. The increase in movement resulted in more time spent outside of *C. eos'* initially established thermal preference, as documented by an overall increase in the thermal selection of 1.7 °C, post *G. affinis'* introduction. A fish's thermal preference, measured through laboratory studies, represents a snapshot of the thermal regulation employed by the fish and has been found to correlate with its optimal physiological performance [54]; an interruption to a fish's natural thermal regulation may reduce its physiological performance. Boltaña et al. [55] reported negative effects of a restricted thermal regime, inhibiting thermal regulation, on the early life-stage growth, survival, and cellular microstructure of Atlantic salmon. In the current study, the introduction of *G. affinis* altered the thermal preference of *C. eos,* which we hypothesize would shift the latter's physiological performance.

The observed increased movement of 21% by *C. eos* in this study could result in an increased mortality risk. For example, the increased energy requirements indicate a need to consume more food [20], which can increase its predation risk [21]. In addition, the increased energetic costs of movement can reduce somatic growth [22]; alternatively, that energy could have been put toward gonadal growth [56]. Trudel and Boisclair [57] found the activity costs of hybrids of northern redbelly dace and finescale dace, *Phoxinus eos* Cope x *Phoxinus neogaeus* Cope, ranged from 36.8 to 153.0 J per 12 h^{-1} in July and 17.0 to 76.2 J 14 h^{-1} in August during daylight hours. Increasing these rates would be a significant cost to *C. eos* and potentially cross an energetic threshold.

G. affinis is widely considered an aggressive nipper and chaser with agonistic behaviors toward other species frequently being documented [8,9,58]. These interactions can lead to increased inter-specific competition and the native fish being displaced from its thermally preferred habitat [38]. However, our results suggest that the effect of *G. affinis* agonistic interactions might be species- or condition-specific. Contrary to our hypothesis, we observed significantly more (six times as many) chases initiated by *C. eos* than *G. affinis*. The aggression by *C. eos* toward *G. affinis* led to the observed thermal displacement and increased movement. Additionally, although it was not a motivating factor in our study, our study design allowed an inference regarding wild and hatchery-reared fish interactions. It might be expected that hatchery fish would be more docile and that wild fish would be more aggressive. However, our results showed the hatchery-reared fish to be more aggressive than the wild-caught fish. The literature on the aggression of wild versus hatchery-reared fish is mixed. For example, Deverill et al. [59] found wild brown trout *Salmo trutta* initiated more aggressive acts than hatchery-reared *S. trutta*. However, Mesa [60] found that hatchery-reared coastal cutthroat trout *Oncorhynchus clarki clarki* were more aggressive than their wild conspecifics.

The lack of *G. affinis* aggression observed here may have been influenced by a variety of factors including the fish's size, prior residency, and water temperature. The *C. eos* were, on average, larger than the *G. affinis*; however, *G. affinis* have been shown to be aggressive toward larger species [58]. Prior residency is the hypothesis that the individual who is already a resident in a habitat has an advantage in territory contests over an intruder [61–63]. In our case, *C. eos* would have prior residency in a natural setting as the native species, so this study design approximates conditions in which these encounters would occur during biological invasions. The range of temperatures tested in this study are closer to the preference of *C. eos* (24 °C) than *G. affinis* (31 °C), but within the range of temperatures *G. affinis* and *C. eos* experience naturally [27,53].

Manipulation effects may also influence the results of behavioral studies and should be accounted for—especially in studies in which fish are netted, removed from water, and transferred to another tank—as these manipulations can affect fish behaviors [64–67]. We attempted to minimize and control manipulation effects by allowing a 1.5 h acclimation period for *C. eos* to establish their thermal preference. This allowed the fish to settle after being transported, time to explore the thermal preference chamber, and time to select their preferred temperature. Once *G. affinis* were introduced, we allowed a 15 min acclimation period to account for any short-term responses to subside.

This study focused on the adult life stage of these species; however, significant knowledge gaps remain in our understanding of how *G. affinis* impact earlier life stages of *C. eos*. Both species are omnivores [16,23,24], so competition for food resources likely occurs at all life stages. For earlier life stages, there is also likely competition for habitats— both preferred thermal habitats and vegetation that provides protection from predators. The potential for competition between *G. affinis* and *C. eos* in their early life stages is compounded by *G. affinis'* reproductive strategy. *G. affinis* is a livebearer that produces prodigious amounts of offspring [16]; these offspring may overwhelm *C. eos* in its early life stages in terms of competition for food and habitat availability. In addition, adult *G. affinis* may predate on *C. eos* in its early life stages.

5. Conclusions

In this study, we observed a native fish shifting its temperature preference in the presence of *G. affinis* in an experimental chamber simulating a thermally heterogeneous habitat, but aggressive behaviors were more frequently observed in the native fish toward *G. affinis* rather than the other direction. We hypothesize that indirect mechanisms (e.g., reduced growth via a thermal displacement and increased movement), not direct agonistic behaviors, may negatively affect *C. eos* in the presence of *G. affinis*. Conservation actions that may help conserve remaining glacial relict populations of *C. eos* include: protecting thermal refugia; promoting habitat enhancement strategies for *C. eos*; the hatchery stocking of *C. eos* into suitable habitats, with priority given to areas that do not include *G. affinis*; and establishing genetic refugia in hatcheries that can be used as source populations for hatchery releases. The thermal experimental chamber used in this study is a promising tool to study the thermal preference, and changes in preference and behavior, of native fish in the presence of invasive species. Additional research is needed to identify which native species are vulnerable to the direct and indirect effects of *G. affinis'* presence, especially given the likelihood of increasing temperatures, which would favor *G. affinis*. Future studies should examine temperatures outside of those included in this study, especially higher temperatures, to test the hypothesis that behaviors change at elevated temperatures. Additional research may also include further investigation into the impacts of *G. affinis* on native fish populations relative to the other ecological traits, especially its reproductive output, of this highly successful invasive species.

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