Social cues can push amphibious fish to their thermal limits

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Abstract

Social context can impact how animals respond to changes in their physical environment. We used an aggressive, amphibious fish, the mangrove rivulus (*Kryptolebias marmoratus*) with environmentally-determined sociality to test the hypothesis that social interactions would push fish to their thermal limits. We capitalized on the propensity of rivulus to emerge from warming water and demonstrated that social stimuli, produced by their reflection, increased emersion threshold without changing critical thermal maximum, effectively diminishing thermal safety margins. When rivulus were denied air access, surface behaviours dramatically increased, supplanting social interactions. This suggests that assessing the terrestrial environment is crucially important. We conclude that social stimulation narrows the scope for survival in naturally stressful conditions.
Introduction

Species living near their thermal limits may be the most vulnerable to climate warming [1, 2] at least in the short-term [3]. Life in the tropics is characterized by warm, relatively stable temperatures and optimal animal performance occurs over a narrow range of temperatures with little variation in maximum thermal tolerance or CT\text{max}. Thus, thermal safety margins, or the difference between maximum thermal tolerance and the warm temperatures regularly experienced, are assumed to be small [1]. Furthermore, stochastic thermal stressors will have a disproportionate impact on animals already living on the edge.

The social (biotic) environment affects the response to the abiotic environment. For example, certain air-breathing fish exhibit synchronous (i.e., social) surfacing behaviours [4]. Since surfacing behaviours in fish are risky, they are more rare in shy fish [5]. Furthermore, the cellular response to environmental stressors is affected by social hierarchy position in juvenile salmonid fish [6]. Finally, individual temperature preferences in sticklebacks can be superseded by social behaviours as schooling fish prefer to belong to a group [7]. Thus, behavioural decisions could have life or death consequences especially at thermal performance maxima where performance drops off precipitously.

The mangrove rivulus is an aggressive [8] fish that lives in a highly variable and warm environment and is one of two known self-fertilizing hermaphroditic vertebrates [9]. This fish is also androdieicious, with natural
populations consisting only of hermaphrodites and a low percentage (usually <3%) of males [10]. They have numerous adaptations to deal with low quality habitat, notably active emersion from water, especially at elevated water temperatures [11]. During the dry season, rivulus can be found out of water, packed inside logs in large groups, whereas during the wet season, fish live at lower densities from one to approximately 10 individuals [12]. Social context, therefore, appears to be environmentally dependent, although whether social cues affect environmentally-driven behavioural decisions is unknown.

We tested the hypothesis that social behaviours interfere with adaptive thermal responses, pushing mangrove rivulus closer to their thermal limits. We capitalized on the amphibious nature of this fish and predicted that social interactions would increase emersion temperature without affecting critical maximum temperatures related to physiological tolerance. We defined critical temperature as the temperature beyond which the animal would incur ecological death and evaluated this using rapid heating and loss of equilibrium [11]. We also observed that prior to emersion, rivulus engaged in repeated visits to the surface. We then hypothesised that these surface excursions were dependent on their opportunity to emerge.

**Materials and Methods:**

**Animals**

We collected adult mangrove rivulus (all hermaphrodites; length range 14-33 mm; mass range 0.022 to 0.536 g) from Long Caye, Lighthouse Reef Atoll,
Belize in April 2018 from a cluster of crab burrows and small pools, using Gee minnow traps and Taylor cup traps [13]. Daily water conditions at all collection sites are reported in Rossi et al. [14] (sites 3, 8 and 10: 27.6 – 33.1°C; 21.6 – 42.9 ppt; 0.0 – 5.8 mg l\(^{-1}\) dissolved O\(_2\); pH 6.3 – 7.8). After collection, we held fish in specimen cups (120 mL) for 24-72 h and fasted before experiments at ambient temperature (28-29°C), 36 ppt salinity, and >80% O\(_2\) saturation.

**Emersion Temperature**

We tested emersion temperature in an opaque, non-reflective chamber (15 x 9.5 x 20 cm) divided into two sections with a mesh barrier. One side of the chamber was fitted with aquarium heaters and a stir bar. The entire chamber sat on a stir plate with water 8.5 cm deep. We placed fish in the opposite side of the chamber, which had a mirror or opaque sham (i.e. taped mirror) placed, vertically below the water surface (Fig S1). We then recorded the number of excursions to the surface, surface breaks, lateral displays, mirror charges and water temperature of emersion (ET). We heated the water at a constant, rapid rate of 1°C min\(^{-1}\) [11] and monitored water temperature with a thermal imaging camera (FLIR T1030), which was calibrated against a blackbody source of known emissivity. This temperature rate is similar to the acute thermal tolerance protocol/test used previously in rivulus [11] and not meant to reflect natural warming. The experiment ended when the whole body of the fish emerged from the water. We tested (N = 15) each fish twice (with or without mirror, order randomised) separated by 4-6 h.

**Loss of Equilibrium Temperature**
We measured the loss of equilibrium (LOE) using the same rate of heating and chamber as above but removed the mesh divider and confined the fish by covering the chamber with mesh screen (11 x 6.5 x 3.5 cm), to prevent access to the surface. The chamber was fitted with either a mirror or an opaque sham. We placed a stir bar below the floating insert and the entire chamber sat on a stir plate, as above. We gave the fish 3-5 min to explore the plastic insert before the trial began. Each fish \( (N = 10) \) was tested twice in experiments separated by 18-24 h.

**Access to Air and Social Activity**

We used the same chamber as above but separated the chamber with a non-reflective, opaque plexi-glass divider, and housed fish at temperatures (~30 to 32°C) well below the ET or LOE temperatures. We tested fish \( (N = 16) \) with and without access to the surface, using a tight-fitting mesh square on the water surface to block surface access, but allow clear observation. We measured surface excursions, surface breaks, lateral displays, mirror charges, as well as mirror approaches over 10 min (the average time of the emersion temperature trials). We also ran preliminary experiments \( (N = 7) \) with access to the surface but the mesh placed ~ 2 cm above the surface to ensure that fish were not simply avoiding the mesh; we did not detect any difference in surface behaviours \( (t_{9.2} = 0.43; \ P = 0.7) \) between this group and the Air Access treatment (Fig. 2). We tested each fish with or without mirror, in experiments separated by 4-6 h.

**Data Analysis**
Behaviours were summed according to category (surface behaviours vs. social behaviours) and normalised to the experiment duration to determine the rate. Since the order of exposure was randomised, potential experience was incorporated into the experimental design, although occasionally some fish only experienced one condition. We used linear mixed effects models to analyse behaviour data, incorporating fish identity as a random intercept [15]. Initially we assessed the influence of experiment order on all parameters and only proceeded if there was no effect (see supplementary results). We determined significance level using the Satterthwaite approximation, obtained from the lmerTest package, to estimate the degrees of freedom [16].

**Results**

Mangrove rivulus showed a higher emersion temperature when exposed to a mirror (41.8 °C) compared to the opaque control object (40.5 °C; $F_{1,14} = 11.8$, $P = 0.0041$; Figure 1a). This delay in emersing from water was accompanied by a lower rate of surface-related behaviours in the mirror group (1.9 ± 0.48 min$^{-1}$) compared to the opaque control group (4.0 ± 0.41 min$^{-1}$; $F_{1,14} = 19.3$, $P = 0.00061$).

In contrast to the emersion temperatures, mangrove rivulus lost equilibrium at the same temperature in both the mirror (43.1°C) and opaque treatment (43.1°C; $F_{1,18} = 0.035$, $P = 0.95$; Figure 1b), even though the mirror treatment led to fewer surface behaviours (3.5 ± 0.5 min$^{-1}$) compared to opaque controls (4.1 ± 0.4 min$^{-1}$; $F_{1,9} = 10.1$, $P = 0.01$).
We assessed the propensity of rivulus to visit the surface by examining the difference in the rate (min\(^{-1}\)) of social behaviours minus the rate of surface behaviours, to normalize for activity. This social-surface behaviour difference was significantly higher in fish with access to air (1.8 min\(^{-1}\)) compared to those without (-0.1 min\(^{-1}\); \(F_{1,10.2} = 22.4\); \(P = 0.00076\); Figure 2).

Discussion

Mangrove rivulus emerged from warming waters at lower temperatures than are considered ecologically lethal (CT\(_{\text{max}}\)). This observation suggests that this behavioural indicator may correspond to the pejus temperature, the point on a thermal performance curve where physiological perform begins to decline [c. 17]. In tropical species, behavioural thermoregulation, including emersion, would help to buffer the effects of high temperature [18]. The thermal safety margin (which we defined as the difference between the CT\(_{\text{max}}\) and the emersion threshold) is clearly context-dependent, dramatically decreasing when social stimulation was present: 2.6°C with no social cues, compared to 1.3°C with social cues. Some fish (7 of 15) receiving social cues voluntarily emerged at temperatures within the range of observed CT\(_{\text{max}}\) values. Thus, we conclude that the presence of conspecific cues can push an animal to its ecological thermal limits.

Behavioural trade-offs often result from conflicting environmental challenges or complexities, and animals may alter the priority of specific behaviours and physiological functions [19, 20]. In the context of mangrove
rivulus, however, the emersion response is traded-off in favour of dealing with conspecific information, with potentially deleterious, immediate fitness consequences. In our study, animals were faced with two distinct, extrinsic stressors – their reflection (i.e., image of a conspecific) and high temperature, and thus were forced to compromise their behavioural thermoregulation. Although winning the contest is impossible with the mirror test, engaging with social cues at warm temperatures could lead to a high mortality risk. In the case of two fish in a dyadic contest where there would be a winner and a loser, we would expect the loser to emerge, if possible. We show that emersion temperature is plastic and can be modulated by conspecific cues; this is not the case for $CT_{\text{max}}$.

Rivulus were clearly more interested in investigating the water surface when they could not emerge, and were not experiencing a stressor (i.e., heat) sufficient to induce emersion. It is possible that blocking access to the surface induces an anxiolytic response [21], resulting in elevated surface behaviour responses. Alternatively, surfacing may be a surveillance behaviour. Spatial memory and cognitive maps [22] are essential to the formation of time-place learning, a type of anticipatory learning where fish associate temporal events with spatial positions [23]. Since emersion is a key adaptive trait of rivulus, it is reasonable to expect that they maintain an awareness of their surroundings in preparation to emerge.

In conclusion, social information can modulate behavioural and physiological responses to warming temperature and will be important for
predicting how individuals, and ultimately populations, respond to acute and long-term environmental disturbances.
Ethics
Mount Allison University Animal Care Committee approved experimental protocols (101864).

Data Accessibility
Data [24] are made available at the following:

Authors’ Contributions
Conceptualization, Methodology, Investigation, Writing: SC, GJT; Formal Analysis, GJT. All authors agree to be held accountable for the content therein and approved the final version of the manuscript.

Competing Interests
The authors have no competing interests.

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Figure 1. Emersion threshold and surfacing behaviours (mean ± 95% confidence limits) in mangrove rivulus (K. marmoratus) (panels a and b). Rivulus emerge from water at a higher temperature (P = 0.0041) and show a lower rate of surfacing (P = 0.00061) in the presence of a mirror. Loss of equilibrium temperatures and surfacing behaviours are shown in panels c and d. Rivulus show no difference in LOE (P = 0.95), but still show fewer surfacing behaviours (P = 0.011) when exposed to a mirror. Pairwise raw data (N = 15 in a,b; N = 10 in c,d) are depicted for each fish.
Figure 2. Difference between the rate of mirror behaviours and the number of surface behaviours (i.e. mirror behaviours normalized to surfacing activity) in the mangrove rivulus (*K. marmoratus*) exposed to 10 minutes with and without access to the air (mean ± 95% confidence limits). Rivulus with air access engaged in fewer surface-related behaviours (*P* = 0.00076) relative to mirror-related behaviours. Data (N = 16) are depicted for each fish.
Supplementary Information

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Supplementary Methods

Thermal Imaging of Surface Water

In order to minimise instrument interference with fish behaviour and ensure sufficient thermal mixing, we measured water surface temperatures with a thermal imaging camera (FLIR T1030K). One experimenter continually monitored the camera and recorded the average temperature within a prescribed region of the water surface from the region of interest for each behavioural occurrence. We set object parameters to the appropriate conditions (reflected environment temperature was ~30°C, emissivity for water was 0.95, distance was 1.5 m) using on-board settings available in the camera. The camera was calibrated by the manufacturer (<6 months prior to use) and verified to be within 0.5°C of a standard NIST bulb thermometer.

Potential order effects

Given the paired nature of the experiments, we examined the potential for immediate, prior experience to influence results by including procedure order for all the relevant behaviours in a linear mixed effect model. Order was nearly fully balanced, such that ~50% of replicates were conducted with the opaque object first, and 50% with the mirror object first.

Detailed behaviour threshold summaries
To visualise the pattern of behavioural thresholds, we combined results from the emersion experiments and the loss of equilibrium experiments into a linear mixed effects model, with threshold temperature as the response variable and behaviour as the predictor variable. We classified 10 potential behaviour categories: mirror display, mirror charges, surface excursions, surface breaks, emersion, and loss of equilibrium, where the latter four occurred under both mirror and opaque conditions. The final model allowed for model predictions for each category to be estimated and visualised.

**Statistical Analysis**

We analysed all behaviours using linear mixed effects models with the lmerTest package (Kuznetsova et al., 2017) in R v. 3.5.1 (R Core Team, 2018). This approach was selected rather than paired t-tests since it can accommodate missing data, although yields identical conclusions to paired t-test for fully balanced experimental designs. An additional advantage of the linear mixed effects approach is the ability to include multiple response variables in the final model. For the main paper, we report on the primary treatment effects, since these were the only significant results and the primary focus. In the supplementary material, we include experimental order and treatment and report on the results for the order effect. Degrees of freedom estimation was achieved using the Satterthwaite approximation as outlined in the lmerTest package (Kuznetsova et al., 2017). This approach is less anti-conservative than likelihood ratio tests for small or unbalanced datasets and, thus more closely estimates the
true p value (Kuznetsova et al., 2017). Residuals were verified for normality and homoskedasticity, and data log transformed for responses where necessary (only the rates of emersion). Model effects and standard errors were obtained using the Effects package in R (Fox, 2013). The alpha for statistical significance was set to 0.05. Figures were produced using the ggplot2 package in R (Wickham, 2016).
Supplementary Results

Order effects

For all behaviours assessed, order had no significant effect (Fig. S2):

- Emersion temperature threshold: $F_{1,27} = 0.01$, $P = 0.92$; emersion experiment
- Emersion surface behaviours: $F_{1,13} = 0.14$, $P = 0.72$; loss of equilibrium temperature: $F_{1,8} = 3.14$, $P = 0.11$; and loss of equilibrium experiment surface behaviours: $F_{1,8} = 0.29$, $P = 0.69$. 
References


Supplementary Figure 1. Schematic of the tank used to assess behaviours related to rapid warming. We separated the test portion of chamber from the stir bar and submersible water heater by a mesh screen to allow for water circulation and thermal mixing. The fish interacted with a mirror (or opaque, size-matched object) under water along one edge of the test chamber. For experiments involving loss of equilibrium or to assess the effects of blocking access to the aerial environment, we placed an additional mesh screen on the water surface.
Supplementary Figure 2. Threshold temperatures from the emersion and loss of equilibrium experiments (combined), categorised for each respective behaviour in Mangrove rivulus (N=26; see methods for precise breakdown within each experiment) exposed to a mirror or an opaque object. The large circles depict the model (± se) predictions from a linear mixed effects model with fish identity incorporated as a random intercept. Individual responses in smaller circles represent the first observation for each behaviour within each experiment. Abbreviations: LD, lateral display; MC, mirror charge; SE, surface excursion; BS, break surface; ET, emersion; LOE, loss of equilibrium.