



Research

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Physiology

Out of the frying pan into the air—emersion behaviour and evaporative heat loss in an amphibious mangrove fish (*Kryptolebias marmoratus*)

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Amphibious fishes often emerse (leave water) when faced with unfavourable water conditions. How amphibious fishes cope with the risks of rising water temperatures may depend, in part, on the plasticity of behavioural mechanisms such as emersion thresholds. We hypothesized that the emersion threshold is reversibly plastic and thus dependent on recent acclimation history rather than on conditions during early development. *Kryptolebias marmoratus* were reared for 1 year at 25 or 30°C and acclimated as adults (one week) to either 25 or 30°C before exposure to an acute increase in water temperature. The emersion threshold temperature and acute thermal tolerance were significantly increased in adult fish acclimated to 30°C, but rearing temperature had no significant effect. Using a thermal imaging camera, we also showed that emersed fish in a low humidity aerial environment (30°C) lost significantly more heat (3.3°C min⁻¹) than those in a high humidity environment (1.6°C min⁻¹). In the field, mean relative humidity was 84%. These results provide evidence of behavioural avoidance of high temperatures and the first quantification of evaporative cooling in an amphibious fish. Furthermore, the avoidance response was reversibly plastic, flexibility that may be important for tropical amphibious fishes under increasing pressures from climatic change.

1. Introduction

Behavioural flexibility may be critical for animals facing rising temperatures [1,2]. In aquatic systems, behavioural avoidance is often the first line of defence for fishes. Amphibious fishes inhabiting warm tropical waters may leave water (emerge) to avoid aquatic temperature extremes, but few studies have quantified the thermal characteristics of emersion [3], and there is no knowledge of whether emersion thresholds are plastic.

Phenotypic plasticity is the capacity of an organism to express a range of phenotypes in response to environmental stimuli and can be reversible or irreversible [4]. Irreversible plasticity is often the result of environmental factors experienced during early development. If conditions during development are accurate predictors of the adult environment, irreversible plasticity should be favoured by selection [5,6]. In unpredictable environments however, reversible plasticity (flexibility) should be favoured. Both types of plasticity are important for determining the physiological tolerances of adult arthropods [7–9], frogs [10], birds [11] and zebrafish [12]. Although extensive research has investigated the thermal acclimation of physiological traits [13], the effects of thermal history on avoidance behaviour, the first line of defence for many animals, has been overlooked. We tested the hypothesis that in amphibious fishes inhabiting unstable thermal environments the emersion threshold is reversibly plastic

and thus influenced primarily by their recent thermal environment. We investigated emersion plasticity and evaporative cooling in a eurythermal (7–38°C) amphibious fish, the mangrove rivulus (*Kryptolebias marmoratus*) that typically inhabits small pools or crab burrows in mangrove forests [14].

Plasticity in thermal tolerance would help fish cope with high water temperatures, but there is still the risk of overheating at temperature extremes. After observing fish emerge in the laboratory and in the field, we hypothesized that cutaneous evaporation could quickly lower body temperature and minimize thermal damage. This hypothesis predicts that fish emerging into low relative humidity (RH) would lose more body heat than fish in high RH environments even if water and air temperatures were similar.

2. Methods

(a) Emersion plasticity

Newly hatched *K. marmoratus* hermaphrodites from a single lineage were separated into four temperature groups in a fully crossed thermal history experimental design: (i) reared at 30°C and later tested at 30°C, (ii) reared and tested at 25°C, (iii) reared at 30°C and one week prior to experimentation, transferred to 25°C, and (iv) reared at 25°C and one week prior to experimentation, transferred to 30°C ($n = 9–16$ per group). Fish were fed *Artemia* nauplii three times per week.

To determine the emersion threshold temperature, individual fish in experimental containers (100 ml) were placed in a circulating water bath behind a visual barrier, and behaviour was recorded with a webcam (Logitech C905®, Newark, CA, USA). Temperature was increased by 1°C min⁻¹ starting at the acclimation temperature of each group. Containers were aerated to ensure uniform oxygenation and temperature.

We also tested whether emersion temperature was correlated with the acute thermal tolerance of each group. The experiment was repeated, except a mesh barrier was placed at the air–water interface to prevent emersion ($n = 11–18$ per group). Acute thermal tolerance was defined as the temperature at which fish lost equilibrium, which provides a proxy for the physiological upper temperature limit [15]. All fish recovered fully.

(b) Evaporative cooling

To determine the role of evaporative cooling after emersion, emersion was induced by increasing water temperature (approx. 1°C min⁻¹) as described above, but in these experiments, fish emersed onto a moist substrate. The entire experimental apparatus was contained within an incubator (Pelt-5, Sable Systems, NV, USA) to control air temperature (30°C) and RH. Emersed fish experienced one of two treatments, high (95 ± 5%) or low (65 ± 5%) RH (each $n = 7$). RH was regulated by aerating a separate water chamber within the incubator and continuously measured using a hygrometer (SperScientific, Scottsdale, AZ, USA). Thermal images were captured through a transparent Germanium window (10 cm diameter × 4 mm thickness; > 97% infrared (IR) transmittance) every second post-emersion using a thermal imaging camera (Mikron Instruments, Model 7515, Vista, CA, USA). Images were analysed using MikroSpecRT (Mikron) by taking the average temperature of the emersed fish along a line down the centre of the body. The total number of movements (jumps) per fish was also measured for 60 s after emersion to determine whether fish enhance cooling by increasing convection.

Finally, we measured RH in the field using the same hygrometer (see above). Daily RH measurements were taken

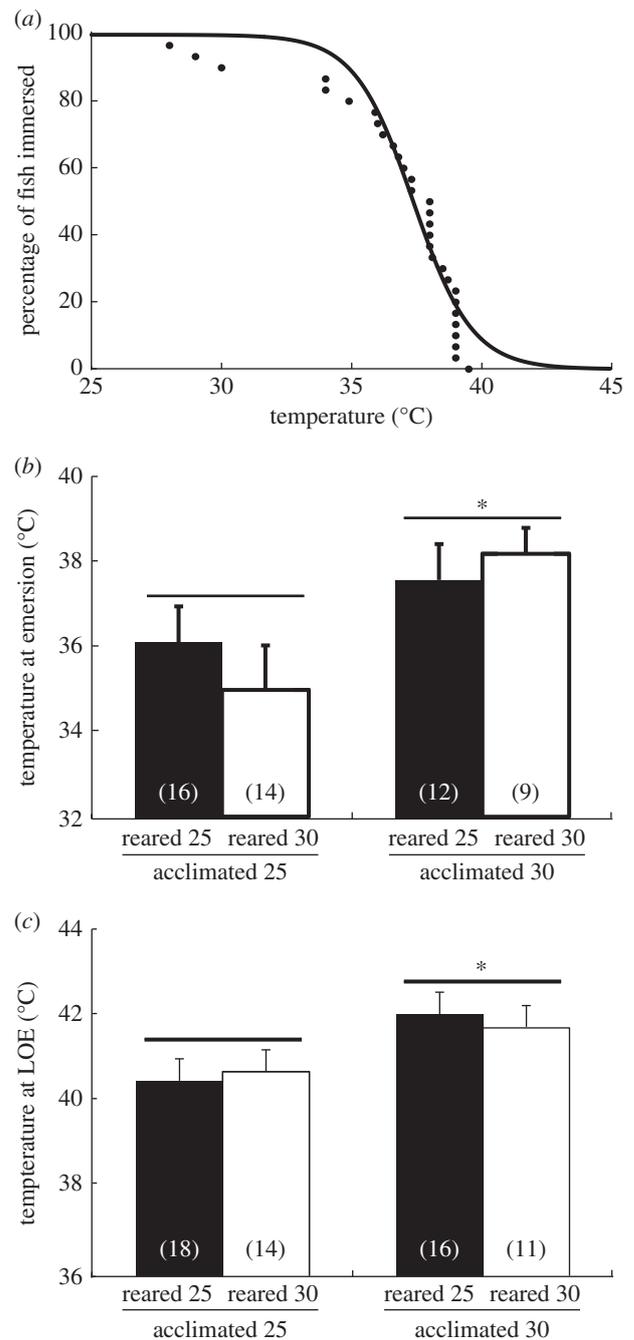


Figure 1. (a) Percentage of immersed (submerged) *Kryptolebias marmoratus* (control, reared and acclimated to 25°C) when exposed to rising water temperatures (approx. 1°C min⁻¹). The line of best fit follows the sigmoidal curve $y = 100/(1 + e^{-((x-37.37)/-1.13)})$. (b) Emersion temperatures and (c) loss of equilibrium (LOE) temperatures of *K. marmoratus* reared or acclimated to 25 or 30°C. Significant differences are indicated by an asterisk (means ± s.e.). Sample sizes are given in parentheses.

between 15 and 20 December 2012 immediately adjacent to crab burrows from which *K. marmoratus* had been observed to emerse (Long Caye, Belize; N 17°13.08'; W 087°35.66'). Data were analysed using two-way ANOVA or two-way repeated measures ANOVA as appropriate (critical $\alpha = 0.05$).

3. Results

Acutely increased water temperature caused emersion in all mangrove rivulus tested (65 out of 65 fish). Control fish (reared and acclimated to 25°C) emersed at temperatures between 28°C and 39.5°C (figure 1a). Fish acclimated to 30°C

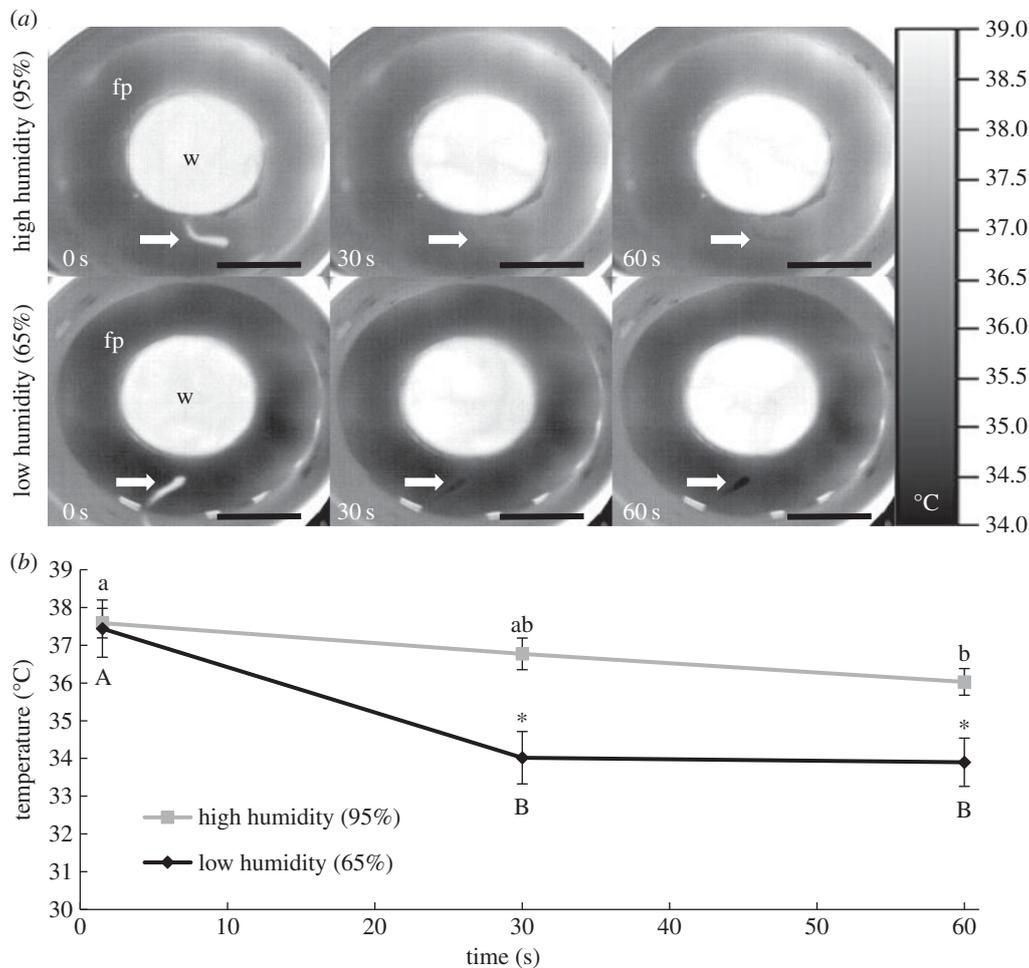


Figure 2. (a) Representative thermal images showing the decrease in temperature of *Kryptolebias marmoratus* (arrows) after 30 and 60 s of emersion. W, water; fp, filter paper. Scale bar, 5 cm. (b) Mean (\pm s.e.) body temperature of emersed fish in high and low humidity treatments (each $n = 7$). Starting water temperature was 25°C and air temperature was constant at 30°C. Different letters indicate significant differences within a treatment over time ($p < 0.001$). Asterisks denote significant differences between treatments ($p < 0.01$).

for 7 days emersed at significantly higher temperatures (approx. 2.3°C; $p < 0.05$) than fish acclimated to 25°C (figure 1b), but at a lower differential between acclimation and emergence temperature (25: 10°C versus 30: 8°C). Similarly, the acute thermal tolerance of fish acclimated to 30°C was significantly higher than that of fish acclimated to 25°C ($p < 0.001$; figure 1c), but again at a lower differential between acclimation and loss of equilibrium temperature (25: 15.5°C versus 30: 11.5°C). In addition, we performed an assessment of thermal tolerance using standard critical thermal maximum methods (heating rate of 0.3°C min⁻¹) and the results were similar (electronic supplementary material, table S1). The duration of acute heating was different between 25°C and 30°C groups in both the emersion and thermal tolerance experiments. There was no effect of rearing temperature on the emersion threshold or thermal tolerance ($p > 0.05$; figure 1b,c). Overall, fish lost equilibrium at water temperatures that were 4–5°C warmer than the emersion threshold.

Emersed fish quickly cooled; by 30 s, it was difficult to distinguish fish from background in thermal images (figure 2a). By 60 s, the fish appeared to be slightly cooler than the surrounding filter paper, a difference that was more pronounced at low RH. The surface temperature of fish decreased significantly in both treatments, but cooled faster under low RH ($p < 0.001$; figure 2b). Terrestrial activity (jumps) post-emersion was not affected by RH (6.0 ± 2.8 at low RH versus 3.8 ± 1.4 at high RH, each $n = 7$, $p > 0.05$).

In the field, RH immediately above crab burrows occupied by mangrove rivulus was $83.8 \pm 1.5\%$ (range 79–92%).

4. Discussion

Kryptolebias marmoratus reap the benefits of an amphibious lifestyle because they can escape stressful thermal conditions through emersion and evaporatively cool to prevent overheating. In the field, RH was below 100% (and between our test conditions of 65–95% RH) and therefore body heat would be lost to the environment in the wild through evaporation even if water and air temperatures were similar. Fish appeared cooler than the substrate, suggesting that evaporative cooling was more rapid than conductive loss of heat through direct contact. Although emersion appears to be a thermoregulatory avoidance response, we found no evidence to suggest that fish continued to actively thermoregulate by jumping to increase convection. Evidence for behavioural thermoregulation has been reported in amphibians that move to cooler environments to reduce water loss under arid conditions [16]. Behavioural thermoregulation in amphibious fishes was suspected in earlier studies [17,18], but ours, to our knowledge, is the first direct evidence.

Our results demonstrate that emersion behaviour is reversibly plastic in adult *K. marmoratus*. Increased emersion temperatures after acclimation to 30°C were associated with an increased acute thermal tolerance, suggesting a parallel

response in behaviour and physiological traits. The ability for *K. marmoratus* to safely remain in relatively warm water after acclimation would allow fish to continue to forage for aquatic prey and avoid terrestrial predators.

Interestingly, we found no evidence of irreversible plasticity in acute thermal tolerance as has been reported in other fishes [12,19]. Plasticity acts at many levels of biological organization, often interactively, and thus it is possible that different developmental temperatures irreversibly altered some physiological traits in *K. marmoratus* but these are hidden by other, reversible phenotypes [11,20]. Overall, our results suggest that amphibious fishes may be at an advantage during peak aquatic temperatures, as behavioural avoidance by emersion and subsequent rapid evaporative cooling provide a mitigation strategy unavailable to fully aquatic species.

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