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Rapid heat hardening in embryos of the lizard *Anolis sagrei*

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Adaptive thermal tolerance plasticity can dampen the negative effects of warming. However, our knowledge of tolerance plasticity is lacking for embryonic stages that are relatively immobile and may benefit the most from an adaptive plastic response. We tested for heat hardening capacity (a rapid increase in thermal tolerance that manifests in minutes to hours) in embryos of the lizard *Anolis sagrei*. We compared the survival of a lethal temperature exposure between embryos that either did (hardened) or did not (not hardened) receive a high but non-lethal temperature pre-treatment. We also measured heart rates (HRs) at common garden temperatures before and after heat exposures to assess metabolic consequences. 'Hardened' embryos had significantly greater survival after lethal heat exposure relative to 'not hardened' embryos. That said, heat pre-treatment led to a subsequent increase in embryo HR that did not occur in embryos that did not receive pre-treatment, indicative of an energetic cost of mounting the heat hardening response. Our results are not only consistent with adaptive thermal tolerance plasticity in these embryos (greater heat survival after heat exposure), but also highlight associated costs. Thermal tolerance plasticity may be an important mechanism by which embryos respond to warming that warrants greater consideration.

1. Introduction

Anthropogenic climate change is increasing the frequency and magnitude of extreme temperatures [1], with potentially severe physiological consequences [2,3]. One mechanism by which organisms may mitigate the negative effects of high temperatures is adaptive plasticity in heat tolerance [4–7]. It has been proposed that the evolution of thermal tolerance plasticity is influenced by the ability of organisms to behaviourally thermoregulate ([8], *sensu* the 'Bogert Effect' [9]). Specifically, selection may favour physiological flexibility in taxa or life stages that either cannot move or that live in spatially homogeneous thermal environments such that they cannot dampen environmental temperature fluctuations through microclimate choice.

Within this context, heat tolerance plasticity may be particularly beneficial in immobile embryonic stages such as eggs that have little opportunity to behaviourally thermoregulate [10–15]. Embryos are also sensitive to heat exposure, with effects including increased mortality, decreased energy reserves, changes in development rate and structural birth defects [16–20]. However, our knowledge of embryonic heat tolerance plasticity is lacking. A recent systematic meta-analysis found estimates of embryonic heat tolerance plasticity for only five ectotherm species, all of which were aquatic taxa ([21], but see also, [22,23]). This is in stark contrast our knowledge of thermal tolerance plasticity in later life stages, for which we have hundreds of estimates across many taxa [8,24].

The few existing data paint a varied picture of embryonic thermal plasticity, with examples of exposure to warm temperatures increasing [25–27], decreasing [23,28] or having no effect [22,23,29] on heat tolerance.

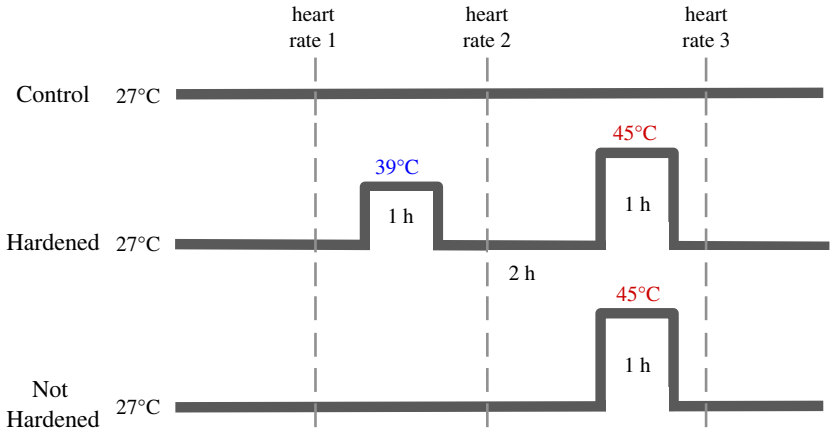

Figure 1. Schematic of experimental treatments administered on day 12 embryos. Grey dashed lines indicate when HRs were measured across all groups.

Broadening our understanding of embryonic plasticity is crucial because the embryonic stage can play a key role in population responses to environmental thermal stress [12,30–33]. For example, an analysis of *Sceloporus* fence lizards found that predicted decreases in fitness with anthropogenic warming were much more severe when embryonic thermal sensitivity was included (−33%) versus when it was not (−2%) [22].

To help deepen our understanding of embryonic thermal biology, we tested for heat tolerance plasticity in embryos of the lizard *Anolis sagrei*. We focused on heat hardening, a rapid plastic response to heat that manifests in minutes to hours [34–36]. We compared survival after an episodic extreme heat event between brown anole embryos that either did or did not receive a high but non-lethal temperature pre-treatment. We predicted higher survival of embryos in the heat pre-treatment group, indicative of heat hardening ability. To address potential metabolic consequences of heat events, we measured heart rates (HRs) throughout embryonic development as well as before and after heat exposures.

2. Methods

Approximately 300 female *A. sagrei* were collected from Miami-Dade County, FL during May of 2022 and transported to Loyola University Chicago, where they were housed following established protocols. Briefly, females were housed in groups of up to six individuals per cage with perches and moist potting soil available for egg laying [16,37]. Crickets were provided daily as food and enclosures were misted with water five or six times per day with an automatic misting system (MistKing, Jungle Hobbies Ltd). Eggs were collected daily between 09.00 and 10.00 h and were incubated at 27°C, within the natural range of *A. sagrei* nests [16,38], in a sealed Petri dish filled with moist vermiculite (−150 kPa) [16]. Given that multiple females were housed together, we could not determine the source of each egg. However, we assume that each egg was from a different female due to our sampling design: *A. sagrei* lay one egg at a time at an interval of 7–10 days, and all eggs used in the study ($N = 89$) were collected over 4 consecutive days (13–16 June 2022). Each egg was randomly assigned to one of three groups: Hardened, Not Hardened or Control (see below). Starting on day 7, the HR of each embryo was measured between 09.00 and 13.00 h every other day using an infrared egg monitor (Avitronics, Cornwall, UK) [10]. HRs were recorded for 30 s, and if a HR could not be found the egg was gently rotated up to four times and reassessed [18].

(a) Experimental protocol

Heat tolerance and heat hardening ability were measured on day 12 (figure 1). Not all embryos survived to day 12, and we moved forward with the following sample sizes: $N = 25$ Hardened, $N = 29$ Not hardened and $N = 28$ Control. Heat tolerance was measured as survival after 1 h at 45°C, a temperature known to be potentially lethal to developing embryos of this species from previous studies [18,39]. Eggs in the Hardened group experienced a heat pre-treatment at 39°C for 1 h before exposure to 45°C 2 h later (figure 1). The Not Hardened group experienced only the 45°C exposure, while the Control group did not experience any elevated temperatures. The HR of all eggs was measured three times on experimental days, corresponding to times before and after heat exposures in the groups that received them (figure 1). There was approximately 1.25 h between the first and second HR measurements, and 2 h between the second and third HR measurements. At the conclusion of data collection, eggs were placed back in their chambers at 27°C and raised until hatching or egg failure as determined by fungal growth.

(b) Statistical analysis

We tested for differences in mortality between control and treatment groups with a G-test. We tested for differences in HR change before and after heat exposure (i.e. HR 2 − HR 1 and HR 3 − HR 2; figure 1) using paired *t*-tests. Data were analysed using R [40].

3. Results

Embryos in the Hardened group were more likely to survive exposure to 45°C than embryos in the Not Hardened group (G-test, $p = 0.033$). Although not included in the statistical analysis, embryos in the Control group had the highest survival rate overall (figure 2*a*). Embryos that experienced the 39°C pre-treatment (Hardened group) increased their HR afterwards ($\bar{X}$ (95% CI); $\Delta HR = 7.6$ (1.7–13.5) bpm; *t*-test, $p = 0.014$) while embryos that did not experience the 39°C pre-treatment (Not Hardened and Control groups) did not change over the same time frame ($\Delta HR = 0.8$ (−2.3–3.9) bpm; *t*-test, $p = 0.601$; figure 2*b*). Exposure to 45°C led to a decrease in the HR of Hardened embryos ($\Delta HR = -9.4$ (−15.9 – −2.9) bpm; *t*-test, $p = 0.008$; figure 2*c*). The mean ΔHR of embryos in the Not hardened group was negative (−5.7 (−17.7–6.4)) but was not significantly different from

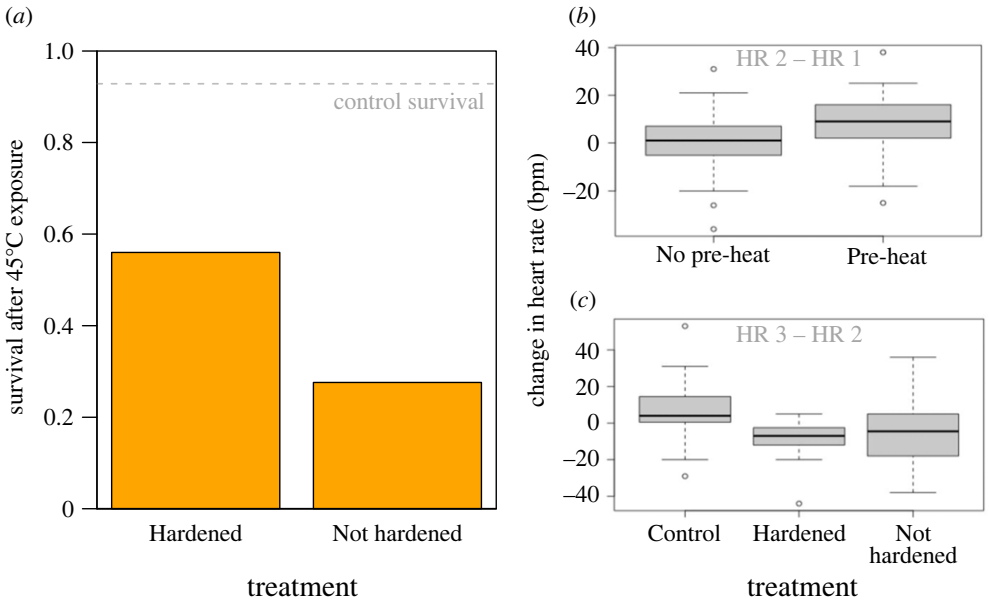

Figure 2. (*a*) Survival of brown anole embryos after exposure to 1 h at 45°C (figure 1). *Hardened*: embryos that experienced a 1 h heat pre-treatment at 39°C prior to exposure to 1 h 45°C. *Not Hardened*: embryos only exposed to 1 h at 45°C. *Dashed line*: survival of 'Control' embryos that did not experience any elevated temperatures. (*b*) Change in the HR of individual embryos over time (HR 2 – HR 1; figure 1) showing the effect of the heat pre-treatment. *No pre-treat*: embryos that did not experience the 39°C heat pre-treatment ('Not hardened' and 'Control' groups combined). *Pre-treat*: embryos that experienced the 39°C heat pre-treatment ('Hardened' group only). (*c*) Change in the HR of individual embryos over time (HR 3 – HR 2; figure 1) showing the effect of the 45°C exposure. All HRs (HR 1, HR 2 and HR 3, figure 1) were measured at a common temperature (27°C).

0 (*t*-test, $p = 0.323$; figure 2*c*). Meanwhile, embryos in the Control group that did not experience 45°C demonstrated an increase in HR over the same time interval (6.8 (0.4–13.2) bpm; *t*-test, $p = 0.008$; figure 2*c*).

4. Discussion

Embryonic thermal tolerance plasticity is understudied, and the few data that exist paint an unclear picture as to whether embryos can increase their heat tolerance in response to environmental warming [21]. We provide clear evidence of embryonic heat hardening capacity in brown anoles: embryos exposed to a high but non-lethal temperature were more likely to survive lethal heat just 2 h later (figure 2*a*). Embryos may thus have the capacity to rapidly adjustment to extreme heat excursions. However, the generality of this finding is yet to be determined. Heat hardening is well-known in later life stages of ectotherms [35,41–43], including lizards [34,44,45], but most studies of embryonic plasticity focus on longer term acclimation. Notably, adult brown anoles from another invasive, higher latitude (New Orleans) population cannot heat harden [45]. This species may therefore undergo ontogenetic change in the capacity for heat tolerance plasticity commensurate with the ability to behaviourally thermoregulate [8]. However, more population-specific analyses are necessary to test that hypothesis directly.

Induction of the heat hardening response is expected to increase energetic expenditure, as it can involve cell membrane remodelling, higher expression and function of energetically expensive molecular chaperones, and large-scale changes in the metabolome (e.g. [46–48]). Consistent with this expectation, embryos in the Hardened group increased their HRs at a common temperature (27°C) after the high-temperature pre-treatment (figure 2*b*). By contrast, the HRs of the Not Hardened and Control groups, which did not experience the pre-treatment, did not change over the same time period when measured at the same temperature (figure 2*b*). HR is positively associated with metabolic rate in *A. sagrei* embryos [18], and therefore the higher HRs of Hardened embryos likely reflect an energetic cost of heat hardening [49].

Exposure to a potentially lethal temperature had the opposite effect on HRs: embryos in the Hardened and Not Hardened groups generally experienced a decrease in HR after experiencing 45°C (e.g. HR 3 – HR 2; figure 2*c*). Embryos in the Control group did not decrease their HR over the same time interval and measured at the same common temperature (figure 2*c*). A similar response to high temperature was seen in *A. sagrei* embryos from a different population [18,19]. Why embryonic HRs decrease after extreme heat exposure is unknown, but may be due to cell and tissue damage associated with stressful temperatures [50].

Predicting the effects of climate change depends on knowledge of thermal responses across the lifespan [51–55], but data on the thermal physiology of embryos is relatively sparse [21]. Our data are consistent with lizard embryos having the capacity for adaptive plasticity under a warming scenario [56]. That said, the induction of said plasticity appears to carry an energetic cost in the form of an increased metabolic rate. More work is required to understand how embryonic thermal plasticity relates to thermal adaptation and population responses to global change more broadly [21–23]. However, it is clear that one must consider the possibility of flexibility in embryo thermal tolerance when projecting population responses to thermal variation.

Ethics. All research was conducted with the approval of the Loyola University Institutional Animal Care and Use Committee (protocol no. 1992).

Data accessibility. Data and a description of the data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.pzgmsbcs1 [57].

The data sheet and data are provided in the electronic supplementary material [58].

Authors' contributions. G.S.G.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft and writing—review and editing; K.S.: data curation, investigation and writing—review and editing; T.S.: conceptualization, funding acquisition, project administration and writing—original draft and writing—review and editing; A.G.: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, visualization, writing—original draft and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare no competing interests.

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