1 **Title: Heat Hardening of a Larval Amphibian is Dependent on Acclimation Period and**

2 **Temperature**

3 **Running Title: Hardening in a Larval Amphibian**

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8 **Keywords:** heat hardening, CT_{max}, wood frogs, thermal acclimation, thermal plasticity

9 **Summary Statement:** A larval amphibian follows the trade-off hypothesis such that the group

10 with the highest basal heat tolerance displays the lowest hardening response and vice-versa.

11

12   **Abstract**

13   The thermal tolerance–plasticity trade-off hypothesis states that acclimation to warmer

14   environments increases basal thermal tolerance in ectotherms but reduces plasticity in coping

15   with acute thermal stress characterized as heat hardening. We examined the potential trade-off

16   between basal heat tolerance and hardening plasticity, measured as critical thermal maximum

17   (CT_{max}) of a larval amphibian, *Lithobates sylvaticus*, in response to differing acclimation

18   temperatures (15° and 25°C) and periods (3 or 7 days). A hardening treatment applied 2 hours

19   before CT_{max} assays induced pronounced plastic hardening responses in the cool, 15°C treatment

20   after 7 days of acclimation, compared to controls. Warm acclimated larvae at 25°C, by contrast,

21   exhibited minor hardening responses, but significantly increased basal thermal tolerance. These

22   results support the trade-off hypothesis and fill a knowledge gap in larval amphibian thermal

23   plasticity. Elevated environmental temperatures induce acclimation in heat tolerance yet

24   constrains ectotherm capacity to cope with further acute thermal stress.

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Introduction

Environmental temperature is one of the most important abiotic drivers of organismal physiology (Angilletta Jr, 2009). As temperatures increase due to climate change, ectotherms will be under greater risk of approaching their upper thermal limit that will lead to shifts in species distributions, altered biological interactions, and reduced activity periods, all of which can result in extinction (Bellard et al., 2012; Blois et al., 2013; Cox et al., 2022; Somero, 2010). Global declines in amphibians have been linked to climate change (e.g., Blaustein et al., 2010; Campbell Grant et al., 2020; Lowe et al., 2021; Rollins-Smith, 2017), highlighting the need for continued research on how they respond to warming and thermal extremes.

As thermal traits generally evolve slowly in herpetofauna (Bodensteiner et al., 2020), phenotypic plasticity is likely a primary response to climate change and increasing thermal stress. Thermal acclimation represents reversible plasticity in basal heat tolerance and develops over days to weeks of chronic exposure to altered environmental temperatures (e.g., Cupp Jr, 1980; Lapwong et al., 2021b; Li et al., 2009; Rohr et al., 2018; Sgro et al., 2010). However, acclimation does not necessarily protect organisms against acute exposure to short-term heat events such as heat waves which are projected to increase in frequency (Seneviratne et al., 2021). The related heat hardening response is another form of thermal plasticity that, by contrast, develops rapidly over minutes to hours of exposure to acute heat stress (Bowler, 2005). Heat hardening is generated by exposing organisms to temperatures near or at their upper thermal limit. While hardening rapidly increases heat tolerance, these increases are transient and disappear within 36 hours (Deery et al., 2021; Maness and Hutchison, 1980; Phillips et al., 2016; Rutledge et al., 1987; but see Moyen et al., 2020), highlighting its role as a short-term protection mechanism. Therefore, plasticity in heat tolerance occurs at two different levels: basal thermal tolerance, measured as the limits of thermal performance curves (Huey and Stevenson, 1979), following an acclimation period, and hardening, which temporarily increases basal thermal tolerance following an acute heat stress.

Under an ideal scenario, both high basal thermal tolerance and hardening would improve ectotherm persistence under climate change. However, there appears to be a physiological limitation such that elevated basal thermal tolerance constrains the capacity of an organism to further increase their heat tolerance. For example, Stillman (2003) found a negative relationship between basal thermal tolerance and acclimation capacity in *Petrolisthes* crab populations across

58 a latitudinal gradient. Building upon this, van Heerwaarden and Kellermann (2020) identified

59 that this negative link was widespread across ectothermic clades and named this pattern the

60 tolerance–plasticity trade-off hypothesis. Heat shock proteins (HSPs) may underlie the trade-off

61 hypothesis because of the central role they play in maintaining homeostasis during extreme

62 temperatures (Feder and Hofmann, 1999; Sørensen et al., 2003) and improving basal thermal

63 tolerance (Bahrndorff et al., 2009; Blair and Glover, 2019; Gao et al., 2014; Krebs and Feder,

64 1998; but see Easton et al., 1987; Jensen et al., 2010). Because HSPs are energetically expensive

65 to produce and maintain (e.g., Hoekstra and Montooth, 2013), populations from warm

66 environments may be 'preadapted' to favor relatively high constitutive HSP expression to elevate

67 basal thermal tolerance but exhibit less flexibility in upregulation following an acute heat shock

68 compared to cool environment populations (Gleason and Burton, 2015). Therefore, under the

69 trade-off hypothesis, hardening may be more useful to species that are less likely to experience

70 chronic heat stress but receive greater benefits in combating acute stress (van Heerwaarden and

71 Kellermann, 2020; but see Sgro et al., 2010). Thus, acute upper thermal limits that are near or

72 pushed near adapted thermal maxima restrict additional plasticity for further increased thermal

73 tolerance through acclimation (Somero, 2010). While a meta-analysis on ectotherms by Barley et

74 al. (2021) provided support for the trade-off hypothesis, there is mixed evidence in larval

75 amphibians (e.g., Menke and Claussen, 1982; Simon et al., 2015; Turriago et al., 2022)

76 suggesting a need for further exploration.

77 　　The role of heat hardening in adult (Maness and Hutchison, 1980) and larval amphibians

78 (Sherman and Levitis, 2003; Sørensen et al., 2009) is understudied. We aimed to investigate the

79 trade-off hypothesis by testing how acclimation temperatures (low or high) and duration (short or

80 long acclimation periods) affect interactions between heat hardening and basal thermal tolerance

81 – estimated via critical thermal maximum (CT_{max}). These tests were conducted on larval wood

82 frogs, *Lithobates sylvaticus* (LeConte 1825). Because larval anurans display a positive

83 relationship between acclimation temperature and CT_{max} (e.g., Cupp Jr, 1980; Ruthsatz et al.,

84 2022), we predicted that longer acclimation to warmer temperatures would increase basal heat

85 tolerance compared to those acclimated to cooler temperatures. In line with the trade-off

86 hypothesis, we also expected the hardening effect would be most pronounced in larvae with the

87 lowest CT_{max} suggesting greater acute thermal plasticity under these environments.

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Materials and Methods

Field Collection and Husbandry

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91   Freshly laid (< 36 hours old) wood frog egg masses were collected from wetlands in

92   Jackson Co., IL under an Illinois Department of Natural Resources permit (HSCP 19-03). The

93   egg masses were maintained in 60 L plastic containers with aerated, carbon-filtered water. After

94   hatching, larvae were initially fed autoclaved algal flakes (Bug Bites Spirulina Flakes, Fluval

95   Aquatics, Mansfield, MA, USA), followed by crushed alfalfa pellets at two weeks after hatching.

96   Animals were fed twice weekly, and water was changed weekly. All experimental procedures

97   were approved by the Southern Illinois University Institutional Animal Care and Use Committee

98   (22–008).

Critical Thermal Maximum Assay

100   After larvae reached early pro-metamorphic stages, 64 individuals were randomly

101   selected and staged, weighed, and transferred to individual 750 mL plastic containers filled with

102   600 mL of aged (>24 hours) aerated, carbon-filtered water. Larvae were split (N=32/treatment)

103   into low ($15°C \pm 0.2$) and high ($25°C \pm 0.3$) acclimation temperatures. There were no differences

104   in initial Gosner (1960) stage (range = 27 – 35) or mass (0.25 – 0.55 g) between these groups (P

105   > 0.3). The larvae were further randomly split into four groups (n=8 per group) that differed in

106   acclimation period and heat hardening treatment: 1) 3-day control, 2) 3-day hardened, 3) 7-day

107   control, and 4) 7-day hardened. Larvae were acclimated to low or high temperatures for either

108   three or seven days. On the last day of acclimation, the CT_{max} of control groups was measured.

109   The hardened groups were heated for 10 minutes at 2–4°C below the CT_{max} of control groups,

110   following the protocol of Sherman and Levitis (2003). After this heat hardening treatment, the

111   animals were returned to their acclimation temperatures for 2 hours, after which their CT_{max} was

112   measured. Sample sizes were reduced to seven for the 7-day hardened low and high temperatures

113   groups, and the 7-day control low temperature group due to mortality.

114   CT_{max} was measured between 1000 – 1600 hrs to minimize potential diel effects on heat

115   tolerance (Healy and Schulte, 2012; Maness and Hutchison, 1980). Larvae were staged, weighed,

116   and then placed in individual 125 mL flasks filled with 75 mL of aged, aerated, carbon-filtered

117   water and submerged in a hot water bath (Isotemp 220, Fischer Scientific) and given 5 minutes

118   to acclimate prior to beginning the assay. Water temperatures increased $0.6 \pm 0.01°C$ per minute

119   from a starting temperature of $19.9 \pm 0.2°C$. Beginning at ~34°C, larvae were prodded with a

120   spatula every 30 seconds until they did not respond to the stimulus. At this point, a thermocouple

121   probe (Physitemp BAT-12) was placed in the flask, water temperature was recorded which

122   represented the larval CT_{max}. Flasks were then placed in a water bath at room temperature to

123   facilitate larval recovery, and all larvae recovered $\leq$ 5 minutes. Upon completion of CT_{max}

124   measurements, all larvae were euthanized via snap-freezing in -80°C ethanol.

125   *Statistical Analyses*

126   We assessed how larval CT_{max} shifted in response to our various treatments using a

127   general linear model. While Gosner stage recorded prior to the CT_{max} measurement was normally

128   distributed, mass was log-transformed to achieve normality, and both were included as covariates

129   in the model. Fixed effects included acclimation period (3 or 7 days), acclimation temperature

130   (low or high), hardening treatment (control or hardened), and their interactions. Post-hoc

131   analyses were conducted using Tukey tests. All analyses were conducted in R Studio v.

132   2022.02.3 (https://www. Rstudio.com/) and significance values were set as $\alpha = 0.05$.

Results

Across all treatments, wood frog larvae displayed a moderate degree of variation in their CT_{max} (range = 35.8° – 39.6°C; Table 1). Two individuals were dropped from analyses due to abnormally low CT_{max} values ($\leq 34.9°C$) in relation to their group mean. Of the main effects, only acclimation temperature ($F_{1,49} = 6.52$, P = 0.014) had a significant effect (Table 2) with those in the high acclimation temperature treatment exhibiting greater heat tolerance (Fig. 1). While neither hardening ($F_{1,49} = 0.088$, P = 0.77) nor acclimation period ($F_{1,49} = 2.55$, P = 0.12) had significant effects on CT_{max}, there was significant hardening by acclimation period ($F_{1,49} = 6.11$, P = 0.017) and acclimation period by acclimation temperature ($F_{1,49} = 18.71$, P < 0.0001) interactions. The former was driven by a more pronounced hardening effect for day 7 individuals, while the latter was the outcome of a pronounced increase in CT_{max} among larvae in the high acclimation treatment on day 7 (Fig. 1). Lastly, a significant three-way interaction was found for acclimation period, acclimation temperature, and hardening treatment ($F_{1,49} = 4.47$, P = 0.040). Larvae in the low acclimation treatment on day 7 showed the largest hardening effect of 0.9°C, which was more than double the hardening effect of any other group (Fig. 1). Larval mass and Gosner stage were unrelated to CT_{max} (P $\geq$ 0.29).

150   **Discussion**

151   Phenotypic plasticity of heat tolerance provides ectotherms the ability to counter the

152   threat of overheating due to temperature extremes associated with climate change. Heat

153   hardening, a form of thermal plasticity, represents the "first line of defense" against heat stress

154   (Deery et al., 2021) through rapid upregulation of HSPs and/or changes to cellular structure in

155   response to an acute thermal shock that can increase short-term heat tolerance (Bowler, 2005).

156   However, the tolerance–plasticity trade-off hypothesis (van Heerwaarden and Kellermann, 2020)

157   proposes that basal heat tolerance and thermal plasticity are negatively correlated; such that

158   individuals with high CT_{max} have limited hardening (Gilbert and Miles, 2019). While numerous

159   studies have demonstrated that amphibians exhibit plastic basal heat tolerance (e.g., Cupp Jr,

160   1980; Ruthsatz et al., 2022), hardening remains understudied.

161   In our study, we found evidence in support of the trade-off hypothesis for larval wood

162   frogs, although the effect was minor (Fig. 1), potentially due to low sample sizes. The group with

163   the lowest mean CT_{max} (36.5°C) had the greatest hardening effect (0.9°C), while the group with

164   the highest mean CT_{max} (39.0°C) had a minimal hardening effect (0.1°C). While the 0.9°C

165   hardening effect was comparable to larval American toads (*Anaxyrus americanus*) and African

166   clawed frogs (*Xenopus laevis*) (Sherman and Levitis, 2003), the remaining groups had a minor

167   hardening response (≤ 0.4°C) that was similar with values for larval bullfrogs (*L. catesbeianus*)

168   (Menke and Claussen, 1982). Additionally, the bullfrogs showed no evidence of the trade-off

169   hypothesis as CT_{max} increased positively with acclimation temperatures while hardening effect

170   was unchanged. Hardening effects in lizard, salamander, and fish species are variable ranging

171   from –0.4°C (*Anolis sagrei*) to 2.1°C (*A. carolinensis*) (Deery et al., 2021; Lapwong et al.,

172   2021a; Maness and Hutchison, 1980; Phillips et al., 2016; Rutledge et al., 1987). In relation to

173   other species, larval wood frogs acclimated to cooler conditions have a relatively strong

174   hardening effect indicating significant plasticity in heat tolerance to improve their tolerance of

175   overheating. This may benefit wood frogs as ephemeral pond breeding species are threatened by

176   climate change (Blaustein et al., 2010) during the larval stage (Enriquez-Urzelai et al., 2019).

177   We can only speculate on the mechanism that drove the observed results, but we propose

178   that HSPs represent an intriguing answer. This is because they are intimately tied to

179   environmental temperature (Dalvi et al., 2012; Gu et al., 2019; Jin et al., 2019) and basal

180   thermotolerance (Bahrndorff et al., 2009; Blair and Glover, 2019). Warm-tolerant ectotherms

181 often express higher constitutive levels of *hsp70* relative to less-tolerant populations, but that an

182 acute heat-stress results in greater *hsp70* expression in those with lower basal thermal tolerance

183 (Gleason and Burton, 2015; Zatsepina et al., 2000; Zatsepina et al., 2001). Zatsepina et al. (2000)

184 proposed that this provided temperate populations the capability to rapidly and intensely

185 synthesize HSPs after brief exposure to heat shock that was absent in low latitude populations.

186 We propose a similar pattern in the wood frog larvae, such that higher constitutive HSP levels in

187 warm-acclimated larvae provided increased basal heat tolerance compared to cold-acclimated

188 larvae, yet hardened larvae from the latter group greatly upregulated HSP expression following a

189 heat shock enhancing their hardening response. This is in line with *Drosophila* acclimated to

190 cooler temperatures (Bettencourt et al., 1999), which exhibited pronounced hardening plasticity

191 that was absent in the warm-acclimated group. Quantifying constitutive and heat-shocked *hsp70*

192 mRNA of larval liver and gill tissues would offer support to this conclusion. Additionally, many

193 ectotherms appear to have hard upper-limits to thermal tolerance after which their pejus range

194 constraints any further plastic responses (Denny and Dowd, 2012). Thus, the warm acclimated

195 larvae in our study could have approached their physiologically and evolutionarily determined

196 upper limit that constrained any further plastic responses. Future tests are required to understand

197 1) if there is a degree of plasticity to hard upper limits of thermal acclimation, 2) the cellular and

198 physiological mechanisms underlying these limits, 3) how these mechanisms determine the

199 trade-offs between hardening and acclimation to chronic heat stress, and 4) how these

200 mechanistic interactions are shaped by evolution in comparative studies.

201 Wood frog larvae with low basal heat tolerance demonstrated a large hardening effect

202 suggesting a trade-off between the two traits. There is an inherent link between CT_{max} and

203 hardening which may bias the presence of the trade-off hypothesis (van Heerwaarden and

204 Kellermann, 2020), and Deery et al. (2021) proposed that correlative evidence of the trade-off

205 hypothesis is a statistical artifact. However, we believe our methodology of using different

206 individuals for CT_{max} and hardening removed the risks of spurious correlation and strengthened

207 our analyses. Based on our results, we propose that larval wood frogs support the trade-off

208 hypothesis after a relatively short acclimation period. Hardening benefits cool-acclimated

209 populations in response to acute heat stress but plasticity in basal heat tolerance in response to

210 prolonged warming are likely to be more beneficial in reducing overheating risk.

211

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371

372 **Table 1**: **Mean critical thermal maximum (CT$_{max}$) of control and hardened larval wood**

373 **frogs at different acclimation temperatures and acclimation periods.**

Group	Control (°C)	Hardened (°C)
15°C Day 3	36.8 ± 0.1 (7)	36.9 ± 0.3 (8)
25°C Day 3	37.4 ± 0.1 (8)	37.8 ± 0.2 (7)
15°C Day 7	36.5 ± 0.2 (7)	37.4 ± 0.2 (7)
25°C Day 7	39.0 ± 0.1 (8)	39.1 ± 0.1 (7)

374 Data are means ± standard error, with sample size in parentheses.

375

376 **Table 2: Effects of body mass, Gosner stage, hardening treatment, acclimation period, and**

377 **acclimation temperature on larval wood frog critical thermal maximum from a generalized**

378 **linear model.**

Source of Variation	S. S.	d. f.	F	*P*
Log-Transformed Body Mass	0.14	1	0.73	0.40
Gosner Stage	0.22	1	1.15	0.29
Hardening	0.017	1	0.088	0.77
Acclimation Period	0.49	1	2.55	0.12
Acclimation Temperature	1.25	1	6.52	**0.014**
Hardening x Acclimation Period	1.17	1	6.11	**0.017**
Hardening x Acclimation Temperature	0.094	1	0.49	0.49
Acclimation Period x Acclimation Temperature	3.57	1	18.71	**< 0.001**
Hardening x Acclimation Period x Acclimation Temperature	0.85	1	4.47	**0.040**
Residuals	9.35	49		

379 Values in bold indicate significant differences, $p < 0.05$.

380

381 **Figure Legend**

382 **Figure 1: Heat tolerance of larval wood frogs across differing acclimation conditions and**

383 **hardening.** Larval wood frog critical thermal maximum (CT_{max}) exposed to two different

384 acclimation temperatures (15° and 25°C), two different acclimation periods (3 and 7 days), and a

385 hardening treatment (control vs. hardened). Points represent individual larvae. Center lines

386 within boxplots represent the median and the boxes denote the interquartile range with whiskers

387 representing 1.5x the upper or lower quartile. Letters indicate significant differences between

388 post hoc pairwise comparisons (Tukey HSD, $P < 0.05$).

389