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1 **Heat hardening capacity in *Drosophila melanogaster* is life stage specific and juveniles show**  
2 **the highest plasticity**

3  
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15 **Keywords:** thermal sensitivity, hardening, heat resistance, life stage specific plasticity, climate  
16 change

17 **Abstract**

18 Variation in stress resistance and adaptive plastic responses during ontogeny have rarely been  
19 addressed, despite the possibility that differences between life stages can affect range margins and  
20 thermal tolerance of species. Here we assessed the thermal sensitivity and hardening capacity of  
21 *Drosophila melanogaster* across developmental stages from *larval* to the adult stage. We observed  
22 strong differences between life stages in heat resistance with adults being most heat resistant followed  
23 by *puparia*, *pupae* and *larvae*. The impact of heat hardening (1h at 35 °C) on heat resistance changed  
24 during ontogeny with the highest positive effect of hardening observed in *puparia* and *pupae* and the  
25 lowest in adults. These results suggest that immobile life stages (*puparia* and *pupae*) have evolved  
26 high plasticity in upper thermal limits whereas adults and *larvae* rely more on behavioral responses  
27 to heat stress allowing them to escape from extreme high temperatures. While most studies on the  
28 plasticity of heat resistance in ectotherms have focused on the adult life stage, our findings emphasize  
29 the crucial importance of juvenile life stages of arthropods in understanding the thermal biology and  
30 life stage specific physiological responses to variable and stressful high temperatures. Failure to  
31 acknowledge this complication might lead to biased estimates of species' ability to cope with  
32 environmental changes, such as climate change.

### 33 **Introduction**

34 Adaptive phenotypic plasticity is a mechanism enabling organisms to adjust their phenotype to  
35 changing conditions and this is proposed to be especially important in fluctuating environments [1,  
36 but see 2]. The induction of plastic responses can occur through hardening where a brief exposure to  
37 a non-lethal condition triggers changes, that can increase the ability of organisms to tolerate  
38 subsequent more extreme conditions [3]. For example, heat or cold hardening induces plastic  
39 physiological and behavioral responses that significantly affect the ability to tolerate subsequent more  
40 extreme high or low temperatures and this seem to be a general phenomenon across a wide range of  
41 organisms [4–6].

42 In holometabolous insects, each life stage may have a different capacity for plasticity due to variation  
43 in the thermal sensitivity of life stages and/or morphological and physiological differences between  
44 them [7]. For example, low mobility and lack of fully functional organs during pre-adult stages may  
45 increase the selection pressure on plastic responses that improve the thermal tolerance in the  
46 juveniles. However, adults may show a lower thermal plasticity as a consequence of their high  
47 dispersal ability that allow them to avoid extreme conditions [8].

48 The influence of physiological or morphological changes induced by hardening or acclimation on  
49 thermal tolerance is a well-studied phenomenon, particularly in ectotherms [9]. However, most  
50 published studies on insects focus on adults, whereas plasticity of other life stages and its importance  
51 in mediating responses to daily and seasonal thermal fluctuations has rarely been addressed [10–12].  
52 Such information is however key to understanding the range- and tolerance limits of species, as  
53 knowledge from a single life-stage could over- or underestimate species tolerance. Thus, this can  
54 hinder our ability to correctly predict the consequences of altered environments, for example due to  
55 climate change, on distributions and future prospects of species [7]. Here, we conducted an  
56 experiment with *Drosophila melanogaster* in which the heat resistance of hardened and non-hardened  
57 individuals was assessed across seven developmental stages (3 larval, puparium, pupa, and 2 adult

58 stages). We hypothesized that sessile life stages (*puparium* and *pupa*) or stages with low mobility  
59 (*larva*) show higher plasticity in response to heat hardening compared to adults, which are better able  
60 to evade adverse conditions by dispersal.

## 61 **Materials and Methods**

### 62 *Population*

63 A *D. melanogaster* population was set up in 2010 using the offspring of 589 inseminated females  
64 caught at Karensminde fruit farm in Odder, Denmark (55°57' N, 10°09' E). The population was  
65 maintained on standard *Drosophila* agar-sugar-yeast-oatmeal medium at  $25 \pm 1^\circ\text{C}$  and on a 12h  
66 light:12h dark cycle [13]. For the sample collection, adult flies (6 to 7 days old) were placed into 300  
67 mL plastic bottles containing a plastic spoon filled with 5 mL standard medium (50 to 60 flies per  
68 bottle, 20 bottles per sampling period). Unless otherwise stated, flies were allowed to lay eggs for 2h,  
69 thereafter eggs were collected at a controlled density (15 eggs per 35 mL plastic vial containing 7 mL  
70 standard medium) and kept at  $25 \pm 1^\circ\text{C}$  and on a 12h L:12h D cycle until they reached the specific  
71 life stage being investigated (see below).

72 *Larvae* (1<sup>st</sup>, 2<sup>nd</sup> & 3<sup>rd</sup> instar *larvae*): *larval* stages were defined by the time after oviposition. The first,  
73 second and third instar *larvae* were collected 24, 48 and 72h after oviposition, respectively. The  
74 selected stages are physiologically, morphologically, and behaviorally different from each other. The  
75 first two *larval* stages mainly search for food and eat while the third instar *larvae* crawl out of the  
76 food source to search for a suitable pupation site. At each stage, 10 *larvae* were collected into each  
77 of 180 vials with 7 mL standard *Drosophila* medium.

78 *Puparia and pupae*: for both *puparial* and *pupal* stages, 15 eggs were collected into each of 180 35  
79 mL vials containing 7 mL standard *Drosophila* medium. 96h after egg collection the vials were  
80 inspected and the few early-formed *puparia* (rarely observed) were gently removed from vials and

81 discarded to control the age of samples. 122h (*puparium*) or 168h (*pupa*) after oviposition, the number  
82 of *puparia* or *pupae* in all vials was counted.

83 Adult (1- & 3-day old): The flies were collected 24h after the first emergence and placed into 35 mL  
84 plastic vials containing 7 mL standard *Drosophila* medium. For both ages, we placed 10 flies per vial,  
85 pooled sexes. We did not separate male and female adult flies, to match the handling of juvenile life  
86 stages where we did not know the distribution of males and females in the test samples.

### 87 *Thermal sensitivity*

88 Heat tolerance was tested for all life stages using heat mortality assays exposing flies to six different  
89 test temperatures (25, 37, 38, 39, 40 and 41 °C) with or without prior hardening (1 h at 35 °C). Pilot  
90 studies were conducted to determine appropriate hardening and test temperatures as well as their  
91 duration (data not shown). The selected heat hardening temperature and duration were sufficient to  
92 induce a heat stress response [14] but did not cause mortality in any of the life stages. The test  
93 temperatures reduced survival markedly, at least at the highest test temperature, after one hour  
94 exposure. All individuals were tested in 35 mL plastic vials containing 7 mL standard *Drosophila*  
95 medium providing an environment where the temperature changed gradually to reach the test  
96 temperature. At each life stage half of the collected samples (90 vials out of 180) were placed in a  
97 water bath set at 35 °C for 1h (heat hardening) and the rest of the vials were kept at 25 °C. Thereafter,  
98 equal numbers of hardened and non-hardened vials with individuals were randomly assigned to six  
99 water baths (15 replicate vials per treatment) set at 25, 37, 38, 39, 40 or 41 °C. The samples were  
100 exposed to the test temperature for 1h and then placed in a climate room (25 ± 1°C and 12h L:12h D  
101 cycle). Adult flies were scored for survival 24h after the heat treatment. For the remaining life stages  
102 vials were kept in the climate room (25 ± 1°C and 12h L:12h D cycle) until adults emerged. Upon  
103 emergence flies were counted (not sexed) and removed each day until no new flies had emerged for  
104 3 consecutive days.

## 105 **Data analysis**

106 For all life stages, the proportion of survivors from each vial was calculated as the number of live  
107 flies divided by the sum of dead and alive flies in each vial. The mortality rate at 25 °C and 37 °C test  
108 temperatures with or without hardening displayed a similar pattern throughout ontogeny (Table S1).  
109 Therefore, data on survival at 25 °C was removed from the dataset, to improve the data fit. The  
110 influence of hardening on thermal resistance of individuals throughout ontogeny was investigated  
111 using a linear model with hardening and life stage as fixed factors, with test temperature as a  
112 continuous variable, and including all interactions between fixed and continuous factors. We also  
113 removed the hardening factor from the model and analyzed the heat resistance of only non-hardened  
114 flies to test the life stage-specific basal thermal tolerance. In both analyses, the test temperature was  
115 mean centered (mean temperature minus each of the test temperatures) and the survival proportion  
116 was arcsine-square-root transformed. P-values were adjusted for multiple pairwise comparisons using  
117 a false discovery rate at the 5% level [15]. All analyses were performed with R (version 3.4) and  
118 RStudio (version 1.1.44).

## 119 **Results**

120 The impact of hardening on heat resistance varied significantly between life stages and test  
121 temperatures (hardening  $\times$  life stage  $\times$  test temperature:  $F = 23.67$ ,  $df = 6$ ,  $p < 0.0001$ ). *Puparium* and  
122 *pupa* responded most to hardening illustrated by a relatively constant survival across different test  
123 temperatures (~ 97% survival on average) while the non-hardened groups displayed a reduction in  
124 survival from 39 °C onwards (Fig. 1, Table 1). The hardened and non-hardened *larvae* (all three  
125 stages) showed a similar survival pattern with significantly higher resistance of the hardened group  
126 mainly at temperatures above 37 °C. Hardening did not affect the thermal resistance of 1-day old  
127 adults while at 3 days of age, hardening significantly reduced the thermal resistance of flies at 40 and  
128 41 °C. Within hardened or non-hardened groups, the heat resistance varied between life stages in a  
129 temperature-specific manner (non-hardened:  $F = 5.64$ ,  $df = 6$ ,  $p < 0.0001$ ; hardened:  $F = 40.51$ ,  $df =$

130 6,  $p < 0.0001$ , Table S2). In general, the non-hardened adults showed a significantly higher survival  
131 than *puparia* and *pupae* especially at 40 and 41 °C. The hardened *puparia* and *pupae* were more heat  
132 resistant than the hardened adults (both ages) across the test temperatures except at 38 °C, where no  
133 difference was observed between adults (both ages) and *puparia* as well as *pupae* (Table S2).

## 134 **Discussion**

135 As hypothesized, we observed that adaptive hardening responses were most pronounced in more  
136 sessile life stages compared to mobile adults. Under the hardening and test conditions we used,  
137 *puparia* and *pupae* followed by *larvae* (all three stages) had very strong hardening capacity compared  
138 to adults, where hardening either had no (1-day old adults) or negative (3-day old adults) effect on  
139 thermal resistance. These findings may arise from the ability of adults to evade critically extreme  
140 temperatures through behavioural responses and hence dismissing the need for responding plastically  
141 to quickly changing temperatures. Therefore, our data suggest, that in thermal variable environments  
142 natural selection will favor individuals / genotypes that are plastic as juveniles and less plastic but  
143 good dispersers at adult life stages [16]. The basal heat resistance was higher in adults than in other  
144 life stages (Fig. 1), which may be linked to the stage-specific energy allocation strategies in  
145 holometabolous insects and difference in energy requirement during ontogeny [17].

146 The increased survival of the hardened compared to the non-hardened juveniles points to their high  
147 dependence on plastic responses in the face of sudden temperature changes. Low plasticity of adults  
148 in upper thermal limits is a common observation in the literature [2,18], which can be a strategy to  
149 prevent the costs of physiological adjustments in response to thermal variation [4]. The absence of  
150 this pattern in juvenile stages, at the conditions that we have tested, highlights the need to perform  
151 studies on pre-adult stages to get a more complete picture of the thermal biology of a species. This is  
152 currently not a common practice as at least in *Drosophila*, where most studies focus on the adult life  
153 stage [but see 19].

154 Our findings provide evidence that different life stages have different thermal sensitivity and  
155 hardening capacity. The results suggest that the ability to cope with adverse thermal conditions has  
156 evolved in a life stage-specific manner. Such life-stage specificity in key adaptation mechanisms  
157 suggest that concentrating studies on a single life-stage, or single trait, in determining the range limits,  
158 or evolutionary potential of a species can bias the predictions concerning the ability to cope with  
159 environmental changes, such as climate change.

## 160 **Ethics**

161 NA

## 162 **Data accessibility**

163 DOI: <https://doi.org/10.5061/dryad.0908bq0>

164

## 165 **Author contributions**

166 NNM, CP, SB and TNK designed and NNM performed the experiment. NNM and TK analyzed the  
167 data. NNM, TNK, and TK wrote the manuscript, CP and SB provided useful comments on the  
168 manuscript and all authors approved the final version. All authors agree to be held accountable for  
169 the content of this manuscript.

## 170 **Competing interests**

171 We have no competing interests.

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177 **References**

- 178 [1] Lande R. Evolution of phenotypic plasticity and environmental tolerance of a labile  
179 quantitative character in a fluctuating environment. *J Evol Biol* 2014;27:866–75.  
180 doi:10.1111/jeb.12360.
- 181 [2] Sørensen JG, Kristensen TN, Overgaard J. Evolutionary and ecological patterns of thermal  
182 acclimation capacity in *Drosophila*: is it important for keeping up with climate change? *Curr Opin*  
183 *Insect Sci* 2016;17:98–104. doi:10.1016/j.cois.2016.08.003.
- 184 [3] Wilson RS, Franklin CE. Testing the beneficial acclimation hypothesis. *Trends Ecol Evolut*  
185 2002;17:66–70. doi:10.1016/S0169-5347(01)02384-9.
- 186 [4] Kristensen TN, Hoffmann AA, Overgaard J, Sørensen JG, Hallas R, Loeschcke V. Costs  
187 and benefits of cold acclimation in field-released *Drosophila*. *PNAS* 2008;105:216–21.  
188 doi:10.1073/pnas.0708074105.
- 189 [5] Guyot S, Pottier L, Hartmann A, Ragon M, Hauck Tiburski J, Molin P, et al. Extremely  
190 rapid acclimation of *Escherichia coli* to high temperature over a few generations of a fed-batch  
191 culture during slow warming. *MicrobiologyOpen* 2014;3:52–63. doi:10.1002/mbo3.146.
- 192 [6] Schou MF, Kristensen TN, Pedersen A, Karlsson BG, Loeschcke V, Malmendal A.  
193 Metabolic and functional characterization of effects of developmental temperature in *Drosophila*  
194 *melanogaster*. *Am J Physiol Regul Integr Comp Physiol* 2017;312:R211–22.  
195 doi:10.1152/ajpregu.00268.2016.
- 196 [7] Kingsolver JG, Arthur Woods H, Buckley LB, Potter KA, MacLean HJ, Higgins JK.  
197 Complex life cycles and the responses of insects to climate change. *Integr Comp Biol* 2011;51:719–  
198 32. doi:10.1093/icb/icr015.
- 199 [8] Marais E, Chown SL. Beneficial acclimation and the Bogert effect. *Ecol Lett* 2008;11:1027–  
200 36. doi:10.1111/j.1461-0248.2008.01213.x.
- 201 [9] Maness JD, Hutchison VH. Acute adjustment of thermal tolerance in vertebrate ectotherms  
202 following exposure to critical thermal maxima. *J Therm Biol* 1980;5:225–33. doi:10.1016/0306-  
203 4565(80)90026-1.
- 204 [10] Loeschcke V, Hoffmann AA. Consequences of heat hardening on a field fitness component  
205 in *Drosophila* depend on environmental temperature. *Am Nat* 2007;169:175–83.  
206 doi:10.1086/510632.
- 207 [11] Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M. Changes in membrane  
208 lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J Insect Physiol*  
209 2005;51:1173–82. doi:10.1016/j.jinsphys.2005.06.007.
- 210 [12] Alemu T, Alemneh T, Pertoldi C, Ambelu A, Bahrndorff S. Costs and benefits of heat and  
211 cold hardening in a soil arthropod. *Biol J Linn Soc* 2017;122:765–73.  
212 doi:10.1093/biolinnean/blx092.
- 213 [13] Kristensen TN, Henningsen AK, Aastrup C, Bech - Hansen M, Bjerre LBH, Carlsen B, et  
214 al. Fitness components of *Drosophila melanogaster* developed on a standard laboratory diet or a  
215 typical natural food source. *Insect Sci* 2016;23:771–9. doi:10.1111/1744-7917.12239.
- 216 [14] Malmendal A, Overgaard J, Bundy JG, Sørensen JG, Nielsen NC, Loeschcke V, et al.  
217 Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *Am J*  
218 *Physiol-Regul, Integr Comp Physiol* 2006;291:R205–12. doi:10.1152/ajpregu.00867.2005.

- 219 [15] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful  
220 approach to multiple testing. *J R Stat Soc Series B* 1995;57:289–300.
- 221 [16] Bahrndorff S, Gertsen S, Pertoldi C, Kristensen TN. Investigating thermal acclimation  
222 effects before and after a cold shock in *Drosophila melanogaster* using behavioural assays. *Biol J*  
223 *Linn Soc* 2016;117:241–51. doi:10.1111/bij.12659.
- 224 [17] Merkey AB, Wong CK, Hoshizaki DK, Gibbs AG. Energetics of metamorphosis in  
225 *Drosophila melanogaster*. *J Insect Physiol* 2011;57:1437–45. doi:10.1016/j.jinsphys.2011.07.013.
- 226 [18] Heerwaarden B van, Kellermann V, Sgrò CM. Limited scope for plasticity to increase upper  
227 thermal limits. *Funct Ecol* 2016;30:1947–56. doi:10.1111/1365-2435.12687.
- 228 [19] MacLean HJ, Kristensen TN, Overgaard J, Sørensen JG, Bahrndorff S. Acclimation  
229 responses to short-term temperature treatments during early life stages causes long lasting changes  
230 in spontaneous activity of adult *Drosophila melanogaster*. *Physiol Entomol* 2017;42:404–11.  
231 doi:10.1111/phen.12212.
- 232

233 **Table and Figure Legends**

234 Table 1. Results from the ANCOVA analysis testing heat resistance of hardened vs. non-hardened  
235 groups at different test temperatures throughout ontogeny. The table shows the  $F_{df}$  ratio and the p-  
236 values with  $p < 0.05$  in bold.

237 Fig.1. Fitted regression lines of the survival proportion of hardened (1h at 35 °C, dark blue line) vs.  
238 non-hardened (light blue line) *D. melanogaster* at different life stages from *larval* to adult after 1h  
239 exposure to 37, 38, 39, 40 or 41 °C. The dashed red line shows the basal thermal tolerance (average  
240 survival proportion of non-hardened flies across the test temperatures).

241 Table 1

	Test temperature (°C)									
	37		38		39		40		41	
	F <sub>1</sub>	p	F <sub>1</sub>	p	F <sub>1</sub>	p	F <sub>1</sub>	p	F <sub>1</sub>	p
<i>Larva 1</i>	5.27	0.06	26.63	< <b>0.0001</b>	75	< <b>0.0001</b>	80.68	< <b>0.0001</b>	59.33	< <b>0.0001</b>
<i>Larva 2</i>	7.29	<b>0.04</b>	16.81	<b>0.0003</b>	28.80	< <b>0.0001</b>	21.75	< <b>0.0001</b>	12.23	<b>0.002</b>
<i>Larva 3</i>	6.62	0.05	14.28	<b>0.0008</b>	23.04	< <b>0.0001</b>	16.48	<b>0.0001</b>	8.81	<b>0.006</b>
<i>Puparium</i>	6.46	0.05	3.30	0.28	78.39	< <b>0.0001</b>	159.80	< <b>0.0001</b>	162.97	< <b>0.0001</b>
<i>Pupa</i>	8.70	<b>0.02</b>	2.87	0.28	85.72	< <b>0.0001</b>	180.25	< <b>0.0001</b>	186.09	< <b>0.0001</b>
Adult 1	0.01	1.00	0.09	1.00	0.27	0.60	0.31	0.57	0.24	0.62
Adult 2	0.42	1.00	0.18	1.00	4.75	0.06	9.80	<b>0.003</b>	10.03	<b>0.005</b>

242

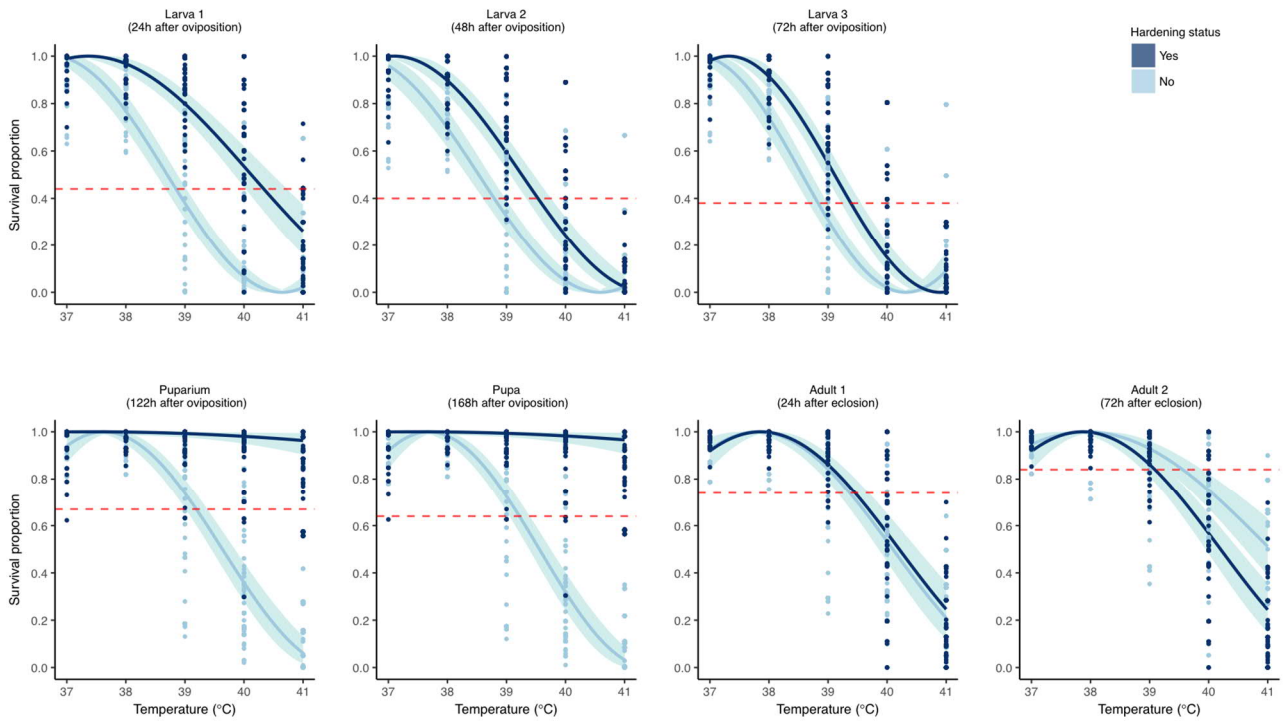


Fig. 1

Table S1. Tukey's post-hoc test results after false discovery rate correction to compare the heat resistance of hardened and non-hardened individuals at different life stages exposed to 25 °C compared to corresponding 37 °C test temperature. The table shows the sum of square (SS),  $F_{df}$  ratio and the p-values.

Temperature (°C)	Life stage	Hardening status	SS	$F_1$ ratio	p value
25 vs. 37	<i>Larvae 1</i>	Yes	0.6583	3.20	0.09
25 vs. 37	<i>Larvae 2</i>	Yes	0.0007	0.36	1
25 vs. 37	<i>Larvae 3</i>	Yes	0.0035	1.87	1
25 vs. 37	<i>Puparia</i>	Yes	0.0026	1.43	1
25 vs. 37	<i>Pupae</i>	Yes	0.0025	1.34	1
25 vs. 37	Adult 1	Yes	0.0000	0	1
25 vs. 37	Adult 2	Yes	0.0000	0	1
25 vs. 37	<i>Larvae 1</i>	No	0.0043	2.34	1
25 vs. 37	<i>Larvae 2</i>	No	0.7208	1.18	0.06
25 vs. 37	<i>Larvae 3</i>	No	0.0000	0	1
25 vs. 37	<i>Puparia</i>	No	0.0028	1.53	1
25 vs. 37	<i>Pupae</i>	No	0.1424	4.21	1
25 vs. 37	Adult 1	No	0.0000	0	1
25 vs. 37	Adult 2	No	0.0035	1.87	1

Table S2. Tukey’s post-hoc test results after false discovery rate (FDR) correction to compare the heat resistance of life stage at different test temperatures. The table shows the  $F_{df}$  ratio and the p-values with  $p < 0.05$  in bold.

		Test temperature (°C)									
		37		38		39		40		41	
		$F_1$	p	$F_1$	p	$F_1$	p	$F_1$	p	$F_1$	p
Non-hardened group	<i>Larva1 vs. Larva2</i>	1.61	1.00	3.53	1.00	1.26	0.84	2.06	1.00	0.15	0.91
	<i>Larva1 vs. Larva3</i>	0.10	1.00	1.61	1.00	6.52	0.66	1.51	0.30	6.91	0.34
	<i>Larva2 vs. Larva3</i>	3.58	1.00	3.84	1.00	0.65	1.00	3.84	0.64	3.58	0.34
	<i>Larva1 vs. Puparium</i>	5.56	<b>0.00</b>	29.45	<b>0.00</b>	47.28	<b>0.00</b>	33.66	<b>0.00</b>	7.23	<b>0.00</b>
	<i>Larva1 vs. Pupa</i>	1.11	<b>0.00</b>	31.52	<b>0.00</b>	43.53	<b>0.00</b>	26.63	<b>0.00</b>	1.67	<b>0.00</b>
	<i>Larva2 vs. Puparium</i>	2.00	<b>0.00</b>	44.25	<b>0.00</b>	63.37	<b>0.00</b>	40.29	<b>0.00</b>	2.10	<b>0.00</b>
	<i>Larva2 vs. Pupa</i>	27.43	<b>0.00</b>	46.78	<b>0.00</b>	59.01	<b>0.00</b>	32.55	<b>0.00</b>	1.93	<b>0.00</b>
	<i>Larva3 vs. Puparium</i>	6.30	<b>0.00</b>	34.90	<b>0.00</b>	68.33	<b>0.00</b>	57.62	<b>0.00</b>	35.55	<b>0.00</b>
	<i>Larva3 vs. Pupa</i>	1.13	<b>0.00</b>	37.15	<b>0.00</b>	63.81	<b>0.00</b>	48.29	<b>0.00</b>	27.20	<b>0.00</b>
	<i>Larva1 vs. Adult1</i>	2.30	<b>0.00</b>	43.91	<b>0.00</b>	85.61	<b>0.00</b>	71.96	<b>0.00</b>	44.28	<b>0.00</b>
	<i>Larva1 vs. Adult2</i>	5.27	<b>0.00</b>	53.98	<b>0.00</b>	139.89	<b>0.00</b>	143.21	<b>0.00</b>	101.92	<b>0.00</b>
	<i>Larva2 vs. Adult1</i>	26.38	<b>0.00</b>	61.65	<b>0.00</b>	106.85	<b>0.00</b>	81.51	<b>0.00</b>	46.24	<b>0.00</b>
	<i>Larva2 vs. Adult2</i>	6.42	<b>0.00</b>	73.49	<b>0.00</b>	166.72	<b>0.00</b>	156.56	<b>0.00</b>	104.87	<b>0.00</b>
	<i>Larva3 vs. Adult1</i>	2.49	<b>0.00</b>	50.52	<b>0.00</b>	113.27	<b>0.00</b>	105.51	<b>0.00</b>	70.25	<b>0.00</b>
	<i>Larva3 vs. Adult2</i>	6.25	<b>0.01</b>	61.29	<b>0.00</b>	174.71	<b>0.00</b>	189.22	<b>0.00</b>	139.77	<b>0.00</b>
	<i>Puparium vs. Pupa</i>	1.26	1.00	0.24	1.00	0.54	1.00	2.86	1.00	3.88	0.91
	<i>Puparium vs. Adult1</i>	0.73	1.00	3.09	1.00	4.70	0.09	1.59	<b>0.04</b>	6.13	0.08
	<i>Puparium vs. Adult2</i>	0.14	1.00	4.91	3.46	24.52	<b>0.00</b>	38.01	<b>0.00</b>	34.34	<b>0.00</b>
	<i>Pupa vs. Adult1</i>	0.07	1.00	0.22	1.00	0.63	0.06	0.74	<b>0.01</b>	0.60	<b>0.01</b>
	<i>Pupa vs. Adult2</i>	2.24	1.00	3.00	4.65	27.35	<b>0.00</b>	46.34	<b>0.00</b>	43.65	<b>0.00</b>
<i>Adult1 vs. Adult2</i>	1.52	1.00	3.61	1.00	4.63	0.06	1.47	<b>0.00</b>	6.28	<b>0.00</b>	
Hardened group	<i>Larva1 vs. Larva2</i>	4.61	1.00	2.04	0.14	2.65	<b>0.00</b>	27.05	<b>0.00</b>	4.04	<b>0.00</b>
	<i>Larva1 vs. Larva3</i>	1.28	1.00	0.40	0.36	32.07	<b>0.00</b>	52.41	<b>0.00</b>	48.56	<b>0.00</b>
	<i>Larva2 vs. Larva3</i>	3.84	1.00	1.69	1.00	6.22	1.00	1.24	1.46	3.39	0.10
	<i>Larva1 vs. Puparium</i>	6.77	1.00	3.23	0.14	62.74	<b>0.00</b>	108.14	<b>0.00</b>	102.69	<b>0.00</b>
	<i>Larva1 vs. Pupa</i>	6.18	1.00	6.06	0.12	64.62	<b>0.00</b>	110.51	<b>0.00</b>	104.56	<b>0.00</b>
	<i>Larva2 vs. Puparium</i>	0.21	1.00	25.37	<b>0.00</b>	159.71	<b>0.00</b>	243.37	<b>0.00</b>	218.03	<b>0.00</b>
	<i>Larva2 vs. Pupa</i>	0.12	1.00	26.18	<b>0.00</b>	162.70	<b>0.00</b>	246.91	<b>0.00</b>	220.75	<b>0.00</b>
	<i>Larva3 vs. Puparium</i>	2.01	1.00	5.33	<b>0.00</b>	184.52	<b>0.00</b>	311.11	<b>0.00</b>	292.47	<b>0.00</b>
	<i>Larva3 vs. Pupa</i>	6.18	1.00	3.47	<b>0.00</b>	187.74	<b>0.00</b>	315.12	<b>0.00</b>	295.63	<b>0.00</b>
	<i>Larva1 vs. Adult1</i>	6.01	0.40	1.08	0.22	3.01	2.81	2.25	1.00	0.27	1.00
<i>Larva1 vs. Adult2</i>	0.52	0.38	2.29	0.22	0.54	2.81	2.13	1.00	0.35	1.00	

<i>Larva2 vs. Adult1</i>	0.99	<b>0.04</b>	5.85	<b>0.00</b>	41.62	<b>0.00</b>	33.29	<b>0.00</b>	5.44	<b>0.00</b>
<i>Larva2 vs. Adult2</i>	2.92	<b>0.04</b>	1.49	<b>0.00</b>	41.81	<b>0.00</b>	33.12	<b>0.00</b>	3.66	<b>0.00</b>
<i>Larva3 vs. Adult1</i>	1.07	0.93	2.51	<b>0.00</b>	54.73	<b>0.00</b>	60.97	<b>0.00</b>	45.85	<b>0.00</b>
<i>Larva3 vs. Adult2</i>	2.21	0.91	4.79	<b>0.00</b>	54.94	<b>0.00</b>	60.74	<b>0.00</b>	45.46	<b>0.00</b>
<i>Puparium vs. Pupa</i>	0.00	1.00	0.01	1.00	0.10	1.00	0.09	1.00	0.01	1.00
<i>Puparium vs. Adult1</i>	1.53	<b>0.03</b>	0.51	1.00	38.27	<b>0.00</b>	96.64	<b>0.00</b>	106.72	<b>0.00</b>
<i>Puparium vs. Adult2</i>	3.57	<b>0.03</b>	0.38	1.00	38.09	<b>0.00</b>	96.92	<b>0.00</b>	107.32	<b>0.00</b>
<i>Pupa vs. Adult1</i>	6.50	<b>0.03</b>	0.85	1.00	39.74	<b>0.00</b>	98.87	<b>0.00</b>	108.63	<b>0.00</b>
<i>Pupae vs. Adult2</i>	1.61	<b>0.03</b>	0.68	1.00	39.56	<b>0.00</b>	99.16	<b>0.00</b>	109.24	<b>0.00</b>
<i>Adult1 vs. Adult2</i>	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00

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