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

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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

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

Materials and Methods

Population

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

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

Puparia and pupae: for both *puparial* and *pupal* stages, 15 eggs were collected into each of 180 35 mL vials containing 7 mL standard *Drosophila* medium. 96h after egg collection the vials were 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 ($25 \pm 1^\circ\text{C}$ 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 ($25 \pm 1^\circ\text{C}$ 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 ($\sim 97\%$ 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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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).

	Test temperature (°C)									
	37		38		39		40		41	
	F ₁	p	F ₁	p	F ₁	p	F ₁	p	F ₁	p
<i>Larva 1</i>	5.27	0.06	26.63	< 0.0001	75	< 0.0001	80.68	< 0.0001	59.33	< 0.0001
<i>Larva 2</i>	7.29	0.04	16.81	0.0003	28.80	< 0.0001	21.75	< 0.0001	12.23	0.002
<i>Larva 3</i>	6.62	0.05	14.28	0.0008	23.04	< 0.0001	16.48	0.0001	8.81	0.006
<i>Puparium</i>	6.46	0.05	3.30	0.28	78.39	< 0.0001	159.80	< 0.0001	162.97	< 0.0001
<i>Pupa</i>	8.70	0.02	2.87	0.28	85.72	< 0.0001	180.25	< 0.0001	186.09	< 0.0001
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	0.003	10.03	0.005

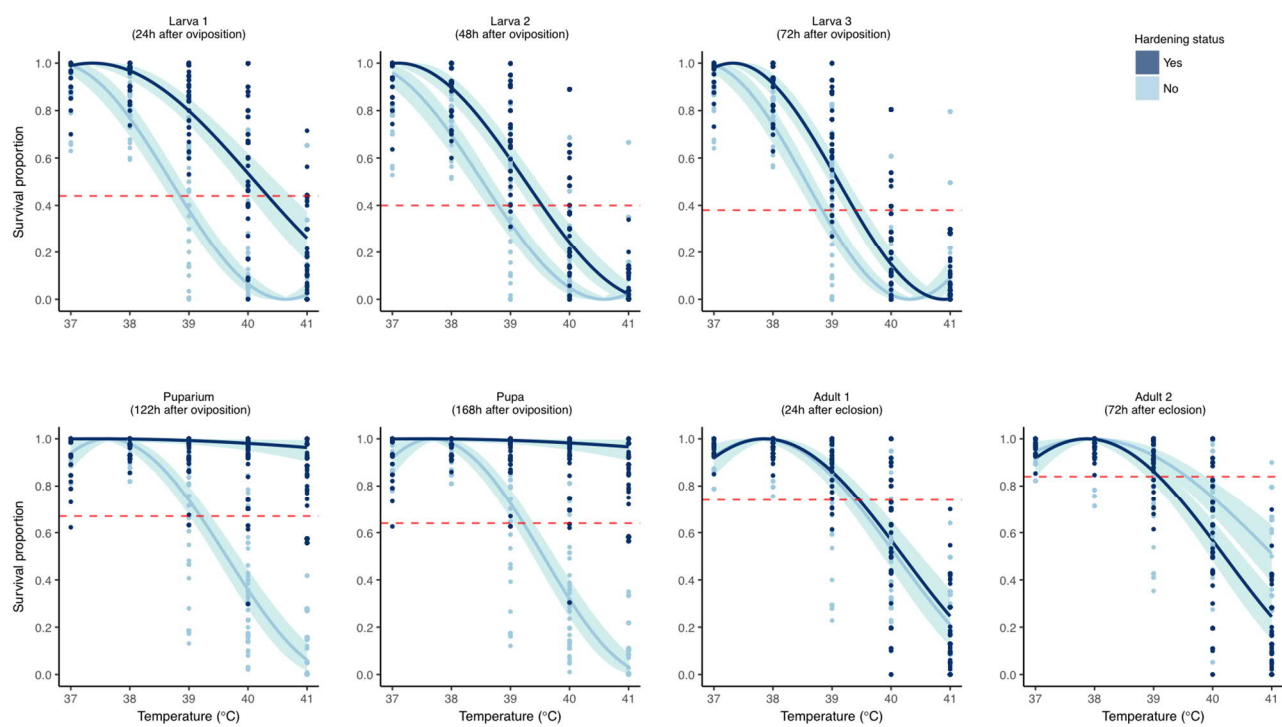


Fig. 1

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

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