Acclimation responses to short-term temperature treatments during early life stages causes long lasting changes in spontaneous activity of adult *Drosophila melanogaster*

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Abstract

Ecotherms adjust their physiology to environmental temperatures. Long-term exposures to heat or cold typically induce acclimation responses that generate directional, but reversible shifts in thermal tolerance and performance. However, less is known about how short exposure in different life stages will affect the adult phenotype. Here we compared the effects of long-term temperature exposure to 15, 19 and 31°C to that of brief (16 hour) exposure periods at the same temperatures in *Drosophila melanogaster* eggs, larvae, pupae, or adults, respectively. The acclimation responses were evaluated using activity measurements at 11, 15, 19, 27, 31, 33°C and measuring upper and lower thermal limits (CT_{max} and CT_{min}) in five-day-old adult males. As expected, long-term cold exposure reduced relative CT_{min} while long-term heat exposure increased relative CT_{max}. In contrast, we found little effect on thermal limits when using short-term exposures at different life stages. Long-term exposures to 31°C and 15°C both suppressed activity relative to the 19°C control suggesting that development at high and low temperatures may lead to reduced activity later in life. Short-term cold exposure early in development reduced activity in the adult stage while effects of short-term heat exposure on behaviour was dependent on life stage and test temperature. Together, our results highlight how the thermal sensitivity of the trait measured determines the ability to detect acclimation responses.

Introduction:

Temperature fluctuations pose a challenge for insects, that have limited ability to regulate their body temperature above and below environmental temperatures. Nevertheless, many insect species thrive in thermally heterogeneous environments where they use physiological and behavioural means to cope with changes in temperature (Cossins and Bowler, 1987; Hoffmann and Parsons, 1991). Such responses are often refered to as acclimation responses or phenotypic plasticity.

For insects with complex life-cycles, the length and ontogenetic timing of exposure to different temperatures may determine the potential cost and benefit of an induced plastic response (Hoffmann, Sørensen and Loeschcke, 2003; Bowler and Terblanche, 2008). For example, developmental (long-term) exposure to cold or hot temperatures has been shown to directionally affect survival under stressful thermal conditions later in life (Kristensen *et al.*, 2008; Colinet and Hoffmann, 2012). Studies have shown that ectotherms react to both long- and short-term exposure to cold (Kelty and Lee, 2001; Teets and Denlinger, 2013) and hot temperatures via acclimation within a specific life stage (Kingsolver *et al.*, 2011). Thus exposing e.g. young adults to low or high temperatures typically increases cold and heat resistance when assessed later in life. Moreover, individual life stages have different capacities for thermal acclimation (Loeschcke and Krebs, 1996; Slabber and Chown, 2005; Lee *et al.*, 2006; Powell and Bale, 2006; Jensen, Overgaard and Sørensen, 2007; Marais, Terblanche and Chown, 2009) and these differences can be linked to ecological (i.e. the specific microclimate experienced by that life stage) or behavioural factors (i.e. the ability for that life stage to behaviourally thermoregulate) (Mitchell et al., 2013; Potter et al., 2011, Loeschcke and Krebs 1996; Pincebourde and Casas 2015, Terblanche et al 2007).

59 Within life stages the cost and benefit of acclimation responses changes on a temporal
60 scale, it is highly trait specific and depends on the duration and intensity of the temperature
61 exposure. However, less is known about how, or if, cost and benefit of acclimation responses at
62 different life stages affect the performance and behaviour of adults. The reversibility of
63 acclimation responses has been shown to depend on a number of factors including the
64 temperature and length of exposure (Davison, 1971; Geister and Fischer, 2007; Weldon,
65 Terblanche and Chown, 2011; Allen, Clusella-Trullas and Chown, 2012). For example, in
66 *Drosophila melanogaster*, increased cold tolerance induced by long-term cold acclimation lasts
67 less than 24 hours when returned to standard rearing temperatures (25°C) whereas long-term heat
68 acclimation increases heat resistance for several days, even when transferred to lower
69 temperatures (15°C) suggesting that cold-acclimation is more labile (Slotsbo et al. 2016).
70 However, evidence from the hawkmoth, *Manduca sexta*, suggests the length of exposure and,
71 potentially, life stage during exposure to high temperature may also determine reversibly;
72 specifically, short-term exposure to high temperatures during the egg stage had little to no effect
73 on size or developmental rate in later life stages (Potter et al. 2011). Taken together, these data
74 demonstrate that acclimation to high and low temperatures have different consequences,
75 reversibility, and that the life stage during exposure may determine the degree of plastic
76 response.
77 Additionally, the ability to detect acclimation may depend on the traits and methods used
78 to assess both the immediate plastic response and the rate of its reversibility. For example,
79 behavioural traits, such as locomotor activity, flight or mating, exhibit greater thermal sensitivity
80 than critical thermal performance endpoints, including survival and small changes in temperature
81 can result in large differences in behaviour particularly when approaching the organism's

thermal limits (Patton and Krebs, 2001; Krebs and Thompson, 2006; Kjærsgaard *et al.*, 2015). As a result, spontaneous or voluntary locomotion has been used to detect potential benefits of long-term thermal acclimation in crickets (Lachenicht *et al.*, 2010) and fruit flies (Loeschcke and Hoffmann, 2007), as well as short-term heat acclimation in ants (Clusella-Trullas, Terblanche and Chown, 2010). These differences in behaviour can have direct fitness consequences, and are important when considering population dynamics generally (e.g. Berrigan and Partridge 1997).

In the present study, we used the model holometabolous insect *D. melanogaster* to investigate how short and long-term exposure to different temperature treatments during different life stages might alter adult thermal tolerance and performance. Specifically, we ask 1) can we induce any of the benefits of long-term acclimation on thermal tolerance with short-term acclimation during different life stages? and 2) can we draw similar conclusions about short- and long-term temperature exposures using thermal tolerance traits and thermal performance of spontaneous activity? To do this, we exposed flies to high, low or intermediate temperatures either through a long-term exposure for their whole life or through a short-term exposure consisting of 16 hour (h) at either the egg, larval, pupal, or adult life stage. With this design, we investigated how both short- and long-term acclimation affected the critical thermal limits (CT_{max} and CT_{min}) and the spontaneous activity (tested across six test temperatures: 11, 15, 19, 27, 31, and 33°C).

Material and Methods

Experimental design: We used a mass-bred population of *D. melanogaster* established from 25 isofemale lines collected from the Danish peninsula of Jutland in 2013. Prior to the experiment flies had been maintained at 19°C at a 12/12 light dark cycle on a standard *Drosophila* media

105 (agar, oatmeal, yeast, sugar) for approximately 70 generation. Experimental animals were
106 prepared by allowing parental flies to oviposit on food media. Eggs were picked within 12h of
107 being laid and were then transferred to 7mL vials at a density of approximately 40 eggs per vial.
108 Both the long-term and short-term temperature regimes are summarized in Figure 1. For the
109 long-term temperature treatment, vials were placed at 15, 19, or 31°C throughout development
110 such that the vials were kept at these constant temperature regimes until 1h before the flies were
111 tested. For experiments using short-term temperature exposure the flies were generally
112 maintained at 19°C but then transferred briefly (16h) to either 15 or 31°C. Short-term exposure
113 occurred at one of the following four stages, egg, 2^{nd} instar larvae, 24-hour-old pupae, or 3-day-
114 old adults. Upon emergence, two-day old adults were sexed briefly (less than 5 min) under CO_2
115 anaesthesia and then returned to 19°C for 3 days before tests of thermal tolerance (CT_{min} and
116 CT_{max}) or measurements of activity. To avoid complications from mating status and maturity,
117 only males were used for subsequent experiments.
118
119 *Thermal limits:* 20 adult males from each treatment were placed in individual 5 mL glass vials
120 and placed in a circulating water/glycol bath at 19°C, after which they were exposed to a slow
121 ramp down or up (rate of 0.1°C per minute) for assessment of critical thermal minimum (CT_{min})
122 or maximum (CT_{max}) respectively. The temperature at which all movement ceased was recorded
123 as the thermal limit for that individual.
124
125 *Activity*: Locomotor activity of flies was scored using a Drosophila Activity Monitor (DAM;
126 Trikinetics, Waltham, MA, USA). Single male flies were transferred to vials that were positioned
127 horizontally in the DAM. Each vial was 50 mm long and 0.5 mm wide and was centred

according to the light beam in the DAM. Vials were filled with fly medium at one end to provide food and were subsequently sealed with Parafilm. The other end of the vials was sealed with a 0.5-cm-long piece of pipe cleaner, soaked in water, to provide moisture. Each monitor was placed inside a temperature cabinet (Binder, Tuttlingen, Germany) set to one of six temperatures (11, 15, 19, 27, 31, or 33°C). Monitors were placed at ~10am and removed at ~8am the following day. Twenty-one flies were assessed for each short- and long-term acclimation treatment (in total eleven treatments) and at each of the six test temperatures. Total activity was tallied for the 12h period excluding the first 5 minutes of recording.

Statistical analyses: All statistical analyses were conducted in R (version 2.15.2).
For all measures of performance, we analysed short-term heat exposure, short-term cold exposure, and long-term exposure separately. For the thermal limits experiments, critical temperature was analysed in a linear model framework, with life stage at the time of exposure as the main effect in the model. For activity, we modelled the sum total of 12h of movement as a function of life stage at exposure and assay temperature. Since the relationship between activity and temperature is non-linear, we fit this model using a third order polynomial. For long-term exposure (development at 15,19, or 31°C), all three temperatures were analysed together.

Results:

Thermal Limits:

Long-term acclimation resulted in directional shift of CT_{min} (slope = 0.36 °C°C^{-1}, change in CT_{min} in degree Celsius per change in exposure temperature in degree Celsius, $P < 0.01$, Figure 2A) such that CT_{min} was reduced 0.36 °C per degree lowering of temperature exposure.

Acclimation also induced a shift in CT_{max} but this effect was less dramatic (slope = 0.05 °C°C^{-1}, $P < 0.01$, Figure 2C).

For short-term temperature exposure, life stage was ordered as a predictor variable and as such, a positive slope would indicate that short-term exposure closer to the focal life stage conferred a greater benefit. We see this in the short-term heat exposure where exposure to 31°C during the adult stage significantly increase $CT_{min,}$ (slope= 0.04, $P < 0.01$, light grey squares in Figure 2B). A similar but not significant response was seen with respect to CT_{max} (slope = 0.024, $P = 0.06$, light grey squares in Figure 2D). For short-term cold exposure, we see no significant change in slope indicating that short-term exposure to 15°C did not change CT_{min} or CT_{max} regardless of which life stage the treatment was given (CT_{min} slope= 0.01, $P = 0.49$ or CT_{max} slope= -0.003, $P = 0.75$, dark circles in Figure 2B&D).

Activity:

Long-term temperature exposure at each of three different constant temperatures significantly influences activity (Table 1, Figure 3). While all three treatments are significantly different from each other, long-term exposure to both 15°C and 31°C decreased overall activity relative to the 19°C control. Here we found that flies exposed to 31°C throughout development and at the adult life stage show the lowest activity, even at the highest temperatures.

The effects of short-term temperature exposure during development on adult activity depended on the temperature to which they were exposed. Short-term exposure to 15°C suppressed activity relative to activity of flies reared at 19°C throughout life ($P < 0.01$, Figure 4), particularly when exposed during the egg stage (slope = -0.95, $P < 0.01$, Figure 4A, Table 1). The effect of short-term exposure to 31°C had no significant effect on adult activity ($P = 0.17$,

Figure 4). However, the analyses revealed an interaction between life stage and assay temperature, such that short-term heat exposure at the egg, pupal and larval life stages increased relative activity at low temperatures and decreases it at high temperatures ($P < 0.05$, Table 1, Figure 4D).

Discussion:

In this study, we compared the effects of long-term and short-term temperature exposure on critical thermal limits and locomotor activity. While much attention has been paid to long-term developmental acclimation and to short-term acclimation processes during the adult life stage, the main focus of our study is on behaviour and thermal resistance in flies experiencing short-term exposures to hot or cold temperatures at different life stages. Unsurprisingly, long-term exposure to hot or cold temperatures directionally shifted critical thermal limits in-line with what has previously been reported in *Drosophila* (Hoffmann, Sørensen and Loeschcke, 2003; Sørensen, Kristensen and Overgaard, 2016). This is typically used as evidence for beneficial acclimation, particularly for organisms who live in seasonal environments (Levins, 1969; Prosser, 1986). However critical thermal maxima of the flies did not increase linearly across exposure temperatures, which could indicate that exposure to 31°C, is stressfully high. This is supported by Schou et al. (2017) who assessed thermal tolerances of *D. melanogaster* developed at temperatures spanning the full range that allowed successful development. They showed that flies that developed at very high or very low temperatures did not follow the linear expectation, i.e. they had lower CT_{max} and higher CT_{min} than expected based on linear predictions. However, since the thermal environment can change with the ecology and mobility of a given life stage it is important also to investigate short-term acclimation responses throughout ontogeny. Here we

found that short-term exposure to heat or cold, even in the adult stage, does not shift thermal tolerance limits. Only adult short-term heat exposure seems to have a small negative effect on adult CT_{min} suggesting a cost of acclimation rather than a benefit (Figure 2). However, the lack of benefit may be due to the 48 hours of recovery between the exposure and scoring of adult thermal performance, as others have shown that brief (hardening) exposures immediately prior to experimentation can have significantly positive effects (Loeschcke and Hoffmann, 2007; Arias *et al.*, 2012; Slotsbo *et al.*, 2016).

Functionally, the reversibility of short-term acclimation responses may account for the lack of an effect of short term exposures on adult thermal tolerance to heat or cold. Previous studies have indicated that long-term cold acclimation is more reversible (>24h) than long-term heat acclimation (ca. 5days) (Slotsbo *et al.*, 2016), suggesting that cold-acclimation is more rapidly reversed. In our study, short-term exposure of the adult life stage was given 2-3 days post-eclosion and assays were conducted following 48h of recovery so it is possible that any benefit of the cold treatment had been reversed in that time frame. Thus, we conclude that short-term exposures, regardless of life stage, does not confer lasting beneficial shift in thermal tolerance. Based on these results, our study suggests that short-term exposure to heat or cold during development has little effect on the adult life stage. However, behaviour can demonstrate greater thermal sensitivity than thermal limits (Kjærsgaard *et al.*, 2015), and therefore we also measured the thermal performance of a behavioural trait in the form of spontaneous movement.

Activity, as measured by number of spontaneous crossings in the activity monitor, revealed an effect of both long- and short-term exposures to hot or cold temperatures. While the effect of long-term exposure on critical thermal limits can be seen as evidence for beneficial acclimation, the effect on activity measurements show a more complicated story. In measurements of

stimulated locomotion, there is typically a positive linear relationship between temperature and activity (Angilletta, 2009). Here we saw a similar increase at low temperatures but also a reduction in activity between 27-31°C before we observed a sharp increase in spontaneous activity between 31-33°C. We hypothesize that the behavioural response between 27 and 31°C is indicative of an energetic conservation strategy and we speculate that temperatures above 31°C then elicit a form of escape behaviour. With long-term exposures, we expected to see horizontal shifts in thermal performance such that heat acclimated flies would be more active at high temperatures (right-shifted) and cold acclimated flies would perform better at low temperatures (left-shifted) as is typically seen in measurements of stimulated movement in other taxa (Huey and Kingsolver, 1989; Angilletta, 2009).We did see a shift in activity with long-term exposure, but this shift was in the vertical position of the performance curve such that long-term exposure to both heat and cold resulted in reduced activity across all six temperatures. While this was somewhat unexpected for stimulated movement, it is in accordance with other studies looking at spontaneous movement using DAMs, where flies that develop at intermediate temperatures showed higher behavioural activity across test temperatures (Zamudio, Huey and Crill, 1995; Gilbert, 2001; Kjærsgaard *et al.*, 2015; Bahrndorff *et al.*, 2016). One possible explanation for the lower activity could be that we measured locomotor activity as spontaneous movement and animals developed at temperatures above or below optimum may be more likely to conserve their energy.

We find that activity following short-term heat exposure was dependent on life stage and test temperature as evident from the significant interaction observed between assay temperature and life stage. Short-term cold acclimation in early life stages reduced the spontaneous activity of the emerging adults. Specifically, we see that short-term cold exposure in early life stages (egg and

larva) significantly lowered adult activity at all test temperatures relative to the 19°C control. Here we find evidence that there may be critical developmental windows where cold temperatures can change an aspect of the adult phenotype. These data might explain why the conclusions of field recapture studies can deviate from conclusions of laboratory activity studies (see e.g. Kristensen et al. 2008; Bahrndorff et al. 2016). Moreover, this study highlights the importance of measuring traits with different thermal sensitivities. While spontaneous movement in the lab can be difficult to interpret in a traditional context, these data provide clear evidence that by looking at both traditional measures of thermal acclimation, and highly sensitive traits, like spontaneous activity, researchers will gain more information about the effects of temperature on adult fitness components.

In conclusion, we find evidence that long-term cold and heat exposure respectively reduced CT_{min} and slightly increased CT_{max}. In contrast, short-term exposures to cold or hot temperatures at one life stages had little effect on thermal limits at a different life stage. However long-term exposures to heat and cold both suppressed activity suggesting that development above or below an optimal temperature might have long-lasting behavioural consequences. Short-term cold exposure at one life stages led to reduced activity in the emerging adults. Thus, benefits of acclimation are dependent on the life stage and duration of exposure and interestingly behavioural assays might be more sensitive in detecting responses to short term thermal changes than traditionally used measures of thermal tolerances (summarized in graphical abstract) . These results are interesting in relation to thermal acclimation currently being investigated in applied insect biology. For example, acclimation to low or high temperatures may impact the efficiency of biological control agents used in integrated pest management (Sørensen, Toft and Kristensen, 2013). While long-term exposure to high or low temperatures may facilitate beneficial

acclimation when insects are released in greenhouses or on open land, this could be a time-intensive and costly change to apply to mass-rearing. If short-term acclimation at adult or juvenile stages could furnish similar benefits, such treatments might be a useful and more feasible tool in rearing of insects for pest management. Here we see no benefit of short-term acclimation on thermal limits but reveal a critical developmental window for thermally sensitive traits, in this case activity, for cold treatments. Further research is needed to investigate potential mechanisms and applications.

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Table 1: For cumulative activity over 12h, we analysed short-term heat exposure, short-term cold exposure, and long-term exposure separately. Using a linear model, we tested the total of movement as a function of life stage at exposure and assay temperature. Since the relationship between activity and temperature is non-linear, we fit this model using a third order polynomial. For long-term exposure (development at 15,19, or 31°C), all three temperatures were analysed together.

Table 1:

	Short-term cold exposure (15°C)			**Short-term heat exposure (31°C)**	
	DF	*F-value*	*P-value*	*F-value*	*P-value*
Stage of exposure	4	4.972	< 0.001	1.623	0.167
Assay Temperature	1	106.398	< 0.001	70.419	< 0.001
Assay Temperature ^2	1	0.271	0.603	2.222	0.136
Assay Temperature ^3	1	59.454	< 0.001	128.780	< 0.001
Stage:Assay Temperature	4	2.349	0.053	2.408	0.048

	Long-term exposure		
	DF	*F-value*	*P-value*
Exposure Temperature	1	16.487	< 0.001
Assay Temperature	1	25.264	< 0.001
Assay Temperature ^2	1	0.197	0.657
Assay Temperature ^3	1	12.581	< 0.001
Exposure Temp:Assay Temp	1	2.674	0.103

Figure 1: Experimental design for long-term and short-term exposure. Long-term exposure flies were reared from egg to adult at their exposure temperature. Short-term exposure flies were taken from 19°C at a designated life stage and exposed to 16h at 15 or 31°C and then returned to 19C for the rest of development. Experiments were performed on 5-6 day old adults.

Figure 2: Mean (±SE) critical thermal minimum, CTmin (A and B) and maximum, CTmax (C and D) for 5-days-old adult male flies of *Drosophila melanogaster* fitted with a linear regression line. On the x-axes, long-term exposure temperatures are presented in A and C and age at the time of short-term exposure are presented in B and D with the mean of the 19°C control flies represented as a dashed grey line.

Figure 3: Effect of long-term exposure on the mean (±SE) number of crossings in *Drosophila* activity monitors (DAM) over 12h for 5-day old adult male *Drosophila melanogaster*. Shown here, flies given long-term heat exposure (31°C, light grey squares), long-term cold exposure (15°C, dark grey circles) and the 19°C control (black triangle).

Figure 4: Effect of short-term exposure on the proportional difference in mean (±SE) number of crossings in DAM over 12h for 5-day old adult male *Drosophila melanogaster* compared to the 19°C control (dashed grey line). Short-term cold exposure (15°C, dark grey circles) and short-term heat exposure (31°C, light grey squares) are plotted by life stage during exposure; egg (A), larva (B), pupa (C), and adult (D).

Figure S1: Effect of short-term exposure on the mean (±SE) number of crossings in *Drosophila* activity monitors (DAM) over 12h for 5-day old adult male *Drosophila melanogaster*. Dark blue and red represent the activity of flies given short-term cold or heat exposures respectively. These are compared with in each plot to flies given long-term cold (15°C, light blue line), long-term heat exposure (31°C, pink line) and the 19°C control (grey line).

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