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RESEARCH ARTICLE

Reversibility of developmental heat and cold plasticity is asymmetric and has long-lasting consequences for adult thermal tolerance

Stine Slotsbo^{1,*‡}, Mads F. Schou^{1,*}, Torsten N. Kristensen², Volker Loeschcke¹ and Jesper G. Sørensen¹

ABSTRACT

The ability of insects to cope with stressful temperatures through adaptive plasticity has allowed them to thrive under a wide range of thermal conditions. Developmental plasticity is generally considered to be a non-reversible phenotypic change, e.g. in morphological traits, while adult acclimation responses are often considered to be reversible physiological responses. However, physiologically mediated thermal acclimation might not follow this general prediction. We investigated the magnitude and rate of reversibility of developmental thermal plasticity responses in heat and cold tolerance of adult flies, using a full factorial design with two developmental and two adult temperatures (15 and 25°C). We show that cold tolerance attained during development is readily adjusted to the prevailing conditions during adult acclimation, with a symmetric rate of decrease or increase. In contrast, heat tolerance is only partly reversible during acclimation and is thus constrained by the temperature during development. The effect of adult acclimation on heat tolerance was asymmetrical, with a general loss of heat tolerance with age. Surprisingly, the decline in adult heat tolerance at 25°C was decelerated in flies developed at low temperatures. This result was supported by correlated responses in two senescence-associated traits and in accordance with a lower rate of ageing after low temperature development, suggesting that physiological age is not reset at eclosion. The results have profound ecological consequences for populations, as optimal developmental temperatures will be dependent on the thermal conditions faced in the adult stage and the age at which they occur.

KEY WORDS: Longevity, Climate change, Heat tolerance, Cold tolerance, Phenotypic plasticity, *Drosophila*

INTRODUCTION

In insects, temperature is a key environmental factor, influencing almost all ecological and evolutionary processes (Addo-Bediako et al., 2000; Chown and Terblanche, 2007). Phenotypic plasticity enables many ectotherms to continuously adjust their thermal tolerance to the prevailing conditions and is therefore central for their distribution and abundance (Angilletta, 2009; Hoffmann et al., 2003; Van Dooremalen et al., 2013). The fitness benefits of plasticity are dependent on trait- and environment-specific costs and

constraints at various timescales, and might bridge life stages and even generations (reviewed by Sgrò et al., 2016). Generally, cues from the developmental environment will lead to adjustments of the phenotype that are mostly irreversibly in the adult life stage (developmental plasticity). Conversely, physiological adjustments based on cues received during the adult stage are generally assumed to lead to continuously adjusted phenotypes and may also change over the course of a lifetime (adult acclimation) (Piersma and Drent, 2003).

Several studies of thermal tolerance in ectotherms have investigated developmental plasticity (Gibert and Huey, 2001), adult acclimation (Allen et al., 2012; Davison, 1971; Lyons et al., 2012) or the combination of the two (Colinet and Hoffmann, 2012a; Maynard Smith, 1957; Terblanche and Chown, 2006). Across life stages, developmental plasticity and adult acclimation might interact in two different ways. First, the environment experienced during development might affect adult acclimation responses, including the capacity and rate of acclimation (Beaman et al., 2016), and second, some developmentally induced phenotypic adjustments might be partly or fully reversible during adult thermal acclimation, as is the case for egg size in butterflies (Fischer et al., 2003).

There are few studies investigating to what extent developmental plasticity for thermal tolerance is reversible in the adult stage of insects (Fischer et al., 2003). Given the adaptive significance of a continuous alignment of the phenotype to the environment as well as the associated costs, an understanding of the detailed adult acclimation response (capacity and rate of acclimation) and its dependence on the developmental environment has received surprisingly little attention. Most noteworthy is the work on adult cold and heat acclimation in butterflies developed at different temperatures (Fischer et al., 2010; Geister and Fischer, 2007; Zeilstra and Fischer, 2005).

In *D. melanogaster*, as well as in other insects, low temperature acclimation improves cold tolerance, but with a trade-off in heat tolerance and vice versa (Allen et al., 2012; Chidawanyika and Terblanche, 2011; Hoffmann et al., 2003). The trade-off between cold and heat tolerance induced by developmental plasticity or adult acclimation seems not to rely on a genetic correlation between these traits (Hoffmann et al., 2003) and is not found in all species (Nyamukondiwa et al., 2013; Sørensen et al., 2015). If the trade-off is a consequence of a mechanistic or physiological link, it may drastically influence the ability of ectotherms to attain adaptive phenotypes for cold and heat tolerance in temporally changing thermal environments. However, the time course of acclimation and acclimation reversibility for high and low temperature tolerance is rarely followed simultaneously across life stages and longer time spans (but see Terblanche et al., 2006; Weldon et al., 2011).

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The aim of the present study was to investigate the reversibility of developmental plasticity in the adult stages of *D. melanogaster*. We assayed critical thermal maximum (CT_{max}) and critical thermal minimum (CT_{min}) (Gibert et al., 2001; MacMillan and Sinclair, 2011) as ecologically relevant proxies for heat and cold tolerance in drosophilids (Andersen et al., 2015; Terblanche et al., 2011). We applied a full factorial design with developmental plasticity and adult acclimation at either 15 or 25°C and measured the progression in high and low temperature tolerance during the first day after relocation of young flies and every second day for the subsequent 23 days. To investigate potential age effects, we measured correlated responses in longevity and locomotor activity in all treatment groups (Bowler and Terblanche, 2008).

MATERIALS AND METHODS

Animals

Drosophila melanogaster used in this study came from a mass-bred laboratory population established in 2010 from 589 wild-caught females from Denmark (Schou et al., 2014). The laboratory population was maintained in the laboratory at an approximate population size of 750 individuals in five bottles with 35 ml medium on 12 h:12 h light:dark cycles at 20°C for approximately 65 generations prior to the experiment. The medium used for rearing of the laboratory population and throughout this experiment was a standard oatmeal–sugar–yeast–agar *Drosophila* medium.

Thermal acclimation

Adult flies from the laboratory population were allowed to lay eggs for 12 h. Deposited eggs were transferred in batches of 40 eggs per 7 ml food vial, such that larval density during development was controlled. Vials with eggs were placed at either constant 15°C or constant 25°C. The eggs placed at 25°C were collected 19 days later than the eggs placed at 15°C, ensuring a synchronized emergence of flies from the two temperatures such that the maximum age difference between experimental flies was less than 1 day. All experiments were performed on males to reduce potential confounding effects of reproductive status. On the day of emergence, male flies were placed in fresh vials (25 flies per vial) and female flies were discarded. Less than 24 h after emergence, the male flies were relocated to their adult temperature regime, thus generating four combinations of developmental and adult temperatures (developmental temperature/adult temperature: 15/15°C, 15/25°C, 25/25°C and 25/15°C). The flies were provided with fresh food vials every second day. Even if some mortality occurred during the acclimation experiment, there was no noticeable difference within adult acclimation treatments and any bias would thus have a negligible effect on our overall results.

Heat and cold tolerance

Heat and cold tolerance were measured using the metrics CT_{max} and CT_{min} , respectively. Individual flies were continuously monitored and CT_{max} and CT_{min} were assigned as the temperature at which all capacity for movement ceased (Gibert et al., 2001; MacMillan and Sinclair, 2011).

Critical thermal limits of flies from the four treatments were investigated in both a short- and a long-term study. The two studies were performed in different generations, but were in all other aspects performed identically unless otherwise mentioned. In the short-term study, we measured CT_{max} and CT_{min} at 3, 6, 12 and 24 h after relocating the flies to their adult acclimation temperatures. After assaying thermal limits, the sex of the assayed flies was verified. In the long-term study, we measured CT_{max} and

CT_{min} every second day, from when the flies were 2–3 days old until they were 24–25 days old, resulting in a total of 12 time points. Separation of sexes prior to assessment of thermal limits in *Drosophila* is usually done while flies are anaesthetized with CO₂. As studies of the effects of CO₂ anaesthesia have shown physiological and phenotypic effects up until 24 h after exposure (Colinet and Renault, 2012b; Nilson et al., 2006), the consensus across *Drosophila* studies is to allow flies to recover for 2 days prior to testing, to ensure they are fully recovered. In accordance with this practice, all flies used for the long-term study were anaesthetized and separated shortly after emergence before transfer to the adult acclimation temperature and therefore given a minimum of 2 days to recover before the tolerance assessment. Conversely, flies for the short-term experiment were separated without anaesthesia, as the effects of CO₂ anaesthesia would have been a confounding factor in this 24 h experiment.

Both the starting temperature and the rate of temperature change affect the estimate of critical thermal limits (Terblanche et al., 2007). In consequence, we used the same starting temperature and absolute rate of temperature change for all assessments. For each tolerance assessment, 20 male flies from each treatment were transferred individually to small screw-top glass vials (5 ml) without the use of anaesthesia. The transfer was done at 20°C and lasted no more than 20 min. Vials were randomly placed in a rack and submerged in a temperature-controlled water tank set to 20°C. The water in the tank was continuously stirred by a pump to ensure homogeneity of the water temperature. For CT_{max} , the temperature was gradually increased at a rate of 0.1°C min⁻¹ and the flies were continuously monitored to register the temperature at which they lost the ability to move any body part. When assessing CT_{min} , ethylene glycol was added to the water tank (1:1 v/v) to avoid freezing and the temperature was decreased at a rate of 0.1°C min⁻¹.

Ageing effects of thermal acclimation

Negative geotaxis is an innate escape response in which flies climb the wall of a container after being tapped to its bottom, and was used in this study as a proxy for potential physiological ageing effects (Gargano et al., 2005). Negative geotaxis was measured on male flies at age 9 and 23 days for the four different acclimation treatments, and additionally at age 72 days for flies maintained at 15°C as adults. Measurements were performed at 20°C, and all flies were acclimated to this temperature for 10 min prior to testing. Five adults were placed in a clean standard shell vial (25×95 mm) and tapped to its bottom. The median vertical climbing length of the flies was determined from a picture taken 3 s after knockdown. This was repeated five times for each vial. We measured 10 vials for each of the temperature treatments. All the measurements were performed in five blocks, using a rack containing eight vials per block (two vials with flies from each of the four treatments randomly placed in the rack). The protocol was a modification of the rapid iterative negative geotaxis (RING) assay described by Gargano et al. (2005) and Nichols et al. (2012).

In addition to negative geotaxis, we estimated longevity of male flies from the four acclimation treatments. Longevity was measured as the time from emergence to the day of death. At the time of relocation between developmental and adult acclimation temperatures, 10 males were placed in each of 10 fresh food vials per treatment. Fresh food vials were provided and the number of deaths was counted every second and every fourth day for flies maintained at 25 and 15°C, respectively.

Table 1. Analysis of CT_{min} and CT_{max}

Source	Short-term CT_{min}			Long-term CT_{min}			Short-term CT_{max}			Long-term CT_{max}		
	Estimate	$F_{d.f.}$	P	Estimate	$F_{d.f.}$	P	Estimate ($\times 10^{15}$)	$F_{d.f.}$	P	Estimate ($\times 10^{15}$)	$F_{d.f.}$	P
Intercept	2.99±0.10	–	–	1.80±0.07	–	–	90.1±12.9	–	–	128.9±12.8	–	–
Time	0.01±0.01	–	–	–0.02±0.00	–	–	–0.04±0.99	–	–	–1.8±0.8	–	–
Adult	0.08±0.14	–	–	2.07±0.09	–	–	–2.9±16.2	–	–	77.6±18.2	–	–
Dev	3.59±0.14	–	–	2.35±0.09	–	–	220.1±16.1	–	–	243.3±18.3	–	–
Adult×time	0.05±0.01	–	–	0.09±0.01	276.11 _{1,850}	<0.001	3.0±0.9	9.19 _{1,304}	0.003	–2.1±1.2	–	–
Dev×time	–0.05±0.01	–	–	–0.06±0.01	118.42 _{1,850}	<0.001	–2.9±0.9	9.03 _{1,304}	0.003	–7.1±1.2	–	–
Dev×adult	0.07±0.20	–	–	–0.35±0.08	20.11 _{1,850}	<0.001	–39.7±16.0	6.18 _{1,304}	0.013	–27.8±25.6	–	–
Dev×adult×time	–0.03±0.01	4.22 _{1,293}	0.041	–	0.48 _{1,849}	0.481	–	1.51 _{1,303}	0.221	–3.9±1.7	5.53 _{1,776}	0.019

Estimates are presented \pm s.e.m.

All four combinations of developmental plasticity (Dev) and adult acclimation (Adult) at either 15 or 25°C were investigated using general linear models. We used sequential model reduction to find the minimal adequate model, such that model reductions were halted in the case of significant interactions. Time is the effect of progressively longer time at the adult acclimation regimes, measured in hours (short term, 0–24 h) or days (long term, 2–24 days) (see Figs 1 and 2). All model coefficients are at 25°C and thus the deviation is from 15°C. Note that CT_{max} data were transformed such that the coefficients are not directly transferable to changes in the CT_{max} .

Statistical analyses

The temperature tolerance data (CT_{max} and CT_{min}) were analysed separately for the short-term study (3–24 h after relocation) and the long-term study (2–24 days after relocation). CT_{max} and CT_{min} were analysed by general linear models containing a three-way interaction between developmental acclimation regime, adult acclimation regime and time (day or hour). We performed sequential model reduction and model comparisons with F -tests to find the minimal adequate model (Crawley, 2013). In the case of a significant interaction, model reduction was halted for the involved predictor variables. All models of CT_{min} fulfilled assumptions of homogeneity of variances and normality of residuals. CT_{max} from both studies was anti-log transformed to fulfil the assumption of normality of residuals. However, the full model of CT_{max} from the long-term study still showed some deviance from normality of residuals, which was driven by a small group of outliers, all originating from adult acclimation at 25°C at day 6. We removed all data from day 6, which did not change the conclusions of the analysis, but improved the normality of residuals as well as the model fit. To assist the interpretation of the statistical output of the complex models, we performed pairwise comparisons of rates of change in thermal tolerance over time (slopes). This was done by assessing the significance of the interaction between time and treatment, for each possible treatment pair using the model framework described above. These analyses were done in the standard R package ‘stats’ (<http://www.R-project.org>).

To investigate whether negative geotaxis changed as the flies aged, we performed two separate analyses, one for flies experiencing an adult acclimation temperature of 15°C and one for flies experiencing

an adult acclimation temperature of 25°C. We found this approach appropriate as a consequence of the very different rates of ageing affecting longevity at 15 and 25°C. Each of two datasets was analysed using general linear mixed models in the R package ‘lme4’ (v.1.1-5; <https://cran.r-project.org/web/packages/lme4/index.html>), with the fixed effects developmental acclimation regime and age. Position of the vial in the rack, picture number and assay block were incorporated as random effects. We performed sequential model reduction and model comparisons using maximum likelihood ratio tests. Because of zero being a lower limit, the assumption of homogeneity of variances was not fulfilled in the 25°C subset (where average values got close to zero). The data were square-root transformed, which improved homogeneity of variances.

Longevity data were analysed with the non-parametric Cox proportional hazards (Crawley, 2013) in the R package ‘survival’, without censoring (<https://cran.r-project.org/web/packages/survival/index.html>). With this approach, we used likelihood ratio tests to compare median longevity between developmental acclimation temperatures for flies reared at the same adult acclimation temperature. Models were performed separately for each adult acclimation temperature. All statistical analyses were completed in R (<http://www.R-project.org>).

RESULTS

Cold tolerance

Low temperature development induced strong benefits to low temperature tolerance, giving adult flies developed at 15°C a 4°C lower CT_{min} than flies developed at 25°C (see Fig. 1; see Tables S1 and S2 for raw data). The analysis of the short-term CT_{min} study

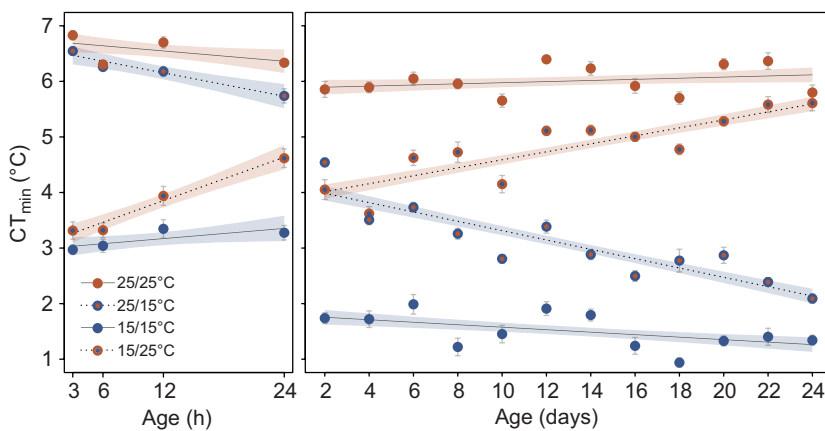


Fig. 1. Short-term and long-term critical thermal minimum (CT_{min}) of individual *Drosophila melanogaster* at the four combinations of developmental and adult acclimation. Mean \pm s.e.m. values are presented ($N=20$). The shaded area represents the 95% confidence interval (CI) of the fit performed in the statistical analysis of the data. The short-term measurements (left) ranged from 3 to 24 h, while the long-term measurements (right) ranged from 2 to 24 days.

Table 2. Pairwise comparisons of slopes

Pairwise comparison (°C)	Short-term CT _{min}		Long-term CT _{min}		Short-term CT _{max}		Long-term CT _{max}	
	F _{d.f.}	P	F _{d.f.}	P	F _{d.f.}	P	F _{d.f.}	P
25/25°C vs 25/15°C	6.15 _{1,149}	0.014	142.62 _{1,424}	<0.001	1.41 _{1,154}	0.238	19.06 _{1,387}	<0.001
25/25°C vs 15/25°C	53.21 _{1,144}	<0.001	66.49 _{1,424}	<0.001	8.18 _{1,152}	0.005	67.32 _{1,387}	<0.001
25/25°C vs 15/15°C	9.80 _{1,148}	0.002	16.61 _{1,421}	<0.001	0.00 _{1,151}	0.965	123.35 _{1,386}	<0.001
25/15°C vs 15/25°C	82.86 _{1,145}	<0.001	373.87 _{1,428}	<0.001	15.77 _{1,152}	<0.001	17.39 _{1,390}	<0.001
25/15°C vs 15/15°C	26.52 _{1,149}	<0.001	52.24 _{1,425}	<0.001	1.73 _{1,151}	0.190	48.36 _{1,389}	<0.001
15/25°C vs 15/15°C	15.61 _{1,144}	<0.001	134.76 _{1,425}	<0.001	11.49 _{1,149}	<0.001	4.46 _{1,389}	0.035

As a supplement to the full models presented in Table 1, we performed pairwise comparisons of slopes to ascertain statistically significant differences in slopes.

showed a significant three-way interaction between developmental plasticity, adult acclimation and time (hours) (Table 1), showing that the difference in the rate of acclimation between the two adult acclimation treatments is dependent on the developmental treatment. Pairwise comparisons between slopes showed distinct rates of acclimation in each treatment group (Table 2). While CT_{min} for flies not exposed to any thermal relocation remained largely constant, CT_{min} for flies developed at 25°C and moved to 15°C decreased slightly, while CT_{min} for flies developed at 15°C and moved to 25°C increased more than 1°C within the first 24 h of relocation.

In the long-term CT_{min} study, the three-way interaction between developmental plasticity, adult acclimation and time (days) was not significant, but all two-way interactions were highly significant (Table 1). The rate of change in adult CT_{min} is thus dependent on both developmental plasticity and adult acclimation regime. However, the rate at which the two 25°C adult acclimation treatments approached each other is the same as the rate at which flies from the two 15°C adult acclimation treatments approached each other (as indicated by the lack of a three-way interaction; Fig. 1). In other words, the relative interaction between developmental plasticity and adult acclimation does not change with time. Pairwise comparisons between slopes showed distinct rates of acclimation in each treatment group (Table 2). Overall, the increased cold tolerance achieved by developmental plasticity was almost completely reversible over the course of 23 days.

Heat tolerance

Developmental plasticity led to an approximately 1°C increase in CT_{max} (Fig. 2; see Tables S1 and S2 for raw data). For short-term CT_{max}, the three-way interaction between developmental plasticity, adult acclimation and time (hours) was not significant, but all two-way interactions were significant (Table 1, Fig. 2). No short-term acclimation response was observed for CT_{max} for 15/15°C and 25/25°C flies, as these retained a constant CT_{max} within the first 24 h.

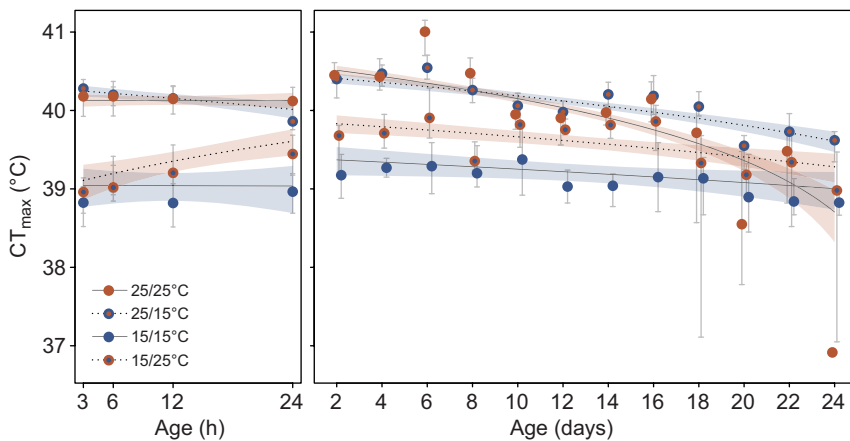


Fig. 2. Short-term and long-term critical thermal maximum (CT_{max}) of individual *D. melanogaster* at the four combinations of developmental and adult acclimation. Median values are presented \pm 95% CI, obtained by bootstrapping ($N=20$). As CT_{max} data were negatively skewed, also illustrated by the necessity for an anti-log transformation, we have chosen to represent data as medians to avoid giving too much weight to the first few flies succumbing to the heat. The shaded area represents the 95% CI of the back-transformed fit performed in the statistical analysis of the data. The short-term measurements (left) ranged from 3 to 24 h, while the long-term measurements (right) ranged from 2 to 24 days. The error bars of the 25°C treatment on day 24 were omitted to improve the resolution of the y-axis (lower and upper limits were 35.1 and 38.8°C, respectively).

Table 3. Analysis of negative geotaxis in temperature-acclimated flies

Adult acclimation	Source	Estimate	χ^2 d.f.	P-value
25°C	Intercept	4.76±0.32	–	–
	Age	−0.034±0.005	–	–
	Dev	1.31±0.31	–	–
	Dev×age	−0.015±0.007	4.53 ₁	0.033
15°C	Intercept	4.52±0.38	–	–
	Age	−0.17±0.02	–	–
	Dev	2.19±0.46	–	–
	Dev×age	−0.092±0.027	6.73 ₁	0.009

Estimates are presented ±s.e.m.

Developmental acclimation (Dev) was at either 15 or 25°C. Age refers to the progressing age of flies (see Fig. 3). We used sequential model reduction to find the minimal adequate model, such that model reductions were halted in the case of significant interactions. All model coefficients are at 25°C and thus the deviation from 15°C.

The longevity of flies was influenced by adult acclimation but also by the developmental plasticity regime. Flies from the 25/25°C treatment (median=26 days, $N=99$), had a significantly lower longevity compared with that of flies from the 15/25°C treatment (median=30 days, $N=97$; $\chi^2_1=3.87$, $P<0.05$). No statistically significant difference was observed between flies acclimated at 15°C as adults (25/15°C: median=54 days, $N=94$; 15/15°C: median=48 days, $N=81$; $\chi^2_1=0.26$, $P=0.61$).

DISCUSSION

The analyses of the thermal acclimation effects on upper and lower critical thermal limits produced in all cases significant three-way or two-way interactions, which may challenge the biological interpretation of the results. Our interpretation of these complex interactions is therefore supported by more simple pairwise comparisons of the change of thermal limits over time.

Development at 15°C led to more cold-tolerant flies than development at 25°C. Such beneficial acclimation to low temperatures has previously been found in both larvae and adults of *D. melanogaster* (Kostal et al., 2011; Overgaard et al., 2008) and in other insects (e.g. Terblanche and Chown, 2006). Previous studies on other insects found that complete adult acclimation is reached or developmental plasticity is reversed within a few days (Allen et al., 2012; Geister and Fischer, 2007; Zeilstra and Fischer, 2005). Here, we found developmental plasticity of cold tolerance to be readily, but incompletely, reversible across the first 24 h of adult acclimation. The reversibility continued at a decelerated rate following 2 days of adult acclimation and was incomplete after 24 days of adult acclimation (see also Terblanche and Chown, 2006). The trajectory of the observed changes, however, suggests that the developmental acclimation in cold tolerance is fully reversible.

In accordance with earlier studies in drosophilids, acclimation at 25°C increased heat tolerance compared with acclimation at 15°C, as measured by CT_{max} (Hoffmann et al., 2003; but see Gunderson and Stillman, 2015, for a comprehensive analysis of thermal plasticity responses across ectotherms). The increased heat tolerance induced during developmental acclimation at 25°C was not lost when adult flies were acclimated at 15°C. However, in the reverse treatment combination, young adults that were acclimated at 15°C during development quickly increased their heat tolerance when moved to 25°C. Thus, the response was asymmetrical, as CT_{max} was reversible (in the short term) for developmental acclimation at 15°C, but not at 25°C. This asymmetric response can only be investigated by a combination of several developmental

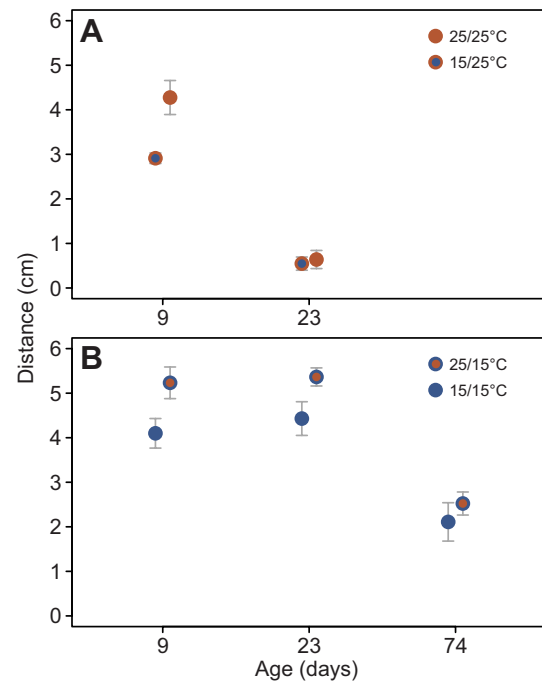


Fig. 3. The effect of age on negative geotaxis of *D. melanogaster* at the four combinations of developmental and adult acclimation. Flies were acclimated as adults to 25°C (A) or 15°C (B), after development at either 25 or 15°C. Negative geotaxis was measured as the vertical distance crawled (in cm). Data are presented as means±s.e.m. The mean climbing length was determined by measuring the median vertical climbing length of five flies in a vial; this was repeated five times for each of the 10 vials per treatment. Flies reared as adults at 15°C were tested at ages 9, 23 and 74 days, while flies reared as adults at 25°C were only tested at ages 9 and 23 days because of their shorter longevity. The rate of decrease of negative geotaxis with age was higher in flies developing at 25°C than in flies developing at 15°C, irrespective of the adult acclimation temperature at which they were compared.

and adult regimes, and by high resolution of the adult acclimation through time. Although other studies have used such a design, most often adults are not followed through a significant proportion of the adult life span as in this study (Geister and Fischer, 2007; Zeilstra and Fischer, 2005). The asymmetric response could indicate that several independent or independently controlled mechanisms contribute to heat tolerance across life stages and that some mechanisms activated during development are truly irreversible as often suggested (e.g. Angilletta, 2009), while other mechanisms seem readily reversible (Allen et al., 2012; Beaman et al., 2016; Fischer et al., 2010; Zeilstra and Fischer, 2005). The major changes in heat tolerance in response to adult acclimation occurred within the first 24 h as also observed in other species (Allen et al., 2012; Geister and Fischer, 2007; Zeilstra and Fischer, 2005). However, in contrast to findings for CT_{min} , no further increase in CT_{max} was observed despite continued adult acclimation at 25°C for 24 days. Rather, all treatment groups seemed to show a slight and varying decrease in heat tolerance with progressing age.

Generally, the age-dependent susceptibility of adult insect thermal tolerance is dominated by a loss of tolerance due to ageing rather than a gain due to continued adult acclimation (reviewed by Bowler and Terblanche, 2008). Furthermore, in accordance with the rate-of-living theory (Pearl, 1928), flies at higher temperatures will have an increased rate of development as well as an increased rate of senescence and loss of physical performance (e.g. Gibert et al., 2001; Norry and Loeschcke, 2002). Studies have shown that newly emerged drosophilids can have

much higher heat tolerance, measured as heat knockdown time and heat shock survival, than older flies (Pappas et al., 2007; Sørensen and Loeschcke, 2002). In the present study, using CT_{max} as a measure of heat tolerance, we did not see any decrease in heat tolerance within the first couple of days, indicating that age-dependent effects on heat tolerance may depend on the exact trait measured. However, after the first 2 days of acclimation, we observed a general decrease in heat tolerance across all acclimation treatments, indicating an effect of ageing on heat tolerance. More specifically, flies acclimated at 25°C as both juveniles and adults showed the highest rate of decrease in heat tolerance, while development at 15°C prevented this in adults acclimated at 25°C. We interpret this as an effect of developmental temperature on the rate of ageing in the adult stage. We found no indication of a decrease in cold tolerance caused by ageing/senescence as previously suggested by Geister and Fischer (2007), assayed by chill coma recovery time. Instead, flies at low temperatures (15°C) throughout juvenile and adult life stages maintained or slightly increased their cold resistance as assayed by CT_{min} , which matches results of long-term cold acclimation in the sub-Antarctic wingless fly *Anatalanta aptera* (Lalouette et al., 2010).

To further investigate the interaction between age and adult acclimation, we assessed negative geotaxis as well as longevity in flies from the different acclimation treatments. Negative geotaxis has repeatedly been shown to decline with age in *Drosophila* (Gargano et al., 2005; Miquel et al., 1976; Orr and Sohal, 1994), a pattern that we confirmed. We found a faster rate of decrease in negative geotaxis of flies acclimated during development at 25°C compared with those acclimated at 15°C, irrespective of the adult acclimation temperature at which they were compared. Similarly, we found a decreased lifespan at 25°C in flies developed at 25°C compared with that of flies developed at 15°C. Thus, the results of this study show strong carry-over effects from the developmental environment, in addition to the direct effect of adult temperature on the rate of ageing (Bowler and Terblanche, 2008; Fischer et al., 2010). It is commonly assumed that the physiological age of flies at eclosion is reset, regardless of the developmental conditions. In the present study, both lifespan and geotactic behaviour, two traits reflecting senescence, indicate that the maintenance of physiological capacity with age and perhaps ageing itself is not solely determined by adult maintenance temperature, but is also strongly affected by developmental conditions. In conclusion, physiological age is not reset at eclosion in these insects, a result with important implications for a wide range of studies.

It has been suggested that *D. melanogaster* developed at 21–25°C may be physiological superior to flies developed at lower temperatures (Cohet and David, 1978). For example, flies raised at 25°C had much higher maze-running success than did flies raised at 13°C (Cohet, 1974). In spite of theoretical predictions, it is unclear to what extent plasticity of thermal tolerance traits is affected by costs and constraints (Sgrò et al., 2016). The present study suggests that an optimal temperature strongly depends on the interaction between developmental and adult environmental conditions. Thus, development at 15°C gave superior cold tolerance, increased longevity and decreased rate of senescence, compared with development at 25°C, although at the expense of a prolonged duration of development. However, flies developed at 25°C were able to acquire cold tolerance during adult acclimation at 15°C, without the loss of increased heat tolerance. Across all assayed traits, a combination of warm development (25°C) and colder adult temperatures (15°C) seemed to promote the best overall thermal performance and longest lifespan, possibly reflecting an

important component of overwintering adaptation (Sørensen et al., 2015). Furthermore, these results show that climate-related susceptibility will be dependent on age structure and the distribution of developmental temperatures across individuals in a population (Bowler and Terblanche, 2008).

Conclusions

In this study, we used a 3 year old laboratory population of *D. melanogaster* collected at a fruit orchard in Denmark. In this population, the results showed that the cold tolerance attained during development is readily adjusted to the prevailing conditions during adult acclimation, without a detectable developmental constraint. While most of the adult acclimation response is reached within a few days, cold tolerance is only gradually and incompletely acquired during the following weeks of adult life. In contrast, the effects of acclimation on heat tolerance are only partly reversible and are constrained by the developmental temperature regime. Thus, the developmental regime influences the acclimation of adults to changing temperatures, particularly affecting the ability to adjust adult heat tolerance when faced with higher temperatures. The results further showed a remarkable strong and complex carry-over effect of the developmental regime on ageing, which affected high but not low temperature tolerance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization and methodology: S.S., M.F.S., T.N.K., V.L. and J.G.S.; investigation: S.S., M.F.S., T.N.K. and J.G.S.; formal analysis: M.F.S. and J.G.S.; writing – original draft preparation: S.S., M.F.S. and J.G.S.; writing – review and editing: S.S., M.F.S., V.L., T.N.K. and J.G.S.; visualization: M.F.S.; resources: J.G.S. and V.L.

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Supplementary information

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