Author/s:
Hangartner, S;Dworkin, I;Denieu, M;Hoffmann, AA

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Does increased heat resistance result in higher susceptibility to predation? A test using *Drosophila melanogaster* selection and hardening

Sandra Hangartner[12], Ian Dworkin[3] , Michael DeNieu[4] and Ary A. Hoffmann[2]

[1]School of Biological Sciences, Monash University, Clayton 3800, Australia

[2]School of BioSciences, University of Melbourne, Bio21 Institute, Parkville 3010, Australia

[3]Department of Biology, McMaster University, Hamilton, Canada L8S 4K1

[4]Department of Biological Sciences, George Washington University, Washington DC 20052, USA

Corresponding author: Sandra Hangartner phone (03) 9905 2718

email: sandra.hangartner@monash.edu

Running headline: Predation costs of increased heat resistance

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Abstract

Heat resistance of ectotherms can be increased both by plasticity and evolution, but these effects may have trade-offs resulting from biotic interactions. Here we test for predation costs in Drosophila melanogaster populations with altered heat resistance produced by adult hardening and directional selection for increased heat resistance. In addition, we also tested for genetic trade-offs by testing heat resistance in lines that have evolved under increased predation risk. We show that while 35/37°C hardening increases heat resistance as expected, it does not increase predation risk from jumping spiders or mantids; in fact there was an indication that survival may have increased under predation following a triple 37°C compared to a single 35°C hardening treatment. Flies that survived a 39°C selection cycle showed lower survival under predation, suggesting a predation cost of exposure to a more severe heat stress. There was however no correlated response to selection because survival did not differ between control and selected lines after selection was relaxed for one or two generations. In addition, lines selected for increased predation risk did not differ in heat resistance. Our findings suggest independent evolutionary responses to predation and heat as measured in laboratory assays, and no costs of heat hardening on susceptibility to predation.

Keywords: biotic interactions, climate change, genetic correlations, phenotypic plasticity, thermal adaptation, trade-offs

Introduction

Resistance to thermal extremes including high temperatures is an important factor influencing the distribution and abundance of animal species (Cossins and Bowler 1987, Hoffmann et al. 2013). As high temperature stresses are expected to become more common in severity and frequency, species are increasingly at risk of exposure to conditions exceeding their upper thermal limit (IPCC 2014). However, upper thermal limits of ectotherms can be increased to

some extent by both plastic and evolutionary responses (Hoffmann and Parsons 1991, Angilletta 2009, Hoffmann et al. 2013).

Plastic responses are rapid, occur within an organism's lifetime, and have been predicted to play a major role in thermal adaptation (Charmantier et al. 2008, Chevin et al. 2013). In particular, many studies have examined the benefits of hardening responses to heat exposure, whereby heat resistance is enhanced by prior exposure to a moderate heat stress (Hoffmann et al. 2003, Sinclair et al. 2003, Angilletta 2009). Moreover, plasticity may itself evolve in response to environmental change (Scheiner 1993, Kingsolver et al. 2007, but see Sørensen et al. 2016). In addition to plastic responses, upper thermal limits can be altered by selection, resulting in populations that differ in levels of resistance to heat stress (Hoffmann et al. 2013). Selection responses have been particularly well studied in Drosophila melanogaster, where artificial selection can increase resistance to heat stress (McColl et al. 1996, Gilchrist and Huey 1999, Bubliy and Loeschcke 2005, Hangartner and Hoffmann 2016) and populations can be differentiated along climatic gradients as well (Hoffmann et al. 2002). These results demonstrate that standing genetic variation and new mutations are sufficient to drive the evolutionary response. However, evolutionary changes in heat responses might nevertheless be limited (Schou et al. 2014, Hangartner and Hoffmann 2016).

Although plastic and evolutionary changes can allow insects to adapt to stressful, hot conditions, their benefits are likely to be curtailed by costs of plastic and genetic shifts. Hardening responses may incur costs that become evident in terms of growth rates (e.g., Feder et al. 1992), longevity (e.g., Bubliy et al. 2012, Bubliy et al. 2013) or fecundity (e.g., Krebs and Loeschcke 1994, Hercus et al. 2003, Huang et al. 2007). However, the costs and benefits of hardening have been defined mainly in laboratory assays of fitness, making the ecological significance of hardening unclear (Loeschcke and Hoffmann 2007). Field release studies have revealed costs associated with acclimation or hardening that laboratory-based assays did not detect, highlighting the importance of integrating ecological parameters that affect costs and benefits of phenotypic plasticity (Loeschcke and Hoffmann 2007, Kristensen et al. 2008). In addition, genetic constraints may act to limit evolutionary responses. Genetically correlated traits do not evolve independently, and the covariances between traits can either facilitate or hamper adaptation (Walsh and Blows 2009).

To date, costs associated with heat resistance have mostly been considered through potential trade-offs with performance measures under favorable conditions or under opposing thermal extremes (e.g., Huey and Kingsolver 1993, Willett 2010, Karl et al. 2014). On the other hand, biotic interactions around predation, parasitism and competition can also form an important component of resistance costs. Because species interactions are expected to significantly alter climate change responses, interspecific relationships should be incorporated into the predictive framework of climate change (Sanford 1999, Harley 2011, Miller et al. 2014). The effects of heat stressors may be magnified by biotic stressors such as predators (e.g., Relyea and Mills 2001, Alton et al. 2010). Most natural populations are likely to experience selection from multiple abiotic and biotic selective pressures concurrently, however, our understanding of adaptation to multiple selective agents is still limited (but see Ghalambor et al. 2004, Schulte 2007, Eränen et al. 2009, Rogell et al. 2009, Egea-Serrano et al. 2014).

Predation is an important selective force in natural systems, and can impose strong selection on anti-predator traits. Adaptive responses to predation risk may be modified by other stressors, as environmental stress can increase the costs (Hanazato 2001, Huber et al. 2004, Teplitsky et al. 2005) or decrease investment in defenses (Barry 2000, Relyea 2004, Teplitsky et al. 2007). A few studies have tested for heritability in predator avoidance traits and usually found substantial genetic variation (Grant and Mettler 1969, Stirling et al. 2002, Relyea 2005, Brokordt et al. 2012, DeNieu et al. 2014, but see Blumstein et al. 2010). Whether predator avoidance is genetically correlated with other types of stress resistance is largely unknown (but see Jansen et al. 2011). Predation and heat stress resistance might influence the same behavioural or physiological aspects of an organism's biology (Miller et al. 2014). Exposure to heat stress has for example been shown to reduce metabolic rates (Dinh et al. 2016), which potentially could affect survival under predation risk (Rovero et al. 1999, Beckerman et al. 2007, Slos and Stoks 2008). Whether predation pressure alters the costs and benefits of heat hardening and evolved heat resistance has never been tested.

We first tested for the ability of flies to avoid predators in D. melanogaster populations with altered heat resistance produced by adult hardening and directional selection for heat resistance. We measured the costs and benefits of single and repeated heat hardening treatments by subjecting flies that were exposed to different hardening treatments to predation by juvenile false

garden mantids (Pseudomantids albofimbriata) and jumping spiders (Salticidae spp.). Both predators have excellent eyesight, slowly approach their prey, and capture them with a rapid movement (Gelperin 1968, Jackson and Pollard 1996). Costs (or benefits) of the hardening treatments would be evidenced by increased (or decreased) survival of flies from the hardening treatments compared to controls. Differential survival among the hardening treatments would suggest intensity and/or frequency dependent costs (or benefits) under predation pressure.

Second, lines that have been selected for increased heat resistance were tested for performance under predation by the same two predators. Experiments were performed after zero, one and two generations of relaxed selection to test for costs of stress exposure and genetic trade-offs. In addition, we also tested for genetic trade-offs between heat resistance and predator avoidance using lines that have evolved under predation risk by juvenile Chinese mantids (Tenodera aridifolia sinensis) or zebra jumping spiders (Salticus scenicus). These lines were tested for heat resistance after two generations of relaxed selection. Genetic trade-offs between heat resistance and predator avoidance would be evident if heat resistant lines have lower survival under predation than control lines after two generations without selection (generation 2). In addition, lines evolved under predation risk would be expected to be less heat resistant than control lines after two generations of relaxed selection. Costs (or benefits) of a more severe stress exposure would be evident as stress resistant lines having lower (or higher) survival after the selection cycle (generation 0). Reduced (or increased) survival of heat resistant lines compared to control lines after one generation without selection (generation 1) could include genetic and trans-generational costs (or benefits) such as exerted through maternal effects (Mousseau and Fox 1998).

Material and Methods

Fly cultures

All cultures were held at constant 19°C, under 12 : 12 h light : dark cycle in 250 ml bottles containing laboratory medium composed of dextrose (7.5 % w/v), cornmeal (7.3 % w/v), inactive yeast (3.5 % w/v), soy flour (2 % w/v), agar (0.6% w/v), 4 - methyl 4-hydroxybenzoate

(1.6 %) and acid mix (1.4 % 10:1 proprionic acid:orthophosphoric acid). The experimental flies were reared under controlled density conditions by removing parents from the bottles after 48 h of oviposition.

Testing predator avoidance after heat hardening treatments

Heat hardening treatments

The experimental flies originated from a mass-bred population that was collected near Melbourne in May 2012 and was maintained under standard lab conditions as described above. 2-3 days old flies were separated by sex under light CO_2 anesthesia on 17.12.2013 and held in separate vials according to sex, at a density of 25 individuals per vial (day 1). The hardening experiments were started at 4-5 days post eclosion and flies were randomly allocated to the five heat hardening treatments. Females and males were kept separately throughout the hardening experiments, which enabled us to test for sex effects of the hardening treatments on heat resistance.

For the heat hardening, glass bottles (100 ml) containing 50 females or males were immersed in a circulating water bath at either 35°C or 37°C. Temperature was controlled using a Ratek SP599 thermoregulator with a REXP24 controller (Ratek, Boronia, Vic, Australia). In the control treatment, flies were kept in bottles for 75 min at 19°C on day 3, day 5 and day 7. Flies in the single 35 (35-1) Treatment received one hardening treatment of 75 min at 35°C on day 7. Flies in triple 35 (35-3) Treatment received three hardening treatments of 75 min at 35°C on day 3, day 5 and day 7. The single 37 (37-1) Treatment consisted of one hardening treatment of 75min at 37°C on day 7. Finally the triple 37 (37-3) Treatment involved three hardening treatments of 75 min at 37°C on day 3, day 5 and day 7.

Heat resistance experiments

Flies were tested for heat resistance after the heat hardening treatments to test if the hardening increased resistance. Ten females and males per treatment were tested for heat resistance at static 39.0°C. These experiments were performed on day 8 in two blocks, where five females and

males per treatment were tested in each block. To score heat resistance, flies were placed individually into 5 mL vials submerged into a glass tank with water held at 39.0°C. Each fly was scored for heat resistance, where resistance was defined as the time taken for each fly to be knocked down and become immobile even when exposed to a flashlight.

Predator cultures

Female adult false garden mantids (Pseudomantids albofimbriata) and mantid egg cases were collected between March and May 2013 and juvenile jumping spiders (Salticidae spp.) in June and July 2013 near Melbourne. Juvenile jumping spiders were kept individually in vials. Although it was not possible to identify spiders to species, we exposed all treatments to the spider simultaneously (see below) to ensure that any species differences did not confound the detection of treatment effects. Female adult mantids were kept individually in containers where they laid egg cases. The egg cases were hatched and maintained at 19°C. The hatching mantids were collected and kept individually in vials containing fly medium and Drosophila as a food source. Predators were kept at constant 19°C, under 12 : 12 light : dark cycles. All animals were fed on Drosophila and vials or containers had fly medium (see above) and twigs as a substrate for the spiders and mantids.

Predation experiments

We tested for survival of the flies that had been exposed to heat hardening treatments under predation by jumping spider (Salticidae spp.) and juvenile mantids (Pseudomantids albofimbriata). Flies were exposed to predators after a recovery period of 8 hours after the hardening treatment on day 7. These experiments were performed separately for the two predators, and females and males were tested in separate vials/bottles. Flies originating from different treatments were color-marked by lightly shaking them in a vial containing micronized fluorescent dust (Radiant). Five different color combinations were used to test for any potential effects of a particular color, whereas each treatment had a different color in each color combination. One fly per treatment (total of 5 flies) were exposed to one spider in a vial (28 x 95mm) containing laboratory medium as food for the flies and some branches which provided

structural complexity and shelter for the flies and spiders. Each of the five color combination was replicated eight times resulting in a total of 40 replicates (vials) for both sexes (200 females and males in total) for the spider experiments. For the mantids, the experimental procedure was similar, however, two flies per treatment (total of 10 flies) were exposed to one mantids in a bottle (6 x 13cm). Each of the five color combinations was replicated four times resulting in a total of 20 replicates (bottles) for both sexes (200 females and males in total). Surviving flies were removed from the vial/bottle when about 50% of the flies have been predated (after 1-5 days) and survived flies were scored for treatment origin using the color markings. No natural mortality was observed during the experiments. Survival (yes or no) of each fly was used for the statistical analyses.

Testing heat resistance selection lines for predation avoidance

Heat resistance selection lines

The heat selected lines have been described in detail in Hangartner and Hoffmann (2016). In short, all selected and control lines were founded from D. melanogaster collected near Melbourne in May 2012. The offspring of 60 field-collected females were pooled and mass bred for two generations in the laboratory prior to the first selection at generation F3 for the heat resistant selected lines. The selection experiments were done separately for both sexes and the top 10% most resistant flies were selected and randomly allocated into five replicate lines per selection regime comprised of 90 - 110 flies of each sex (200- 210 in total). Flies were selected for heat knockdown resistance by immersing glass bottles (100 ml) containing 100 flies in a circulating water bath at 39°C. When ca 90% of the flies were knocked down (did not move anymore when flashed with a flash light), bottles were removed from the tank and the remaining 10% of flies that were able to stand up were selected (for further details see Hangartner and Hoffmann 2016). The control lines were established and maintained in the same manner as the heat resistance lines, but these lines were not exposed to any treatment. The heat resistance lines have evolved to have a tolerance level around 0.5°C higher than the control lines after ten generations of strong selection (Hangartner and Hoffmann 2016).

Predation experiments

We scored the heat selection lines for survival under predation by jumping spiders (Salticidae spp.) and juvenile mantids (Pseudomantids albofimbriata) to test for costs or benefits of a severe stress exposure and genetic trade-offs. These were performed on adult flies after one and two generations of relaxed selection, as well as right after the selection experiment (no relaxation). The experiments were performed separately for the two predators, and the sexes were tested in separate vials/bottles. Flies were between 4 - 7 days old at the beginning of the experiment. However, selected flies were slightly older (9-12 days), as selection experiments were performed on them before. The control flies had the same age as the selected flies, which means that any potential age effect of the flies would apply to both, control and selected flies.

Six adult flies were randomly chosen from three different control and selected lines for the spider experiments, whereas 12 adults were chosen for the mantids experiments. Flies were marked with dust colors as described above, where each fly (line) was assigned a color. Ten different color combinations were used to account for potential color effects. Each color combination was replicated five times for the spider experiments resulting in a total of 50 replicates (vials) for both sexes (300 flies in total per sex). For the mantid experiments, each color combination was replicated three times resulting in a total of 30 replicates per sex (360 flies in total per sex). The color marked flies were exposed to one spider or one mantid in a vial (28 x 95mm) or bottle (6 x 13cm) respectively, containing fly food and some twigs which provided structural complexity and shelter for the flies and predators. Surviving flies were removed from the vial/bottle when about half of the flies have been predated, and scored for the line origin based on color markings. Survival (yes or no) of each fly was used for the statistical analyses.

Testing predation selection lines for heat resistance

In addition, we also tested for genetic trade-offs between heat resistance and predator avoidance by scoring heat resistance in lines that have evolved under predation risk by jumping spiders or mantids. These flies derived from the Dworkin lab at Michigan State University, USA. Two sets of selection lines were used (episodic and continuous predation), which are described in detail in the Supplementary material S1. The episodic and continuous predation lines differed in effective

population size and the strength of selection induced by the predators. Including both sets of selection lines allowed us to test whether effective population size and/or strength of selection may affect the detection of an apparent trade-off. The episodic and continuous predation lines were tested for heat resistance after two generations without selection to ensure that any differences found in the subsequent experiments were genetic rather than due to plastic (cross generation) effects (c.f. Schiffer et al. 2013). Ten females and males per line were tested for heat resistance at static 39.0°C. These experiments were performed separately for the episodic and continuous predation regimes and in two blocks per regime, where five females and males per line were tested in each block. Flies were sexed under light CO_2 anesthesia and scored for heat resistance when they were 4-5 days old. To score heat resistance, flies were placed individually into 5 mL vials submerged into a glass tank with water held at 39.0°C. Each fly was scored for heat tolerance, where tolerance was defined as the time taken for each fly to be knocked down and become immobile even when exposed to a flashlight.

Statistical analyses:

All statistical analyses were done with SAS 9.3 (SAS Institute, Inc.) and involved general and generalized linear models. Post-hoc pairwise comparisons were undertaken using Tukey tests comparing least square means and adjusting for multiple comparisons.

Heat resistance of the hardened flies was analyzed using a general linear model with the GLM procedure and Kenward-Roger degrees of freedom method (Littell et al. 2006. Hardening treatment, sex, the hardening treatment × sex interaction, and block were included as fixed factors in this analysis.

Survival under predation after the hardening treatments was analyzed with a generalized linear mixed model with REML estimation, logit link function and a binary distribution using the Proc GLIMMIX. REML specification performs residual (restricted) maximum likelihood, where negative estimates are constrained to zero (Littell et al. 2006). In these analyses, sex, treatment, and their interaction were included as fixed factors. To test for any potential effect of a particular color on survival, color was included as an additional fixed factor. In addition, vial (nested under sex) was included as a random factor.

Survival under predation of the selection lines was analyzed with a generalized linear mixed model with REML estimation, logit link function and a binary distribution using the Proc GLIMMIX (Littell et al. 2006). Separate models were run for each generation (0, 1, or 2 generations of relaxation). In these models, selection regime and sex as well as their interactions were included as fixed factors. In addition, color was included as a fixed factor and vial (nested within sex) and line (nested within selection regime) were included as random factors.

Heat knockdown time of the predation selection lines was normally distributed and was analyzed using linear mixed model analyses of variance with the MIXED procedure and Kenward-Roger degrees of freedom method (Littell et al. 2006). Selection regime and sex were included as fixed factors and line (nested under selection regime) as a random factor.

Results

Testing predator avoidance after heat hardening treatments

Hardening effects on heat knockdown time

The hardening treatments had a significant effect on heat resistance, with all hardening treatments increasing heat knockdown time of the flies by 21-73% compared to the control treatment (Table 1, Fig. 1). Posthoc Tukey tests revealed that all hardening treatments significantly increased heat resistance (not shown). In addition, flies from the triple 37°C hardening treatment had a significantly higher heat resistance than flies from the triple 35°C hardening treatment at (t_{139} = 2.89, adjusted P = 0.004), increasing heat resistance by about 30% (Fig. 1). The sexes did not significantly differ in heat resistance and the sex × treatment interaction was not significant (Table 1, Fig. 1).

Predation avoidance after heat hardening

The hardening treatments had a significant effect on survival under predation by jumping spiders (Table 2A, Fig. 2A). A posthoc Tukey test revealed that flies from the triple 37°C hardening treatment had significantly higher survival than those from the single 35°C hardening treatment at (t_{224}=3.01, adjusted P = 0.024), with a survival difference of around 29%. The sexes did not

differ in survival and the treatment × sex interaction was not significant. In addition, color did not have a significant effect on survival (Table 2A). Heat hardening treatments, sex and color did not have a significant effect on survival under predation by juvenile mantids, but the treatment × sex interaction was significant (Table 2B, Fig. 1B). A posthoc Tukey test revealed that the difference between the sexes in the single 37°C hardening treatment was observed non-significant (t_{254}=318, adjusted P = 0.051) (Fig. 2B).

Testing heat resistance selection lines for predation avoidance

Lines that have been selected for increased heat resistance were tested for predation avoidance immediately after selection (generation 0), as well as after 1 and 2 generations without selection.

Generation 0: The analysis at generation 0 showed that the selection regime had a significant effect on survival under predation risk by spiders and mantids: heat resistant lines had significantly lower survival than control lines after the selection cycle reflecting an average survival difference of 10% under predation by spiders and 17% under predation by mantids (Table 3, Fig. 3). Survival under predation by spiders did not significantly differ between sexes and the selection regime × sex interaction was not significant (Table 3A). The sexes did not differ for survival under predation by mantids, but there was a significant selection regime × sex interaction. Posthoc tests revealed that the selection regimes did not significantly differ for the females (t_{597}= 0.97, P=0.331), but there was a significant difference for the males (t_{597}= 3.75, P<0.001), where survival of the control males was 25% higher than the survival of the selected males (Fig. 3B).

Generations 1 and 2: After one and two generations without selection, there was no significant difference of survival between the selection regimes under both predators (Table S1&S2, Fig. 4). The sexes and the sex × selection regime interaction had no significant effect on survival under predation from either spiders or mantids at generation one and two (Table S1&S2, Fig. 4).

Testing for heat resistance in predation selection lines

316 Next we investigated heat resistance in lines that have evolved under predation risk with both
317 jumping spiders or mantids. The selection regime as well as the selection regime × sex
318 interaction did not have a significant effect on heat knockdown time in both the episodic and
319 continuous predation regimes (Table 4A&B). Heat knockdown time did however differ between
320 the sexes in the episodic and continuous predation selection regimes, whereas males were more
321 heat tolerant than females overall, where heat resistance of males was roughly 30% higher than
322 female resistance (Table 4A&B). In addition, there was significant variation among the lines
323 within the selection regimes in the continuous predation lines (Table 4B).

324

325 **Discussion**

326 To our knowledge this is the first study to consider both the impact of evolutionary changes in
327 heat resistance and heat hardening on susceptibility to predation as well as the impact of
328 selection for predation avoidance on heat resistance. We found that heat hardening with a higher
329 frequency and intensity can increase survival under predation by spiders. However, there was no
330 evidence for a genetic trade-off between heat resistance and predator avoidance because survival
331 did not differ between control and heat selected lines and lines selected for increased predation
332 risk did not differ in heat resistance after selection was relaxed for two generations. Survival
333 after the selection cycle at generation 0 was, however, reduced, which suggests that a severe heat
334 stress can reduce survival under predation. Our finding that frequency and intensity dependent
335 heat exposure, but not evolutionary changes in heat resistance, affects predation avoidance is
336 novel and has implications for taxa in the face of climate change.

337

338 Costs and benefits of heat hardening under predation pressure

339 We did not find any costs of heat hardening under predation by either jumping spiders or
340 juvenile mantids. We suspect that costs associated with hardening found in the field and leading
341 to lower capture rates of released flies (Loeschcke and Hoffmann 2007, Kristensen et al. 2008)
342 are unlikely to reflect increased susceptibility to predation. These results suggest that other costs
343 of hardening are likely to be involved under field conditions.

We did however find benefits of a higher intensity and frequency hardening treatment (triple 37°C) compared to a lower intensity and frequency hardening treatment (single 35°C) under predation by spiders. The triple 37°C hardening treatment therefore increased heat resistance and survival under predation by spiders compared to the single 35°C hardening treatment. Cross resistance, where exposure to one stressor enhances resistance to other stressors, has been found in association with some climatic stressors (Hoffmann et al. 2003). Predation risk and climatic factors may influence the same traits of an organism's biology such as foraging or metabolic rate (Cossins and Bowler 1987, Rovero et al. 1999, Sanford 1999, Trussell and Smith 2000, Hawlena and Schmitz 2010, Trussell and Schmitz 2012). A potential explanation for our results is that heat hardening reduces the metabolic rate and/or the activity level. Reduced metabolic rate, together with accumulation of energy reserves, has been suggested as a general mechanism for stress resistance (Hoffmann and Parsons 1989, Bubliy et al. 2012). Reduced activity is also thought to be one of the most efficient anti-predator defense that reduces the encounter rate with predators, decreases detection by predators and increases time spent hiding (Werner and Anholt 1993, Stoks et al. 2003, Stoks et al. 2005). In addition, predation risk can alter metabolic rates (Rovero et al. 1999, Beckerman et al. 2007, Slos and Stoks 2008) and elevate stress proteins (Kagawa and Mugiya 2002, Pauwels et al. 2005). The heat hardening effects on fly physiology might therefore involve similar physiological responses as predation risk, and decrease vulnerability to predation. Although these responses are beneficial under predation, they might still come with long term costs, as a decreased activity also reduces foraging and usually result in reduced growth and fecundity (Werner and Anholt 1993, Brodin and Johansson 2004).

Costs of extreme heat stress under predation pressure

Our results showed that flies surviving a 39°C selection cycle had a lower survival under predation than control flies. In comparison to the hardening treatments, the selection cycle exposed the flies to a much more severe heat stress which very likely causes cellular and physiological damage. Heat shock has deleterious effect on the internal organization of the cell beyond unfolding of proteins (Sørensen et al. 2003). With increasing temperature and a longer heat exposure than experienced under the hardening treatment, the damage is likely to increase and benefits decrease. These damages are likely to affect the flies` physiological performance

and to impair the detection and escape of the predators. Benefits of short term heat hardening on predation can therefore only be expected as long as the heat hardening is not too harsh (Angilletta 2009).

Frequency and intensity dependent heat hardening effects under predation pressure

We only found significant differences between single 35°C and triple 37°C hardening treatment, but not between the control and the hardening treatments in survival under predation by spiders. In addition, we found costs of severe heat exposure (39°C) under predation pressure by spiders and mantids. These results may suggest that the relationship between heat exposure and predation avoidance is non-linear. One possible scenario could be that metabolic rate (or activity) has an inverse U shaped reaction norm across different levels of heat exposure. The mild single 35°C treatment might increase metabolic rate and might therefore be slightly costly under predation risk compared to the control treatment. The moderate triple 37°C hardening treatment becomes beneficial under predation, due to a decreased metabolic rate (or activity). An extreme heat stress, such as the 39°C exposure at generation 0, becomes costly again, which might be due to a strongly decreased metabolic rate (or activity) and/or due to its deleterious effects on cell functioning as mentioned above. There is some support for this scenario: first, metabolic rates are assumed to increase with temperature up to an optimal temperature in most insects (Angilletta et al. 2010). In addition, evidence for a decrease metabolic rate after an extreme heat stress has been found in damselflies (Dinh et al. 2016). Whether metabolic rate (or activity) indeed follows an inverse U shaped reaction norm across different levels of heat stress remains to be tested.

Sex specific predation avoidance after heat exposure

Our results also revealed some sex effects in predation avoidance after heat exposure. Whereas the hardening effects on survival under predation by spiders were similar in both sexes, we found that the sexes responded differently to the hardening treatments under predation by mantids. In addition, only the males had reduced survival under predation by mantids after the 39°C selection cycle at generation 0. What is driving sex specific predation avoidance is currently not

known, but one explanation could be that the reaction norms of metabolic rate (or activity) differs between the sexes across different levels of heat exposure, which remains to be tested.

No evidence for a genetic trade-off between heat resistance and predation avoidance

Little is known about predation of natural D. melanogaster populations. Insects are generally predated by a wide range of insects, as well as other species, such as vertebrates and birds which can play a significant role in insect population dynamics (Speight et al. 2008). Predation pressure can vary in space and time and can have severe effects on population demography (Speight et al. 2008) as evident from classic life table studies such as those undertaken on the population demography of winter moths (Varley and Gradwell 1960).

At this stage we have no evidence that selection for increased heat resistance decreases survival under predation by jumping spiders or mantids. In addition, both sets of lines selected for decreased predation risk (episodic and continuous predation), did not differ in heat resistance. These experiments suggest that there is no strong genetic covariance between predation avoidance and heat resistance in D. melanogaster and suggests that heat adaptation is not limited by biotic interactions associated with predation. Very few studies have tested for biotic costs of evolved stress resistance. Studies on the water flea have found that the evolution of increased pesticide resistance has costs under predation risk (Jansen et al. 2011). However, pesticide resistance is based on different mechanisms than thermal resistance. Currently we have limited knowledge on genetic constraints associated with predation and their impact on climate adaption, but the results presented here suggest that the observed phenotypic patterns (in terms of hardening effects and stress costs) are not reflected in evolutionary changes. Any impacts of predation costs and benefits on natural population are likely to be complex, as not only prey species, but also predators are exposed to heat stress and predator species may respond differently to such stress (Harmon et al. 2009).

Figure legend

Fig. 1. Mean ± SE heat knockdown time (min) of female and male D. melanogaster after a control treatment, a single 35°C (35-1) and 37°C (37-1) hardening treatment and a triple 35°C (35-3) and 37°C (37-3) hardening treatment.

Fig. 2. Mean ± SE survival (%) of female and male D. melanogaster after different heat hardening treatments when exposed to A) jumping spiders and B) juvenile mantids. Hardening treatments included a control treatment, a single 35°C (35-1) and 37°C (37-1) hardening treatment and a triple 35°C (35-3) and 37°C (37-3) hardening treatment.

Fig. 3. Mean ± SE survival (%) of female and male D. melanogaster under predation by A) jumping spiders and B) juvenile mantids of control lines (Control) and lines selected for increased heat resistance (Heat). Survival was scored at generation 0 (no relaxation).

Fig. 4. Mean ± SE survival (%) of female and male D. melanogaster under predation by A) jumping spiders and B) juvenile mantids of control lines (Control) and lines selected for increased heat resistance (Heat). Survival was scored at generation 1 and 2 (1 and 2 generations of relaxation).

Table 1. General linear model of heat knockdown time (min) of female and male D. melanogaster after five different heat hardening treatments. Eta square is the proportion of total variation accounted for by the effect being tested. Significant values are shown in **bold**.

Effect	df	Eta square	Mean square	F	P
Block	1	0.001	7.14	0.16	0.686

Sex	1	0.006	46.03	1.06	0.305
Treatment	4	0.213	434.58	10.01	**<0.001**
Sex × treatment	4	0.041	83.36	1.92	0.110

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Table 2. Generalized linear mixed model of binary survival (yes or no) of female and male D. melanogaster under predation pressure by A) jumping spiders and B) juvenile mantids. Flies originating from five different heat hardening treatments. For the fixed effects, ndf are numerator degrees of freedom, and ddf are denominator degrees of freedom. For the random effects, var is the random effects variance component. Significant values are shown in **bold.**

	A) Spiders				B) Juvenile mantis			
Fixed factors								
Effect	**ndf**	**ddf**	**F**	**P**	**ndf**	**ddf**	**F**	**P**
Sex	1	57	0.2	0.660	1	28	1.6	0.220
Treatment	4	224	2.5	**0.043**	4	254	0.1	0.976
Sex × treatment	4	224	0.9	0.494	4	254	3.3	**0.012**
Color	4	224	1.1	0.375	4	254	0.7	0.596
Random factors								
Effect	**var**	**se**	**Z**	**P**	**var**	**se**	**Z**	**P**
Vial (sex)	0.00	.	.	.	0.00	.	.	.

Table 3. Generalized linear mixed model of binary survival (yes or no) of female and male D. melanogaster under predation pressure by A) jumping spiders and B) juvenile mantids. Flies originated from control and heat resistant selection lines, and the predation experiment was performed after the selection experiments (generation 0). For the fixed effects, ndf are numerator degrees of freedom, and ddf are denominator degrees of freedom. For the random effects, var is the random effects variance component. Significant values are shown in **bold.**

	A) Spiders				B) Juvenile mantis			
Fixed factors								
Effect	**ndf**	**ddf**	**F**	**P**	**ndf**	**ddf**	**F**	**P**
Selection regime	1	8	5.57	**0.046**	1	8	7.93	**0.023**
Sex	1	98	0.12	0.725	1	52	0.68	0.414
Selection regime x sex	1	476	0.47	0.493	1	574	5.11	**0.024**
Colour	5	476	0.77	0.570	5	574	1.19	0.313
Random factors								
Effect	**var**	**se**	**Z**	**P**	**var**	**se**	**Z**	**P**
Vial (sex)	0.00	.	.	.	0.00	.	.	.
Line (selection regime)	0.00	.	.	.	0.00	.	.	.

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Table 4. Mixed model analyses of variance of heat knockdown time (min) of female and male D. melanogaster. Flies originating from the A) episodic and B) continuous predation lines. For the fixed effects, ndf are numerator degrees of freedom, and ddf are denominator degrees of freedom. For the random effects, var is the random effects variance component. Significant values are shown in **bold.**

	A) Episodic predation lines				B) Continuous predation lines			
Fixed factors								
Effect	**ndf**	**ddf**	**F**	**P**	**ndf**	**ddf**	**F**	**P**
Selection regime	1	2	0.30	0.638	2	9	0.10	0.908
Sex	1	74	25.40	**<0.001**	1	223	37.81	**<.0001**
Selection regime x sex	1	74	2.26	0.137	2	223	1.77	0.172
Block					1	223	0.11	0.743
Random factors								
Effect	**var**	**se**	**Z**	**P**	**var**	**se**	**Z**	**P**
Line (selection regime)	0.99	5	0.21	0.417	24.2	14	1.73	**0.042**
Residual	74.42	12	6.08	**<0.001**	110	10	10.56	**<0.001**

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