Plastic and evolutionary responses to heat stress in a temperate dung fly: negative correlation between basal and induced heat tolerance?

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Abstract

Extreme weather events such as heat waves are becoming more frequent and intense. Populations can cope with elevated heat stress by evolving higher basal heat tolerance (evolutionary response) and/or stronger induced heat tolerance (plastic response). However, there is ongoing debate about whether basal and induced heat tolerance are negatively correlated and whether adaptive potential in heat tolerance is sufficient under ongoing climate warming. To evaluate the evolutionary potential of basal and induced heat tolerance, we performed experimental evolution on a temperate source population of the dung fly Sepsis punctum. Offspring of flies adapted to three thermal selection regimes (Hot, Cold and Reference) were subjected to acute heat stress after having been exposed to either a hot-acclimation or non-acclimation pretreatment. As different traits may respond differently to temperature stress, several physiological and life history traits were assessed. Condition dependence of the response was evaluated by exposing juveniles to different levels of developmental (food restriction/rearing density) stress. Heat knockdown times were highest, whereas acclimation effects were lowest in the Hot selection regime, indicating a negative association between basal and induced heat tolerance. However, survival, adult longevity, fecundity and fertility did not show such a pattern. Acclimation had positive effects in heat-shocked flies, but in the absence of heat stress hot-acclimated flies had reduced life spans relative to non-acclimated ones, thereby revealing a potential cost of acclimation. Moreover, body size positively affected heat tolerance and unstressed individuals were less prone to heat stress than stressed flies, offering support for energetic costs associated with heat tolerance. Overall, our results indicate that heat tolerance of temperate insects can evolve under rising temperatures, but this response could be limited by a negative relationship between basal and induced thermotolerance, and may involve some but not other fitness-related traits.

Introduction

Ongoing rapid climate change has already had significant biological consequences by shifting phenologies

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and distributions of organisms (Hughes, 2000; Bradshaw & Holzapfel, 2001; Parmesan, 2006), influencing physiological, behavioural and life history traits (Gardner et al., 2011; Higgins et al., 2014), altering population structure and dynamics (Post et al., 2009), and changing community composition and biotic interactions (Walther, 2010; Roth et al., 2014; Lõhmus & Björklund, 2015). Global increase in average temperatures (IPCC,

2013) is the most prominent parameter signifying current climate change. Accordingly, most of the attempts to understand and predict organisms' responses to this global warming have focussed on mean annual temperatures. Generally, these studies suggest that because of their smaller thermal safety margins (the difference between mean ambient temperature and optimal temperature for a given species), tropical species are more vulnerable to current climate change than species living at higher latitudes (Deutsch *et al.*, 2008; Huey *et al.*, 2009, 2012; Sunday *et al.*, 2011; Diamond *et al.*, 2012). For temperate ectotherms, in contrast, models based on temperature means even predict an increase in fitness at mid-to-high latitudes due to the prolonged growing season (Deutsch *et al.*, 2008; Vasseur *et al.*, 2014).

In addition to rising mean temperatures, temperature variation and the frequency and intensity of extreme weather events such as heat waves are also increasing (Easterling *et al.*, 2000; Meehl & Tebaldi, 2004; Hansen *et al.*, 2006; Rahmstorf & Coumou, 2011; IPCC, 2012). Actually, the ecological impact of temperature extremes is likely to be much stronger than that of the moderate increase in mean temperatures (McKechnie & Wolf, 2009; Chown *et al.*, 2010; Thompson *et al.*, 2013; Bauerfeind & Fischer, 2014; Fischer *et al.*, 2014). In fact, recent analyses that have incorporated temperature fluctuations and incidence of temperature extremes into the models (see Estay *et al.*, 2014 for a theoretical framework) have yielded quantitatively and qualitatively different conclusions than models based only on mean temperatures. Thus, the impact of climate change on ectotherms is predicted to be greater, but also more heterogeneous than previously thought (Clusella-Trullas *et al.*, 2011; Paaijmans *et al.*, 2013; Sunday *et al.*, 2014; Colinet *et al.*, 2015; Kingsolver & Buckley, 2015), with species living at mid-latitudes (i.e. subtropical and temperate species) likely to be most threatened (Hoffmann *et al.*, 2013; Kingsolver *et al.*, 2013; Vasseur *et al.*, 2014; but see Overgaard *et al.*, 2014).

In response to frequent temperature peaks, organisms may evolve higher *basal heat tolerance* (genetic/evolutionary response), greater plasticity enabling stronger *induced heat tolerance* (plastic response), and/or more efficient behavioural thermoregulation (e.g. preference of microhabitats with milder microclimates). Behavioural thermoregulation is considered to be important in avoiding or reducing heat stress in ectotherms in general (Kearney *et al.*, 2009; Sunday *et al.*, 2014). However, in some ectotherms, insects in particular, the role of behaviour may be less decisive than that of heat tolerance in ameliorating the negative effects of heat stress (Rego *et al.*, 2010; Blackburn *et al.*, 2014; van Heerwaarden *et al.*, 2014; Sunday *et al.*, 2014; but see Andrew *et al.*, 2013). Therefore, the present level of thermotolerance, together with its acclimation and

adaptive potential, largely determines insects' susceptibility to extreme temperature events (Hoffmann, 2010; Somero, 2010; Huey *et al.*, 2012; Kingsolver *et al.*, 2013; Sunday *et al.*, 2014).

Whereas there typically is a negative association between cold tolerance and the temperature of the environment where the organisms live (e.g. Addo-Bediako *et al.*, 2000; Hoffmann *et al.*, 2002; Clusella-Trullas *et al.*, 2011), the relationship between ambient temperature and basal heat tolerance is less clear. For example, even within one genus (*Drosophila*) some studies have documented positive relationships between heat tolerance and mean temperature of the habitat/thermal selection regime applied (Cavicchi *et al.*, 1995; Gilchrist *et al.*, 1997; Hoffmann *et al.*, 2005), whereas others have found more complex patterns (van Heerwaarden *et al.*, 2012; Condon *et al.*, 2015) or no clear relationships (Kellermann *et al.*, 2012; Schou *et al.*, 2014). Moreover, within the same experimental set-up Sgrò *et al.* (2010) found negative, positive, or no associations between latitude and basal heat tolerance in *Drosophila melanogaster* depending on the ontogenetic stage (larva or adult), resistance trait (knockdown time, knockdown temperature or survival), and type of the heat shock assay applied (static or ramping). In addition, the duration and intensity of the thermal stress applied may substantially influence the outcome (Rezende *et al.*, 2014; Castañeda *et al.*, 2015). Results of studies reporting sexual differences in heat tolerance also diverge, as sometimes females (Folk *et al.*, 2006; Franke & Fischer, 2013; Ma *et al.*, 2014; Kjaersgaard *et al.*, 2015) but occasionally males (Williams *et al.*, 2012; Bauerfeind *et al.*, 2014; Condon *et al.*, 2015) are found to be more heat-tolerant.

Plastic increase in heat tolerance by acclimation to rising temperature is common in various organisms (Fischer *et al.*, 2010; Hofmann & Todgham, 2010; Gunderson & Stillman, 2015). Typically it is measured as hardening in response to brief (minutes to hours) exposure to high but not extreme temperatures before the actual heat shock, or acclimation in response to longer (days to weeks) exposure to high temperatures. Currently, there is a lack of consensus whether there exists a negative correlation between basal heat tolerance and such induced heat tolerance. Such a negative relationship would mean that organisms already adapted to tolerate heat stress cannot (because of a trade-off or some physiological, evolutionary or genetic constraint) or should not (because of high accompanying costs of plastic response) substantially improve their heat tolerance by acclimation. Indeed, negative associations between basal and induced heat tolerance have been recorded in porcelain crabs (Stillman, 2003), in acclimation trials with widespread *Drosophila* species (Mitchell *et al.*, 2011), and in a study investigating hardening responses of experimental evolution lines of *D. melanogaster* (Cavicchi *et al.*, 1995). In contrast, positive

relationships were found in diving beetles (Calosi *et al.*, 2008), and in some experiments focussing on hardening responses in *Drosophila* (Kellett *et al.*, 2005; Mitchell *et al.*, 2011; Nyamukondiwa *et al.*, 2011). Moreover, contrasting patterns (Castañeda *et al.*, 2015) or no apparent associations between basal and induced heat tolerance were noted in other intraspecific (van Heerwaarden *et al.*, 2012, 2014) or interspecific (Overgaard *et al.*, 2011) *Drosophila* studies. Related to these discrepancies, there is disagreement on whether the evolutionary potential of basal and induced heat tolerance in insects and other ectotherms is substantial enough (Huey *et al.*, 1991; Cavicchi *et al.*, 1995; Williams *et al.*, 2012; Geerts *et al.*, 2015), or indeed too low (Kellermann *et al.*, 2012; Araújo *et al.*, 2013; Hoffmann *et al.*, 2013; Schou *et al.*, 2014; Gunderson & Stillman, 2015; Kristensen *et al.*, 2015) to enable them to respond effectively to climate change.

Most existing studies that have investigated the consequences of extreme temperatures on organism performance are limited to studying either the evolutionary response or plasticity, a reduced number of physiological or life history traits, and model organisms such as *Drosophila* (Schilthuizen & Kellermann, 2014). However, different organisms and traits may respond differently to temperature stress (Berrigan & Hoffmann, 1998; Fischer & Karl, 2010; Karl *et al.*, 2011; Liang *et al.*, 2014), and the response is likely determined by the interplay between basal thermotolerance, phenotypic plasticity and their potential to evolve (Gienapp *et al.*, 2008; Chown *et al.*, 2010; Hofmann & Todgham, 2010; Crozier & Hutchings, 2014). Therefore, integrated approaches considering both plastic and evolutionary changes and focussing on multiple traits are desired (Williams *et al.*, 2012; van Heerwaarden & Sgrò, 2013; Blackburn *et al.*, 2014; Magozzi & Calosi, 2015).

To evaluate the evolutionary potential of basal heat tolerance, we applied experimental thermal evolution to the temperate black scavenger fly *Sepsis punctum* (Diptera: Sepsidae) resulting with three thermal selection regimes (Hot: 31 °C, Cold: 15 °C and Reference: 23 °C, Fig. S1). To assess condition dependence and induced heat tolerance, these flies were subjected to two developmental stress treatments during their larval period and to two acclimation pretreatments after their emergence and before they were exposed to acute heat stress. During and after the heat shock, two traits that are most widely used to measure heat tolerance (heat knockdown time and mortality) as well as several life history traits that are expected to correlate with heat tolerance (body size, longevity, fecundity and fertility) were measured. Besides the possibility to evaluate the associations between evolutionary and plastic responses to heat stress in different traits, such a set-up also enables a broader approach to studying both short-term and long-term effects of thermal adaptations.

Materials and methods

Study species

Sepsis punctum (Diptera: Sepsidae) occurs widely across the Northern hemisphere and is multivoltine throughout most if not all of its range (Pont & Meier, 2002). The species mainly inhabits open meadows at low-to-mid-altitudes (Pont & Meier, 2002; Rohner *et al.*, 2015). Larvae are coprophagous and females lay eggs into faeces of various larger vertebrates, with cattle dung being the most common breeding substrate (Pont & Meier, 2002). *S. punctum* adults feed both on dung and on nectar of flowering plants. European populations exhibit male-biased sexual size dimorphism, whereas females are the larger sex in North American populations (Puniamoorthy *et al.*, 2012a,b; Dmitriew & Blanckenhorn, 2012, 2014). Adults overwinter, but even nondiapausing adults are relatively long-lived (Puniamoorthy *et al.*, 2012a) for such a small insect (wing length less than 4.5 mm, Pont & Meier, 2002) and thus likely experience substantial temperature variation during their lives. Moreover, as different populations of the species and different generations within populations are exposed to very different climatic conditions, *S. punctum* serves as an excellent model species for studies of thermal adaptations (see Berger *et al.*, 2013, 2014).

Experimental evolution

Experimental thermal evolution was performed to study the evolvability of basal heat tolerance in temperate *S. punctum*. The source flies (F0) emerged from 45 manure baits set out for 1 week in Zurich (47°22′N, 8°33′E), Switzerland in August 2009. Upon retrieval, all colonized baits were singly placed into 1-L plastic containers provided with sugar, cow dung and water and were then transferred into an environmental chamber set to 23 °C and 16 : 8 LD cycle (the same photoperiod as used in all following experiments). To minimize variation in food quality and to eliminate competitors and parasites, cow dung used here and in the following experiments was always homogenized and deep-frozen (at −80 °C) before usage. Emerged F0 adults (typically 30–100 per container) were allowed to mate and produce the F1 generation (200–400 adults per container). Subsequently, five randomly chosen F1 individuals from each of 45 bait-based lines were placed together into a larger (3-L) container to produce one of the nine populations of F2. 50 newly emerged F2 individuals from each of 9 populations were then sampled and mixed to initiate one of the 8 experimental thermal evolution replicates (i.e. $n = 450$ per replicate). In particular, 4 replicates were exposed to constant 31 °C (termed 'Hot' selection regime hereafter), whereas another 4 replicates were exposed to constant 15 °C

('Cold' selection regime). During approximately 2 years of laboratory evolution, Hot populations went through 35 generations and Cold populations 20 generations of experimental evolution at their respective temperature regimen (see Fig. S1). Further details concerning the experimental evolution procedure are described in Berger *et al.* (2014).

Maintenance after experimental evolution

After the experimental evolution regime all replicate populations were transferred to a benign temperature; for acclimation purposes Cold regime flies were first transferred to 18 °C for three generations and thereafter kept at 23 °C for the following seven generations, whereas Hot regime flies were kept at 23 °C for 12 generations (Fig. S1). This relatively long period of relaxed selection was aimed to considerably reduce the possibility that subsequent differences in heat tolerance between the selection regimes were caused by parental effects (Mousseau & Dingle, 1991; Burgess & Marshall, 2014) or by inherited epigenetic changes (Bossdorf *et al.*, 2008), as opposed to genetic differences. To reduce differences in inbreeding level potentially resulting from the different number of generations that Cold and Hot replicate populations went through during and after experimental evolution procedure, within-regime replicate population crosses were performed. Specifically, 20–30 two- to five-day-old virgin males from one replicate population were crossed with the same number of females from another replicate of the same selection regime (see Fig. S1 for details). This resulted in four crossed Hot populations and four crossed Cold populations. The original experimental evolution populations were discarded at this point and only the progeny of crossed populations (8th and 11th generations after crossings) was used in the heat shock experiments described below.

In addition to the Hot and Cold selection regime, a third regime representing wild type flies was introduced ('Reference' selection regime hereafter). Specifically, during the same time when replicate populations were crossed (May 2012), new traps were put out at the same location as before (campus of the University of Zurich). Flies emerging from these 10 traps were treated analogously to those of the experimental evolution protocol above except that emerging F0 flies were randomly distributed among four 3-L containers. F1 and F2 generations were then further mixed, and F8 and F11 offspring of the resulting four replicate populations were used in the heat shock experiments (Fig. S1). The reason why experiments were not performed with earlier generations was to give the Reference regime flies some time to adapt to laboratory conditions and thereby to minimize the possibility that the observed differences between the Reference regime and other regimes were not caused by their different thermal histories, but rather by the presence or absence of adaptations to laboratory environment in general. Reference populations were invariably kept at 23 °C.

Developmental stress treatments and acclimation pretreatments

To obtain flies for heat shock experiments, plastic containers filled with fresh cow dung were exposed for egg laying for 8 h inside all replicate population containers on two occasions in October 2012 (8th generation after crossings/establishing Reference regime) and on three occasions in January 2013 (11th generation after crossings). Thus, two cohorts of flies (with 1-week interval) were obtained from 8th generation and three cohorts (with 3-day interval) from 11th-generation parents. To generate different levels of developmental stress for flies from different generations, the amount of dung presented during the 8-h oviposition period differed between the generations. Specifically, for 8th-generation flies, two small dung containers (both containing 10 mL of dung) were placed into each replicate population container, whereas two large dung containers (both containing 40 mL of dung) were put into each population container for the 11th-generation flies (Fig. S1). As a result 8th-generation flies were experiencing more severe food limitations and higher densities of conspecifics during their juvenile period ('Stressed' treatment afterwards) than individuals from the 11th generation ('Unstressed' treatment hereafter).

The dung containers with eggs were placed into 1-L rearing containers and kept at 23 °C until the first adults emerged. Sugar, water and small amounts of fresh dung (necessary for females to mature) were then provided, and adults were allowed to emerge for 3 days and to copulate for another 2 days. To evaluate the level of induced heat tolerance, emerged adults from all replicate populations were divided among the two acclimation pretreatments (Fig. S1). In the 'Hot-acclimation' pretreatment adults were transferred to 31 °C for 3 days, whereas in the 'Non-acclimation' pretreatment adults were kept at 23 °C. During this period, flies were provided with sugar and water *ad libitum*.

Heat shock experiment

To study heat tolerance, adult flies were subjected to acute heat shock. All adults used in our experiment were 6–9 days old and were transferred to 23 °C at least 2 h prior to the start of the first trial. A total of 10 heat shock trials were carried out, five each with adults from the Stressed and the Unstressed treatment, two-to-three trials per day. Prior to the trial, flies were placed singly into 9-mL glass containers, which were then attached to a plastic holder capable of accommodating 60 vials in total. In all trials flies from all 12

replicate populations (4 Cold, 4 Hot, 4 Reference) were included. The position of the vials on the holder was randomized with regard to their selection treatment (Cold, Hot, Reference), acclimation pretreatment (Hot-acclimation, Non-acclimation) and replicate population. The holder was then submerged into a thermostatically controlled, prewarmed recirculating water bath for 15 min. The targeted water temperature during the heat shock was 42 °C, but the actual temperature deviated up to 0.5 °C within the heat shock trials and ranged from 41.5 °C to 42.3 °C across trials. The flies were video-recorded during the trial, and heat knockdown time, a reliable proxy of heat tolerance (Fischer *et al.*, 2010), was estimated later from the video recordings. Heat knockdown time was defined here as the time from the beginning of the heat shock until the point when the fly had ceased all movements for 30 s.

In addition to the flies that were subjected to heat stress (Heat shock treatment), a control treatment was run with flies not exposed to heat stress, but which were otherwise treated analogously to the heat shock treatment flies (Fig. S1). Control flies were individually placed into 9-mL glass containers and submerged in the water bath at 23 °C for 15 min.

Scoring survival and life history traits

After the procedures in the water bath, vials with both heat-shocked and control flies were transferred to an environmental chamber and kept at 23 °C for 24 h, whereafter survival was recorded. To measure adult longevity, males were subsequently individually placed into 50-mL glass vials containing one 3-mL plastic container for sugar and another for a water-soaked cotton swab, with a dry cotton swab placed on the bottom of the vial to absorb any surplus moisture. To record fertility (measured here as proportion of fertile females) and fecundity (number of adult progeny of females that had at least one adult offspring), females were first transferred for 1 week to individual 50-mL glass vials supplied with 10 mL of defrosted cow dung for egg laying, *ad libitum* sugar and a cotton swab at the bottom. After 1 week, females were transferred to new vials prepared similarly as those for males. All vials were inspected daily to record the longevity of adults. After death, the right hind leg (or left hind leg in the rare cases when the right leg was damaged) of the flies was removed, glued flat on paper and measured for tibia length under 50× magnification. Hind tibia length is a commonly used proxy for body size in *S. punctum* (Dmitriew & Blanckenhorn, 2012; Berger *et al.*, 2013, 2014) and in other sepsid species. Vials containing 10-mL dung containers with eggs and hatched larvae were left at 23 °C until adult offspring emerged, when their number was counted and used as an estimate of fecundity of their mother.

Data analysis

For analysing the dependence of heat knockdown time, adult longevity and fecundity on sex, heat shock treatment, selection treatment, acclimation pretreatment, development stress treatment and body size (hind tibia length), general linear mixed models (PROC MIXED; Littell *et al.*, 2006) were applied. Replicate population nested in selection regime and cohort nested in generation were included as random factors. Because temperature fluctuated slightly during the heat shock, mean water temperature was included as a continuous variable when analysing heat-shocked flies. Flies that died during the first 24 h after the heat shock were excluded from adult longevity analysis. Females that did not produce any adult offspring were included in the fertility analysis but not when analysing fecundity. Position of the fly on the holder during the heat shock and the time of the day when heat shock was performed were omitted from the final analyses as these parameters never attained statistical significance. When analysing binary dependent variables (mortality, female fertility), generalized linear mixed models (PROC GLIMMIX; Littell *et al.*, 2006; Stroup, 2013) with logit link functions were applied; in all other respects these analyses were analogous to those described above. Denominator degrees of freedom were estimated using the Satterthwaite option. Minimum adequate models were constructed by sequentially removing nonsignificant interaction terms. As the assumptions of parametric tests were not violated, all analyses were performed on untransformed data and conducted in SAS 9.2 (SAS Institute Inc., Cary, North Carolina).

Results

Knockdown time

In total 588 *S. punctum* adults were subjected to heat shock (for sample sizes of treatment groups, see Table S1). Heat knockdown times differed significantly between selection regimes and acclimation pretreatments. In particular, individuals from the Hot selection regime had significantly longer knockdown times than those from the other regimes, and hot-acclimated flies had longer knockdown times than non-acclimated flies (Tables 1 and S1; Fig. 1a). Differences in knockdown times between selection regimes were greater in non-acclimated (ca 20% longer in Hot than in Cold and Reference treatment flies) than in hot-acclimated flies (ca 10% longer in Hot treatment flies), as indicated by the significant interaction between selection regime and acclimation pretreatment (Table 1). This is because acclimation notably extended knockdown times in flies from the Cold and Reference regimes, but not from the Hot regime (Fig. 1a). Still, even in hot-acclimated flies the effect of selection regime remained statistically significant ($F_{2, 6, 9} = 6.5$, $P = 0.026$).

Table 1 General linear mixed model results for heat knockdown times and survival* of heat-shocked[†] *S. punctum* adults. Replicate population nested within selection regime[‡] and cohort[§] nested within developmental stress treatment[¶] were incorporated as random effects. Degrees of freedom were estimated by the Satterthwaite method. Only interactions that were statistically significant at the 0.05 level are shown.

Effect	Heat knockdown time			Survival		
	d.f.	F	P	d.f.	F	P
Sex	1, 558	58.7	< 0.0001	1, 560	5.8	0.017
(Sel)ection regime	2, 7.9	14.6	0.0022	2, 8.6	1.3	0.32
(A)cclimation pretreatment**	1, 552	29.2	< 0.0001	1, 560	64.8	< 0.0001
Hind tibia length	1, 331	14.5	0.0002	1, 560	4.9	0.027
Shock temperature††	1, 105	48.4	< 0.0001	1, 200.6	0.5	0.48
(D)ev. stress treatment	1, 2.9	25.8	0.016	1, 4.2	0.01	0.98
Sex × A				1, 560	6.5	0.011
Sex × D				1, 560	4.9	0.035
Sel × A	2, 552	3.3	0.038			
A × D	1, 551	6.7	0.01	1, 560	21.9	< 0.0001
Sex × A × D				1, 560	6.4	0.012

*Assessed 24 h after the flies were transferred to individual containers prior to heat shock trials.

†15 min at ~42 °C.

‡Hot (35 generations at 31 °C), Cold (20 generations at 15 °C) and Reference (10 generations at 23 °C).

§Individuals that emerged from the eggs laid during the same 8-h oviposition event in crossed replicate population containers.

¶Stressed (individuals from the 8th generation after the within-treatment crossings, exposed to food limitation and high rearing densities during their juvenile periods) and unstressed (individuals from the 11th generation after the within-treatment crossings, not exposed to food limitation and crowding during their juvenile life).

**Hot-acclimation (3 days at 31 °C) and non-acclimation (kept at 23 °C) pretreatment.

††Mean temperature during the heat shock trials.
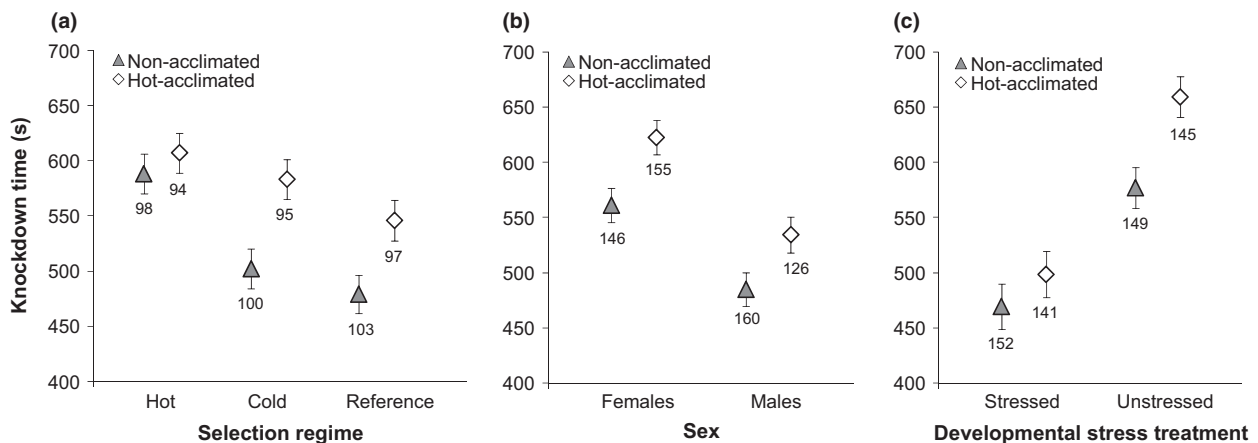
Fig. 1 Heat knockdown times of heat-shocked *S. punctum* flies representing (a) different selection regimes, (b) the sexes and (c) developmental stress treatments. Symbols indicate means ± 1 SE. Values are corrected for the effects of mean temperature during the heat shock, cohort (nested within developmental stress treatment), replicate population (nested within selections regime) and body size (hind tibia length), as well as for the effect of sex in (a) and (c), selection regime in (b) and (c), and developmental stress treatment in (a) and (b) by the SAS, PROC MIXED, least square mean option. Numbers are sample sizes.

Body size positively influenced knockdown time (Fig. 2, significant linear term in Table 1, whereas the quadratic term was not significant in the model: $F_{1, 519} = 2.6$, $P = 0.11$). Nonetheless, males, which are the larger sex, had (15%, on average) shorter heat knockdown times than females (Figs 1b and 2). Differences were also observed between developmental stress treat-ments: flies from the Stressed treatment had significantly shorter knockdown times than those from the Unstressed treatment (Table 1, Fig. 1c). However, hot-acclimation only prolonged heat knockdown times in unstressed and not in stressed flies (Fig. 1c), as indicated by a significant interaction between developmental stress treatment and acclimation pretreatment (Table 1).

Fig. 2 Heat knockdown time as a function of body size of male (dashed line) and female (solid line) *S. punctum* adults that either survived (open symbols) or died (closed symbols) during 24 h after the heat shock. Selection regimes, replicate populations, developmental stress treatments, cohorts and acclimation pretreatments are pooled.
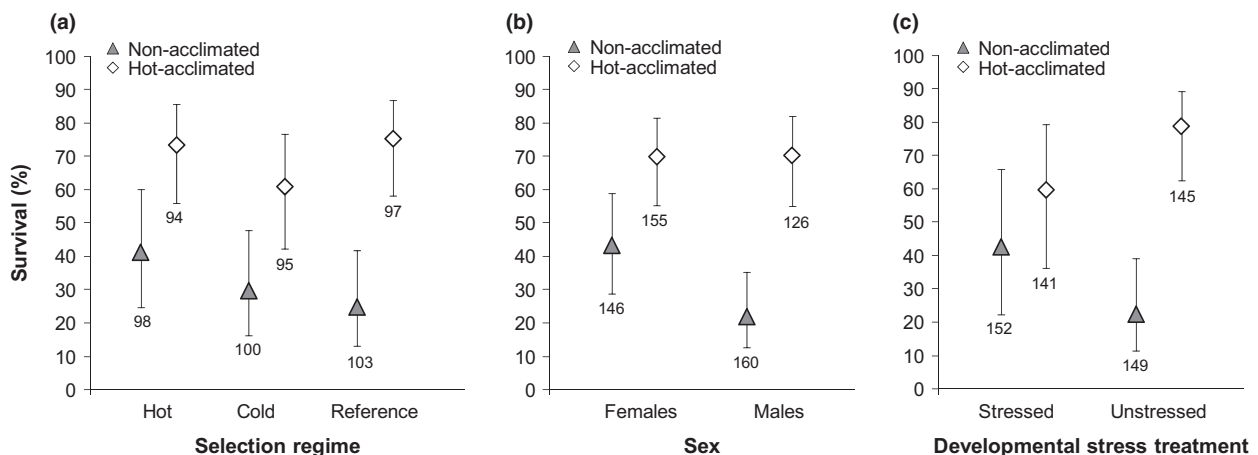
Survival

As average survival was 50% in heat-shocked flies, but as high as 98% (424 individuals of the 434 survived) in control flies (significant effect of the heat shock treatment: $F_{1, 991} = 116.4$, $P < 0.0001$), only the former group was considered in further analyses. Hot-acclimated individuals had, on average, significantly (40%) higher survival than non-acclimated flies (Tables 1 and S1). Males had lower survival than females (Table 1), but only in the Non-acclimation and not in the Hot-acclimation pretreatment (significant interaction between sex and acclimation pretreatment; Table 1,

Fig. 3b). There were no significant differences in survival between selection regimes (Table 1, Fig. 3a) or developmental stress treatments (Table 1, Fig. 3c). However, as indicated by a significant interaction, the difference between acclimation pretreatments was greater in unstressed individuals compared to those experiencing greater developmental stress (Table 1, Fig 3c). The significant interaction between acclimation pretreatment, sex and developmental stress treatment (Table 1) was mainly caused by extremely low survival among non-acclimated unstressed males (ca 10%, on average; Table S1). Larger heat-shocked flies had higher survival than smaller ones (Table 1, Fig. 2), the hind tibias of the surviving individuals being, on average, 5% longer than that of flies that died after heat shock.

Life history traits

Consistent with previous studies of European *S. punctum*, males were the larger sex in our sample ($F_{1, 987} = 98.6$, $P < 0.0001$), having on average 7% longer hind tibiae than females (Fig. 2). Sizes did not differ significantly between heat shock treatments ($F_{1, 987} = 1.2$, $P = 0.27$), selection regimes ($F_{2, 9} = 1.5$, $P = 0.28$), acclimation pretreatments ($F_{1, 987} = 0.3$, $P = 0.61$) or developmental stress treatments ($F_{1, 3} = 2.8$, $P = 0.20$). Sexual size dimorphism was greater in the Unstressed treatment compared to the Stressed treatment, as indicated by the significant interaction between sex and developmental stress treatment ($F_{1, 3} = 4.9$, $P = 0.027$).

Adult life span of non-shocked flies was, on average, 60% (12 days) longer than that of heat-shocked flies ($F_{1, 689} = 89.7$, $P < 0.0001$). Because of multiple significant interactions between heat shock treatment and other variables, results are presented separately for

Fig. 3 Survival of heat-shocked flies from the (a) selection regimes, (b) sexes and (c) developmental stress treatments. Values were calculated using least square means option in SAS, PROC GLIMMIX, with logit link function. Symbols indicate means, and error bars stand for 95% confidence intervals (calculated using the ilink option in PROC GLIMMIX). Numbers stand for sample sizes. See Fig. 1 for further details.

Table 2 Linear mixed model results for the effects of sex, selection regime, acclimation pretreatment, body size and developmental stress treatment on adult longevity* of heat-shocked and non-shocked *S. punctum* flies. Only individuals that lived at least 24 h after they were transferred to individual containers were considered. Replicate nested within selection regime and cohort nested within generation were incorporated as random effects. Only interactions statistically significant at the 0.05 level are shown. For further details, see Table 1.

Effect	Heat-shocked adults			Non-shocked adults (controls)		
	d.f.	F	P	d.f.	F	P
Sex	1, 271	0.8	0.38	1, 412	38.7	< 0.0001
(Sel)ection regime	2, 8.8	0.8	0.48	2, 9.7	9.6	0.005
(A)cclim. pretreatment	1, 269	12.2	0.0006	1, 410	4.8	0.029
Hind tibia length	1, 242	9.3	0.0025	1, 207	0.1	0.79
(D)ev. stress treatment	1, 3.0	35.1	0.0099	1, 3.1	176.3	0.0009
Sex × D				1, 408	22.9	< 0.0001
Sel × A	2, 270	3.7	0.027			

*Measured as time from the transfer of 6- to 9-day-old adults to individual containers until their death (days).

heat-shocked and non-shocked flies. Hot-acclimated heat shock-treated flies had significantly longer adult life spans than non-acclimated flies, but when selection regimes were analysed separately (justified by the significant interaction between acclimation pretreatment and selection regime; Table 2), this difference remained significant only in the Reference regime (Table 2, Fig. 4a). Unstressed flies lived over two times longer than stressed flies (Table 2, Fig. 4c). Larger heat-shocked individuals lived longer than smaller ones (Table 2). Water temperature during the heat shock ($F_{1, 10.4} = 0.1$, $P = 0.8$), sex and selection regime had no significant effects on adult life span in heat-shocked flies (Table 2). Hot-acclimated adults from the Reference selection regime had significantly longer life spans than non-acclimated Reference flies, whereas there were no such differences in other selection regimes (Table 2, Fig. 4a).

Among non-shocked individuals (controls), Reference regime flies had 25–30% longer adult life spans than Hot and Cold regime flies (Table 2, Fig. 4b). Non-acclimated individuals lived 10% (3.3 days) longer than hot-acclimated ones, and unstressed flies lived 2.5 times longer than stressed flies (Fig. 4d). Sexual differences in adult life span of control flies only appeared in Unstressed (females lived 45% longer) but not in Stressed treatment (Fig. 4d), as indicated by a highly significant interaction between sex and developmental stress treatment (Table 2).

Heat shock had a negative impact on both fertility ($F_{1, 362} = 4.0$, $P = 0.046$) and fecundity ($F_{1, 272} = 4.7$,

$P = 0.03$) of females: there were ca 25% more fertile individuals among non-shocked (control) than heat-shocked females, and control females produced approximately 30% more offspring than heat-shocked ones (Table S2, Fig. 5). Longer-lived females were less often infertile and were more fecund than those having a shorter adult life (Table 3). Among heat-shocked females, hot-acclimated individuals produced 35% more offspring than non-acclimated ones (Table 3, Fig. 5a). There was no apparent effect of acclimation pretreatment on fecundity in non-shocked flies (Fig. 5b), as also indicated by the significant interaction between heat shock treatment and acclimation pretreatment ($F_{1, 275} = 4.7$, $P = 0.031$). There was also a tendency that in heat shock treatment, fertility was higher in hot-acclimated than non-acclimated individuals, although this effect remained marginally nonsignificant (Table 3). Larger females produced more offspring than smaller females, but only in the heat shock treatment and not among controls (Table 3). There were no significant differences in fertility and fecundity between selection regimes or developmental stress treatments (Table 3).

Discussion

Using experimental thermal evolution and subsequent laboratory experiments, we showed here that both basal and induced heat tolerance of *S. punctum* flies evolved in response to increased mean temperatures. Hot adapted flies exhibited superior basal tolerance, whereas adult acclimation significantly alleviated heat stress effects only in flies from Cold and Reference but not from the Hot regime, indicating a negative association between basal and induced heat tolerance. However, the results depended on the traits measured as this pattern clearly emerged only for heat knockdown time, but not when survival or other traits closely related to fitness (longevity, fecundity and fertility) were assessed. Whereas flies not acclimated to hot temperatures prior to heat shock generally suffered reduced trait values relative to hot-acclimated flies, these effects were seemingly reversed for adult longevity in the absence of heat shock (i.e. in controls), thereby revealing a potential cost of acclimation. Food limitation and higher rearing density during the juvenile period decreased heat knockdown time but not survival after heat stress, indicating a trait-specific influence of developmental stress on heat tolerance. Females and larger individuals were more heat-tolerant than males and smaller flies.

The intraspecific negative relationship between basal and induced heat tolerance (Fig. 1a) has been also reported by Cavicchi *et al.* (1995) in their experimental thermal evolution experiment on *D. melanogaster*, although both the responding trait (mortality vs. heat knockdown time) and the type of plastic response used
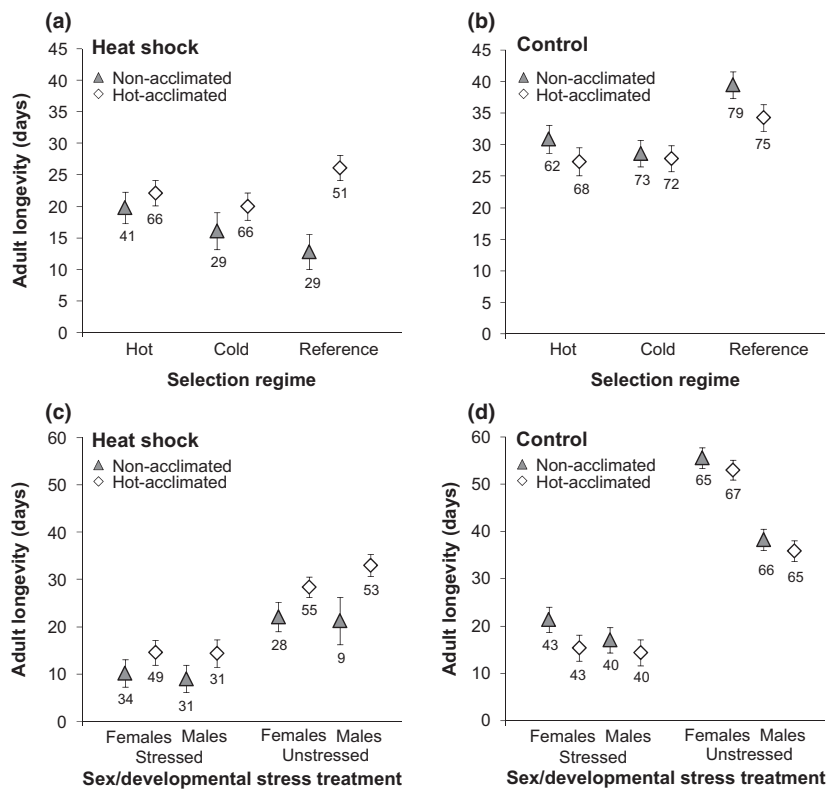
Fig. 4 Adult longevity (measured as time from the transfer of 6- to 9-day-old adults to individual containers until their death) of heat-shocked flies (a, c) and non-shocked controls (b, d) from different selection regimes (a–b), developmental stress treatments (c–d), acclimation pretreatments (a–d) and the sexes (c–d). Values are corrected for the effects of cohort (nested within generation), replicate population (nested within selection regime) and body size (hind tibia length) as well as for temperature during heat shock (in a and c), developmental stress treatment (a–b), experimental evolution treatment (c–d) and sex (a–b) by the SAS, PROC MIXED, least square mean option. Numbers stand for sample sizes. See Fig. 1 for further details.
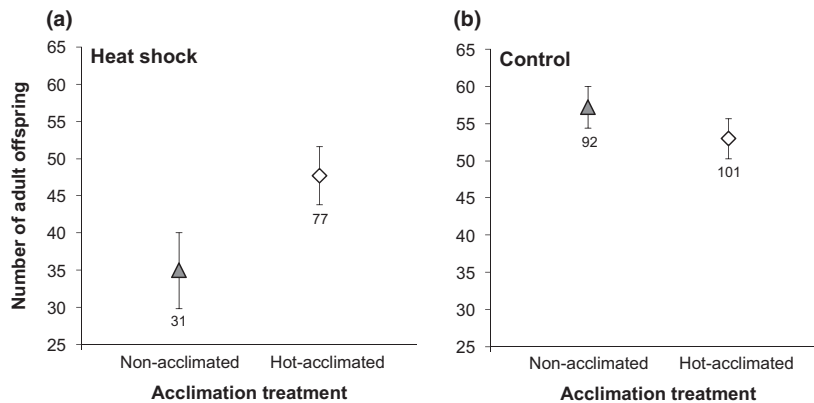
Fig. 5 Fecundity (measured as number of adult progeny produced by individuals that had at least one offspring) of (a) heat-shocked and (b) non-shocked *S. punctum* females. Symbols indicate means ± 1 SE, numbers stand for sample sizes. Acclimation pretreatments differ significantly in (a), but not in (b).

(hardening vs. acclimation) in their experiment differed from ours. Such associations could mean that organisms cannot effectively use the plastic and genetic response simultaneously to increase their thermotolerance, thus limiting their potential to cope with temperature changes and extreme temperature events. The physiological basis of the negative relationship between basal and induced thermotolerance is, however, still not known. It may be that organisms adapted to tolerate heat stress are not able to further improve their heat tolerance by acclimation because they are already close

to their upper limit set by constraints. For example, strong phylogenetic signal in heat resistance in *Drosophila* is proposed to be an indicator of an evolutionary constraint (Kellermann *et al.*, 2012; Hoffmann *et al.*, 2013). The existence of a functional trade-off between achieving high heat tolerance and maintaining its plasticity has also been proposed (Pörtner *et al.*, 2006). However, the alternative hypothesis, that the benefit of acclimation (higher thermotolerance) simply does not outweigh its physiological costs (specified below) when the organism already possesses a high basal tolerance,

Table 3 Generalized linear mixed model results for female fertility* and female fecundity†. Only females that lived at least 24 h after they were transferred to individual containers were considered. Replicate nested within selection treatment and cohort nested within generation were incorporated as random effects. For further details, see Table 1.

	Heat-shocked females						Non-shocked females (controls)					
	Fertility			Fecundity			Fertility			Fecundity		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Selection regime	2, 7.0	0.2	0.83	2, 8.9	1.2	0.36	2, 6.8	0.2	0.81	2, 7.7	2.1	0.19
Acclimation pretreatment	1, 152	3.2	0.075	1, 91.1	5.9	0.018	1, 205	2.8	0.094	1, 172	1.6	0.21
Hind tibia length	1, 152	0.5	0.47	1, 93.8	6.2	0.015	1, 58.2	2.5	0.12	1, 119	0.03	0.87
Developmental stress treatment	1, 2.7	3.2	0.18	1, 3.2	5.2	0.1	1, 5.0	0.1	0.73	1, 6.9	0.3	0.63
Adult longevity	1, 152	31.0	< 0.0001	1, 88	21.7	< 0.0001	1, 205	12.1	0.0006	1, 175	12.2	0.0006

*Measured as proportion of females that had at least one adult offspring.
†Number of adult progeny produced by females that had at least one offspring.

cannot be ruled out. In any case, Gerken *et al.* (2015) recently demonstrated that genetic architectures underlying the basal cold tolerance and acclimation to cold do not overlap in *D. melanogaster*, suggesting that basal and induced thermotolerance could evolve independently from each other. Nevertheless, primarily interspecific studies of drosophilids have found no association between basal and induced heat tolerance (Overgaard *et al.*, 2011; van Heerwaarden *et al.*, 2012, 2014), or the relationship even turned out to be positive (Kellett *et al.*, 2005; Calosi *et al.*, 2008; Nyamukondiwa *et al.*, 2011). Therefore, owing to a low number of studies assessing basal and induced heat tolerance in conjunction, conflicting results and limited taxonomic coverage, it remains to be explored how common negative associations between baseline and inducible responses in ectotherm heat tolerance really are, what the causal agents of these relationships are, and whether systematic differences between intra- and interspecific patterns or between hardening- and acclimation-related plasticity exist.

Our result showing that increased tolerance to heat stress in *S. punctum* follows adaptation to moderately high temperatures (Fig. 1a) is also consistent with some (Huey *et al.*, 1991; Cavicchi *et al.*, 1995; mortality in Gilchrist *et al.*, 1997) but not other previous experimental evolution studies on *Drosophila* (knockdown temperature in Gilchrist *et al.*, 1997; Schou *et al.*, 2014; Condon *et al.*, 2015). Moreover, the relationship reported here was only statistically significant when heat tolerance was measured as heat knockdown time (a physiological response) but not for further downstream life history traits that are presumably more closely linked to fitness (survival, longevity, fecundity, fertility). These findings indicate the complex nature of thermal adaptations: high(er) mean temperatures during previous generations may improve some heat tolerance traits of progeny, whereas others may remain unaffected or even show the opposite pattern (see Gilchrist *et al.*, 1997 for similar results in *D. me-*

lanogaster). Besides differences in statistical power when analysing different traits, another possible reason why we observed evolutionary responses only in one trait of the five may be that heat tolerance was measured only after the selection regimes had been relaxed for ca. 20 generations. During this time possible previously existing thermal adaptations in life history traits may have disappeared from our experimental populations by genetic drift and/or because they turned out to be maladaptive/costly in the relaxed selection environment and were subsequently eliminated by selection. Indeed, complete or almost complete reversal selection over 20 generations has been documented in some traits in *D. melanogaster* (Teotónio & Rose, 2000; Fragata *et al.*, 2014), although usually much longer time is needed (Teotónio & Rose, 2001). Nevertheless, even if in our study qualitatively similar responses in other studied traits were present immediately after the termination of experimental evolution, they were very likely weaker than those in heat knockdown time, and therefore less important in contributing to overall heat tolerance. Moreover, due to low standing genetic variation and low potential for new mutations to arise, even mildly deleterious mutations are well known to sometimes become fixed in small and isolated populations (Lynch *et al.*, 1995; Frankham, 1996; Mattila *et al.*, 2012). Therefore, we consider it unlikely that adaptations to heat stress completely disappeared from our relatively small and inbred experimental populations even if they became neutral or (mildly) deleterious after the flies were transferred to a different thermal regime.

The plastic response to heat stress in *S. punctum* adults was substantial and more pronounced than any evolutionary (genetic) response to experimental evolution: 3 days of acclimation at 31 °C prolonged heat knockdown times and longevity, augmented female fecundity and also increased survival relative to non-acclimated heat-shocked flies (Tables 1–3). This large effect of acclimation relative to the evolutionary responses may be partly caused by the rather long per-

iod of relaxed selection in our experimental set-up. However, positive effects of acclimation and/or hardening on heat tolerance are common in insects (Fischer *et al.*, 2010; Nyamukondiwa *et al.*, 2011; Karl *et al.*, 2014), but depend on the trait in focus and tend to be weaker than in the case of cold tolerance (reviewed in Schilthuizen & Kellermann, 2014). Nevertheless, the relatively few studies that have evaluated the importance of plastic and genetic contributors to high or increasing temperatures also tend to reach the conclusion that the former are more important (Hoffmann *et al.*, 2005; Hoffmann & Sgrò, 2011 and references therein, but see Andrew *et al.*, 2013). Interestingly, flies from the Reference regime, which tended to have the lowest basal heat tolerance, showed the strongest induced response in adult longevity compared to flies from other selection regimes (Fig. 4a). This result further confirms the existence of a negative association between basal and induced thermotolerance in *S. punctum*. Moreover, longer adult life span of Reference flies in the absence of heat shock (Fig. 4b) is probably also related to their different thermal history compared to other selection regimes and may indicate the presence of a cost associated with adaptations to suboptimal temperatures.

We were also able to uncover a potential cost of acclimation, as hot-acclimated flies not exposed to heat shock had significantly shorter life spans than non-acclimated individuals (Table 2). Acclimation to high temperatures can be expected to increase energetic demands, for example due to the production and maintenance of heat-shock proteins (Sørensen *et al.*, 2003; Macario & de Macario, 2007; King & MacRae, 2015), and therefore has been proposed to be costly (Angilletta, 2009). However, experimental evidence for such costs of heat acclimation has remained inconsistent and elusive (Hoffmann & Hewa-Kapuge, 2000; Angilletta, 2009; Bubliy *et al.*, 2012). Our results support the idea that such costs may be quite specific and therefore not always easy to detect, as we only found statistical support for costs of acclimation in one (adult longevity) of the four life history traits studied. Furthermore, it could even be argued that the observed longevity difference between acclimated and non-acclimated control flies was not caused by the acclimation *per se,* but perhaps via negative effects of high temperature on life span of hot-acclimated flies mediated by increased metabolism during the acclimation period (see e.g. Angilletta, 2009). However, the fact that the mean longevity difference between hot-acclimated and non-acclimated control flies even exceeded the duration of the entire acclimation period of hot-acclimated flies (3.3 vs. 3 days) does not support this explanation.

There was a positive effect of body size on heat knockdown time and survival (Table 1, Fig. 2), longevity (Table 2) and female fecundity (Table 3), although significant size effects were only present in heat-

shocked flies and not in the control group. Positive intraspecific associations between body size and stress resistance have been shown repeatedly in insects for cold tolerance (e.g. Renault *et al.*, 2003; Kovacs & Goodisman, 2012), starvation resistance (e.g. Stockhoff, 1991; Gergs & Jager, 2013) and desiccation resistance (e.g. Weldon *et al.*, 2013; Tejeda *et al.*, 2014). By contrast, evidence for a relationship between size and heat tolerance is surprisingly scarce. Moreover, the few studies examining this relationship in insects suggest a lack of significant associations (Jenkins & Hoffmann, 1994; Fischer *et al.*, 2010; Condon *et al.*, 2015; Kjaersgaard *et al.*, 2015). Our findings clearly contrast with the latter results and support the more general view of positive associations between stress (including heat) resistance and body size.

Actually, the positive association between size and heat tolerance may constitute an additional obstacle for ectotherms to cope with global warming. Ectotherms in general, and multivoltine terrestrial arthropods in particular (Horne *et al.*, 2015; *S. punctum* among them: Berger *et al.*, 2014), are known to achieve larger body size when exposed to lower temperatures during juvenile development, a phenomenon known as the temperature–size rule (Atkinson, 1994; Angilletta & Dunham, 2003). Therefore, under the predicted future climatic conditions with higher incidences of heat stress, insects may be simultaneously faced with higher developmental temperatures leading to smaller adult size and the selection pressure towards higher heat tolerance favouring larger size.

Resistance to heat stress and adult longevity, but not survival, body size, fecundity or fertility, differed significantly between developmental stress treatments characterized by differences in juvenile food regime and rearing density. In particular, adults from the Stressed treatment had considerably lower heat knockdown times (Fig. 1c) and shorter life spans (Fig. 4c, d) than those from the Unstressed treatment, thus confirming the anticipated negative effects of developmental stress. Moreover, effects of acclimation on heat knockdown time (Fig. 1c) and post-heat shock survival (Fig. 3c), as well as sexual differences in body size (Table S1), were greater in unstressed than stressed flies, thereby demonstrating the condition dependency of these traits. Low quality larval diet has been previously shown to decrease heat tolerance in insects (Andersen *et al.*, 2010; Fischer *et al.*, 2010; Sisodia & Singh, 2012). None of these studies, however, investigated the influence of diet quality or quantity on acclimation capacity. Our results suggest that besides their direct effects on heat tolerance, suboptimal conditions during the juvenile period may have long lasting indirect effects by lowering the capacity of plastic responses to heat stress during the adult period. As developmental stress treatments were performed with flies from different generations (food restriction/rearing density stress was

applied in the 8th generation after crossings, whereas the 11th generation was used for the Unstressed treatment), factors directly related to any differences between generations could also have caused the observed patterns. However, we consider this unlikely because such factors, for example inbreeding level (Franke & Fischer, 2015), are expected to produce opposite results (i.e. 8th-generation individuals would then be expected to perform better than those from the 11th generation, which was not the case). Moreover, the smaller sexual size dimorphism in the 8th than 11th generation suggests that observed differences between the generations were mainly caused by the differences in food quality as dimorphism in insects tends to decrease on poor diets (Teder & Tammaru, 2005).

In summary, our results, together with results of some previous studies (Cavicchi *et al.*, 1995; Kellett *et al.*, 2005), suggest that even if temperate species of terrestrial ectotherms are most threatened by the current and future climate change (Walters *et al.*, 2012; Hoffmann *et al.*, 2013; Kingsolver *et al.*, 2013; Vasseur *et al.*, 2014), they have a potential to use both evolutionary and plastic responses to cope with heat waves that accompany global warming. However, unlike many other studies (Kellett *et al.*, 2005; Calosi *et al.*, 2008; Nyamukondiwa *et al.*, 2011; Overgaard *et al.*, 2011; van Heerwaarden *et al.*, 2014), we found a negative association between basal and induced thermotolerance, but only for one immediate (physiological) trait studied (heat knockdown time). Such a relationship implies that individuals already adapted to high temperatures have only limited potential to further improve their heat tolerance by costly acclimation responses. However, it remains to be shown by future studies how common and trait-specific negative associations between basal and induced heat tolerance are among insects and other ectotherms, what the physiological basis of this relationship is, and whether laboratory-derived results also hold in natural environments.

Acknowledgments

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Mean hind tibia lengths, knockdown times, adult longevities and survival of experimental flies.

Table S2 Mean fertilities and fecundities of experimental flies.

Figure S1 Flow chart describing experimental design.

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