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A *Drosophila* laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future

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Abstract

The ability to respond evolutionarily to increasing temperatures is important for survival of ectotherms in a changing climate. Recent studies suggest that upper thermal limits may be evolutionary constrained. We address this hypothesis in a laboratory evolution experiment, encompassing ecologically relevant thermal regimes. To examine the potential for species to respond to climate change, we exposed replicate populations of *Drosophila melanogaster* to increasing temperatures (0.3 °C every generation) for 20 generations, whereas corresponding replicate control populations were held at benign thermal conditions throughout the experiment. We hypothesized that replicate populations exposed to increasing temperatures would show increased resistance to warm and dry environments compared with replicate control populations. Contrasting replicate populations held at the two thermal regimes showed (i) an increase in desiccation resistance and a decline in heat knock-down resistance in replicate populations exposed to increasing temperatures, (ii) similar egg-to-adult viability and fecundity in replicate populations from the two thermal regimes, when assessed at high stressful temperatures and (iii) no difference in nucleotide diversity between thermal regimes. The limited scope for adaptive evolutionary responses shown in this study highlights the challenges faced by ectotherms under climate change.

Introduction

Current climate models predict an increase in average surface temperature around 0.3 °C per decade in the 21st century (IPCC, 2007). This temperature change will push many terrestrial ectotherms towards their physiological upper thermal limits, with subsequent negative impacts on survival and reproduction. The enclosing limits are expected to be most pronounced at low to mid-latitudes (Deutsch *et al.*, 2008; Hoffmann, 2010; Clusella-Trullas *et al.*, 2011; Sunday *et al.*, 2011; Diamond *et al.*, 2012; Kellermann *et al.*, 2012; Overgaard *et al.*, 2014). However, current projections of climate change impacts on species survival and distributions often do not account for evolutionary adaptive responses (Bradshaw & Holzapfel, 2006; Parmesan, 2006; Hoffmann *et al.*, 2013 and references herein). This is problematic as the thermal performance curve (Huey & Stevenson, 1979) is likely to be altered through evolution (Huey & Kingsolver, 1993; Chown *et al.*, 2010; Hoffmann, 2010). Understanding the potential for species to adapt to future climate change through evolutionary changes is thus an essential part of predicting their vulnerability and future distributions (Williams *et al.*, 2008; Kearney *et al.*, 2009).

Increasing evidence for conserved upper thermal limits in the genus *Drosophila* and in ectotherms in general

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(Araújo *et al.*, 2013; Hoffmann *et al.*, 2013), coupled with studies suggesting low heritability in *Drosophila melanogaster* for heat resistance assessed under ramping, suggests that evolutionary responses to a warmer climate may be constrained (Krebs & Loeschcke, 1994; Gilchrist & Huey, 1999; Mitchell & Hoffmann, 2010; see Hoffmann *et al.*, 2003 for a review). Artificial selection experiments often assume genetic correlations between traits when predicting restricted evolutionary potential caused by strong trait correlations. However, a recent study investigating trait correlations among > 95 *Drosophila* species in hot and dry environments and in wet and cold environments found trait correlations to be dependent on the environment (Kellermann *et al.*, 2013). In species occupying cold and wet environments, Kellermann *et al.* (2013) did not find evidence for correlations between desiccation resistance and other stress resistance traits such as starvation resistance and heat resistance, whereas strong correlations were found for these traits in species occupying hot and dry environments. This finding indicates strong environmental dependence of trait correlations as opposed to genetic correlations driving trait evolution (Kellermann *et al.*, 2013). Experiments aiming to identify correlations and possible limits in the evolutionary potential of traits should be performed under ecological relevant conditions and avoid simplifying important parameters such as temperature.

Moreover, many studies predicting limited potential of *Drosophila* to respond to increasing temperatures primarily investigate responses to intense selection, despite these selection regimes bearing little resemblance to selection pressures in natural environments. A more ecologically relevant approach in a climate change perspective is a more slowly changing environment which potentially allows for adaptive changes of rare beneficial variants and phenotypes (Huey & Kingsolver, 1993; Brakefield, 2003). To further increase the ecological relevance, the daily thermal regime should mimic the variance experienced by the flies in nature, as there can be great disparities between the stress and heritabilities generated from a constant and a variable temperature (Brakefield & Mazzotta, 1995; Ruel & Ayres, 1999; Mitchell & Hoffmann, 2010; Niehaus *et al.*, 2012). Using a more ecologically relevant approach in laboratory studies of thermal adaptation will likely reveal more reliable estimates of the evolutionary potential of natural populations of ectotherms in a predicted warmer and more variable future climate.

To address these issues, we performed a laboratory evolution experiment with *D. melanogaster*. *Drosophila melanogaster* was exposed to daily temperature fluctuations, with ambient temperatures increasing by 0.3 °C every generation. Evolutionary responses in heat and desiccation resistance and the life history traits, egg-to-adult viability and fecundity were assessed and compared to replicate populations maintained in control climate chambers calibrated to 25 °C. To evaluate whether replicate populations from the two regimes diverged in life history traits, the assessments were performed at temperatures experienced in the generation prior to testing. To ensure results were not driven by strong genetic drift, we monitored the loss of nucleotide diversity (π) throughout the experiment. Based on previous laboratory selection experiments (Service *et al.*, 1988; Hoffmann & Parsons, 1989a; Huey & Kingsolver, 1993; Cavicchi *et al.*, 1995; McColl *et al.*, 1996; Bubli *et al.*, 1998; Bubliy & Loeschcke, 2005), we expected, across the course of the experiment, a gradual increase in heat and desiccation stress resistance coupled with an increase in fitness when tested at high temperatures in the replicate *D. melanogaster* populations kept at temperatures increasing every generation.

Materials and methods

The mass bred population

A mass bred population of *D. melanogaster* was established from the offspring of 589 inseminated females, caught in Karensminde orchard at the Danish peninsula of Jutland (55°56′42.46″N, 10°12′45.31″E) in October 2010. The inseminated females produced offspring in individual vials which developed at 17 ± 1 °C in a 12 : 12 L : D photoperiod. Five virgin males and five virgin females from each inseminated female were used to establish the mass bred population. After establishment, the mass bred population was maintained at 25 ± 1 °C at 12 : 12 L : D photoperiod with a population size of approximately 6000 for two generations before the experimental replicate populations were established. Flies were reared on a standard oatmeal–sugar–yeast–agar *Drosophila* medium throughout the experiment. The use of a newly established population for a laboratory selection experiment has many advantages (Harshman & Hoffmann, 2000; Sgrò & Partridge, 2001). However, in the current experiment, the populations may adapt to laboratory conditions in parallel to adapting to environmental change. Therefore, the focus of this study is not on absolute changes in the phenotypes assessed, but in the divergence between replicate populations of the ramping and control regimes.

Thermal regimes

Three replicate populations were initiated, each from 250 males and 250 females sampled from the mass bred population, such that each replicate population was established to represent the genetic pool of 589 isofemale lines. Two consecutive 24-h egg-laying periods were used to duplicate each replicate population into a ramping and a control replicate. Each replicate population was maintained in ten 100-mL glass bottles containing 35-mL medium, each with 50 flies. The ramping and

control regimes were exposed to daily variation in temperature following a Gaussian function (Fig. 1). The control regime was simulated in an incubator with mean temperature (mean) of 24.8 ± 0.5 °C, a night-time temperature (night) of 23.5 ± 0.5 °C and a daily temperature peak (peak) of 27.5 ± 0.5 °C. This was the same in all experimental generations. In the incubator running the ramping regime, the mean, night and peak temperatures were increased by 0.3 °C in each generation such that in generation 10 the mean, night and peak temperatures were 27.8, 26.5 and 30.5 °C, respectively (Fig. 1 and Supporting Information Table S1 for details), that is, 3 °C higher than in generation 0. Throughout the experiment, we monitored the temperature in each of the two incubators using data loggers (iButton® Data Loggers; Maxim, Sunnyvale, CA, USA). The light intensity followed the same daily pattern as temperature, such that low light intensity and low temperatures coincided. The humidity inside the bottles was high and stable as the bottles were sealed with stoppers allowing very low flux of water vapour. We estimated the humidity inside the bottles in generation 20, where the largest temperature differences between treatments were present, using data loggers (iButton® Data Loggers; Maxim). The average relative humidity ± SD was 91.9 ± 1.8% in the control regime and 91 ± 2.7% in the high-temperature regime. To establish a new generation and to maintain density within each bottle approximately constant across generations, 5- to 8-day-old flies laid eggs in the two thermal environments in a 12-h period in one set of bottles followed by a 24-h laying period in another set of bottles. Low-density bottles were used to establish the next generation. In this way, density was roughly controlled.

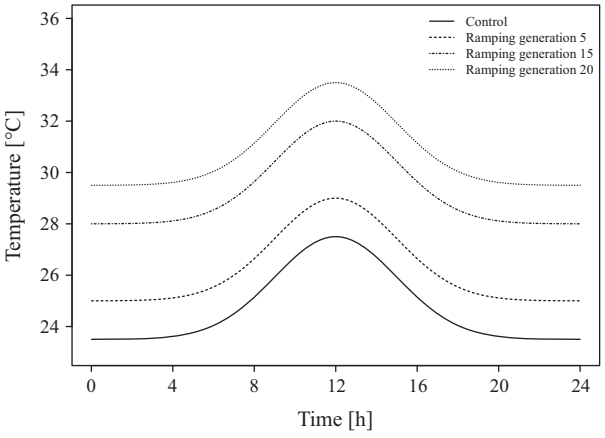

Fig. 1 Illustration of the daily temperature change for the control regime in all generations and for the ramping regime in the three generations where phenotypic tests were performed. The daily temperature followed a Gaussian function $\text{Temperature(time)} = a \times e^{-\frac{(\text{time}-b)^2}{2c^2}} + d$, with the amplitude ($a$ = 4), the time of the peak (b = 12), the night-time temperature (d) and the width (SD) of the distribution (c = 3.16).

When all flies of a replicate population had emerged, flies were sexed under CO_2 anaesthesia to establish equal sex ratios with a population size of 500. Thus, no artificial selection was performed.

Phenotypic assays

In generations 5, 15 and 20, an additional batch of eggs was collected by egg laying for a 24-h period in bottles. These bottles were transferred to 25 °C and 12 : 12 L : D for two generations, to control for cross-generational parental and developmental effects (Levins, 1969; Crill *et al.*, 1996). Hereafter, the flies were density-controlled with 40 eggs per vial, and eclosing adults were subjected to the four phenotypic assays described below. Flies were sexed under CO_2 anaesthesia when necessary and allowed to recover for 2 days prior to testing according to recommendations in Nicolas & Sillans (1989) and Smith & Huey (1991).

Egg-to-adult viability

Egg-to-adult viability for replicate populations from ramping and control regimes was assessed in the thermal regime reached by the ramping regime in the generation of transfer (Supporting Information Table S1). Eggs were moved into vials with 7 mL of food (20 eggs in each of 16 vials in generation 5 or in each of 20 vials in generation 15 and 20, per replicate population). The eggs were then allowed to develop at the selected temperature, and the number of adult flies that emerged was counted.

Fecundity

Fecundity was assessed in all replicate populations, over a 10-day period, in the same thermal regime as the egg-to-adult viability assays. Vials containing 40 eggs were left to hatch in the climate chamber. One day after hatching, 12 vials per replicate population were set up without the use of anaesthesia and transferred to the given test temperature. Each vial contained two males, one female and a plastic spoon with 1.3 mL of food. The flies received a new spoon every day at 1 p.m., and the number of eggs on each spoon was counted. Approximately a quarter of all vials was discarded and removed from the statistical analysis, as either the female or both males were dead or lost during the 10-day egg-laying period. On average, nine vials were analysed from each replicate population.

Desiccation resistance

The desiccation resistance of females (n = 20) from each replicate population was assessed by measuring their ability to cope with low humidity. The females were placed individually into 6-mL vials sealed with gauze and put into a sealed glass tank containing silica gel that lowered the relative humidity to 0–5%. The test was performed with constant light at 24.5 ± 1 °C. Flies were

checked every hour. When no movement could be provoked with a flashlight, a fly was considered dead. Flies were 7–8 days old at the time of testing.

Heat knock-down resistance
Females ($n = 20$) from each replicate population were tested for their heat resistance by measuring their ability to withstand a constant high temperature. The females were placed individually into sealed 6-mL glass vials and lowered into a glass tank containing 37.2 °C water. When no movement could be provoked with a flashlight or with mechanical stress (gently knocking on the vials with a stick), a fly was considered dead or in a coma, and the time since the fly entered the water bath was noted. The flies were 6–7 days old at the time of testing.

Monitoring nucleotide diversity

Genomic DNA from pooled samples of 25 females was extracted using a CTAB-based method (Junghans & Metzlaff, 1990) and by a modified salting out procedure (see Supporting Information Data S1 for details). Samples were obtained from all replicate populations in generation 5, 10, 15 and 22 (20 generations in the given thermal regime and two generations at 25 °C constant). RAD tag libraries for Illumina sequencing were created by digesting the DNA with the 6-cutter enzyme *Eco*RI (New England Biolabs, Ipswich, MA, USA) for 1 h and 12 rounds of PCR amplification (Miller *et al.*, 2007). The 45-bp single-end reads were mapped to the *D. melanogaster* reference genome (v5.38) with BWA using DistMap (v0.5.8c; Li & Durbin, 2009; Pandey & Schlötterer, 2013), and local realignment was performed with GATK (v2.4-7; McKenna *et al.*, 2010). Only reads that mapped in the pattern expected from a restriction site were accepted (two stacks overlapping with four nucleotides). Transposable elements and simple sequence repeats were removed with RepeatMasker 3.29 (Smith *et al.*, 2012). Average coverage per sample was 349–906 (mean ± SD: 578 ± 143). Restriction sites and indels were also removed. Only positions with a minimum coverage of 200 in all samples were used for calculation of π. If the coverage exceeded 200, it was adjusted by downsampling without replacement. In calculation of π, variant sites were called independently for each sample, with the following calling criteria: (i) only nucleotides with phred quality scores at the minimum of 36 were included (Boitard *et al.*, 2012); (ii) minor alleles present in at least four reads; (iii) sites where the sum of the standardized coverage for each stack across all samples exceeded three standard deviations above the median sum were discarded. π was calculated for windows of 3 Mb across the genome. PoPoolation (Kofler *et al.*, 2011a) and PoPoolation2 (Kofler *et al.*, 2011b) were used for the data treatment and calculation of π.

Statistical analysis

The analysis of all traits was performed in R (v3.03; R Core Team, 2014) using generalized linear mixed models in the 'lme4' package (v1.1-5; Bates *et al.*, 2014). For each of the five traits assessed, we produced a full model including the fixed effects: generation (5, 15 and 20) (z-transformed), thermal regime (ramping and control) and their interaction. Replicate population was included as a random effect, with random slopes between generations (Schielzeth & Forstmeier, 2009), and further random effects, such as day, were included if appropriate for the given analysis. When testing for differentiation between the two thermal regimes, we chose a priori to reduce the number of tests by only considering two possible findings: (i) a significant interaction between generation and thermal regime and (ii) a significant effect of thermal regime in the final generation (generation 20). Desiccation and heat resistance (log-transformed), as well as total fecundity and π, were analysed assuming Gaussian distributions. The assumptions of normality of residuals and homogeneity of variances were fulfilled. For desiccation resistance, an ANOVA was performed for the data from generation 20. Here, the data from the three replicate populations were pooled within regimes as no significant difference was observed between replicate populations (data not shown). Egg-to-adult viability data were modelled assuming a binomial distribution with the logit link function. The full models, including all fixed effects, were validated by a likelihood ratio test (assuming a χ^2 distribution with d.f. = $\text{d.f.}_{\text{full}} - \text{d.f.}_{\text{nested}}$) against the null model, which only contained random effects. After validation of the full model, the *P*-values for the relevant terms were obtained by reduction of the full model. If the full model failed to explain the data better than the null model, only the *P*-value for the comparison of the full and null model was reported (Mundry & Nunn, 2009).

Results

Egg-to-adult viability

No significant differentiation across generations and between ramping and control regimes in egg-to-adult viability across generations was detected at high temperatures (Fig. 2). The full model, including the interaction between regime and generation as the higher term, failed to explain the data ($\chi^2_3 = 3.73$, $P = 0.28$). Furthermore, analysis of the 20 generation found no difference in egg-to-adult viability between the controls and the selected replicate populations ($\chi^2_1 = 0.22$, $P = 0.64$).

Fecundity

There was a significant decrease in fecundity across generations and thus across test temperatures for all

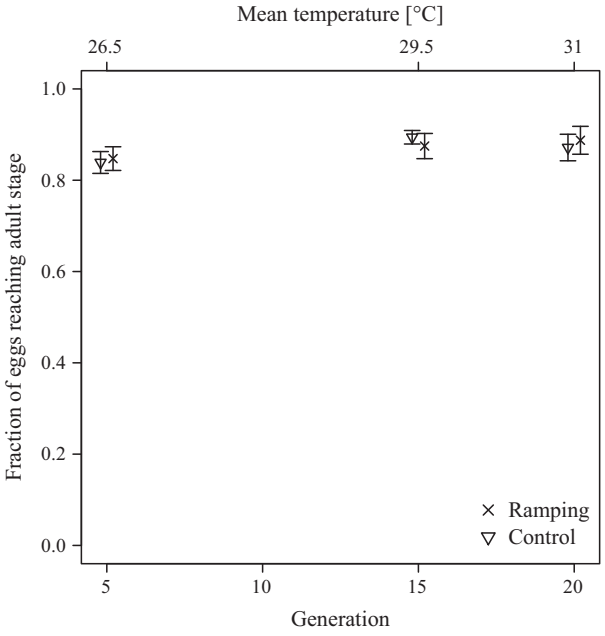

Fig. 2 Egg-to-adult viability across generations. The mean temperature of the thermal regime at which the test was performed is indicated at the top of the figure.

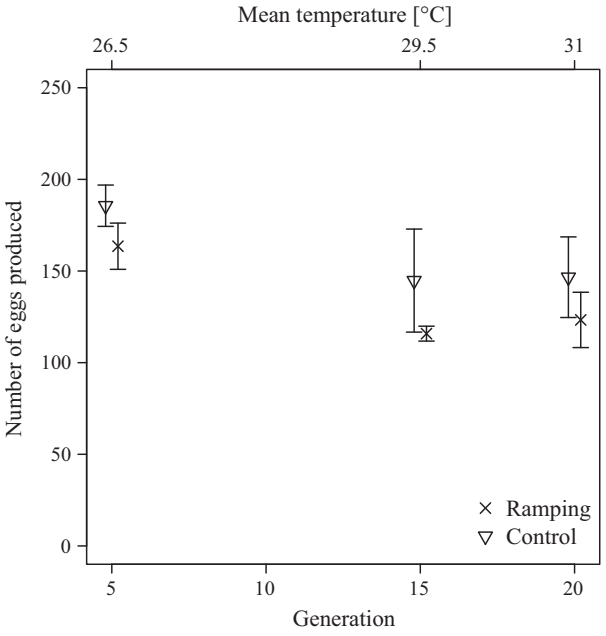

Fig. 3 Total fecundity measured across 10 days in generation 5, 15 and 20. The mean temperature of the thermal regime at which the test was performed is indicated at the top of the figure.

replicate populations ($\chi^2_1 = 5.83$, $P < 0.05$, Fig. 3). However, no significant differentiation between replicate populations from control and ramping regimes was detected in fecundity assessed at high temperatures, neither across all generations ($\chi^2_1 = 0.01$, $P = 0.92$), nor in generation 20 ($\chi^2_1 = 1.08$, $P = 0.30$).

Desiccation resistance

Desiccation resistance was assessed at room temperature, which differed slightly across test days, making direct comparisons of absolute values problematic. Therefore, change in desiccation resistance in replicate ramping populations is reported relative to the mean desiccation resistance of replicate control populations (Fig. 4). In contrast to the control regime, increased desiccation resistance was observed in the selection regime throughout the experiment; however, the interaction between generation and thermal regime was not significant ($\chi^2_1 = 3.34$, $P = 0.067$; Fig. 4). Significantly higher desiccation resistance was detected in the replicate populations exposed to increasing temperatures compared with replicate control populations in generation 20 ($F_{1,114} = 4.05$, $P < 0.05$).

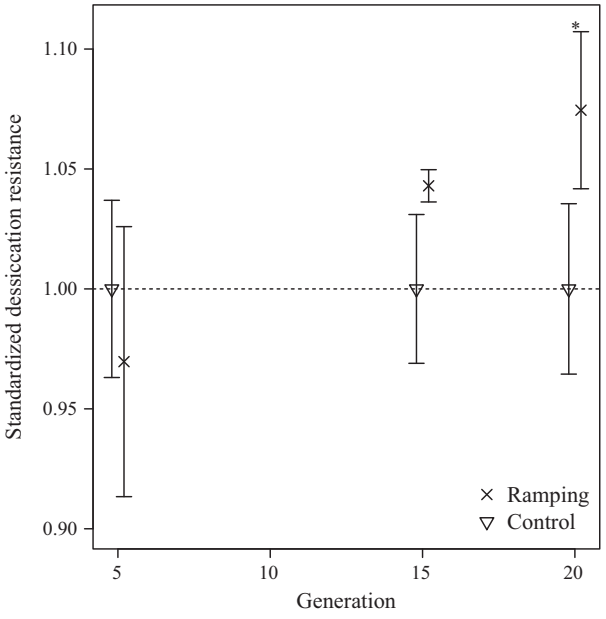

Fig. 4 Desiccation resistance across generations. Data are standardized according to mean value of the replicate control populations. *$P < 0.05$.

Heat knock-down resistance

Figure 5 shows the divergence in heat knock-down resistance between replicate populations from the ramping regime and control regime. Data are presented relative to the replicate control population mean, as small differences in experimental environments between generations (± 0.4 °C) will affect absolute

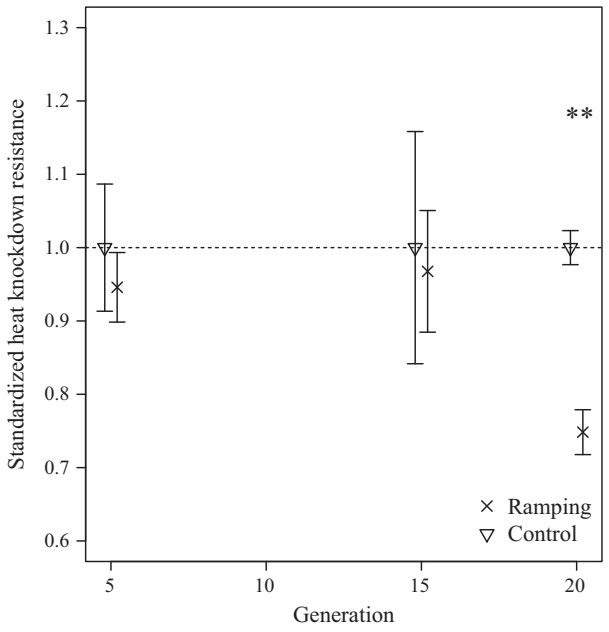

Fig. 5 Heat knock-down resistance across generations. Data are standardized according to mean value of the replicate control populations. $^{**}P < 0.01$.

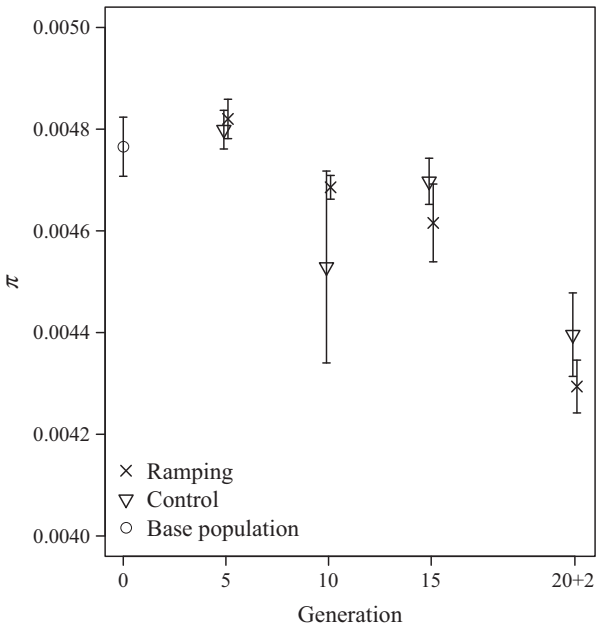

Fig. 6 Change in nucleotide diversity (π) across generations for the base population and the replicate populations of the control and the ramping regime. π was calculated for windows of 3 Mb across the genome.

values making a direct comparison problematic. The replicate control populations were significantly more heat resistant than the replicate ramping populations in generation 20 ($\chi^2_1 = 9.30$, $P < 0.01$, Fig. 5). The strong differentiation in heat resistance in generation 20 drove the interaction between thermal regime and generation close to significance ($\chi^2_1 = 3.12$, $P = 0.077$).

Genomic data

Replicate populations from both regimes experienced a minor gradual decrease in π from the start to the end of the experiment ($\chi^2_1 = 10.44$, $P < 0.01$, Fig. 6). However, we found no significant interaction between generation and ramping regime in π ($\chi^2_1 = 0.52$, $P = 0.47$, Fig. 6), and thus no indication of different rates of change in π between control and ramping regimes. Furthermore, π did not differ between control and ramping regimes at the end of the experiment in generation 22 ($\chi^2_1 = 0.31$, $P = 0.57$, Fig. 6).

Discussion

Based on previous laboratory studies showing adaptive evolutionary responses to selection for increased heat and desiccation stress and a positive phenotypic and genetic correlation between the two traits, we predicted that the ability to tolerate heat and desiccation would increase in replicate populations exposed to increasing temperatures over 20 generations (Service *et al.*, 1988; Hoffmann & Parsons, 1989a; Huey & Kingsolver, 1993; Cavicchi *et al.*, 1995; McColl *et al.*, 1996; Bubli *et al.*, 1998; Bubliy & Loeschcke, 2005). Desiccation resistance did increase in our replicate populations exposed to increasing temperatures but no evidence for increased heat resistance was observed.

An absence of increased heat resistance in replicate populations exposed to increasing temperatures over time is supported by evidence of constrained upper thermal limits in some *Drosophila* populations and species (Gilchrist & Huey, 1999; Kellermann *et al.*, 2012), and a lack of genetic variation for heat shock survival of adults previously observed in a Danish population of *D. melanogaster* (Krebs & Loeschcke, 1994). The DNA decay hypothesis (Hoffmann & Willi, 2008) may explain a lack of adaptive genetic variance for increased heat knock-down resistance in the population investigated in the current study. Under this hypothesis, genetic variation for coping with very high-temperature events will be lost due to a lack of selection maintaining variation for heat resistance in populations. Lack of genetic variation for increased heat resistance in the population may also be explained by strong historical directional selection for increased heat tolerance, whereby the upper evolutionary limit for heat tolerance is reached (Hoffmann, 2010). This hypothesis does, unlike the decay hypothesis, allow for the maintenance of genetic variation for lowered resistance as observed in this study and thus may be a more likely explanation for the observed results.

Another possible explanation for the observed decrease in heat resistance is the difference between the acute stress imposed in the assay used to assess heat resistance and the slow daily ramping of the ramping regime, in which recovery from daily heat stress might be of major importance for survival. Therefore, adaptive plastic physiological responses may have evolved in the ramping regime. These responses will have little time to be induced in the heat knock-down assay. Thus, trade-offs between different types of heat resistance (Hoffmann *et al.*, 1997; Berrigan & Hoffmann, 1998) could influence the outcome of selection (Barton & Turelli, 1989) and may explain the decreased heat resistance observed in our laboratory evolution experiment where fitness is shaped by many interacting traits. In future studies, the use of ramping assays to assess changes in heat resistance might reveal more ecologically relevant information on the potential of natural populations to respond evolutionarily to future climatic conditions.

Trans-generational effects can bias phenotypic estimates of heat resistance (Stephanou & Alahiotis, 1983; Crill *et al.*, 1996). However, the bias is not always in the same direction as the selection target, for example, when selecting for increased cold resistance Watson & Hoffmann (1996) observed a decrease in cold resistance when no generation of relaxed selection was allowed before testing, likely caused by accumulating cross-generational stress-induced damage. This pattern is similar to our heat resistance results; however, the two generations of relaxed selection prior to trait assessments used in our study make cross-generational effects unlikely. Thus, we do not expect trans-generational effects to explain our results.

Similar to heat resistance, we found no evidence of an adaptive response in the two fitness surrogates, egg-to-adult viability and fecundity, both estimated at high temperatures (Figs 2 and 3). Egg-to-adult viability for both regimes remained constant across generations, whereas fecundity decreased at the high temperatures in generation 20. We expected these highly complex life history traits to respond to natural selection as they encompass many morphological and physiological traits with moderate heritabilities in *D. melanogaster* (Mousseau & Roff, 1987; Huey *et al.*, 1991; Gilchrist *et al.*, 1997; Hoffmann, 2000). Slow ramping temperatures, as experienced on a daily basis in the thermal regimes used in this study, unlike previous studies (Huey *et al.*, 1991; Gilchrist *et al.*, 1997), have been suggested to increase the phenotypic variance such that heritabilities for heat resistance are reduced (Terblanche *et al.*, 2007; Chown *et al.*, 2009; Hoffmann, 2010; Mitchell & Hoffmann, 2010). This may explain the lack of evolutionary response in the life history traits studied in this experiment and emphasizes the importance of using temperatures that reflect natural conditions in studies of adaptation to climate change.

In contrast to heat resistance and the fitness traits fecundity and egg-to-adult viability, replicate populations exposed to increasing temperatures across generations were more desiccation resistant than replicate control populations. Although there was no difference in the relative humidity between the two thermal regimes (see Materials and Methods), desiccation stress can arise from temperature differences (Anderson, 1936). At a relative humidity of 91.4% in both temperature regimes in generation 20 (mean of the two regimes), average vapour pressure deficit (VPD) will be higher in the ramping regime (VPD = 2.63 mm Hg) than in the control regime (VPD = 2.01 mm Hg), illustrating why evolution of desiccation resistance might be linked to the evolution of changed temperature resistance. The combined responses of an increase in desiccation resistance and a decrease in heat resistance of the replicate ramping populations compared to the replicate control populations contradicts previous reports of positive genetic and phenotypic correlations between the two traits (Hoffmann & Parsons, 1989b; Bubliy & Loeschcke, 2005; Bubliy *et al.*, 2012). The combined responses could be suggestive of adaptation. However, we find it unlikely that decreased heat resistance is adaptive under the high temperatures of the ramping regime, and as these two responses did not translate into an improved fitness via an increased fecundity, we doubt the adaptive nature of these responses.

The gradual temperature increase in the ramping regime followed the increase in temperature predicted per decade by IPCC (2007). This increase in temperature corresponds to the increase experienced by species with generation times of approximately 10 years. The chosen increase in temperature, and the fact that we have been able to control the population size and the daily variation in temperature and to rule out genetic drift as the explanatory parameter for the differentiation between treatments (Fig. 6) means that our data are relevant in a climate change perspective. Experimental design such as number of generations, population and species used and strength of selection (rate of temperature change) can affect the outcome of experimental evolution. We focused on creating an ecological relevant experimental setup, and the conclusions should always be seen in the light of the specific set-up and its restrictions. Other studies have also taken steps to increase the complexity and ecological relevance of their experiments using slow ramping assays to assess thermal tolerance and investigating experimental animals under fluctuating environments (Mitchell & Hoffmann, 2010; Hallsson & Björklund, 2012). Given the commonly observed strong effect of experimental conditions on the conclusions drawn in studies of thermal adaptation in ectotherms, we argue that a continued focus on ecological relevance in such studies is needed if they are to provide relevant information in respect to impacts of climate change on natural populations.

In summary, we did not find clear-cut adaptive responses to increasing temperatures. We interpret our results as an example of how intricate phenotypic evolutionary responses may arise in ecologically relevant and thereby complex test conditions. Possible adaptive responses to increasing temperatures in natural populations may consist of small changes in a range of traits, as the change in temperature will interact with other environmental parameters such as humidity. Estimating the ability of organisms to adapt to climate change demands not only ecologically relevant laboratory environments during the experiment, but also requires ecologically relevant phenotypic assays to assess the adaptation. Our data provide yet another example that the ability to tolerate high temperatures may be evolutionary constrained in some ectotherms (Kellermann *et al.*, 2012; Araújo *et al.*, 2013; Hoffmann *et al.*, 2013) and highlight the immediate challenges for many species when faced with increases in temperature in the future, as well as for researchers attempting to quantify the evolutionary potential of natural populations.

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Supporting information

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

Table S1 Rearing temperatures at four representative experimental time points.

Data S1 DNA extraction with modified salting out.

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