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Direct and correlated responses to artificial selection for high and low knockdown resistance to high temperature in *Drosophila buzzatii*

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ABSTRACT

Knockdown resistance to high temperature (KRHT) is a genetically variable trait for thermal adaptation in insects. Selection for KRHT may affect a number of fitness components as well as resistance to several forms of environmental stress. To test for heritable (co)-variation in KRHT, we examined direct and correlated responses to bi-directional selection on this trait in *Drosophila buzzatii*. Replicated lines were artificially selected for decreased and increased KRHT. After 12 generations of artificial selection, lines diverged significantly for high KRHT only. Starvation resistance increased in two lines that strongly responded to selection for high KRHT, and these two lines also showed relatively longer chill-coma recovery time. Developmental time and body size showed no correlated responses to KRHT-selection. These results suggest that KRHT is a heritable trait that can evolve towards increased thermotolerance with no genetic trade-offs associated to starvation resistance, developmental time and body size.

1. Introduction

Thermal stress is an increasingly important agent of natural selection because of global warming, and extinction risk can increase with reduced genetic variation for thermal adaptation (Hoffmann et al., 2003; Bochdanovits and De Jong, 2003; Sørensen et al., 2005; Norry et al., 2007; Deutsch et al., 2008; Hoffmann and Willi, 2008; Williams et al., 2008; Bowler and Terblanche, 2008). Consequences to fitness after thermal stress can be related to multiple effects on a number of fitness-related traits (Hoffmann and Parsons, 1991; Hoffmann et al., 2003; Norry et al., 2006; Loeschcke and Hoffmann, 2007; Karl and Fischer, 2009; Steigenga and Fischer, 2009). For instance, it has been suggested that thermal-stress selection can have a major impact on the resistance level to several other stresses (Hoffmann et al., 2003; Bubliy and Loeschcke, 2005; Folk et al., 2006; Mori and Kimura, 2008).

Experiments of artificial selection in *Drosophila* have recently been used as an experimental evolutionary tool to identify the relevant traits that are most likely to be involved in adaptation to environmental temperature in ectotherms (reviewed in Hoffmann et al., 2003; Bowler and Terblanche, 2008). One of the main advantages of artificial selection experiments is the possibility to evaluate not only direct, but also the correlated responses to selection (Harshman and Hoffmann, 2000). Artificial selection experiments have revealed moderate to relatively high levels of heritability for resistance to high-temperature stress in *Drosophila melanogaster* (reviewed in Hoffmann et al., 2003; Reusch and Wood, 2007). Remarkably, most artificial selection programs were mainly performed in *D. melanogaster*, though recent studies have also addressed the question whether or not results in *D. melanogaster* are consistent across species (reviewed in Hoffmann and Willi, 2008). Further, artificial selection on thermal-stress traits was generally performed in only a single direction (mainly, for increased resistance), but selection for decreased resistance to heat stress can also be informative as the selection response can often be asymmetrical for thermal-stress traits (Gilchrist and Huey, 1999; Folk et al., 2006; Norry et al., 2007; Mori and Kimura, 2008; Gomez et al., 2009; Bertoli et al., 2009).

One of the ecologically most relevant traits for measuring the response to selection on heat stress in small insects is knockdown resistance to high temperature, hereafter referred to as KRHT (Huey et al., 1992; Hoffmann et al., 2003; Sørensen et al., 2005). The adaptive significance of KRHT phenotypes is partly based on the fact that heat-knocked insects remain temporarily incapacitated at knockdown (stressful) temperature (Huey et al., 1992). In addition, individuals that are less susceptible to heat stress should not be heat-knocked so often as heat-susceptible individuals, resulting in a demographic advantage for reproduction under relatively high temperature (Loeschcke and Hoffmann, 2007; Kristensen et al., 2007).

In artificial selection experiments, KRHT has the advantage of allowing selection in both directions (low *versus* high resistance) in a simple way, in contrast to other related traits such as survival to heat stress, where the heat-susceptible individuals cannot be directly selected (Huey et al., 1992). This issue of bi-directional

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selection is of major interest to test for genetic correlations between traits, as bi-directional selection on KRHT can increase the probability to detect correlated responses by considering the two possible directions of artificial selection rather than only one single direction of selection.

Here, we examine the response to bi-directional selection for knockdown resistance to high temperature (KRHT) in the cactophilic *Drosophila buzzatii*. This species had colonized diverse geographical areas from lowlands to relatively high elevations with a high habitat specificity by using rotting tissues of *Opuntia* species as trophic and breeding resources on different continents, where KRHT and other stress-related traits exhibit clinal variation with an altitude of population (Sørensen et al., 2005; Sarup et al., 2006; Sambucetti et al., 2006; Norry et al., 2006). Therefore, *D. buzzatii* is an interesting model to test for direct and correlated responses to selection on KRHT. Replicated lines were selected in both possible directions: increased and decreased KRHT. Other replicated lines were maintained without any selective stress treatment (controls). After twelve generations of KRHT-selection, possible correlated responses were examined for multiple traits, including chill-coma recovery, starvation resistance, developmental time and body size. Chill-coma recovery is likely to be affected by KRHT-selection, if there is any strong trade-offs between heat and cold resistance, as an apparently pleiotropic quantitative trait locus (QTL) showed antagonistic associations between heat and cold resistance (Morgan and Mackay, 2006; Norry et al., 2007; 2008). Regarding developmental time, this trait has been shown to vary clinally with an altitude in *D. buzzatii,* suggesting that developmental time can also respond to thermal-stress selection (Sambucetti et al., 2006).

2. Materials and methods

2.1. Base population

The lines used in this study were selected from a laboratory stock that was set up in April 2003 from a single wild population sampled at Chumbicha (28.52°S, 66.15°W), Province of Catamarca, Argentina, as described in Norry et al. (2006). Briefly, wild flies were collected using banana baits. Twenty two isofemale lines were derived from the wild flies and crossed (pooled in a mass population) to set up the laboratory stock used in this study. In order to estimate KRHT in recently derived cultures from wild flies, a sample of flies was measured for KRHT after only seven laboratory generations (see below for measurement method). However, to reduce any possible confounding effects of laboratory adaptation (Sgrò and Partridge, 2000; Baldal et al., 2006; Scannapieco et al., 2009), our laboratory stock was maintained at 25 °C on a 12:12 light:dark cycle for another 30 generations before the start of thermal-stress selection, with five standard bottles and a mean number of about 70 flies per bottle. Standard bottles were 125 ml bottles containing 30 ml of instant *Drosophila* medium.

2.2. Heat-knockdown resistance and selection regime

Three sets of replicated lines were set up from the above described laboratory stock. One set were the control (non-selected) lines which were maintained as three independent replicates (denoted C1, C2 and C3) in four standard bottles each (with 80–100 flies per bottle, 1:1 sex ratio) at 25 °C on a 12:12 light:dark cycle. The other two sets were selected for knockdown resistance to high temperature (KRHT) in three replicated lines. One of these sets was selected for decreased KRHT (denoted K1−, K2−, K3−) and the other set was selected for increased KRHT (denoted K1+, K2+, K3+). Flies aged at 1–2 days were collected from the base stock and placed in standard bottles with fresh medium. At the age of 3–4 days 150–200 flies (1:1 sex ratio) were released within a knockdown tube (65 cm longitude, 3.8 cm diameter) at 37.5 ± 0.1 °C (Norry et al., 2008). KRHT was scored in intervals of 30 s as described in Norry et al. (2008). To select for reduced knockdown time, the first 30% of flies that were knocked-down by heat was used as parents of the next generation. To select for extended knockdown time, the last 30% of flies that were knocked-down by heat was used as parents of the next generation, in every generation of selection.

For each replicate line, 45–60 adult flies were used as parents of the next generation by allowing them to lay eggs for 4 days in standard bottles with fresh instant medium (with 15–20 flies per bottle and three bottles per line). All cultures were maintained at 25 °C on a 12:12 light:dark cycle. After 4 days, all adult flies were removed from each culture bottle. Selection was performed every generation during the first seven generations of selection. Thereafter, selection was continued every other generation to allow the recovery of lines and increase the population size for one generation. In total, 12 generations of KRHT-selection were performed. KRHT (in seconds) was scored both in the G7 and G12 generations of selection, using the same stress temperature (37.5 ± 0.1 °C) as indicated above for the selection regime. After our selection regime was finished (G12), replicated lines for each selection treatment were expanded for one generation and measured for several traits as described above. The direct response on KRHT was also measured in among-replicate hybrid flies to reduce any possible inbreeding effects in the response to selection (Kristensen et al., 2009). To do this, replicated lines were crossed within each selection regime (hereafter referred to as F3-hybrid) by transferring a sample of 30 males and 30 virgin females from each replicate line into one standard bottle with fresh medium in the same environmental conditions as described above throughout the experiment. The F2 was allowed to lay eggs for 4 days in standard bottles and 3–4-days old flies emerged from these cultures (F3-hybrids) were measured for KRHT.

2.3. Chill-coma recovery

Chill-coma recovery (CCR) was measured as the time to recover after an exposure to 0 °C (David et al., 1998). To obtain experimental flies, 20 flies from each line (four bottles per line) were allowed to lay eggs at 25 °C, under the same culture conditions as mentioned above for KRHT. Newly emerged flies were collected within 1 day of emergence and placed in vials with fresh medium at 25 ± 0.5 °C. After 48 h all experimental flies were sexed on ice (anesthesia), transferred to empty vials (30 flies per sex and line) and placed immediately in a cold chamber containing a water/ice mixture in a cold room at 4 °C. After 15 h at 0 °C, the vials were moved back to 25 °C and CCR time was scored in seconds by recording the amount of time until an individual was able to stand on its legs.

2.4. Starvation resistance

Starvation resistance (SR) for each replicated line was measured in all selected and control lines. Adult flies from each replicated line were allowed to lay eggs in the same conditions as described above for other traits and emerged flies were aged to 1–2 days in vials with fresh medium. After 24 h flies were transferred to new vials containing 6 ml of 2% agar–agar water medium, without any other source of food (as no bacterial or fungal growth was present) to induce starvation without

desiccation stress (20 flies per vial and 10 vials per replicate line, with approximately 1:1 sex ratio). All experimental vials were placed at 25 °C on a 12:12 light:dark cycle and checked for dead flies three times a day (09:00, 15:00 and 21:00). Dead individuals were removed and sexed. The number of vials was gradually reduced as deaths occurred with surviving adults being kept at a density as close as possible to 20 per vial.

2.5. Developmental time and body size

Adult flies from each replicated line were allowed to lay eggs in small spoons with agar plus yeast paste medium. First-instar larvae were collected, transferred at a density of 100 into standard bottles (125 ml bottles with 30 ml of culture medium, 2–3 bottles per replicate line) and kept at 25 °C on a 12:12 light:dark cycle. The number of males and females emerging from these standardized cultures was scored every 6 h. Larva to adult developmental time (DT) was estimated over the mid-point of each successive interval. To estimate body size, 15 females plus 15 males were randomly chosen from the total of experimental flies that emerged for each line. Body size was indexed as thorax length (TL) by measuring the distance between the anterior margin of the thorax and the posterior tip of the scutellum at 50× magnification with an ocular micrometer.

2.6. Statistical analysis

A nested two-way ANOVA was performed for each trait using sex and type of line as fixed factors and replicate within line as a random factor. This is a very conservative ANOVA design because only three replicates were used per line. When the line factor was significant, *post-hoc* pair-wise comparisons were performed using the Tukey's Honestly Significant Difference (HSD) test, which is based on the error term from the respective ANOVA. KRHT was not normally distributed even if using different transformations. For analysis, KRHT was ln-transformed, as this transformation improved normality and, more importantly, removed dependence of variances on means (Pearson correlations between means and variances were non-significant across lines after ln-transforming data, $P > 0.20$). Reported analyses for other traits were robust for non-transformed data in exploratory analyses which showed no correlations between variances and means (results not shown). Additionally, non-parametric Mann–Whitney tests were made for KRHT by pairing either Ki− and Ci or Ki+ and Ci replicates according to the line numbers that were assigned arbitrarily to them at the start of the experiment, with all three *P*-values being then combined (Sokal and Rohlf, 1981, p. 779; Roper et al., 1993; Norry and Loeschcke, 2002). All analyses were performed with STATISTICA, 1999 version (StatSoft, 1999).

3. Results

Mean values of KRHT are shown in Fig. 1 for each line both in the G7 and G12 generations of selection. Lines selected for decreased (K−) and increased (K+) KRHT have successfully diverged by bi-directional selection on KRHT. Although the K− and K+ lines were not significantly different in G7, differences in KRHT were statistically significant between K− and K+ lines in G12 (Table 1): the Tukey's test showed that differences were significant between K− and K+ ($P < 0.05$), even though C flies were not significantly different from either K− or K+ in this very conservative test. In spite of substantial variation among replicates within treatments (which is a common result in almost every selection experiments), individual *post-hoc* (Tukey) tests showed homogeneous groups for some lines (Fig. 1).

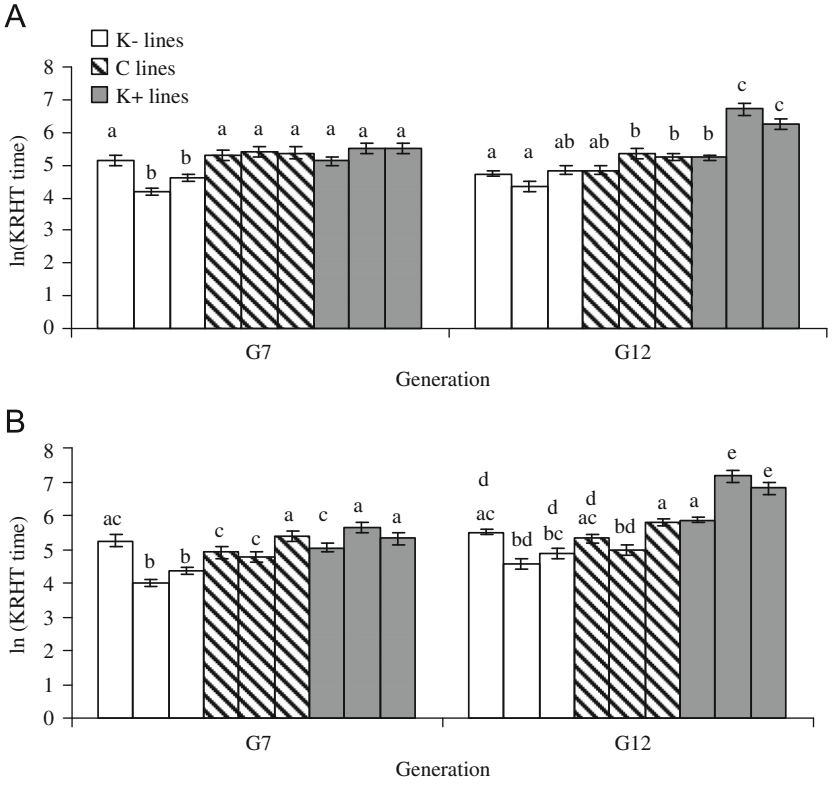

Fig. 1. Mean values (±SE) of knockdown resistance to high temperature (KRHT, in seconds, ln-transformed) for lines selected for decreased (K−) and increased (K+) KRHT and control lines (C). Mean values are presented for generations 7 and 12 of selection. Bars that share a letter are not significantly different ($P > 0.05$). (A) Males and (B) Females.

Specifically, two out of three K+ lines were statistically different from all their control (and K−) lines in the direction expected from selection in G12 (Fig. 1). In addition, Mann–Whitney tests were made by pairing Ki+ and Ci replicates according to the line numbers that were assigned arbitrarily to them at the start of the experiment, with a significant combined *P*-value (<0.05) indicating that K+ lines chiefly diverged from their control lines. Furthermore, F3-hybrid showed that K+ flies were more heat-resistant than both C and K− flies (Fig. 2A,B, ANOVA with (1) line and (2) sex as a fixed factors: (1) $F_{2,\ 290}=17.88^{***}$; (2) $F_{1,\ 290}=0.41$; (1)×(2) $F_{2,\ 290}=0.58$). The Tukey's test was highly significant between K− and K+ ($P<0.001$) as well as between K+ and C ($P<0.001$), but not between K− and C. KRHT of the F3-hybrid lines was also compared with the KRHT of recently derived cultures (RDC) from wild flies (at the G7 laboratory generation). This comparison showed that RDC flies differed only with the K+ line, indicating that laboratory selection for K+ was successful to increase KRHT (Fig. 2A, same ANOVA as above: (1) $F_{3,\ 321}=12.9^{***}$; (2) $F_{1,\ 321}=0.57$; (1)×(2) $F_{3,\ 321}=0.4$. The Tukey's test between K+ and the base population was significant with $P<0.05$).

Chill-coma recovery (CCR) did not differ between K− and K+ individuals (Fig. 3, same ANOVA model as in Table 1: (1) $F_{1,\ 4}=1.07$; (2) $F_{4,\ 646}=10.62^{***}$; (3) $F_{1,\ 4}=1.27$; (1)×(3) $F_{1,\ 4}=0.01$; (2)×(3) $F_{4,\ 646}=4.02^{**}$. $^{**}P<0.01$;$^{***}P<0.001$).

Table 1
Analysis of variance performed to test differences in knockdown resistance to heat among selection and control lines after 12 generations of selection.

Effect	df	MS	F
(1) Line	2	336.62	6.40*
(2) Replicate	6	52.62	23.42***
(3) Sex	1	59.06	8.48*
(1)×(3)	2	4.35	0.62
(2)×(3)	6	6.96	3.10**
Within	1939	2.25	

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

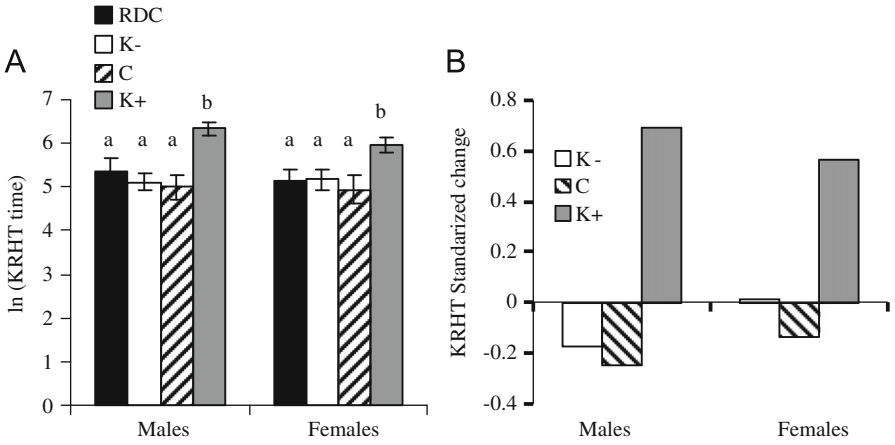

Fig. 2. (A) Mean values (±SE) of knockdown resistance to high temperature (KRHT, in seconds, ln-transformed) for lines originated from crosses between replicated lines (F3-hybrid, see materials and methods) within each selection regime (K− and K+ flies selected for increased and decreased KRHT, respectively), control lines (C) and the recently derived cultures (RDC) from wild flies which were subsequently used to generate our base population (G30) for selection. (B) Standardized differences between each F3-hybrid line and RDC are shown for KRHT (between-line difference in phenotypic units of standard deviation). Bars that share a letter are not significantly different ($P>0.05$).

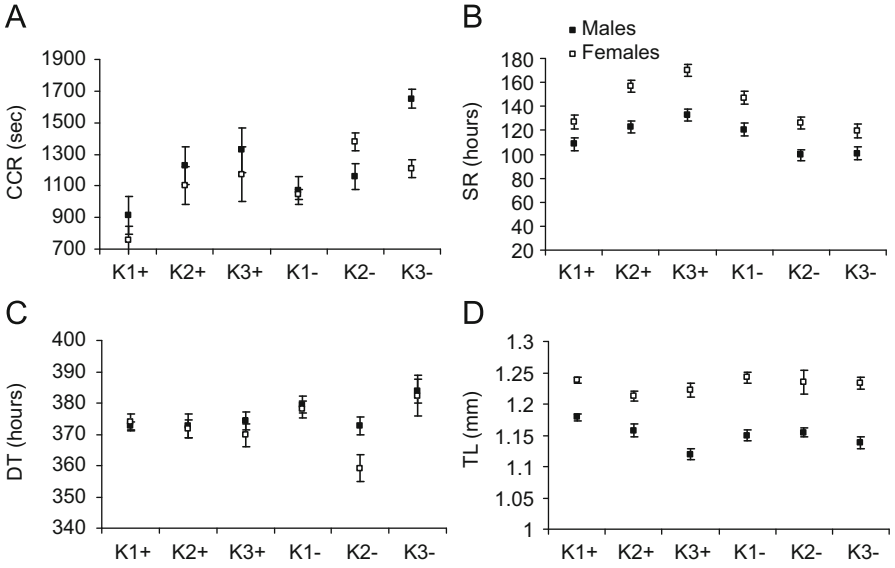

Fig. 3. Mean values (±SE) of (a) chill-coma recovery time (CCR), (b) starvation resistance (SR), (c) developmental time (DT) and (d) thorax length (TL) are shown for lines selected for both decreased (K−) and increased (K+) knockdown resistance to high temperature.

However, CCR time was significantly longer in the two K+ lines that strongly responded to selection for KRHT (K2+ and K3+) than in the K+ line that did not apparently respond to selection (Fig. 3A, Mann–Whitney tests for both K1+ *vs*. K2+ and K1+ *vs*. K3+ for CCR in both sexes in G12: $P<0.05$), perhaps suggesting some within-regime selection trade-off between KRHT and CCR. In addition, starvation resistance (SR) increased in K2+ and K3+ lines when compared to other lines (Fig. 3B, Mann–Whitney tests for SR in females were all significant in pairwise comparisons of both K2+ and K3+ with each of the remaining lines in G12: $P<0.05$), although SR was not significantly different between K− and K+ flies when considering all replicated lines under our experimental conditions (Fig. 3, same ANOVA design as in Table 1: (1) $F_{1,\ 4}=1.93$; (2) $F_{4,\ 1256}=48.15^{***}$; (3) $F_{1,\ 4}=134.15^{***}$; (1)×(3) $F_{1,\ 4}=0.06$; (2)×(3) $F_{4,\ 1256}=1.74$; $^{***}P<0.001$). When analyzing possible correlated responses, it is also useful to know the magnitude of possible shifts in the trait means in phenotypic units of standard deviation. We computed the standardized difference between the more divergent lines averaged over replicates, using $\Delta z'=(X_{k-}-X_{k+})/\mathrm{SD}$ (where X_{k-} is the mean of the trait averaged over replicates within K− lines, X_{k+} is the mean of the trait averaged over replicates within K+ lines, and SD is the standard phenotypic deviation of the trait averaged across all replicate lines). Both CCR and SR resistance exhibited relatively small $\Delta z'$ values when considering the three replicated lines averaged, the sign indicating the direction of the change (−0.153 for males and −0.371 for females for CCR and 0.493 for males and 0.665 for females for starvation, in phenotypic units of standard deviation).

Mean values of developmental time and thorax length are showed for each line in Fig. 3. $\Delta z'$-values computed as above were quite small between K− and K+ lines for both developmental time (−0.236 for males and 0.05 for females) and thorax length (−0.133 for males and 0.289 for females). In addition, neither developmental time nor thorax length showed differences as correlated responses to bi-directional selection on KRHT (same ANOVA design as in Table 1: (1) $F_{1,\ 4}=0.37$; (2) $F_{4,\ 561}=7.52^{***}$; (3) $F_{1,\ 4}=2.1$; (1)×(3) $F_{1,\ 4}=0.76$; (2)×(3) $F_{4,\ 561}=1.31$, for developmental time; (1) $F_{1,\ 4}=0.74$; (2) $F_{4,\ 169}=4.25^{**}$; (3) $F_{1,\ 4}=100.9^{***}$; (1)×(3) $F_{1,\ 4}=1.12$; (2)×(3) $F_{4,\ 169}=2.14$, for thorax length; $^{**}P<0.01$; $^{***}P<0.001$).

4. Discussion

Knockdown resistance to high temperature (KRHT) responded substantially to selection for increased KRHT in *D. buzzatii*, indicating a considerable level of genetic variation for thermal stress in the base population. After 12 generations of selection, lines selected for high KRHT increased heat thermotolerance in both sexes. These results with a cactophilic *Drosophila* are consistent with previous studies in *D. melanogaster*, where KRHT was also found to substantially respond to artificial selection to increase the trait (McColl et al., 1996, Bubli et al., 1998; Gilchrist and Huey, 1999; Bubliy and Loeschcke, 2005; Folk et al., 2006). The selection response was substantial only in one direction of selection, namely to increase KRHT, which was more evident from the analysis with F3-hybrid (Fig. 2A,B). The rationale why K− lines have not responded to selection is unknown. One hypothesis is that higher environmental heterogeneity could be present for KRHT in the less resistant flies, reducing the heritability of the trait when selecting for reduced KRHT. However, this heritability-hypothesis was not supported by the fact phenotypic variances tended to be lower instead of higher in all K− than in C and K+ lines (results not shown). Alternatively, either inadvertent selection (e.g., Gibbs, 1999; MacMillan et al., 2009) or inbreeding depression (Kristensen et al., 2009) could either be possible explanations why K− lines did not respond to our selection regime. In contrast to K− lines, K+ flies diverged from controls which was more evident not only when computing a combined *P*-value (rather than our conservative ANOVA design, Partridge et al., 1995; Norry and Loeschcke, 2002), but also when analyzing F3-hybrid lines. The analysis of the F3-hybrid also showed that selection substantially increased KRHT in the K+ flies as K+ hybrids are more heat-resistant than recently derived cultures (RDC) from wild flies. Furthermore, there is no difference in KRHT between the RDC and C flies (Fig. 2), indicating that the response to selection was not the result of laboratory adaptation (Sgrò and Partridge, 2000; Baldal et al., 2006).

Selection for resistance to any one stress trait is often found to affect other stress resistance traits (reviewed in Hoffman and Parsons, 1991; Hoffmann et al., 2003). However, regarding heat *versus* cold resistance, the present results suggest that selection for knockdown resistance to heat stress has no obvious impact on chill-coma recovery (Fig. 3A). This result with *D. buzzatii* appears to be consistent with some previous observations in *D. melanogaster* (MacMillan et al., 2009), where cold tolerance was not affected as a correlated response to selection on heat resistance (Bubliy and Loeschcke, 2005). Associated changes in KRHT were found neither in *D. melanogaster* nor in *D. buzzatii* selected for chill-coma recovery (Mori and Kimura, 2008; Bertoli et al., 2009), suggesting no obvious trade-off between resistance levels to both extremes of the thermal scale. However, the two replicate lines that strongly responded to increased KRHT in this study (K2+ and K3+) were less tolerant to cold stress by exhibiting significantly longer chill-coma recovery times than the apparently more static line K1+, suggesting some possible within-regime selection trade-off between KRHT and CCR (Figs. 1 and 3A). It is still not possible to conclude that CCR is genetically correlated with KRHT, and cold acclimation might also be an issue to investigate in future analyses on correlated responses to CCR as the cold acclimation was recently found to be substantial in some species (Sisodia and Singh, 2010). In traits that could respond to cold acclimation, a trade-off association was recently found between heat and cold tolerance in a field-caged experiment in *D. melanogaster* (Overgaard and Sørensen, 2008).

Recently, quantitative trait locus (QTL) studies identified a pericentromeric region of the second chromosome affecting both heat and cold resistance in *D. melanogaster* (e.g., Morgan and Mackay, 2006; Norry et al., 2007; 2008; 2009). This region affects both KRHT and chill-coma recovery with antagonistic additive effects between the traits in very different mapping populations, which should be the result of either linkage or pleiotropy (Morgan and Mackay, 2006; Norry et al., 2007, 2008). Likewise, Anderson et al. (2003) found a trade-off between KRHT and CCR within the right arm of chromosome 3 in *D. melanogaster*. As mentioned above, a trade-off association was also recently found between heat and cold tolerance in a field-caged experiment (Overgaard and Sørensen, 2008). In addition, at the geographic level of variation in the same species, KRHT and chill-coma recovery have exhibited opposite latitudinal clines along the east coast of Australia (Hoffmann et al., 2002). However, we found that bi-directional selection on KRHT did not result in correlated responses for chill-coma recovery between K− and K+ selection regimes in *D. buzzatii*, suggesting that there is no simple association between these traits, possibly because of many trait specific genes segregating in populations (Norry et al., 2008). Alternatively, it is possible that alleles with positive effects on KRHT were already in high frequency in the base population for the present experiment (Sørensen et al., 2005), and some alleles in low frequency (e.g., alleles decreasing KRHT) could have been lost by drift, resulting in nearly no responses to artificial selection for reduced KRHT and associated changes in CCR.

Bubliy and Loeschcke (2005) observed that starvation resistance increased in *D. melanogaster* as a correlated response to selection for resistance to several stress traits, including KRHT, although there was no genetic relationship between starvation and KRHT in other lines (Gomez et al., 2009). This pattern was apparent for two lines that substantially responded to selection for increased KRHT in this study. In females, the two more heat-resistant and responsive lines (K2+ and K3+) were in turn more starvation resistant than other lines (Fig. 3B). Besides, Sørensen et al. (2005) have shown that starvation resistance varies along an altitudinal gradient from the north-western Argentina in *D. buzzatii*, where clinal variation was found for KRHT. However, variation in both altitudinal and latitudinal clines of starvation resistance shows a complex relationship between this trait and climatic adaptation in *Drosophila*, as the relationship between starvation resistance and environmental temperature can shift in sign across continents (Karan et al., 1998; Harshman and Hoffmann, 2000; Robinson et al., 2000; Schmidt et al., 2005; Sørensen et al., 2005).

Neither developmental time nor body size showed differences as correlated responses to selection on KRHT (Fig. 3C,D). Previous work in *D. melanogaster* showed similar results for body size (Bubli et al., 1998; Bubliy and Loeschcke, 2005). However, developmental time had exhibited correlated responses to selection on heat-stress resistance in laboratory populations of *D. melanogaster*. For example, Bubliy and Loeschcke (2005) found an increase in developmental time after selection on KRHT, and this increase was independent of changes in body size as thorax length was not modified by selection on this trait. Similar results were obtained when comparing populations of *D. buzzatii* from an altitudinal gradient in north-western Argentina, where thermal adaptation is evident and temperature is the main climatic variation between lowland and highland populations (Sørensen et al., 2005; Sambucetti et al., 2006). Along this elevation gradient, KRHT and developmental time were negatively and positively correlated with altitude, respectively (Sørensen et al., 2005; Sambucetti et al., 2006). However, there was no significant correlation between developmental time and body size, with no altitudinal cline for body size in the mentioned elevation gradient (Sambucetti et al., 2006). The present results further show that developmental time and adult body size do not consistently change by thermal selection for KRHT in adult *D. buzzatii*.

Recent studies in *D. melanogaster* have shown that selection for heat resistance increases the likelihood of locating resources under warm conditions in the field (Loeschcke and Hoffmann, 2007; Kristensen et al., 2007). This observation suggests that direct selection for heat resistance may confer not only an increased survival at high temperature (see Hoffmann et al., 2003 for review), but also an increased ability to locate both feeding and breeding resources (Partridge et al., 1987; Norry et al., 1995). Also mating success had been found to be affected by adaptation to temperature in *Drosophila* (Dolgin et al., 2006). An exploratory study with the same lines used in this study has showed that male mating success could be affected by selection for heat resistance (Sambucetti, P., unpublished, preliminary observation). Lines selected for increased KRHT showed higher mating success at high temperature (31 °C) than lines selected for decreased KRHT. The differences in mating success were not apparent when tested at 25 °C, suggesting that selection for increased KRHT could confer a direct advantage on relevant fitness components such as mating success at elevated temperature in *D. buzzatti*.

In sum, the results in this study show that knockdown resistance to heat stress significantly responds to directional selection in *D. buzzatii*. This trait has been showed to consistently exhibit adaptive thermal clines in different continents and species, indicating its ecological relevance (Hoffmann et al., 2002; Sørensen et al., 2005). The response to selection was also only in one direction, namely to increase KRHT and similar in both sexes. The present results in *D. buzzatii* indicate that knockdown resistance to heat stress can evolve towards increased thermotolerance with no trade-offs associated to starvation resistance, developmental time and body size.

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