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Critical thermal maxima in knockdown-selected *Drosophila*: are thermal endpoints correlated?

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Summary

To explore the correlation of traits linked to thermotolerance, we compared three thermal endpoints (knockdown temperature and two critical thermal maxima) among replicate populations of *Drosophila melanogaster* selected for high, or low, knockdown temperature. The high knockdown flies maintain normal posture and locomotor ability within a knockdown column at temperatures ⩾40°C, whereas the low knockdown flies fall out of the column at much cooler temperatures (~35°C, on average). The critical thermal maximum (CT_{max}) for respiratory control in the selected knockdown populations was determined by analyzing CO_2 output of individuals during exposure to a temperature ramp (from 30°C to >45°C) and was indicated by an abrupt alteration in the pattern of CO_2 release. The CT_{max} for locomotor function was determined by monitoring activity (concurrent with CO_2 analysis) during the temperature ramp and was marked by the abrupt cessation of activity. We hypothesized that selection for high knockdown temperature may cause an upward shift in CT_{max}, whereas selection for low knockdown may lower CT_{max}. Correlations among the three thermal endpoints varied between the high and low knockdown flies. Finally, we compared metabolic profiles, as well as Q_{10} values, among the high and low knockdown males and females during the temperature ramp.

Key words: *Drosophila melanogaster*, laboratory selection, thermotolerance, knockdown, critical thermal maximum, thermolimit respirometry, metabolic rate, Q_{10}.

Introduction

Virtually all organisms have evolved physiological, behavioral and developmental programs, as well as life history strategies, for confronting fluctuations in environmental temperature. As a consequence of global warming, both the minima and maxima of these fluctuations are increasing within many habitats (Houghton et al., 2001). A relevant concern of physiological and evolutionary ecologists is the capacity of organisms to deal with such challenges. The extent to which organisms can respond to thermal stress has a 'profound effect on evolutionary fitness' (Huey and Bennett, 1990).

Drosophilids are model organisms in which thermotolerance has been studied extensively (Hoffmann et al., 2003) by a variety of methods, including assessment of mortality or survivorship following heat shock (Huey et al., 1991; Hoffmann et al., 1997; Berrigan and Hoffmann, 1998; Stratman and Markow, 1998; Berrigan, 2000; Sørensen et al., 2001; Folk et al., 2006; Rashkovetsky et al., 2006), knockdown time (McColl et al., 1996; Hoffmann et al., 1997; Berrigan and Hoffmann, 1998; Berrigan, 2000; Sørensen et al., 2001; Kellett et al., 2005), knockdown temperature (Gilchrist and Huey, 1999; Berrigan, 2000; Folk et al., 2006), and locomotor functioning during or following heat shock (Krebs et al., 2001; Zatsepina et al., 2001; Roberts et al., 2003; Newman et al., 2005).

Although an array of traits linked to thermotolerance in flies has been studied, some of the traits may have different physiological bases and thus may not be correlated with each other. This idea was supported by Hoffmann et al. who examined the correlation of different measures of thermotolerance in replicate lines of *Drosophila melanogaster* selected for increased knockdown time at a high temperature (Hoffmann et al., 1997). They tested the flies for recovery time and survivorship following thermal stress. The flies selected for the highest knockdown times did not recover more quickly or show improved survivorship. In other words, the populations selected for highest knockdown had not evolved enhanced thermotolerance in a broad sense. If traits typically used to gauge thermotolerance lack correlation, the ecological and evolutionary relevance of some of the traits may require re-evaluation.

We are currently using populations of *Drosophila melanogaster* artificially selected for knockdown temperature as a model for investigating the complexities of thermotolerance (Gilchrist and Huey, 1999; Folk et al., 2006). Knockdown temperature (T_{KD}) is the temperature at which flies drop out of a heated knockdown column (Huey et al., 1992). This study system comprises four replicate populations that have undergone directional selection for high T_{KD}, four replicate populations that have undergone stabilizing selection on lower T_{KD} values, and four replicate control populations. The High T_{KD} populations have evolved high knockdown thermotolerance (average T_{KD}=~41°C), whereas the Low T_{KD} populations

experience knockdown at ~35°C. The mechanistic underpinnings of the knockdown phenotypes are unclear.

Critical thermal maximum (CT_{max}) estimations have been used to define the thermal tolerance of vertebrate and invertebrate taxa for over six decades (Cowles and Bogert, 1944; Lutterschmidt and Hutchison, 1997). Historically, CT_{max} is defined as the thermal endpoint at which "*…locomotory activity becomes disorganized and the animal loses the ability to escape from conditions that will promptly lead to its death*" (Cowles and Bogert, 1944). Lutterschmidt and Hutchison assert that "*…CT_{max} is an excellent index and standard for evaluating the thermal requirements and physiology of an organism.*"

The physiological states used traditionally to define CT_{max} are variable and include the 'loss of righting response', 'onset of muscular spasms', 'heat paralysis' or 'heat coma', and even 'knockdown' (Lutterschmidt and Hutchison, 1997; Berrigan and Hoffmann, 1998). Here we define CT_{max} in the flies as the upper temperature at which normal locomotory functions and spiracular control are compromised. The central question addressed in the present study is: are thermal endpoints correlated? Specifically, we question whether selection for high (or low) knockdown temperature has resulted in high (or low) critical thermal maximum for heat paralysis. Exposure to either CT_{max} or T_{KD} would presumably result in disruption of normal neuromuscular functions.

Traditionally, CT_{max} has been estimated by determining body (or ambient) temperature at the onset of one of the physiological states mentioned above (Lutterschmidt and Hutchison, 1997). To estimate CT_{max} in individual flies we used a more objective method, namely thermolimit respirometry, in conjunction with constant monitoring of activity (Lighton and Turner, 2004). Thermolimit respirometry allows the estimation of CT_{max} for involuntary muscle function, as indicated by loss of spiracular control, whereas activity monitoring allows estimation of CT_{max} for voluntary muscle function, as indicated by cessation of movement. The loss of spiracular control is evidenced by a distinct alteration in the pattern of CO_2 output, characterized by a dramatic reduction in the variance. In addition, we explore the feasibility of using thermolimit respirometry and activity monitoring to detect T_{KD}, which would be possible only if the patterns of spiracular and locomotor activity were altered significantly at knockdown.

Materials and methods

Establishment of artificially selected fly lines

The history of the original and new knockdown lines is detailed fully elsewhere (Folk et al., 2006). Briefly, in 1992 a subset of flies from a founding population of *Drosophila melanogaster* (collected by L. Harshmann and M. Turelli in 1991 in Escalon, CA, USA) was used to establish discrete lines. From these lines, six High Knockdown lines ('High T_{KD}'), three Low knockdown lines ('Low T_{KD}'), and six Control lines were established (Fig. 1). These lines were subjected to artificial selection (described below) for 46 generations until September 1997. Selection was resumed for 7 generations in June 2004. In January 2005, we combined flies from all replicate lines within each selection (and control) group and randomly chose individuals to generate new replicate High T_{KD}, Low T_{KD}, and Control lines (Fig. 1), referred to as the $HN_{1–4}$, $LN_{1–4}$ and $CN_{1–4}$ lines, respectively. We generated the new knockdown selection lines to reduce inbreeding by crossing the original lines within each treatment group, and to create equal numbers of lines within the treatment groups.

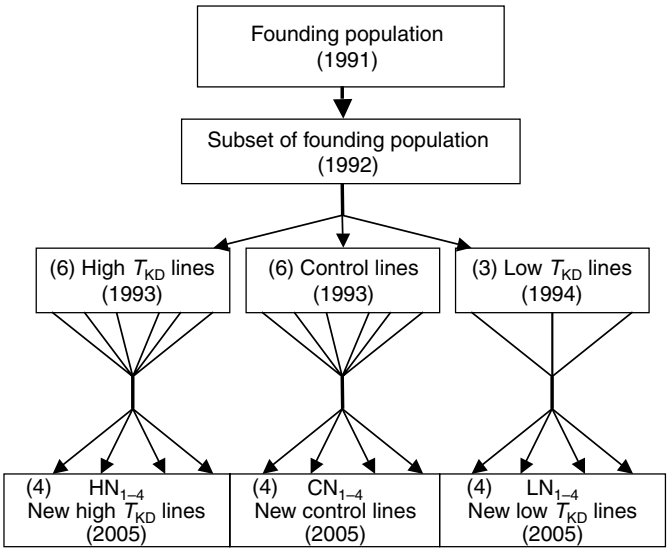

Fig. 1. A diagram depicting the history of the original and newly established High T_{KD} and Low T_{KD} selection lines and the Control lines (from Folk et al., 2006).

Measuring knockdown temperature

The knockdown protocol is detailed fully elsewhere (Folk et al., 2006). To begin a knockdown experiment, ~1000 flies from each line are released into the inner tube of a Weber column (Weber, 1988). The inner tube is surrounded by a water jacket, through which water circulates *via* a heat pump (Haake-Buchler Inc., Paramus, NJ, USA) immersed in a water bath. The flies are transferred immediately from a 25°C incubator into the column, which is initially heated to 30°C. The temperature setting of the heat pump is then increased to 50°C. Consequently, the inner tube heats up (~0.4°C min^{-1}); the flies fall out and are collected at 0.5°C intervals between 32–46°C. After falling out of the heated column, the flies resume an upright posture nearly instantaneously (Gilchrist and Huey, 1999). Following knockdown, the flies are anesthetized with CO_2, sexed and counted. The distribution of knockdown temperatures is computed separately for males and females from each line.

Selection regime and experimental protocols

The selection regime and fly maintenance are detailed fully elsewhere (Folk et al., 2006). Briefly, after each High T_{KD} line ($HN_{1–4}$) is run through the knockdown column, ~30% of the flies with the highest T_{KD} are retained for breeding. For each Low T_{KD} line ($LN_{1–4}$), ~30% of the flies with T_{KD} values of ~35.5–37°C are retained for breeding. After each Control line ($CN_{1–4}$) is run through the column, ~30% of all the flies are randomly chosen for retention and breeding. The selected flies are maintained for 6–7 days at 25°C to ensure remating (Gilchrist and Huey, 1999), following which eggs are collected

for rearing of the subsequent generation. Selection on adults occurs 4–5 days post-eclosion.

For all respirometry experiments, eggs (50 eggs/food vial; 7 vials/line) from each $HN_{1–4}$, $LN_{1–4}$, and $CN_{1–4}$ line were collected from a subset of adults removed from selection. The flies were reared at 25°C. At 2–4 days post-eclosion, 12 flies (6 males, 6 females) from each line were haphazardly chosen, anesthetized with CO_2, weighed to the nearest 0.001 mg, and placed individually into food vials for 20–28 h. We then haphazardly chose four individuals (2 males, 2 females) from the larger group of 12, and measured CO_2 output and activity of individuals across a temperature range from 30°C to >45°C. All respirometry and activity measurements of flies from a line were made on the same day.

Respirometry, activity detection, and monitoring/controlling temperature

To measure CO_2 output, we used a flow-through respirometry system (Sable Systems International, Las Vegas, NV, USA). The respirometer chambers were constructed from 2-dram, glass shell vials (19 mm diameter×51 mm length, Kimble Glass, Inc., Vineland, NJ, USA), each fitted with a two-holed, solid-rubber stopper (no. 2). A piece of aluminum tubing (~50 mm length, 4.76 mm i.d.; K&S Engineering, Chicago, IL, USA) was pushed into each hole of the stopper until the end of the tube was flush with the bottom surface. A piece of fine mesh was glued to the bottom surface of the stopper to keep flies from escaping. Space between the stopper holes and the aluminum tubes was filled with silicone sealant (Devcon, Riviera Beach, FL, USA). The two sections of aluminum tubing (~23 mm length) protruding from the top of the stopper were used to attach tubing (1.5875 mm i.d., PharMed, Cole-Parmer Instrument Co., Vernon Hills, IL, USA), which directed air flow to an RM8 Intelligent Multiplexer (Sable Systems International). A thermocouple (Type T) wire was inserted into the PharMed tubing and threaded into the respirometer chamber, allowing us to record the chamber temperature at 1 s intervals. [The thermocouple wire was connected to a digital thermometer (Omega HH509R, Stamford, CT, USA).] The wire insertion site was sealed with silicone sealant.

An air pump (Topfin, XP-125, Pacific Coast Distributors, Inc., Phoenix, AZ, USA) pulled air into the respirometry system. The air stream was then pushed through three scrubbing columns: (1) silica gel (Type III, Sigma-Aldrich, St Louis, MO, USA), (2) silica gel and (3) Drierite/Ascarite II/Drierite (Drierite^{TM}: Fisher Scientific, Pittsburgh, PA, USA; Ascarite II^{TM}: Thomas Scientific, Swedesboro, NJ, USA), and then through a mass flow control valve (Side-Trak, Sierra Instruments, Inc., Monterey, CA, USA) at a mass flow rate of 50 ml min^{-1}, maintained by a Mass Flow Controller (Sable Systems International, Version 1.1). The air stream flowed next through tubing and a coil of aluminum tubing (350 mm length; 3 mm i.d.) held in a temperature-control incubator (DigiTherm Incubator, Tritech Research, Los Angeles, CA, USA), to promote thermal equilibration of the air (see Lighton and Turner, 2004). The temperature-equilibrated air then flowed through the respirometer chamber and into the Li-Cor 7000 CO_2 infrared gas analyzer (LI-COR, Inc., Lincoln, NE, USA). (Before the air stream reached the CO_2 analyzer, H_2O released by the fly was scrubbed with magnesium perchlorate.)

In order to measure CO_2 output within a temperature-controlled environment, we kept the RM8 Intelligent Multiplexer (Sable Systems International, the RM8 controls the air flow through different respirometer chambers) inside the incubator. We used only two respirometer chambers in each experiment: no. 1 for obtaining the zero-CO_2 baseline, and no. 2 for measuring CO_2 output from a fly. The temperature of the incubator was set initially at 30°C. When the temperature inside chamber no. 2 reached 30±0.5°C, we inserted a fly and allowed it to equilibrate for 5 min, during which time we set zero-CO_2 baseline from the empty chamber (no. 1). After equilibration, we started measuring CO_2 output; and at the same time, we increased the incubator temperature setting to 50°C. The temperature within the fly respirometer chamber increased at an average rate of 0.24±0.02°C min^{-1}. We continued to measure CO_2 output of each fly until the temperature inside the chamber reached >45°C, well beyond the lethal temperature. ExpeData software (Sable Systems International, Version 1.01) was used to control the respirometry system and for data acquisition.

An AD-1 activity detector (Sable Systems International, Version 2) was used to monitor fly activity in conjunction with the CO_2 data acquisition. The respirometer chamber fit securely into the cradle of the AD-1, which generates an infrared light (880 nm) field. Movements of the fly cause fluctuations in the light field intensity, which are detected and recorded (by ExpeData software) as deviations from a zero-activity baseline. Activity was recorded at 1 s intervals.

Data analysis and CT_{max}

We used the methods of Klok et al. to estimate both CT_{max} endpoints (Klok et al., 2004). CT_{max} for spiracular function was indicated on each respirometry tracing by a distinctive and abrupt alteration in the pattern of CO_2 output, characterized by a dramatic reduction in the variance (Fig. 2). Using the data analysis functions of ExpeData software, we were able to select the transitional point on each tracing at which the CO_2 output was altered. The CO_2 data were aligned with the temperature data (using the timing of both recordings), allowing us to estimate the temperature at which spiracular function was compromised (CT_{max}). The CO_2 data were transformed using *R*, version 2.2.1 (R Development Core Team, 2006) by: (1) correcting for baseline drift, (2) converting from p.p.m. to μl h^{-1} (mass flow rate=50 ml min^{-1}) and (3) normalizing with body mass (μg). CT_{max} for locomotor function was estimated in a similar manner. Using the data analysis functions of ExpeData software, we marked the point on each activity tracing beyond which activity was no longer apparent (Fig. 2). The activity data were aligned with the temperature data (as above), allowing us to estimate this CT_{max}.

All statistical analyses were performed using *R*, version 2.2.1, and are discussed in detail in the Results.

Results

Knockdown thermotolerance

The evolved responses of our *Drosophila melanogaster* populations to selection for high and low knockdown temperature (T_{KD}) are described in full elsewhere (Folk et al.,

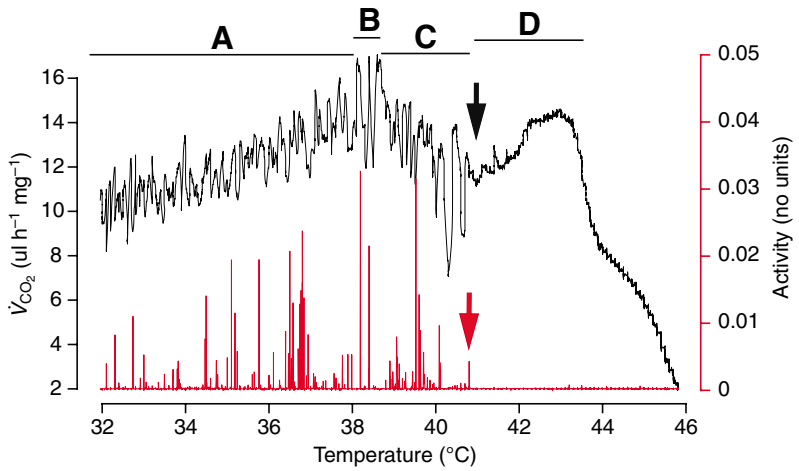

Fig. 2. Thermolimit respirometry and activity data from a single fly during a temperature ramp. The CO_2 output profile (in black) and the activity tracing (in red) of an LN male are shown. The black arrow indicates CT_{max} for spiracular control, whereas the red arrow indicates CT_{max} for locomotor function. The respirometry tracing shows the typical phases seen in other insect species: (A) exponential phase, (B) plateau, (C) mortal fall and (D) postmortal peak.

2006). Essentially, the High T_{KD} lines have evolved significantly enhanced knockdown thermotolerance with an overall mean T_{KD} that is >5°C above that of the Low T_{KD} lines, and >4°C above that of the control lines. Populations of *D. melanogaster* typically have a bimodal distribution for T_{KD} (Gilchrist and Huey, 1999). We show in Table 1 the mean T_{KD} values of the upper and lower modes of the knockdown populations (Folk et al., 2006). In response to selection, the HN lines have a significant reduction in the numbers of individuals in the lower mode; conversely, the LN lines have relatively few individuals in the upper mode (see Table 1 legend).

Metabolic profile and CT_{max} endpoints

The metabolic profiles of the flies during the temperature ramp resemble those observed in a tenebrionid beetle (*Gonocephalum simplex*) and in ants (*Pogonomyrmex rugosus* and *P. californicus*) (Klok et al., 2004; Lighton and Turner, 2004). During the initial phase of the profile, CO_2 output increases exponentially and reaches a maximum, followed by a short plateau (Fig. 2). The profile then shows a 'mortal fall' (Lighton and Turner, 2004), during which CO_2 output drops, and oscillates dramatically in some flies. Following the mortal fall, the pattern of CO_2 release is distinctively altered by an abrupt reduction in variance, indicating CT_{max} for spiracular control (Fig. 2). At temperatures >CT_{max}, the ability to modulate spiracle opening is apparently lost and CO_2 is released continually. The release of CO_2 during this phase, termed the 'postmortal peak', may be due to the discharge of bound and dissolved CO_2 or to mitochondrial respiration that continues beyond CT_{max} (Lighton and Turner, 2004). Interestingly, preliminary observations indicate that the flies seemingly recover when switched to a milder temperature (~23°C) following 2–4 min into the postmortal peak phase, suggesting that physiological processes are not irreversibly damaged at the onset of the postmortal peak phase. These few minutes may constitute a window of recovery, beyond which death is inevitable if the fly is not moved to milder thermal conditions. Following this final peak of CO_2 release, exponential decay in CO_2 output was observed in all flies (Fig. 2).

The critical thermal maximum for locomotor function was estimated from the activity patterns of individual flies and was defined as the highest temperature beyond which movement was no longer detectable (Fig. 2).

The effect of knockdown selection on both CT_{max} endpoints, as well as on T_{MetMax} (the temperature at which maximal metabolic rate occurred) among the HN, LN and CN groups was examined using Model I ANOVA. CT_{max} for locomotor function did not differ among the groups ($F_{[2,45]}$=0.1228,

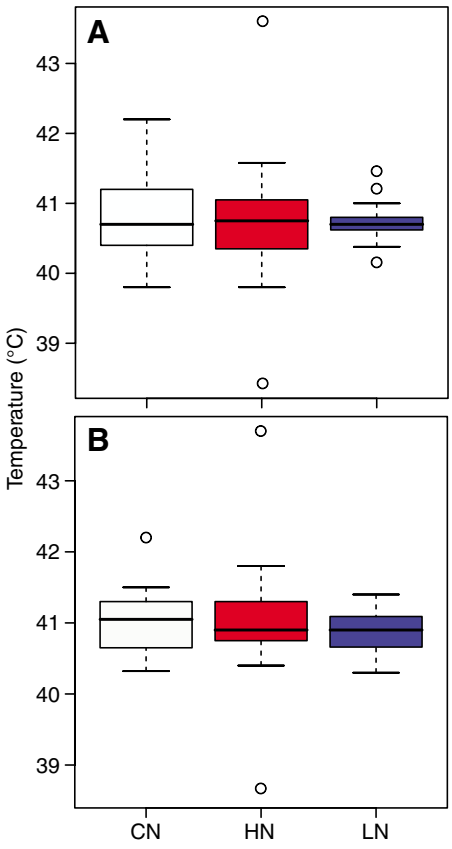

Fig. 3. Boxplots showing CT_{max} statistics for (A) locomotor function and (B) spiracular control in the HN, LN and CN flies (N=16 for each group). The horizontal line within each box indicates the median response, whereas the box outline denotes the range for the middle 50% of the data. The 'whiskers' show the dispersion of the data (i.e. 1.5 times the interquartile range). Points beyond this range are shown as open circles. CT_{max} for locomotor function or spiracular control did not differ significantly among the groups. The Low T_{KD} flies had a significant reduction in the variance for CT_{max} for locomotor function (P=2.257×10^{-5}).

Table 1. *Critical thermal endpoints and CO_2 output*

Thermal endpoints	T_{KD} line	Temperature (°C) Lower mode	Upper mode
T_{KD}	HN	37.1±0.2	41.0±0.1
	CN	35.2±0.1	38.6±0.3
	LN	34.7±0.1	38.1±0.2
CT_{max}			
Spiracular control	HN	41.0±0.3	
	CN	41.0±0.1	
	LN	40.9±0.1	
Locomotor function	HN	40.8±0.3	
	CN	40.8±0.2	
	LN	40.7±0.1	
T_{MetMax}	HN	38.4±0.3	
	CN	38.3±0.3	
	LN	38.2±0.2	

$\dot{V}_{CO_2}$ parameters	T_{KD} line	$\dot{V}_{CO_2}$ (μl h^{-1} mg^{-1})
At CT_{max}		
Spiracular control	HN	10.8±0.7
	CN	10.4±0.8
	LN	11.3±0.5
Locomotor function	HN	10.8±0.6
	CN	10.7±0.6
	LN	11.3±0.7
At T_{MetMax}	HN	13.9±0.8
	CN	13.3±0.8
	LN	14.1±0.7

Critical thermal endpoints for knockdown (T_{KD}), spiracular control, locomotor function and T_{MetMax}, which is the temperature at which maximum CO_2 output occurred. $\dot{V}_{CO_2}$ is listed at all critical temperatures, except T_{KD}. Values are means ± s.e.m.

T_{KD} values are maximum likelihood estimates for the upper and lower mode of a bimodal distribution for T_{KD}, measured in ~4000 flies per selection group (Folk et al., 2006). Nearly all HN females (98%) and HN males (93%) are within the upper mode of the T_{KD} distribution, whereas 66% of LN females and 90% of LN males are in the lower mode.

Mean CT_{max} and $\dot{V}_{CO_2}$ parameters were estimated from thermolimit respirometry analysis of four individuals from each of the four replicate populations within the HN, LN or CN groups (N=16 per group). While the populations show significant divergence in T_{KD}, no significant differences were found among the groups for all other parameters. (See Results for test statistics.)

P=0.8848), nor did CT_{max} for spiracular control ($F_{[2,45]}$=0.2122, P=0.8096) nor T_{MetMax} ($F_{[2,45]}$=0.1262, P=0.8817). We used boxplots to summarize graphically the data for the CT_{max} thermal endpoints (Fig. 3A,B). Homogeneity of variances for CT_{max} of locomotor function among the groups was examined using a Bartlett's test, which showed that the LN lines had a significantly lower variance than the CN and HN lines for CT_{max} of locomotor function (P<0.001).

To determine whether the dual CT_{max} measures are functionally related within individual flies we used least squares-linear regression analysis (Fig. 4). Specifically, we tested whether CT_{max} for locomotory function of a fly is

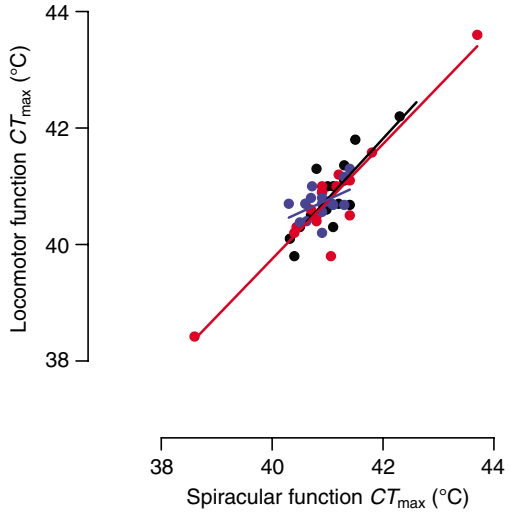

Fig. 4. CT_{max} for locomotor function is predictive of CT_{max} for spiracular control in the HN and CN groups. The slopes of the regressions were 0.99 (HN) and 1.04 (CN). The slope for the LN group was not significantly different from zero, due probably to the extreme lack of variability among the CT_{max} endpoints. The red symbols and line, HN values; blue, LN; black, CN. N=16 for each group.

predictive of CT_{max} for its spiracular control. The relationship between the two CT_{max} endpoints was essentially unity for the HN and CN lines: the slopes of the regression lines were 0.99 (P=1.28×10^{-14}) and 1.04 (P=3.72×10^{-7}), respectively. The slope of the regression line for the LN group was not significantly different from zero (P=0.1233). The lack of significance is likely due to the exceedingly low variance in CT_{max} in the LN group.

To test for the effects of selection and sex on the pattern of mass-specific $\dot{V}_{CO_2}$ (μl h^{-1} mg^{-1}) over a temperature range of 32°C to CT_{max} (~41°C), we used a second-order orthogonal polynomial analysis for repeated measures. Both $\dot{V}_{CO_2}$ and the temperature data were log-transformed to attain a normal distribution. We tested for homogeneity of the second-order orthogonal polynomial coefficients among the sex X selection groups and were unable to reject the null hypothesis of homogeneity of slopes ($F_{[4,42]}$=0.0088, P=0.9998). We then fit common second-order orthogonal polynomial coefficients to the sex X selection groups and tested for homogeneity among the intercepts using ANCOVA. Knockdown selection did not have a significant effect on mass-specific $\dot{V}_{CO_2}$ over the experimental range of temperatures ($F_{[2,42]}$=0.0048, P=1.0000), whereas sex had a strong effect ($F_{[1,42]}$=32.5884, P=1.042×10^{-6}). All males had a significantly higher mass-specific $\dot{V}_{CO_2}$ when heated than the females (Fig. 5).

The effect of selection on mass-specific $\dot{V}_{CO_2}$ at the critical thermal endpoints, namely CT_{max} for spiracular control, CT_{max} for locomotor function and T_{MetMax}, was tested using Model I ANOVA. $\dot{V}_{CO_2}$ of the treatment groups did not differ significantly at any of the critical thermal endpoints (CT_{max} for spiracular control: $F_{[2,45]}$=0.4528, P=0.6387; CT_{max} for locomotor function: $F_{[2,45]}$=0.2634, P=0.7696; and T_{MetMax}: $F_{[2,45]}$=0.2501, P=0.7798). Mean $\dot{V}_{CO_2}$ at each critical temperature for all selection groups is listed in Table 1.

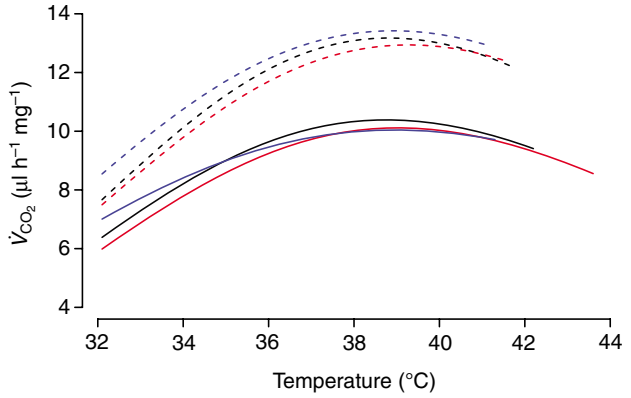

Fig. 5. Metabolic profiles of the HN (red), LN (blue), and CN (black) males (broken lines) and females (solid lines) during a temperature ramp, ending at CT_{max} for locomotor function (N=8 for each group). Knockdown selection treatment did not affect significantly the mass-specific $\dot{V}_{CO_2}$ over the experimental range of temperatures (P=1.000), whereas sex had a strong effect (P=1.042×10^{-6}). Specifically, males had a higher mass-specific $\dot{V}_{CO_2}$ throughout the experiment.

Q_{10} of metabolic rate

We used Model I ANOVA to test for the effects of sex and selection on Q_{10} for CO_2 production during the temperature ramp, focusing on sensitivity of metabolic rate to increasing temperature during the exponential phase (32–38°C). When calculating Q_{10}, we corrected for the <10°C change in temperature. Although sex did not affect Q_{10} ($F_{[1,42]}$=0.3399, P=0.5630), selection treatment had a significant effect ($F_{[2,42]}$=4.2193, P=0.0214). The LN flies had a lower Q_{10} than the HN flies (Tukey HSD, P=0.043) and the CN flies (Tukey HSD, P=0.039). Q_{10} did not differ significantly between the CN and HN flies (Tukey HSD, P=0.999). These data suggest that the thermal sensitivity of metabolic rate in the LN flies was relatively reduced. Q_{10} values for all groups are listed in Table 2.

Table 2. *Q_{10} values for $\dot{V}_{CO_2}$ for males and females from the selection and control lines*

	Q_{10}	
Knockdown treatment	Females	Males
HN	2.4±0.3	2.5±0.2
CN	2.4±0.2	2.5±0.3
LN	1.8±0.2*	1.9±0.2*

Values are mean Q_{10} (± s.e.m.) for $\dot{V}_{CO_2}$ (µl h^{-1} mg^{-1}) for males and females from the selection and control lines during the exponential phase of CO_2 output (32–38°C). For each value, N=8 flies.

Q_{10} values show the sensitivity of metabolic rate to increasing temperature. For example, a Q_{10} of 2 denotes a doubling of $\dot{V}_{CO_2}$ for each 10°C increase in temperature.

Asterisks denote that LN females and LN males have a significantly lower Q_{10}, thus less thermal sensitivity to the increase in temperature. (See Results for statistical analysis.)

Discussion

Lack of correlation between T_{KD} and CT_{max}

The response to knockdown selection of the HN and LN lines has been well-characterized (Gilchrist and Huey, 1999; Folk et al., 2006). The HN lines have evolved the ability to maintain normal posture within a heated knockdown column at temperatures ⩾40°C. In contrast the LN lines fall down and out of the column at much cooler temperatures (~35°C). A primary aim of this study was to estimate the critical thermal maxima for locomotor and spiracular function in the divergent knockdown lines in order to explore the correlation, or lack thereof, between various thermal endpoints. Our data indicate that, although T_{KD} has diverged between the HN and LN lines, the CT_{max} endpoints have not shifted in accordance. These findings underscore the importance of the notion that the "*choice of indices for study (of thermotolerance) could be critical*" (Berrigan, 2000).

Correlation between thermal endpoints

The CT_{max} endpoints for locomotor function and spiracular control of each fly are essentially equivalent (Fig. 4). The thermal ceiling of both the locomotory and spiracular control systems are strikingly similar. We propose that a shared physiological constraint may dictate the upper thermal boundary of the dual CT_{max} endpoints. Hochachka and Somero (Hochachka and Somero, 2002) proposed that all physiological systems participate in setting thermotolerance limits of an organism, but that 'weak links' assert the strongest influence. They further propose that the 'weakest links' in thermotolerance are cellular membranes. Alternatively, Pörtner proposed that at high temperatures metabolic rate may be limited by inadequate delivery of oxygen to the mitochondria (Pörtner, 2002). Klok et al. provide evidence that this may not be the case in terrestrial insects (Klok et al., 2004). Thermal stress can disrupt membrane homeostasis by altering composition, permeability, ion channel activity, etc. A membrane property that is highly sensitive to thermal stress is synaptic transmission, the thermal impairment of which would compromise both effective locomotion and control of ventilation (Robertson, 2004). The narrow range of the CT_{max} endpoints in our flies supports the hypothesis of a common 'weakest link', such as synaptic transmission (Fig. 3A,B). The LN lines, in particular, had very little variability for CT_{max} of locomotor function. (Stabilizing selection on a limited range of lower T_{KD} values appears to have eliminated most of the variability for this trait.) Overall, our findings are suggestive of a mechanistic correlation between both measures of CT_{max}.

High and low knockdown phenotypes: different traits?

Knockdown temperature has been defined generally as the "*upper temperature at which a fly falls from a Weber column*" (Huey et al., 2003). In natural populations, the distribution for knockdown temperature is typically bimodal (Gilchrist and Huey, 1999). Selection for High T_{KD} causes flies to lose the lower mode of the distribution, whereas selection for Low T_{KD} tends to eradicate the upper mode. The simplest explanation for the shift to a unimodal distribution is that we are selecting for enhanced (in HN lines) or diminished (in LN lines) performance of a complex polymorphic trait. We propose otherwise. Specifically, we propose that the upper and lower modes of knockdown may represent altogether different traits. Selection for High T_{KD} may be targeting CT_{max}, the thermal ceiling for

heat paralysis. Interestingly, both T_{KD} of the HN flies and CT_{max} of the HN *and* LN flies are set at ~41°C (Table 1). If High T_{KD} is essentially CT_{max}, then the same mechanisms (discussed above) involved in establishing CT_{max} would be involved in setting the upper limits of knockdown temperature.

Selection on Low T_{KD} may be targeting something altogether different, possibly behavior. When we first place the LN lines into the knockdown column during an experiment, the column temperature is moderately high (30°C). As the column temperature rises, the LN flies tend to walk along the surface of the baffles, move to the lower edge, and then drop to a lower baffle. They continue to drop to successively lower baffles, until eventually they drop out of the column. The HN flies do not display these behaviors. They localize within the top half of the column when first transferred to the column and do not tend to travel downwards during knockdown. These behavioral peculiarities have led us to question whether we are selecting for a behavioral phenotype in the LN flies. Specifically, are we selecting for negative phototaxis, positive geotaxis, or escape behavior? If this were the case, it might explain the lack of correlation between T_{KD} and CT_{max} in the LN flies.

Historical selection experiments, using *D. melanogaster* and *D. pseudoobscura*, generated flies with strong positive or negative geotactic (also phototactic) behaviors (Hirsch and Erlenmeyer-Kimling, 1961; Dobzhansky and Spassky, 1962; Del Solar, 1966). The flies had sufficient genetic variation for these behaviors and responded quickly to selection. Interestingly, two desert species (*D. mettleri* and *D. nigrospiracula*) share photonegative behavior, but display contrasting geotactic behaviors (Markow and Fogleman, 1981). Toma et al. have identified a number of genes involved in phototaxic and geotaxic responses, providing evidence that they are highly complex polygenic behaviors (Toma et al., 2002). We speculate that our selection lines, in response to selection for high or low knockdown performance, may have diverged in the expression of such behaviors.

Evolution of thermal sensitivity of metabolism

The composite metabolic profile during the mounting heat stress was similar among the HN, LN and CN lines (Fig. 5). Yet, as indicated by the Q_{10} values, the thermal sensitivity of metabolism was significantly reduced in the LN lines (Table 2). We are now presented with a paradox: why do flies selected for a lower knockdown temperature have reduced metabolic thermal sensitivity? Selection for Low T_{KD} performance may have resulted in photo- and geotactic behaviors (discussed above) genetically correlated to metabolic performance. Selection on phototaxis and geotaxis in *Drosophila* results in correlated changes in multiple traits, including eye size, testis color, wing venation and mating behavior (Del Solar, 1966; Dobzhansky and Spassky, 1969). Toma et al. compared gene expression of 250 genes in populations of *D. melanogaster* selected for negative or positive geotaxis and found that ~5% of the genes were differentially expressed (Toma et al., 2002). Further testing strongly implicated three candidate genes, whose mechanistic involvement in geotaxis is unclear. We speculate that selection on photo- or geotaxis (*via* selection on low knockdown) may lead to genetic changes influencing the thermal sensitivity of metabolism. An informative study would be to select on photo- or geotaxis and monitor metabolism, particularly Q_{10} values during heat stress.

In summary, ambient temperature of small ectotherms, such as *Drosophila melanogaster*, influences directly all physiological networks. We have explored here the thermal limits of respiratory and locomotor performance of flies. We hypothesized that the critical thermal maxima of these performances would co-evolve with knockdown temperature. The dual CT_{max} values were strongly correlated with each other and with knockdown temperature in the HN lines, suggesting a shared physiological 'weakest link' in thermal sensitivity. The CT_{max} values were not correlated with knockdown temperature in the LN flies, leading us to conclude that some other 'weakest link', perhaps behavioral, sets knockdown in these populations. Finally, respiratory and gross activity patterns were not altered significantly in the LN flies at T_{KD}, resulting in an inability to detect T_{KD} using thermolimit respirometry or activity monitoring.

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