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RESEARCH ARTICLE

Survival of heat stress with and without heat hardening in *Drosophila melanogaster*: interactions with larval density

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SUMMARY

Survival of a potentially lethal high temperature stress is a genetically variable thermal adaptation trait in many organisms. Organisms cope with heat stress by basal or induced thermoresistance. Here, we tested quantitative trait loci (QTL) for heat stress survival (HSS) in *Drosophila melanogaster*, with and without a cyclic heat-hardening pre-treatment, for flies that were reared at low (LD) or high (HD) density. Mapping populations were two panels of recombinant inbred lines (RIL), which were previously constructed from heat stress-selected stocks: RIL-D48 and RIL-SH2, derived from backcrosses to stocks of low and high heat resistance, respectively. HSS increased with heat hardening in both LD and HD flies. In addition, HSS increased consistently with density in non-hardened flies. There was a significant interaction between heat hardening and density effects in RIL-D48. Several QTL were significant for both density and hardening treatments. Many QTL overlapped with thermotolerance QTL identified for other traits in previous studies based on LD cultures only. However, three new QTL were found in HD only (cytological ranges: 12E–16F6; 30A3–34C2; 49C–50C). Previously found thermotolerance QTL were also significant for flies from HD cultures.

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INTRODUCTION

Heat stress survival (HSS) of organisms in the wild depends not only on basal but also on inducible thermotolerance (Hoffmann and Parsons, 1991; Hoffmann et al., 2003; Hoffmann and Daborn, 2007; Reusch and Wood, 2007). Inducible tolerance to heat stress is an adaptive response of organisms to mild stress, including improved thermotolerance as a result of previous exposure to a sub-lethal high temperature stress (for reviews, see Hoffmann et al., 2003; Loeschcke and Sørensen, 2005). Under changing climatic conditions the persistence of populations may often depend on their potential to respond to thermal stress (Reusch and Wood, 2007; Hoffmann and Willi, 2008).

Drosophila melanogaster is a model insect for thermal adaptation studies as it is found over wide geographical areas from tropical to temperate regions around the world. Adaptive geographical patterns of thermotolerance have evolved in this cosmopolitan model organism (e.g. Weeks et al., 2002; Svetec et al., 2011). Larval density is an important component of the environment and can vary dramatically for *Drosophila* (Bubli et al., 1998; Leips and Mackay, 2000; Baldal et al., 2005). Density is a factor potentially interacting with both inducible and basal thermotolerance. However, few studies have tested whether genetic variation in thermotolerance depends on crowding conditions such as larval density. It is well known that HSS usually increases with previous exposure(s) to high temperature (Krebs and Loeschcke, 1994), but it remains unknown whether other ecological factors such as density interact with heat hardening and thermotolerance.

The analysis of quantitative trait loci (QTL) is a useful approach to identify genome regions in which the relevant genes for adaptive changes in thermotolerance are localized (Morgan and Mackay, 2006; Norry et al., 2008). All three major chromosomes of *D. melanogaster* were found to contain QTL for basal (non-induced) thermotolerance (Norry et al., 2004; Norry et al., 2007a; Norry et al., 2007b; Norry et al., 2008; Morgan and Mackay, 2006). Recently, Takahashi and colleagues used a genome-wide deficiency screen to find deletions affecting knockdown resistance to high temperature (Takahashi et al., 2011). Chromosome regions that showed significant effects were mostly included within QTL regions (Takahashi et al., 2011) previously found by recombination mapping (Norry et al., 2004; Norry et al., 2008; Morgan and Mackay, 2006). Recombinant inbred lines (RIL) are useful tools for genetic analysis of possible interactions between larval density and the ability to resist heat, as QTL mapping in RIL populations can be performed for multiple conditions of larval crowding and thermotolerance phenotypes. For instance, knockdown resistance to heat stress can be increased by a single, short-term heat-hardening treatment (Norry et al., 2008), but the possible effects of repeated and longer treatments of heat hardening and larval density remain to be tested for thermotolerance QTL. The genetic architecture of heat resistance could differ in flies that developed under very different larval densities, as other traits such as longevity have also shown interactions between QTL and larval density (Leips and Mackay, 2000).

Here, we performed a QTL-based scan for HSS with and without a repeated heat-hardening treatment at different culture densities

using a set of RIL that were previously constructed from heat stress-selected flies (Norry et al., 2008). Several hypotheses were addressed. First, we tested whether HSS interacts with larval density and hardening treatment across RIL populations. Second, we tested whether thermotolerance QTL generally overlaps between heat-hardened and non-hardened flies. Third, we explored the effects of larval density on the genetic basis of thermotolerance, as QTL for HSS might only be apparent for either low (LD) or high (HD) density conditions because of possible interactions with larval density. Finally, we also explored whether QTL for HSS co-localize with previously identified QTL for heat knockdown resistance in the same sets of intercontinental RIL, as earlier studies have used different traits of heat resistance to search for thermotolerance QTL in adult flies.

MATERIALS AND METHODS

Recombinant inbred lines

Lines in this study are two sets of RIL described previously (Norry et al., 2008). In short, two inbred lines denoted SH2 and D48 were the parental stocks used to construct RIL. Prior to the original cross of the lines, SH2 (derived from Melbourne, Australia) was selected for high knockdown resistance to heat stress whereas D48 (derived from eastern Jutland, Denmark) was selected for low knockdown resistance to heat stress. To increase the statistical power to detect QTL if compared with design based on single-way introgression to construct RIL, two sets of RIL, denoted RIL-D48 and RIL-SH2, were constructed from the two reciprocal backcrosses (Norry et al., 2008). The genetic map associated with these RIL was based on 36 microsatellite markers throughout all major chromosomes (Norry et al., 2008).

Thermotolerance phenotypes

All RIL were maintained at 25±1°C in replicate 95×20 mm vials containing 6 ml of a culture medium prepared with instant mashed potato plus water and nipagin (hereafter standard vials). To obtain the parents of experimental individuals, RIL were expanded for one generation from all stocks using 3–4 125 ml glass bottles containing 40 ml of the instant culture medium (hereafter standard bottles) per RIL, with 25 males plus 25 females per bottle.

Two sets of experimental cultures were set up with flies from the expanded RIL. One set was the HD condition using 3–4 standard vials per RIL with 50 males plus 50 females per vial at 25±1°C. The other set was the LD condition using 3–4 standard bottles per RIL with 25 males plus 25 females per bottle at 25±1°C. These two conditions of fly density resulted in a much higher number of emerged flies (>4 times higher per ml of culture medium) in HD than in LD as expected from the higher density of flies used per culture volume. Sixty flies from each RIL that emerged within the emergence peak from each of these HD and LD cultures were transferred to new vials with fresh medium at 1 day of age. Half (30) of the total of 60 flies per RIL were used for heat-hardening treatment (HH; see below) and the other half were maintained without any heat-hardening treatments (non-heat hardening, NHH) at 25±1°C until 4 days of age when all flies were measured for HSS.

To measure HSS we used a semi-lethal stress of 39°C for 35 min, using glass vials placed within a water bath. HSS was measured as the proportion of flies surviving 24 h after exposure of the four different sets of experimental 4 day old flies described above (HD, LD, HH and NHH). Our hardening pre-treatment consisted of exposing flies at 32°C (water bath) for 4 h (12:00 h to 16:00 h) every day until the age of 3 days (i.e. 1 day less than the age used for HSS measurement). Flies received no anesthesia treatment throughout the experiment. The experimental test was replicated three times (i.e. 3 LD and NHH × 3 LD and HH × 3 HD and NHH × 3 HD and HH × 53 RIL × 2 sexes). ANOVA was performed on HSS to test for fixed effects of hardening and density treatments in each RIL panel and sex because of significant interactions involving RIL panel and sex in another ANOVA (not shown) including all these factors.

QTL analysis

Composite interval mapping was used to test the hypothesis that an interval flanked by two adjacent markers contains a QTL. This test was performed using model 6 in QTL-Cartographer Windows version 2.5, for Ri2 design (RIL, sib mated), initially with five control markers and a window size of 10 cM. We explored the effects of altering this initial combination of parameters. QTL positions that were found by using 10 cM as window size and five control markers were consistent across a wide range of parameter combinations. Significance thresholds were determined by 1000 random permutations. QTL analysis was performed for each replicate as well as for averaged replicates both for each RIL panel (RIL-D48 and RIL-SH2) and for pooled RIL panels (results not shown). Because the number of QTL was higher in the analysis based on each replicate separately than in the analyses based on both mean values across replicates and pooled RIL, and because QTL peaks otherwise overlapped across analyses, results are presented for each replicate separately. Confidence intervals were calculated for significant QTL by using 1.5 LOD ($\log_{10}$ of odds) for confidence intervals >95% (see Dupuis and Siegmund, 1999).

Additionally, multi-trait composite interval mapping (MCIM) can increase statistical power by considering the correlations between traits (Jiang and Zeng, 1995). Although across-RIL correlations were rather small or even non-significant either between LD and HD or between NHH and HH (see supplementary material Fig. S1), significant QTL were detected by MCIM. We explored whether significant QTL in MCIM co-localized with QTL detected by single-trait composite interval mapping (see supplementary material Fig. S2).

Additionally, pairwise epistatic interactions were evaluated by using a linear model, with $y=m_x+m_y+m_xm_y+e$, where m_x and m_y are the genotypes of markers x and y (Morgan and Mackay, 2006).

RESULTS

Mean values of HSS are shown in Fig. 1 for heat-hardened and non-hardened flies from cultures at both low and high density. Heat-hardening treatment significantly increased HSS in both sexes (Fig. 1; Table 1). In non-hardened flies, HSS significantly increased with larval density (Fig. 1; Table 1). Hardening effects at low density were more evident in RIL-D48 than in RIL-SH2 (Fig. 1; Table 1). Females were the more heat resistant sex in both heat-hardened and non-hardened RIL, and at both low and high larval density (Fig. 1). Basal thermotolerance as measured by HSS in non-hardened females was higher in RIL-SH2 than in RIL-D48 (Fig. 1). Interestingly, this HSS differentiation between RIL panels was shifted by heat hardening at low density (Fig. 1), with a significant interaction between heat-hardening and density treatments in RIL-D48 only (Table 1), suggesting substantial effects from the genetic background.

Heat shock survival was influenced by large-effect QTL from all major chromosomes, with 10 QTL that explained between 17% and 33% of the phenotypic variance in either trait (Fig. 2; Table 2). The number of significant QTL was higher in the analysis based on each replicate separately than in the analysis based on mean values across

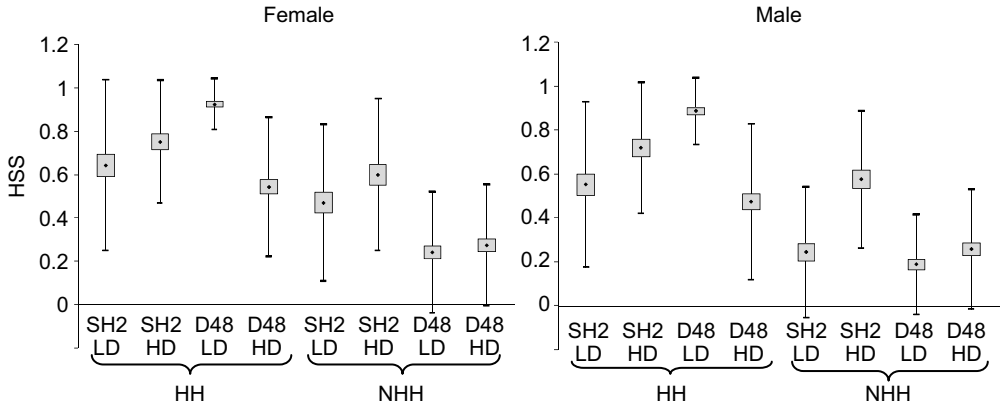

Fig. 1. Mean values (±s.e.m.) are shown for heat stress survival (HSS) in two recombinant inbred lines (RIL) of *Drosophila melanogaster* (RIL-D48 and RIL-SH2). Flies that were reared at either low (LD) or high (HD) density were scored for HSS with (HH) and without (NHH) a heat-hardening pre-treatment.

replicates (see supplementary material Fig. S3), which can be related to environmental variation between replicates. In fact, the stability of the within-treatment phenotypic correlations between replicates was not always apparent (supplementary material Table S1). In addition, a few QTL were not consistent with the sign of their additive effects across replicates within either RIL panel (Q6, Q7 and Q15; Table 1), and because of this we discarded these QTL peaks as relevant for HSS.

Basal thermotolerance was affected by several QTL that co-localized with thermotolerance QTL detected in previous studies (Table 2). However, new QTL were identified in this study. For example, Q9 and Q18 are new large-effect QTL that explain more than 17% of the phenotypic variance in survival of heat stress (Table 2). Some QTL were detected in flies reared under high density only (e.g. Q14; Table 2). In addition, Q18, Q19 and Q20 were significant in non-hardened flies at high density only (Table 2). Even though environmental variance in quantitative traits usually increases with larval density, some QTL were also found in flies that were reared at high larval density explaining a large proportion (between 10% and 30%) of the phenotypic variance in HSS (Q10 and Q16; Table 2). In total, the number of significant QTL was greater at high density than at low density, with eight QTL that were significant at high density against six QTL that were significant at low density (Table 2).

Both the number of QTL and the phenotypic variance explained by each QTL were lower in heat-hardened than in non-hardened flies (Table 2). One of the most important QTL for thermotolerance in adult flies is Q1 (partially overlapping with Q15), as this QTL was consistently found in very different mapping populations (Table 2). This QTL was the most dynamic in the sign of additive effects, with a shift in sign in heat-hardened flies at high larval density, as this well-known QTL has been shown to have a positive additive effect on thermotolerance in non-hardened flies in the present and previous studies based on the same mapping populations as in the present study (see Table 2 for references). The above-mentioned QTL were significant in either RIL panel (RIL-SH2 or RIL-D48) but many such QTL were also significant when all replicates were pooled (see supplementary material Fig. S3).

Table 1. Results of ANOVA performed to test for fixed effects of (1) heat hardening and (2) density in two panels of RIL lines of *Drosophila melanogaster* used in this study

	RIL-D48			RIL-SH2		
Source of variation	d.f.	MS	*F*	d.f.	MS	*F*
Females						
(1) Heat hardening	1	21.26	315.51***	1	1.51	12.37***
(2) Density	1	2.85	42.32***	1	0.81	6.61***
(1) × (2)	1	4.01	59.58***	1	0.05	0.82
Error	372	0.07		226	0.12	
Males						
(1) Heat hardening	1	19.54	286.18***	1	2.91	27.79***
(2) Density	1	2.78	40.67***	1	3.55	33.92***
(1) × (2)	1	5.45	79.80***	1	0.40	3.70
Error	372	0.07		226	0.10	

RIL, recombinant inbred lines.
**P<0.01; ***P<0.001.

Epistasis was tested between all pairs of markers used in the genetic map associated with our RIL [references for all markers are given in Norry et al. (Norry et al., 2008)]. Epistasis was apparent on HSS when testing pairwise interactions involving the marker AC008198 (66D10–E2), particularly between AC008198 and DMU43090 (99D6–D9) ($F_{1,46}$=12.57; P=0.0009) as well as between AC008198 and either DMU56661 ($F_{1,46}$=11.58; P=0.001) or AC009392 (23A–E) ($F_{1,46}$=11.56; P=0.001) in hardened flies at low density. Other interactions involving other markers were also significant but only before correcting for multiple comparisons (AFO47180, DROEXPAND, DMRHOb, DS06577 and AC004759).

DISCUSSION

QTL mapping in HD flies was successful in finding new genomic regions involved in thermotolerance in environments that were not previously tested for QTL (Table 2). The genetic basis of both basal and induced thermotolerance was dependent on density, with four new QTL in non-hardened flies that were significant at high density only, involving chromosomes X and 2. There was a substantial genotype-by-environment interaction, involving both density and heat-hardening treatments (Table 1).

In one previous QTL study on heat-induced thermotolerance only a short-term (<1 h) heat treatment was applied and only one QTL was detected for the heat-hardening effect on heat knockdown resistance, which explained only a small fraction of the phenotypic variance and co-localized with the region where the small heat-shock protein genes map (Norry et al., 2008). In contrast, in the present study a much longer and repeated heat stress treatment was used to induce heat-hardening effects on thermotolerance. In this case, we tested the possible beneficial effects of a longer term mild stress pre-treatment on HSS and detected new QTL with large effects on HSS (e.g. explaining more than 15% of the phenotypic variance in HSS) but only in flies reared at high density (Table 2). These QTL include many candidate genes (see supplementary material Table S2). This is an interesting result, suggesting that the interaction

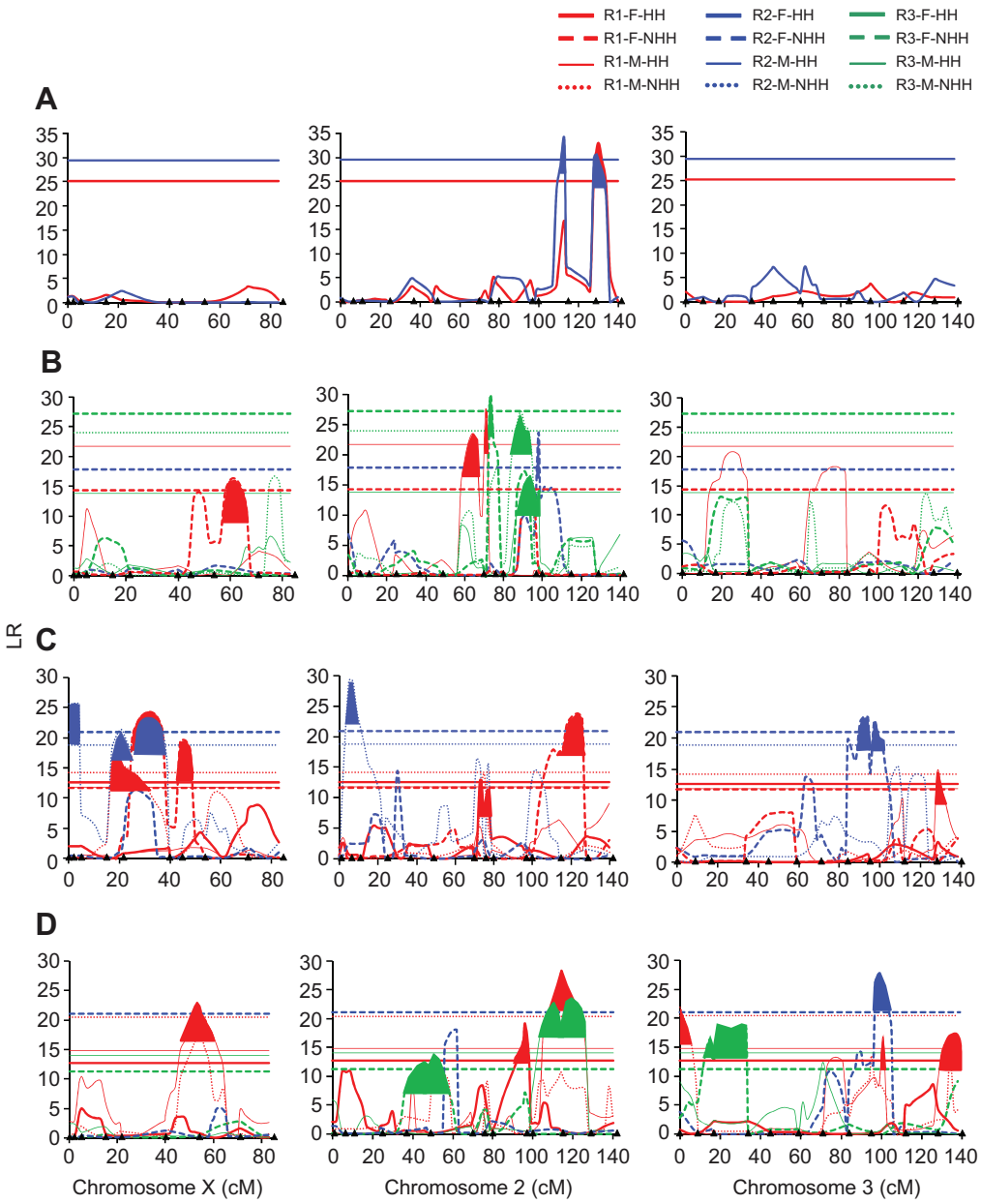

Fig. 2. Plots of likelihood ratio (LR) scores against map position (in cM) from composite interval mapping for survival of heat stress is shown for adult RIL-D48 flies at low density (A), RIL-D48 at high density (B), RIL-SH2 at low density (C) and RIL-SH2 at high density (D). The analysis was performed for three independent replicates (R1, R2, R3) in heat-hardened (HH) and non-hardened (NHH) females (F) and males (M). Significance thresholds were determined by 1000 random permutations (horizontal lines). Approximate intervals for a confidence level higher than 95% are shown for significant quantitative trait loci (QTL) peaks (maximum width of marked QTL peak), using 1.5 $\log_{10}$ of odds (LOD)=6.9 LR (Dupuis and Siegmund, 1999). To avoid overlapping of lines, only cases that were significant are shown.

of heat stress and high larval density may increase the number of QTL for HSS in heat-hardened flies.

Given that the number of emerged flies was about 4 times higher in our high density cultures, larval density was a major factor in this study. Some of the major QTL that were previously identified in other studies of thermotolerance in flies reared at low density were also significant at high density (Table 2). For instance, Q1 is a well-known QTL for thermotolerance both in the laboratory (Table 2) and in field-released flies (Loeschcke et al., 2011). Q8 and Q12 were also previously detected for heat knockdown resistance in low density studies (Table 2), and apparently these QTL were also significant for HSS at high density (Table 2). All these QTL co-localizing both between thermotolerance traits and between high and low density could be suggested to be general rather than environment-specific QTL for thermal adaptation in adult *D. melanogaster*.

High larval density could be more important in summer than in winter in temperate populations, as population size would increase during summer when heat stress should be more frequent than in winter. Larval density consistently increased HSS in RIL-SH2 (both in heat-hardened and in non-hardened flies) but the lines that derived from the backcross to the more cold-adapted population of origin (RIL-D48) exhibited a beneficial effect of larval density only in non-hardened flies. Such an interaction between density and heat-hardening effects was significant by ANOVA for RIL-D48, whereas RIL-SH2 showed no significant interactions between these factors (Table 1), perhaps reflecting an adaptive response to both temperature and population density simultaneously. For instance, high larval crowding is a stress that can induce Hsp70 expression, leading to increased adult thermal stress resistance in *Drosophila* (e.g. Bubli et al., 1998; Sørensen and Loeschcke, 2001). Although no QTL in this study was significant at the region of the Hsp70 genes, three QTL were specific for lines that were reared at high density (Q14, Q16 and Q17; Table 2).

The beneficial effect of both heat acclimation and long-term heat hardening is an important component of thermotolerance as it

Table 2. QTL for survival of heat stress for adult flies from RIL-D48 and RIL-SH2

Treatment	QTL name	R; Sex; RIL	QTL range	*a*	% Variance	QTL overlapping with previous LD studies
LD; HH	Q1	R1 F RIL-SH2	34C4–42A	0.006	11.85	a, b, c, d
	Q2	R2 F RIL-D48	50C–54B2	0.317	12.94	b
	Q3	R1 F RIL-D48	56D11–59A2	–0.178	1.72	
	Q3	R2 F RIL-D48	56D11–59A2	–0.248	7.42	
	Q4	R1 M RIL-SH2	97F–99D9	–0.284	9.78	
LD; NHH	Q5	R2 M RIL-SH2	1B8–3C6	0.207	12.99	d
	Q6	R2 M RIL-SH2	4F1–7B3	–0.193	3.38	
	Q6	R1 M RIL-SH2	4F1–7B3	0.248	13.89	
	Q7	R2 M RIL-SH2	7B3–10A1	–0.193	3.98	d
	Q7	R1 F RIL-SH2	7B3–10A1	0.147	15.4	d
	Q8	R1 F RIL-SH2	10A1–12E	0.146	10.69	c, d
	Q9	R2 M RIL-SH2	21C3–23E	–0.243	17.39	
	Q10	R1 F RIL-SH2	50C–56E6	–0.195	21.83	b
	Q11	R2 F RIL-SH2	90B1–90E	–0.378	32.78	a, c
	Q12	R2 F RIL-SH2	90E–95C8	–0.378	29.35	a, c
HD; HH	Q13(8)	R1 M RIL-SH2	10A1–16F6	–0.257	2.62	c, d
	Q14	R1 M RIL-D48	30A3–34C2	0.254	3.13	
	Q1	R1 M RIL-D48	34C2–42A	0.255	0.73	a, b, c, d
	Q15	R3 M RIL-D48	42A–49C	0.238	6.71	b, d
	Q15	R1 F RIL-SH2	42A–49C	–0.223	25.74	b, d
	Q16	R3 M RIL-D48	49C–50C	0.243	10.25	d
	Q10	R1 M RIL-SH2	50C–56E6	–0.328	12.19	b
	Q10	R3 M RIL-SH2	50C–56E6	–0.123	28.53	b
	Q12	R1 M RIL-SH2	90E–95C8	–0.114	1.69	a, c
	Q4	R1 M RIL-SH2	97F–99D9	–0.165	12.77	
HD; NHH	Q17	R1 F RIL-D48	12E–16F6	0.276	1.88	
	Q18	R3 F RIL-SH2	28A1–30A6	0.116	19.96	
	Q14	R3 F RIL-SH2	30A3–34D2	0.135	12.05	
	Q1	R3 F RIL-D48	34C4–38E9	0.240	23.42	a, c
	Q15	R3 M RIL-D48	42A–49C	0.201	6.57	b, d
	Q16	R2 F RIL-D48	49C–50C	0.194	13.28	d
	Q19	R1 M RIL-SH2	62A–63D2	–0.382	20.23	b
	Q20	R3 F RIL-SH2	63D2–66E2	–0.127	11.24	b
	Q12	R2 F RIL-SH2	90E–95C8	–0.333	18.04	a, c

Flies were reared in cultures at low (LD) and high (HD) density and were subsequently either heat hardened (HH) or non-heat hardened (NHH).
Significant quantitative trait loci (QTL) are indicated for each replicate (R) in females (F) and males (M). Underlined QTL were specific for HD.
% Variance is the percentage of total phenotypic variance explained by the QTL. Additive effects (*a*) are also given.
References: (a) Norry et al., 2004; (b) Morgan and Mackay, 2006; (c) Norry et al., 2008; (d) Rand et al., 2010.

generally increases tolerance to heat stress (reviewed in Hoffmann et al., 2003). No previous studies have investigated QTL for HSS in hardened flies. Consistent with a heat knockdown study on short-term heat hardening (Norry et al., 2008), we detected that the number of QTL as well as the phenotypic variance explained by each QTL were lower in hardened than in non-hardened flies (Table 2). This general trend might suggest that heat-hardening effects are generally related to genes that are not functionally variable between individuals or populations.

Importantly, new QTL for thermotolerance were detected in the present study on HSS. This trait was not previously mapped in our RIL lines. In this study, we used different larval densities and hardening treatments and detected approximately 20 QTL for HSS across the different environments (low and high larval density with and without hardening). In contrast, previous studies detected less than 10 QTL considering diverse traits of heat stress resistance in flies reared at relatively low density (Morgan and Mackay, 2006; Norry et al., 2008). Q9 at low density as well as Q14, Q16 and Q17 at high density are new QTL that explain a significant portion of the phenotypic variance in HSS in this study (Table 2). These QTL include several candidate genes that were differentially expressed in response to regimes of thermal selection (Sørensen et al., 2007).

Overall, the present results illustrate how the genetic architecture of thermotolerance in adult insects may strongly depend on larval density, with some large-effect QTL being significant at either low or high density only. New QTL for thermotolerance in this study were specific for heat-hardened flies, indicating that the genetic basis for heat hardening is not identical to the genetic basis for basal thermotolerance. This is the first QTL study to consider both hardening and larval density when studying the genetic basis of thermotolerance in *D. melanogaster*. Taken together, the results are consistent in indicating that both heat hardening and larval density are crucial aspects of thermotolerance QTL.

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