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Quantitative genetic analysis suggests causal association between cuticular hydrocarbon composition and desiccation survival in *Drosophila melanogaster*

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Survival to low relative humidity is a complex adaptation, and many repeated instances of evolution to desiccation have been observed among *Drosophila* populations and species. One general mechanism for desiccation resistance is Cuticular Hydrocarbon (CHC) melting point. We performed the first Quantitative Trait Locus (QTL) map of population level genetic variation in desiccation resistance in *D. melanogaster.* Using a panel of Recombinant Inbred Lines (RILs) derived from a single natural population, we mapped QTL in both sexes throughout the genome. We found that in both sexes, CHCs correlated strongly with desiccation resistance. At most desiccation resistance loci there was a significant association between CHCs and desiccation resistance of the sort predicted from clinal patterns of CHC variation and biochemical properties of lipids. This association was much stronger in females than males, perhaps because of greater overall abundance of CHCs in females, or due to correlations between CHCs used for waterproofing and sexual signalling in males. CHC evolution may be a common mechanism for desiccation resistance in *D. melanogaster*. It will be interesting to compare patterns of CHC variation and desiccation resistance in species which adapt to desiccation, and rainforest restricted species which cannot.
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Introduction

The genetics of complex adaptation, particularly the degree to which populations follow either parallel or divergent evolutionary trajectories under common selective pressures is an area of active interest in evolutionary biology see (Gompel and Prud'homme, (2009); Stern and Orgogozo, 2009). Desiccation resistance in *Drosophila* is an interesting candidate complex trait in which to study repeated patterns of evolution among species and populations (Hoffmann and Harshman, 1999; Gibbs, 2002). Many *Drosophila* encounter periods of low humidity during their life-history and must balance water content against integumentary water loss owing to a high surface area/volume ratio. In doing so, numerous *Drosophila* species have successfully colonized arid habitats including deserts and high altitudes as well as tropical and temperate zones, providing an excellent model to study adaptation to desiccation at the intra- and inter-population level (Parsons, 1991; Gibbs, 2002; Parkash *et al*., 2005). By contrast several rainforest restricted species show little ability to evolve resistance to desiccation (Hoffmann *et al*., 2003; Kellermann *et al*., 2009), although they show ample genetic variation for many other traits. Little is known about the mechanisms underlying natural genetic variation for desiccation survival in *Drosophila* at the population scale, and thus why some species adapt easily whereas others are limited by low adaptive variation for desiccation stress.

To date, most studies on the evolution of desiccation resistance have focused on populations derived from experimental evolution, with emphasis on the physiological, correlated and life-history responses associated with increased desiccation reviewed in (Hoffmann and Harshman 1999; Telonis-Scott *et al*., 2006). Comparative physiology among different *D. melanogaster* lines suggests that multiple evolutionary solutions can arise from a common selection pressure, although reducing water loss by water retention is a common mechanism underlying survival to desiccation (Gibbs *et al*., 1997; Hoffmann and Harshman, 1999; Gibbs, 2002; Telonis-Scott *et al*., 2006). Although artificial selection experiments are not without limitations, trait variation in natural populations may be inferred if enough alleles are sampled from the founding population. Signatures of natural selection for desiccation resistance are also evidenced in latitudinal clines, where survival can vary markedly among *Drosophila* according to local climatic conditions. Substantial variation in survival between populations

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suggests local adaptation and the presence of ample genetic variation (Coyne *et al*., 1983; Blows and Hoffmann, 1993; Kennington *et al*., 2001; Hoffmann *et al*., 2005). Opposing clines for desiccation and starvation resistance have been observed for seven Drosophilid species of the Indian subcontinent including *D. melanogaster*, where desiccation survival increased with latitude (Parkash *et al*., 1994; Karan and Parkash, 1998; Karan *et al*., 1998; Parkash and Munjal, 1999)

In *D. melanogaster*, geographic clines in Cuticular Hydrocarbon (CHC) profiles parallel climatic clines and correlate with patterns of desiccation resistance (Rouault *et al*., 2001, 2004). CHCs are the main constituent of the insect epicuticle, which functions primarily as a barrier against desiccation in nature (Hadley, 1981). Hydrocarbon chains in *D. melanogaster* range in length from 20–34 carbons, and there is evidence to suggest that expression of different chain lengths can affect survival to abiotic stresses such as desiccation and temperature (Toolson and Kupersimbron, 1989; Gibbs *et al*., 1991, 1997; Gibbs, 1998). Whether genetic variation in CHC expression at the population level in *Drosophila* correlates with variation in desiccation resistance remains to be seen, and it is unclear whether, despite the dimorphism in males and females, they use similar genetic mechanisms to adapt to common stresses.

Here, we use Quantitative Trait Locus (QTL) analyses in a set of recombinant inbred lines (RILs) to explore associations between survival to desiccation stress and CHCs at the population level in male and female *D. melanogaster*. QTL methodology permits statistical analyses of the associations between phenotype and genotype to characterize a minimum set of genomic regions that affect complex traits (Doerge, 2002). Quantitative trait loci for many fitness-related, stress resistance and sexually dimorphic traits have previously been mapped in this particular panel of RILs, which originate from two wild caught heterozygous *D. melanogaster* isofemales (Kopp *et al*., 2003; Wang *et al*., 2004; Mezey *et al*., 2005). High levels of CHC variation have also been identified in these lines, and a large number of CHC QTL identified (Foley *et al*., 2007). Here, we use these RILs to characterize QTL underlying natural genetic variation for survival to desiccation, and report the first QTL data for this trait in *D. melanogaster*. Furthermore, we explore the causal relationship of CHC expression on desiccation resistance using a Structural Equation Modelling (SEM) framework (Li *et al*., 2006; Jansen *et al*., 2009).

Materials and methods

Recombinant inbred lines (RILs) and CHC analysis

A set of 144 RILs was generated from the cross between a single pair of F_1 progeny derived from two wild isofemales trapped at the same location (Winters, California). One hundred and fifty-two markers were retained for the analysis. The cross schematics and line genotyping are described in detail elsewhere see (Kopp *et al*., 2003). CHCs were assayed as described in Foley *et al*., 2007. Rearing and assay conditions were similar—12/12 light/dark, 25C—for both the CHC assays and desiccation (below). Representative CHC traces for each sex are shown in Supplemental Figure 1 (reproduced, with permission from Foley *et al*., 2007).

Desiccation survival assays

Density was standardized in the RILs for two generations before the desiccation assays by placing five pairs of flies on dextrose cornmeal medium for 3 days (line, $n = 103$). For the assays, flies from each RIL were collected across 2–3 vials within 24–48 h of eclosion, matured for 2 days in a mixed sex cohort, then separated by sex and using CO_2 anaesthesia and held at a density of five flies per vial for another day. Survival to desiccation was assessed by desiccating three replicates of five females and five males (sexes tested separately). The flies were placed in empty vials sealed with gauze and were affixed with Parafilm to another vial containing approximately 10 g of silica desiccant. Vials were scored at hourly intervals until all flies in a group had died (LT_{100}), and the time for half the flies to die (LT_{50}) was determined by linear interpolation. QTL mapping was performed for both sexes and time-points.

Analysis of mean survival to desiccation

The data for each trait was log transformed before analysis to normalize them (tested with proc UNIVARIATE, SAS Institute, Cary NC, USA, v9.1). Two-way ANOVAs were performed with sex as the fixed effect, and the line and line-by-sex as random effects. Genetic correlations between the traits and sexes were estimated (Proc CORR) after averaging between replicates.

Composite interval mapping (CIM)

QTL mapping software is designed to analyse mapping populations with two alleles derived from isogenic parents. As outbred founders were used in this study, up to four parental autosomal haplotypes, and 3 of the X, may be segregating among RILs. However, by adapting standard mapping software to the RILs used in this study, we can test whether an allele from one chromosome codes for a trait value significantly different from the average trait because of the weighted average of the other chromosomes described in detail in (Wang *et al*., 2004). Separate analyses were performed for each of the linkage groups; three for the X and third chromosomes (two of the parental third chromosomes appear identical see Kopp *et al*., 2003), and four for the second chromosome. Female and male survival at 50% mortality (LT_{50}) and 100% (LT_{100}) mortality were analysed using the CIM procedure in Windows Version 2.0 QTL Cartographer (Basten *et al*., 1994, 1999). The CIM Model 6 of QTL Cartographer was used, with number of background markers = 2, window size of 30 cM and Kosambi mapping function. Significance thresholds of $\alpha = 0.05$ were determined by 1000 permutations for each trait and chromosome. QTL analyses on other traits considered for estimation of pleiotropy with desiccation were performed in a similar way using the same software using identical marker sets.

Results

Analysis of mean survival to desiccation

The average time (± s.d.) of 50% mortality to desiccation was 12. 1 (4.25) and 7.4 h (1.93) for females and males, respectively. The average time (± s.d.) for 100% mortality was 15.4 h (4.90) for females and 9.6 h (2.60) for males. Considering the sexes separately, the proportion of

variance in desiccation resistance due to line (similar to broad sense heritability) was very high. For females, line explained 90.6% of the variance in LT_{50}, and 90.7% of the variance in LT_{100}, and in males 89.7 and 85.5%, respectively. Table 1 presents the ANOVA for survival to desiccation. For both survival traits, there was a highly significant line and sex term in the ANOVA ($P<0.0001$), as well as a significant line by sex interaction ($P<0.0001$). All traits were genetically correlated both among and between the sexes, (Pearson correlations significant at $P<0.0001$): $r=0.73$ for male and female LT_{50}; $r=0.68$ for male and female LT_{100}; $r=0.95$ female LT_{50} and LT_{100}, and $r=0.89$ for male LT_{50} and LT_{100}.

QTL analysis

Composite interval mapping was employed to estimate the position of QTL affecting survival to desiccation in both males and females. The log-likelihood ratios (LOD) were plotted against the cytological position in Centimorgans (cM) for the 10 linkage groups across the three major chromosomes (Supplemental Figure 2). The separate CIM analysis of each LG for 50% (LT_{50}) and 100% (LT_{100}) female and male mortality are presented in (Supplemental Figure 2). As up to four alleles may be segregating among the RILs, QTL Cartographer was used to test if an allele significantly affected survival to desiccation compared with the weighted average of several alleles, and the positive or negative effects of a QTL on survival were estimated and expressed as the additive effect (Table 2).

Approximately 95% confidence intervals were constructed for all significant peaks using the two-LOD support rule (Lynch and Walsh, 1998). As a conservative estimate, we assumed that significant peaks across adjacent marker intervals with overlapping confidence intervals were potentially a single QTL and where two QTL had non-overlapping confidence intervals, a minimum of two QTL were assumed to be in the region. Significant LOD maxima with wide confidence intervals spanning the entire linkage group were considered uninformative, and excluded from this analysis. The same QTL may be identified by the separate analysis of each chromosome, therefore each significant peak may not necessarily reflect a unique QTL (Mezey *et al*., 2005). Following the selection criteria outlined in Mezey *et al*. (2005), a single putative QTL was declared for a trait if significant peaks were observed for overlapping marker regions in different linkage groups. A total of 40 analyses were performed for 10 linkage groups and four traits. To express all QTL and confidence intervals for desiccation survival on the cytogenic scale, they were plotted according to the proportion of their distance between flanking markers (following Foley *et al*., 2007).

An approximate minimum set of 15 putative QTL for survival to desiccation was identified by these criteria (Table 2). Nine QTL co-localized between the sexes with concordant effects for all traits, suggesting substantial non sex-specific genetic co-regulation for survival to desiccation with only minor differences between 50 and 100% mortality. Three sex-specific QTL were observed on the X chromosome. The largest effects on desiccation resistance mapped to the 2nd chromosome—according to our criteria there were at least 8 QTL, of 15,

Table 1 Analysis of variance for survival to desiccation

Trait	*Source*	*d.f.*	*Mean squares*	*F-value*	*Significance*
Female LT_{50}	Line	103	42.417	9.63	$P<0.0001$
	Error		4.403		
Female LT_{100}	Line	103	57.222	9.74	$P<0.0001$
	Error		5.873		
Male LT_{50}	Line	102	8.965	8.67	$P<0.0001$
	Error		1.035		
Male LT_{100}	Line	102	14.544	5.88	$P<0.0001$
	Error		2.474		
LT_{50}	Line	103	0.071	14.43	$P<0.0001$
	Sex	1	6.898	1395.77	$P<0.0001$
	Line × sex	102	0.013	2.65	$P<0.0001$
	Error	397	0.005		
LT_{100}	Line	103	0.064	13.12	$P<0.0001$
	Sex	1	6.708	1376.60	$P<0.0001$
	Line × sex	102	0.013	2.70	$P<0.0001$
	Error	397	0.005		

Table 2 Summary of putative QTL and effects on survival to desiccation in males and females

Putative QTL	*Linkage group*	*LOD max (CI) female LT_{50}*	*LOD max (CI) female LT_{100}*	*LOD max (CI) male LT_{50}*	*LOD max (CI) male LT_{100}*
QTL1	X-1	—	—	—	↑ **12B (6E-17E)**
QTL2	X-2	↓ 7F (6B-8C)	—	—	—
QTL3	X-2	↓ 8F (8D-9A)	—	—	—
QTL4	2–2	↓ 23C (22E-35F)	↓ 23C (22E-35F)	—	—
QTL5	2–1	↑ **25B (21A-30A)**	↑ **25B (21C-32C)**	↑ **25B (22C-33F)**	↑ **27D (21C-33F)**
QTL6	2–3	—	—	↑ **28C (25B-31E)**	↑ **28C (25B-31E)**
QTL6	2–2	↓ 28D (26D-34E)	↓ 28D (26D-34E)	↓ **28D (23C-36B)**	↓ **28D (23C-36B)**
QTL7	2–2	↓ **31C (28F-33B)**	↓ **31C (27C-33B)**	↓ **31C (25B-34B)**	↓ **31C (25B-34B)**
QTL8	2–2	↓ **37C (22A-47C)**	↓ **37C (22A-45C)**	↓ **37C (22A-55A)**	↓ **37C (23C-49F)**
QTL9	2–1	↑ 45C (36A-53E)	↑ 44A (22C-53E)	↑ **40A (21C-53E)**	↑ **40A (21C-48D)**
QTL10	2–3	↓ **47D-E (43E-47E)**	↓ **47D-E (45D-47E)**	↓ **47D-E (42A-47E)**	↓ **47D-E (22B-47E)**
QTL11	2–1	↑ **51C (39E-52F)**	↑ **51C (50A-52F)**	↑ **51C (21C-52F)**	—
QTL12	3–2/3	—	—	—	↑71E-76C (66D-77D)
QTL13	3–2/3	↑ **84D-F (77C-85D)**	↑ **84D-F (77C-84F)**	↑ 84D-F (83C-85D)	↑ 84D-F (83C-85F)
QTL13	3–1	—	—	—	↓ 84DE (69A-84DE)
QTL14	3–1	↓ 87A (87A-89AB)	↓ 87A-F (87A-97B)	↓ **87A (87A-94D)**	↓ **87F-F (87A-94D)**
QTL15	3–1	—	—	—	↓ **89AB (86D-94D)**

The LOD maxima for each interval and overlapping 2-LOD support intervals (in brackets) are given for all traits. Arrows indicate the QTL effect on survival. Desiccation QTL with overlapping CHC QTL (Foley *et al*, 2007) are indicated in bold.

segregating on chromosome 2, 6 of which co-localized between males and females (Table 2, Supplemental Figure 2). The largest effect QTL were identified on the distal end of 2L for linkage groups 2–1, 2–2 and 2–3 between cytological positions 22–33, but for both sexes, there were QTL on all the major chromosomes.

There was qualitative QTL agreement between several desiccation loci and CHC expression (Foley *et al*., 2007) across chromosome 2L, and many of these QTL colocalized in both sexes (Table 2, Supplemental Figure 2). On chromosome 3 and the X chromosome, male and female CHC expression did not colocalize well with each other, or with desiccation QTL. Although there is a large desiccation QTL on 3L on linkage group 3–2&3 for both males and females, we observed no significant CHC QTL on male 3–2&3. Similarly, while on the 3R chromosomal arm of linkage group 3–1 there are QTL for desiccation resistance in males and females, there are no female CHC QTL. Because confidence intervals for CHC traits and desiccation were so broad, however, it is impossible to assess pleiotropy based on colocalization.

CHC and desiccation pleiotropy

Principal Components (PC) analysis is commonly used as a factor reduction method to study large numbers of correlated traits (Stevens, 1996). Because PCs are constrained to be uncorrelated, they are also ideal for use in multiple regression analysis, to avoid overfitting and because multicollinearity can make it difficult to assign significance to particular factors. We conducted PC analyses on the covariance matrix of male and female CHC logcontrasts (Supplemental Table 1). Logcontrasts are a standard transformation used in the previous analysis of CHCs in these lines (Foley *et al*., 2007). We plotted the eigenvalues on a scree plot to determine, which PCs explained the majority of the non-error variance in CHC expression (Stevens, 1996). In males, the top three PCs were retained, and in females the top five. Multiple regression of the major PCs on desiccation showed that CHCs and desiccation resistance were highly correlated in both sexes, and that CHCs explained from 31–45% of variation in desiccation resistance in these lines. For female LT_{50} both PC2 and PC3 were highly significant ($PC2_{1,101}$ $t=4.94$, $P<0.001$; $PC3_{1,101}$, $t=-5.696$, $P<0.001$) with a model adjusted r^2 of 0.341. For LT_{100}, PC2 and PC3 were also both significant ($PC2_{1,101}$ $t=-3.97$, $P<0.001$; $PC3_{1,101}$ $t=-5.96$, $P<0.001$) with a total model adjusted r^2 of 0.324. In males PC1, PC2 and PC3 were all significantly associated with both LT_{50} ($PC1_{1,103}=-2.38$, $P=0.019$; $PC2_{1,103}$, $t=-3.57$, $P=0.001$; $PC3_{1,101}$, $t=-3.24$, $P=0.002$) with an adjusted r^2 of 0.203, and LT_{100} ($PC1_{1,103}=-4.10$, $P<0.001$; $PC2_{1,103}$, $t=-4.44$, $P<0.001$; $PC3_{1,101}$, $t=-3.80$, $P<0.001$) and an adjusted $r^2$of 0.32. Lower PCs were not significantly associated with desiccation resistance in either sex (data not shown).

Correlations between traits

It can be difficult to interpret loadings of PCs when looking for causal relationships between traits (Li *et al*., 2006). We do, however, have *a priori* knowledge of the chemical classes of CHCs, and some idea of the expected mechanism by which they affect desiccation resistance in insects c.f. (Rouault *et al*., 2004). Therefore, to determine whether particular chemical classes of CHCs contribute to increased desiccation resistance, subsets of the CHCs of the most abundant categories were selected and their relationship with desiccation estimated. Because male and female CHC profiles qualitatively differ in *D. melanogaster*, somewhat different chemical classes were considered in the two sexes. In males, linear alkanes (linear), 7:Cn alkenes (7C), and 2MeCn methylated alkanes (methyl) were evaluated, and in females linear alkanes (linear), 7:Cn alkenes (7C) and 7,11:Cn alkadienes (7,11). Besides the chemical group, the relative abundance of long and short chain CHCs was calculated for each sex. For females, compounds with 27 carbons or more represented approximately half the CHC blend, and were considered long chain CHCs (long). For males, the compounds with 25 or more carbons represented approximately half the total amount, and were considered long chain CHCs for males (long). This is consistent with long/short ratios considered in other studies in this species (Rouault *et al*., 2004). Other metrics of chain length, such as mean CHC carbon number, gave very similar results (data not shown). We opted for this binning-metric as it is a proportion calculated in an identical manner to the other chemical-class metrics, allowing us to analyse all CHC traits similarly. The proportion of the total CHC abundance for these separate categories was calculated, and arcsine transformed to approximate a normal distribution.

Linear regression on the arcsine transformed traits shows that the proportion of long-chain CHCs expressed is a good predictor of desiccation resistance in the RILs, whereas the chemical class of the compound is evidently less important (Table 3a and b). The proportion of long-chain hydrocarbons positively correlates with desiccation resistance in both sexes. Correlations ranged from 19% for male desiccation lt_{50} (marginally non-significant) to 54% for female lt_{100} (highly significant: $P<0.0001$). In females, the proportion of both alkanes and 7,11:Cn alkadienes were also significantly associated with desiccation

Table 3 Correlations (r) between desiccation and CHC traits measured in the RILs and their significance in female (a) and male (b) *D. melanogaster*

	lt50	*lt100*	*Long*	*Linear*	*7,11*	*7C*	*Melt*
(a)							
lt50	1.000	**0.960**	**0.525**	**0.269**	**−0.273**	0.093	**0.377**
Lt100	<0.001	1.000	**0.540**	**0.249**	−0.237	0.076	**0.379**
Long	<0.001	<0.001	1.000	0.033	0.080	−0.115	**0.867**
Linear	0.007	0.012	0.723	1.000	**−0.747**	−0.135	**0.304**
7,11	0.006	0.017	0.382	<0.001	1.000	**−0.494**	**−0.372**
7C	0.356	0.448	0.208	0.141	<0.001	1.000	−0.035
Melt	<0.001	<0.001	<0.001	0.002	<0.001	0.731	1.00
	lt50	*lt100*	*Long*	*Linear*	*7C*	*Methyl*	*Melt*
(b)							
Lt50	1.000	**0.854**	0.193	0.182	0.017	0.005	**0.314**
Lt100	<0.001	1.000	**0.293**	0.184	−0.006	0.120	**0.444**
Long	0.051	0.003	1.000	**−0.241**	**−0.258**	**0.634**	**0.846**
Linear	0.067	0.063	0.007	1.000	**−0.456**	**−0.312**	−0.016
7C	0.869	0.954	0.004	<0.001	1.000	**−0.347**	−0.094
Methyl	0.963	0.227	<0.001	<0.001	0.000	1.000	**0.680**
Melt	0.001	0.000	0.000	0.877	0.345	0.000	1.000

Correlations are shown above the diagonal, and significant correlations are in bold. *P*-values are indicated below the diagonal.

resistance. Multiple regression and model selection using AIC suggests that while the proportion of long-chain hydrocarbons is the largest contributor to desiccation resistance in both sexes, other aspects of CHC expression profile were important contributors to overall ability to resist desiccation. For females, the model including the proportion of long-chain CHCs and 7,11:Cn alkadienes best explained the data for both LT_{50} ($F_{2,97}=27.93$, $r^2(adj)=0.352$, $P \ll 0.0001$), and LT_{100} ($F_{2,97}=27.91$, $r^2(adj)=0.352$, $P \ll 0.0001$). For males, the model including the proportion of long-chain CHCs, linear alkanes and 7:Cn alkenes best explained the data for both LT_{50} ($F_{3,\ 98}=5.65$, $r^2(adj)=0.121$, $P=0.001$) and LT_{100} ($F_{3,\ 98}=9.32$, $r^2(adj)=0.198$, $P<0.0001$). In females, the correlation of long-chain hydrocarbons and desiccation resistance was similar to the model adjusted r of PCs on desiccation traits (0.584 and 0.569 for lt_{50} and lt_{100}, respectively), whereas in males the correlation was less strong than found in the PC regression. There were also significant correlations between different chemical CHC classes in females, and especially in males, as well as between male and female CHC traits (Supplemental Table 2). In particular, the correlation between male and female proportion of long-chain CHCs was relatively high, with an r of 0.292.

For females, the effect of chain length on desiccation resistance was positive, whereas that for 7,11:Cn alkadienes was negative. For males, the effect of all variables were positive. For a given chain length, the melting point of CHCs decreases with the number of double bonds or methyl groups (Supplemental Figure 1, reproduced from Foley *et al*., 2007). The direction of effects in the best-fit models were consistent with an interpretation that overall CHC melting point was important in determining desiccation resistance. Accordingly, we constructed a metric of melting point by taking the average retention time of CHCs (which is proportional to melting point) weighted by compound abundance, for each sex and genotype. Average melting point was a significant predictor of desiccation resistance for female LT_{50} ($F_{1,98}=16.21$, $r^2(adj)=0.133$, $P=0.0001$), and LT_{100} ($F_{1,98}=16.44$, $r^2(adj)=0.135$, $P=0.0001$); as well as male LT_{50} ($F_{1,100}=10.92$, $r^2(adj)=0.089$, $P=0.001$), and LT_{100} ($F_{1,100}=24.59$, $r^2(adj)=0.189$, $P \ll 0.0001$). These r^2 values are somewhat less than the best-fit PC models. Model selection using AIC, including the other CHC traits, agrees that while the melting point metric explains the majority of the contribution of CHCs to desiccation resistance, there are other important interactions between CHC expression and desiccation resistance not captured by this metric. For females, the best fit models did not include melting point at all, and instead the model described above was favoured by AIC. For males, AIC favoured models, which included melting point and the proportion of methylated alkanes for both LT_{50} ($F_{1,100}=10.81$, $r^2(adj)=0.17$, $P<0.0001$), and LT_{100} ($F_{1,100}=18.12$, $r^2(adj)=0.25$, $P \ll 0.0001$). In both cases, the proportion of methylated alkanes contributed significantly and negatively to desiccation resistance—this is in contrast with the univariate correlation (Table 3b).

Mapping-based evidence of pleiotropy

To verify the pleiotropy of shared QTL, we modelled the effect of CHCs on desiccation in a linear regression framework, and conducted CIM mapping of the residuals (Schadt *et al*., 2005). In complex networks of associated traits, within a Structural Equation Modelling (SEM) framework, mapping of residuals in this way may be used to determine the effect of one trait on the expression of another, and to infer the direction of causality throughout regulatory networks (Jansen *et al*., 2009, Li *et al*., 2006). As our network consists of only two traits, and we have functional knowledge of the relationship between CHC expression and desiccation resistance, we did not explore the inverse function—the effect of desiccation resistance on CHC expression. We separately examined both the relationship between CHC PCs, and the melting point CHCs on desiccation. For both sexes we modelled only the PCs with a significant effect on desiccation resistance. For females, we used the residuals of the model containing PCs 2 and 3; for males the model containing PCs 1, 2 and 3. We examined the effects of CHC melting point as opposed to other classes of CHC traits (such as the proportion of linear alkanes), because this was the best univariate contributor to desiccation resistance, and we have *a priori* theoretical reasons to expect CHC melting point to mechanistically affect desiccation resistance.

A 2 LOD drop between an original trait and its residual at a QTL peak is suggested as a general significance threshold for determining whether there is a significant causal relationship between a dependent trait and its predictor (Li *et al*., 2006). As our data set differed in many ways from the simulated data sets used to establish the significance of a 2 LOD drop, we empirically tested for the significance of the LOD dropoff between the original traits and their residuals by permutation. We permuted CHC traits relative to desiccation among the lines 1000 times, and modelled the effects of the permuted CHC traits on desiccation as with the original traits, and extracted the residuals. We mapped the residuals in QTL cartographer, using the same settings as above. Within the marker intervals flanking each of the original QTL LOD maxima, we took the highest LOD score for each of the permuted residuals and ranked them. An LOD drop between the original trait and residuals more extreme than 95% of the residuals from the permuted data set is taken to indicate the drop is significant at $P=0.05$. An LOD drop more extreme than among all of the residuals from the permuted data set is taken to indicate the drop is significant at $P<0.001$.

We found pervasive effects of CHC expression on desiccation resistance throughout the genome in both sexes (Table 4, Figure 1). The majority of desiccation QTL, were in fact significantly reduced in effect when the effects of CHCs were removed (Table 4, Supplemental Figure 3). Across all QTL, we found that the two LOD dropoff criterion was very conservative relative to permutation. For females, CHCs were found to contribute to all desiccation QTL and the melting point of CHCs correlated positively with desiccation resistance at all QTL, apart from the QTL on 3–1. In males, the association between CHCs and desiccation somewhat less straightforward. Regression of CHC PCs on desiccation resistance indicated that CHCs contribute significantly to all identified QTL, similar to the case in females. At some QTL however, the association between desiccation and CHC melting point was in the opposite

Table 4 The LOD maxima for the original desiccation QTL, as well as the LOD maxima in the same marker interval of their residuals, for both regression of CHC PCs and long-chain CHCs in *D. melanogaster* females (a) and males (b)

		flt$_{50}$	*PC dropoff*	*Melt dropoff*	*flt*$_{100}$	*PC dropoff*	*Melt dropoff*
(a)							
QTL1	X-1	—	—	—	—	—	—
QTL2	X-2	2.02	**1.08 (1000 = 1.14)**	**1.48 (*P* = 0.008)**	—	—	—
QTL3	X-2	2.08	1.73 (*P* = 0.069)	1.92 (***P*** = 0.092)	—	—	—
QTL5	2–1	6.27	***1.69 (1000 = 3.93)***	**4.83 (*P* = 0.002)**	6.96	***1.95 (1000 = 4.69)***	***5.19 (P = 0.001)***
QTL9	2–1	6.46	**4.76 (*P* = 0.001)**	**4.63 (1000 = 5.04)**	6.74	***4.62 (P = 0.002)***	**4.75 (*P* = 0.002)**
QTL11	2–1	6.66	***3.54 (1000 = 4.67)***	***3.52 (1000 = 4.96)***	6.77	**3.31 (1000 = 4.59)**	***1.90 (1000 = 4.90)***
QTL4	2–2	2.05	2.52 (*P* = 0.605)	1.98 (*P* = 0.161)	3.92	**2.35 (*P* = 0.015)**	**2.03 (*P* = 0.003)**
QTL6	2–2	4.21	**3.81 (1000 = 4.02)**	**3.81 (1000 = 4.42)**	9.00	***5.29 (P = 0.002)***	***4.98 (1000 = 5.54)***
QTL7	2–2	7.18	***2.82 (1000 = 5.25)***	***3.50 (1000 = 5.76)***	8.38	***3.67 (1000 = 5.81)***	***4.32 (1000 = 6.74)***
QTL8	2–2	2.36	**1.28 (1000 = 1.52)**	**0.91 (1000 = 2.29)**	2.56	**1.34 (*P* = 0.002)**	**1.04 (1000 = 1.77)**
QTL6	2–3	—	—	—	—	—	—
QTL10	2–3	2.79	**2.07 (*P* = 0.005)**	**2.10 (1000 = 2.11)**	2.87	**2.11 (*P* = 0.004)**	**2.19 (*P* = 0.003)**
QTL13	3–1	—	—	—	—	—	—
QTL14	3–1	2.46	***0.39 (1000 = 0.72)***	2.96 (*P* = 0.993)	2.16	**0.28 (1000 = 0.51)**	2.74 (0 = 2.94)
QTL15	3–1	—	—	—	—	—	—
QTL12	3–2/3	—	—	—	—	—	—
QTL13	3–2/3	3.23	***0.18 (1000 = 0.81)***	***0.70 (1000 = 1.45)***	3.82	***0.27 (1000 = 2.42)***	*1.00 (1000 = 2.56)*
		mlt$_{50}$	PC dropoff	Melt dropoff	mlt$_{100}$	PC dropoff	Melt dropoff
(b)							
QTL1	X-1	—	—	—	2.15	**1.59 (*P* = 0.003)**	**1.06 (1000 = 2.52)**
QTL2	X–2	—	—	—	—	—	—
QTL3	X–2	—	—	—	—	—	—
QTL5	2–1	4.11	***1.25 (1000 = 2.22)***	3.75 (*P* = 0.100)	4.11	**1.26 (1000 = 2.18)**	3.96 (*P* = 0.310)
QTL9	2–1	2.77	**1.11 (1000 = 1.18)**	2.42 (*P* = 0.573)	2.08	**0.66 (1000 = 1.22)**	**1.82 (*P* = 0.032)**
QTL11	2–1	1.99	**0.62 (1000 = 0.90)**	1.64 (*P* = 0.272)	—	—	—
QTL4	2–2	—	—	—	—	—	—
QTL6	2–2	2.05	**1.60 (1000 = 1.64)**	**1.70 (*P* = 0.009)**	3.49	**1.72 (1000 = 2.256)**	**1.70 (1000 = 2.84)**
QTL7	2–2	2.94	**1.49 (1000 = 1.08)**	**1.73 (1000 = 1.92)**	3.71	**1.64 (1000 = 2.578)**	**1.72 (1000 = 2.71)**
QTL8	2–2	1.93	**0.82 (1000 = 0.92)**	**1.19 (1000 = 1.25)**	2.59	**1.08 (1000 = 1.337)**	**1.52 (*P* = 0.001)**
QTL6	2–3	2.20	**0.71 (*P* = 0.003)**	1.22 (0 = 1.10)	1.37	**0.11 (1000 = 0.823)**	1.23 (0 = 1.07)
QTL10	2–3	1.84	1.54 (*P* = 0.587)	**0.57 (1000 = 0.62)**	1.03	**0.45 (*P* = 0.004)**	0.73 (*P* = 0.261)
QTL13	3–1	—	—	—	3.45	***0.26 (P = 0.016)***	**2.72 (*P* = 0.024)**
QTL14	3–1	1.78	**0.04 (1000 = 0.13)**	**0.13 (1000 = 1.04)**	3.67	***0.24 (P = 0.005)***	**1.14 (*P* = 0.001)**
QTL15	3–1	—	—	—	2.73	***0.18 (P = 0.002)***	***0.40 (P = 0.005)***
QTL12	3–2/3	—	—	—	2.83	**1.02 (1000 = 1.309)**	**2.39 (*P* = 0.048)**
QTL13	3–2/3	3.37	***1.16 (1000 = 2.16)***	**2.68 (*P* = 0.001)**	4.09	***1.18 (1000 = 2.342)***	**2.80 (*P* = 0.001)**

P-values determined by 1000 permutations of CHC traits on desiccation resistance, and QTL mapping of the residuals are shown in brackets in which the LOD maxima of residuals are more extreme than any of the permuted-trait residuals, the most extreme values of the 1000 are noted in brackets.
Significant values as determined by permutation are indicated in bold.
Significant values as determined by 2 LOD dropoff are indicated in italics.

direction to that we would expect—notably on chromosome 2, linkage group 3, where the LOD of the residuals are significantly higher than the original trait. This indicates that the effect of CHCs at these loci were in the opposite direction as the overall trend of the regression (Li *et al*., 2006).

The association between female desiccation resistance and CHC expression was higher for almost all metrics, and this was supported by the results of the QTL mapping. Females expressed significantly more CHCs overall, however—1.27 times the total amount of CHCs as males ($F_{1,246}$, F=15.98, $P > 0.001$).

Candidate genes

We considered genes from the literature known to affect hydrocarbon profile as candidate genes (Supplemental Table 3), as well as genes identified through FlyBase <http://flybase.org/> containing Gene Ontology (GO) terms with the words 'fatty acid' or 'lipid', and 'metabolism', 'transport', 'synthase' or 'synthesis', and their equivalents. We also searched for the genes annotated with 'elongase', 'desaturase' and 'adult fat body'. These terms will necessarily miss many genes which, for instance, underlie fatty-acid or other resource allocation between tissues, or the many other possible ways in which energy and resource use might affect CHC profile. Given how broad many of the QTL are, each QTL is likely to contain thousands of genes, and multiple candidate and non-candidate genes potentially contribute to each QTL. Several candidates did colocalize with QTL (Supplemental Table 3).

Discussion

We present the first systematic study of population level genetic variation for survival to desiccation in *D. melanogaster*. Variation in resistance to desiccation was abundant among the RILs and was associated with multiple loci throughout the genome. We found high

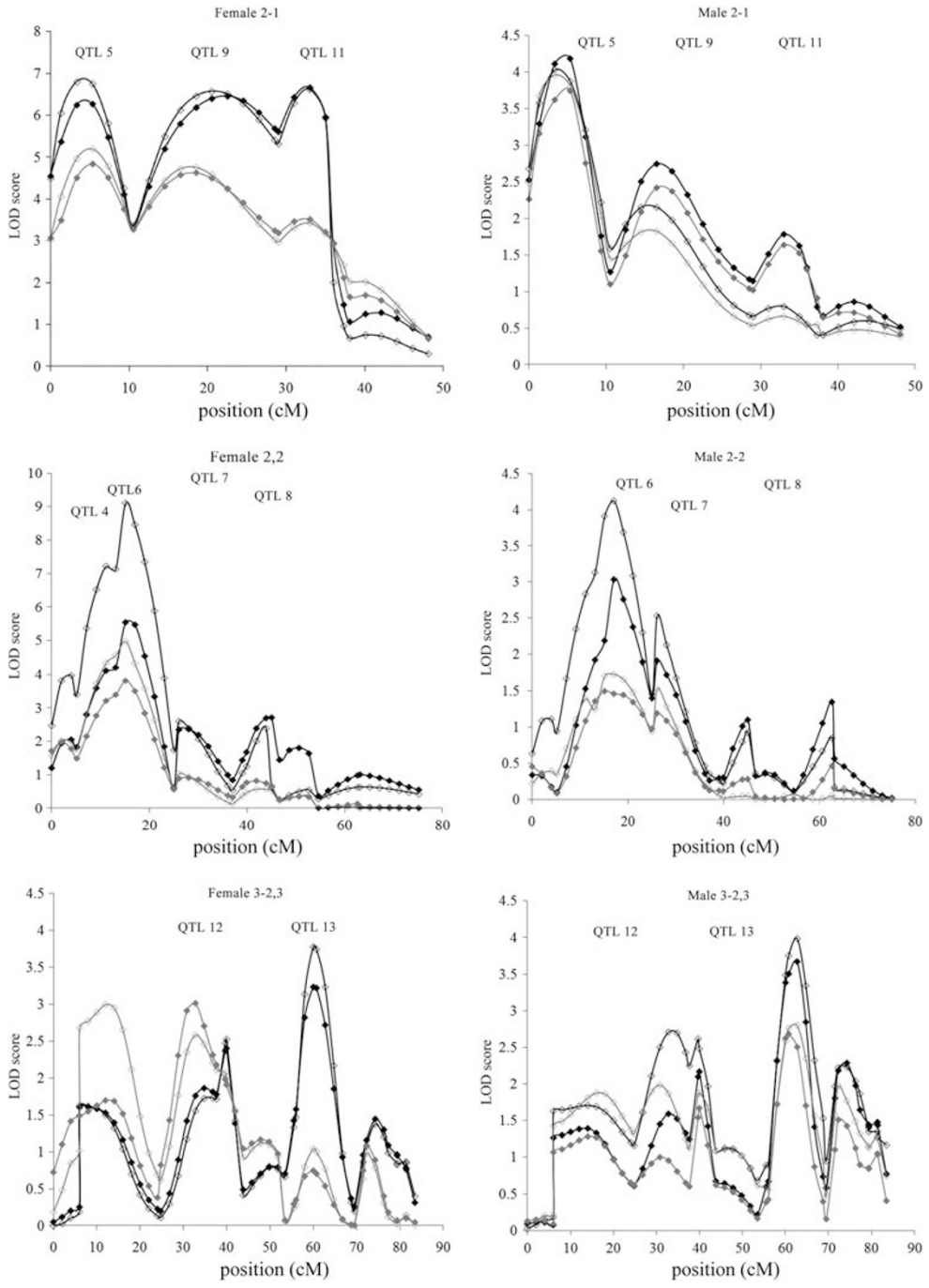

Figure 1 LOD plot of CIM map of desiccation traits (black lines) lt50 (solid symbols) and lt100 (open symbols); and their associated residuals (grey lines) of the regression of CHC melting point for RILs of *D. melanogaster*. Shown are the three linkage groups where >2 LOD drop-offs were found.

correlations between desiccation resistance and a previously hypothesized mechanistic trait (CHCs) in both sexes. This correlation was evident overall, and at specific loci throughout the genome.

CIM mapping identified at least 15 desiccation QTL, which most likely underestimates the number of genes underlying this trait discussed in (Mezey *et al*., 2005). Resistance to desiccation stress had a similar genetic basis in males and females, reflected in highly significant Pearson correlations of approximately 70% between the sexes. There was also evident co-localization of QTL between the sexes. The locus with the strongest effect was restricted to a region on the distal end of chromosomal arm 2L in both sexes, although with a much stronger effect in females, and large regions across the 3rd chromosome had effects on desiccation resistance with similar directionality of effects in males and females. The number of broad, but very significant regions of the genome associated with desiccation resistance is consistent with previous studies, which have shown a great deal of variation for resistance to low humidity in *D. melanogaster* selection experiments, with

both X-linked and autosomal effects (Hoffmann and Parsons, 1989b; Telonis-Scott *et al*., 2006). Heritability estimates of around 60% for desiccation resistance (Hoffmann and Parsons, 1989b) are known, and at least in *D. melanogaster*, rapid responses to selection are usual (Hoffmann and Parsons, 1989aHoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005; Telonis-Scott *et al*., 2006).

Differences between desiccation QTL at the two time points (LT_{50} and LT_{100}) were negligible, with only one QTL at 7F specific to female survival at 50% mortality. In the other cases where QTL are listed for only one time point, the corresponding time-point showed a significant peak but was not included in the overall summary owing to low statistical support from wide confidence intervals (Table 2). Although the traits have a similar genetic basis in almost all cases, these loci do not have identical effects on both traits, and it may be that different alleles are associated with different stages during desiccation.

Water retention is a key adaptation to desiccation stress in Drosophila, where water is lost via excretion across the spiracles or cuticle; (Hoffmann and Harshman, 1999; Gibbs *et al*., 2003). In highly desiccation-resistant xeric species, water is retained primarily via lowered metabolic rate reducing respiration across the spiracles and changes in cyclic CO_2 release (Gibbs *et al*., 2003). Changes in CO_2 release via the spiracles was observed in selected lines of *D. melanogaster* (Williams *et al*., 1998), although the effect on desiccation was less clear. Initial associations between desiccation resistance and lowered metabolic rate in Drosophila selected lines tended to disappear when corrected for energy stores (Hoffmann and Parsons, 1989a b; Djawdan *et al*., 1998). Other mechanisms known to affect desiccation resistance include greater dehydration tolerance, increased bulk water, greater metabolic stores increased body size see (Telonis-Scott *et al*., 2006). In some cases several mechanisms have been found to contribute to desiccation resistance; that is, *adipose* variants when combined with wild-type alleles survived desiccation in part because of larger size, reduced transpiration and greater tolerance of low water content (Clark and Doane, 1983).

In terms of cuticular transpiration, it is known that CHCs are important for maintaining the impermeability of the cuticle, and that the melting point of the hydrocarbons, which increases with carbon number, is a factor in reducing water exchange through the cuticle (Gibbs *et al*., 1997; Gibbs 1998). Desiccation susceptibility owing to rapid water loss across the cuticle was documented in a desiccation sensitive mutant as well as lines selected for postponed senescence (Kimura *et al*., 1985; Graves *et al*., 1992). Here, consistent with theoretical predictions (Toolson and Kupersimbron, 1989) and global clinal patterns of CHC expression variation as well as plastic responses to temperature (Rouault *et al*., 2004), there were strong correlations between desiccation resistance and CHC melting point. In both sexes, these correlations were strong, up to 48% in females and 40% in males for desiccation lt_{100}. In neither sex did the proportion of other chemical classes of CHCs predict desiccation resistance as well.

We found strong effects of CHCs on desiccation resistance at individual QTL. There were significant drops in the likelihood of nearly all desiccation QTL when the overall effects of CHC expression were removed. This significantly reduced the genomic landscape specific to desiccation and reflects that genetic variation for CHCs may be causal to the desiccation survival phenotype, itself a composite trait. The effect was clearer in females, where the magnitude of the drop in LOD was much larger than in males. Male desiccation LOD maxima were not generally as high, however, and this might simply reflect higher error variance in male measurements, consistent with overall higher LOD maxima in female CHC QTL (Foley *et al*., 2007), or the greater abundance of female CHCs. Although the 2 LOD significance threshold indicated effects of CHCs only at female desiccation QTL, permutation tests indicate the 2 LOD threshold was far too conservative in our panel of RILs. Permutation indicated that both male and female desiccation survival were affected by CHCs at many loci throughout the genome. Given that we have found many regions throughout the genome with effects on CHC expression (Foley *et al*., 2007), and given the predicted association between CHCs and desiccation resistance, this perhaps should not be surprising. We evidently have stronger power to detect the effects of genetic loci on CHC expression when considering multivariate measures such as PCs or CHC chemical classes than when mapping CHCs as univariate traits. Significant associations between CHC expression and desiccation were found even in haplotypes such as 3–2,3, in which single CHCs mapped poorly (Foley *et al*., 2007).

The predicted association between CHC melting point and increased desiccation resistance was robustly evident at an individual QTL in our lines. This is true particularly in females where at nearly every QTL the melting point of CHCs correlate with desiccation resistance. In males, the association is somewhat less consistent across QTL. This sex-specificity of effects at the genomic level is consistent with the overall lesser correlation of male CHC melting point with desiccation resistance. This may be due to interactions between traits that we have not measured. Notably in our lines, methyl-alkane and long-chain CHC expression are strongly positively correlated and methyl-alkanes are present in high levels in males but not in females, suggesting they are involved with sexual signalling in *D. melanogaster*, as they are found to be in other *Drosophila* (Chenoweth and Blows, 2005). It might be that males who exert strong signalling effort are subject to tradeoffs for desiccation survival. The various correlations between methyl-alkane expression, CHC melting point and desiccation resistance might explain why at one locus on 2–3, the association between male CHC melting point and desiccation resistance is significantly in the direction *opposite* to that expected. Alternatively, *D. melanogaster* has been found to facultatively modify CHC profile in response to stresses, like heat, which are likely to increase the risk of desiccation (Rouault *et al*., 2004), with males strongly upregulating their long chain CHCs. As we measured all our CHCs at 25 °C, and at relatively high relative humidity, we likely missed all sex-specific facultative responses that may have affected desiccation survival.

Given our number of potential candidate genes, it was notable which of several strong candidates did not contribute to any QTL. We might expect elongases—which increase CHC chain length—to be involved in desiccation resistance. One elongase, *Elo68α*, might

potentially contribute to QTL12, but this desiccation QTL did not seem to be affected by CHC expression, and the association thus seems dubious. Another elongase, *EloF*, did not colocalize with a strong LOD maxima. *Fst* (Sinclair *et al*., 2007), which has been shown to respond to environmental stress including desiccation and cold, did not colocalize with any QTL. Although *Desat2* (Greenberg *et al*., 2003)—potentially implicated in stress resistance—colocalizes with QTL14, there are effects in both sexes of CHC expression on desiccation resistance at this locus. As *desat2* expression is female specific, it seems unlikely to be involved in the RILs. We did not measure gene expression in this study, however, thus cannot detect *trans* effects on gene expression for our candidates. Of the strongest desiccation QTL most influenced by CHC expression, those at the distal end of 2L had several potentially interesting candidates, including *FatP*, a fatty acid transport molecule, and *smoq* (Ferveur and Jallon, 1996). Another noted CHC-expression gene, *sept* (Ferveur and Jallon, 1996), also falls within the 95% confidence interval of several QTL. However, the transition from QTL to QTN (quantitative trait nucleotide) requires fine-scale molecular dissection (that is, complementation tests, transgenic studies, gene expression, cloning) to elucidate the contribution of individual genes to desiccation survival.

Conclusion

Given the extent of variation in CHCs found in even this small genetic sample, and the robust association of this variation with desiccation resistance in our lines, it seems plausible that within *D. melanogaster* and other *Drosophila*, CHC evolution is likely to be a common adaptation to desiccation stress. It will be interesting to test this association in species, which do not evidence an ability to adapt to desiccation. Although rainforest species like *D. bunnanda* have a great deal of genetic variation in their CHCs at a population level (Van Homrigh *et al*. 2007), it will be necessary to test whether they have any variation in mean hydrocarbon chain length to begin to understand why they cannot avail of this mechanism of desiccation resistance

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)