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RESEARCH ARTICLE

Physiological basis of starvation resistance in *Drosophila leontia*: analysis of sexual dimorphism

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ABSTRACT

Geographically varying starvation stress has often been considered as a natural selector that constrains between-population differences for starvation resistance (SR) in *Drosophila* species. On the Indian subcontinent, a dozen *Drosophila* species have shown clinal variations in SR across latitude, but the evolved physiological basis of such contrasting adaptations is largely unknown. In the present study, I untangled the physiological basis of sex-specific as well as between-population divergence for SR in *D. leontia*, collected across a latitudinal transect of the Indian subcontinent (11°45′–31°19′N). Secondly, I tested the assumptions that hardening to starvation stress facilitates an increased survival under subsequent lethal levels of starvation, and such plastic effects differ between the sexes. I observed several interesting results. In contrast to a steeper cline of starvation-related traits with latitude in females, a shallower gradient was observed for males. Females stored higher (~1.3-fold) dry-mass-specific levels of body lipids and glycogen contents, and utilized these both of these energy resources under starvation stress, whereas the starved males metabolized only body lipids as a source of energy. Conversely, the rate of body lipid utilization and threshold need were considerably higher in females as compared with males. Between-population differences were significant for storage levels of energy reserves only, but not for other avenues (rate of metabolite utilization and threshold need) of SR for both sexes. These findings indicate that multiple pathways shape the physiological basis of sexual dimorphism for SR in *D. leontia*. Further, single or multiple bouts of starvation hardening conferred an increased longevity (~4–9 h; $P<0.001$) under subsequent lethal levels of starvation stress for females only, and such plastic responses were consistent with a decrease in rate of metabolite utilization. Nevertheless, between-population effects were non-significant for absolute hardening capacity (AHC=K_{SR}–C). Altogether, these findings suggest that similar evolutionary constraints have resulted in divergent genetic as well as plastic responses to evolve adaptations under starvation stress, and account for the observed sexual dimorphism for basal SR in *D. leontia*.

KEY WORDS: Starvation resistance, Sexual dimorphism, Energy metabolites, Starvation hardening, *D. leontia*

INTRODUCTION

Abiotic stress is an environmental hazard that affects fitness and survival, and restricts the physiological boundaries of a species (Randall et al., 1997; Chown and Nicolson, 2004; Hoffmann, 2010). Therefore, environmental stresses act as potential natural selectors that constrain multiple physiological mechanisms to enhance physiological adaptations (Hoffman and Parsons, 1991; Hoffmann, 2010; Kellermann et al., 2012). One of the most ubiquitous causes of stress in animals is the shortage or suboptimal quality of food, i.e. a period of starvation stress (Hoffmann and Harshman, 1999; Rion and Kawecki, 2007; McCue, 2010). To understand the physiological basis of starvation resistance (SR) in the evolutionary landscape, evolutionary biologists and eco-physiologists have used *Drosophila* as a model organism (Hoffmann and Harshman, 1999; Hoffmann and Weeks, 2007; Goenaga et al., 2013).

Drosophila seems to harbor ample genetic variation for SR, as evident from clinal variations in wild habitats (Hoffmann and Harshman, 1999; Rion and Kawecki, 2007). However, the patterns of clinal changes were inconsistent when geographical populations originating from different continents were compared for SR (Rion and Kawecki, 2007). For example, in contrast to an opposite cline of SR with latitude in India (Karan et al., 1998; Parkash et al., 2012), Australian populations of *D. melanogaster* have shown either a weak cline (Hoffmann et al., 2001) or no cline (Hallas et al., 2002). Similarly, latitudinal variations in SR are evident in eastern North American populations (Schmidt et al., 2005), but not in South American populations of *D. melanogaster* (Robinson et al., 2000). Thus, evolutionary trajectories might have constrained SR, but the outcome has often varied among populations as well as species (Hoffmann and Harshman, 1999; Harshman and Hoffmann, 2000; Parkash and Munjal, 1999; Parkash and Munjal, 2000).

In general, three avenues have been considered as basic mechanisms that may enhance survival under starvation stress: (1) storage of higher energy reserves, especially in the form of lipids, (2) a reduced rate of metabolite utilization, and (3) a lower threshold need of energy metabolites for survival under starvation stress (Rion and Kawecki, 2007; Ballard et al., 2008; Parkash and Aggarwal, 2012). Molecular analyses (*P*-element survey and whole genome transcript abundance) suggested that starvation stress has induced upregulation of genes associated with biosynthesis of lipid in *D. melanogaster* (Harbison et al., 2005; Arrese and Soulages, 2010). Nevertheless, debates are still ongoing regarding the implication of empirical observations of physiological studies analyzing correlated changes in SR and body lipid contents in *Drosophila* species. For example, within- and between-population variations in SR were not associated with changes in body lipid reserves in *D. melanogaster* (Hoffmann et al., 2001). Increased larval density in the culture of *D. melanogaster* resulted in a higher storage of body lipids, but a lack of changes in SR (Baldal et al., 2005). In contrast, higher levels of body lipid reserves have been shown to facilitate increased SR in geographical populations of *D. simulans* (Ballard et al., 2008) and *D. melanogaster* (Parkash and Aggarwal, 2012; Goenaga et al., 2013). Similarly, a comparative analysis of ecologically diverse *Drosophila* species (van Herrewege and David, 1997) and laboratory selection experiments based on directional selection of SR (Hoffmann et al., 2005) or life span (Service, 1987; Zwaan et al., 1995; Vermeulen et al., 2006) have also indicated the association of

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Received 9 September 2013; Accepted 16 February 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.096792

storage levels of body lipids and SR. Further, another possible mechanism, changes in the rates of metabolic utilization and their threshold need, has been analyzed for field populations of *Drosophila* species (Marron et al., 2003; Parkash and Aggarwal, 2012).

Besides the genetic adaptations, non-genetic effects, i.e. phenotypic plasticity, may further provide an opportunity to alleviate or buffer the consequences of nutritional stress (Berrigan and Scheiner, 2004; Rion and Kawecki, 2007; Bubliy et al., 2012). For example, seasonal fluctuations in temperature have influenced metabolic rates under starvation stress in the butterfly *Bicyclus anynana* (Pijpe et al., 2007). A complex interaction of growth and starvation treatment temperatures substantially affected starvation survival curves in geographically distinct populations of *D. melanogaster* and *D. ananassae* (Karan and David, 2000). Calorically restricted (yeast) or diapaused phenotypes of *D. melanogaster* showed increased SR compared with their *ad-libitum*-fed (Leroi et al., 1994; Kapahi et al., 2004; Piper et al., 2005) or non-diapaused (Tatar and Yin, 2001; Schmidt et al., 2005) counterparts, respectively. Further, increased SR has been evident under a lemon diet in genetically evolved SR lines of *D. melanogaster*, whereas control lines lack such effects (Harshman et al., 1999). Nevertheless, a single study has explored the consequences of direct hardening on starvation stress in *Drosophila* (Bubliy et al., 2012). To my knowledge, no previous study has explored the physiological basis of starvation hardening and sex-specific effects in any *Drosophila* species so far.

In the present study, I examined the trends of clinal variations and sex-specific divergence for starvation-related traits in *D. leontia*. Secondly, I examined between-population and sex-specific differences in the physiological basis of SR, i.e. storage levels, utilization rate and threshold need of energy metabolites. Further, I assessed whether basal tolerance level buffers plastic capacities (starvation hardening) and whether the effects differ between sexes and/or populations. Finally, I analyzed whether repeated bouts of starvation stress facilitate an increased tolerance towards starvation stress in *D. leontia*.

RESULTS

Wild populations of *D. leontia* were sampled from eight localities, showing a steep latitudinal gradient (south to north: 11°45′–31°19′N), but no particular trends for altitude and longitude across the Indian subcontinent. Notably, mean monthly thermal variations were more prominent in northern than southern localities of the Indian subcontinent (Fig. 1A). Average temperature (T_{ave}) correlates poorly with the latitude of low altitudinal localities, whereas the coefficient of mean monthly temperature variations was positively correlated to latitude (Fig. 1B,C). Interestingly, minimum, maximum and average relative humidity (RH_{min}, RH_{max} and RH_{ave}, respectively) were significantly correlated to latitude of origin of *D. leontia* populations (Fig. 1D–F).

Analysis of clinical patterns

A comparison of slope values (*t*-test) (Zar, 1999) of starvation-related traits (SR, body lipids, carbohydrates and protein contents) in wild versus laboratory conditions, and between sexes (wild as well as laboratory) is shown in Table 1. In females, slope values of SR were significantly higher for field populations as compared with laboratory-reared populations (6.17, $P<0.001$; Fig. 2A), which suggests the possible influence of plasticity on trait values in the

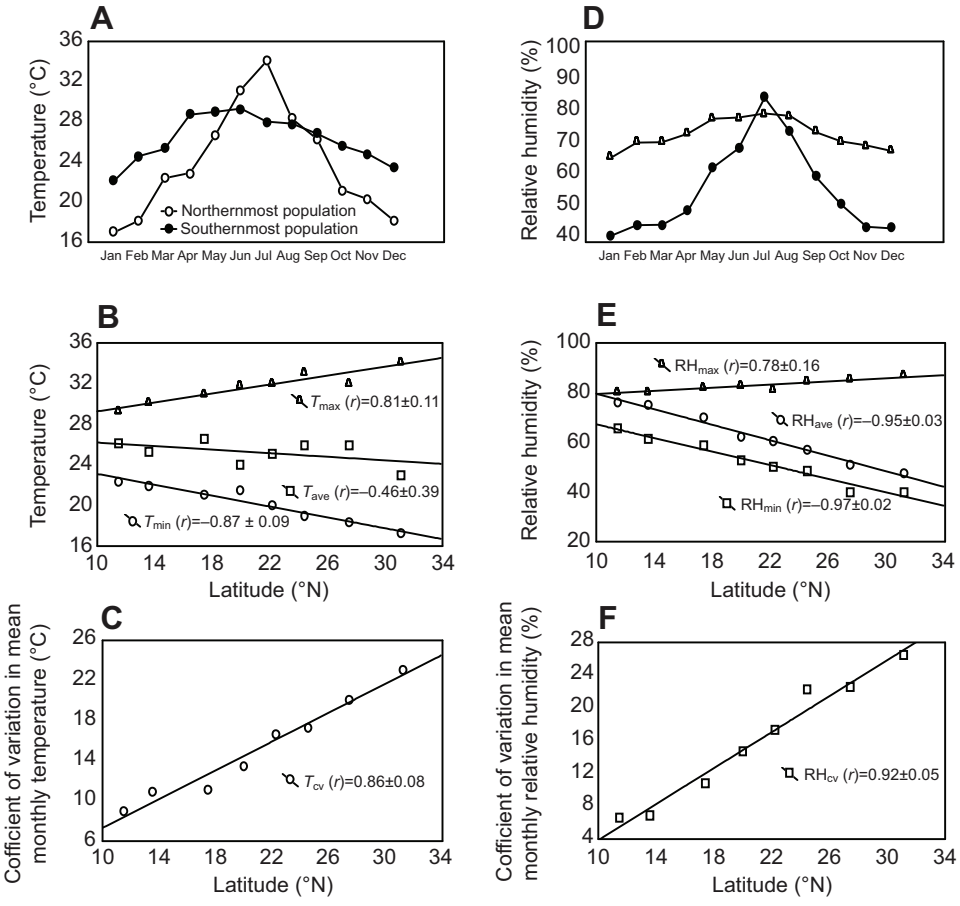

Fig. 1. Temperature and relative humidity variations across the collection sites of *Drosophila leontia*. Monthly variations in temperature (A) and relative humidity (%; D) in one northernmost and one southernmost population of *Drosophila leontia*. Latitudinal variations in multiple variables of temperature (T_{min}, T_{max}, T_{ave} and T_{cv}: B,C) and relative humidity (RH_{min}, RH_{max}, RH_{ave} and RH_{cv}; E,F) in collection sites of *D. leontia* populations.

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Table 1. Comparions of elevational slope values (*b* ± s.e.m.) for starvation resistance and dry-mass-specific levels of energy metabolites (body lipids, carbohydrates and protein contents) in wild versus laboratory conditions in *Drosophila leontia*

	Female			Male			Female vs male (*t*-test)	
Trait	Wild	Laboratory	*t*	Wild	Laboratory	*t*	Wild vs wild	Laboratory vs laboratory
Starvation resistance (h)	–2.022±0.07	–1.404±0.06	6.17***	–0.583±0.04	–0.573±0.04	0.18$^{n.s.}$	17.74***	11.54***
Body lipid content (mg mg^{-1} dry mass)	0.0061±0.00025	0.0047±0.00018	6.13***	0.0021±0.00009	0.0020±0.00009	0.34$^{n.s.}$	14.72***	15.33***
Carbohydrate content (mg mg^{-1} dry mass)	0.0076±0.00013	0.0035±0.00009	21.51***	0.0033±0.00011	0.0015±0.00010	8.11***	17.65***	5.94***
Protein content (mg mg^{-1} dry mass)	0.0007±0.0003	0.0007±0.0002	0.12$^{n.s.}$	0.0006±0.0002	0.0005±0.0002	0.34$^{n.s.}$	0.19$^{n.s.}$	0.71$^{n.s.}$

Slopes for starvation-related traits were also compared between sexes.
Slopes were compared using Student's *t*-test. n.s., non-significant; ***P<0.001.

field habitats. In contrast, differences in slopes of SR in males under two different regimes (wild versus laboratory) were non-significant (0.18, P>0.05; Fig. 2D). Similarly, females showed a contrast in slope values of body lipid contents in wild versus laboratory-reared conditions (6.13, P<0.001; Fig. 2B), but males lack such effects (0.34, P>0.05; Fig. 2E). However, slopes of carbohydrate contents exhibited significant differences in wild versus laboratory conditions for both sexes (female=21.51, male=8.11, P<0.001; Fig. 2C,F). However, an opposite trend was observed for protein contents (female=0.12, male=0.34, P>0.05). Further, sex-specific differences in slopes of SR in wild (W) as well as laboratory (L) conditions were significant (W=17.74, L=11.54, P<0.001). Trends were similar for body lipids (W=14.72, L=15.33, P<0.001) and carbohydrate contents (W=17.65, L=5.94, P<0.001) but not for protein contents (W=0.19, L=0.71, P>0.05). Thus, these results indicate that plasticity for starvation-related traits influences the slopes in females but not for males from the wild populations. Secondly, there was a steeper slope in wild as well as laboratory populations of females as compared with males, suggesting that the outcome of evolutionary trajectories constrained by SR differs between the sexes in *D. leontia*.

Sexual dimorphism and between-population divergence for starvation-related traits

Partial crossed and partial nested [isofemale (IF) lines nested into populations] analysis of covariance (ANCOVA; with dry body mass as a covariate) was executed to explain sex-specific (S) and between-population (P) differences for SR (LT_{50} and LT_{100}; hours)

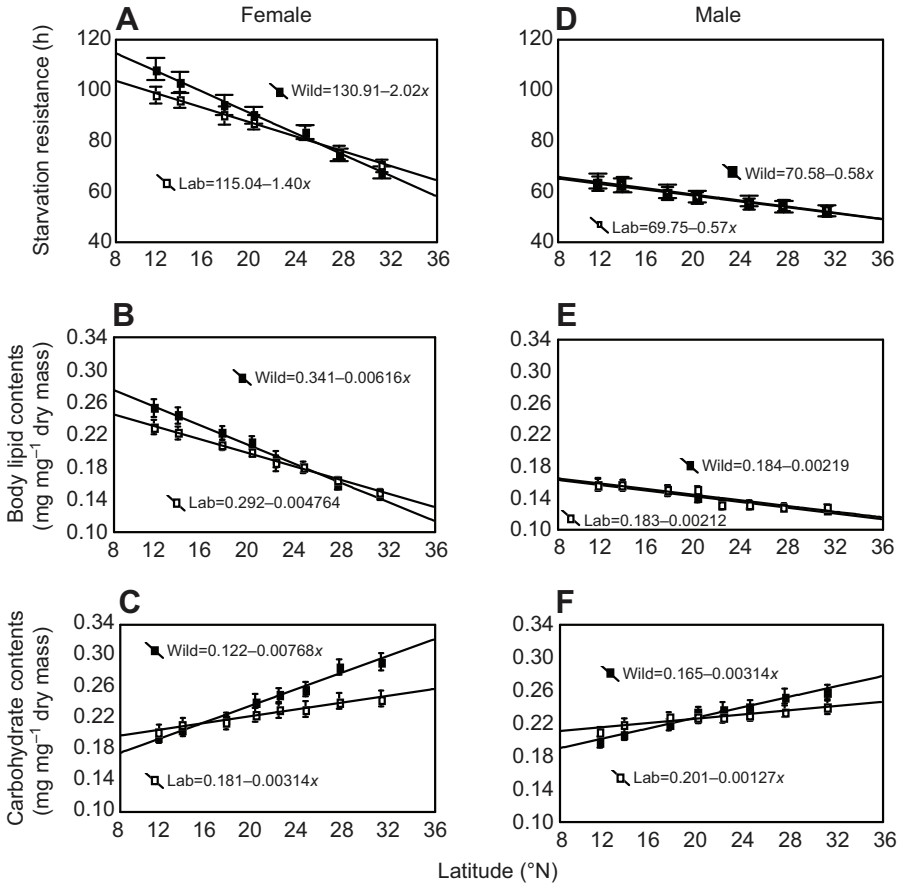

Fig. 2. Analysis of clinal patterns of starvation-related traits across latitude. Clinal variations in starvation resistance (A,D), dry-mass-specific body lipid contents (B,E) and dry-mass-specific carbohydrate contents (C,F) in field-collected and laboratory-reared populations of *D. leontia*. Sexes of *D. leontia* differ in the patterns of variation in starvation-related traits with latitude.

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Table 2. Results of ANCOVA (with body mass as a covariate) explaining trait variability due to sex, population, isofemale (IF) line and their interactions for starvation-related traits in *D. leontia*

		Sex	Population	IF line	Sex × Population	Sex × IF line	Error
Trait	d.f.	1	7	152	7	152	2879
Starvation resistance (LT_{50} hours)	MS	50,193.11	4519.94	87.05	657.79	22.63	0.28
	F	179,261.10	16,142.64	310.89	2349.25	80.82	
	% Variance	48.29***	30.44***	12.73****	4.43***	3.31***	0.79
Starvation resistance (LT_{100} hours)	MS	41,949.04	4331.58	64.13	646.64	16.58	0.46
	F	91,193.56	9416.47	139.41	1405.73	36.04	
	% Variance	46.43***	33.56***	10.79***	5.01***	2.79***	1.48
Body lipid content (μg fly^{-1})	MS	57,222.67	5697.29	55.92	630.69	20.69	0.13
	F	440,174.38	43,825.30	430.15	4851.46	156.76	
	% Variance	50.42***	35.14***	7.49***	3.89***	2.73***	0.33
Trehalose content (μg fly^{-1})	MS	31,380.91	4337.34	65.86	621.24	10.32	0.54
	F	58,112.79	8032.11	121.96	1150.44	19.11	
	% Variance	39.40***	38.12***	12.57***	5.46***	2.48***	1.97
Glycogen content (μg fly^{-1})	MS	37,497.42	4444.98	50.62	506.94	16.54	0.25
	F	149,989.68	17,779.92	202.48	2027.76	65.80	
	% Variance	45.12***	37.49***	9.26***	4.27***	3.01***	0.90
Protein content (μg fly^{-1})	MS	5924.07	0.52	6.07	0.21	1.03	0.13
	F	45,569.53	4.01	46.69	1.61	7.92	
	% Variance	80.12***	0.05$^{n.s.}$	12.49***	0.02$^{n.s.}$	2.11*	5.21

Homogeneity of the slopes was checked in order to validate body mass as a covariate in the factorial models.
LT_{50} hours were calculated using the Probit method.
n.s., non-significant; ****P*<0.001.

and energy metabolites (body lipid, carbohydrates and protein contents) in *D. leontia* (Table 2). For SR (LT_{50}), all effects, i.e. sex (*F*=179261.10, 48.29% variation, *P*<0.001), population (*F*=16142.64, 30.44% variation, *P*<0.001), IF line (*F*=310.89, 12.73% variation, *P*<0.001) and their interactions (3.31–4.43% variation, *P*<0.001), were highly significant. ANCOVA showed similar trends of variability for SR (LT_{100}) (sex: *F*=91193.56, 46.43% variation, *P*<0.001; population: *F*=9416.47, 33.56% variation, *P*<0.001; IF line: *F*=139.41, 10.79% variation, *P*<0.001; and their interactions: ~3–6% variation, *P*<0.001). Likewise, all these effects were significant for body lipids (S: *F*=440174.38, 50.42% variation, *P*<0.001; P: *F*=43825.30, 35.14% variation, *P*<0.001), trehalose (S: *F*=58112.79, 39.40% variation, *P*<0.001; P: *F*=8032.11, 38.12% variation, *P*<0.001) and glycogen contents (S: *F*=149989.68, 45.12% variation, *P*<0.001; P: *F*=17779.92, 37.49% variation, *P*<0.001; Table 2). However, between-population differences for protein contents were non-significant, despite sex and IF line having shown significant effects (Table 2).

Analysis of energy metabolites

Results of nested ANCOVA (IF lines nested into treatment) for the utilization of energy metabolites (dry-mass-specific levels) under starvation stress in one northern (N) and one southern (S) population are shown in Table 3. In both sexes, body lipids were metabolized under starvation stress until death (female N: $F_{1,38}$=178.03; S: $F_{1,38}$=165.29; male N: $F_{1,38}$=206.81; S: $F_{1,38}$=191.23; *P*<0.001). In contrast, glycogen contents decreased significantly in females (N: $F_{1,38}$=156.73; S: $F_{1,38}$=152.46; *P*<0.001) but not in males (N: $F_{1,38}$=2.48; S: $F_{1,38}$=1.90; *P*>0.05) under starvation stress. Therefore, sexes have shown divergence in utilization of glycogen under starvation stress. However, the levels of trehalose (females N: $F_{1,38}$=1.31; S: $F_{1,38}$=1.52; males N: $F_{1,38}$=0.96; S: $F_{1,38}$=1.50; all *P*>0.05) as well as protein contents (females N: $F_{1,38}$=2.11; S: $F_{1,38}$=2.73; males N: $F_{1,38}$=2.98; S: $F_{1,38}$=1.96; all *P*>0.05) did not reduce significantly under starvation stress in either sex of *D. leontia*. For changes in body lipids, carbohydrates and protein contents, significant *F*-values of IF lines represent between-line variability.

Table 3. Nested ANCOVA (IF line nested in treatment) explaining changes in levels of energy metabolites (body lipids, trehalose, glycogen and protein contents) as a consequence of starvation stress until death in one northern and one southern population of *D. leontia*

			Females				Males			
			Northern		Southern		Northern		Southern	
Trait		d.f.	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Body lipids	Treatment	1	16,089.20	178.03***	10,059.71	165.29***	12,695.93	206.81***	9949.89	191.23***
	IF line	38	90.37	77.91***	60.86	55.03***	61.37	74.84***	52.03	38.71***
	Error	359	1.16		1.10		0.82		1.344	
Trehalose content	Treatment	1	62.11	1.31$^{n.s.}$	62.47	1.52$^{n.s.}$	15.45	0.96	17.05	1.50$^{n.s.}$
	IF line	38	47.06	15.53**	41.06	48.88***	16.05	69.78***	11.30	59.47***
	Error	359	3.03		0.084		0.23		0.19	
Glycogen content	Treatment	1	9938.63	156.73***	5801.16	152.46***	25.72	2.48	12.36	1.90$^{n.s.}$
	IF line	38	63.41	26.53***	38.05	20.34***	10.35	52.01***	6.21	62.10***
	Error	359	2.39		1.87		0.19		0.10	
Protein content	Treatment	1	49.16	2.11$^{n.s.}$	40.95	2.73$^{n.s.}$	48.19	2.98	22.09	1.9$^{n.s.}$
	IF line	38	23.20	28.64***	15.02	30.65***	16.12	48.89***	11.23	56.15***
	Error	359	0.81		0.49		0.33		0.20	

n.s., non-significant; ****P*<0.001.

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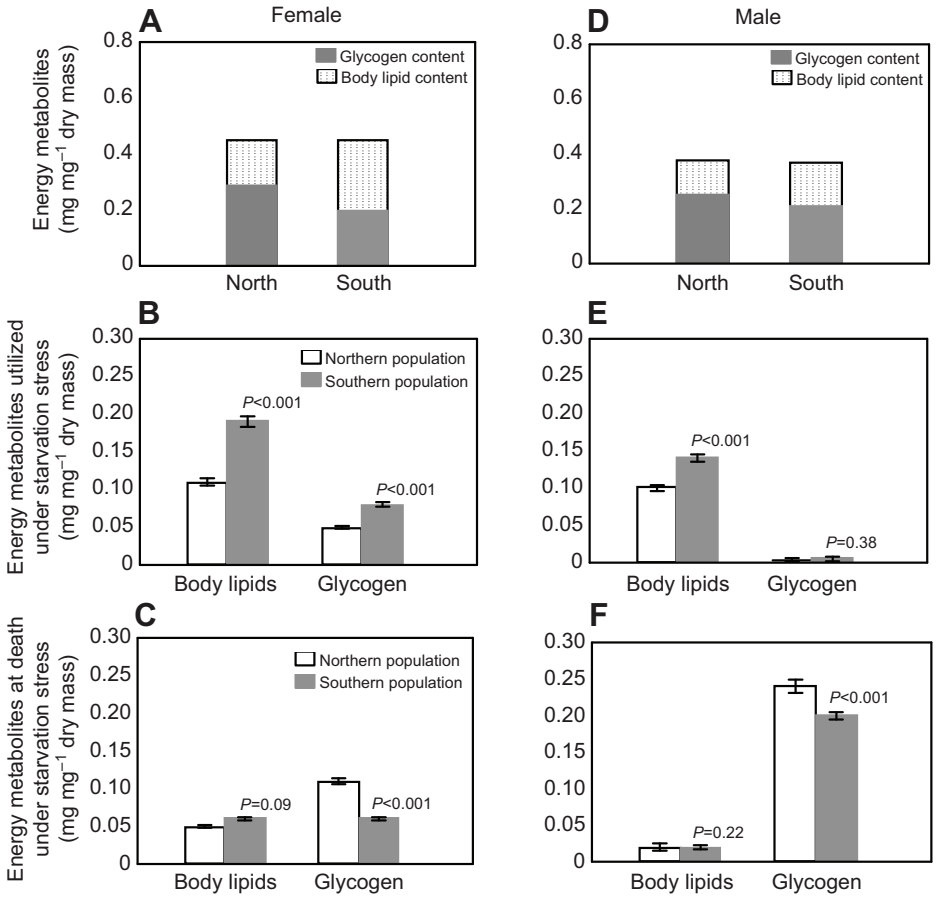

Fig. 3. Analysis of energy metabolites (basal storage levels, utilization and threshold need under starvation stress) in one northern as well as one southern population of *D. leontia*. Between-population differences (southern versus northern) in the dry-mass-specific storage levels of body lipids and carbohydrate contents of female and male *D. leontia* (A,D). The levels of body lipids were significantly higher in the southern population, but the northern population has stored greater levels of glycogen contents. The southern population utilized a greater dry-mass-specific proportion of body lipids as compared with the northern population (B,E); trends were similar in both sexes. However, sexual dimorphism occurred for the utilization of glycogen contents. Threshold needs, i.e. levels of energy reserve soon after death under starvation stress, differ between sexes (C,F), but not between populations.

Fig. 3 illustrates the dry mass storage levels of energy metabolites (body lipids and carbohydrates) in one northern and one southern population of *D. leontia*. Storage levels of body lipids were significantly higher in the southern as compared with the northern population, whereas the reverse trend was observed for carbohydrates (Fig. 3A,D). The southern population utilized more body lipids as compared with the northern population (Fig. 3B,E), but the rate of metabolite utilization of body lipids did not differ between them (females: $F_{1,38}$=2.05; males: $F_{1,38}$=3.42; both P>0.05). The levels of glycogen contents decreased significantly under starvation stress in females only, whereas between-population differences for rate of glycogen utilization were non-significant (Fig. 3B). In contrast, males did not utilize glycogen contents under starvation stress (Fig. 3E, Tables 3, 4). However, protein contents were remaining unutilized in northern as well as southern populations in females as well as males in *D. leontia* (Table 3, 4). Interestingly, the levels of body lipids (threshold need) at death did not differ between the northern and southern populations in females (Fig. 3C) and males (Fig. 3F). However, the threshold need in females was higher as compared with males (Fig. 3C,F).

Changes in energy budget

The analysis of total energy budget as well as net energy budget utilized during starvation stress in one northern and one southern population of *D. leontia* is shown in Table 4. For both sexes, the southern population showed a higher energy budget derived from lipids than the northern population (females: F=1688.72; males: F=1350.55; P<0.001), consistent with between-population differences in the consumption of the lipid energy budget. However, higher energy content due to carbohydrates was found in the northern population compared with the southern population for both sexes (Table 4). Females used energy obtained from glycogen during starvation stress, but males consumed only body lipids. However, the total energy budget used under starvation was higher in females than males (Table 4). Therefore, the energetic basis of starvation resistance differs between the sexes in *D. leontia*.

Climatic associations

The results of multiple regression analysis to explain trait variability as a simulation function of temperature as well as relative humidity are shown in Table 5. For both sexes, SR was significantly correlated to latitudinal variations in T_{min}, T_{max} and T_{cv}, whereas no such correlation was observed for T_{ave} (Table 5). Interestingly, similar trends were observed for correlated changes in energy metabolites (body lipids and carbohydrate contents) and multiple variables of temperature for both sexes. However, all the measured variables of relative humidity (RH_{min}, RH_{max}, RH_{ave} and RH_{cv}) along the latitudinal transect of *D. leontia* collection sites were significantly correlated to starvation-related traits (Table 5).

Effects of hardening on starvation resistance

Fig. 4 illustrates the changes in SR (hours) due to multiple bouts (one to four bouts) of pre-treatment to starvation stress. Females showed increased longevity starvation stress due to pre-treatment (Fig. 4A), but males lack such plastic responses (Fig. 4C). Interestingly, females respond to one and two bouts of pre-treatment (Fig. 4A), but a non-significant increase was evident for the third and forth bouts. Further, repeated-measures GLM ANCOVA was

Table 4. Data on rate of metabolite utilization, net energy budget for carbohydrates, lipids and proteins, and net energy budget used under starvation stress in female and male *D. leontia*

	Females			Males		
	Northern	Southern	*F*-value	Northern	Southern	*F*-value
Rate of metabolite utilization						
Body lipid content	0.780±0.022***	0.763±0.024***	3.95$^{n.s.}$	0.691±0.018***	0.679±0.019***	2.60$^{n.s.}$
Trehalose content	0.010±0.007$^{n.s.}$	0.013±0.005$^{n.s.}$	0.66$^{n.s.}$	0.008±0.005$^{n.s.}$	0.012±0.003$^{n.s.}$	3.26$^{n.s.}$
Glycogen content	0.293±0.035***	0.302±0.029***	3.80$^{n.s.}$	0.011±0.006$^{n.s.}$	0.009±0.004$^{n.s.}$	1.48$^{n.s.}$
Protein content	0.015±0.06$^{n.s.}$	0.009±0.04$^{n.s.}$	2.41$^{n.s.}$	0.006±0.002$^{n.s.}$	0.008±0.005$^{n.s.}$	3.68$^{n.s.}$
Metabolite energy budget						
Body lipid content	2.751±0.09	3.860±0.11	1688.72***	1.696±0.05	2.088±0.07	1350.55***
Trehalose content	0.669±0.03	0.528±0.03	2102.68***	0.814±0.03	0.660±0.02	540.89***
Glycogen content	1.201±0.02	0.880±0.02	648.20***	0.666±0.02	0.557±0.03	568.20***
Protein content	1.215±0.04	1.217±0.03	3.85$^{n.s.}$	1.129±0.03	1.135±0.04	410.77***
Energy budget used in starvation						
Body lipid content	2.051±0.06	3.074±0.10	987.82***	1.004±0.03	1.762±0.05	1913.06***
Trehalose content	0.017±0.01	0.020±0.01	7.16$^{n.s.}$	0.019±0.02	0.015±0.01	1.34$^{n.s.}$
Glycogen content	0.354±0.02	0.528±0.02	261.50***	0.023±0.01	0.028±0.02	8.30$^{n.s.}$
Protein content	0.015±0.01	0.021±0.01	5.23$^{n.s.}$	0.013±0.01	0.017±0.01	3.22$^{n.s.}$

GLM ANCOVA was used for the comparisons.
Slope values represent rate of metabolite utilization as a function of time. Units are μg h^{-1} for rate of metabolite utilization, and J mg^{-1} for energy budget.
Conversion factors: 17.6 J mg^{-1} for carbohydrates, 39.3 J mg^{-1} for lipids, and 17.8 J mg^{-1} for proteins (Schmidt-Nielsen, 1990; Marron et al., 2003).
Asterisks on slope values indicate significant changes in the levels of energy metabolites with an increase in desiccation stress duration.
n.s., non-significant; ***P<0.001.

undertaken to explain geographical differences (northern versus southern) and effects of repeated bouts of starvation hardening in *D. leontia*. In order to determine between-population differences in absolute changes in SR, the storage levels of body lipids, the rate of energy budget utilization and the absolute hardening capacity (AHC=K_{SR}–C; see Materials and methods, Plasticity in starvation resistance) for each trait were calculated. Females showed a significant effect of starvation hardening (F=711.70, P<0.001), whereas males lacked such effects (F=0.095, P>0.05). However, between-population differences were not significant for either sex (female: F=0.014; male: F=0.024; P>0.05). Further, between-population differences (female: F=0.684; male: F=0.250; P>0.05) as well as hardening effects (female: F=2.807; male: F=0.751; P>0.05) in both sexes were non-significant for changes in energy budget derived from lipids. In contrast, females showed a decrease in the rate of body lipid utilization due to hardening (F=327.41, P<0.001), but such changes were not evident in males (F=0.340, P>0.05). Further, differences for the threshold need of energy reserves were non-significant in hardened versus non-hardened flies (female: F=3.08; male: F=1.213; P>0.05). In all cases, interaction effects between population and hardening were non-significant. These results suggest that starvation hardening affects SR capacities in females, but such plastic responses did not differ between geographical populations of *D. leontia*.

Table 5. Multiple regression analysis of the effects of simultaneous variation in climatic variables (temperature and relative humidity) and starvation-related traits in eight *D. leontia* populations

	Starvation resistance		Body lipid content		Carbohydrate content	
	b ± s.e.m.	*r* ± s.e.m.	b ± S.E.	r ± S.E.	b ± S.E.	r ± S.E.
Female						
T_{min}	2.11±0.07***	0.30±0.006***	1.86±0.05***	0.36±0.008***	–2.64±0.12***	–0.27±0.007***
T_{max}	–2.01±0.09**	–0.22±0.011*	–1.68±0.03**	–0.21±0.07*	2.12±0.07*	0.25±0.005**
T_{ave}	0.13±0.36$^{n.s.}$	0.04±0.007$^{n.s.}$	0.19±0.25$^{n.s.}$	0.02±0.03$^{n.s.}$	–0.30±0.26$^{n.s.}$	–0.01±0.03$^{n.s.}$
T_{cv}	–2.56±0.11***	–0.35± 0.010***	–2.14±0.09**	–0.41±0.018**	2.29±0.15**	0.46±0.012***
RH_{min}	1.92±0.06**	0.26±0.008**	2.88±0.13**	0.29±0.05*	–2.88±0.09**	–0.21±0.004**
RH_{max}	–0.86±0.04*	–0.21±0.006**	–1.63±0.12**	–0.19±0.02*	1.62±0.06*	0.19±0.003*
RH_{ave}	1.22±0.08**	0.29±0.009*	0.99±0.8**	0.26±0.06**	–1.28±0.05**	–0.29±0.006**
RH_{cv}	–2.05±0.13**	–0.29±0.009**	–2.20±0.19**	–0.25±0.05**	2.36±0.08**	0.31±0.008***
Male						
T_{min}	0.36±0.12**	0.38±0.012**	0.72±0.05**	0.32±0.04**	–1.69±0.07**	–0.28±0.009**
T_{max}	–0.31±0.05*	–0.25±0.004*	–0.56±0.02*	–0.28±0.03*	1.22±0.06**	0.25±0.005*
T_{ave}	0.09±0.18$^{n.s.}$	0.06±0.09$^{n.s.}$	0.11±0.19$^{n.s.}$	0.07±0.09$^{n.s.}$	–0.16±0.17$^{n.s.}$	0.03±0.07$^{n.s.}$
T_{cv}	–0.68±0.11**	–0.31±0.011$^{n.s.}$	0.85±0.07**	0.27±0.02**	1.35±0.05**	0.33± 0.012*
RH_{min}	0.87±0.03**	0.25±0.009**	0.80±0.07**	0.26±0.02**	–1.85±0.09**	0.23±0.005**
RH_{max}	–0.62±0.08*	–0.22±0.007*	–0.51±0.05**	–0.19±0.01**	1.70±0.06**	0.28±0.007**
RH_{ave}	0.66±0.05*	0.28±0.05*	0.34±0.09*	0.29±0.03**	–1.36±0.08**	0.22±0.005*
RH_{cv}	–0.82±0.14*	–0.41±0.016**	–0.37±0.07**	–0.31±0.04*	1.91±0.11***	0.31±0.008**

T_{min}, minimum temperature; T_{max}, maximum temperature; T_{ave}, average temperature; T_{cv}, coefficient of mean monthly variations in temperature; RH_{min}, minimum relative humidity; RH_{max}, maximum relative humidity; RH_{ave}, average relative humidity; RH_{cv}, coefficient of mean monthly variations in relative humidity.
n.s., non-significant; *P<0.05; **P<0.01; ***P<0.001.

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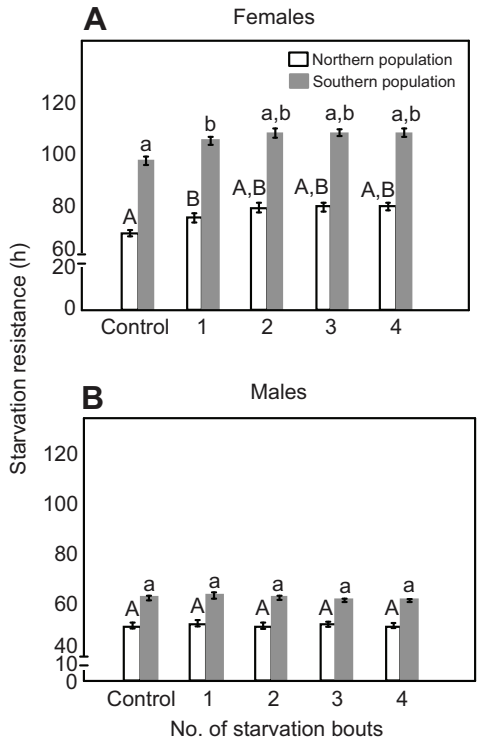

Fig. 4. Sexual dimorphism for starvation-hardening in *D. leontia*. Analysis of starvation-hardening responses due to a single and multiple (up to four) bouts of starvation pre-treatment in females (A) and males (B) of *D. leontia*. Starvation-hardening responses (up to two bouts) were evident in the southern as well as the northern population of females, but males lack such effects. A Bonferroni *post hoc* test was used for multiple comparisons.

DISCUSSION

Variation in availability and quality of food is one of the ubiquitous abiotic stresses in animals (Rion and Kawecki, 2007; McCue, 2010). All organisms face a period of nutritional stress during their lifespan; therefore, various physiological adaptations might have evolved in the course of phylogeny (Hoffmann and Harshman, 1999; Rion and Kawecki, 2007). Nutritional challenges may be constrained by acquisition, utilization and threshold need of energy metabolites to endure an enhanced survival (Rion and Kawecki, 2007; Ballard et al., 2008; Parkash and Aggarwal, 2012). Besides the genetic adaptations, non-genetic effects, i.e. phenotypic plasticity, may further facilitate improved survival, and buffer the consequences of food stress to some extent (Bubliy et al., 2012). In the present study, these empirical data precisely demonstrate sex-specific divergence for the mechanistic basis of physiological adaptations (genetic as well as plastic responses) to starvation stress in *D. leontia*. These results illustrate that the outcomes of long-term (genetic) as well as short-term (plasticity) adaptations to starvation stress differ between sexes in *D. leontia*. Females exhibited a greater response to evolutionary constraints, which resulted in steeper clinal variations for starvation-related traits as compared with males. Further, repeated bouts of pre-treatment to starvation stress resulted in a reduced rate of energy budget consumption in females, which in turn promotes survival after subsequent exposure to starvation stress, whereas males lack such plastic responses. Finally, these results suggest that sex-specific divergence to evolve genetic and plastic capacities under similar evolutionary constraints contribute to sexual dimorphism for basal SR levels in *D. leontia*.

Clinal patterns of starvation resistance: analysis of sex-specific differences

Clinal variations are considered as changes in trait values or genes over space (Endler, 1986; Hoffmann and Weeks, 2007). The repeatability of clines for quantitative traits on different continents reflects the role of natural selection, instead of genetic drift (Endler, 1986; Mousseau et al., 2000). Nevertheless, latitudinal clines for SR have shown inconsistent patterns, or reverse trends in *Drosophila*, which might have been associated with variations in stress factors occurring in the natural habitats. For example, in contrast to a negative association of SR with latitude for Indian populations (Parkash and Munjal, 1999; Parkash and Munjal, 2000), SR was positively correlated with latitude for North American populations of *D. melanogaster* (Schmidt et al., 2005). However, no such clinal variations were evident for SR in South American populations of *D. melanogaster* (Robinson et al., 2000). Further, *P*-element analysis has suggested that the candidate genes involved in SR showed sex-specific effects on the phenotype (Harbison et al., 2005; Wang et al., 2005). Consequently, sexes might have asserted variable levels of adaptation to starvation stress under similar evolutionary forces, which might have resulted in sexual dimorphism in clinal patterns of SR. Nevertheless, sex-specific variations in starvation-related traits for geographical populations have been least explored so far (Goenaga et al., 2013). In the present study, SR between sexes was compared through a common garden experiment to eliminate the possible influence of phenotypic plasticity on trait values. Females showed a stepper latitudinal cline for SR, whereas a shallower gradient was evident in males (Table 1, Fig. 2). Trends of sexual dimorphisms for SR were consistent in both wild and laboratory conditions. Interestingly, SR clinal patterns were significantly steeper in wild habitats as compared with laboratory conditions for females, but such plastic effects were non-significant for males (Table 1). Results of ANCOVA suggest highly significant sex-specific effects for SR in geographical populations of *D. leontia* (Table 2). Moreover, the presence of significant genotype and population interactions indicates segregation of genetic variation for sexual dimorphism in SR (Table 2). Finally, these results suggest that sexes of *D. leontia* differ in their genetic potential to evolve SR under similar evolutionary constraints.

Climatic associations

Fluctuating temperature and relative humidity have been often considered as natural selectors shaping the evolution of stress-related traits (Hoffmann and Harshman, 1999; Hoffmann, 2010; Chown et al., 2011; Kellermann et al., 2009; Kellermann et al., 2012). SR clines have been shown to be repeatedly correlated to climatic variables across the latitudinal transect of the Indian subcontinent (Karan et al., 1998; Parkash and Munjal, 1999; Parkash and Munjal, 2000). In the present study, yearly average temperature poorly correlated to latitude, but a positive correlation was evident for the coefficient of variation in mean monthly temperature due to more thermal fluctuations in northern as compared with southern localities of the Indian subcontinent (Fig. 1). Further, yearly average relative humidity and amplitude of humidity variations vary significantly with latitude. Data on multiple regression analysis as a simultaneous function of changes in trait values and thermal or humidity variations show that thermal (T_{min}, T_{max} and T_{cv}) as well as humidity variations (RH_{min}, RH_{max}, RH_{ave} and RH_{cv}) in the natural habitats significantly affected the evolution of starvation-related traits in *D. leontia* across the latitudinal transect of the Indian subcontinent (Table 5).

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Role of energy metabolites in SR

Clinal patterns are well documented for various *Drosophila* species across latitude in India (Karan et al., 1998; Karan and Parkash, 1998; Parkash and Munjal, 1999; Parkash and Munjal, 2000), but the underlying energetic basis of such adaptations has not yet been much explored (Parkash and Aggarwal, 2012; Parkash et al., 2012). Previous studies based on laboratory selection of SR have demonstrated unequivocally that the accumulation of higher body lipids acts as a chief metabolite in starvation-resistant lines of *D. melanogaster* (Chippindale et al., 1996; Hoffmann et al., 2005; Schwasinger-Schmidt et al., 2012). In contrast, debates are ongoing for field populations. For example, a positive correlation of percentage body lipids with SR has been evident in diverse *Drosophila* species (van Herrewege and David, 1997; Ballard et al., 2008; Parkash et al., 2012), but such a pattern was not found to be consistent for populations of *D. melanogaster* across latitudes in Australia (Hoffmann et al., 2001). The results of the present study depict a positive correlation between the levels of body lipid and SR capacities, which forge the basis of between-population differences in SR in *D. leontia*. Indeed, the trends were similar in the both sexes. Finally, these findings suggest that geographically varying starvation stress has been constrained by levels of body lipids to confer increased SR in *D. leontia*.

Furthermore, the differences in SR between xeric versus mesic *Drosophila* species have been associated with rate of metabolite utilization, although the dry-mass-specific levels of energy metabolites did not differ between them (Marron et al., 2003). In general, one may assume that the rate of metabolite utilization might be constrained, inconsistently, by geographically varying starvation stress. However, the GLM ANCOVA results suggest that rates of metabolite utilization and threshold need did not differ between southern and northern populations in both sexes. Conversely, males have shown a reduced rate of metabolite utilization and also a lower threshold need to survive under starvation stress as compared with females. Conclusively, these results suggest that the sexes differ in actual storage levels, rate of utilization as well as threshold need of energy metabolites to adapt under starvation stress.

Analysis of starvation-hardening effects

The eco-physiological adaptations to fluctuating water and food stress have often been considered as key factors affecting the physiological boundaries of a species (Kellermann et al., 2009; Benoit, 2010; Hoffmann, 2010). Therefore, multiple strategies might have developed to respond to such non-static stress conditions. In *D. melanogaster*, hardening to water stress (0% RH) has conferred an increased longevity because of plastic changes in cuticular permeability and levels of body sugar (Bazinet et al., 2010). Similarly, improved survival after an exposure to ecologically relevant multiple bouts of dehydration stress have been associated with depletion of energy reserves as well as reduced fecundity in the mosquito *Culex pipiens* (Benoit et al., 2010). In *Drosophila*, the induced plasticity for SR has been mostly elucidated in caloric restriction experiments (Leroi et al., 1994; Kapahi et al., 2004; Piper et al., 2005). A single study has analyzed starvation acclimation (plastic) effects by complete deprivation of food in alternate modes for *D. melanogaster* (Bubliy et al., 2012). Nevertheless, the physiological basis of plasticity in SR has not been explored in any *Drosophila* species so far.

In the present study, repeated bouts of starvation stress have conferred an enhanced longevity under subsequent lethal levels of starvation stress for female *D. leontia*, but males lack such plastic effects. During the severe starvation stress in dipterans, the mobilization of lipids from the fat body to the hemolymph occurs by stimulation of hormonal control lapses. The mobilized lipids are transported to various tissues, and β-oxidation of fatty acids facilitates further growth and survival (Chapman, 2013). In order to expedite the survival under starvation stress, the mobilization of lipids from fat body to hemolymph might have occurred in starvation-hardened individuals. However, it is reasonable to argue that if mobilization of lipids from fat body is a key mechanism that confers an improved survival in starvation-hardened flies, the hardened flies should have lower threshold levels of energy reserves compared with their non-hardened counterparts. Conversely, the present data suggest a significant reduction in metabolic rate, and a lack of changes in the net energy budget as well as threshold levels of energy reserves in starvation-hardened individuals (Table 6). Presumably, the mobilization of lipids from fat body to hemolymph might have occurred in both non-hardened and hardened flies under lethal levels of starvation stress; therefore, the observed changes in threshold need in flies of two different regimes (hardened versus non-hardened) were non-significant. Altogether, these results posit that the reduced rate of metabolite utilization may often be an important component of plastic changes in SR, rather than other possible avenues of increased SR. However, future studies are needed to support such contentions.

Conclusions

In the present study, I tested whether: (1) sexes of *D. leontia* differ in their physiological basis of SR, and (2) do multiple bouts of starvation stress improve survival after a subsequent exposure to lethal levels of starvation stress until death, and whether patterns of such responses differ between the sexes and populations. Results show a steeper clinal gradient for SR females than males of *D. leontia*, which indicates sexual dimorphism in the potential mechanisms to evolve SR in field habitats. Higher SR resistance of females relied on greater proportional body lipid contents, whereas the rate of body lipid consumption and threshold need under starvation stress was significantly lower in males. Furthermore, females showed improved SR by reducing the rate of metabolite utilization after one or two bouts of starvation pre-treatment (hardening), whereas the between-population differences in absolute hardening capacity were not significant. In contrast, such plastic responses were not evident in males. Finally, these results suggest that sexes in *D. leontia* differ in their genetic and plastic responses to starvation stress, consistent with the observed divergence in the SR capacities.

MATERIALS AND METHODS

Collections and cultures

Wild-occurring individuals of *Drosophila leontia* (n=500–600 flies from each site) were sampled in October 2010 using the net-sweeping method in a single trip from eight latitudinal sites (listed southernmost to northernmost): Forst S David (11°45′N, 79°50′E; altitude 268 m); Hiriyur (13°57′N, 76°40′E; altitude 630 m); Homnabad (17°43′N, 77°12′E; altitude 632 m); Penganga (20°0′N, 78°15′E; altitude 329 m); Nagod (22°33′N, 80°19′E; altitude 310 m); Bhadaura (24°48′N, 77°98′E; altitude 390 m); Firozpur Jhirka (27°37′N, 76°59′E; altitude 672 m); and Bilaspur (31°19′N, 76°60′E; altitude 673 m). I examined the patterns of clinal variations for starvation resistance (SR) in the field-collected flies. However, an isofemale (IF) line approach (n=20 IF lines per population) was used to examine sex-specific as well as between-population divergence for starvation-related traits in laboratory-reared populations of *D. leontia*. All cultures were maintained at low density (60–70 eggs per vial; 40×100 mm size) on cornmeal-yeast-agar medium at 25°C. For experimentation, 4-day-old virgin flies of the G_5 and G_6 generations were used. Data for temperature and

Table 6. Results of repeated measures GLM ANCOVA (with dry mass as a covariate) explaining between-population differences (northern versus southern) and the effects of four bouts of starvation pre-treatment (hardening) on net changes in starvation resistance, body lipid energy budget and rate of body lipid utilization in *D. leontia*

		Female		Male	
Source	d.f.	MS	*F*	MS	*F*
Starvation resistance					
Population	1	0.059	$0.014^{n.s.}$	0.039	$0.024^{n.s.}$
Error	394	4.224		1.642	
Hardening	3	2569.22	711.70***	0.359	$0.095^{n.s.}$
Population × Hardening	3	0.075	$0.019^{n.s.}$	0.422	$0.112^{n.s.}$
Error	1184	3.610		3.770	
Energy budget					
Population	1	0.013	$0.684^{n.s.}$	0.009	$0.250^{n.s.}$
Error	394	0.019		0.036	
Hardening	3	0.073	$2.807^{n.s.}$	0.027	$0.751^{n.s.}$
Population × Hardening	3	0.022	$0.846^{n.s.}$	0.013	$0.361^{n.s.}$
Error	1184	0.026		0.036	
Rate of metabolite utilization					
Population	1	2.944	$0.488^{n.s.}$	0.375	$0.386^{n.s.}$
Error	394	6.022		0.971	
Hardening	3	1366.29	327.41***	1.021	$0.340^{n.s.}$
Population × Hardening	3	0.230	$0.055^{n.s.}$	0.290	$0.096^{n.s.}$
Error	1184	4.173		3.001	
Threshold level of energy budget					
Population	1	1.227	$1.316^{n.s.}$	0.734	$2.924^{n.s.}$
Error	394	0.932		0.251	
Hardening	3	1.639	$3.080^{n.s.}$	0.937	$1.213^{n.s.}$
Population × Hardening	3	0.237	$0.445^{n.s.}$	0.227	$0.294^{n.s.}$
Error	1184	0.532		0.771	

In males, energy budget, rate of utilization of energy budget and threshold levels of energy budget represent the analysis of body lipid data, but body lipid and glycogen content data were pooled for these calculations in females, because both glycogen and body lipids act as fuel under starvation stress in females.
Net changes in starvation resistance are absolute values: the trait value after starvation pre-treatment was the substrate before pre-treatment.
Homogeneity of slopes was checked in order to validate body mass as a covariate in the factorial models.
n.s., non-significant; ***P<0.001.

relative humidity in *D. leontia* sampling habitats were obtained from 'Climatological Tables' published by the Indian Meteorological Department, Government of India, New Delhi (Indian Meteorological Department, 2010).

Trait analysis

For analysis of clinal trends of SR, body lipid, carbohydrate and protein content in the field populations, 50 individuals of each sex per population were used. However, 20 IF lines (×10 replicates each) per population were used for the analysis of sex-specific clinal patterns as well as plastic responses of starvation-related traits. A group of individuals per replicate (10 replicates × 20 IF lines) was used for estimation of SR and energy metabolites.

Starvation resistance

A group of 10 adult female or male flies was placed in a dry plastic vial that contained a foam sponge impregnated with 2 ml of water + 2 mg sodium benzoate (to prevent bacterial growth); these vials, with muslin wraps, were placed in a humidity chamber (www.metrexinstruments.com; MEC-30) that was maintained at 80–85% relative humidity at 25±0.5°C. The number of immobile flies was scored three times on the first day, but eight times in the subsequent days until all survivors died as a result of starvation stress.

Plasticity in starvation resistance

I moderately modified the protocol suggested by Bubliy et al. (Bubliy et al., 2012) for analyzing starvation hardening time in *Drosophila*. A group of 10 adult female or male flies (10 replicates × 20 IF lines) from one northern as well as one southern population was subjected to starvation stress until 5% failure time was reached, which was calculated using the Probit method. Starvation hardening time was estimated to be 32 h for the southern population but 20 h in the northern population for females. However, males were exposed to pre-treatment to starvation for 18 and 13 h in the southern and northern populations, respectively. Thereafter, flies were subjected to laboratory food medium for 12 h for recovery from starvation stress. Further, I explored the effects of repeated bouts of starvation stress by imposing a starvation stress period alternating with a recovery period on laboratory food medium. The effects of four successive bouts of starvation–pre-treatment on changes in SR potential females as well as males of *D. leontia* were analyzed.

In order to determine absolute changes in SR, I calculated the absolute hardening capacity (AHC) using the formula AHC=K_{SR}–C, where C and K_{SR} represent trait values (SR) without the pre-treatment and after the pre-treatment, respectively.

Analysis of body lipid content

A group of 10 adult female or male flies was dried in 2 ml Eppendorf tubes (www.tarsons.in) at 60°C for 48 h and then weighed on a Sartorius microbalance (model CPA26P, 0.001 mg precision; www.sartorious.com). Thereafter, 1.5 ml di-ethyl ether was added to each Eppendorf tube and kept for 24 h under continuous shaking (200 rpm) at 37°C. Finally, the solvent was removed and individuals were again dried at 60°C for 24 h and re-weighed. Lipid content was calculated per individual by subtracting the lipid-free dry mass from the initial dry mass per fly.

Estimation of trehalose and glycogen

For trehalose and glycogen content estimation, 10 adult flies from each IF line were homogenized in a homogenizer (Labsonic® M; www.sartorious.com) with 300 µl Na_2Co_3 and incubated at 95°C for 2 h to denature proteins. An aqueous solution of 150 µl acetic acid (1 mol l^{-1}) and 600 µl sodium acetate (0.2 mol l^{-1}) was mixed with the homogenate. Thereafter, the homogenate was centrifuged (Fresco 21, Thermo-Fisher Scientific, Pittsburgh, PA, USA) at 9660 ***g*** for 10 min. This homogenate was used for independent estimations of trehalose and glycogen as given below.

For trehalose estimation, aliquots (200 µl) were placed in two different tubes; one was taken as a blank and the other was digested with trehalase at 37°C using the Megazyme trehalose assay kit (K-Treh 10/10, www.megazyme.com). In this assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6-phosphate and ADP, which was further coupled with glucose-6-phosphate dehydrogenase and resulted in the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was measured at 340 nm (UV-2450-VIS, Shimadzu Scientific Instruments, Columbia, MD, USA).The pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and subtracted from total glucose concentration.

For estimation of glycogen, a 50 µl aliquot was incubated with 500 µl *Aspergillus niger* glucoamylase solution (8.7 U ml^{-1} in 200 mmol l^{-1} of acetate buffer) for 2 h at 40°C with constant agitation and the suspension was centrifuged at 1073 ***g*** for 5 min. It has mainly hydrolyzed alpha-(1,4) and alpha-(1,6) glycosyl linkages and was suited for breakdown of glycogen. Glucose concentration was determined with 20 µl of supernatant from the suspension and added to 170 µl of a mixture of G6-DPH (0.9 U ml^{-1}), ATP (1.6 mmol l^{-1}) and NADP (1.25 mmol l^{-1}) in triethanolamine hydrochloride buffer (380 mmol l^{-1} TEA-HCl and 5.5 mmol l^{-1} $MgSO_4$) and 10 µl of hexokinase solution (32.5 U ml^{-1} in 3.2 mol l^{-1} ammonium sulphate buffer), and absorbance was measured at 340 nm.

Protein assay

Protein levels were determined using the bicinchoninic acid method as followed by Marron and co-workers (Marron et al., 2003). For the protein assay, 10 female flies per IF line (n=10 replicates × 20 IF lines of each species) were homogenized in 3 ml distilled water and centrifuged at 6708 ***g*** for 5 min. Further, 50 µl of aliquot was taken from the supernatant and treated with 2 ml of Sigma BCA reagent and incubated at 25°C for 12 h. Absorbance was recorded at 562 nm and protein concentration was determined by comparing with a standard curve.

Utilization of energy metabolites

I quantified the changes in the levels of energy metabolites (glycogen, trehalose, body lipids and proteins) as a consequence of an increase in the duration of starvation stress in males as well as females of one northern and one southern population of *D. leontia*. Multiple replicates of each IF line (n=20 IF lines) were run simultaneously, and were exposed to starvation stress for different time durations, until all survivors reached LT_{100}. Each replicate experienced 5 h more starvation stress than the counter-replicate. The rate of metabolite utilization was analyzed as the regression slope values obtained by plotting changes in the levels of energy metabolites (y-axis) as a simultaneous function of increase in the durations of starvation stress on the x-axis (suggested by Marron et al., 2003).

Statistical analyses

For each trait, mean ± s.e.m. values (20 IF lines, 10 replicates each) were used for illustrations and tables. Slope values between wild versus laboratory conditions, and between the sexes were compared with a Student's t-test (Zar, 1999). The Probit method was used to analyze the lethal time to starvation death (50%; LT_{50}) in females as well as males of *D. leontia*. Partial crossed and partial nested (IF lines nested into populations) ANCOVA (with body mass as a covariate) was used to explain the sex-specific and between-population differences for starvation-related traits. Homogeneity of slopes was checked to validate the use of body mass as a covariate in the factorial analysis models. To examine whether body lipids, glycogen, trehalose and protein contents act as metabolic fuel under starvation stress, nested ANCOVA was used (fixed effect: treatment; random effect: IF lines). Further, multiple regression analysis was used to explain the trait variability as a simultaneous function of latitudinal variations in temperature and relative humidity. Rates of metabolite utilization under starvation stress were compared with GLM ANCOVAs. For the analysis of absolute changes in starvation-related traits due to hardening effects, repeated-measures ANOVA was performed (population: between effects; pre-treatment to starvation stress: within effects). However, multiple bouts in starvation hardening experiments were compared using a Bonferroni *post hoc* test. Standard conversion factors were used for calculations of energy budget derived from carbohydrates, lipids and proteins (following Schmidt-Nielsen, 1990; Marron et al., 2003). SPSS (Version 19.0, IBM, Armonk, NY, USA) and Statistica (Statsoft Inc., Release 5.0, Tulsa, OK, USA) software were used for calculations as well as illustrations.

Acknowledgements

I am indebted to two anonymous reviewers for several helpful comments which improved the paper.

Competing interests

The author declares no competing financial interests.

Funding

The Council of Scientific and Industrial research (CSIR) is gratefully acknowledged for a senior research fellowship (SRF) [File no. 10/2(5)-2007(ii)EMU. II]. Most of the chemicals/reagents were purchased using my personal SRF fellowship, and no other funding was received for this study.

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