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Climatic adaptation of *Drosophila buzzatii* populations in southeast Australia

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Variation in 19 traits possibly relevant for thermal adaptation was studied in 11 populations of *Drosophila buzzatii* collected in southeast Australia. Using stepwise multiple regression, the variation was compared to variation in geographic coordinates and to a set of climatic variables estimated for each collection site. For 13 of the traits, a significant part of the variation was explained by climatic variables and/or geographic coordinates, suggesting directional selection for adaptation to the environment in the majority of traits studied. In 10 of the traits, both geographic coordinates and climatic variables explained significant proportions of the variation, with R^2 ranging from 0.075 to 0.58. Although larvae, pupae and adults of *D. buzzatii* share a common habitat, the measured traits were not correlated across life stages and gender. Also, there seemed to be special conditions in marginal populations near species borders, giving rise to nonlinear relations with latitude. Climate apparently does influence the adaptive evolution of the traits studied, but they also are affected by other factors that vary with latitude, longitude and distance to coast. These results highlight the complex challenges imposed by the environment on the adaptive process.
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Introduction

The distribution and abundance of species is for a large part determined by environmental variables of which temperature is generally considered to be one of the most important (Cossins and Bowler, 1987; Hoffmann *et al*, 2003a). Thus, the study of variation in thermal resistance is relevant when looking for adaptive variation among populations and species. From a biodiversity perspective, thermal resistance also is potentially relevant, as climate is predicted to be even more variable and changing in the near future (IPCC, 2001). In order to persist, species must adapt to, or avoid, stressful conditions imposed by the ever-changing climate. A well-established model system for the study of thermal adaptation is *Drosophila buzzatii* (Loeschcke *et al*, 1994; Sørensen *et al*, 2001, 2003). This cactophilic species is especially well suited to the study of adaptation, because of its well-defined ecology. All life stages live on and in rotting cladodes of *Opuntia* spp. (Barker and Mulley, 1976), a habitat that is often exposed to high temperatures (Gibbs *et al*, 2003).

Some studies of thermal resistance focus on resistance at the adult stage, but this gives an incomplete picture of the thermal resistance of an organism that has four very different life stages, as thermal extremes may not affect all life stages in the same way (Loeschcke and Krebs, 1996). Even the seemingly homogeneous habitat of a rotting cactus cladode can provide very different microhabitats for small organisms such as *Drosophila*. Sun and shade will alternate and different areas of the rot will offer different degrees of thermal stress (Feder, 1997; Sørensen *et al*, 2003). The immobile life stages (ie eggs and pupae) cannot actively avoid high temperatures, as can the adults and to a lesser degree the larvae. This means that eggs, pupae and to some degree the larvae may be more exposed to thermal stress than adults and therefore might be a more likely target for thermal adaptation (Krebs and Loeschcke, 1995b). In addition, the different life stages may very well react differently to the same environmental conditions; this also makes it important to study heat resistance in life stages other than the adult.

One tool in the study of thermal adaptation is to compare resistance traits among populations from different geographic regions, which are assumed to differ in thermal characteristics. Most studies have compared a small number of traits in a small number of populations. With only two to three populations studied along a climatic gradient (eg latitudinal), it is difficult to distinguish between real adaptation and random variation (Garland and Adolph, 1994). Davidson's (1990) study of geographical variation in desiccation resistance in *D. melanogaster* exemplifies the problem. The results showed a population from a temperate region to be more resistant than a tropical

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population, but this difference was not consistent when 13 populations from the two regions were compared (Hoffmann *et al*, 2001). Another important issue for successfully identifying natural adaptation is to investigate resistance traits that are relevant under natural conditions. However, as the thermal environment that *Drosophila* adults and larvae experience in the field is poorly known, it can be difficult to predict whether natural adaptation should lead to clinal variation in specific stress resistance traits. Local conditions might also disturb the overall pattern of adaptation, especially when only a small number of populations are studied. However, for some traits there is evidence that links climatic variability with variation in stress resistance traits. For example, survival following high temperature heat exposure (Hsp induction) was strongly related to Hsp70 expression, which itself was inversely related to high temperature adaptation in adult *D. buzzattii* (Sørensen *et al*, 2001). For heat adaptation, knockdown resistance has been suggested to be important and to correlate with natural adaptation to high-temperature environments (Hoffmann *et al*, 2002; Sørensen *et al*, 2001, 2005). In nature, high temperature is correlated with desiccation, but many studies have failed to show clinal variation in desiccation resistance (Hoffmann *et al*, 2001, 2002), whereas others have found presumably adaptive patterns in desiccation resistance (Karan *et al*, 1998; Hoffmann *et al*, 2003b).

The aim of the present study was to identify traits important for thermal adaptation, that is, those showing some kind of clinal variation. We investigated the variation in 19 traits potentially involved in thermal adaptation of *D. buzzatii*. The 11 populations studied were selected on the basis of extensive climatic data to ensure that the sites were maximally different with regard to most climatic variables. Resistance traits in three of the four life stages, larvae, pupae and adult, were studied, with the majority of traits in the larval or adult stage. Several heat-resistance traits and developmental time at 25 and 30°C were measured. We found clinal variation in 13 of the 19 traits studied. Ten of these traits were influenced by several of the variables used in the analysis, indicating a complex relationship between geographic coordinates, climatic variables and trait response.

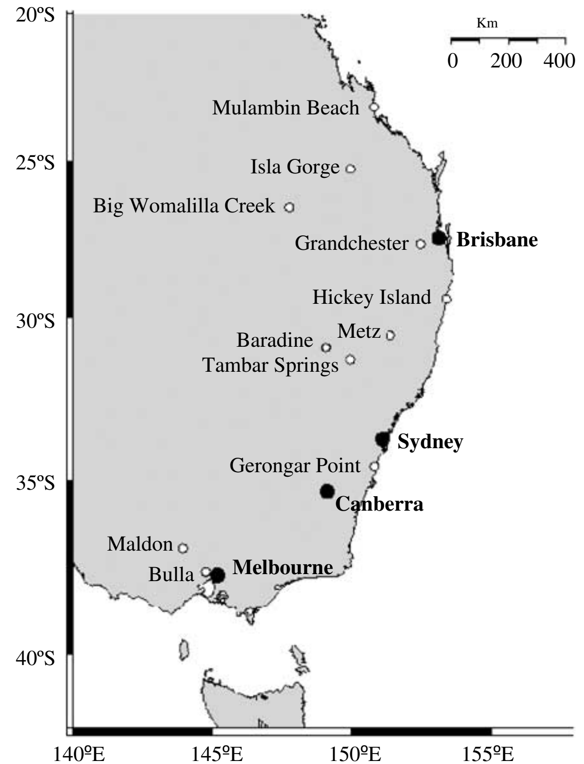

Figure 1 Map of southeast Australia showing the locations where *D. buzzatii* were collected.

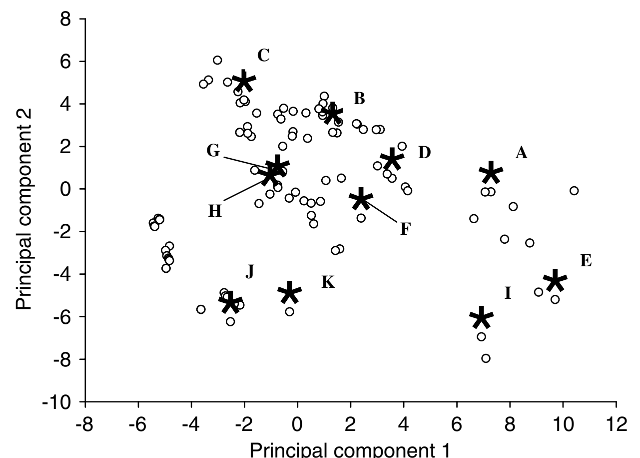

Figure 2 Graphic presentation of the first two principal components from a PCA analysis of 35 climatic variables from 97 known *D. buzzatii* collection sites. The collected populations are marked with stars and letters: A, Mulambin Beach; B, Isla Gorge; C, Big Womalilla Creek; D, Grandchester; E, Hickey Island; F, Metz; G, Tambar Springs; H, Baradine; I, Gerongar Point; J, Maldon; and K, Bulla.

Materials and methods

Origin and maintenance of flies

Flies were collected in April 2002 at 11 sites in southeast Australia (Figure 1) – Mulambin Beach (150°47′E, 23°11′S); Isla Gorge (149°56′E, 25°15′S); Big Womalilla Creek (147°46′E, 26°29′S); Grandchester (152°27′E, 27°41′S); Hickey Island (153°21′E, 29°26′S); Metz (151°53′E, 30°35′S); Baradine (149°04′E, 30°57′S); Tambar Springs (149°57′E, 31°18′S); Gerongar Point (150°49′E, 34°33′S); Maldon (Baringhup) (143°57′E, 36°59′S); and Bulla (144°46′E, 37°39′S). These sites were selected from 97 where *D. buzzatii* had been collected previously (Barker *et al*, 2005). With the position of a locality described by latitude, longitude and elevation, the BIOCLIM program of the ANUCLIM 5.1 package (Houlder *et al*, 2000) estimated 35 climatic variables for that locality. Principal component analysis was then applied to these data for each of the 97 localities (SAS Institute, 1985) to provide a summary of the climatic environment for each. The first PC describes a gradient of increasing precipitation and moisture indices and decreasing temperature variability and radiation. The second component describes a gradient of increasing temperature, precipitation and moisture index season-

ality, and decreasing moisture indices in the lowest week and quarter, precipitation in the driest quarter and radiation seasonality. Selection of the 11 sites for this study was based on the plot of principal component 1 and principal component 2 (Figure 2), so as to maximise climatic differences among them.

Twelve isofemale lines were established from each population. Each isofemale line was maintained before and during the experiment with approximately 25 pairs in each of two bottles (200 ml) with 14 ml of instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC, USA). Throughout the experiments, instant *Drosophila* medium was used, unless stated otherwise. All experiments were conducted within three to 10 generations after collection.

Hsp70 expression

Hsp70 expression was assayed in larvae, pupae and adult males and females. In all cases, five random isofemale lines from each of the 11 populations were assayed. Hsp induction took place in preheated waterbaths for 1 h with the water above the bottom of the stoppers, followed by a 1 h recovery at 25°C before freezing at −70°C. The vials were placed in racks in preheated waterbaths and spaced evenly to ensure homogeneous heating. Third instar larvae for heat treatment were collected by inserting paper into the bottles and leaving for 6 h. The paper with wandering third instar larvae was then transferred to empty plastic vials with moistened stoppers to prevent desiccation and heated at 37°C. On another set of papers from different bottles, the larvae were allowed to pupate and then transferred to empty plastic vials on the moist paper. The pupae were heat exposed at 38°C 2–3 days after pupation. Adult flies less than 24 h old were collected, sexed under light CO_2 anaesthesia and transferred to food vials at a density of 15 individuals per vial. On day 3, they were transferred to fresh vials. When flies were 5–6 days old, they were placed in empty glass vials and heat exposed. To prevent desiccation, the stoppers were in all cases moistened with tap water. For adults, two temperatures were used, 37 and 39°C. Later, larvae, pupae and adults were homogenised and the level of Hsp70 expression assayed using a monoclonal inducible Hsp70 antibody (7.FB; Velazquez *et al*, 1980, 1983). Enzyme-linked immunosorbent assay was conducted in five replicate microwell plates following the protocol described in Sørensen *et al* (1999).

Knock down resistance

Flies less than 24 h old from five random lines per population were collected and transferred to food vials at a density of approximately 50 (equal number of both sexes). Every second day, they were transferred to fresh vials. When flies were between 5 and 7 days old, the knockdown test (Huey *et al*, 1992) was conducted at a constant temperature of 40.0°C±0.1, as described in Sørensen *et al* (2001).

Sterility period

Sterility period was scored after males developed at one of two experimental temperature regimes: 25 and 25°C (18 h)/38°C (6 h). Twenty-five pairs from 12 lines per population were allowed to lay eggs in 200 ml bottles for 24 h at 25°C. Bottles were placed at the appropriate temperature 2 days after the beginning of oviposition, where they remained until emergence of adults. To prevent desiccation of the bottles at the high temperature, a tray with water was placed in the bottom of the incubators and stoppers were wetted every second day.

After hatching, males (0–12 h old) were placed on sugar–agar–yeast medium with 6-day-old virgin females, raised at 25°C, from their respective population. For each combination of line and treatment, one vial with 10 males and four females was placed at 25°C. Every 12 h, the flies were transferred to new vials. Vials were kept at 25°C and later evaluated for the presence of larvae. Time until sexual maturity was reached, for at least one of the 10 males in a vial, was calculated from the mean of the period where the males eclosed to the mean of the period where the first viable eggs were laid. This measure of heat-induced sterility includes the period where males naturally are sexually immature.

Developmental time

Developmental time was measured at two experimental temperatures: 25 and 30°C. From each of 10 random lines from each population, first instar larvae (raised at 25°C) were collected, and 25 transferred to each of two vials. These vials were placed at the temperature regimes of 25 and 30°C, respectively, and the number of eclosed flies was scored every 12 h. To prevent desiccation of the vials at the high temperature, a tray with water was placed in the bottom of the incubator and stoppers were wetted every second day. Mean developmental time of each sex emerging in each vial, estimated as the mean time from larval collection to adult eclosion, was $\log_{10}$ transformed to give a better fit to the normal distribution.

Heat-shock survival

Heat-shock survival was assayed in larvae and adult flies. First instar larvae were collected from 10 lines from each population with one replicate/line, and transferred to food vials at a density of 25 larvae/vial. The larvae were kept at 25°C until heat shocked. Two experimental groups were used and a control was kept at 25°C throughout the development. At day 4 after collection, one group was hardened for 1 h at 37°C followed by 2 h recovery at 25°C before being heat shocked at 42°C for 1 h. The other group was placed directly at 40.5°C for 1 h. After heat shock, the larvae were returned to 25°C for development and survivors were scored as number of eclosing flies.

Adult flies less than 24 h old were collected from eight lines per population, set up (sexes separate) at a density of 20 per vial, and heat shocked according to the protocol described for Hsp70 expression in adults. One group was hardened at 37°C for 1 h, followed by 1 h at 25°C to allow the flies to recover before being heat shocked for 1 h at 41.5°C. The other group was directly heat shocked at 40.5°C for 1 h. After the heat shock, flies were transferred to fresh food vials, which were turned upside down to prevent the flies from getting stuck in the medium, and allowed 24 h of recovery at 25°C before being scored as either alive or dead. Flies were considered as alive if they were able to walk after a light touch with a brush. As survival was calculated as proportions, arcsine-square-

root transformation was applied to improve normality and homogeneity of variances.

Statistical analyses

ANOVA was used when appropriate to establish general effects of treatment and sex within experiments using JMP v.5 (JMP, 2002), thereby determining if separate regressions for males and females were necessary. For each trait, effects of geographical location and climate were analysed, with climate characterised by the scores for the first four principal components (PC1, PC2, PC3 and PC4), which accounted for 93% of the variation.

Latitude, longitude and distance from the coast were recorded for each locality, but as the relationships with latitude and longitude were not necessarily linear, quadratic and cubic terms for these were included. Distance from the coast provides a further coordinate to latitude and longitude for geographical location, and was included because climatological zones in eastern Australia tend to run parallel to the coast (Nix, 1982). That is, localities on the same longitude but at different distances from the coast could be in quite different climatological and ecological regions.

Multiple regression analysis of each trait was carried out using a backwards stepwise procedure to suggest which terms may be required in the final model, using the statistical package R (R Development Core Team, 2003), and its stepwise procedure, STEP. For adding and deleting terms, this procedure uses the Akaike Information Criterion (Akaike, 1974), which is based on log-likelihood but also takes into account the number of parameters fitted. If two models give similar fits, the model using the least number of parameters is selected. All tests for individual terms were adjusted for other terms in the model of the same or lower order (Type II tests).

Results

Means and standard errors of all traits in all populations are presented in Table 1.

Hsp70 expression

No comparisons of Hsp70 expression level in larvae, pupae, and male and female adults were possible as Hsp70 expression level in each 'Hsp70 expression group' was assayed in different experimental blocks. However, it is possible to compare Hsp70 expression between life stages and sex by correlating Hsp70 expression level in the different groups. After correcting the probability levels by the Dunn–Sidak method (Sokal and Rohlf, 1995), the only two groups that were significantly correlated were Hsp70 expression level in females at 37 and 39°C (one-tailed Pearson's correlation; $r = 0.76$; $P = 0.0034$).

Within each experimental group, population variation was investigated by multiple regression. For third instar larvae, there was a significant regression with latitude squared ($\beta = 0.0002$; $P < 0.01$) explaining 16% of the variation in Hsp70 expression between populations (Table 2, Figure 3a). No significant regression was detected for pupal expression level. For females at 37°C, latitude squared ($\beta = -1.4$; $P < 0.01$), latitude cubed ($\beta = -0.028$; $P < 0.05$), longitude ($\beta = 2022$; $P < 0.01$), longitude squared ($\beta = -135$; $P < 0.\ 01$), longitude cubed ($\beta = 0.3$; $P < 0.\ 01$), PC1 ($\beta = -8.9$; $P < 0.01$), PC2 ($\beta = -1.8$; $P < 0.01$) and PC4 ($\beta = -9.9$; $P < 0.01$) explained 58% of the variation in Hsp70 expression (Table 2). At 39°C, latitude ($\beta = 25$; $P < 0.01$), PC1 ($\beta = -12$; $P < 0.05$), PC2 ($\beta = -32$; $P < 0.01$) and PC4 ($\beta = -8.8$; $P < 0.05$) explained 31% of the variation in female Hsp70 expression (Table 2). For Hsp70 expression in males at 37°C, latitude ($\beta = 298$; $P < 0.01$), latitude squared ($\beta = 8.3$; $P < 0.05$), latitude

Table 1 Means and SE for all traits measured

Pop	*Hsp70 expression (abs)*						*KD (s)*	*Sterility (h)*		*Developmental time (h)*				*Heat-shock survival rate*					
	Larvae	*Pupae*	♀		♂					♀		♂		*Larvae*		♀		♂	
	37°C	*38°C*	*37°C*	*38°C*	*37°C*	*38°C*		*25°C*	*38°C*	*25°C*	*30°C*	*25°C*	*30°C*	*No H*	*H*	*No H*	*H*	*No H*	*H*
Mub	0.34	0.047	0.30	0.71	0.16	0.43	124	48	111	316	274	318	289	0.46	0.51	0.74	0.72	0.45	0.53
SE	0.1	0.02	0.07	0.2	0.04	0.1	21	4	14	3	3	3	4	0.08	0.07	0.07	0.06	0.1	0.1
Isg	0.37	0.042	0.40	0.78	0.39	0.55	70	38	99	308	282	309	281	0.38	0.31	0.75	0.74	0.41	0.65
SE	0.06	0.01	0.06	0.1	0.1	0.04	8	2	13	3	4	2	4	0.08	0.08	0.05	0.08	0.08	0.07
Bwc	0.41	0.054	0.22	0.65	0.10	0.40	68	43	84	333	296	336	300	0.54	0.56	0.94	0.80	0.63	0.64
SE	0.07	0.01	0.04	0.2	0.01	0.07	9	4	19	3	4	5	5	0.08	0.1	0.02	0.06	0.09	0.07
Grd	0.35	0.053	0.13	0.35	0.14	0.27	75	40	120	313	287	311	288	0.57	0.38	0.69	0.82	0.50	0.67
SE	0.05	0.009	0.02	0.1	0.03	0.05	10	2	8	4	4	2	3	0.09	0.08	0.09	0.06	0.1	0.07
His	0.34	0.023	0.46	0.92	0.26	0.63	60	43	104	313	270	310	283	0.34	0.41	0.61	0.74	0.44	0.53
SE	0.06	0.009	0.04	0.2	0.03	0.08	6	2	7	3	4	2	4	0.08	0.06	0.07	0.06	0.06	0.08
Metz	0.44	0.040	0.17	0.41	0.20	0.48	50	40	101	317	290	330	299	0.55	0.54	0.85	0.84	0.52	0.74
SE	0.04	0.01	0.04	0.1	0.05	0.1	5	2	10	4	4	3	3	0.08	0.1	0.05	0.07	0.1	0.08
Bar	0.37	0.036	0.26	0.75	0.10	0.56	61	41	108	311	289	314	305	0.53	0.41	0.91	0.78	0.41	0.69
SE	0.1	0.001	0.06	0.2	0.002	0.01	12	2	11	3	4	3	5	0.08	0.08	0.04	0.09	0.1	0.05
Tam	0.55	0.054	0.14	0.45	0.12	0.28	98	40	84	330	304	335	301	0.25	0.30	0.80	0.84	0.58	0.68
SE	0.05	0.007	0.03	0.08	0.03	0.05	20	3	13	3	4	3	3	0.08	0.08	0.03	0.03	0.1	0.1
Ger	0.50	0.057	0.33	0.40	0.09	0.26	55	36	93	310	290	311	284	0.61	0.39	0.42	0.73	0.43	0.63
SE	0.08	0.02	0.09	0.08	0.01	0.03	6	2	18	4	5	3	4	0.06	0.1	0.08	0.05	0.09	0.1
Bul	0.49	0.045	0.24	0.43	0.25	0.65	51	40	85	312	295	315	300	0.58	0.32	0.79	0.78	0.50	0.58
SE	0.01	0.01	0.02	0.08	0.02	0.09	5	2	13	3	5	2	4	0.02	0.08	0.1	0.08	0.08	0.06
Mal	0.51	0.027	0.18	0.56	0.24	0.35	68	36	67	315	278	320	296	0.38	0.29	0.76	0.67	0.40	0.48
SE	0.02	0.008	0.02	0.07	0.07	0.08	17	4	12	4	4	2	6	0.05	0.08	0.07	0.09	0.06	0.09

KD, knockdown resistance; sterility, heat-induced male sterility.

Table 2 Sign and significance level of the regressions of traits on latitude (lat), latitude squared (lat^2), latitude cubed (lat^3), longitude (long), longitude squared ($long^2$), longitude cubed ($long^3$), the first four principal components from a PCA of 35 climatic variables (PC1–4) and distance to coast (DSCT)

Trait	*HspL*	*Hspf37*	*Hspf39*	*Hspm37*	*Hspm39*	*KD*	*Steril25*	*Steril38*	*Dm25*	*HSf*	*HHSm*	*HSL*	*HHSL*
lat			+**	+**	+***	−**					−**	+***	
lat^2	+***	−**		+**			+**		+***	+***	−**	−**	
lat^3		−*		+*					+***	+***		+***	
long		+**			+***			+*					−*
$long^2$		−**			−***				−***			+***	+*
$long^3$		+**			+***							−***	−*
PC1		−**	−*	−***	−***				+***			+**	+**
PC2		−**	+**		−***				+***			−**	−*
PC3				+***		−*			+**			−***	
PC4		−**	+**	−***						+*	+*	+***	+**
DSCT				−***					−**				+**
R^2	16%	58%	31%	52%	49%	14%	5.6%	7.5%	16%	28%	11%	22%	10%

HspL, Hsp70 expression level in larvae; Hspf37, Hsp70 expression level in females at 37°C; Hspf39, Hsp70 expression level in females at 39°C; Hspm37, Hsp70 expression level in males at 37°C; Hspm39, Hsp70 expression level in males at 39°C; KD, knockdown resistance; Steril25, male sterility period at 25°C; Steril38, male sterility period at 25°C (18 h)/38°C (6 h); dm25, male developmental time at 25°C; Hsf, heat shock resistance without hardening in females; HSL, heat shock resistance without hardening in larvae; HHSm, heat shock resistance with hardening in males; HHSL, heat shock resistance with hardening in larvae. Not presented in this table are nonsignificant regressions for pupal Hsp70 expression, female developmental time at 25 and 30°C, male developmental time at 30°C, male heat-shock survival without hardening and female survival with hardening.
$^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

cubed ($\beta=0.076$; $P<0.05$), PC1 ($\beta=-12$; $P<0.001$), PC3 ($\beta=14$; $P<0.001$), PC4 ($\beta=-26$; $P\ll0.001$) and distance to coast ($\beta=-0.25$, $P\ll0.001$) explained 52%. At 39°C, latitude ($\beta=26$; $P\ll0.001$), longitude ($\beta=3410$; $P<0.001$), longitude squared ($\beta=-229$; $P<0.001$), longitude cubed ($\beta=0.51$; $P<0.001$), PC1 ($\beta=-15$; $P<0.001$) and PC2 ($\beta=-37$; $P\ll0.001$) explained 49% (Table 2).

Knockup down resistance

A two-way ANOVA showed no effect of sex and no significant population by sex interaction. However, the effect of population was significant (Table 3). Latitude ($\beta=-2.7$; $P<0.01$) and PC3 ($\beta=-6.2$; $P<0.05$) explained 14% of the variation in knockdown time (Table 2).

Sterility period

Two-way ANOVA showed no significant population by rearing temperature interaction. However, there was an effect on sterility period of both temperature and population (Table 3). Latitude squared explained 5.6% of the variation in the sterility period at 25°C ($\beta=0.0077$; $P<0.01$). In all, 7.5% of the variation in sterility period at 25/38°C ($\beta=3.3$; $P<0.05$) could be explained by longitude (Table 2). Figure 4 shows the relation between sterility period and latitude.

Developmental time

A three-way ANOVA showed no significant two or three-way interactions, but population, sex and environment all had significant effects on developmental time (Table 3).

Female developmental time showed no significant regressions at either temperature, nor did male developmental time at 30°C. However, 16% of the variation in male developmental time at 25°C could be explained by latitude squared ($\beta=3.22$; $P<0.001$), latitude cubed ($\beta=0.067$; $P<0.001$), longitude squared ($\beta=-0.12$; $P<0.001$), PC1 ($\beta=16$; $P<0.001$), PC2 ($\beta=27$; $P<0.001$), PC3 ($\beta=8.1$; $P<0.01$) and distance to coast ($\beta=-0.29$; $P<0.01$) (Table 2).

Heat-shock survival without hardening, adults

A two-way ANOVA revealed no interaction between the effects of population and sex on survival after 1 h at 40.5°C. However, the effects of both population and sex were significant (Table 3). Latitude squared ($\beta=0.0087$; $P<0.001$), latitude cubed ($\beta=0.0002$; $P<0.001$), longitude squared ($\beta=-0.00032$; $P\ll0.001$) and PC4 ($\beta=0.073$; $P<0.05$) explained 28% of the variation in survival in females (Table 2). No significant regression was detected in male survival after heat shock.

Heat-shock survival with hardening, adults

A two-way ANOVA revealed no interaction between the effects of population and sex on survival after 1 h at 41.5°C. The effect of sex was significant, but the effect of population was not (Table 3). However, latitude ($\beta=-0.3$; $P<0.01$), latitude squared ($\beta=-0.0049$; $P<0.01$) and PC4 ($\beta=0.064$; $P<0.05$) explained 11% of the variation in male heat-shock survival (Table 2). No significant regression was detected in female survival after hardening and heat-shock.

Heat-shock resistance without hardening, larvae

For larval survival after heat shock, latitude ($\beta=28$; $P<0.001$), latitude squared ($\beta=-0.86$; $P<0.001$), latitude cubed ($\beta=0.0087$; $P<0.001$), longitude squared ($\beta=0.08$; $P<0.001$), longitude cubed ($\beta=-0.00036$, $P<0.001$), PC1 ($\beta=0.19$; $P<0.01$), PC2 ($\beta=-0.36$; $P<0.01$), PC3 ($\beta=-0.48$; $P<0.001$) and PC4 ($\beta=0.53$; $P<0.001$) ex-

plained 22% of the variation in survival (Table 2, Figure 3b).

Heat-shock resistance with hardening, larvae

For larval survival after hardening and heat shock, longitude ($\beta = -782$; $P < 0.05$), longitude squared ($\beta = 5.2$; $P < 0.05$), longitude cubed ($\beta = -0.012$; $P < 0.05$), PC1 ($\beta = 0.19$; $P < 0.01$), PC2 ($\beta = -0.15$; $P < 0.05$), PC4 ($\beta = 0.44$; $P < 0.01$) and distance to coast ($\beta = 0.0085$; $P < 0.01$) explained 10% of the variation in survival (Table 2).

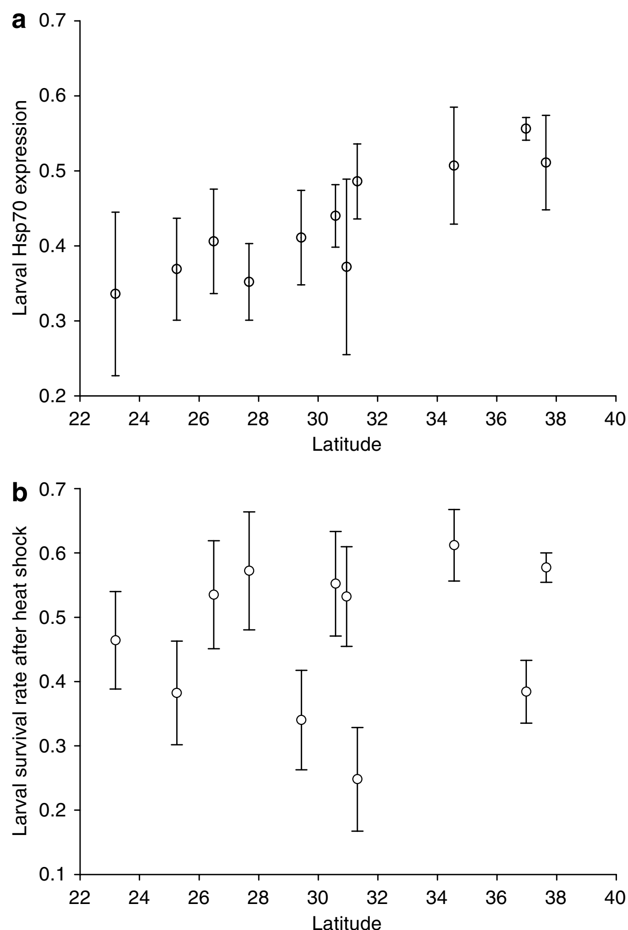

Figure 3 (**a**) Larval Hsp70 expression level after 1 h heat treatment at 37°C and then 1 h at 25°C, plotted against latitude. Hsp70 expression level increases in lines collected at more southern sites, indicating that larvae from northern sites were less stressed by the heat treatment. (**b**) Survival after heat shock at 40.5°C for 1 h without hardening in larvae, plotted against latitude.

Discussion

We found clinal variation in 13 of the 19 traits studied (Table 2). Ten of these traits were influenced by several of the variables used in the analysis indicating a complex relationship between geographic coordinates, climatic variables and trait response, a fact that complicates interpretation. These traits also demonstrated simultaneous significant effects of both geographic coordinates and climatic variables (Table 2), meaning that important factors other than the climatic variables accounted for by ANUCLIM vary along latitudinal and/or longitudinal gradients. These factors might be variation in coexisting species (eg competitors, predators and food plants) or different frequencies of rare catastrophic events (eg droughts, fires).

The immobile life stage, pupae, showed no variation among populations in the one trait studied (Hsp70 expression level). The temperature tested induced only a low expression level in this comparably heat-resistant life stage (Krebs and Loeschcke, 1995b), but a higher

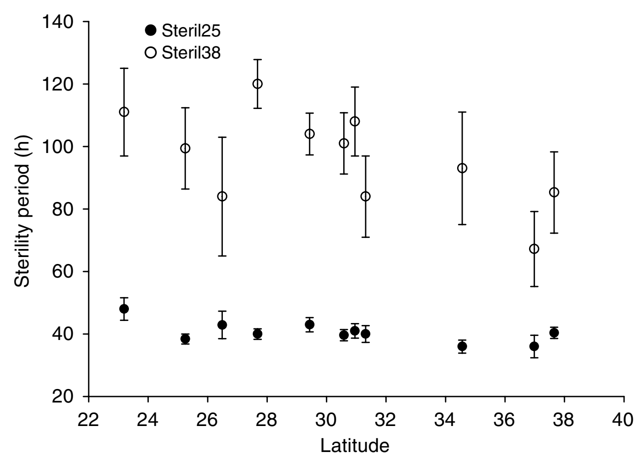

Figure 4 Plot of sterility period after development at 25°C (Steril25) or 25(18 h)/38(6 h) (Steril38) and transfer to 25°C at eclosure, plotted against latitude, showing longer sterility periods in lines originating from northern more heat-exposed localities.

Table 3 ANOVA results (mean squares) for tests of resistance in *D. buzzatii* from Australia, with degrees of freedom given in parentheses

Source	*KD*	*Sterility*	*Dev. time*	*HS adults*	
				No H	*H*
Pop (1)	4690**(10)	1119**(10)	1678**(10)	0.2**(10)	9.6E-02(10)
Sex (2)	2103(1)		2383*(1)	4.5***(1)	1.3***(1)
Temp (3)		1.4E05***(1)	6.5E04***(1)		
(1) × (3)		749(10)	321(10)		
(1) × (2)		631(10)	239(10)	9.7E-02(10)	1.2E-02(10)
(2) × (3)			280(1)		
(1) × (2) × (3)			237(10)		
Error	1629(81)	421(175)	597(374)	7.9E-02(163)	6.3E-02(138)

Traits are abbreviated as follows: KD, knock down; sterility, male sterility period; dev. time, developmental time; HS, heat shock; no H, without hardening; H, with hardening.
$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

temperature might have revealed differences between populations. All larval traits investigated showed clinal variation (Figure 3), as did most adult traits (Table 2). Generally, a greater proportion of the variation in the adult traits could be explained by the environmental and geographic variables (higher R^2) than in the larval traits. This might be owing to larval samples containing a mixture of males and females, whereas the adults were separated according to sex. In adults, the sexes can differ in their response to heat stress (eg this study and Krebs and Loeschcke, 1995a; Sørensen *et al*, 2001; Norry *et al*, 2004), and this also may be the case in larvae. Although there are no striking visual differences between the sexes at this stage, high rearing temperatures can induce sterility in males when hatched but not in females. Thus, patterns specific to or differing between sexes might be reduced or overlooked because larvae were treated as a single unit.

As mentioned above, some studies have shown that different life stages and sexes differ in their reaction to heat stress. This is also the case in our study where males and females showed different response patterns in most traits. Only knockdown resistance did not display a significant effect of sex (Table 3). With regard to Hsp70 expression level, even though only two of the six measurements of Hsp70 expression level were correlated with each other, five of the six showed clinal variation confirming that the variation found between populations was not random. The independence of Hsp70 expression level between life stages and sexes is confirmed by the different pattern in the regressions (Table 2), for example, larval, adult female and male expression level at 37°C were all influenced by latitude squared, but opposed to larval and male expression, female Hsp70 expression level had a negative relation with latitude squared indicating lower Hsp70 expression at both high and low latitudes. As is seen in Figure 3a, larval Hsp70 expression showed a clear tendency towards higher levels in lines originating from colder environments. This pattern has previously been shown in adults (Sørensen *et al*, 2001; Zatsepina *et al*, 2001) and may indicate that the northern more heat-exposed populations were less affected by the Hsp70 inducing temperature.

Knockdown resistance is considered a very important heat resistance trait and clinal variation in this trait has been found in several studies, showing greater resistance in populations originating in warmer areas (for a review see Hoffmann *et al*, 2003a; Sørensen *et al*, 2005). In our study, knockdown time was affected negatively by both latitude and PC3, which means that knockdown resistance increases the further north a collection site is positioned. This is consistent with previous findings by Hoffmann *et al* (2002) in *D. melanogaster*, and suggests selection towards longer knockdown times in hot environments.

Earlier studies of *D. buzzatii* have found a prolonged duration of heat-induced male sterility in populations originating in hot environments (Vollmer *et al*, 2004). Rohmer *et al* (2004) found the opposite pattern in *D. melanogaster*. In the present study, northern populations had longer sterility periods than temperate populations, at least at 25°C, confirming the previous findings in *D. buzzatii* (Table 2, Figure 4).

Developmental time has been found to vary with latitude in *D. melanogaster*, being slightly faster at high latitudes (James *et al*, 1995; James and Partridge, 1995). In *D. buzzatii*, however, different studies have found increased (Bubliy and Loeschcke, 2005), unchanged (Loeschcke *et al*, 2000) and decreased developmental time (Norry *et al*, 2001) in flies originating from colder environments. In this study, only developmental time in males at 25°C showed clinal variation, but in a complex manner with developmental time related to both geographic and climatic variables.

Survival after a heat shock with or without prior hardening is an often-used measure of heat resistance. In *D. buzzatii*, some studies have found evidence of greater heat resistance in populations from hot environments (Krebs and Loeschcke, 1995a, b), whereas others did not (Sørensen *et al*, 2001, 2005). In this study, we found clinal variation in heat shock without hardening in adult females and larvae (Figure 3b), but not in males. However, in both life stages the proportion of the variation that was explained by the regressions was only half in hardened individuals compared to flies/larvae that were not hardened. Hardening seems to some degree to level out the differences between populations. The mechanism behind this might be that the flies most stressed by the hardening treatment had a higher activation of the heat-shock response and thereby were comparably more protected against the following heat shock.

In many traits we see a significant effect of latitude squared, which might reflect a general response of the trait to stress, as both high and low latitudes could represent stressful conditions at the species boundaries. The collection sites span the whole distribution of *D. buzzatii* in Australia (Barker *et al*, 2005). The relations with latitude squared and cubed could be interpreted as a result of the joint effect of several evolutionary forces that vary differently along the gradient and therefore influence the traits in a complex manner. This complexity stems, among other things, from the fact that as latitude varies many climatic variables will vary simultaneously and most of them will be correlated. A correlation analysis of the 35 climatic variables provided by ANUCLIM (Houlder *et al*, 2000) revealed 33 correlations with $r > 0.9$. Thus, significant and causal associations with individual climatic variables cannot be untangled by statistical analyses.

In conclusion, we found clinal variation in most traits studied, and in 10 of these, both geographic coordinates and climatic variables explained significant proportions of the variation (Table 2). As the principal components represent a combination of climatic variables, the results are not interpretable in terms of specific selection pressures. However, a few results are pointing in the expected direction, as for example the negative correlation of knockdown resistance with latitude discussed above. Moreover, this study shows indisputably that climate does influence the evolution of stress-related traits in both larvae and adults.

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