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Evolution of total net fitness in thermal lines: *Drosophila subobscura* likes it 'warm'

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Abstract

Fisher's fundamental theorem states that heritable variation for net fitness sets a limit to the rate of response to natural selection. How will temperate (i.e. cold-tolerant) species cope with contemporary rapid global warming? Using three-fold replicated lines of *Drosophila subobscura* that had been allowed to evolve for 4 years (between 32 and 59 generations) at 13 °C (cold), 18 °C (the supposed optimum temperature), and 22 °C (warm) I assess here how net fitness changes according to thermal environments. Net fitness was estimated following the classical approach in population genetics of competing over a number of generation in outbred experimental populations multiple wild-type O chromosomes (homologous to arm 3R in *D. melanogaster*) independently derived from each base thermal stock in an otherwise homogeneous genetic background against a balancer chromosome. Warm-adapted populations ('warm-adapted O chromosomes') performed comparatively well at all tested temperatures. However, net fitness was severely reduced in cold-adapted populations when transferred to warmer conditions. It seems, therefore, that thermal fitness breath for *D. subobscura* flies is positively associated to temperature. These findings are discussed in relation to the fast world-wide clinal shifts in the frequency of genetic markers correlated with current climate change.

Introduction

Development, survival and reproduction in ectothermic animals are closely linked to environmental temperature. As a result, many morphological, physiological and behavioural adaptations in these animals have evolved within a given range of thermal conditions (Cossins & Bowler, 1987; Huey & Kingsolver, 1989; Huey & Berrigan, 2001; Angilletta *et al.*, 2002), and new challenges like the current human-induced climate warming are already having profound consequences on their biology (Parmesan *et al.*, 1999; Balanyá *et al.*, 2004, 2006; Møller & Merilä, 2004; Levitan & Etges, 2005; Umina *et al.*, 2005; Parmesan, 2006). To which extent there is heritable variation for temperature-dependent performance (e.g. any physiological, behavioural or life-history trait associated with the capacity of an individual to grow, mate and reproduce), and what is the relationship between the performance and fitness profiles (*sensu* Robertson, 1955) associated with temperature, have been largely debated questions. Evolutionary physiologists mostly rely on comparative studies to examine how historical constraints and natural selection in the wild have shaped the standing genetic and phenotypic variability in natural populations (Feder *et al.*, 2000), but ambiguous results are often obtained as environmental variation may obscure the underlying genetic effects. A useful complement to comparative studies is experimental evolution, in which evolving populations are sufficiently replicated (Rose *et al.*, 1996). Experimental evolution is a powerful way to explore the evolutionary potential of organisms and to uncover trade-offs that arise because of negative genetic correlations between performance at different temperatures, or between different performances at a given temperature. The drawback is, however, that selection studies are impractical for many model species that interest evolutionary

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physiologists and, in addition, the conclusions obtained from these studies are not straightforwardly extrapolated to natural populations (Harshman & Hoffmann, 2000).

To easily visualize the potential evolution of thermal sensitivity it is usually assumed that the relationship between relative performance and environmental (body) temperature follows an approximate Gaussian function (which is typically found to be skewed in real data) where performance peaks at the thermal optimum, and the breath of the distribution is the difference between the maximum and minimum temperatures at which performance is greater than zero (Huey & Stevenson, 1979; Huey & Kingsolver, 1993; Gilchrist, 1995). The impact of temperature on Darwinian fitness can be depicted similarly, and classical fitness measures as the intrinsic rate of increase (r) or the net reproductive rate (R_0) have been widely used (Huey & Berrigan, 2001; Frazier *et al.*, 2006). The thermal tolerance of an organism is therefore its ability to maintain functionality at extreme temperatures (Johnston & Bennett, 1996). The basic question behind these graphical and mathematical models is: can selection alter both the position and the shape of the thermal performance curve (Huey & Kingsolver, 1989)? Hertz *et al.* (1983) discussed two alternative views: the conservative or constrained view, and the labile or unconstrained view. Under the constrained view altering the performance function incurs a major cost and, hence, trade-offs impair the evolution of thermal sensitivity. Under the unconstrained view, however, a long-term change in performance can occur through acclimation or evolution.

Empirical studies of evolution in thermally variable environments have generally focussed on the optimum and the breath of performance as a function of temperature for life-history traits such as growth rate and fecundity, physiological traits such as heat and cold tolerance, and locomotor traits such as running speed. For instance, Gilchrist *et al.* (1997) studied the thermal sensitivity of walking speed in thermal lines of *Drosophila melanogaster* that had been maintained at three constant temperatures (16.5, 25 and 29 °C) during a minimum of 100 generations of selection. They showed that the thermal optimum for walking speed was weakly but positively correlated with the past thermal regime, but performance breadth was inversely related to selection temperature with flies maintained in the cold regime (16.5 °C) performing comparatively well at intermediate or high temperatures and better than the two other thermal lines when tested at low temperatures. Therefore, thermal sensitivity seems to be unconstrained in cold-adapted lines of *D. melanogaster* as both the position and breath of the thermal walking speed curve changed as a result of selection. Conversely, natural selection at high temperature apparently resulted in a trade-off in walking speed at low temperature. But the question naturally arises: what is the relationship between walking speed and fitness in these lines?

The thermal dependence of life-history traits had also been studied in the original Partridge's lines used by Gilchrist *et al.* (1997) to derive their populations. Thus, Partridge *et al.* (1994) found that cold-adapted flies had a faster growth rate and a shorter larval development period than their warm-adapted (25 °C) counterparts at all tested temperatures, thus suggesting that *D. melanogaster* flies in cold environments use the food resources more efficiently to attain a bigger size. This is a somewhat counterintuitive result in view of the standard within-population positive association between adult body size and larval development time in *Drosophila* (e.g. Santos *et al.*, 1994; Betrán *et al.*, 1998). On the other hand, longer preadult developmental times for cold-adapted populations were consistently observed in thermal lines of *D. subobscura* maintained at three constant temperatures (13, 18 and 22 °C) without a concomitant increase in body size (Santos *et al.*, 2006), which apparently agrees with the lower levels of gene expression for the overrepresented category of genes involved in metabolic processes observed in cold-adapted lines relative to their warm-adapted counterparts (Laayouni *et al.*, 2007). Egg-to-adult viability was also studied in the same *D. subobscura* lines and some slight evidence of beneficial adaptation was observed in that for each line this life-history trait tended to be the highest at the developmental temperature at which the stock was selected.

So far the only experimental evolution studies that have directly looked at the thermal dependence of fitness have been carried out with organisms such as *Escherichia coli*, where the Malthusian fitness parameter can be easily determined by the frequency change of a given line or genotype (Hartl & Clark, 1997). Bennet *et al.* (1992) showed that *E. coli* lines selected at constant 32, 37, or 42 °C improved fitness at their own temperatures without a necessary loss of fitness at other temperatures, indicating that thermal adaptation was largely temperature specific. In addition, the thermal niche (i.e. the breath of the thermal fitness curve) was not affected by selection history (Bennet & Lenski, 1993), although later experiments suggested a small shift in the lower thermal minimum and a concomitant decrease near the thermal maximum (Mongold *et al.*, 1996). Although microbial models offer a number of advantages for experimental evolution such as the possibility of preserving genotypes by freezing, many organisms of interest reproduce sexually and it is desirable to quantify how their thermal dependence of fitness evolves. In addition, it is also well understood that adaptation between prokaryotes and eukaryotes differs profoundly (Poole *et al.*, 2003).

Here I study the relationship between total net fitness and temperature by using the three-fold replicated lines of *D. subobscura* referred to above. At the time of the experiments the lines had evolved for 4 years (between 32 and 59 generations) at constant temperatures covering much of the physiologically tolerable range of the species (cold: 13 °C; optimum: 18 °C; warm: 22 °C). We

have previously shown that the thermal populations had already diverged for chromosome inversion frequencies, preadult life-history components, morphological traits and gene expression levels for a sizeable number of genes located approximately at random with respect to chromosomes (Santos *et al.*, 2005, 2006; Laayouni *et al.*, 2007). To estimate total net fitness I used the classical approach in population genetics of competing over a number of generations in outbred experimental populations a lethal gene (i.e. a balancer chromosome that carries a dominant morphological marker that is homozygous lethal and multiple inversions to suppress recombination) against multiple wild-type O chromosomes independently derived from each base thermal stock in an otherwise homogeneous genetic background. Chromosome O (homologous to arm 3R in *D. melanogaster*; Powell, 1997) is the longest chromosome in *D. subobscura* (*c.* 23.3% of the euchromatic portion; i.e. 28 Mb out of a total of 120 Mb; see Laayouni *et al.*, 2007) and is the only one for which a balancer stock is available.

Materials and methods

Extraction of O chromosomes from the thermal stocks

The derivation and maintenance of the thermal selection lines have been described elsewhere (Santos *et al.*, 2004). Briefly, all thermal lines were initiated from an ancestral population of *Drosophila subobscura* derived from a large outbred stock collected in November 1999 at the estimated Chilean epicentre of the original New World invasion (Puerto Montt, Chile, 41°28′S, 73°00′W; Brncic & Budnik, 1980). From that ancestral population three sets (P13, P18 and P22) of three replicate populations each (R1, R2 and R3) were set up in May 2001 and have since kept at three experimental temperatures covering much of the physiologically tolerable range in this species (Krimbas, 1993) on a discrete generation, controlled breeding under constant larval density (*c.* 5 larvae mL^{-1} of food) and constant 12 : 12 light : dark period: cold (13 °C), optimum (18 °C) and warm (22 °C) respectively. The number of breeding adults per population is typically well over 1500 flies.

After 4 years of thermal evolution, 20 nonlethal O chromosomes were obtained from each thermal line (i.e. P13R1, P13R2, …, P22R3) following a procedure similar to that shown in Fig. 1 to guarantee a homogeneous genetic background for all chromosomes but chromosome O (obviously the male Y chromosomes was also derived from the thermal lines). The *ch-cu* marker strain in Fig. 1 (labelled as O_{+cu+ch}/O_{+cu+ch}) has a highly homogeneous genetic background and is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu*) (Koske & Maynard Smith, 1954). The *Va/Ba* (*Varicose/Bare*) stock (labelled as $O_{Va\ cu+ch}/O_{++Ba+)}$ is the only balancer stock available in *D. subobscura* and was derived from the *ch-cu* strain (Sperlich *et al.*, 1977). Both marker strains are routinely maintained in the lab at 18 °C. Wild-type flies were first crossed with the *ch-cu* stock because the expression of the *Ba* gene is highly variable and affected by modifiers located on the O chromosome (Alvarez *et al.*, 1981). All crosses were carried out at 18 °C. It is important to notice that the replication scheme for sampling chromosomes from a thermal stock does not cause reverse evolution on the cold- or warm-temperature lines to the optimum temperature (i.e. barring mutations the O chromosomes were carried intact all along the crosses) if it may occur (Teotónio & Rose, 2000). Chromosomes that were lethal or with viability < 5% ('quasi-lethal'), according to the recorded proportions of wild-type flies raised in the viability tests (Fig. 1 and below), were excluded, which amounted to *c.* 12% of a total of 247 extracted chromosomes (the proportion of lethal and quasi-lethal chromosomes was about the same for all thermal stocks when grouped according to their past temperature regime). Viability tests were performed as described in Fernández Iriarte *et al.* (2003). Thus, two $O_{Va\ cu+ch}/O^{1}$ males were crossed to three $O_{Va\ cu+ch}/O^{1}$ females in each of two replicated 2×8 cm vials containing 6 mL of food (David's killed-yeast *Drosophila* medium; David, 1962). Twelve days after the crosses were made all parents were discarded. The progeny raised from each vial was counted and the viabilities were estimated as the ratio, number of wild-type files/total number of files, after pooling the progeny from the two replicated vials. The viability distribution of all lines had the usual pattern in this kind of experiments (e.g. Lewontin, 1970, pp. 49–50; Fernández Iriarte *et al.*, 2003), with a bimodal distribution with one mode at lethality and the other around the expected 33% of wild type flies (results not shown). All fly handling was carried out at room temperature using CO_2 anaesthesia when necessary on flies not < 12 h after eclosion.

Experimental populations

The heterozygous flies $O_{Va\ cu+ch}/O^{i(i=1,\ldots,20)}$ obtained from each thermal selection stock (amounting to 95% of the standing genetic variation for chromosome O) were used to set up the experimental populations by dumping *c.* 40 pairs of virgin flies from each chromosomal line into Plexiglas cages ($27 \times 21 \times 16$ cm^3). The expectation was that the lethal balancer would speedily reduce its frequency and eventually be eliminated from the cages because of the known selection against the *Va/Va* genotype and the probable disadvantage of the *Va*/+ genotype in competition with heterozygous O chromosome genotypes appearing after random mating (see Sved & Ayala, 1970).

Eggs were sampled from the cages and placed in 130-mL bottles (at a high density of *c.* 1000–1200 eggs per bottle) containing *c.* 40 mL of food. A total of 36

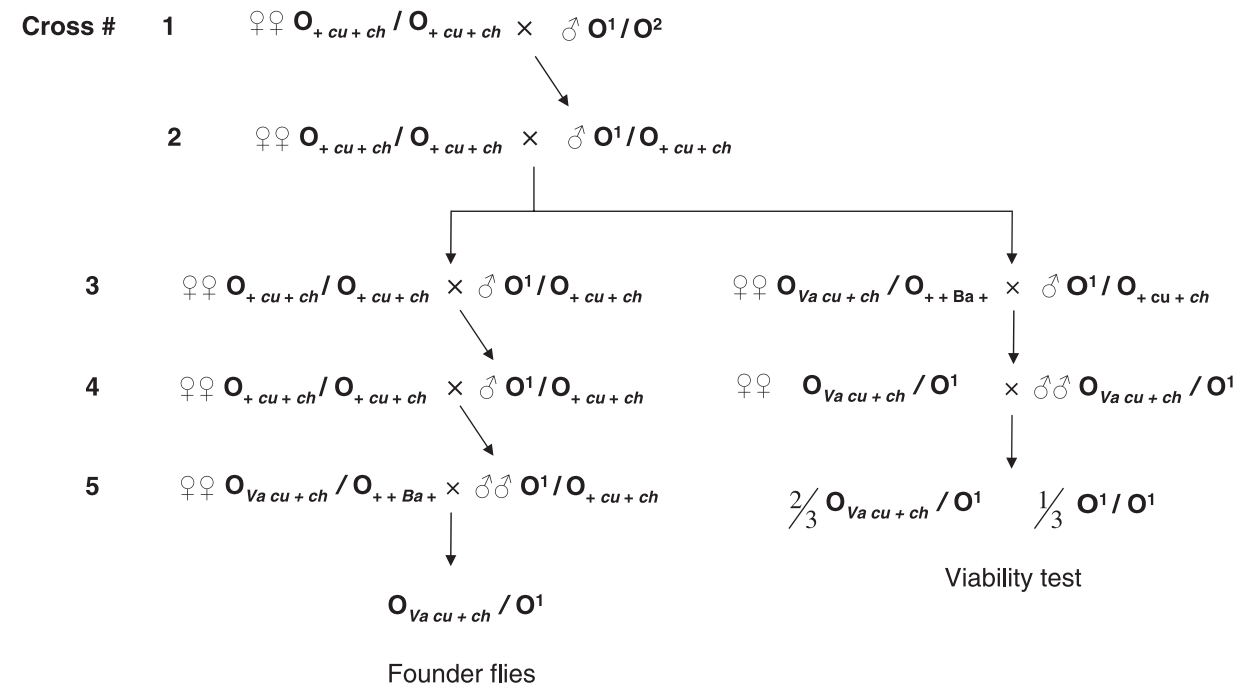

Fig. 1 Mating scheme to obtain the heterozygous flies $O_{Va\ cu+ch}/O^{i\ (i=1,\ldots,20)}$ from each thermal stock. The minimum number of crosses with the *ch-cu* marker strain to homogenize the genetic background was four, but in a number of cases and additional fifth cross with the *ch-cu* was necessary in order to synchronize the lines before the last cross with the *Va*/*Ba* balancer stock. Only wild-type O chromosomes with viabilities $\geqslant$ 5% were sampled (see text for details).

bottles were set up for each population and randomly distributed into three groups with 12 bottles each to be allocated at each of the three experimental temperatures (13, 18 and 22 °C). All populations were subsequently maintained on a discrete generation regime as follows. Emerging flies were dumped into Plexiglas cages (the number of breeding adults never dropped below 1500) and supplied with liberal amounts of food (two 90 mm Ø Petri dishes with *Drosophila* medium supplemented with active dried yeast) until females reached their peak of fecundity. Thereafter, eggs were collected over an 8-day period at 13 °C, 6-day at 18 °C and 4-day at 22 °C, and placed in twelve 130-mL bottles at high density. Samplings from the cages to estimate wild-type and lethal-carrying genotype frequencies were always made simultaneously by placing eggs under near-optimal conditions (100–110 eggs per bottle) so that selection was minimized and, therefore, the frequency of the balancer chromosome among zygotes could be inferred.

The balancer O chromosome with the homozygous lethal *Va* carries the gene arrangement $O_{\underline{3+4}+\underline{VIII+210}}$ Following the usual convention in *D. subobscura* (Krimbas & Loukas, 1980), overlapping inversions are underlined and VIII+210 denotes the small (VIII) and long (210) X-ray-induced overlapping inversions, and 3+4 the naturally occurring ones located at the distal tip of chromosome O (the marker *Va* is outside the region covered by 3+4). Therefore, some recombination occurs between the balancer and those chromosome arrangements in the thermal stocks that bear 3+4; namely, $O_{\underline{3+4}}$, $O_{\underline{3+4}+2}$ and $O_{\underline{3+4}+7}$. Overall, these arrangements occur at approximately the same frequency in the thermal stocks (Santos *et al.*, 2005) and, hence, it seems unlikely that recombination could have been an important shortcoming of the present experiments. Recombination could reduce the selective differential between homozygotes and heterozygotes but would scarcely increase it (Sved & Ayala, 1970).

Estimation of net fitness

The procedure used to estimating total net fitness involves comparing the frequencies of lethal-carrying flies recorded at the zygotic stage in generation $t(H_t)$ with the frequencies of the same genotype at generation $t + 1(H_{t+1})$:

$$\hat{W}_t = \frac{2H_{t+1}(1-H_t)}{H_t(2-3H_{t+1})};$$

$$\mathrm{var}(\hat{W}_t) = \hat{W}_t^2\left(\frac{1}{N_tH_t(1-H_t)} + \frac{4(1-H_{t+1})}{N_{t+1}H_{t+1}(2-3H_{t+1})^2}\right),$$

(caret denotes 'an estimator of') where $N_t(N_{t+1})$ is the sample size (Anderson, 1969). As total fitness was estimated at all time intervals (generation $g_t \rightarrow g_{t+1}$)

from g_1 to g_{12} the final result in the cages was not obtained from one but for a number of generations and, therefore, fluctuations in selective values (either because selection was not constant, because of sampling errors, or both) is unlikely to be of critical importance here. As the information from each estimate of ($\hat{W}_t$) is the reciprocal of its variance, a weighted average net fitness was computed for each population as:

$$\tilde{W} = \frac{\sum_t \hat{W}_t/\mathrm{var}(\hat{W}_t)}{\sum_t 1/\mathrm{var}(\hat{W}_t)}.$$

Results

Starting with a maximum frequency of 50% for the lethal balancer, heterozygous net fitness was assessed for all thermal stocks at all three temperatures (amounting to 27 independent experimental populations in total) after 12 nonoverlapping generations of selection, when the frequency of the balancer dropped below 5% in most cases (Fig. 2a–c). At each generation the frequencies of wild-type and lethal-carrying genotypes were monitored at the zygotic stage (the harmonic mean of number of flies counted was 475.25; Appendix 1), and net fitnesses

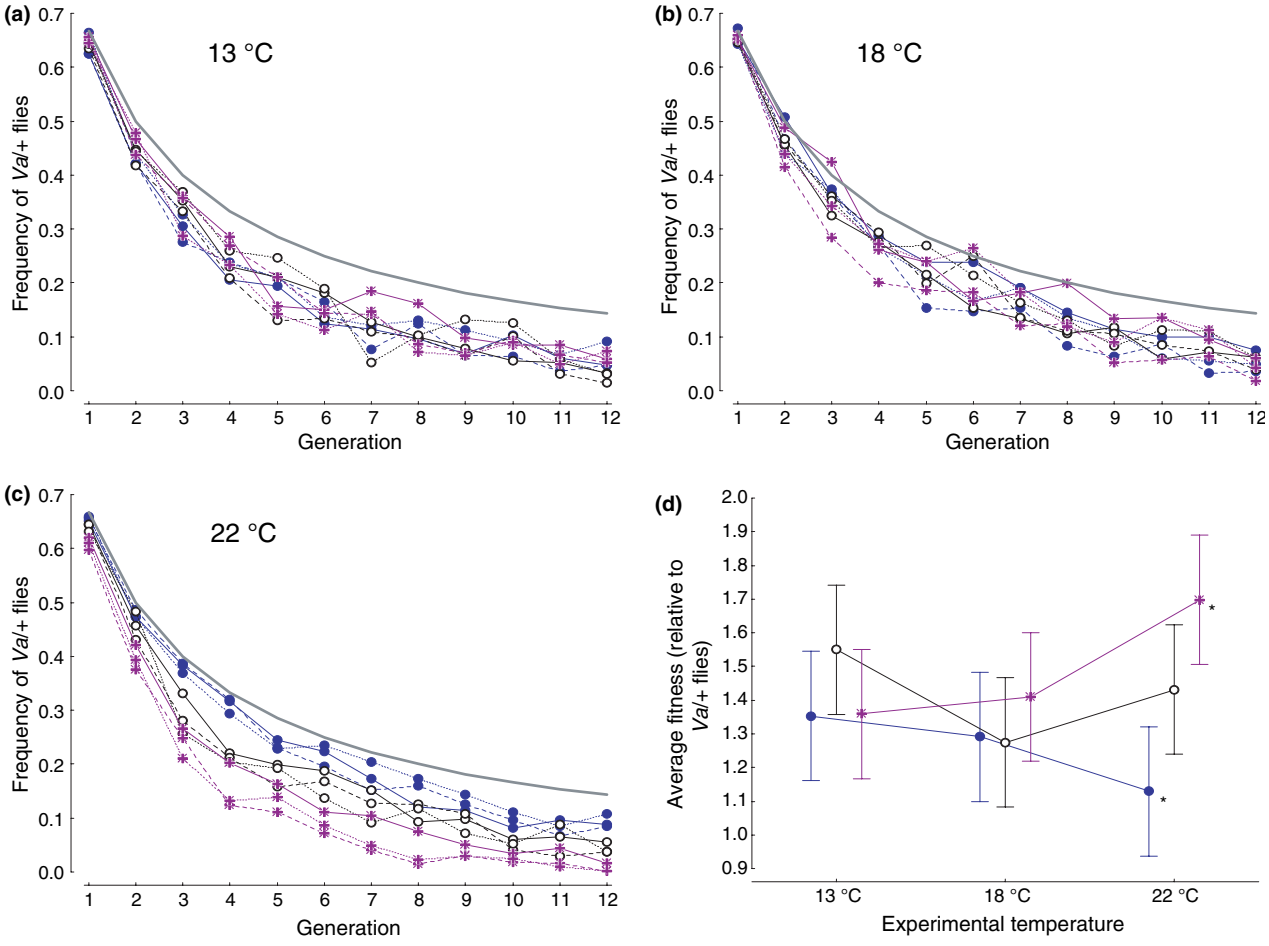

Fig. 2 Temperature-related net fitness evolution in thermal-adapted lines of *Drosophila subobscura*. Panels (a)–(c) plot the rate of decay over 12 nonoverlapping generations of *Va*/+ (*Varicose* is homozygous lethal and the dominant morphological marker of the balancer O chromosome) heterozygotes derived from 20 nonlethal O chromosomes sampled from each of the three-fold replicated cold- (P13 in blue and filled circles), optimum- (P18 in black and open circles) and warm-adapted (P22 in purple and crosses) populations (amounting to 95% of the standing genetic variation in each population for that chromosome) when transferred to different experimental temperatures. (a) Constant temperature of 13 °C; (b) 18 °C; (c) 22 °C. The thick grey lines indicate the expected rate of decay for a recessive lethal; the continuous lines the populations derived from replica 1 in the thermal stocks, the dashed lines from replica 2, and the dotted lines from replica 3. Generation 1 is the offspring of the founding populations, which were started with 100% *Va*/+ flies. Panel (d) plots the average net fitness (with 95% confidence intervals computed from the pooled variance; Levene's $F_{8,18} = 1.73$, $P > 0.05$) of wild-type homozygotes relative to *Va*/+ heterozygotes (a faster rate of decay for the lethal balancer in panels (a)–(c) obviously means a higher relative fitness of the wild-type genotype). Within each experimental temperature, asterisks indicate those averages that were statistically different as indicated in the text.

Appendix 1 Frequency of nonlethal (+/+) and lethal-carrying genotype (*Va*/+) estimated from samples of eggs taken at the beginning of each generation and raised under nearly optimal conditions. Experimental temperatures are 13, 18 and 22 °C respectively.

	Generation																							
	1		2		3		4		5		6		7		8		9		10		11		12	
Population	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+	+/+	*Va*/+
13 °C																								
P13R1	164	273	293	213	357	157	394	102	344	83	426	60	393	51	384	41	440	32	386	44	453	29	435	22
P13R2	178	297	255	186	330	126	394	123	419	111	413	82	423	35	430	61	426	30	413	28	515	19	475	24
P13R3	167	331	268	210	303	147	383	118	381	102	393	62	456	63	443	67	428	54	435	44	431	31	397	40
P18R1	175	320	264	214	350	190	416	124	368	98	390	86	423	62	461	51	420	36	414	24	496	27	497	17
P18R2	152	265	287	206	289	144	333	88	373	56	468	72	423	52	476	52	505	35	423	48	412	13	426	6
P18R3	160	288	277	221	290	169	398	140	378	124	373	87	403	22	439	50	434	66	391	56	410	26	495	16
P22R1	164	313	263	230	281	158	309	124	361	67	421	74	437	99	370	71	453	49	422	39	386	36	446	28
P22R2	163	310	303	236	286	159	379	140	390	104	435	73	463	77	467	44	435	33	418	43	418	30	435	24
P22R3	157	285	282	258	345	139	385	117	379	63	407	52	377	65	463	36	402	28	428	42	439	23	399	32
18 °C																								
P13R1	157	294	243	250	304	175	376	151	390	122	362	113	342	81	444	75	420	54	445	49	456	50	463	38
P13R2	171	350	242	212	343	195	331	123	384	70	371	64	358	65	442	40	397	27	398	38	424	14	492	18
P13R3	172	309	246	193	316	188	343	124	421	112	436	87	398	94	415	65	470	58	488	31	440	26	473	24
P18R1	175	320	249	208	290	139	322	123	386	106	377	68	430	66	460	55	393	52	478	30	405	31	411	27
P18R2	173	322	280	236	342	193	341	142	421	105	331	110	465	73	403	50	403	48	472	44	468	37	390	16
P18R3	146	275	253	222	307	167	340	123	321	118	395	107	422	82	442	66	438	40	432	55	387	48	446	29
P22R1	177	331	232	221	260	191	387	137	341	107	431	86	434	96	359	89	389	60	368	58	389	41	448	29
P22R2	162	305	300	212	354	140	342	86	414	95	437	98	441	61	423	60	404	22	435	26	520	35	457	8
P22R3	163	316	286	223	306	160	307	115	358	113	376	135	422	94	468	63	475	47	402	63	439	56	467	21
22 °C																								
P13R1	154	287	248	223	291	181	347	161	381	123	396	114	400	84	421	58	429	55	405	36	395	42	423	41
P13R2	155	296	252	238	289	182	349	164	385	115	376	92	358	64	425	81	408	59	438	47	469	34	472	44
P13R3	151	293	252	226	336	197	375	156	363	107	404	124	340	87	383	80	444	74	375	47	400	37	397	48
P18R1	162	293	287	241	330	164	377	107	338	84	434	100	404	72	455	47	425	46	436	28	472	33	483	28
P18R2	166	302	264	200	330	129	353	95	441	83	410	83	372	54	426	61	444	54	431	18	440	13	420	16
P18R3	185	318	268	250	333	115	395	101	401	96	413	66	419	42	428	57	467	36	400	22	490	47	514	20
P22R1	168	274	262	190	379	137	363	92	371	72	406	51	483	56	423	34	488	26	499	18	454	21	422	7
P22R2	200	296	299	180	347	114	374	53	392	49	468	36	487	21	448	7	412	13	497	9	419	7	474	1
P22R3	183	286	260	169	373	99	393	60	415	67	439	42	460	24	480	11	520	16	487	12	426	4	445	1

Generation 1 is the offspring of the founding populations, which were started with 100% *Va*/+ flies. The observed frequencies (close to expectation) were used to estimate fitness in the time interval $g_1 \rightarrow g_2$.

were estimated at all time intervals to obtain a weighted average net fitness for each experimental population (Fig. 2d).

A repeated-measures design ANOVA using the frequencies of the lethal-carrying genotype (transformed as arcsin $\sqrt{p}$) at each generation as the dependent variable, and two subject factors (past thermal regime and experimental temperature) as fixed effects, revealed different rates of decay of the balancer chromosome for the different thermal stocks according to experimental temperature (P × T interaction in Table 1), essentially due to the poor performance of cold-adapted (P13) populations ('cold-adapted O chromosomes' would be a more rigorous characterization) when tested at the highest temperature of 22 °C as compared with their warm-adapted (P22) counterparts (Scheffé *post hoc* test: $P < 0.05$). This translated into a huge loss in relative net fitness of 33% for the former, which is likely to be a minimum estimate as all chromosomes but the tested one had been homogenized (Fig. 1). On the other hand, the results indicated that P22 populations performed comparatively well at all experimental temperatures, although their fitness at 22 °C improved relative to that estimated at low temperatures (Fig. 2d). Finally, optimum (P18) populations ranged in between as an average but never significantly differed from P13 or P22 populations within each experimental temperature. Note, however, that Scheffé *post hoc* test comparing P18 vs. P22 at 22 °C (Fig. 2c) is marginally nonsignificant ($P = 0.085$). In sum, it seems that thermal fitness breath for *D. subobscura* flies is positively related to temperature.

Discussion

If the O chromosome is representative of all chromosomes in *D. subobscura* – which seems to be the case when

Table 1 Repeated-measures ANOVA using the frequencies of the lethal-carrying genotype (transformed as arcsin $\sqrt{p}$) at each generation as the dependent variable, and past thermal regime (P13, P18 and P22) and experimental temperature (13, 18 and 22 °C) as fixed effects (data plotted in Fig. 2a–c).

Source	d.f.	SS	MS	*F*	*P*
Between populations	26	2355.49			
Past thermal regime (P)	2	388.71	194.36	6.95	0.0058
Experimental temperature (T)	2	362.45	181.23	6.48	0.0076
P × T	4	1101.31	275.33	9.85	0.0002
Error*	18	503.02	27.95		
Within populations	297	46566.47			
Generations (G)	11	45368.87	4124.44	1284.07	< 0.0001
G × P	22	125.22	5.69	1.77	0.0217
G × T	22	131.59	5.98	1.86	0.0138
G × P × T	44	304.81	6.93	2.16	0.0002
Error†	198	635.98	3.21		

*Error term For P, T and P × T.
†Error term for G, G × P, G × T and G × P × T.

considering the chromosome distribution of candidate genes for thermal adaptation in this species obtained after contrasting the gene expression profiles of the two furthest apart thermal selection regimes (i.e. 13 °C vs. 22 °C; Laayouni *et al.*, 2007) – the present results show that thermal-induced relative net fitness evolution can have dramatic consequences when cold-adapted populations of *D. subobscura* are relocated to warmer conditions. Conversely, net fitness of warm-adapted populations remains about the same at cold and optimum temperatures relative to the other thermal lines, but their fitness improved at their own temperature. Hence, even in the simple and temporally stable thermal environments that these flies have experienced the patterns of temperature-related net fitness evolution are relatively complex. The thermal dependence of total net fitness has not been investigated in previous laboratory natural selection studies using *Drosophila* and, therefore, no generalizations can be made. However, we have already reported some inconsistent results for body size and preadult life-history traits and have suggested possible reasons for why conflicting patterns have arisen with earlier experiments in this taxon (Santos *et al.*, 2005, 2006).

The scheme employed to maintain the thermal lines used as base stocks to derive the O chromosomes entails identical treatment of all populations. However, the 9 °C range between cold- and warm-adapted lines obviously imposes a difference in their demographic regimes: *c.* 46 days generation cycle of P13 populations against the *c.* 25 days generation cycle of P22. Although the number of breeding adults per thermal population is typically well over 1500 flies, the degree of inbreeding might be expected to be higher in P22 populations as 59 generations have elapsed in these lines in comparison with the 32 generations in P13 at the time the present experiments started. But the finding that the O chromosomes derived from P22 populations performed better as an average than the O chromosomes derived from P13 populations suggests immediately that thermal adaptation, rather than differences in demography, has cause the huge loss of relative net fitness for the latter at warm temperature.

Besides the obvious limitations of experimental selection studies in that they cannot duplicate the complexity and diversity of natural habitats along a latitudinal gradient, some caveats have to be added here before discussing the possible implications of the results. Thus, in a strict sense there is no control available to estimate total net fitness in the present experiments. The method could be extended by competing wild-type chromosomes against a pair of balancers and the heterozygous genotype containing the two balancers would act as a yardstick against which the fitness of the wild-type homozygotes could be assessed, but problems arise if the balancers differ markedly in fitness (Barton & Partridge, 2000). In any case, this approach is impossible with *D. subobscura* as there is only one balancer chromosome available: the chromosome with the *Ba* gene in the *Va/Ba* balancer stock does not carry X-ray induced inversions (Sperlich *et al.*, 1977). A further potential problem is that eggs were sampled from the population cages over a relatively short time window after females reached their peak of fecundity. The sampling schedule could, therefore, have biased the fitness estimates if death rates between cold- and warm-adapted flies were different. With all these caveats in mind, what can we conjecture from the present data? The key point to be made below is that some testable predictions arise after speculating about the direct and indirect consequences of climate change on *D. subobscura* populations. I will first shortly review the population biology of the species and summarize several sets of empirical evidence. Finally, I will dare to challenge some recent claims suggesting that the exceptionally fast world-wide clinal genetic shifts in response to increasing temperature (Rodríguez-Trelles & Rodríguez, 1998; Solé *et al.*, 2002; Balanyá *et al.*, 2004, 2006) are mainly due to local adaptation, and propose instead that (empirically testable) range shifts of more equatorial populations towards higher latitudes have already occurred and might partially explain the current patterns.

The native Palearctic fly *D. subobscura* spans more than 30° latitude in the Old World: from North Africa to Scandinavia – where it has recently expanded some 500 km to the north (Saura, 1995). As a result, its populations experience a strong climatic gradient (Krimbas, 1993). In the late 1970s and early 1980s the species invasively spread in, respectively, South and North America and nowadays spans about 15° latitude on each continent (Prevosti *et al.*, 1988; Huey *et al.*, 2005). Latitudinal clines in the frequency of many chromosomal

inversions were well documented in the original Palearctic populations (Krimbas & Loukas, 1980), and the discovery of parallel clinal patterns a few years after the American invasion (Balanyà *et al.*, 2003) provided compelling evidence that the clines evolved by natural selection. Many environmental factors change with latitude, but a reasonable first guess for the selective agent at least partially responsible for the clines obviously points to temperature. Several recent studies strongly suggest that this is indeed the case: clinal shifts in the frequency of chromosomal inversions correlated with current climate change have been detected on three independent continents (Balanyá *et al.*, 2006). These shifts provide fascinating data and have been interpreted as ongoing adaptation to global warming trends, but in their paper Balanyá *et al.* (2006) were cautious enough and admitted that they cannot tell whether the shifts at the different populations represent local selection or invasion from more equatorial populations. This latter alternative is not at all unreasonable because we already know that gene flow in this species is high and no genetic differentiation between European populations has been detected using microsatellite markers (Pascual *et al.*, 2001), thus reinforcing the notion that the clinal patterns in inversion frequencies are because of a balance between gene flow and selection. How to distinguish between local adaptation and migration as the two potential causes underlying the shifts in the genetic composition of *D. subobscura* populations worldwide?

Drosophila subobscura certainly has high adaptive potential and genetically responds to thermal changes and latitudinal gradients after a few generations (Prevosti *et al.*, 1988; Gilchrist *et al.*, 2001, 2004; Santos *et al.*, 2005; Laayouni *et al.*, 2007), which favours the view that rapid local adaptation in response to climate warming is indeed possible. However, the present data suggest that the relative net fitness of warm-adapted populations remains about the same at low temperatures than that of their cold-adapted counterparts but substantially increases at higher temperatures. Together with recent analyses showing that an insect's maximum rate of population growth is also enhanced at warmer temperatures (Frazier *et al.*, 2006), both pieces of evidence are consistent with the notion that range shifts of more equatorial populations towards higher latitudes (probably due to demographical asymmetries correlated with climate change) could easily result in local (high latitude) populations being competitively displaced prior to their selective response to climate warming.

Native Palearctic populations offer a wonderful (and probably unique) scenario to test, at least qualitatively, whether the clinal shifts in chromosomal inversion frequencies mainly represent local selection or invasion from further south. Thus, many chromosomal arrangements (recall that in the original Palearctic region approximately 92 chromosomal arrangements – produced from 66 inversions – have been recorded; Krimbas, 1992) were known to be restricted to northern Africa or southern Europe in historical samples (Krimbas & Loukas, 1980; Menozzi & Krimbas, 1992). If invasion from 'warm-climate' populations is at least partially responsible for the clinal shifts, an easily testable prediction comes up: 'private' (Neel, 1973) chromosome inversions formerly restricted to southern Palearctic populations should now be scored northwards at relatively high frequencies. If this happens, proof of migration would be unquestionable because overwhelming evidence favours the traditional view that chromosomal inversions in *Drosophila* are monophyletic (Powell, 1997).

Some data clearly indicate that various 'southern' chromosomal arrangements are currently found at high latitudes where they were not present before (L. Serra & J. Balanyà, personal communication). In any case, even if migration is partly or mostly responsible for the latitudinal shifts the conclusion that *D. subobscura* polymorphic inversions are good indicators of climate change still remains.

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