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Heat and cold-induced male sterility in *Drosophila buzzatii*: genetic variation among populations for the duration of sterility

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Here we studied three phenotypic traits in *Drosophila buzzatii* that are strongly effected by temperature, and are expected to be closely associated with fitness in nature. The traits measured were thermal threshold of male sterility, time for males to gain fertility when reared at a sterility-inducing temperature and transferred to 25°C on eclosion and survival after development. The last two traits were measured under four temperature regimes, constant 12°C, 25°C, 31°C, and fluctuating 25°C (18 h) and 38°C (6 h). We looked for genetic variation in these traits and relations among them in four lines of *D. buzzatii* originating from Argentina and Tenerife. The thermal threshold of heat-induced male sterility was found to lie within the range of 30.0–31.0°C. When measuring the time for males to gain fertility, males reared at a nonstressful temperature (25°C) were fertile 58–67 h after emergence with only minor differences among lines. When reared constant 31°C, males were fertile 174–225 h after hatching. The Argentinean lines were significantly faster in recovering from sterility than were the lines from Tenerife. When reared in a fluctuating temperature regime, differences among lines increased, dividing the lines into three significantly different groups, with a sterility period of 135–215 h. When reared at 12°C from the pupal stage, males were fertile after 106–130 h with significant difference in the variance but not in the mean duration of sterility. Significant differences in viability were found among development temperatures, but not among lines, and viability and the duration of sterility seem to be genetically independent.
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Introduction

Temperature plays an important role in the life history of insects and has a significant influence on fitness. An example of this is male fitness, as thermal-induced male sterility seems to occur in most *Drosophila* species studied (Jean David, personal communication). When kept at a constant temperature, *Drosophila melanogaster* can complete development, from egg to adult, only at temperatures between 11°C and 32°C (Chakir *et al*, 2002), and males are fertile only when reared at temperatures between 13°C and 29°C. At temperatures just above and below this interval, males become sterile (eg if reared at constant 12°C (Cohet, 1973) or 30°C (David *et al*, 1971).

The narrow temperature interval, which constitutes the limit between fertility and sterility, is fixed at a specific temperature in *D. melanogaster* and *D. simulans* (David *et al*, 1983; Chakir *et al*, 2002). Although this borderline temperature may vary between species (Chakir *et al*, 2002) and lines within species (Kuznetsova, 1994), attempts to raise the temperature limit with artificial selection have been without success (Chakir *et al*, 2002). Although males will remain sterile if kept at a sterility-inducing temperature, a return to 25°C restores fertility within days (David *et al*, 1983). This means that males reared at temperatures above the sterility threshold will gain fertility several days later than flies not stressed in this way. Given the average life span of wild *Drosophila* being estimated to be from a few days to a few weeks (Rosewell and Shorrocks, 1987; Turelli and Hoffmann, 1995), a delay of a few days in sexual maturity should have profound effects on male fitness. Age at sexual maturity is a principal life history trait (Stearns, 1992), that is a trait which directly influences survival and reproduction. In this context, the thermal threshold for inducing male sterility seems an obvious target for selection, and it could be a trait with great importance for fitness in nature. A higher value of the threshold would give a reduced sensitivity to sterilising temperatures, and consequently a longer proportion of fertile life span in thermal stressful environments.

In spite of this, the thermal threshold in *D. melanogaster* and *D. simulans* seems narrow and lies within an interval of 2°C at the lower temperature and within only 1°C at the high temperature (Chakir *et al*, 2002). However, the duration of the sterile period does not seem to be fixed at a specific length. Instead, the sterility period seems plastic, meaning that an increase in the temperature above the thermal threshold for sterility is associated

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with a prolonged duration of the sterility period (Chakir *et al*, 2002).

In this paper, we suggest the hypothesis that natural populations may be genetically variable for the duration of the sterility period. Ample genetic variation exists among populations in the degree to which specific temperatures are experienced as stressful, indicated by differences in for example Hsp70 expression and survival after heat shock (Krebs and Loeschcke, 1996; Sørensen *et al*, 2001). The association between the stressfulness of the environment and the duration of the sterility period thus potentially implies that natural populations may be genetically variable for the latter.

The aim of this study was to look for genetic variation in the duration of temperature sterility and to compare this to other resistance traits and the thermal history of the lines used in the study. We examined the sterility period after development at 12°C, 25°C, 31°C or at a fluctuating temperature regime (25°C for 18 h and 38°C for 6 h) in four different lines of *D. buzzatii* originating from different thermal environments, and previously shown to differ in heat-shock resistance, egg laying activity and knockdown resistance (Sørensen *et al*, 1999, 2001; Dahlgaard *et al*, 2001). Two of the lines had experienced high temperatures, one in the natural environment where from it originated (a lowland site in Argentina), and the other in the laboratory where it had completed larval development at a high fluctuating temperature (38°C (6 h)/25°C (18 h)). Two corresponding lines came from comparatively low temperature regimes (without daily high temperatures), one from the Argentinean highlands and one kept at 25°C in the laboratory. Since longer duration of sterility increases the risk of males dying before gaining fertility, the line exposed to high temperatures in nature could be expected to have evolved towards a shorter heat-induced sterility period than the corresponding low temperature line. The line selected in a high temperature environment in the laboratory was expected to have the same heat-induced sterility period as the corresponding low temperature line, as the former was only exposed to the high thermal regime until the time of pupation and then allowed 10 days at 25°C between generations to restore fertility. These lines served as a control of whether larval selection influenced the sterility period. Only the Argentinean populations were used in the 12°C experiment since these have experienced a wide range of temperatures in the wild, from extreme cold to extreme warm temperatures. In contrast, the two lines from Tenerife originally were sampled in the same environment and have never been exposed to cold temperatures during maintenance. Following the same argument as given above, we expected the highland line to have the shortest duration of cold-induced sterility.

To compliment the testing for genetic variation in the duration of thermal-induced sterility, we characterised the temperature threshold for male sterility in *D. buzzatii*, as has been done for *D. melanogaster* at 30°C (David *et al*, 1971) and *D. simulans* at 28°C (Chakir *et al*, 2002). Finally, in order to measure the stressfulness of the temperature treatments administered in this study, larval to adult survival was measured at the four experimental temperatures, and compared to the lines' duration of thermal-induced sterility. We expected low larva to adult survival in the environments where the lines had a long duration of sterility.

Materials and methods

Origin of lines

Four lines were used, two originating from Argentina and two from the island of Tenerife, Spain off the North African west coast. The Argentinean lines were collected at two different localities near to Tilcara and Catamarca. Tilcara lies in a highland area (23°35′S, 65°24′W; 2460 m elevation) with monthly average maximum temperatures in the range 23–24°C and monthly average minimum temperatures from 11–12°C during summer and autumn (averaged from data obtained from two close by weather stations). Catamarca is located in the lowland (28°29′S, 65°39′W; 590 m elevation) with monthly average maximum temperatures in the range 30–35°C and monthly average minimum temperatures from 20–21°C during summer and autumn, that is experiencing higher overall temperatures than Tilcara (Dahlgaard *et al*, 2001; Sørensen *et al*, 2001). The line from Tilcara is here named Til(L) for low temperature and the line from Catamarca, Cat(H) for high temperature. These lines had been kept in the laboratory for approximately 30 generations under standard laboratory conditions at 25°C and a 12 h light/dark cycle, but still differed in heat knockdown response at the time of the experiment (Sørensen and Loeschcke, 2002). The two lines originating from a single collection from Tenerife had spent about 100 generations in the lab. Here, one line had been kept at constant 25°C and a 12 h light/dark cycle and is named Ten(L). The other line, Ten(H), had experienced a fluctuating temperature regime in the laboratory, with 25°C from the egg stage until day two and thereafter it was kept at fluctuating temperatures: 25°C for 18 h and 38°C for 6 h, with a 18/6 h dark/light cycle, until the beginning of pupation, where it was returned to constant 25°C for pupal to adult development, mating and egg laying.

Instant *Drosophila* medium (Carolina Biological Supply) with a little live dry yeast added was used for the entire experiment, unless stated otherwise. All collections were performed using light CO_2 anaesthesia.

Temperature threshold for heat-induced male sterility

Virgin males and virgin females from the four lines were collected and held on a sugar–agar–yeast medium at 25°C, separated by sex. At 6 days after eclosion, that is at maturation, males and females were joined in vials with instant medium. These were immediately placed in water-baths at 30.0°C, 30.5°C or 31.0°C in order to control the temperature during the entire development from fertilisation to imago. The flies were allowed to lay eggs at a density of four pairs per vial with five vials per population per temperature treatment for 24 h, after which the flies were removed. As progeny developed in all the vials, this was testing that all aspects of reproduction, except for gametogenesis, could be carried out at the study temperatures. After completing development from the fertilised egg to imago in the water-baths, males were collected and placed with 6-day old virgin females raised at 25°C, in fresh vials that were placed at the respective temperatures. This procedure ensured that it was only male fertility and not some other

aspect of reproduction (eg courtship) that was tested in this experiment, we therefore assigned lack of offspring production to failure in spermatogenesis. For each combination of temperature and line, we had five vials with 10 males and 10 females. These flies were transferred to new vials every third day and kept in the water-baths for a minimum of 12 days after the males eclosed. After flies had been transferred, vials were stored at 25°C for evaluation for offspring after at least 7 days.

Duration of heat-induced male sterility

Three experimental temperatures were used: 25°C, 31°C and a fluctuating regime with 38°C (6 h)/25°C (18 h). The temperature 31°C is just above the limit for male fertility in *D. buzzatii* and the cycling regime is an attempt to reflect natural temperature conditions (Dahlgaard and Loeschcke, 1997). For each combination of line and temperature treatment, bottles with 20 pairs (approximately 7 days old) were set up at 25°C for egg laying. The flies were transferred to new bottles after 24 h to obtain enough flies for the experimental set up. This was done three or four times to assure that an adequate number of flies emerged at approximately the same time, regardless of development temperature. Bottles were placed in the appropriate temperature chambers 2 days after the beginning of oviposition, where they remained until emergence of adults. To prevent desiccation of the vials at the high temperatures, a tray with water was placed in the bottom of the incubators and stoppers were wetted every second day.

After hatching, males (on average 6 h old) were placed in vials with 6-day-old virgin females from their respective line that had been raised at 25°C. For each combination of line and treatment, 13 replicate vials with four males and four females were placed at 25°C. Four males per vial were used to exclude any effect of random permanent sterility, which had been observed in earlier experiments at 25°C where 7.5% of the males failed to become fertile. Every 12 h, the flies were transferred to new vials. Time until sexual maturity was reached, for at least one of the four 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. Vials were allowed a minimum of 7 days at 25°C before they were evaluated for the presence of pupae, larvae or adults. This measure of heat sterility includes the period where males naturally are sexually immature.

Two-way ANOVA tested for line and treatment interaction on duration of temperature-induced sterility (using SPSS 10.0). To test for effect of lines and treatment, one-way ANOVA and subsequently Tukey test (SPSS 10.0), where appropriate, were performed.

Duration of cold-induced male sterility

Only the two Argentinean lines were used in this experiment, as Til(L) was the only population that regularly had experienced 12°C in its original environment. To test for variation in the duration of cold-induced sterility, sterility was induced at 12°C with a control group at 25°C. Procedures were similar to those for duration of heat male sterility, except that larval development was completed at 25°C and pupae were collected when on average 12 h old and placed at 12°C until adults emerged. To account for much higher mortality among eclosed males reared at 12°C than at the higher temperatures, a minimum of 40 vials with four males and four virgin females was set up for each line. Vials were combined as males died to ensure that vials always contained between two and four males. Differences between time to fertility due to development temperatures (ie 12 and 25°C) were analysed by Welch's approximate t-test (Sokal and Rohlf, 1995), as were differences between the two lines at 12°C. Differences in variance where tested using Levene's test (SPSS 10.0).

Viability

Four experimental temperatures were used: 12°C, 25°C, 31°C and a fluctuating regime with 38°C (6 h)/25°C (18 h). The effect of temperature was determined on preadult viability, the proportion of first instar larvae reaching adult stage, for each line under all temperatures. For all lines, first instar larvae from 25°C were collected, with 45 larvae per vial into 10 vials per combination of line and treatment. These were placed at the respective temperature regimes and eclosion percentage was monitored. To prevent desiccation of the vials at the high temperatures, a tray with water was placed in the bottom of the incubators and stoppers were wetted every second day.

The data were recorded as percentages and arcsine transformed prior to a two-way ANOVA, comparing survival between lines and development temperatures. Subsequently a Tukey test (SPSS 10.0) was performed where appropriate based on results from one-way ANOVA.

Results

Temperature threshold for heat male sterility

At 30.0°C all vials from all lines produced offspring, at 30.5°C only the lines Til(L) and Cat(H) produced a fertile vial each, and at 31.0°C no lines showed any fertile vials, here all males were permanently sterile. These observations therefore establish that the threshold temperature for inducing sterility in male *D. buzzatii* lies between 30.0°C and 31.0°C.

Duration of heat-induced male sterility

The results for the duration of heat-induced male sterility are presented as the cumulative percentages of vials where offspring could be detected (Figure 1), and as mean duration of sterility in Table 1. It is evident that under heat-induced sterility, some line-treatment combinations never reached 100% fertility (Figure 1b, c). The results show significant interaction between temperature and line (two-way ANOVA, $P<0.001$).

A temperature of 25°C is nonstressful for *D. buzzatii* (Figure 1a), and the results show that the duration of sexual maturation in *D. buzzatii* males at 25°C is 62.5 h (calculated from Table 1), with a significant difference only among Ten(L) and Cat(H) in mean duration of the sterile period, Tukey test $P<0.05$ (Table 1).

After development at 31°C, the average duration of sterility for all lines is longer than at 25°C (Tukey test, $P<0.05$, Table 1). The mean values for this period vary between lines (one-way ANOVA, $P<0.001$). The Spanish lines (Ten(L) and Ten(H)) remain sterile for about 2 days

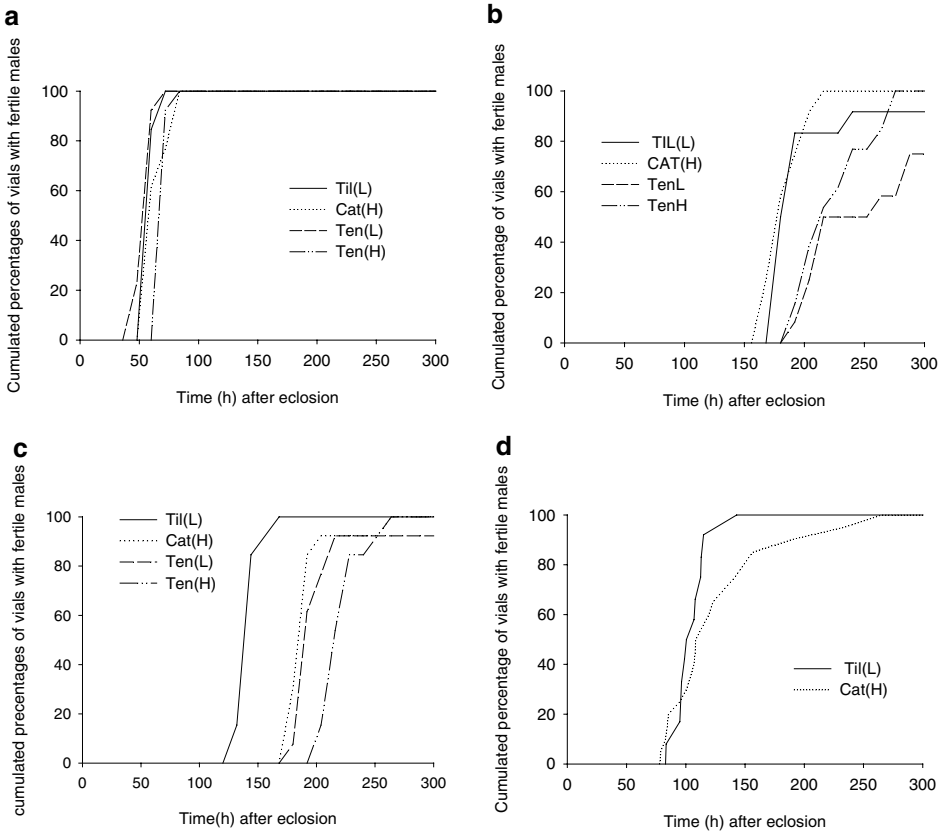

Figure 1 Cumulated percentage of vials with at least one fertile male as a function of time after eclosion. (**a**) Males were reared at 25°C from egg to adult. (**b**) Males were reared at 31°C from first instar larvae to adult. (**c**) Males were reared at fluctuating temperatures with 25°C for 6 h and 38°C for 18 h, from first instar larvae to adult. (**d**) Males were reared at 12°C from pupae to adult.

Table 1 Mean duration of male sterility for males reared at different temperatures and transferred to 25°C after eclosion (h ± SD (sample size))

Line	*Temperature treatment*			
	12°C	*25°C*	*31°C*	*25/38°C*
Ten(L)		58 ± 7 (13)$^{a;1}$	225 ± 47 (9)$^{b;3}$	187 ± 12 (12)$^{b;2}$
Ten(H)		63 ± 7 (13) $^{a,b;1}$	217 ± 34 (13)$^{b;2}$	215 ± 24 (13)$^{c;2}$
Til(L)	106 ± 15 (12)^{x}	62 ± 5 (13)$^{a,b;1}$	178 ± 18 (11)$^{a;3}$	135 ± 13 (13)$^{a;2}$
Cat(H)	130 ± 50 (20)^{x}	67 ± 10 (13)$^{b;1}$	174 ± 16 (12)$^{a;2}$	183 ± 22 (13)$^{b;2}$
Mean of lines	121 ± 42 (32)	62 ± 8 (52)	197 ± 37 (45)	179 ± 34 (51)

Line mean sterility periods were significantly different, within temperatures 25°C, 31°C and 25°C/38°C, (one-way ANOVA). Means significantly different when tested by Tukey test ($\alpha = 0.05$) are indicated by letters (a,b and c). Within all lines, mean sterility periods were significantly different between temperatures 25°C, 31°C and 25°C/38°C (one-way ANOVA). Means significantly different when tested by Tukey test ($\alpha = 0.05$) are indicated by numbers (1,2 and 3). Mean sterility period at 12°C was significantly different from the parallel processed control (data not shown; Welch's approximate *t*-test; $P < 0.001$). At 12°C, line mean sterility periods were not significantly different as indicated by the letter x (Welch's approximate *t*-test; $P > 0.05$).

longer than the Argentinean lines Til(L) and Cat(H) (Tukey test $P < 0.05$, Table 1). At this temperature, offspring were detected in only 90% of the vials from Til(L) and 75% of the vials from Ten(L) (Figure 1b).

The results from the 25°C/38°C treatment are presented in Figure 1c. For this treatment also the average duration of heat-induced sterility is significantly longer than at 25°C (Tukey test *P*, < 0.05, Table 1), and within the treatment, mean values for the lines are different (one-way ANOVA, $P < 0.001$). There was a larger difference between the four lines with 3 days between the first and last line to reach fertility. Til(L) being first, Cat(H) and Ten(L) intermediate and Ten(H) last (Tukey test $P < 0.05$ Table 1). Under this thermal regime, only Ten(L) never reached 100% fertility.

Cold-induced male sterility

At 12°C only the Argentinean lines were used. After development from pupae to imago, males also remained infertile for a significantly longer time than after development at 25°C (data not shown; Welch's approximate

Table 2 Preadult viability expressed as hatching percentage ± SD (sample size)

Line	*Temperature treatment*			
	12°C	*25°C*	*31°C*	*25°C / 38°C*
Ten(L)	0 (10)	50 ± 11 (9)[2]	55 ± 11 (11)[2]	3 ± 2 (10)[1]
Ten(H)	0 (10)	63 ± 15 (9)[2]	60 ± 9 (10)[2]	1 ± 1(10)[1]
Til(L)	0 (10)	52 ± 17 (10)[2]	44 ± 14 (10)[2]	3 ± 1(10)[1]
Cat(H)	0 (10)	57 ± 15 (10)[2]	54 ± 15 (10)[2]	1 ± 1(10)[1]

Viability did not differ between lines (two-way ANOVA, $P = 0.13$). Within lines, viability was significantly different (one-way ANOVA). Means significantly different when tested by Tukey test ($\alpha = 0.05$) are indicated by numbers (1 and 2).

t-test, $P < 0.001$). At 12°C, Til(L) had a lower mean duration of sterile period than the line Cat(H), although not significantly (Welch's approximate *t*-test, $P > 0.05$, Table 1, Figure 1d). Variances for Cat(H) and Til(L) were 2510 and 221, respectively, and significantly different (Levene's test, $P = 0.009$). Additionally, high mortality among adult males was observed, with Cat(H) having the highest mortality (data not shown).

Viability

The hatching percentages are shown in Table 2. Flies never hatched at 12°C. The reason for this was probably that first instar larvae, rather than pupae, as in the sterility set-up, were placed at 12°C, and this temperature is too low for the flies to complete development from the first instar to imago. No interaction between lines and temperature was detected (two-way ANOVA, $P = 0.28$), and there were no differences between lines (two-way ANOVA, $P = 0.13$) in viability. The treatment 25°C/38°C had a marked effect on viability for all populations (Tukey test, $P < 0.05$, Table 2), resulting in a significant difference between temperature treatments (two-way ANOVA, $P < 0.001$).

Discussion

The results show that *D. buzzatii* has a temperature threshold between 30.0°C and 31.0°C for heat-induced male sterility. This is higher than the threshold for heat-induced male sterility found in both *D. melanogaster* and *D. simulans* (David *et al*, 1971; Chakir *et al*, 2002), and this may contribute to *D. buzzatii's* ability to inhabit warmer habitats than the two other species.

The results also show that there is substantial genetic variation for the duration of sterility among populations of *D. buzzatii.* In addition, we have confirmed that the developmental thermal environment affects the duration of the heat-induced male sterility. The results demonstrate that both environmental and genetic factors (ie temperature and line) influence duration of sterility in males of *D. buzzatii* exposed to high temperatures during development. The shortest duration of male maturation was observed at 25°C, which is not surprising, as this is considered a nonstressful condition. This time is probably close to the fastest sexual maturation experienced by male *D. buzzatii* in nature. Both the developmental period and the time from hatching to fertility at nonstressful temperatures is longer in *D. buzzatii* compared to both *D. simulans* and *D. melanogaster*. Both may be influenced by a lower metabolic rate (Hoffmann and Parsons, 1989), and/or larger body size of *D. buzzatii.*

After development at 31°C, the difference among lines in time to fertility was increased, and the pattern changed from that observed at 25°C. At this temperature, the Argentinean lines (Til(L) and Cat(H)) were faster than the Tenerifean lines Ten(L) and Ten(H) resulting in a significant interaction term in the two-way ANOVA, so the rise in development temperature did not just exaggerate differences already present at 25°C.

When males were reared under the fluctuating temperature regime, differences among lines increased even further. The high temperature lines (Cat(H) and Ten(H)) had a similar sterility period as after development at constant 31°C, but the low temperature lines (Til(L) and Ten(L)) gained fertility sooner after development at 25°C(18 h)/38°C(6 h) than at 31°C. This was not expected, as longer duration of heat-induced sterility increases the risk of males dying before being able to mate. Since Cat(H) has been exposed to high temperatures in nature, we expected Cat(H) to be selected for a shorter sterility period at high temperatures than Til(L), which originates from a habitat with comparably lower temperatures. Regarding the laboratory lines, the high temperature line, Ten(H), again showed a longer sterility period than the corresponding low temperature line, Ten(L). However, in this case no difference was expected, as Ten(H) had been allowed 10 days at 25°C, each generation, to complete the pupal stage and gain fertility before mating. In summary, these results raise questions as to why lines selected at high temperature regimes had longer sterility periods than the corresponding low temperature lines at the fluctuating temperature regime, and emphasises the importance of future studies of heat-induced sterility in nature.

At 12°C, Til(L) was expected to have shorter sterility period than Cat(H), as Til(L) had experienced low temperatures in nature, and as longer duration of sterility increases the risk of males dying before being able to mate. A difference in mean time to male maturation after pupal development at 12°C was not found between Cat(H) and Til(L), but this result may be due to lack of power in the analysis. The numerical difference was in the direction expected (Table 1), with Til(L) having a shorter sterility period. As seen in Figure 1d, Til(L) has a significantly lower variance than Cat(H), due to near absence of late matured males. This could be a result of directional selection of Til(L) towards lower sterility period after cold-induced male sterility.

Chakir *et al* (2002) found a trade-off between low and high temperature tolerance with respect to the duration of male sterility, in that *D. melanogaster* was both more resistant to heat and less resistant to cold than *D. simulans.* In contrast, we found that Til(L) in some environments had shorter duration of heat-induced sterility than Cat(H), but no tendency towards lower resistance against cold-induced sterility. This means that a pattern seen between species is not necessarily repeated between lines within species (for discussion see Hoffmann *et al*, 2003). As the duration of sterility in *D. buzzatii* was measured using four males per vial instead of one male per vial as in Chakir *et al* (2002), we cannot, however, directly compare the duration of sterility in the three species.

In terms of viability, 12°C seemed to be the harshest treatment compared to 31°C and 25°C/38°C, as no first instar larvae reached adulthood after development at 12°C. Further, in the sterility experiment, pupal development at 12°C caused severe adult mortality, especially in the Cat(H) line (data not shown). In contrast, the sterility period after development at 31°C or 25°/38°C seemed longer than the sterility induced by pupal development at 12°C, and there was negligible mortality among adult males after development at 31°C or 25°C/38°C. In addition, the fluctuating temperature was more stressful than constant 31°C when measured in terms of viability, but the opposite when measured in terms of time to fertility (Tables 1 and 2). This suggests that these traits, viability and time to fertility, are genetically independent in *D. buzzatii*.

The mechanism behind the temperature sterility is not known, neither for heat nor cold. It is only known that spermatids do not develop into spermatozoa. The narrow interval indicates that the mechanism may be biochemically based. This could mean that the determining mechanism that causes sterility is simple like the denaturation of a protein as proposed by Cohet and David (1978). The cell defends itself from protein denaturation by expressing molecular chaperones called heat-shock proteins (Hsps). These bind to the unfolded protein and help it refold or direct it to degradation, thus preventing large aggregations of denatured protein in the cell (Parsell and Lindquist, 1993). Not very much is known about the role of Hsp in temperature-induced male sterility. The expression of Hsp is tissue specific, and there is no expression in sperm cells, but in testes Hsp70 is expressed (Michaud *et al*, 1997). Since Hsp90 is involved in spermatogenesis (Yue *et al*, 1999), and heat-adapted populations have been shown to downregulate Hsp70 production (Sørensen *et al*, 2001), the variation in sterility period could be correlated to Hsp expression, a question that is currently being addressed in our laboratory.

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