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Heat induced male sterility in *Drosophila melanogaster*: adaptive genetic variations among geographic populations and role of the Y chromosome

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Summary

We analyzed genetic variation among geographically diverse populations of *Drosophila* and showed that tropical flies are more tolerant than temperate ones to heat-induced male sterility, as assessed by the presence of both motile sperm and progeny production. In tropical populations, the temperature inducing 50% sterility (median threshold) is 1°C above the value for temperate populations (30.4 *vs.* 29.4°C). When transferred to a mild permissive temperature (21°C), males recover fertility. Recovery time is proportional to pre-adult culture temperature. At these temperatures, recovery time is greater for temperate than for tropical populations. Crosses between a temperate and a tropical strain (F_1, F_2 and successive backcrosses) revealed that the Y chromosome was responsible for much of the geographic variation. Sterile males exhibited diverse abnormalities in the shape and position of sperm nuclei. However, impairment of the spermatid elongation seems to be the major factor responsible for sperm inviability. Heat-induced male sterility seems to be quite a general phenomenon in Drosophilid species and variation of threshold temperatures may be important for explaining their geographic distributions.

Key words: heat stress, geographic race, heat tolerance, spermatogenesis, climatic adaptation, *Drosophila.*

Introduction

Temperature is a critical determinant of the distribution and abundance of ectotherms (Andrewartha and Birch, 1954, 1960), affecting most physiological functions, and numerous mechanisms compensating for temperature variation are known (Cossins and Bowler, 1987; Precht et al., 1973; Leather et al., 1993). Recently, the emphasis has been on investigating extreme conditions and tolerance of cold or heat stress (Hoffmann and Parsons, 1991, 1997; Zatsepina et al., 2001; Hoffmann et al., 2003).

The drosophilid family, with diverse species living under different climatic regimes, has been a superb model for ecological, ecophysiological, genetical and evolutionary analyses, especially because several hundred species tolerate laboratory culture and are amenable to experimentation. Here we focus on a poorly investigated trait, the induction of male sterility by chronic exposure to high temperature, and its genetic variability in *Drosophila melanogaster*. Most strains are continuously fertile at a 29°C but not at 30°C (Parsons, 1973). David et al. (1971) established that this upper limit was due to the sterilization of males, and that sterile males could recover fertility after a few days at a lower, non-stressful temperature. This phenomenon, although mentioned in reviews (David et al., 1983), has attracted little attention and was considered mainly as a physiological curiosity analogous to the male sterility in mammals with undescended testes. Surprisingly, a strain collected from a very hot locale, N'Djamena (Chad Republic), by L. Tsacas, was fertile at 30°C and could tolerate permanent culture at that temperature (J. R. David, unpublished). Although this observation was not published, the strain was provided to various Russian laboratories where it was further selected for increased heat tolerance, so that permanent culture could be kept at 31–32°C (see Zatsepina et al., 2001).

Here we characterize the geographic pattern of male sterility thresholds and show that most tropical populations from different continents produce fertile males when grown at 30°C, while temperate populations do not. We investigate this variation further in selected strains by characterizing the frequency of male sterility and cytological abnormalities of spermatogenesis after development at high temperatures, and the recovery process after a return at a mild temperature. The results establish that male sterility thresholds are genetically variable and are consistent with adaptation, as populations living under hot climates are more tolerant to heat stress. Unexpectedly, much of the divergence between tropical and temperate strains appears due to the Y chromosome.

Materials and methods

Populations investigated and general overview

Table 1 lists populations screened for male sterility/fertility at 30°C. In each case, wild-collected females were isolated in culture vials and isofemale lines established. These lines (a minimum of 10) were eventually pooled to make a mass culture kept at 20°C. Experiments were generally performed a few months after the establishment of the laboratory strain. In several cases, the same strain was studied two or three times after 1, 2 or 3 years in the laboratory and the same result was obtained. Male sterility temperature appears to be fairly stable, insensitive to genetic drift or laboratory adaptation.

From data in Table 1, two thermosensitive (Draveil and Prunay) and two thermotolerant (Niamey and Delhi) populations were chosen for more detailed investigation in which we measured the fertility of males grown at various temperatures in 0.5°C steps. Two of these populations (Prunay and Delhi) were then chosen for a genetic analysis involving crosses (F_1 and F_2 males) and repeated backcrosses towards the female parent. The aim of backcrosses was to introduce the Y chromosome of each strain into the genetic background (X chromosome and autosomes) of the other strain.

Table 1. *List of populations investigated for male fertility (+) or sterility (–) at 30°C*

Population	Country	Collection date	Latitude	Fertility
Sao Tomé	São Tomé e Príncipe	2001	0.2° (N)	+
400 m	Kenya	2001	1.0° (N)	+/–
1600 m	Kenya	2001	1.2° (S)	+/–
Brazzaville	Congo	2001	4.2° (S)	–
Pointe Noire	Congo	1999	4.5° (S)	+
Abidjan	Ivory Coast	2000	5.2° (N)	+
Cotonou	Bénin	2000	6.2° (N)	+
Weipa	Australia	1998	12.4° (S)	–
Mayotte Island	France (DOM)	1999	12.5° (S)	+
Bahia	Brazil	1999	13.0° (S)	+/–
Niamey	Niger	1997	13.3° (N)	+
Martinique Island	France (DOM)	1999	14.4° (N)	+
Mananara Reserve	Madagascar	2000	15.5° (S)	+
Réunion Island	France (DOM)	2002	20.5° (S)	+/–
Delhi	India	1997	28.3° (N)	+
Nainital	India	1997	29.2° (N)	+
Porto Alegre	Brazil	2001	30.0° (S)	–
Marrakech	Morocco	2001	31.4° (S)	–
Athens	Georgia, USA	2002	33.6° (N)	–
Montevideo	Uruguay	2000	34.5° (S)	–
Bordeaux	France	2000	44.5° (N)	–
Foissac	France	2001	45.0° (N)	–
Draveil	France	2001	48.4° (N)	–
Prunay	France	2001	48.5° (N)	–

Dates of collection are given, as well as latitude of origin.

Experiments were conducted between 1997 and 2002. In some cases, results were variable (‘uncertain’ strains, see Materials and methods) and indicated as +/–.

Fertility recovery (time needed for males to produce progeny at 21°C) was investigated in two populations, Bordeaux (from France) and Delhi (from India). Recovery was also investigated by counting the proportion of fertile males after a return at 21°C. Finally the cytological abnormalities of spermiogenesis in males grown at high temperature were investigated in the populations of Prunay and Delhi.

Fertility/sterility after development at 30°C: progeny production

Male fertility/sterility at 30°C was analysed using the following procedure. Several sets of 10 pairs from a mass culture were isolated as parents of the experimental flies. These parents oviposited at 20–21°C in culture vials containing a killed yeast, high nutrient medium (David and Clavel, 1965). Oviposition lasted 6 h and then the culture vials were transferred at 30°C until emergence of adults. These adults, grown at 30°C, were kept at the same temperature and mass-transferred to fresh food every 2–4 days, for at least 2 weeks. Males were considered as fertile when progeny larvae appeared in vials so that a permanent culture could be established at 30°C. When no progeny appeared in a sample of several hundreds of flies, males were classified as fully sterile. In all cases, females laid numerous eggs and, when mated with males grown at 25°C, and kept at 30°C always produced numerous progeny. In some cases, fertility after development at 30°C was not clearcut. A few larvae could appear in one vial but not in others, and this low fertility was insufficient to establish a permanent culture. For such populations, the experiment was repeated, generally with the same conclusion. The threshold for absolute (100%) sterility in these cases was presumably slightly greater than 30°C (e.g. 30.1°C or 30.2°C). Such strains are classified as ‘uncertain’ in Table 1.

Proportion of fertile males observed after dissection

To estimate the proportion of sterile males, we followed the procedure of Chakir et al. (2002). Flies were cultured in incubators regulated at 0.5°C intervals (±0.1°C) between 28.5° and 31.5°C. After emergence, males were separated from females and kept at their developmental temperature on cornmeal–sugar medium seeded with live yeast. After 5–6 days, males were dissected in *Drosophila* Ringer solution, seminal vesicles were opened with tiny needles and their content examined for the presence of motile sperm. A complete absence of sperm, or the presence of a few non-motile sperm in seminal vesicles, was the criterion for male sterility. At a critical temperature, when for example 50% of males were scored as sterile, the amount of sperm among fertile ones appeared variable. We did no try to quantify this variability.

At each temperature, 50 males grown in several vials were scored for motile sperm. Percentage of sterile males increases with temperature and the response curve has a sigmoid shape. These response curves were adjusted to a logistic function (STATISTICA software) as described in Chakir et al. (2002). This adjustment estimates two parameters: the temperature at the inflection point (TIP), which gives the value at which 50%

of males are sterile (median threshold), and a slope coefficient at the inflection point (SIP), which reflects the steepness of the curve.

Genetic analysis: male sterility in crosses and backcrosses

A thermotolerant (Delhi) and a thermosensitive (Prunay) strain were selected. Preliminary observations on crosses between Indian and French strains revealed that reciprocal F_1 males were clearly different and resembled the male parent, suggesting a role of the Y chromosome. We precisely investigated reciprocal F_1 and F_2 crosses between Delhi and Prunay. F_1 males were also backcrossed to the females of their maternal parent, and the same kind of backcross was repeated for six successive generations to introduce the Y chromosome of a given strain into the genetic background (X chromosome, autosomes and cytoplasm) of the other strain. All crosses and backcrosses were done with flies grown at 25°C. Eggs of each investigated generation were transferred at the various experimental temperatures, and male fertility was analyzed by dissection as described above.

Recovery after a return at a mild temperature

Sterile males may recover when returned to a mild, permissive temperature. Ten young males aged 0 to 1-day post-eclosion, grown at various high temperatures, were isolated in culture vials each with three normal virgin females grown at 21°C. These vials were kept at 21°C, changed daily and then examined for the appearance of progeny. A male was classified as fertile when at least one larva appeared in a vial. Progeny number was not counted and males were discarded after recovery. This procedure was simultaneously applied to Bordeaux and Delhi populations.

We also analyzed male recovery by dissecting the testis and observing the presence of motile sperm in the seminal vesicles. This experiment was done on two strains, Prunay and Delhi, grown at their sterility temperatures of 30°C and 31°C, respectively. Young males of both strain were distributed into several culture vials and transferred to 21°C. These males were dissected regularly up to the age of 9 days. For a given age, 50 males were analyzed.

Cytological observations

Developmental factors leading to male sterility were investigated by analyzing spermiogenesis in males reared at the threshold of absolute sterility, i.e. 31°C for the thermotolerant (Delhi) and 30°C for the thermosensitive (Prunay) strain. Testes were dissected and opened in a drop of Ringer solution, and their content dispersed by gentle movements. Spermatozoa are produced within cysts of 64 spermatocytes arising from four mitotic and two meiotic divisions of a primary gonial cell. These 64 spermatocytes develop synchronously, due to incomplete cytokinesis, through eight postmeiotic stages of spermatogenesis (Lindsley and Tokuyasu, 1980). Four traits were investigated to evaluate the pre- or post-meiotic abnormalities involved in sterility: (1) the number of spermatocytes per cyst, after DAPI staining, to confirm normal

Table 2. *Parameters of the sterility curves obtained after a logistic adjustment*

Crosses	Generation	% of Prunay genes	SIP	TIP (°C)	r^2
Delhi strain		0	9.12±2.41	30.54±0.01	99.9
Female × Male					
Prunay × Delhi	F_1	50	5.91±1.01	30.50±0.02	99.6
$F_1 \times F_1$	F_2	50	9.97±4.91	30.56±0.03	99.7
Prunay × F_1	G_2	75	5.86±1.83	30.09±0.04	98.6
Prunay × G_2	G_3	87.5	3.64±0.87	30.21±0.07	97.7
Prunay × G_3	G_4	93.75	2.79±0.46	30.24±0.07	97.8
Prunay × G_4	G_5	96.88	4.08±0.37	30.23±0.03	99.6
Prunay × G_5	G_6	98.44	4.35±0.31	30.23±0.02	99.8
Prunay strain		100	4.58±0.61	29.47±0.03	99.3
Female × Male					
Delhi × Prunay	F_1	50	3.37±0.97	29.95±0.11	95.7
$F_1 \times F_1$	F_2	50	5.87±1.77	29.88±0.04	98.5
Delhi × F_1	G_2	25	5.86±0.47	29.80±0.02	99.8
Delhi × G_2	G_3	12.5	6.05±0.44	30.15±0.01	99.9
Delhi × G_3	G_4	6.25	10.68±6.19	30.08±0.05	99.7
Delhi × G_4	G_5	3.12	4.45±0.35	30.11±0.02	99.8
Delhi × G_5	G_6	1.56	4.99±0.35	30.15±0.02	99.8

Results of crosses and backcrosses between Prunay and Delhi strains. The proportion of Prunay genes on X and autosomes is given for each generation. Backcrosses introduce the Y chromosome of Delhi in a Prunay background, and the Y chromosome of Prunay in the Delhi background.

SIP, slope coefficient at inflection point (mean ± S.D.); TIP, temperature at inflection point (mean ± S.D.); r^2: value of the logistic adjustment × 100.

cell division; (2) the length of the cyst, which is species specific (Joly et al., 1989) and slightly greater than that of the sperm; (3) the localisation of spermatocyte nuclei along the cyst, as an indicator of the elongation process of each spermatid; (4) the chromatin condensation within sperm nuclei, as abnormal condensation is expected to produce a non-functional, sterile gamete.

Cyst length was measured in parental males (N=50) and in males (N=25) from three successive odd generations (i.e. F_1, G_3 and G_5, see Table 2) reared at 21°C and at the sterility temperature. The numbers, positions and condensations of spermatocyte nuclei within the cyst were counted in 20 cysts in males of each generation investigated. Two categories were distinguished for sperm nuclei position along the cyst: 'apical', when all nuclei were found in the apical part of the cyst as usual in fertile males, and 'distal', when sperm nuclei were dispersed along the cyst or even found only in the distal part of the cyst. Chromatin condensation within cysts was classified as 'maximal' when nuclei were needle-shaped with intense fluorescence, 'minimal' when nuclei were round-shaped with low fluorescence, and 'variable' when the shape of nuclei and their respective fluorescence varied from one spermatocyte to another within the same cyst.

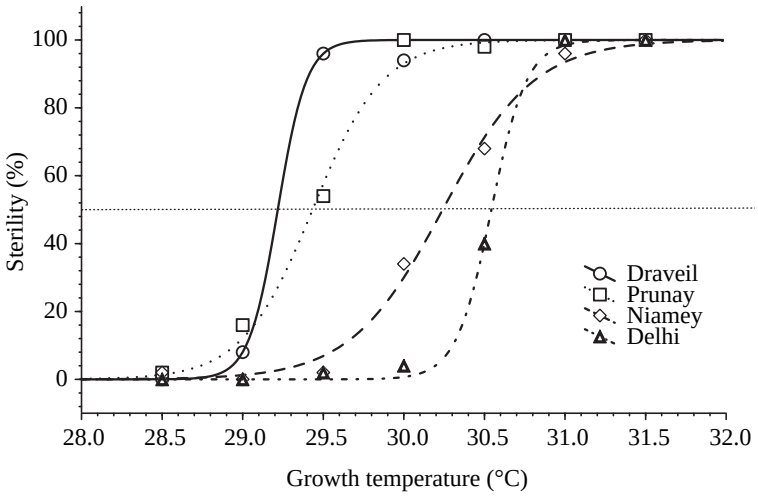

Fig. 1. Sterility curves, after logistic adjustment as a function of growth temperature for two temperate (Prunay and Draveil) and two tropical (Niamey and Delhi) populations. Each point is based on the data from 50 males grown in at least three different vials. Characteristic values for Prunay and Delhi are given in Table 2. For Draveil and Niamey, the slope coefficients at inflection point (SIPs) are, respectively, 11.21±1.56 and 3.51±0.32, and the temperatures at inflection point (TIPs) are 29.22±0.03°C and 30.24±0.03°C.

Results

Comparison of geographic populations grown at 30°C

Table 1 shows the results obtained after a development at 30°C for 24 geographic populations from different origins including Europe, Asia, Australia, the Afrotropical region and North and South America. Ten populations produced fertile males. Ten populations were completely sterile, without any offspring even when, in some cases, adults were kept at 30°C for a month. Four populations were classified as 'uncertain'. A few larvae were observed in some vials, not in others, and the number was too low to sustain the population.

As seen in Table 1, fertile strains were from the Afrotropical region, the Caribbean and India. No geographic pattern is evident other than a strong relationship with latitude. The average latitude of origin for the three kinds of strains is: fertile at 30°C: latitude=12.93±3.89° (N=10; mean ± S.E.M.); sterile at 30°C: latitude=33.25±3.86° (N=10); uncertain: latitude= 8.92±6.11° (N=4).

A statistical analysis of variance (ANOVA; not shown) revealed a strong latitudinal effect ($F_{2,21}$=9.133, P<0.01) and a *post-hoc* test suggested two groups: sterile strains are from high latitudes and fertile and uncertain strains from low latitudes. This pattern shows that populations from temperate places are more thermosensitive than those from tropical climates.

Male sterility as a function of growth temperature in two temperate and two tropical populations

The response curves of two temperate (Draveil and Prunay, France) and two tropical (Niamey, Africa and Delhi, India) populations are shown in Fig. 1 and the parameters obtained after logistic adjustments are given in Table 2. In all four cases, the experimental points are close to the adjusted curves with very high r^2 values, indicating the validity of the model. For TIP (median threshold), which estimates the temperature that produces 50% of sterile males, the mean of the two tropical populations is 30.39±0.15°C (mean ± S.E.M.), while it is 1.04°C less (29.35±0.13°C) for the two temperate populations. The slopes at inflection point (SIP) are quite variable among populations and not related to the geographic origin. Slopes are steeper for the Draveil and the Delhi populations. A smoother slope, as observed with Prunay and Niamey (see Fig. 1), indicates a higher phenotypic variability among males (Sokal and Rohlf, 1995) and might reflect a genetic heterogeneity.

Genetic analysis between a temperate and a tropical population

Preliminary experiments revealed a difference between reciprocal F_1 males, suggesting an effect of the Y chromosome. This problem was investigated in more detail, using a larger set of growth temperatures, in crosses between the French Prunay and the Indian Delhi strains. The design was to introduce the Y chromosome of Delhi into the Prunay background, and *vice versa*. All these crosses were at 25°C, and progeny transferred at various experimental temperatures, to estimate the median sterility threshold (TIP).

The results are given in Table 2. The validity of logistic adjustments is, as previously, shown by very high r^2 values. SIPs were quite variable among generations with no regular pattern.

The TIP values are more interesting. In the first series (Y chromosome of Delhi introgressed into the Prunay background), the three values of parents F_1 and F_2 are almost

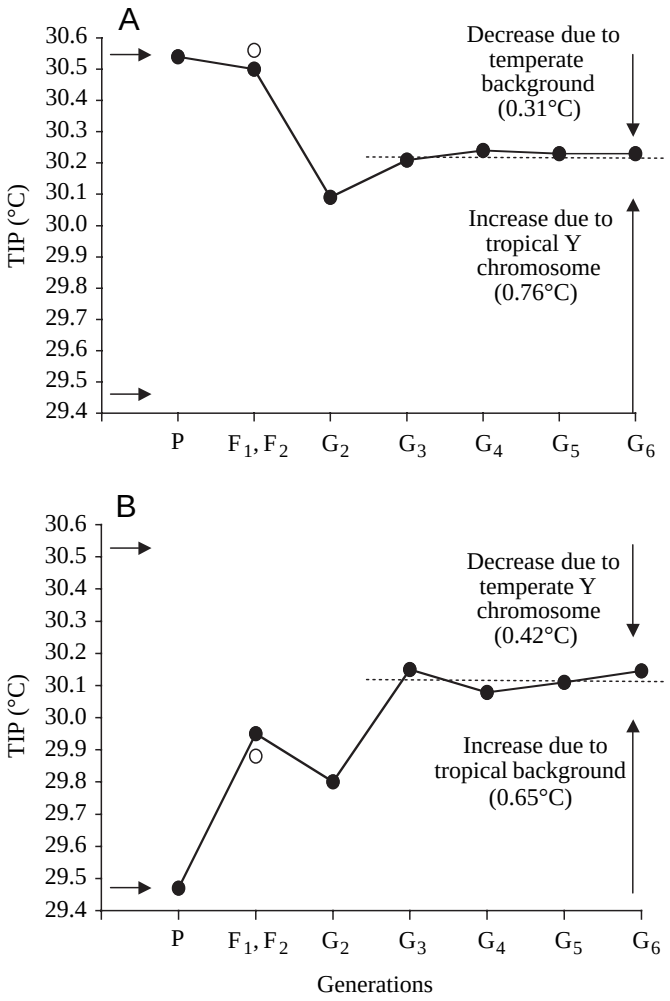

Fig. 2. Temperatures at inflection point (50% sterile males) in parents, F_1, F_2 (white circle) and of successive backcrosses (see Table 2). (A) The Y chromosome of Delhi is introduced into the Prunay background. (B) The Y chromosome of Prunay is introduced into the Delhi background. Horizontal arrows in each graph show the temperatures at inflection point of the two parental lines: Delhi (top) and Prunay (bottom). From G_3 to G_6, values are not different and the mean is illustrated by a dotted line. The respective roles of Y and genetic background are indicated.

identical (30.53±0.02, mean ± S.E.M.) suggesting a complete dominance of thermotolerance (Fig. 2A). This stability is followed by a decrease of 0.44°C in G_2. The decrease does not proceed further in the following generations, however, and indeed the values increase slightly. The average for backcross generations 3–6 is 30.23±0.01°C. This corresponds to a slight but highly significant decrease of 0.31°C with respect to the three parental values (t=18.5, d.f.=5, P=<0.001), which is due to the replacement of the Delhi background by the Prunay background. Most interesting, however, is that, although we almost completely replaced the Delhi by the Prunay background in G_6, the TIP remained much higher than in the pure French strain. The difference, which is 0.76°C, must be explained by the Indian Y chromosome.

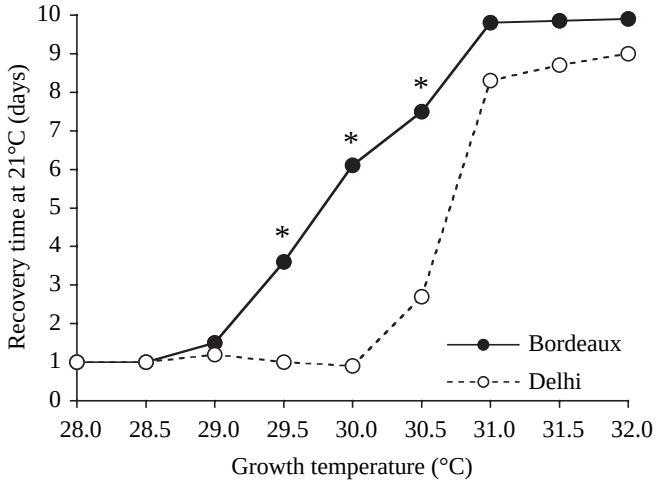

Fig. 3. Variation of male recovery time as a function of their developmental temperature. Young males were isolated with three normal virgin females and transferred at 21°C. Recovery is the mean age at which the first progeny was observed. Differences between the two populations are highly significant for growth temperatures of 29.5, 30 and 30.5°C (Student's t-test, P=0.001).

In the second series of backcrosses, the Y chromosome of Prunay was introduced into the Delhi background (Table 2 and Fig. 2B). The first generations (F_1 and F_2) were intermediate between the parental values. TIP decreased slightly in G_2, matching a similar phenomenon in G_2 of the reciprocal crosses. This parallelism suggests some accidental phenomenon, which might be a slight perturbation in growth temperatures. Starting from G_3 onward the values are very similar and stable, with a mean of 30.12±0.02°C (N=4). This value is close to that obtained in the reciprocal series (30.23±0.01). The difference is, however, significant (paired data; d=0.105±0.022, P<0.05). Again the interpretation of the results is straightforward: introducing a temperate Y chromosome in the Indian background has decreased the TIP of 0.42°C. The difference of 0.65°C above that of the temperate parental strain corresponds to an increase of the thermotolerance due to the Indian genetic background.

Progeny production after a return at a permissive temperature

Males of two different populations (Bordeaux and Delhi) were grown at nine different constant temperatures from 28 to 32°C. Upon emergence, males were isolated with three normal virgin females at 21°C, and vials were changed daily. The first appearance of a larva in a vial defines the age at recovery. For each temperature, ten males were investigated.

Results for Bordeaux and Delhi are shown in Fig. 3. For the three lowest pre-adult temperatures (28, 28.5 and 29°C), males were all normally fertile after emergence in both populations. Such was also the case for the Indian males up to 30°C, and these results match the data shown in Fig.1. For higher growth temperatures, the recovery time is greater in each population, from 1 up to 9–10 days. At very high temperatures, only a

fraction of the males recovered. In all cases, the recovery time was longer in the temperate than in the tropical population. The difference between the two strains is significant for three growth temperatures, 29.5, 30 and 30.5°C (Student *t* test, $P<0.001$). In other words, males of the heat-sensitive, temperate population always required a longer time to recover and produce their first viable spermatozoa.

Proportion of fertile males after a return at a permissive temperature

Fig. 3 shows that French males grown at 30°C recovered on average in 6 days, while a quite similar duration (8 days) was evident for Indian males grown at 31°C. In other words, we expect that French males grown at their absolute sterility threshold (30°C) will show the same functional alterations as Indian males grown at 31°C. This expectation was further tested by comparing Prunay and Delhi populations. Males, grown at 30 and 31°C, respectively, were transferred to 21°C after emergence, and sets of 50 males were dissected regularly up to 9 days post-eclosion. Initially all males were sterile (no sperm visible) but at the end almost all were fertile, with highly motile sperm (Fig. 4). For young males, there was a slight difference between the two populations: fertile males appeared after 3 days in the Delhi strain but after 5 days in Prunay. The overall curves have similar shapes, however, which were adjusted to a logistic model. The ages at the inflection point were similar: 4.97±0.13 and 5.32±0.37 days for Prunay and Delhi, respectively. These values, which indicate a mean recovery time, are slightly less than those obtained by direct progeny observation (Fig. 3). Such a small difference is not surprising and we already know that the presence of sperm, even motile, in the seminal vesicles is not a certitude for offspring production (Araripe et al., 2004).

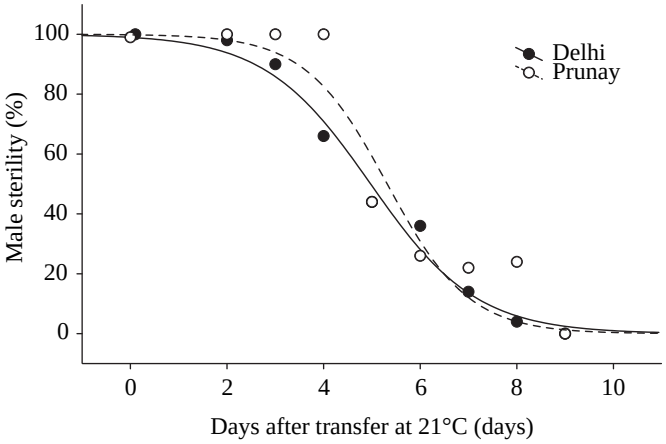

Fig. 4. Fertility recovery (estimated after dissection) when males, grown at a sterilizing temperature, are transferred at a mild permissive temperature. Males of the Delhi strain were grown at 31°C, those of the Prunay strain at 30°C. Data are adjusted to a logistic function. r^2= 0.992 for Delhi and r^2=0.960 for Prunay.

Spermatogenesis defects due to high temperature

Cyst length

In control males grown at 21°C, neither strain nor generation affected cyst length (ANOVA, not shown), which averaged 1.915±0.010 mm (mean ± S.E.M., N=250) (Table 3). Cysts produced at a sterilizing temperature (30°C for Prunay, 31°C for all other cases) were shorter than in controls, the reduction varying between 24 and 44%. Cyst length varied among generations (ANOVA, not shown), but in no regular pattern.

Sperm heads per cyst

Control flies (21°C) differed between generations in both the Prunay and Delhi series (ANOVA, not shown; Table 3).

Table 3. *Influence of growth temperature upon cyst length and sperm head number in parental strains Prunay or Delhi and several backcross generations*

	Cyst length (mm)			Sperm head number		
Generation	21°C	High T (°C)	% Reduction	21°C	High T (°C)	% Reduction
Prunay	1.933±0.012	1.429±0.023	26.1	58.15±1.10	58.25±1.18	NS
F_1	1.918±0.028	1.278±0.019	33.4	61.35±0.87	48.75±1.98	18.9***
G_3	1.956±0.015	1.289±0.020	34.1	57.45±0.82	58.95±1.11	NS
G_5	1.878±0.019	1.426±0.016	24.1	61.25±0.86	55.15±1.18	10.0***
Delhi	1.892±0.021	1.125±0.012	40.5	59.05±1.25	54.45±1.76	7.8*
F_1	1.891±0.029	1.252±0.038	33.7	61.45±0.57	55.15±1.62	10.3**
G_3	1.942±0.040	1.102±0.023	43.3	54.95±1.28	58.05±0.99	NS
G_5	1.908±0.014	1.066±0.018	44.1	56.70±1.24	54.00±1.65	NS

Values are means ± S.E.M.

For details of backcross generations, see Table 2.

High T, developmental temperature was 31°C in all cases except Prunay (30°C).

For cyst length, 50 cysts were measured for parents, but only 25 for generations F_1–G_5. The reduction in cyst length due to a development at high temperature is highly significant in all cases, and % length reduction is given.

For sperm head number, 20 cysts were investigated in all cases. A slight reduction in sterile males is often observed, and significant cases are indicated. *P<0.05, **P<0.01, ***P<0.001; NS, non significant.

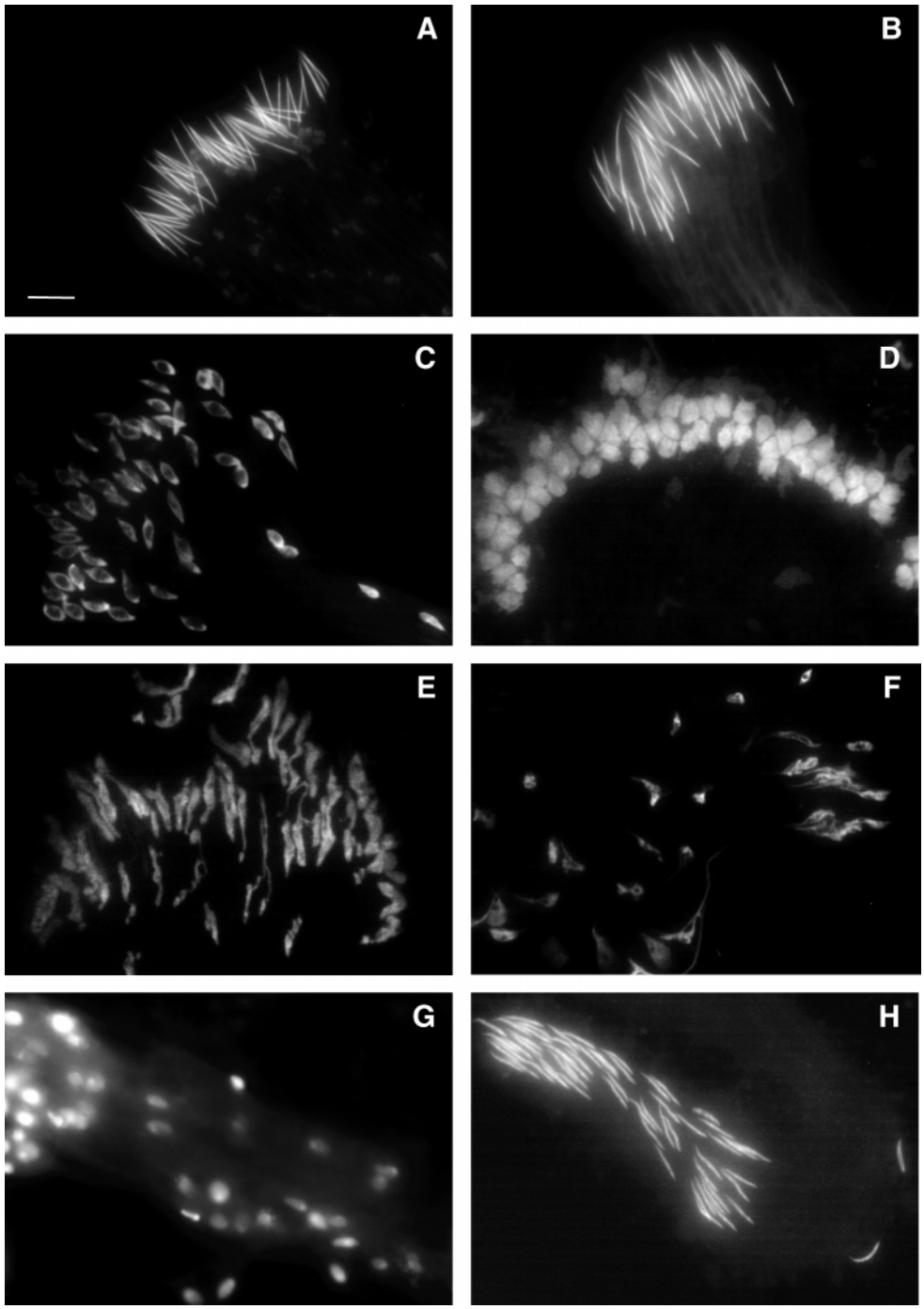

Fig. 5. Variability of sperm nuclei in cysts at their maximum elongation. (A,C,E,G) Delhi series; (B,D,F,H) Prunay series. (A,B) Males grown at 21°C; (C–H) males grown at 31°C. The various abnormalities of nucleus elongation and condensation observed at 31°C were found in the two populations but at different frequencies (see text). (A,B) Normal cysts with condensed chromatin in all apical nuclei (21°C); (C,D) rounded nuclei with variable levels of chromatin condensation (31°C); (E,F) irregular-shaped nuclei with variable levels of chromatin condensation (31°C); (G) rounded nuclei located in abnormal position along the cyst (31°C); (H) normal condensation of nuclei located in abnormal position along the cyst (31°C). Scale bar, 10 μm.

However, the overall means in the two series were similar (59.80±0.49 in Prunay and 58.04±0.6 in Delhi). These numbers are less than the expected number (64), suggesting that during cyst maturation some spermatids die and do no produce a visible nucleus. In the heat-grown males, the number of sperm heads decreased in four of eight cases. In three cases, however, the heated cysts had more nuclei than the controls. We suggest that development at a high temperature tends to increase spermatid mortality, but the effect is small.

Nuclear position

Cysts with at least one abnormal nucleus were more numerous in heat-grown flies, from 1.25% (controls) to 8.75% in the Prunay series, and from 6.25% to 12.5% in the Delhi series (compare Fig. 5A,G and B,H). The difference between the two temperatures, however, was not significant (χ^2=0.163, d.f.=1, P=0.685). Development at a high temperature does impair cyst elongation, but the heterogeneity of nucleus position among spermatids in the same cysts increases only slightly.

Chromatin condensation of sperm heads

In normal cases, most sperm heads in a cyst are elongated with strong DAPI staining (Fig. 5A,B). Some abnormalities are evident, however, even after development at 21°C. These abnormalities correspond to variation either in the strength of condensation or in the proportion of non-condensed nuclei. At 21°C, the proportion of cysts harboring such abnormalities was 26.25% and 32.50% in the Delhi and Prunay series, respectively. But abnormalities were significantly more frequent (χ^2=1.285, d.f.=1, P=0.256) and often more pronounced in males grown at 31°C (see Fig. 5C–H). The frequencies of such cysts was 67.50% in Prunay and 77.50% in Delhi (N=80 in each case).

All these cytological observations suggest that sterility arises mainly from incomplete elongation of the spermatids, and also from abnormalities in the chromatin condensation of the sperm heads. However, heat treatment has only a marginal effect on heterogeneity among spermatids in the same cyst and on their mortality.

Discussion and conclusion

Our two indicators of male sterility induced by development at high temperature, motile sperm in the seminal vesicles and capacity to produce progeny, gave convergent results. Also, both the absolute thermal threshold, i.e. the lower temperature that produces 100% sterility, and the median threshold, which is lower and produces 50% sterility, are informative parameters. Determining the median threshold requires several precisely regulated incubators, but is more informative and can be defined with a logistic adjustment. The median threshold gives very homogeneous results when comparing several populations from the same climatic region. For example, the values for three temperate populations in the Palearctic region (Prunay, 29.47°C, Draveil, 29.22°C, and Marrakech, 29.14°C) give a mean of 29.28±0.10 with a very low coefficient of variation (CV) of 0.59. A similarly low CV has been observed in the genetic analyses, so that even a very small difference (0.2°C) appears reliable and significant.

Populations from diverse geographic regions differed in progeny production at the absolute threshold (30°C) in a direction consistent with climatic adaptation: tropical populations are more heat tolerant than temperate ones. Whether there is latitudinal clinal pattern awaits more precise measurement of the median threshold. In *D. melanogaster*, ancestral populations occur in the Afrotropical region (David and Capy, 1988). In other words, a significant loss of heat tolerance has accompanied the geographic expansion of the species into colder regions. Previously the heat tolerance of the N'Djamena strain (see Introduction) appeared unique, possibly related to some idiosyncratic mutations in a very hot and arid place. We now show that the heat tolerance of spermatogenesis, which permits permanent laboratory culture at 30°C, is widespread. To our knowledge, the most tolerant populations occur in the African Sahel, south of Sahara, and in Tropical India. Both regions experience extremely hot summers, with daily maximum temperature above 38°C (see Gibert et al., 1998, for more climatic information in the Delhi region). If heat tolerance is considered an ancestral trait, we have to explain why this tolerance disappeared when *D. melanogaster* extended its range toward colder, temperate countries. In other words, why did thermotolerance disappear when it was no longer subjected to selection? A general response is that thermotolerance implies a cost, due to unknown pleiotropic effects. A possibility is that heat-tolerant flies might be less cold-tolerant, but the occurrence of a functional trade-off requires further investigation.

Laboratory selection seldom breaches the limit of 30°C for continuous laboratory culture (Parsons, 1973; David et al., 1983; Zatsepina et al., 2001). We report here one more unsuccessful attempt (J. R. David, unpublished). Two French mass populations were submitted to the following selection procedure. Development proceeded at 31°C. After emergence, adults were transferred at 20°C for recovery. About 2 weeks later, numerous progeny were obtained and the larvae transferred back to 31°C. The selection was repeated for 30 generations but, at the end, tolerance of heat-induced male sterilization had not increased: the recovery time at the permissive temperature was not shortened. Against this background, the ability of the N'Djamena strain to grow initially at 30°C and later at 31–32°C (see Zatsepina et al., 2001) is remarkable. This occurrence suggests that the thermotolerance of spermatogenesis was increased by selection, although the thermal thresholds were, to our knowledge, never determined. Other positive responses to laboratory selection have been obtained. Several strains from Niamey and Delhi region have now been cultured permanently at 30°C. At the beginning and when population size was too low, strains had to be put at 29°C for one generation to recover. These difficulties disappeared after about 100 generations. Indeed, the Indian strains were eventually cultured permanently at 30.5°C, and fertile males may occur at 31°C. Thus, permanent culture at a high temperature increased thermotolerance of male reproduction, at least in the already heat-tolerant populations of Sahel and tropical India.

Our crosses between a French and an Indian population demonstrate that much of the genetic difference is due to the Y chromosome. Whether the same phenomenon exists for the Sahel populations is still unknown. Cytological abnormalities at the absolute threshold appeared to result mainly from a perturbation of the elongation process, which may explain why motile sperm are not produced. Chromatin condensation abnormalities increase in sperm heads, suggesting that heat may also affect several different physiological processes. After transfer to a mild, permissive temperature, males may eventually recover fertility. In several species, recovery time is proportional to the sterilizing temperature (Chakir et al., 2002; Vollmer et al., 2004; Araripe et al., 2004) and may take up to 10 days, which is equivalent to the duration of spermatogenesis (Lindsley and Tokuyasu, 1980). Possibly, therefore, heat-induced perturbations are expressed at the level of germ cells themselves. Interestingly, cytological abnormalities related to deletions of Y fertility genes were often observed in early stages of spermatogenesis (Hardy et al., 1981). In this respect, the cytological abnormalities produced by different temperatures above the absolute thermal threshold remain to be investigated.

The importance of the Y chromosome was unexpected because this mostly heterochromatic chromosome bears only a few genes which, however, are generally necessary for male fertility (Kennison, 1981; Carvalho et al., 2000, 2001). Genes on the Y chromosome generally have a low level of molecular polymorphism (Zurovcova and Eanes, 1999). Within a population, all Y chromosomes might be identical, which would leave little variation on which selection could act. On the other hand, we now have evidence that Y chromosomes in distant geographic populations can differ, and in ways that are relevant to spermatogenesis. Three out of nine genes identified on the Y chromosome belong to the dynein family (Carvalho et al., 2001) and may be related to sperm motility. Possibly heat inactivation of at least one such protein is responsible of the elongation abnormality, and also this protein in tropical populations is more tolerant of high temperature. This hypothesis is, however, difficult to reconcile with the differing time courses of recovery. Also unclear is how dynein inactivation might affect the function of germ cells. Perhaps a complete explanation may lie in genes on the Y chromosome whose function remains unknown (Carvalho et al., 2001).

The significant role of genetic background (genes on autosomes and X), as suggested by backcrosses and presumably also by selection, is also difficult to explain. A role for increased Hsp70 is unlikely because the N'Djamena strain has comparatively low expression of this heat-shock protein (Zatsepina et al., 2001). But many other genes are involved in spermiogenesis and interact with temperature (Yue et al., 1999; Rajendra et al., 2001; Rockett et al., 2001).

Interestingly, male sterility at high temperature is evident in all species investigated so far (Chakir et al., 2002; Vollmer et al., 2004; Araripe et al., 2004). The sterility threshold, however, varies among species, and most temperate species cannot be grown at 30°C. For example, for the European *D. subobscura* the upper development limit is 26°C (Moreteau et al., 1997), but males are sterile at 25°C (J. R. David, unpublished observation). Possibly this sterility phenomenon is general among drosophilids and plays an important role in determining the climatic distribution of species, as proposed by Araripe et al. (2004).

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