Correlated response in reproductive and life history traits to selection on testis length in *Drosophila hydei*

SCOTT PITNICK* & GARY T. MILLER

Department of Biology, Syracuse University, 108 College Place, Syracuse, NY, 13244-1270, U.S.A.

Flies in the genus *Drosophila* have undergone striking evolutionary divergence in the size and number of sperm produced. Based on comparative studies of sperm length, testis length, and other reproductive and life history traits, including body size, age at first reproduction, and the number of sperm produced, macroevolutionary trade-offs resulting from the need to produce high-investment testes have been postulated. To understand better the microevolutionary processes underlying these interspecific patterns, we imposed replicated bidirectional selection for testis length for 11–12 generations on *D. hydei*, a species with 23.5 mm-long sperm and 30 mm-long testes. Testis length exhibited realized heritabilities ranging from 0.45 to 0.72. Following selection, traits were assayed for correlated responses. Thorax length, testis mass, sperm length, egg-to-adult development time, and posteclosion maturation time showed consistent positive correlated responses. Numbers of sperm produced and transferred to females, male longevity, female egg productivity, and seminal receptacle length did not show consistent correlated responses to selection on testis length. The pattern of correlated responses to testis length reveal the potential for the evolution of reproductive strategies to alter important life history attributes.

Keywords: artificial selection, body size, pleiotropy, reproduction, sperm, spermatogenesis.

Introduction

Among species, males vary in how much energy they devote to gamete production (Pitnick, 1996 and references therein). Such variation is often manifested as variation in testis size, as this trait positively correlates with sperm production and transfer rates (Amman, 1970; Møller, 1988, 1989). Among species, absolute testis size has been shown to increase with body size in mammals, birds, anurans and insects (Pitnick, 1996). Also, numerous studies have demonstrated a clear relationship between testis size and breeding system, with males of species in which females are mated by more than one male within a reproductive episode (e.g. oestrus, clutch, season) having larger testes for their body size than do males of monogamous species (reviewed in Pitnick, 1996; Kappeler, 1997; Stockley *et al.*, 1997). This is because sperm competition, or the competition between sperm of different males for fertilization of a female's ova, often functions as a raffle

favouring males transferring the greatest number of sperm (Parker, 1970).

Understanding the evolutionary basis of testis size variation for some species or lineages may be more complicated, however, than simply analysing variation in body size and selection on sperm production rates. Among *Drosophila* species, testis mass shows a positive relationship with body mass (Pitnick, 1996). However, this relationship may be a consequence of the combined positive relationships between sperm length and body size (Pitnick *et al.*, 1995a; Pitnick, 1996) and between testis length and sperm length (Joly & Bressac, 1994; Pitnick, 1996). The nature of spermatogenesis in *Drosophila* dictates that testes be longer than the sperm they manufacture (Lindsley & Tokuyasu, 1980). Thus, because the genus *Drosophila* has undergone dramatic divergence in sperm length, with flagellum lengths varying across species by more than 180 times (Pitnick *et al.*, 1995b), testis lengths vary to an equal degree. Moreover, in a comparative study of 11 species, most of the variation in absolute and relative testis mass (72.0% and 60.4%, respectively) was explained by variation in sperm length, whereas little of the variation (0.2% and

*Correspondence. E-mail: sspitnic@syr.edu

7.1%, respectively) was explained by variation in the number of sperm produced (Pitnick, 1996).

Fitness trade-offs of producing long sperm, such as the production and transfer to females of relatively few sperm (Pitnick, 1996) and delayed male maturity (Pitnick *et al.*, 1995a), may be mediated through the testes. Relatively large testes of many *Drosophila* species may therefore represent a cost or physiological trade-off of producing long sperm. For example, it has been suggested that protracted adult male maturation time in species with relatively long sperm is a consequence of the time required to grow their giant testes (Pitnick, 1993; Pitnick *et al.*, 1995a).

The present study employed a programme of artificial bidirectional selection on testis length to explore the relationship between testis length and other reproductive and life history traits within a single population of *D. hydei*, a species with gigantic (i.e. 23.5 mm) sperm (Pitnick & Markow, 1994a). Because across species there is nearly a perfect relationship between testis length and sperm length (Reduced Major Axis slope = 1.00, $r^2 = 0.99$; Pitnick, 1996), we predicted that any response to selection by testis length would also produce a strong correlated response in sperm length. As such, the testis length selection lines would provide material for comparing microevolutionary trade-offs among traits for which macroevolutionary patterns have already been established through comparative analyses of *Drosophila* (Pitnick *et al.*, 1995a; Pitnick *et al.*, 1999; Pitnick, 1996). If successful selection for increased and decreased testis length results in a minimal correlated response in sperm length, then the selection lines would permit discrimination of microevolutionary trade-offs attributable to testis length from those attributable to variation in sperm length. The latter scenario was realized.

Materials and methods

Culturing and selection protocol

The strain of *D. hydei* used was derived from a parent stock of 235 gravid females collected in June 1993 from rotting strawberries at the South Coast Agricultural Research Station, CA and were supplied courtesy of Dr Joe Graves. Following collection, the flies were maintained in population cages containing approximately 3000 flies for six generations, then in a single population cage of approximately 500 flies for 25 generations before initiating the artificial selection programme.

A selection replicate consisted of one line (high) selected for increased and one line (low) selected for decreased testis length. Two replicates ('A' and 'B') were initiated from the base population, and these were started three generations apart. Both within and between replicates, lines were exposed to common environmental conditions.

Throughout the experiment, virgin males and females were collected under light ether anaesthesia and maintained for 12 days in single-sex 8-dram vials containing cornmeal–molasses–agar medium and live yeast. To begin each selection replicate, 100 male–female pairs were aspirated into vials containing medium and yeast. After 24 h, males were transferred to separate vials to await dissection and females were transferred to fresh vials to continue oviposition for an additional 2 days, after which they were discarded. Beginning when males were 20 days old (by which time testes no longer grow; Pitnick, unpubl. data) males were dissected to determine testis length. Progeny from the 20 males with the longest testes and the 20 males with the shortest testes were used to initiate the high and low lines, respectively. In all successive generations, 75 males were dissected per line to determine testis length, and approximately seven sons and daughters from each of 15 selected males were randomly mated, avoiding pairings between full-siblings, to begin the next generation. In all progeny vials, some larvae were transferred to fresh vials when necessary to maintain low rearing densities and thus optimize adult body size.

Testis length was determined by dissecting both testes into phosphate buffered saline (PBS) on a glass slide. Following separation from the seminal vesicles, each testis was uncoiled by gentle tugging with forceps to sever the tracheae that bind the loops of the testis together. A 24×60 mm cover slip with clay at the corners was placed over the preparation and the clay was then compressed to restrict the testes to a two-dimensional plane without overcompression. A digital image of each testis was captured by a Dage CCD72 camera mounted on a stereomicroscope and length of both testes for each male was measured using the public domain NIH IMAGE program. Selection and analyses were based on the average length of both testes for each male.

Selection continued for 12 and 11 generations in replicates A and B, respectively. To accommodate the workload while correlated responses were measured, selection was relaxed in generations 12–16 (replicate A) and 12–14 (replicate B). Changes in testis length were monitored during this time by measuring testes from 10 randomly selected males per line each generation (Table 1).

Correlated responses

All correlated responses were measured on flies reared under standard conditions by transferring 60 first instar

Table 1 Difference (mm) in testis length between High and Low lines of *Drosophila hydei* for generations during which correlated traits were measured

Generation	Testis length difference	Percentage of wild-type
Replicate A		
14	15.58	50.19
15	14.54	46.84
16	9.54	30.73
Replicate B		
11	10.26	32.96
12	10.34	33.22
13	9.93	31.90

larvae per vial containing 8 mL of medium, which approximates the same density as that encountered during selection. In the analyses of direct and correlated responses to selection, we use the paired high and low lines as controls for one another. Gromko *et al.* (1991) discuss the relative merits of this control vs. alternatives.

Body size Thorax length, which is a robust index of body mass in *D. hydei* (Pitnick & Markow, 1994a), was measured in males from standardized density vials while quantifying other correlated traits listed below (replicate A, high: $N = 156$, low: $N = 136$; replicate B, high: $N = 128$, low: $N = 108$). In addition, total dry body mass was determined for generation 16 replicate A males while assessing testis mass (see below).

Testis mass To determine the absolute and relative dry weight of testes relative to body size, both testes were dissected from anaesthetized, 14-day-old males into distilled H_2O and then transferred to a preweighed piece of aluminium foil. All remaining tissue was placed on another preweighed piece of foil. Samples were then dried at 60°C for 24 h prior to weighing on a Cahn C-31 microbalance accurate to the nearest 1.0 μg. Dry mass was measured for replicate A only (generation 16, $N = 20$ males per line).

Sperm length Using the dissection and measurement technique described by Pitnick & Markow (1994b), sperm length, defined as the mean length of three cysts just beginning to enter the seminal vesicle, was determined for replicate A (generation 14, $N = 10$ per line) and replicate B males (generation 12, $N = 5$ per line).

Sperm production As an index of the number of sperm produced, the number of sperm bundles simultaneously undergoing development within a single testis was determined by counting the number of bundles present

at the mid-testis cross-section of 14–15-day-old males for replicate A (generation 16, $N = 12$ per line) and replicate B males (generation 13, $N = 5$ per line).

Egg-to-adult development time For each line, 15 7-day-old virgin males and females were placed over food plates for 3 h. The following day, 240 recently hatched first instar larvae per line were gently transferred using a pin from the plates into food vials (four vials/line/replicate at a density of 60 flies per vial). Beginning shortly prior to the time of the first adult eclosion, vials were checked hourly until all flies had eclosed.

Adult male maturation time Two experiments were conducted. First, posteclosion testis growth was examined by measuring the length of both testes from each male at 3-day age intervals, beginning with eclosion (day 0) and ending at age 18 days for replicate A and 9 days for replicate B ($N = 7$ males per age per line; replicate A: generation 16; replicate B: generation 13). In addition to the selection line flies, testes growth was simultaneously measured in males from the unselected base population reared under similar standardized conditions. In the second experiment, 100 freshly eclosed males were collected from each line, randomly assigned to age treatments, and stored with 10 males per food vial without live yeast. On each of days 6 through 10 posteclosion, 20 males were anaesthetized and dissected to examine for the presence of mature spermatozoa in their seminal vesicles (replicate A: generation 16; replicate B: generation 13).

Longevity Adult longevity was quantified for both virgin and nonvirgin males. In each case, 70 males per line per replicate were collected on the day of eclosion, using light ether anaesthesia, and henceforth maintained at a density of 10 flies per food vial with live yeast. Fresh vials were provided three times weekly and vials were checked daily for deaths. As deaths occurred, surviving flies were recombined to maintain a density as close to 10 per vial as possible. For nonvirgin longevity, males were paired with 7-day-old virgin female flies from the base population at a density of five males and five females per vial. Females were replaced with reproductively mature virgin females every 10–12 days.

Sperm transfer Each 15-day-old virgin male was aspirated into an 8-dram food vial with a 5-day-old virgin female from the baseline population and observed to copulate. Females were dissected immediately following copulation, at which time the entire sperm mass could be removed from the uterus. Sperm numbers were quantified by staining preparations with diaminophenylindole (DAPI, Sigma) and counting absolute numbers using epifluorescence microscopy as described by Pitnick & Markow

(1994b). The number of sperm transferred per copulation was determined for replicate A only (generation 16; $N = 10$ males per line).

Seminal receptacle length The seminal receptacle in *D. hydei* is extremely long and highly coiled (see fig 37 in Patterson, 1943) and therefore must be uncoiled prior to measurement. The coils are maintained through binding by trachea and tracheoles; these were either removed by forceps or broken by gently tugging on two distal points of the seminal receptacle using two forceps. After uncoiling the seminal receptacle, the entire organ, still attached to the uterus, was transferred from PBS to white paraffin oil, which facilitated straightening the organ out so that it could be measured using the ocular micrometer of a stereomicroscope. Females from replicate A (generation 14; $N = 10$ per line) were 29–31 days old and those from replicate B (generation 11; $N = 10$ per line) were 18–20 days old.

Female fecundity Five-day-old females from the virgin selection line were each paired with two randomly assigned 12-day-old virgin males from the base population in 8-dram food vials with live yeast. All females were observed to copulate. All trios of flies (one female and two males) were subsequently transferred to fresh food vials each day for a total of 5 days and all eggs laid were counted under a stereomicroscope. Mean daily fecundity was calculated for each female (replicate A: generation 15; replicate B: generation 12; $N = 35$ females per line). Females that failed to lay eggs on three or more days were excluded from the analyses.

Results

Direct response to selection

The response in testis length as a function of cumulative selection is shown in Fig. 1 for 12 and 11 generations of selection for replicates A and B, respectively. The response was consistently greater in the high than the low lines, with the increase in testis length outpacing the decrease by 54% and 60% in the two respective lines. Regressions of cumulative response on cumulative selection differential are all highly significant and indicate realized heritabilities (Falconer & Mackay, 1996) that vary from 0.45 to 0.72 (Table 2). Consistent with the high heritabilities, response in testes length to selection was rapid, generating nonoverlapping testis length distributions between lines within both replicates after only six generations. The distribution of testis lengths for all lines following selection is illustrated in Fig. 2.

The testes of *Drosophila* males, including those of *D. hydei*, continue to grow following eclosion (Pitnick,
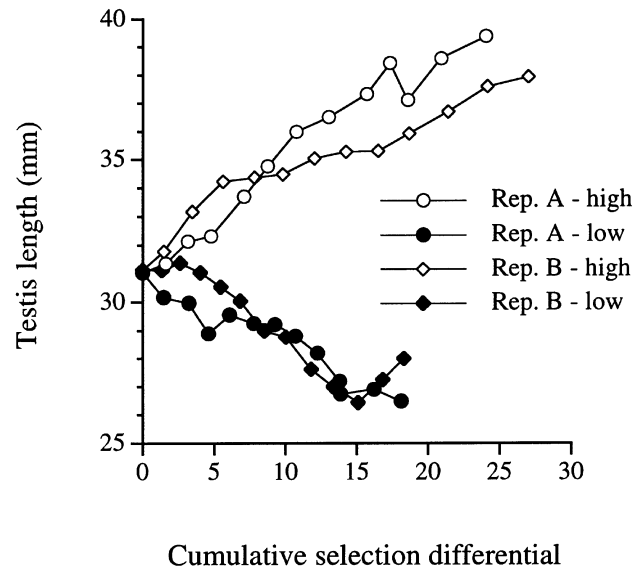

Cumulative selection differential

Fig. 1 Response to selection in two replicate lines of *Drosophila hydei* selected for increased and decreased testis length. Population means are presented as a function of cumulative selection differential.

Table 2 Realized heritabilities (the regression of cumulative selection response on cumulative selection differential) for 12 (replicate A) and 11 generations (replicate B) of *Drosophila hydei*

Replicate	Line	h^2 realized	P
A	High	0.72	0.0001
	Low	0.48	0.0001
B	High	0.45	0.0001
	Low	0.64	0.0001

1993). Examination of Fig. 3 reveals that artificial selection on testis length affected testis length at all stages of development, rather than simply extending the growth period. ANOVAs and Fisher's Protected Least Significant Difference tests (Fisher's PLSD) to compare among all three lines (high, base and low) for each measurement day indicate that, for replicate A, mean testis lengths were significantly different at eclosion ($F_{1,18} = 27.86$, $P < 0.0001$) with high line males having significantly longer testes than the base population males ($P = 0.025$) and both high line and base males having longer testes than low line males ($P < 0.0001$). Differences among all three lines persisted on all successive measurement days (all comparisons, $P < 0.01$). For replicate B, although there was also a significant difference in testis lengths on day 0 ($F_{1,18} = 6.83$, $P = 0.006$), PLSD tests indicate that the low line males had significantly shorter testes than did males from the base population ($P = 0.002$) and the high line
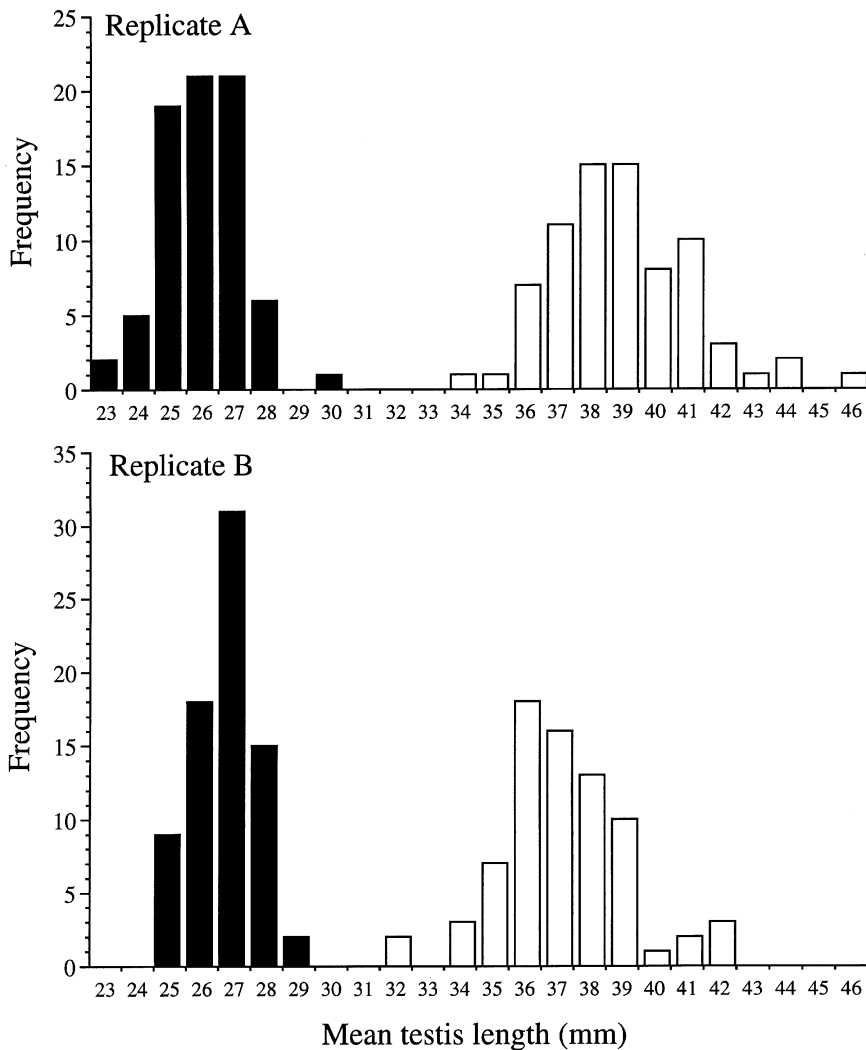
Fig. 2 Frequency distributions of mean testis lengths of *Drosophila hydei* for all selection lines ($N = 75$ males per line) during the last generation of selection.

($P = 0.016$), whereas the base and high line males did not differ ($P = 0.380$). High line male testes were not longer than base male testes until day 3, and not significantly so ($\alpha = 0.05$) until day 9.

ANOVA tests for differences in mean testis length among days within lines revealed significant differences between days 0 and 3 ($F_{1,12} = 30.59$ to 126.84, $P < 0.0001$) and between days 3 and 6 ($F_{1,12} = 52.31$ to 110.75, $P < 0.0001$) for all lines. Mean testis length was significantly longer on day 9 than on day 6 for all lines ($F_{1,12} = 8.92$ to 37.04, $P < 0.01$) except the replicate A high line ($F_{1,12} = 2.19$, $P = 0.16$). In none of the lines (only replicate A examined) were testes of 12-day-old males significantly longer than 9-day-old males ($F_{1,12} = 0.06$ to 0.84, $P > 0.164$). In summary, selection for increased testes length strongly influenced the size of testes at eclosion and the rate of testis growth during the first 6–9 days of adult life, but had little impact on subsequent growth (Fig. 3).

Correlated responses

Body size and testis mass Measurement of thorax length for males reared from standardized cultures following selection revealed a consistent pattern of high line males being significantly larger than low line males, with a greater response in replicate B than A (Fig. 4a; replicate A: $F_{1,290} = 4.46$, $P = 0.036$; replicate B: $F_{1,234} = 46.72$, $P < 0.0001$). However, measurement of total dry mass of standard-reared males following selection revealed no difference between replicate A high and low line males (Fig. 4b; $F_{1,38} = 0.146$, $P = 0.704$).

With no change in total dry body mass between the replicate A lines (Fig. 4b), the significant increase in dry testis mass in the high line relative to the low line (Fig. 4b; ANCOVA of testis mass treating body mass as a covariate: $F_{1,37} = 22.98$, $P < 0.0001$) indicates that high line males were making a greater total investment
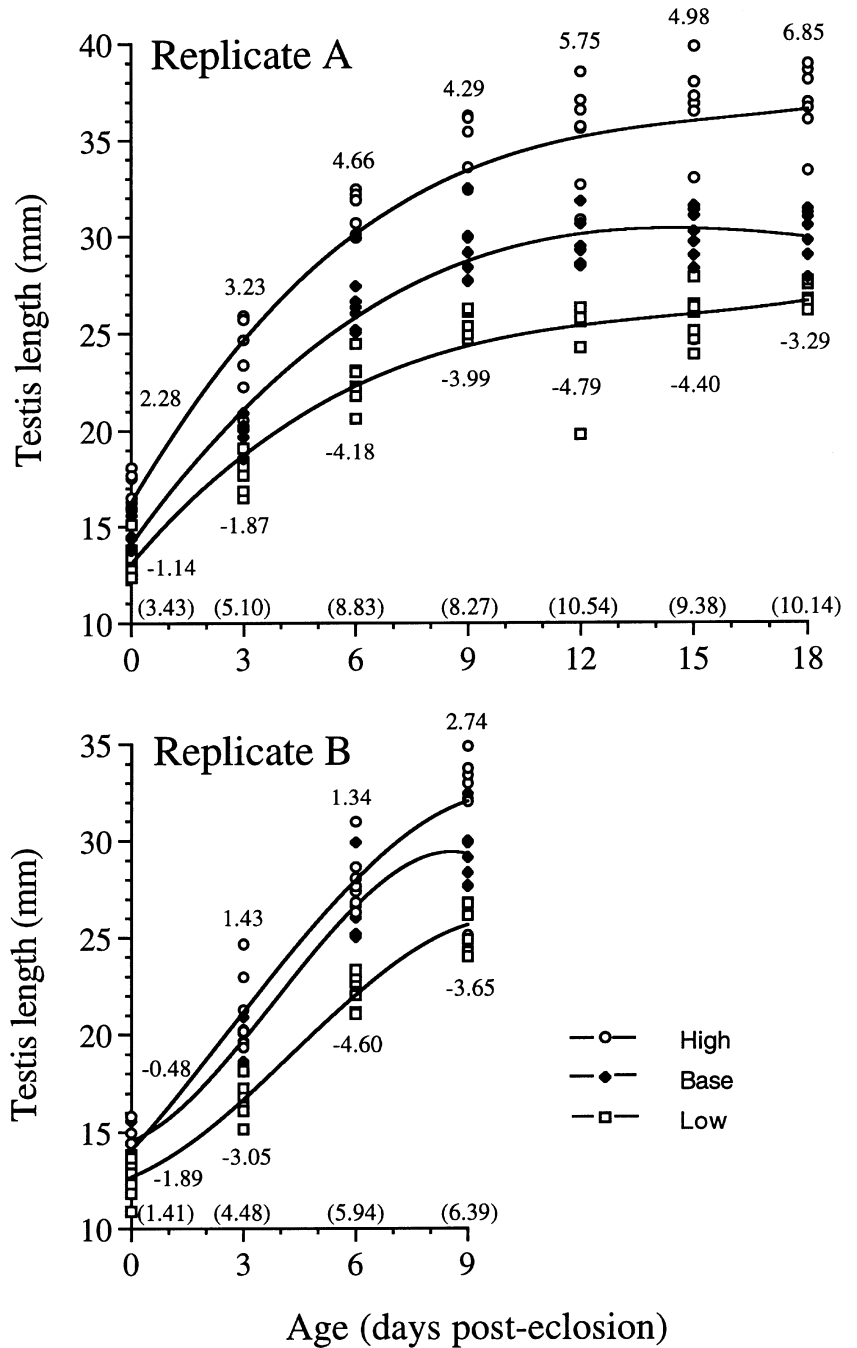
Fig. 3 Ontogeny of testis length in *Drosophila hydei*, beginning with eclosion (day 0), for males from all selection lines and the base population. Numbers above and below the curves indicate the difference in mean testis length between the high line and base population and between the base population and low line, respectively, for each 3-day measurement interval. The numbers in parentheses indicate the difference in mean testis length between the high and low lines.

in testes than were low line males. Dry testes mass represented 10.3% and 8.9% of total dry body mass in the high and low lines, respectively.

Sperm production and transfer Despite the great change in testis length realized through selection, there was very little correlated response in sperm length. Because sperm length showed a positive relationship with male thorax length ($F_{1,28} = 6.37$, $R^2 = 0.185$, $P = 0.018$), we contrasted sperm length among lines by ANCOVA, treating thorax

length as a covariate. Mean sperm length was longer in each high line than in the respective low line (Fig. 5; difference of 1.66 mm and 0.98 mm in replicates A and B, respectively), but only significantly so in replicate A ($F_{1,1,17} = 17.04$, $P = 0.0007$; replicate B: $F_{1,1,7} = 3.30$, $P = 0.11$).

The number of sperm bundles developing in the mid-testis cross-section is known to correlate positively with male size in *D. hydei*, and so differences in this variable between lines was analysed by ANCOVA with thorax
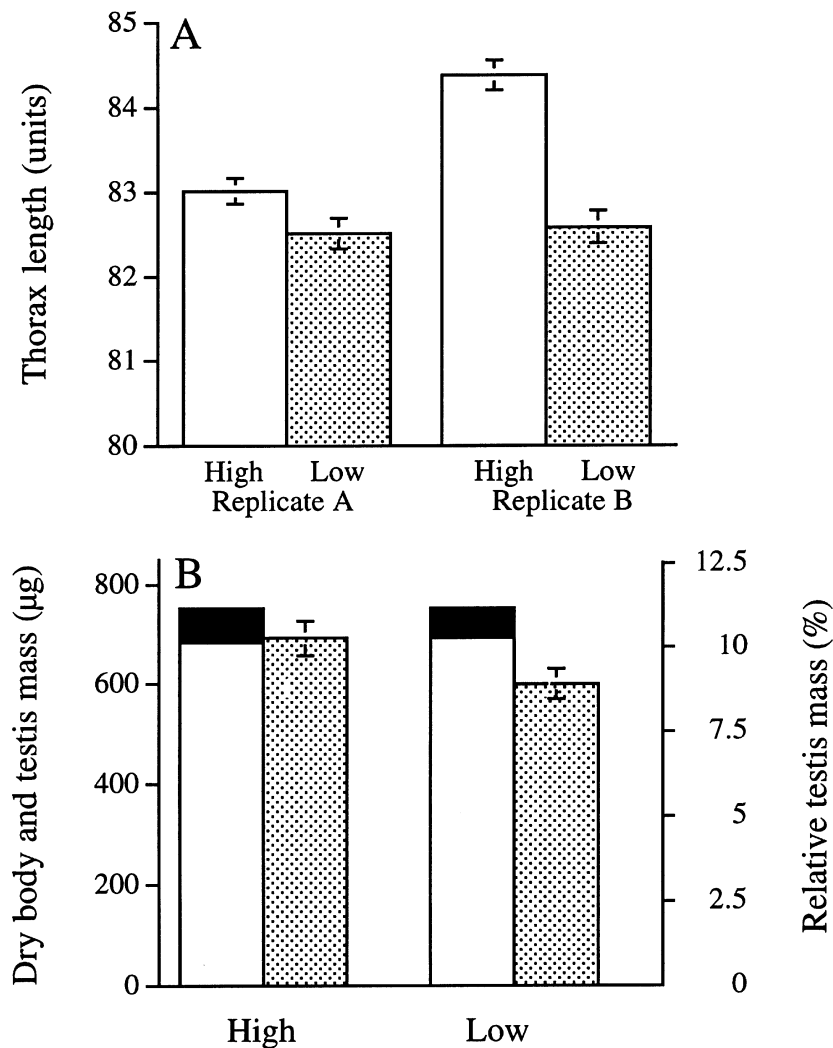
Fig. 4 Correlated responses in body size of *Drosophila hydei*: (a) mean thorax length for standard-reared flies following selection; (b) mean dry body mass (white), dry testis mass (black), and relative dry testis mass (hatched) for standard-reared flies from replicate A following selection. Bars indicate 1 standard error.

length treated as a covariate. No difference in sperm production between lines was found in either replicate (replicate A: $F_{1,1,21} = 0.02$, $P = 0.88$; replicate B: $F_{1,1,7} = 0.06$, $P = 0.82$).

The number of sperm transferred per copulation was examined in replicate A only. There was no significant difference among lines in the size of males or the size of the females inseminated. Neither copulation duration ($F_{1,18} = 1.88$, $P = 0.19$) nor the number of sperm transferred ($F_{1,18} = 0.79$, $P = 0.39$) differed between lines.

Development time, maturity, and longevity Egg-to-adult development time was examined for both sexes from all lines. Comparison of sex-specific development times (h) among lines reveals consistent, highly significant differences, with both sexes of high line flies taking longer to complete development than low line flies (Fig. 6, Table 3). Relative to the low line, high line development

time was delayed in males by 5.75 h (1.42%) and 5.18 h (1.27%) and in females by 14.01 h (3.52%) and 20.50 h (5.28%) in the two respective lines. Both sexes responded to the same degree in replicate A (no line × sex interaction), whereas females showed a significantly greater response than males in replicate B (significant line × sex interaction; Table 3). In most *Drosophila* species, males take longer to complete larval development than do females. This was found to be true in replicate B but, surprisingly, not in replicate A (Table 3). There were no significant vial effects (Table 3).

Examination of the proportion of males having mature spermatozoa in their seminal vesicles at different days following eclosion revealed that high line males exhibited a more protracted pattern of sexual maturation than did low line males (Table 4). Because the variable quantified in this experiment is the proportion of mature males for each day, the sample size is determined by the number of days, not by the number of
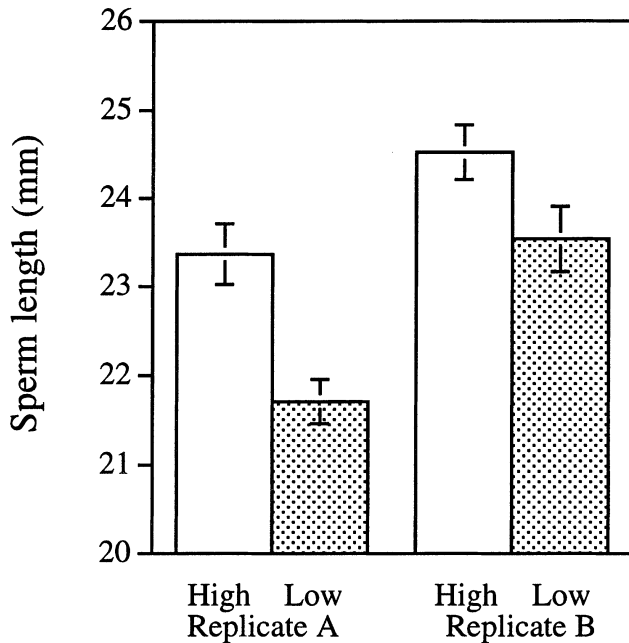
Fig. 5 Correlated responses in sperm length in *Drosophila hydei*. Bars indicate 1 standard error.

males. To achieve adequate statistical power therefore it was necessary to combine data from the two replicates, which did not statistically differ from one another. ANOVA comparing the arcsin (sqrt [% males mature]) on successive days revealed highly significant line ($F_{1,8} = 25.58$, $P = 0.001$) and age ($F_{3,8} = 18.29$, $P = 0.0006$) effects, with a nonsignificant line × age interaction effect ($F_{3,8} = 3.055$, $P = 0.092$).

Correlated responses in longevity were examined for males only, with separate tests of males maintained as virgins for life and those provided *ad libitum* access to females. Contrary patterns were observed in the two

replicates. In replicate A, virgin high line males lived 4.96 days (6.51%) longer than did virgin low line males ($F_{1,127} = 0.74$, $P = 0.39$) and nonvirgin high line males lived 17.11 days (20.46%) longer than did nonvirgin low line males ($F_{1,128} = 13.19$, $P = 0.0004$). In replicate B, virgin low line males lived 31.23 days (35.06%) longer than did virgin high line males ($F_{1,125} = 31.43$, $P < 0.0001$) and nonvirgin low line males lived 8.98 days (9.36%) longer than did nonvirgin high line males ($F_{1,129} = 3.49$, $P = 0.064$).

Female traits Female productivity did not differ among lines (replicate A: high line mean ± SE 233 ± 11, low line mean ± SE = 255 ± 12, $F_{1,62} = 1.88$, $P = 0.17$; replicate B: high line mean ± SE = 329 ± 18, low line mean ± SE = 293 ± 17, $F_{1,63} = 2.18$, $P = 0.14$). Although across the entire experiment the expected positive relationship between female size and productivity was found ($F_{1,127} = 18.42$, $R^2 = 0.127$, $P < 0.0001$), there were no significant differences in size between high and low line females within replicates.

Length of the female's seminal receptacle showed no significant relationship to female thorax length ($F_{1,38} = 2.37$, $R^2 = 0.059$, $P = 0.13$) and exhibited no correlated response to selection (replicate A: high line mean ± SE = 48.58 ± 0.61, low line mean ± SE = 48.24 ± 0.66, $F_{1,18} = 0.144$, $P = 0.71$; replicate B: high line mean ± SE = 51.51 ± 1.25, low line mean ± SE = 52.89 ± 0.67, $F_{1,18} = 0.938$, $P = 0.35$).

Discussion

Testis length and sperm production

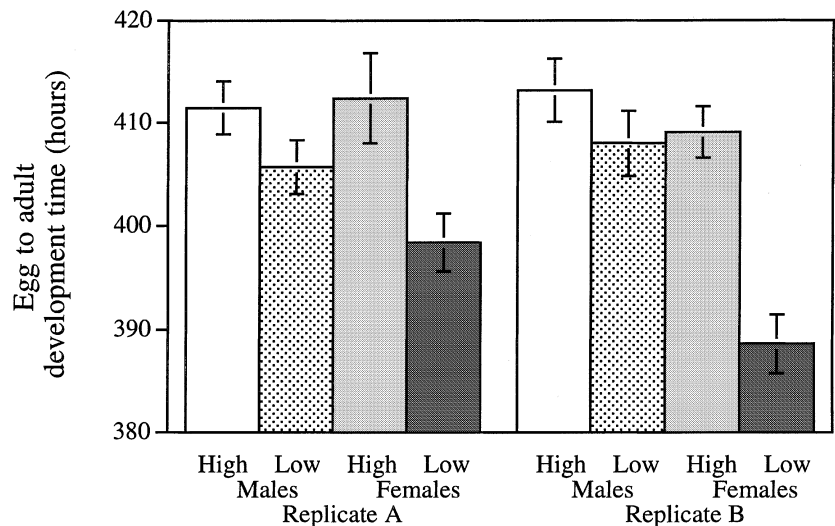We infer from the steady, continuous response to selection that testis length is polygenic in *D. hydei*, a

Fig. 6 Correlated responses in egg-to-adult development time of *Drosophila hydei*. Bars indicate 1 standard error.

Table 3 Analysis of variance of the correlated response in egg-to-adult development time of *Drosophila hydei*

Source	d.f.	Replicate A			Replicate B		
		MS	F	P	MS	F	P
Line	1	8178.6	12.84	0.0004	8216.4	18.30	0.0001
Sex	1	632.0	0.99	0.3201	6840.9	15.24	0.0001
Line × Sex	1	1141.8	1.79	0.1818	3107.3	6.92	0.0092
Vial	3	1789.1	0.94	0.4237	375.6	0.84	0.4751
Residual	A: 272	173288.9	—	—	448.9	—	—
	B: 204						

Table 4 Percentage of male *Drosophila hydei* with mature spermatozoa in seminal vesicles relative to days posteclosion ($N = 20$ males per line per age)

Male age†	Replicate A		Replicate B	
	High	Low	High	Low
6	0	0	0	0
7	45	35	45	55
8	75	100	55	90
9	75	100	60	95
10	85	100	90	100

† Days posteclosion.

conclusion supported by analysis of testis and sperm length in hybrids of *D. simulans* and *D. sechellia* (Joly *et al*., 1997). The relatively high heritability of testis length, approximately 0.57, is consistent with other reported heritabilities of testis size (Coulter *et al*., 1976; Islam *et al*., 1976).

The nearly perfect correlation between sperm and testis length across species suggests a possible genetic relationship between these two traits (Joly & Bressac, 1994; Pitnick & Markow, 1994b; Pitnick, 1996). However, the analysis of *D. simulans* and *D. sechellia* hybrids concludes that the two traits are genetically independent. In *D. hydei*, consistent across both replicates, high line males had significantly longer sperm than did low line males (Fig. 6a), demonstrating the existence of pleiotropy or gametic phase disequilibrium (Lynch & Walsh, 1998) between sperm and testis length for this species. It is notable, however, that changes in sperm length were minuscule compared to the divergences in testis length (Figs 1 and 2). We therefore conclude that some genes for testis length in *D. hydei* are genetically independent of genes for sperm length, and these were the genes that predominantly responded to our selection programme.

The functional relationship between sperm and testis length probably contributed to the consistently greater response for increased over decreased testis length. For example, an equivalent response by the low lines would have resulted in testes that are shorter than the sperm. Our inability to generate substantially shorter sperm despite intense selection suggests either a lack of genetic variation in sperm length or a greater strength to selection maintaining the wild-type sperm length. Because our breeding design included random mate assignment and enforced monogamy, any selection imposed by sperm competition was precluded. To the extent that selection prevented sperm length from diverging, therefore, this selection was derived from noncompetitive sperm–female (Pitnick *et al*., 1999; Presgraves *et al*., 1999) or sperm–egg interactions (Karr, 1996; Pitnick & Karr, 1998).

The number of sperm produced and the number transferred to females both show significant positive relationships with sperm length (and therefore testis length) across species (Pitnick, 1996). In addition, the number of sperm produced and transferred shows significant positive phenotypic correlations with male thorax length (as does testis length) in *D. hydei* (Pitnick & Markow, 1994a). The current study suggests that these relationships are not the result of pleiotropy between testis length and sperm production and use.

Correlated evolution of life history traits

Numerous hypotheses have been forwarded to explain the dramatic divergence in sperm length in the genus *Drosophila* (Pitnick & Markow, 1994b; Pitnick *et al*., 1999); still, the adaptive significance of giant sperm remains a mystery. In sharp contrast to the benefits, numerous costs of producing relatively long sperm have been identified, primarily through comparative analyses. Specifically, after controlling for phylogenetic effects, there are positive interspecific relationships between sperm length and the variables: relative testis mass (Pitnick, 1996) and male age at first reproduction (Pitnick *et al*., 1995a) and negative relationships between sperm length and the numbers of sperm produced and transferred to females (Pitnick, 1996).

Sperm length is also significantly positively correlated with body size (Pitnick *et al*., 1995a; Pitnick, 1996); it has been postulated that this relationship is the selective result of larger size mitigating the costs of producing relatively long sperm (Pitnick & Markow, 1994a; Pitnick *et al*., 1995a; Pitnick, 1996).

The observed pattern of correlated responses to testis length selection reveals that some of these macroevolutionary relationships probably result from genetically determined microevolutionary trade-offs. First, a consistent relative increase in body size in the high lines, as measured by thorax length, was observed in both replicates (Fig. 4a). This correlated response could result from either pleiotropy or selection, as male size may have placed energetic limits on the response in testis length to selection. It is clear from the divergence between the high and low lines in dry testis mass (Fig. 4b), which was independent of dry body mass, that high line males had evolved to make a greater total energetic investment in testes production and maintenance. This difference between the lines may be pivotal to interpreting the correlated responses in egg-to-adult development time and adult maturation time.

Selection influenced the size of testes at all stages of development. In *D. hydei*, the testes begin as small elliptic organs in third instar larvae with germ cells developed only to the primary spermatocyte stage. During pupation, the testes transform into a pair of coiled tubes and the first meiotic divisions occur. By eclosion, elongating spermatids can be found within the testes (Hennig & Kremer, 1990). The consistently greater length of high line relative to low line testes at eclosion (Fig. 3) indicates that a greater proportion of larval-derived nutrition was dedicated to testicular tissue development in third instar larvae and/or pupae in the high lines. This finding is consistent with the significant increase in egg-to-adult development time observed in the high vs. low line flies (Fig. 5). The *a priori* prediction of this response was made for males only; we were therefore surprised to observe not only the same pattern, but differences of greater magnitude, among females.

Similarly, divergence among lines in testis length resulted in a consistent correlated response in posteclosion development time. The testes of most *Drosophila* species are not fully developed at eclosion and thus continue to grow during an adult maturation period that is highly variable among species. The amount of growth required is positively correlated with sperm length (Pitnick *et al*., 1995a). In *D. hydei*, which has 23.5 mm-long sperm (Pitnick & Markow, 1994a), the testes approximately double in length in the week following eclosion (Fig. 3). We found that high line

males consistently exhibited a pattern of more protracted maturation than did low line males (Table 4). Thus, both egg-to-adult and posteclosion maturation times contribute to delayed reproduction in flies with relatively large testes. According to Stearns (1992) 'Age at maturity is pivotal, for fitness is often more sensitive to changes in this trait than to changes in any other.' These microevolutionary trade-offs are consistent with the macroevolutionary patterns observed in the *Drosophila* lineage (Pitnick *et al*., 1995a). No consistent correlated response in longevity was observed.

Correlated evolution of female traits

Independent of phylogenetic effects, across *Drosophila* species there are highly significant positive relationships between the length of sperm (and hence of testes) and length of the female's primary sperm-storage organ, the seminal receptacle (Pitnick *et al*., 1999). This relationship has been suggested to result from correlated evolution caused by sexual selection on sperm–female interactions (Pitnick *et al*., 1999). Lack of any correlated response in seminal receptacle length in any of the selection lines indicates a lack of pleiotropy between testis and receptacle length.

In summary, comparative studies of *Drosophila* species have revealed phenotypic correlations among key reproductive and life history traits. However, there is no necessary pattern of genetic variation and covariation underlying these phenotypic patterns (Rose & Charlesworth, 1981; Falconer & Mackay, 1996). Here we detected through the analysis of correlated responses to direct selection on testis length a pattern of genetic covariation among testis-length and critical life-history traits including body size, development time and adult maturation time. These patterns reveal consistency between microevolutionary process in one species and macroevolutionary pattern across a phylogeny (Hansen & Martins, 1996) representing a large portion of the entire *Drosophila* lineage (Pitnick *et al*., 1995a). Results of this study therefore indicate the potential for testis length evolution to facilitate or constrain the evolution of sperm length in *Drosophila* (Lande, 1979, 1982).

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