Genetic and environmental effects on sperm size in *Drosophila melanogaster*

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Summary

Sperm length is a quantitative genetic trait, important to sperm competition, which varies widely across taxa, as well as within species. There is currently no evidence of an environmental effect on sperm length, which has led to the conclusion that sperm length is maintained under harsh conditions and traded-off against other things, such as sperm numbers. In addition, sperm size has a high heritability and it is therefore important to account for genotype when investigating condition effects.

I used hemiclonal males of *Drosophila melanogaster* to test for a condition effect as well as a genetic effect on sperm length, and to examine the genotype × environment interaction on this trait.

There was a large effect of genotype on sperm size, and smaller but significant effects of environment and genotype × environment. However, the genetic effect on sperm size was largely due to a single hemiclonal line. Finally, there was also a weak relationship between body size and sperm length.

The genotype by environment interaction found may explain why the large variation in sperm size is maintained, even though some types may have a selective advantage. This is since different genotypes may be favored in different conditions.
Background

Sperm competition
When Parker (1970) first described the phenomenon of sperm competition, the competition within a single female between the sperm from two or more males for the fertilization of the ova, the thought revolutionized the whole field of evolutionary biology. Birkhead and Möller (1998) summarized the numerous studies in the field of sperm competition since, and explained why it was such a ground-breaking idea. Earlier the focus had been on precopulatory sexual selection, where traits such as territory, mating rituals and ornaments of males were considered. The theory of sperm competition instead recognized that sexual selection can also take part after mating itself. Thus, a whole variety of new traits, including sperm morphology and behaviour, as well as morphology of the female reproductive tract, became the subject of interest. Gametes and other postcopulatory traits have been studied in a wide diversity of taxa, in organisms with separate sexes as well as hermaphrodites. External as well as internal fertilizers have been investigated and even pollen competition in flowering plants.

Selection and sperm morphology
Several characteristics of sperm have been suggested to play a role in sperm competition. Parker (1982) argued that sperm should be tiny and numerous to have a selective advantage. The explanation for this is that sperm competition can be compared with a lottery or a raffle: the more tickets you have, the more chance you have of winning the prize. The relative number of sperm from each male in the competition plays a great role, but cannot fully explain variation in fertilization success (Birkhead and Möller 1998, Simmons 2001). Variation in sperm behaviour, such as swimming speed and sperm morphology also determine the outcome of the competition (Ward 1998, Snook 2001, Pitnick et al. 2003). Sperm size can vary enormously across species of the same genus, especially Drosophila (Pitnick et al. 1995), but also in the same species - variation between individuals has been observed for species from virtually every corner of the animal kingdom (Ward 1998, Morrow and Gage 2001). However, the fertilization environment is also an important factor to consider here. For example, the size of the female sperm storage-organs has rapidly evolved among species in the same way as sperm, and has been shown to co-evolve with sperm length (Pitnick et al. 1999, Pattarini et al. 2006). Within-species variation of this trait may therefore account for some of the maintenance of variation in sperm length.

One argument for why longer sperm could be better in sperm competition is that they may have higher swimming speeds, since longer flagella are predicted to generate greater flagellar forces (Katz and Drobnis 1990, Gomendio and Roldan 1991). Another hypothesis is that longer sperm may be better at displacing and resisting displacement by smaller sperm in the female reproductive tract (LaMunyon and Ward 1998, Pattarini et al., 2006). There is evidence for a positive relationship between sperm length and intensity of sperm competition in many diverse animal taxa such as butterflies (Gage 1994), mammals (Gomendio and Roldan 1991), birds (Briskie and Montgomerie 1992), nematodes (LaMunyon and Ward 1999) and frogs (Byrne et al. 2003). In addition, there have been some experiments showing that long sperm may be favoured in post-copulatory sperm competition (Joly et al. 1991, Radwan 1996, Pattarini et al. 2006).

Evolution of quantitative traits
For evolution in a quantitative trait, such as sperm size, to occur, there must be both genetic variation and either sexual or natural selection or drift (Futuyma 2005). One of the aims of
this project was to establish whether there is genetic variation in sperm length. The phenotype is a result of both genetic and environmental effects. When the same genotype produces different phenotypes in different environments it is called phenotypic plasticity (Conner and Hartl 2004). A second aim of the project was therefore to examine the environmental effect on sperm size, as well as the genotypic $\times$ environment interaction.

Environmental effects on sperm size
When organisms develop with limited resources, they have to decide where in the body to put the energy. With limited resources, sizes of traits may be traded-off against one another. With limited resources, many organisms put more investment in reproductive activity which can reduce body size. (Stearns 1992). Internal organs and hence sperm size and number may also be affected. Pitnick and Markow (1994) showed that smaller males of *Drosophila hydei* had shorter and thinner testes compared to larger individuals, and that this meant that the males may have less energy to put in to testicular tissue growth as well as less space in the abdominal cavity. They also showed that smaller males invest relatively more energy in testicular tissue than bigger males and the energy loss is compensated by producing fewer numbers of sperm. This trade-off has been indicated further in similar studies (Gage and Cook 1994). Hellriegel and Blanckenhorn (2002) studied gametic investments in yellow dung flies and found an increase in testis size with body size, although the relationship was non-linear, but no evidence of a trade-off between testis size and sperm length.

A clear interspecific relationship between sperm length and body size in *Drosophila* (Pitnick 1996) has been found. However, several studies did not find a similar intraspecific relationship (e.g. Ward and Hauschteck-Jungen 1993, Pitnick and Markow 1994, Hellriegel and Blanckenhorn 2002) although recent work of Amitin and Pitnick (2007) did find a significant positive relationship between thorax length and sperm length as well as seminal receptacle length in *Drosophila melanogaster*.

Gage and Cook (1994) tested for sperm length in male moths reared in different environmental conditions. They found that with limited resources, sperm length tends to stay the same and the males trade this off by sacrificing sperm numbers. The conclusion here is that longer sperm have a competitive advantage and is an important trait to maintain even under harsh conditions. Amitin and Pitnick (2007) tested for the same trait in *Drosophila melanogaster*, again with different larval densities as the environmental factor. Although there was no statistically significant change, a trend for reduced sperm length was found at higher larval densities. In both these cases, the experiments have been carried out with a random sample from the population. Hellriegel and Blanckenhorn (2002) found a change in sperm size in the dung fly *Scathophaga stercoraria* with temperature change, but not with restricted resources. They used brothers to minimize genetic variation and also found that family of origin influences sperm length. This is further evidence that the relatedness of individuals from which sperm length is measured should be accounted for in the experiments.

Hemiclonal analysis
In this study, I used a method called hemiclonal analysis. It is a relatively new method where flies are modified and crossed so they will share at least half their genome in common, but they are also expressed in a random genetic background (see materials and methods for detailed description). They are analogous to a group of brothers with a relatedness of 0.5. The difference is that brothers share half their genome on average, but hemiclones will always share 50% or more. The advantage of using hemiclones is that we know the relatedness of the flies and can investigate the genetic component in a trait. Many individuals can be generated
from the same hemiclone line which gives multiple observations of the phenotype of one genome. With the relatedness known, it is possible to partition total phenotypic variance among and between hemiclone lines. Some variation is due to differences among hemiclones and some is due to differences between the lineages (Chippindale et al. 2000).

**Aims**

I examined the environmental effect on sperm length by rearing *Drosophila melanogaster* on normal and limited food supplies respectively. By using hemiclones (see below for description), I examined genetic variation for sperm length in different lineages, and whether these lineages were affected by the change in larval density. Furthermore, I investigated whether these lineages reacted differently to the environmental changes, and whether body size correlated with sperm size.
Results

**Pilot experiment on treatment effect**

Wing length and dry mass of LHm-male *Drosophila* reared at normal and limited food supplies were significantly different from one another (wing length \( t = 12.39; \) D.F. = 78; \( p < 0.0001 \); dry mass \( t = 17.97; \) D.F. = 17; \( p < 0.0001 \)). Males in low-food vials had wings that were 7.49% smaller than males from normal-food vials and had 37.17% less dry body mass (Fig. 1).

![Figure 1](image1.png)

**Figure 1.** Plot of (a) mean winglength and (b) dry mass per individual of *Drosophila melanogaster* by low- and high-food treatments. Green diamonds represent 95% confidence intervals around the mean.

**Mean sperm size for LHm-population**

Mean sperm size, estimated from flies of the wild-type LHm stock, was 1882.6 microns ± 1 S.D. 51.0 (n = 20) and was normally distributed (Shapiro-Wilk \( W = 0.98; \) P = 0.94). Sperm size ranged from 1635.7 to 2023.4 microns. There was significant difference in sperm length among males (REML male variance component = 1737.4; upper and lower 95% C.I. = 45.7-3429.2; total variance = 5181.5) (Fig 2).

![Figure 2](image2.png)

**Figure 2.** Means and standard error bars for sperm length in 20 flies from the LHm *Drosophila*-population (n = 4 sperm per male).
Condition experiment
There was a similar treatment effect in the main experiment as in the pilot experiment. Wings were on average 8.10% shorter on flies from low-food vials.

There was a significant relationship between body size (measured as wing length) and sperm size in the normal-food treatment \( (t = 2.09; P = 0.0401; R^2 = 0.058; \text{Fig. 3a}) \). Conversely, there was no significant relationship between body size and sperm size in the low-food treatment \( (t = 0.29; P = 0.7699; R^2 = 0.001; \text{Fig. 3b}) \).

Figure 3. Linear regression of sperm size on wing size in hemiclonal *Drosophila melanogaster*, in (a) high-food and (b) low-food treatments.

There was a significant effect of both hemiclone and male identity on sperm length (Table 1). There was also a significant effect of treatment on sperm length (fixed effect \( F = 7.08; \text{D.F.} = 13.7; p = 0.0189 \)). Furthermore, there was a significant hemiclone \( \times \) treatment interaction (genotype \( \times \) environment) (Fig. 4). Hemiclone 7 was found to be unusually short, and when excluded, the significant hemiclone effect disappeared. However, mean sperm size from this hemiclone falls within the range of sperm lengths found in the wildtype LHm-population.

Data for mean sperm length of this hemiclone from a previous replicate was also found to be of a similar size.

**Table 1.** REML (restricted maximum likelihood) variance component estimates of sperm length in *Drosophila melanogaster*.

<table>
<thead>
<tr>
<th>Random Effect</th>
<th>Variance Component</th>
<th>Std Error</th>
<th>95% Lower</th>
<th>95% Upper</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemiclone</td>
<td>1898.0</td>
<td>822.5</td>
<td>943.7</td>
<td>5592.8</td>
<td>48.0</td>
</tr>
<tr>
<td>Hemiclone*treatment</td>
<td>332.3</td>
<td>153.8</td>
<td>159.0</td>
<td>1077.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Male</td>
<td>hemiclone]</td>
<td>303.0</td>
<td>90.1</td>
<td>182.4</td>
<td>600.0</td>
</tr>
<tr>
<td>Residual</td>
<td>1424.8</td>
<td></td>
<td></td>
<td></td>
<td>36.0</td>
</tr>
<tr>
<td>Total</td>
<td>3958.0</td>
<td></td>
<td></td>
<td></td>
<td>100.0</td>
</tr>
</tbody>
</table>
Figure 4. Genotype × environment interaction plot of *Drosophila melanogaster*. Each point (in the legend as well as in the plot) represents the least square means sperm length from a hemiclone line measured in low- and high-food environments. The genotype effect is represented by the separation of the points on the vertical axis. The treatment effect is shown by the general trend of the lines downwards from right to left. The genotype by environment effect is represented by the crossing of the lines, i.e. the reaction gradient is not the same for each hemiclone.
Discussion

There is significant genetic variation for sperm size across hemiclone lines, which confirms the thesis of Hellriegel & Blanckenhorn (2002) that genetic family has a major influence on the sperm morphology characters. The hemiclone effect is estimated to account for 48 % of the total phenotypic variance (Table 1). The heritability for hemiclones, who share half their genome in common, is estimated as 2 times the variance component out of the total phenotypic variance, which gives a very high heritability for sperm length ($h^2 = 0.96$). This high level of heritability is consistent with previous estimates of the heritability of sperm characters (Beatty 1970, Simmons and Kotiaho 2002).

Sperm length from one of the hemiclones was unusually short, and when removed from the analysis, the significant hemiclonal effect disappeared. The shortness may be due to unknown mutations in the lineage affecting sperm length, rather than experimental error, since it still falls within the range of normal population sperm length (see results on mean sperm size for LHM-population as well as Amitin and Pitnick 2007). This indicates that more hemiclone lines may have to be added to have a greater sample size of genomes. This may fill in the sample gaps and show a normal distribution of sperm length across genomes.

The change in larval density was carried out to assure that this factor would create a significant change in the physiology of Drosophila. The tested treatment effect had the desired effect in the pilot experiment, where body size was effectively changed. Furthermore, using a consistent and repeatable measuring technique, as well as a high number of replicates, I was able to see a significant treatment effect on sperm size, indicating it is a plastic trait, something which previous authors either did not see (Gage and Cook 1994, Hellriegel and Blanckenhorn 2002) or did not find a significant trend for (Amitin and Pitnick 2007).

I found a weak but significant correlation between body size and sperm size. Only one experiment has shown this before (Amitin and Pitnick 2007). Body size only accounts for 5% of the variation in sperm length so it cannot fully explain the large intraspecific variation in this trait. The correlation between the traits only exists with normal food supplies, so the fact that the body size gets smaller is inconsistent with the change in sperm length I observed. The genetic component seem to have a much greater effect.

I also found a significant genotype × environment interaction that accounts for 8% of the total variance in sperm length. This interaction indicates that different genotypes produced different sperm lengths under different environmental conditions. If there is an advantage of having longer sperm, which has been implicated by earlier studies of sperm competition (Joly et al. 1991, Radwan 1996, Pattarini et al. 2006), then selection may favour different genotypes in varying environments. This would also be true if shorter sperm would be favoured. If the larval environment frequently varies in quality then this interaction could be one explanation to why the great variation in sperm length is maintained (e.g. Morrow and Gage 2001). Directional selection would otherwise eliminate genetic variation in this quantitative trait.

Since no-one has tested for this interaction before, I can neither confirm nor question earlier results. More work needs to be done in terms of a greater sample of hemiclones, but also, most interestingly, if sperm numbers were to be looked at as well. There is a predicted sperm size/sperm number trade-off (e.g. Gage and Cook 1994) which could not be examined in this project.
Materials and Methods

Measuring sperm and body size
Hemiclone males were dissected in PBS-buffer. The reproductive system was extracted (Fig. 5) and the seminal vesicles cut off and placed on a microscope slide with buffer, where they were punctured with fine forceps. The slide was agitated with more buffer to increase the dispersion of sperm, and when dry, rinsed with deionised water. This will wash off the salt crystals but keep the sperm stuck to the slide. Sperm were photographed using a Micropublisher 3.3 digital camera attached to a phase-contrast microscope at 40x magnification. These images were then measured using segmented-line tool in the software ImageJ (Rasband 1997-2006). 4-5 sperm from 4-5 males per hemiclone were measured (as recommended by Pattarini et al. 2007), for 15 hemiclone lines. This method is highly repeatable (repeatability $R = 0.99$ for 50 sperm) Data were analyzed in JMP (SAS Institute 1989-2003).

Figure 5. The reproductive tract of the male fruit fly, with the arrow pointing to the sac-like seminal vesicles.

Body size was estimated by measuring wing size and dry mass. Wings were cut off and placed with adhesive tape on a microscope slide and photographed as above (using bright field-illumination). Length was measured using the straight line tool in ImageJ (Rasband 1997-2006), from the intersection of the anterior cross vein and longitudinal vein 3 (L3) to the intersection of L3 with the distal wing margin (Fig. 6) (Partridge et. al. 1987). For the dry mass measurement, groups of flies were dried in the oven at 60°C for two days and then weighed 28-57 flies at a time using an electronic analytical balance accurate to five decimal places.

Figure 6. The two landmarks used for measuring wing length in *Drosophila melanogaster*. 
Mean sperm size for LHm-population
20 randomly sampled males from an LHm-population of *Drosophila melanogaster* were dissected as described above. 4 sperm from each male were measured. Fragmented sperm were excluded by not including measurements less than 1600 microns (Amitin and Pitnick 2007).

Pilot experiment on treatment effect
16 males and 16 females from a large, outbred population (LHm) of *Drosophila melanogaster* were placed together in an egg-chamber and allowed to lay eggs on a petridish with cornmeal-molasses-agar food covering the opening. 200 eggs were cut from the cookie and 100 eggs were placed in a vial with normal food-supplies and 100 in a vial with 1 millilitre of food (Byrne and Rice 2006). This was replicated 10 times. On the 12th day, the adult flies were collected and both wings taken off. Wings were measured using the technique described above. Another replicate of the experiment was made, where dry mass was estimated of males and females separately, see technique above.

Hemiclone culturing
When creating hemiclones, the aim is to capture a whole haplotype of the genome. This is done by using a mutated form of *Drosophila melanogaster* (DX-CG), that have a double fused X-chromosome and a Y-chromosome. Since insects have a dosage-effect sex determination, these flies are female despite the Y-chromosome. When crossed with wild-type males, the fused double-X forces the single X-chromosome from the male gamete to be inherited to the sons. The DX-CG flies also have multiple-translocations of their second and third autosomes (*Drosophila melanogaster* only has four chromosomes where the fourth is a dot-chromosome making up less than 1% of the genome). They are illustrated as one single chromosome in the appendix A, but are in reality two chromosomes. The heterozygous males resulting from the first cross can again be crossed with DX-CG females to generate more of the same clones (as shown in the appendix). All the male and female offspring will be of the same genotype since triple-X and double-Y combinations are lethal. The same thing is true for genomes with a split-up multiple translocation chromosomes, where there will be too few or too many copies of some genes.

For generating the actual hemiclones, the single haplotype of interest (red chromosomes in appendix A) needs to be expressed in a random genetic background. The clone males are crossed with DX-LHm females, that is a female with fused X-chromosome where the rest of the genome is wild-type. 75% of the genotypes are lethal due to the same factors as described above. Out of the surviving flies, males will be of two types: target hemiclones and males with multiple-translocation chromosomes. These are separated with the help of dominant brown eye-colour markers on the multiple translocation chromosomes (wild-type flies have red eyes).

Hemiclone experiments
40 virgin DX-LHm females and 15 clone males were placed in egg-chambers on a petridish with cornmeal-molasses-agar food. This was done for 15 separate hemiclone lines. After 16 hours, 800 eggs were taken from each cookie and placed 400 in a vial of normal food level and 400 in a vial of 1 ml of food. 400 eggs were used rather than 100 as in the pilot experiment, due to a 75% death rate due to lethal genotypes arising with unbalanced numbers of sex chromosomes or proportions of autosomes. Plastic sleeves were inserted in the vials on the 1st day, where the larvae were allowed to pupate, and the sleeves were transferred to fresh vials on the 8th day. This procedure was carried out to improve the yield of flies from these
larval-competition vials, particularly for the high-larval density treatment where the food becomes very dry. Flies were harvested and sorted (Fig. 7) under light CO₂ anaesthesia on the 12th day. One wing per fly was taken off for measuring. Dissections and measurements were carried out as described above. See figure 7 for a simplified illustration of the procedure.

**Figure 7.** Experimental design. Target red-eye males are separated from brown-eyed which still have the multiple translocation chromosome.

**Statistical analysis**
The model used to examine the hemiclone and treatment effects on sperm length included treatment as a fixed effect, and hemiclone, hemiclone × treatment and male nested within hemiclone as random effects. The factor “Male” was nested within hemiclone because males from each hemiclone all shared the same larval and adult environments.
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References


Appendix A: Capturing and amplifying a hemicleone.