Sperm length is not influenced by haploid gene expression in the flies Drosophila melanogaster and Scathophaga stercoraria

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Recent theoretical models have postulated a role for haploid–diploid conflict and for kin selection favouring sperm cooperation and altruism in the diversification and specialization of sperm form. A critical assumption of these models—that haploid gene expression contributes to variation in sperm form—has never been demonstrated and remains contentious. By quantifying within-male variation in sperm length using crosses between males and females from populations that had been subjected to divergent experimental selection, we demonstrate that haploid gene expression does not contribute to variation in sperm length in both Drosophila melanogaster and Scathophaga stercoraria. This finding casts doubt on the importance of haploid–diploid conflict and kin selection as evolutionary influences of sperm phenotypes.

Keywords: haploid selection; sperm competition; sperm variation; Diptera; Scathophaga stercoraria

1. INTRODUCTION

Throughout the Metazoa sperm are evolutionarily labile and diverge rapidly, exhibiting a bewildering array of morphological specializations (reviewed by Pitnick et al. 2009a). Although understanding of the adaptive significance of variation in sperm form is meager, post-copulatory sexual selection, occurring through competition to fertilize ova, is widely regarded as the primary agent for sperm diversification (reviewed by Pitnick et al. 2009a; Pizzari & Parker 2009). Recent theoretical treatments of sperm adaptation, with a focus on sperm conjugal and heteromorphism, have postulated roles of kin selection and multi-level selection (Immler 2008; Pizzari & Foster 2008; Pizzari & Parker 2009).

Sperm conjugal occurs when two or more sperm physically unite to enhance motility and/or transport through the female reproductive tract, with sperm typically disassociating from one another only after reaching the site of sperm storage or fertilization (reviewed by Immler 2008; Pitnick et al. 2009a). Sperm heteromorphism is the tightly regulated production by individual males of more than one morphological class of sperm, with the distinct sperm types specialized for performing different reproductive functions and only one type typically participating in fertilization (reviewed by Buckland-Nicks 1998; Swallow & Wilkinson 2002; Till-Bouttraud et al. 2005). Applying social evolution theory, Immler (2008), Pizzari & Foster (2008) and Pizzari & Parker (2009) consider the role of haploid–diploid conflict, arising from the disparate fitness interests of males and of individual sperm they produce, and postulate adaptive variation in sperm form arising through selection for sperm altruism, cooperation and spite at the level of individual spermatozoa (also see Parker & Begon 1993). These authors correctly point out that critical testing of these ideas includes demonstration that some among-sperm morphological variation is attributable to haploid gene expression.

The extent to which within-ejaculate variation in sperm form and function is a consequence of haploid gene expression, and the target of haploid selection, is an open question (Joseph & Kirkpatrick 2004). It is widely held that spermatozoa are terminally differentiated cells (i.e. transcriptionally inert, Hecht 1998). Nevertheless, it is now clear that post-meiotic gene expression occurs (Erickson 1990; Wang et al. 2001; Schultz et al. 2003; Dadoune et al. 2004; Namekawa et al. 2006). The precise timing of expression has not, however, been determined for most of the numerous genes required for spermatogenesis and for sperm function (but see Barreau et al. 2008). Such determination is complicated by the fact that, due to DNA condensation and repackaging during spermatogenesis, some genes required for spermatid function will be transcribed in primary spermatocytes, with the mRNAs stored in spermatids to be translated later during sperm elongation (reviewed by Schäfer et al. 1995; Hecht 1998; Dorus & Karr 2009). Consequently, intra-male variation in sperm phenotypes may predominantly be determined by testicular gene expression and hence the diploid genome of the male (Eddy 2002).

Even with post-meiotic gene expression, it is altogether unclear how much of the phenotypic variation among sperm within an ejaculate is attributable to allelic variation among haplotypes, and hence amenable to haploid selection (Joseph & Kirkpatrick 2004). A phenomenon of incomplete cytokinesis throughout spermatogenesis, resulting in all of the cells derived from each spermatogonial cell developing within a common syncytium, is taxonomically widespread, occurring even in diploblastic...
basal metazoan lineages (Gaino et al. 1984). Large intercellular bridges connect the cells and permit the sharing of cytoplasmic constituents. This arrangement has been experimentally demonstrated with mice to result in phenotypically diploid spermatids, despite haploid expression of genetic differences between developing sperm cells (Erickson 1973; Braun et al. 1989). To date, no characteristic of sperm form or function has been demonstrated to exhibit natural variation resulting from haploid gene expression.

The sperm attribute most widely investigated with regard to post-copulatory sexual selection is flagellum length (reviewed by Pitnick et al. 2009a; Pizzari & Parker 2009). Here we use crosses between discrete laboratory populations of both Drosophila melanogaster and Scathophaga stercoraria that had been subjected to divergent experimental selection for sperm length (i.e. long-sperm and short-sperm populations, Miller & Pitnick 2002; Dobler & Hosken in press) to definitively determine whether or not haploid gene expression contributes to sperm length in these two species. Specifically, given the segregation of alleles at multiple loci contributing to sperm length (Miller et al. 2003), the hypothesis of haploid gene expression generates the prediction that sperm length should be more variable within ‘hybrid’ among-line males than within males from either pure (long-sperm or short-sperm) population. On the other hand, if sperm length is strictly determined by each male’s diploid genome, then no difference between hybrid and ‘pure’ crosses in within-male variation should be observed.

2. MATERIAL AND METHODS

(a) Experimental populations and culturing

All flies were from experimentally evolved populations of Drosophila melanogaster and S. stercoraria selected bi-directionally for sperm length, with one replicate pair of short-sperm and long-sperm populations for D. melanogaster and three replicates for S. stercoraria. Control sperm length selection lines were not used in the present investigation. Details of the source populations and selection protocols are provided in Miller & Pitnick (2002) and Dobler & Hosken (in press).

(i) Drosophila melanogaster

This investigation was conducted in the 40th generation following the inception of selection on sperm length. Although experimental selection for sperm length ceased following generation 17, as demonstrated by data presented herein, no appreciable regression of sperm length had occurred in the experimental lines. All flies, including sires and dams to be crossed and their experimental progeny, were reared at moderate density on standard cornmeal molasses agar medium at 25°C and a 12L:12D light cycle. Flies were collected as virgins following light ether anaesthesia and stored 10 flies per 8-dram vial with medium inoculated with live yeast until reaching experimental age. For each of the four crosses (♀:♂—long × long, long × short, short × long, short × short), 30 pairs were mated and eight sperm were measured from one son per pair. Results are thus based on measures of 960 sperm: eight sperm per male × 30 males per line cross × four line crosses.

(ii) Scathophaga stercoraria

This investigation was conducted in the sixth generation after the inception of selection on sperm length. Although experimental sperm selection only lasted for four generations, sperm length had significantly diverged between treatments and this difference remained at generations five and six. All flies were kept at low densities, with 20 eggs placed on approximately 75 ml of cow dung in each 100 ml plastic container. Emerged flies were housed singly with ad libitum food in 100 ml glasses until maturation. Rearing conditions were 20°C, 66 per cent relative humidity and a 12L:12D light cycle. Dams and sires originated from three independent replicates for both the short-sperm and long-sperm populations. Results are based on measures of 1155 sperm: 15 sperm per male × 77 males, with 15–22 males for each of the four line crosses.

(b) Sperm morphometry

(i) Drosophila melanogaster

Sperm of each anaesthetized male were measured following dissection of the seminal vesicles into phosphate-buffered saline (PBS) on a subbed slide. After passively releasing a few hundred sperm into the saline, preparations were dried in a 60°C oven, fixed in methanol:acetic acid (3:1) and then mounted with glycerol:PBS (9:1) under a glass coverslip. Using digital images of sperm viewed under dark-field optics at a magnification of 200×, the total length of each sperm was measured using NIH IMAGE public domain software (http://rsb.info.nih.gov/nih-image).

(ii) Scathophaga stercoraria

Sperm of each male (killed by freezing at −20°C) were measured following thawing and dissection of the testis into distilled water on a microscope slide. To obtain only mature sperm, the testis was pierced at the proximate end (close to the ejaculatory duct). Released sperm were gently diluted in the droplet of distilled water and dispersed on the slide. After the slide was air-dried, sperm length was measured using microscope images (magnification 160×) conveyed to a PC running ZEISS KS300 software (Carl Zeiss AG, Switzerland).

(c) Statistical analyses

Means, s.d. and coefficients of variation (CVs) in sperm length were calculated independently for each male based on n = 8 and 15 sperm per male for D. melanogaster and S. stercoraria, respectively. Data were analysed with ANOVA and post hoc multiple range tests (Bonferroni Dunn and Tukey’s, where appropriate) using a generalized linear measures approach using SAS (SAS Institute Inc.). All analyses compared values among four ‘genotypes’ (S × S, S × L, L × S, L × L). Analyses of sperm length variation additionally compared between two ‘cross types’ (within line: S × S and L × L versus among line: S × L and L × S). Because dams and sires of S. stercoraria originated from multiple replicate sperm-length selection lines, all analyses of this species were by mixed model ANOVAs with male nested within ‘replicate’ (the unique combination of dam and sire source populations) and replicate nested within genotype or cross type. For both species, statistically identical results were obtained from analyses of within-male standard deviations in sperm length as with CVs in sperm...
length; thus, only analyses of the latter are presented. Analyses of both species revealed no significant differences ($p > 0.10$) between populations in body size (i.e. thorax length for *D. melanogaster* and left hind tibia length for *S. stercoraria*) and no significant main effects or interaction effects when entering body size as a covariate into models of sperm length. Hence, body size was not included in the final analyses presented.

### 3. RESULTS

As expected from previous analyses of the divergence in sperm length among the experimental evolution populations (Miller & Pitnick 2002; Dobler & Hosken in press), ANOVAs examining variation in mean male sperm length revealed a significant effect of population genotype in both *D. melanogaster* and *S. stercoraria* (table 1; figure 1). In both species, males with hybrid cross genotypes had sperm that were intermediate in length relative to pure short- and long-sperm line males (figure 1). However, Bonferroni (Dunn) and Tukey’s Studentized range tests both indicated that all four genotypes differed significantly in mean sperm length only in *D. melanogaster*. In *S. stercoraria*, only the S/C2 S cross males differed in mean sperm length from all other crosses, with males from the S/L, L/S and L/L crosses not being statistically different from one another (figure 1).

Table 1. Analysis of variance comparing mean male sperm length and mean male CV in sperm length among crosses using short- and long-sperm selection lines of *D. melanogaster* and *S. stercoraria*.

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>model component</th>
<th>MS (error)</th>
<th>F</th>
<th>d.f.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>mean sperm length</td>
<td>genotype</td>
<td>0.43 (0.006)</td>
<td>73.19</td>
<td>3,116</td>
</tr>
<tr>
<td></td>
<td>CV sperm length</td>
<td>genotype</td>
<td>14.55 (15.64)</td>
<td>0.93</td>
<td>3,116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cross type</td>
<td>0.65 (15.74)</td>
<td>0.04</td>
<td>1,118</td>
</tr>
<tr>
<td><em>S. stercoraria</em></td>
<td>mean sperm length</td>
<td>genotype</td>
<td>272.55 (49.28)</td>
<td>5.53</td>
<td>3,12.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>replicate (genotype)</td>
<td>54.99 (17.01)</td>
<td>3.23</td>
<td>11,62</td>
</tr>
<tr>
<td></td>
<td>CV sperm length</td>
<td>genotype</td>
<td>2.52 (3.54)</td>
<td>0.71</td>
<td>3,13.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>replicate (genotype)</td>
<td>3.70 (2.66)</td>
<td>1.39</td>
<td>11,62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cross type</td>
<td>2.96 (3.40)</td>
<td>0.87</td>
<td>1,16.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>replicate (cross type)</td>
<td>3.61 (2.63)</td>
<td>1.37</td>
<td>12,63</td>
</tr>
</tbody>
</table>

$^a$Degrees of freedom (model component, error term).
$^b$MS and error are both $\times 10^{-05}$.
Contrary to the haploid gene expression hypothesis, there were no statistically significant differences among either genotypes (S x S, S x L, L x S, L x L) or cross type (within line: S x S and L x L versus among line: S x L and L x S) in CV in sperm length in either D. melanogaster or S. stercoraria (table 1; figure 1). For the analyses of cross type, the difference between mean CVs in D. melanogaster sperm length (within line–among line) was 0.0047, with effect size 95 per cent confidence limits overlapping zero but having a narrow range (95% CIs = −0.0050 to 0.0041). For S. stercoraria, the same difference in CVs was 0.0017, with 95 per cent confidence limits also overlapping zero and having a narrow range (95% CIs = −0.0007 to 0.0040).

Whenever there is a failure to reject the null hypothesis (e.g. no haploid gene expression contribution to variation in sperm length), it is prudent to consider the statistical power of the tests and hence the probability of committing a type II error. Such consideration is frequently based upon post hoc power analyses and determination of detectable effect size. However, these approaches have been shown to be flawed (Hoening & Heisey 2001; Colegrave & Ruxton 2003). In support of this study’s failure to find a significant difference in sperm-length variation in our treatments, we point out that the difference in mean CVs in sperm length between within-line and among-line crosses was very small (2.12%) in D. melanogaster and among-line crosses was very small (0.36%) in S. stercoraria (table 1; figure 1). For S. stercoraria, the same difference in CVs was 0.0017, with 95 per cent confidence limits also overlapping zero and having a narrow range (95% CIs = −0.0007 to 0.0040).

4. DISCUSSION

Transcription of numerous genes, presumably encoding components of the mature spermatozoan, has been shown to occur in the spermatids of mammals (e.g. Schultz et al. 2003). Although post-meiotic transcription may be less common in Drosophila (based on the relative lack of empirical evidence), there is convincing evidence for its occurrence (Barreau et al. 2008). Nevertheless, no variation in sperm form or function in any species has been demonstrated to result from haploid gene expression. On the other hand, there is no empirical evidence that haploid gene expression does not contribute to variation in sperm phenotypes, either within ejaculates or among males.

Sperm length is a trait of great interest as it varies dramatically across species (reviewed by Pitnick et al. 2009a), and comparative studies have demonstrated associations between sperm dimension and sperm function (e.g. Lüpold et al. 2009; Fitzpatrick et al. 2009). Although this relationship within species may be more complex (e.g. Birkhead et al. 2005), investigations of several taxa, including D. melanogaster, have shown a relationship between sperm size and competitive fertilization success (Radwan 1996; LaMunyon & Ward 1998; Miller & Pitnick 2002; Malo et al. 2006), although this is not always the case (Hosken et al. 2001). Nonetheless, the results of the present study suggest, at least for the two very distantly related species of flies examined, that sperm length is strictly a male genome-mediated trait, with no contribution from haploid gene expression. Consistent with this conclusion, analysis of segregation ratios of offspring of heterozygous males, derived from chromosome-extracted D. melanogaster lines with a range of sperm precedence phenotypes, found that sperm competition success depends on the diploid male genome and is not a property of the haploid sperm (Clark et al. 2000).

Moore & Moore (2002) postulated that morphological variation in the pronounced apical hook on the sperm head of mice, which appears to have diversified in response to post-copulatory sexual selection (Immler et al. 2007) and is critical to the formation of sperm trains (Moore et al. 2002), is a result of haploid gene expression. In support of this claim, some genes involved in the patterning of the hooked heads of mouse sperm have been shown to exhibit post-meiotic expression (Kim et al. 1989; Xu et al. 1999; Kleene 2001). However, it is not clear whether any variation among sperm in apical hook form or function is influenced by haploid allelic variation, and thus whether or not this trait may be subject to haploid selection (Keller 2002). Although haploid–diploid conflict and a role for kin selection and social selection in the diversification of sperm form as adaptations for evolutionary cooperation and altruism present interesting intellectual fodder (Immler 2008; Pizzari & Foster 2008; Pizzari & Parker 2009), we suggest caution until the influence of haploid gene expression on relevant variation in sperm form has been demonstrated.

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