Repeatability of sperm size in outbred and inbred *Scathophaga stercoraria* (Diptera: Scathophagidae)

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Abstract—Variability in male gametic traits can depend on several genetic and environmental factors such as developmental instability as a consequence of inbreeding, developmental noise during spermatogenesis, or age- or condition-dependent changes in allocation to sperm cells. Variation in sperm size is particularly evident in species that produce more than one sperm morph but also occurs among males in sperm-monomorphic species. Both discrete and continuous sperm size variation have been implicated in male fertilization success when the sperm of several males directly compete for fertilization of the same set of ova. In this study, we investigated among-male variation in sperm length in field-collected, outbred male *Scathophaga stercoraria* (L.) flies, as well as in flies from the same natural population that had been subjected to 15 and 16 generations of inbreeding under laboratory conditions. Among-male variation in sperm length was significant and repeatable over subsequent matings in both inbred and outbred flies. We conclude that sperm length can be used as an individual male marker in sperm competition studies and that significant repeatability of sperm length supports heritability for this trait.

Résumé—La variation dans les caractéristiques des gamètes peut dépendre de plusieurs facteurs génétiques et environnementaux, tels que l'instabilité du développement causée par la consanguinité, les perturbations (bruit) dans le développement durant la spermatogenèse et les changements reliés à l'âge ou à la condition dans l'allocation des ressources aux cellules spermatiques. La variation dans la taille des spermatozoïdes est particulièrement évidente chez les espèces qui produisent plus d'un morphe de spermatozoïdes, mais elle est présente aussi chez les mâles à sperme monomorphe. Des variations, tant discrètes que continues, dans la taille des spermatozoïdes ont été invoquées pour expliquer le succès de la fécondation lorsque plusieurs mâles sont en compétition pour féconder la même masse d'oeufs. Dans notre étude, nous avons déterminé la variation d'un mâle à l'autre de la longueur des spermatozoïdes chez des mouches mâles *Scathophaga stercoraria* (L.) non consanguines et récoltées en nature, ainsi que chez des mouches provenant de la même population naturelle, mais élevées en laboratoire dans des conditions de consanguinité pendant 15 et 16 générations. La variation de la longueur des spermatozoïdes chez les mâles est significative et c'est un caractère qui se retrouve lors des accouplements subséquents, tant chez les mouches consanguines que non consanguines. La longueur des spermatozoïdes peut donc servir de marqueur mâle individuel lors des études de compétition

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spermatique et le fait que la longueur des spermatozoïdes se retrouve dans les générations suivantes est un indice qui appuie l'hypothèse qu'il s'agit d'une caractéristique héritable.

[Traduit par la Rédaction]

Introduction

Inbreeding has been found to affect fitness and fitness-related traits in plants and animals (Keller and Waller 2002). Inbreeding may negatively affect traits associated with reproduction and reproductive success, such as gametic quality, if increased homozygosity adversely affects spermatogenesis. Low genetic diversity has been found to correlate with sperm morphology in a number of free-living vertebrates, with structurally abnormal sperm being significantly more prevalent in inbred populations (*e.g.*, lions, Wildt *et al.* 1987*a*; cheetahs, Wildt *et al.* 1987*b*). This increased frequency of sperm abnormalities leads to reduced reproductive performance in comparison with more genetically diverse populations (Packer *et al.* 1991). While we are not aware of any study of the effects of inbreeding on sperm features in invertebrates, environmental stressors such as diet and disease have been implicated in abnormal spermatozoal morphology and size variation in several insects (Sait *et al.* 1998; Blanckenhorn and Hellriegel 2002; Hellriegel and Blanckenhorn 2002).

Sperm length is a highly variable trait across species (*e.g.*, Cummins and Woodall 1985; Pitnick and Markow 1994; Gage *et al.* 1998), although an adaptive explanation for the variation remains elusive (Gage *et al.* 1998). Across some taxa, higher levels of polyandry and thus sperm competition risk are associated with longer sperm (*e.g.*, butterflies, Gage 1994; nematodes, LaMunyon and Ward 1998; birds, Briskie *et al.* 1997), but this does not appear to be the case in all taxa (see *e.g.*, Parker 1993).

There is also considerable intraspecific variation in sperm size at several levels. For example, some taxa produce several sperm morphs (*e.g.*, snails, Oppliger *et al.* 1998; flies, Snook and Karr 1998; Joly *et al.* 2004; butterflies, Silberglied *et al.* 1984; Cook and Gage 1995; insects, Bernasconi and Hellriegel 2005). The adaptive functions of the different morphs are not entirely understood, but in moths, for example, they may play a role in delaying female receptivity (Cook and Gage 1995). Variation in sperm length is also found within sperm morphs or in sperm-monomorphic species (Levitan 1993; Radwan and Siva-Jothy 1996; Oppliger *et al.* 1998; Sait *et al.* 1998; Simmons *et al.* 1999; Ward 2000*a*; Morrow and Gage 2001). For some taxa, it has been shown that among-male variation is repeatable across ejaculates (Morrow and Gage 2001; Simmons *et al.* 2003).

Unfortunately variation at this level has been the subject of few studies, and its evolutionary consequences are largely conjectural (Ward 1998; Simmons and Kotiaho 2002). However, intraspecific, interindividual variation is unlikely to be selectively neutral. Indeed, larger amoeboid sperm are more successful at sperm competition in mites (Radwan and Siva-Jothy 1996) and nematodes (LaMunyon and Ward 1998).

The yellow dung fly (*Scathophaga stercoraria* (L.); Diptera, Scathophagidae) is sperm monomorphic but shows significant interindividual variation in sperm length (Ward and Hauschteck-Jungen 1993). Interindividual variation in sperm length appears to be consistent across several ejaculates (Hellriegel and Bernasconi 2000), but the repeatability of this character has not been directly addressed. There is some evidence that variation in sperm length influences sperm storage (Otronen *et al.* 1997).

The aims of this study were to assess among-male variation and within-male stability of sperm length in yellow dung flies and to investigate the effect of inbreeding on sperm length. The results have bearing on the variability of a gametic trait and its plasticity. Moreover, they support the applicability of sperm length as a marker of individual males, *e.g.*, for sperm competition studies.

Materials and methods

The dung fly *Scathophaga stercoraria* belongs to a mainly Holarctic family with over 250 species (Bernasconi *et al.* 2001) and has been an important model for studies of sexual selection for over 30 years (Parker 1970; Ward 2000*b*; Simmons 2001; Martin *et al.* 2004). Here, we investigate the effect of inbreeding on among-male variation and within-male repeatability of sperm length in dung flies kept in the

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laboratory for 1, 15, and 16 generations. Flies were collected at Feraltorf, near Zurich, Switzerland, in 1996 and 1998. Flies from the first collection were kept for 16 generations in the laboratory and selected for the three phosphoglucomutase (*Pgm*) alleles most common in Swiss populations (Ward 2000*b*). The lines were derived from a limited number of individuals and were thus inbred. During selection, brother–sister matings were avoided, ensuring that as much genetic variability as possible was maintained. By the time they were used in the experiments described here, these inbred flies were homozygous for one *Pgm* allele. Flies from the second collection were kept for one generation under the same laboratory conditions (20 °C, 13L:11D; Simmons and Ward 1991). All adult flies were housed individually (females) or in pairs (males) and supplied ad libitum with water, sugar, and prey (*Drosophila melanogaster*) for 2 to 6 weeks after emergence to ensure full sexual maturation (Foster 1967). To elicit mating behaviour, flies were placed in glass bottles containing oviposition subtrate (a smear of cow dung on a filter paper; see Simmons and Ward (1991) for full descriptions of housing and mating procedures). After experimental matings, female flies were killed under anaesthetic (CO_2).

To determine variation in sperm length of individual males, each male was mated to four virgin females. In total, we investigated sperm length for replicate males (*n* between 14 and 19 per generation, Table 1), each of them mated to four independent replicate females. On day 1, males were allowed one copulation, which was interrupted after 20 min, and on day 2 they were allowed three to four full copulations in sequence. This mating regime is directly comparable to that used in an experiment addressing differential storage of sperm from competing males (Hellriegel and Bernasconi 2000).

We measured copulation duration as observable genitalic contact. Singly mated females were dissected after copulation and their three spermathecae were removed, transferred to a microscope slide with a drop of insect saline, and opened under a microscope. The released sperm was spread out. Dried microslides were briefly washed with distilled water to remove salt crystals. We measured the length of 30 sperm per mating using a dark-field microscope (400× magnification) and a camera that conveyed images to a computer running Optimas™ image

Fig. 1. Repeatability of sperm length measurement (μm) in the yellow dung fly, *Scathophaga stercoraria*. For each of eight males, 30 sperm on microslides were blindly re-measured. Measurement was highly repeatable, indicating that repeatabilities as calculated over variance components (Results) reflect biologically relevant trait stability.
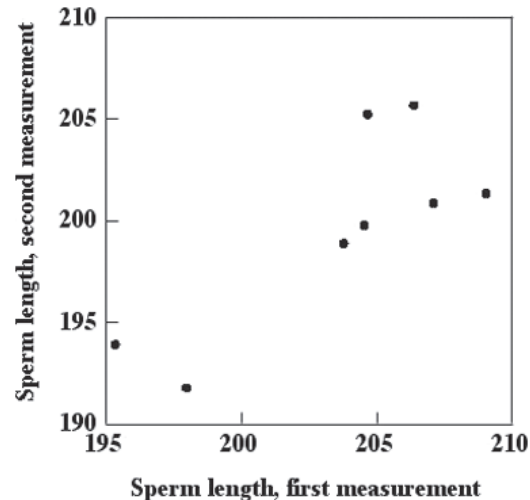
analysis software (BioScan, Edmonds, Washington). We verified that measurement itself was repeatable by re-measuring eight randomly chosen slides (30 sperm per slide) blindly (Fig. 1). Regression between first and second measurements indicated that measurement was repeatable ($\beta \pm$ SE $= 0.76 \pm 0.23$, $F_{1,6} = 10.9$, $P = 0.016$). The mean sperm length of each male over four subsequent copulations was then analysed with a repeated-measures ANOVA to estimate among- and within-male variation over time. We calculated the repeatability (*R*) of sperm length over subsequent matings as $R = V_{\text{among males}}/(V_{\text{among males}} + V_{\text{within males}})$, where *V* is variance. The variance components were calculated as follows: $V_{\text{within males}} = \text{MS}_{\text{within}}$ and $V_{\text{among males}} = (\text{MS}_{\text{among}} - \text{MS}_{\text{within}})/k$, where MS is mean squares and *k* is the number of replicates corrected for unbalancing (Lessells and Boag 1987). The analysis was carried out using restricted maximum likelihood (Patterson and Thompson 1971), which is equivalent to ANOVA but allows for unbalanced data sets (REML in Genstat 5.4.1, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, United Kingdom). The data and residuals met the requirements of normality and homoscedasticity.

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Table 1. Short-term variation in sperm length and copulation duration of individual *Scathophaga stercoraria* males over four matings.

Generation	Mating	Sperm length (μm)	Copula duration (min)	Body mass (mg)
F_1 ($n = 18$)	1st	206.3±4.6	—	40.6±6.0
	2nd	204.5±4.8	29.7±9.3	
	3rd	205.2±5.1	26.3±8.6	
	4th	205.7±6.0	23.7±8.6	
F_{15} ($n = 14$)	1st	201.7±5.0	—	42.7±8.9
	2nd	201.6±5.5	36.0±9.2	
	3rd	202.6±4.2	26.8±7.3	
	4th	201.5±4.3	37.0±20.0	
F_{16} ($n = 19$)	1st	196.7±5.9	—	42.6±2.8
	2nd	198.6±5.8	36.3±12.2	
	3rd	198.3±5.5	31.4±7.1	
	4th	198.6±5.8	30.4±12.1	

Note: For each mating and each male, 30 sperm cells were measured. The first mating was on day 1 and all subsequent matings were on day 2 of the experiment.

Unless otherwise specified, data are reported as $\bar{x} \pm$ SD. Analyses were carried out using SYSTAT® 7.0.1 (Systat Software Inc., Richmond, California) and Genstat 5.4.1.

Results

Sperm tended to be longer in F_1 males than in F_{15} and F_{16} males (Table 1). The length of individual sperm cells was highly repeatable across subsequent matings in all generations, as indicated by significant differences among males (Tables 2 and 3). When the analysis was done on mean sperm length per mating as opposed to individual sperm lengths, there were significant differences among males, and the repeatability of mean sperm length was over 70% in all generations (ANOVA: F_1, $F_{17,54} = 14.7$, $P < 0.001$; F_{15}, $F_{12,33} = 14.8$, $P < 0.001$).

The effect of time (i.e., repeated copulations) on sperm length was not significant in any of the generations. In contrast, the interaction of time with variation among males was significant, indicating that the amount of change in sperm length over time (and repeated copulations) differed among males. However, these changes were only slight, and the variance component attributable to differences among males over time was substantially lower than among-male variation in sperm length (Table 3).

Male body mass, mean sperm length, and mean copulation duration were not significantly correlated, neither in the whole data set (Spearman's rank correlation, all $P > 0.123$, $n = 41$) nor within generations. Thus, sperm length seems to vary independently of male body mass and mating or mate-guarding behaviour.

Discussion

We found that individual male dung flies have a characteristic sperm length, with differences among males being significant and consistent over four successive matings. This was the case also for flies subject to several generations of inbreeding: individual males differed consistently in both the F_1 and the inbred (F_{15} and F_{16}) generations. We found significant repeatability of sperm length within ejaculates (variance component of male effect) but we also found plasticity in sperm length across ejaculates over time (variance component of male × temporal sequence effect). Plasticity within males was substantially smaller than differences among males. Thus, although inbreeding is known to affect fitness-related traits in many species (Keller and Waller 2002), it did not erode among-male differences in sperm length.

Inbred flies tended to have shorter sperm. Inbred lines were maintained by crossing females with one male each (monandry). Thus, the inbreeding regime in fact relaxes conditions favouring male adaptations to counter sperm competition risk. If longer sperm are more successful at sperm competition, but also costly to produce, relaxing the selection pressure may lead to a decrease in sperm length. However, the fitness effect of variation in male sperm length remains to be investigated. Also, shorter

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sperm in inbred flies may be due to inbreeding and, in addition, may be influenced by environmental conditions in the laboratory (Hellriegel and Blanckenhorn 2002), although every effort was made to standardize conditions.

These results confirm that sperm length can be used as a reliable marker for individual males, for instance to investigate processes of differential sperm storage in multiple spermathecae in studies of male–male competition (Otronen *et al.* 1997; Hellriegel and Bernasconi 2000). In a study involving doubly mated females allowed 1 day between the first and the second mating, 80% of the sperm from the spermathecae could be assigned to the competing males by sperm length (Hellriegel and Bernasconi 2000). Given that other sperm-monomorphic species have both substantial among-male variability and within-male stability (Morrow and Gage 2001), this method may be applicable to other species as well. A potential caveat is that sperm length may not be neutral with respect to storage. In studies investigating differential storage, this has been accounted for in the experimental design. For instance, randomizing male mating order with respect to relative sperm length and mating the same male pair repeatedly to replicate females are designs that avoid any potential bias due to differences in sperm length (Lewis and Austad 1990; Birkhead 1998; Hellriegel and Bernasconi 2000). Thanks to the increasingly widespread availability of molecular markers, an interesting research avenue would be to follow up paternity success in situations involving sperm competition using both knowledge of sperm size distribution and molecular methods (*e.g.*, quantitative PCR). Such a combined approach should make it possible to determine whether sperm size affects paternity success and thus male fitness. In addition, such an approach would enable the exploration of the relative success of males with very small sperm size differences, while using sperm size alone necessarily requires large between-male differences for identification of individuals.

In conclusion, male dung flies differ consistently in individual sperm length, even when subject to inbreeding for several generations. Thus, although inbreeding has been found to affect fitness-related traits in many taxa, sperm length variation in this species seems to be unaffected. This supports experimental designs using sperm length as an individual male marker

Table 2. Accumulated ANOVA table for sperm length of individual *Scathophaga stercoraria* males over a sequence of matings with different females (seq, linear contrast; S, deviation from linearity).

	F_1				F_{15}				F_{16}			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
Sequence	3, 51	345.98	2.08	0.114	3, 30	190.55	2.44	0.084	4, 66	10.26	0.189	0.943
seq	1, 51	28.37	0.17	0.682	1, 30	294.22	3.76	0.062	1, 66	8.23	0.151	0.699
S	2, 51	504.78	3.03	0.057	2, 30	138.71	1.77	0.187	3, 66	10.94	0.201	0.895
Among males (M)	17, 51	2685.44	16.12	0.000	12, 30	1805.43	23.09	0.000	18, 66	1321.89	24.32	0.000
Males × sequence	51, 2063	166.6	4.33	0.000	30, 1164	78.18	2.17	0.000	66, 2500	54.36	2.434	0.000
seq.M	17, 2063	312.47	8.12	0.000	11, 1164	79.34	2.2	0.012	18, 2500	46.86	2.099	0.004
S.M	34, 2063	93.66	2.43	0.000	19, 1164	77.5	2.15	0.003	48, 2500	57.17	2.56	0.000
Residual	2063	38.47			1164	35.99			2500	22.33		
Total	2134	63.05			1209	54.98			2588	32.17		

Table 3. Estimated variance components (μm^2, $\bar{x} \pm SE$) and repeatability of sperm length in *Scathophaga stercoraria* males.

	F_1	F_{15}	F_{16}
Variance component			
Male	21.04±7.65	17.71±7.86	9.42±3.28
Male × temporal sequence (M.S)	4.35±1.13	1.47±0.75	1.10±0.33
Residual	38.47±1.20	36.11±1.50	22.35±0.63
Repeatability			
Male		0.33	0.32	0.29
Male × temporal sequence (M.S)	0.07	0.03	0.03

in sperm competition studies and supports heritable variation in sperm length in this species.

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