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ORIGINAL PAPER

Sperm competition affects sex allocation but not sperm morphology in a flatworm

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Abstract Sperm competition has been shown to be an important evolutionary agent affecting the behaviour, physiology, and morphology of both males and females. One morphological trait that is particularly likely to be affected by sperm competition is sperm size because it is thought to influence the competitiveness of sperm by determining sperm longevity, motility, and/or their ability to displace competing sperm. Most comparative studies across taxa have found a positive relationship between the level of sperm competition and sperm length, but very few studies have tested for a phenotypically plastic adjustment of sperm morphology in response to sperm competition. In this study, we experimentally tested for an effect of sperm competition on phenotypic plasticity in sperm morphology in an obligately outcrossing simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*, by either raising worms in monogamous pairs (no sperm competition) or in promiscuous groups (intense sperm competition). Worms in groups produced larger testes and smaller ovaries as predicted by sex allocation theory and as previously documented in this species. However, we found no evidence for an effect of group size on sperm morphology, measured as total sperm length, sperm body length, and the length of two different sperm appendages. We conclude that *M. lignano* may either be incapable of adjusting the sperm morphology in a phenotypically plastic way and/or that there might be no benefit of phenotypic plasticity in sperm traits in this species.

Keywords *Macrostomum lignano* · Phenotypic plasticity · Sperm competition · Sperm length · Testis size

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Introduction

Sperm competition occurs when sperm from different males compete to fertilize the same set of ova (Parker 1970, 1998). Over the last decades, this form of postcopulatory sexual selection has emerged as one of the most important processes to explain the evolution of reproductive traits in animals. It is now clear that sperm competition is a potent evolutionary agent that can affect the behaviour, morphology, and physiology of both males and females (Wigby and Chapman 2004; Pizzari and Parker 2009). The most prominent trait that is affected by sperm competition is the number of produced sperm. Assuming that sperm production is costly and that sperm compete numerically, theoretical models predict that males should invest proportionally more in spermatogenesis at high levels of sperm competition to gain a higher paternity share (reviewed in Parker 1998). This is supported by numerous comparative studies, which show that males of species that generally experience high levels of sperm competition have relatively larger testes (e.g., Hosken 1997; Stockley et al. 1997; Byrne et al. 2002; Pitcher et al. 2005). Similarly, intraspecific studies have demonstrated evolutionary responses in testis size to different levels of sperm competition (e.g., Hosken and ward 2001; Pitnick et al. 2001), and there is also evidence that sperm competition induces a phenotypically plastic response in testis size (e.g.,

Schärer and Ladurner 2003) or sperm production rate (e.g., Schärer and Vizoso 2007; Ramm and Stockley 2009).

Beyond an increased sperm production rate, sperm competition might also select for other sperm or ejaculate traits that enhance the paternity share of a sperm donor (in this article, we preferentially use the term *sperm donor* instead of *males* because it also applies to hermaphrodites). By far, the most frequently studied sperm trait assumed to be under selection by sperm competition is sperm size (Snook 2005). On the one hand, sperm competition may select for smaller sperm if sperm size trades off with sperm number and if sperm of different donors compete in a fair raffle (Parker 1982). However, empirical evidence for this trade-off is equivocal (for reviews, see Snook 2005; Pizzari and Parker 2009). On the other hand, sperm competition may select for bigger sperm, if sperm size is positively linked to sperm competitiveness through, for example, a higher longevity, motility, and/or ability to displace smaller sperm from other males out of the female's reproductive tract (Parker 1993; Snook 2005).

There are many comparative studies that support a positive relationship between the level of sperm competition and sperm size (e.g., Gomendio and Roldan 1991; Gage 1994; Lüpold et al. 2009; Montgomerie and Fitzpatrick 2009). However, there are also many studies that have found no effect of sperm competition on sperm size across species (e.g., Briskie and Montgomerie 1992; Gage and Freckleton 2003; Minder et al. 2005), which suggests that there is no general pattern even within taxa such as insects, birds, and mammals (Pizzari and Parker 2009). Similarly, evidence for a link between sperm competition and sperm size derived from experimental evolution studies is also equivocal. Although it has been demonstrated experimentally that sperm competition can lead to the evolution of larger sperm in *Caenorhabditis elegans* (LaMunyon and Ward 2002), no such response has been found in four insect species (Hosken et al. 2001; Pitnick et al. 2001; Crudgington et al. 2009; Gay et al. 2009).

In contrast to the large body of evidence outlined above, studies focussing on a phenotypically plastic response in sperm morphology to different levels of sperm competition are very scarce (but see Awata et al. 2008; Crean and Marshall 2008; Immler et al. in press). If sperm competitiveness increases with sperm size, we may expect that under certain conditions individuals should produce bigger sperm when facing higher levels of sperm competition (Parker 1993; Snook 2005).

In this study, we tested whether sperm competition affects the sperm morphology in a simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. Individuals of this species are capable of adjusting their sex allocation (i.e., the reproductive investment into the male versus the female sex function) in response to the social group size (i.e., the number of potential mates) that they experience. Such an adjustment is in agreement with a central prediction of sex allocation theory for simultaneous hermaphrodites (for a review, see Schärer 2009), and several studies have demonstrated for *M. lignano* that individuals that were raised in larger groups have bigger testes (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Schärer and Vizoso 2007; Schärer and Janicke 2009).

One of these studies hypothesised that the change in sex allocation is accompanied by a phenotypically plastic response in sperm morphology (Schärer and Vizoso 2007). Specifically, it has been shown that individuals raised in groups (i.e., intense sperm competition) not only have larger testes but also produce a bigger total sperm mass compared to worms in pairs (i.e., no sperm competition). Sperm production rate in this study was inferred from an increase over time in the size of the sperm mass in the seminal vesicle, which is the organ containing the sperm that are ready to be transferred to mating partners (Schärer and Vizoso 2007). Interestingly, worms that had grown up under high levels of sperm competition refilled their seminal vesicle at a faster rate, even after statistically controlling for the effect of testis size. From this, the authors concluded that the phenotypically plastic adjustment of sperm production rate includes a component that is independent of testis size. Beside the possibility that sperm competition led to a faster spermatogenesis, it was hypothesised that this unknown component could be the production of bigger sperm under high levels of sperm competition (Schärer and Vizoso 2007). In the current study, we aimed to test this hypothesis. Specifically, we predicted that individuals that are raised in groups produce bigger sperm compared to individuals that are raised in pairs.

Sperm cells of *M. lignano* carry several unusual appendages, including a rapidly undulating feeler anterior to the sperm body, a pair of stiff lateral bristles anchored at the junction of the sperm body and the shaft, and a terminal brush posterior to the shaft (Fig. 1; Vizoso et al. 2010). The nucleus is located inside the shaft (Willems et al. 2009). So far, the function of the feeler, the bristles, and the brush are not well understood. It has been hypothesised that the feeler allows the sperm to anchor itself in the epithelium of the female sperm-receiving organ and that the bristles prevent the removal of sperm out of the sperm-receiving organ during a postcopulatory behaviour (Vizoso et al. 2010). After insemination, sperm become anchored in the epithelium of the sperm storage organ close to the site where fertilization is likely to take place (Vizoso et al. 2010). Therefore, it seems possible that sperm are competing for access to the anchoring site with the highest likelihood of fertilization. In this study, we focussed on phenotypic plasticity in four morphological traits of the sperm, namely, total sperm length, sperm body length, sperm bristle length, and sperm brush length.

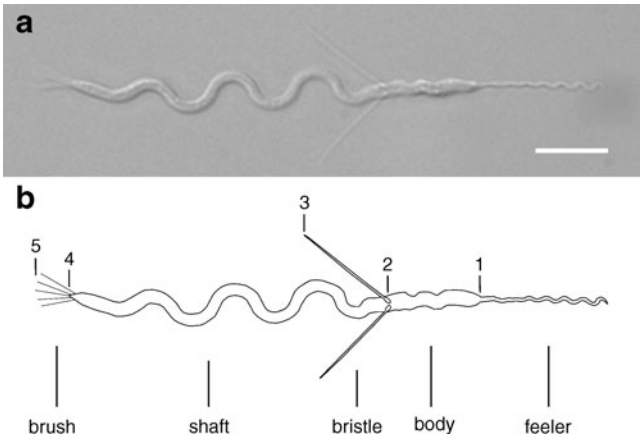

Fig. 1 Micrograph (**a**) and schematic illustration (**b**) of a mature sperm of *M. lignano*. The scale bar represents 10 μm. Numbers in panel (**b**) indicate the landmarks used for the measurement of the morphological sperm traits. "Total sperm length" was defined as the length of a segmented line drawn along the outline of the sperm between the basis of the feeler (1) and the basis of the brush (4), "sperm body length" as the length of a segmented line drawn along the outline between the basis of the feeler (1) and the basis of the bristles (2), "sperm bristle length" as the straight-line distance between the basis (2) and the tip of the bristle (3), and "sperm brush length" as the straight-line distance between the basis of the brush (4) and the central tip of the brush (5)

Methods

Study organism

The free-living flatworm *M. lignano* (Macrostomorpha, Platyhelminthes) is an obligately outcrossing simultaneous hermaphrodite, which belongs to the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). In our laboratory mass cultures, adult worms reach approximately 1.5 mm in body length and have a generation time of about 18 days. In mass cultures, worms are maintained at 20°C in glass Petri dishes containing f/2 medium (Andersen et al. 2005) and fed with the diatom *Nitzschia curvilineata*. Under laboratory conditions, worms mate frequently and are highly promiscuous when kept in groups (Schärer et al. 2004; Janicke and Schärer 2009a). The worms are transparent, allowing noninvasive measurement of morphological traits, such as testis and ovary size (Schärer and Ladurner 2003). Spermatogenesis takes about 6 days (Schärer et al. 2007) after which the sperm is stored in the seminal vesicle, which is located in the tail plate of the worm, before it is transferred to mating partners via the copulatory stylet.

Manipulation of the sperm competition level

To manipulate the level of sperm competition, we raised worms in different social group sizes, namely, in groups of two individuals (hereafter called pairs) and in groups of eight individuals (hereafter called octets). Given that *M. lignano* is an obligately outcrossing simultaneous hermaphrodite (Schärer and Ladurner 2003), there is no sperm competition in pairs. In contrast, a previous study demonstrated that worms in octets experience a high level of sperm competition (Janicke and Schärer 2009a).

On day 1 of the experiment, we collected 1,200 adult worms from our mass culture and distributed them equally to 12 Petri dishes filled with f/2 medium and a dense layer of algae where they could lay eggs. After 48 hours, we removed all adult worms, which limited the range in laying date to 2 days. On day 11, we collected all produced hatchlings and allocated them randomly in pairs and octets into wells of 24-hole well plates. We balanced the number of treatments per plate and alternated the positions of the treatments on the plate to control for position effects. All wells contained 2 ml of f/2 medium and a dense algae layer that guaranteed ad libitum food conditions. We transferred all worms three times (i.e., on days 21, 28, and 35) to fresh wells to ensure that the manipulated social group size was not influenced by the produced offspring (worms usually hatch 5 days after egg laying and do not mature before 13 days after hatching; Schärer and Ladurner 2003). Each treatment was replicated 50 times.

Morphological measurement of sex allocation and sperm morphology

We had to verify if worms actually responded to the manipulation of the sperm competition level by shifting their sex allocation, as previously shown for *M. lignano* (see "Introduction"). For this, we measured body size, testis size, and ovary size of worms in vivo by randomly selecting one individual out of each pair and each octet. The remaining worms were used for another experiment (published elsewhere; Janicke and Schärer 2009b). Image acquisition was carried out from day 36 to 41 according to the standard protocol (Schärer and Ladurner 2003; Janicke and Schärer 2009b). Afterwards, we amputated the tail plate of each worm with a scalpel to make the sperm that is stored in the seminal vesicle accessible for imaging. For this, we ruptured the tail plate by transferring it with only 1 μl medium on a microscope slide and covered it with a cover slip (21×26 mm) causing sperm to flow out of the seminal vesicle. The small amount of medium led to a very thin water film, in which the sperm cells were strongly restricted into two dimensions, greatly facilitating the measurement of the sperm morphology.

We used a Leica DM 2500 microscope (Leica Microsystems, Germany) to which we connected a digital video camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) and took digital micrographs at ×40 for

body size, ×400 for gonad size, and ×1,000 for sperm morphology. For image acquisition, we used the software BTV Pro 6.0b1 (http://www.bensoftware.com/), and we analysed micrographs using ImageJ 1.42 k (http://rsb.info.nih.gov/ij/). Morphological measurements of each sperm included total sperm length, sperm body length, the mean length of the two sperm bristles, and sperm brush length using the "Segmented Lines" tool in ImageJ. Total sperm length included the length of the sperm body and the length of the sperm shaft (for terminologies and description of the sperm measurements, see Fig. 1). The length of the feeler was not included into the analysis because the rapid movement of this structure did not allow for accurate measurements. Sperm traits from 48 individual sperm (each from a different individual worm) were measured twice to assess the repeatability of our measurements. This revealed a high repeatability for total sperm length (r_i=0.96, $F_{47,48}$=46.23, P<0.001) and moderate but significant repeatabilities for the other sperm traits (sperm body length, r_i=0.47, $F_{47,48}$=2.80, P<0.001; sperm bristle length, r_i=0.46, $F_{47,48}$=2.72, P<0.001; sperm brush length, r_i=0.49, $F_{47,48}$=2.90, P<0.001).

Statistical analyses

We first assessed the number of sperm per individual that needs to be measured to obtain a reliable estimate for each individual in all morphological sperm traits. For this, we used a random subset of 15 individuals from which we measured 20 sperm each. Following the method described by Pattarini et al. (2006), we calculated Pearson correlation coefficients of correlations between the individual means of the complete dataset (n=20) and a randomly reduced dataset (n=1 to n=19) and then iterated this procedure 10 times for each individual. This analysis indicated that within-individual variation in all measured traits was low compared to the between-individual variation, a result that is commonly found in other species (e.g., Morrow and Gage 2001; Pattarini et al. 2006). Based on this assessment, we decided to include only those individuals from which we had measured at least 10 sperm in the final analysis, since this sample size is sufficient to capture more than 97% of the within-individual variation in all sperm traits inferred from measuring 20 sperm per individual (Fig. 2). This reduced our final sample size to 48 individuals (24 from pairs, 24 from octets). From these individuals, we randomly selected 10 sperm and used the mean values of each sperm trait in the final analysis. Including individuals for which we had measured less than 10 sperm (n=30) did not change our results qualitatively.

To test if the worms from pairs and octets were comparable in their overall resource budget (cf., Schärer et al. 2005), we tested if our treatment had an effect on body size using a Student's t test. We then assessed whether our treatment induced a phenotypically plastic response in sex allocation, as already shown for *M. lignano* (e.g., Schärer and Ladurner 2003; Janicke and Schärer 2009b). For this, we used analysis of covariances (ANCOVAs), with testis size and ovary size as dependent variables, social group size as a fixed factor, and body size as a covariate (because testis size and ovary size are usually correlated with body size). In all these analyses, the interaction terms between social group size and body size were not statistically significant and were therefore excluded from the final models.

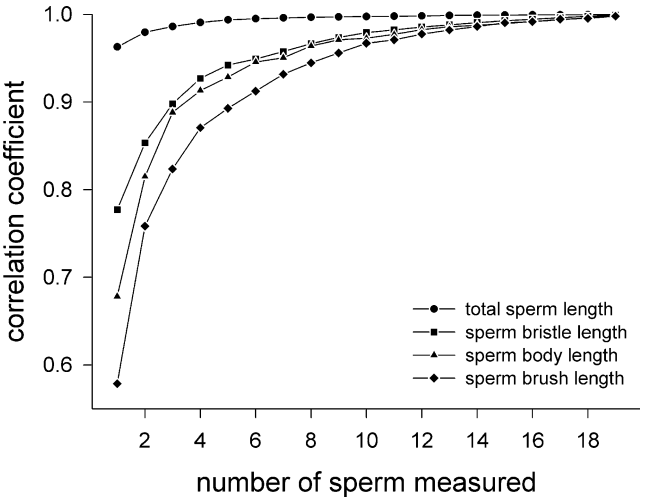

Fig. 2 Relationship between the number of sperm measured and the accuracy of the estimation as described by Pearson correlation coefficients for all sperm traits measured. Note that measuring more than 10 sperm per individual does only marginally improve the accuracy of the estimates. See the "Method" section for a detailed description of the analysis

In addition, we assessed the relationships between sperm morphology, body size, and gonad size by correlating all sperm traits with body size as well as residual testis size and residual ovary size (both calculated from a linear regression fit on body size; testis size: R^2=0.15, $F_{1,46}$=8.24, P=0.006; ovary size: R^2=0.12, $F_{1,46}$=6.16, P=0.017). To test the main hypothesis of this study, we compared all sperm traits between the treatment groups using Student's t tests.

Finally, a power analysis was performed to explore the differences in each sperm trait that would have been detectable between the treatments using our experimental setup. This was done using the pwr package in R v.2.9.1 (R Development Core Team 2009). Based on the overall mean values and the standard deviations of all sperm traits (calculated from individual means), we assessed the relatively smallest significant differences between pairs and octets that we were able to detect with our sample size (n=48, α=0.05, power=0.8, two-tailed t test).

All statistical tests were carried out in R v.2.9.1 (R Development Core Team 2009). Assumptions of normality and homogeneity of variance were met for all parametric tests

presented. All statistical tests were carried out at α=0.05. Values are given as means ± SE unless otherwise stated.

Results

Worms that were raised in pairs and octets were comparable in body size (*t* test: *t*=-0.22, *df*=46, *P*=0.825; see Table S1 for descriptive statistics), suggesting that the overall resource budget was similar between the two treatments. As intended, the manipulation of the social group size had a significant effect on the sex allocation: individuals raised in octets had larger testes (ANCOVA: social group size, $F_{1,45}$=10.60, *P*=0.002; body size, $F_{1,45}$=9.96, *P*=0.003) and smaller ovaries (ANCOVA: social group size, $F_{1,45}$=24.04, *P*<0.001; body size: $F_{1,45}$=9.25, *P*=0.004) compared to worms from pairs (Table S1 for descriptive statistics). Thus, worms from octets clearly had a more male-biased sex allocation compared to worms from pairs.

We found significant between-individual variation in all sperm traits measured (Table 1; for total sperm length, see also Fig. 3), but none of the sperm traits were significantly correlated with body size, residual testis size or residual ovary size (Table 2). However, total sperm length, sperm body length, and sperm bristle length all covaried positively with each other (Table 2), whereas sperm brush length was not correlated with any sperm trait.

Finally, there was no effect of social group size on total sperm length (*t* test: *t*=-0.62, *df*=46, *P*=0.540; Fig. 4a), sperm body length (*t* test: *t*=0.67, *df*=46, *P*=0.505; Fig. 4b), sperm bristle length (*t* test: *t*=0.601, *df*=46, *P*=0.551; Fig. 4c), or sperm brush length (*t* test: *t*=0.11, *df*=46, *P*=0.916; Fig. 4d).

The power analysis revealed that we had sufficient statistical power (0.8) to detect a difference of 5, 4, 4, and 8% in total sperm length, sperm body length, sperm bristle length, and sperm brush length, respectively, between individuals raised in pairs and octets (*n*=48, α=0.05, two-tailed *t* test).

Discussion

Our study suggests that a phenotypically plastic adjustment of the sex allocation in response to varying levels of sperm competition is not accompanied by phenotypically plastic changes in sperm morphology in *M. lignano*. We could show that worms in octets produced larger testes and smaller ovaries and that they were therefore more male-biased compared to individuals in pairs, as previously documented for *M. lignano* (e.g. Schärer and Ladurner 2003; Schärer et al. 2005; Schärer and Vizoso 2007; Janicke and Schärer 2009b). Despite this increase in male reproductive investment, there was no effect of the level of sperm competition on any of the sperm morphology traits we did measure. Neither total sperm length nor sperm body, sperm bristle, or sperm brush length differed between worms that were raised in pairs and octets. Based on the power analysis, the sample size was high enough to detect relatively small differences in sperm morphology between both treatment groups (i.e., 4–8%) compared to the large variation that we observed between individual worms (relative maximum differences: total sperm length 25%; sperm body length 19%; sperm bristle length 16%; sperm brush length 42%). However, it is unclear whether our power was sufficient to capture the smallest biologically relevant differences because we currently lack any empirical data on the relationship between these sperm traits and the siring success in *M. lignano*.

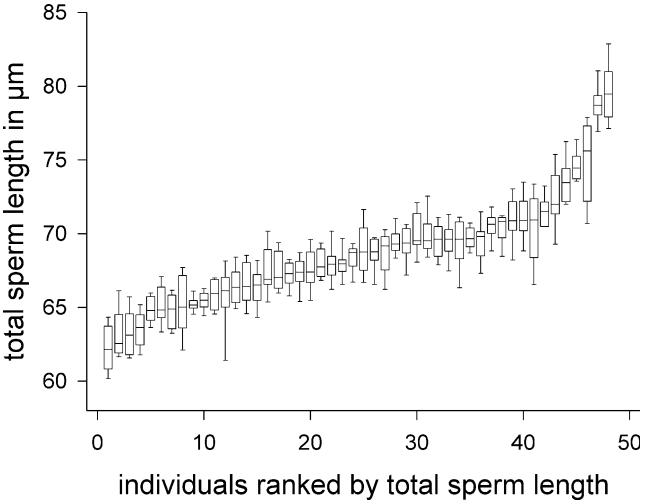

Fig. 3 Box plots showing the between-individual variation in total sperm length among 48 adult worms (10 sperm measured per individual). Individuals are ranked in order of the median in total sperm length. For statistics, see Table 1

Table 1 Descriptive statistics of sperm traits (based on all sperm cells measured, *n*=480) and Kruskal–Wallis analysis of variances on ranks testing for variation in sperm morphology between individuals (*n*=48)

	Mean ± SD	Minimum	Maximum	Chi-square	*Df*	*P*
Total sperm length (µm)	68.6±3.8	60.2	83.0	410.5	47	<0.001
Sperm body length (µm)	14.1±0.9	11.8	16.9	192.1	47	<0.001
Sperm bristle length (µm)	13.2±0.7	11.3	15.7	232.4	47	<0.001
Sperm brush length (µm)	4.6±0.7	2.8	6.9	174.6	47	<0.001

Table 2 Pearson correlation matrix showing correlation coefficients for body size, gonad sizes and all sperm traits based on individual mean values (n=48)

	Body size	Residual testis size	Residual ovary size	Total sperm length	Sperm body length	Sperm bristle length
Residual testis size	–					
Residual ovary size	–	0.070				
Total sperm length	-0.031	0.186	0.315†			
Sperm body length	-0.156	0.041	-0.005	0.713 ***		
Sperm bristle length	-0.084	0.107	0.044	0.584 ***	0.655 ***	
Sperm brush length	0.195	0.034	0.097	0.272	0.180	0.102

***P<0.001, after correcting for multiple testing using the Benjamini–Hochberg method (Benjamini and Hochberg 1995).

† P<0.05 before correcting for multiple testing, but >0.05 after correction.

Schärer and Vizoso (2007) found a positive effect of group size on sperm production rate, which was independent of testis size in *M. lignano*. They hypothesised that a phenotypically plastic increase in sperm size in larger groups might explain the observed effect. However, given that the group size effect (corrected for testis size) on sperm production rate was rather strong in that study and that we should have been able to detect relatively small differences in sperm length between groups in the current study, it seems unlikely that changes in sperm length are responsible for the increased sperm production rate that the authors observed in the larger groups. Consequently, the alternative hypothesis proposed by Schärer and Vizoso (2007), saying that it is a faster spermatogenesis in larger groups that causes the additional effect of group size on sperm production rate, probably represents a better explanation for the observed pattern. Experiments to test this hypothesis are currently ongoing.

The absence of phenotypic plasticity in sperm size in response to sperm competition is consistent with an experimental study in the cooperatively breeding cichlid *Julidochromis transcriptus*, which also found a positive effect of sperm competition risk on testis size but not on sperm size (Awata et al. 2008). Likewise, larval density (a proxy for a higher sperm competition level in the future) had no effect on sperm length in the bruchid beetle *Callosobruchus maculatus* (Gay et al. 2009; but note that in this study, there was no effect of larval density on testis size either). Nevertheless, there are a few studies that suggest a phenotypically plastic response in sperm length to the level of sperm competition. Morrow et al. (2008) studied sperm length in *Drosophila melanogaster* and showed that besides a large additive genetic component, some variation in sperm length could be explained by the larval environment. Male flies that were exposed to a higher level of larval competition produced slightly smaller sperm (Morrow et al. 2008). In addition, Immer et al. (in press) reported pronounced within-individual plasticity in sperm morphometry in Gouldian finches (*Erythrura gouldiae*). Among other effects, the authors could show that males increase the relative length of the sperm midpiece when placed from an intermediate into a highly competitive social environment (Immler et al. in press). The currently best evidence for a phenotypically plastic adjustment of sperm length in response to sperm competition comes from the broadcast spawning ascidian *Styela plicata* (Crean and

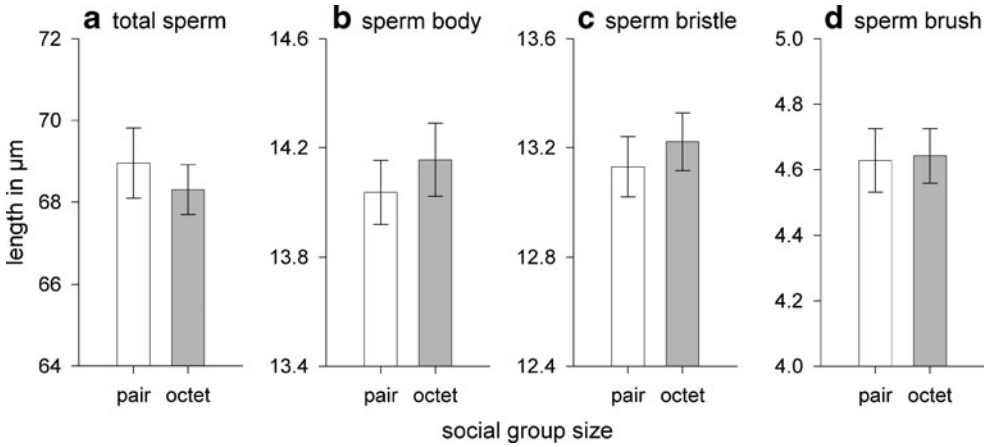

Fig. 4 Comparison of the individual means of (**a**) total sperm length, (**b**) sperm body length, (**c**) sperm bristle length, and (**d**) sperm brush length between worms raised in pairs (n=24) and octets (n=24). See text for statistics

Marshall 2008). Individuals of this simultaneous hermaphrodite produce longer sperm heads when experimentally exposed to high densities, with a relative difference in head length between individuals from high and low densities of about 7% (Crean and Marshall 2008). According to our power analysis, a difference of that magnitude would have been detectable with our experimental setup.

We found relatively low within- but high between-individual variation in all sperm traits we measured. This is consistent with other studies, indicating that sperm length is often male-specific (e.g. Ward and Hauschteck-Jungen 1993) and repeatable between successive ejaculates (e.g. Morrow and Gage 2001). None of the sperm traits covaried with body size or the residual testis size. Such an absence of allometric relationships between sperm traits and body size has been found in both interspecific (e.g., Ward and Hauschteck-Jungen 1993; for review, see Pitnick et al. 2009a) and intraspecific studies (e.g., Minoretti and Baur 2006; Gay et al. 2009; but see Amitin and Pitnick 2007). If we assume that body size is a fitness-related trait in *M. lignano*, our findings suggest that sperm morphology is not strongly condition-dependent in this species, confirming findings in other organisms (reviewed in Pitnick et al. 2009a).

One potential reason for a lack of a phenotypically plastic adjustment of sperm morphology in response to a varying sperm competition level is that most of the between-individual variation is due to genetic variation rather than environmental factors (e.g., Morrow et al. 2008). In agreement with this notion, a recent meta-analysis across many animal taxa found a relatively high average heritability for sperm morphology compared to other sperm traits such as sperm motility (Simmons and Moore 2009). Detailed studies focussing on the heritability of sperm morphology traits in *M. lignano* are now needed.

Another explanation for the absence of an effect of sperm competition on sperm morphology could be that the length of the sperm and its body, bristles, and brush only play a minor role for the outcome of sperm competition in *M. lignano*. So far, we lack any data showing a direct relationship between sperm morphology and sperm competitiveness or cryptic female choice in *M. lignano*. However, a recent study demonstrated that within the genus *Macrostomum*, sperm bristles only occur in species in which sperm from different donors interact in the female sperm receiving organ, but not in species with hypodermic impregnation, suggesting that the bristles and the brush are important traits in postcopulatory sexual selection (Schärer et al. unpublished data). To test directly how sperm morphology affects sperm competitiveness in *M. lignano*, one could use the large between-individual variation in all sperm morphology traits and assess paternity shares from mating experiments in which individuals producing consistently different sperm phenotypes compete for fertilization against each other.

Furthermore, selection on sperm morphology can also be driven by sperm–female interactions (Pitnick et al. 2009b). For instance, it has been shown that the relationship between sperm length and the rate of extrapair paternity (a proxy for the level of sperm competition) in birds arises only indirectly through a positive correlation of extrapair paternity with the length of sperm storage tubules in females (Briskie et al. 1997). Indeed, there are many comparative studies that show that sperm length covaries with the morphology of the female sperm storage organ (reviewed in Pitnick et al. 2009a, b). Moreover, an artificial selection experiment in *D. melanogaster* revealed that the evolution of sperm length can occur as a correlated response to selection on the morphology of the female reproductive tract (Miller and Pitnick 2002).

Finally, ejaculate traits other than sperm morphology might be more important for the outcome of sperm competition. For instance, in the Atlantic Salmon (*Salmo salar*), sperm velocity, but not sperm length, is positively correlated with fertilization success (Gage et al. 2004). Therefore, *M. lignano* may not adjust the sperm morphology in response to sperm competition but may instead adjust dynamic sperm traits, such as sperm velocity or longevity, some of which could be mediated by adjusting the composition of seminal fluids rather than the morphology of the sperm (Poiani 2006).

In conclusion, we found no phenotypically plastic effect of sperm competition on sperm morphology in *M. lignano* despite the presence of a phenotypically plastic response in male reproductive investment. Although our data reveal considerable between-individual variation in sperm morphology, none of the sperm traits were correlated with the gross morphology of the sperm-producing individual, as measured by body size, residual testis size, and residual ovary size. The functional significance of sperm length variation and of the various sperm appendages for sperm competition in *M. lignano* remains unclear and should be addressed in further studies.

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