Bulletin of Insectology ***71*** *(1): 51-56, 2018*
ISSN 1721-8861

Sperm morphometric in males of the paper wasp *Polistes simillimus*

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Abstract

Intraspecific variation in sperm morphometric is widespread across animal taxa and it might be related to different aspects of sexual selection. Within social insects, it has only been documented for some bee and ant species. Here we provided a detailed description of the variation in sperm morphometric in the paper wasp *Polistes simillimus* Zikan (Hymenoptera Vespidae). Light microscopy, digital photography and digital analyses were used to measure sperm (total length, head and flagellum length). Considering a single population from which we sampled four colonies, seven males of each colony and 30 sperm cells of each male (total 840 cells), the overall dimensions of *P. simillimus* spermatozoon is: total length 107 ± 7 (80-129) µm, coefficient of variation, CV = 8%; flagellum length 91 ± 7 (62-114) µm, CV = 7%; head length 16 ± 2 (9-30) µm, CV = 9%. We found that variation in the sperm constituent parts (head and flagellum) contribute to explaining the variation in total sperm length. However, the size of these parts varies independently. Besides, sperm total length and flagellum length differed between males, but not between nestmates and between colonies, while variation in sperm head was similar across all these levels of analyses. Finally, sperm morphometric is not associated with male body size. We discussed implications of our results for the study of sperm morphometric in insects, sampling procedures for estimating species-typical sperm size in social insects and the possibility of variation in male sperm quality in social wasps.

Key words: paper wasps, sampling procedure, sperm size evolution, sperm quality, variation in sperm length.

Introduction

Intraspecific variation in sperm length is a common phenomenon, and there are different ways by which such variation can occur. For example, in some species of *Drosophila* flies (Snook, 1997) and lepidopterans (Gage and Cook, 1994; Morrow and Gage, 2000), the intraspecific distribution of sperm length is bimodal and this has been explained as a specific adaptation for sperm competition, this is, a result of sexual selection. In other species, however, intraspecific variation in sperm length is found to be continuous (e.g. yellow dung flies: Ward and Hauschteck-Jungen, 1993; bumblebees: Baer *et al.*, 2003; ostracods: Smith *et al.*, 2016; reviewed in Ward, 1998), but the adaptive reasons for this kind of variation, if they exist, remain poorly known.

The role of sexual selection in social insects is a topic of growing interest (reviewed in Baer, 2003; Boomsma *et al.*, 2005; Beani and Zuk, 2014; Beani *et al.*, 2014; Baer, 2014; 2015; Heinze, 2016). Indeed, most eusocial Hymenoptera have a number of life history traits that make them unique for this kind of study. Specifically, females have a single mating opportunity at the beginning of adult life (e.g. the nuptial flight in ants). During this short time window, they generally mate with only one male (Boomsma and Ratnieks, 1996; Strassmann, 2001). The sperm obtained from the male is stored in a specialized organ, the spermatheca. The stored sperm must be sufficient for life, which may be up to several decades in some ants (Pamilo, 1991). Each spermatozoon obtained from a single-mated female is a clone, as males are haploid and produce gametes without meiosis and recombination (Crozier, 1975). Thus, it is worth to note that, different from vertebrates and many other invertebrates, social wasps, bees and ants do not have the possibility to mate throughout most of their adult life and they present a considerable delay between insemination and fertilization. How these peculiarities relates to the variation in sperm traits, if so, remain lesser understood. Because sperm characteristics are thought to be the result of selection in the environment where fertilization and competition occur (Jamieson, 1987; Simmons, 2001), investigating the variation in sperm size may be a promisor way of revealing the evolutionary forces underlining reproductive traits in social insects.

To date, detailed information about variation in sperm length in social insects has been only reported for a few bumblebees (Baer *et al.*, 2003) and, recently, an ant (Schrempf *et al.*, 2016). Here, we examine the intraspecific variation in sperm length in the Neotropical primitively eusocial paper wasp *Polistes simillimus* Zikan (Hymenoptera Vespidae). These wasps, like other eusocial Hymenoptera, have a very particular reproductive biology. In the spring, new colonies are started by one (solitary nest founding) or a few (cooperative nest founding) inseminated females (Prezoto *et al.*, 2015). Foundresses in cooperative foundations are highly related (Simokomaki and Del Lama, 2001). Regardless of the mode of foundation, colonies produce generations of workers and at the end of colony cycle (late summer) they produce males and reproductive females (future

foundresses). Within a few weeks after emergence, these future foundresses presumably mate with a single male (Strassmann, 2001) outside the nest (Beani, 1996). Female mate choice through male visual ornamentation occurs (De Souza *et al*., 2014), even though males provide females with nothing but the ejaculate (e.g., lack of male parental care). Females never remate and thus, they store sperm cells in the spermatheca for the rest of their lives. After mating, females wait for favourable environmental conditions to start their colonies. As a result, right after the mating season, they join to winter aggregations characterized by no nest building or egg laying activities, till the beginning of spring (Prezoto, 2001). At this time, the inseminated females found new colonies and start using the stored sperm cells to produce generations of workers and, at the end of colony cycle, reproductive females.

First, we describe sperm general morphology under light microscopy. Then, we examine morphometric relationships between sperm constituent parts: head, flagellum. Also, we assessed variation in sperm morphometric across different organizational levels, specifically: within males, between males, between nestmates and between colonies. Finally, we assessed variation in sperm morphometric according to male body size.

Methods

Study place, male collection and rearing

A total of four male producing colonies of *P. similimus* (but not the adult wasps) were collected during February 2015 in the municipality of Viçosa, Minas Gerais State, Southwestern Brazil (20°48'S 42°51'W, 800 m a.s.l.). We consider that these nests came from a single population, as they were around 10-50 m from each other. Each nest was kept in a plastic aerated cage of 5 L. Daily inspections were conducted to check for the presence of newly emerged individuals. At the day of emergence, each male was removed from the nest and confined individually in a plastic aerated container of 1 L, provided with food and water, till they reach 25 days. Males at this age have already filled their sperm storage organs -the seminal vesicles-, so that they are sexually mature.

Sampling sperm

To collect sperm, males were killed by freezing, and dissected under a stereomicroscope. Paper wasps males store their mature sperm in specialized organs, a pair of seminal vesicles (e.g. Araujo *et al.*, 2010; Moreira *et al.*, 2012). Using fine forceps, the pair of seminal vesicles was dissected, transferred to a few drops of insect buffer (0.1 M phosphate buffer, pH 7.2) and carefully opened at the distal end so that the out-flowing sperm could be mixed with the buffer. A drop of this solution containing the sperm was subsequently spread on clean glass microscope slide and fixed with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Then the slide was washed with running water and dried at room temperature.

Staining procedure

The limits between the constituent parts of the spermatozoon are not always distinguishable in unstained samples of insect sperm. To overcome this limitation, we merged each glass slide in Harris hematoxylin for 10 min. This procedure improved the visibility so that the head and flagellum could be readily discriminated.

Image acquisition and measurements

We capture images of non-coiled and non-damaged sperm, using a light microscope Olympus BX-60 (40× objective lens) connected to an Olympus Q-Color3 digital camera. The total length of sperm, as well as its constituent parts (head and flagellum) was subsequently measured by analyzing the images on the Image-J software (available at http://rsbweb.nih.gov/ij/). Repeated measurements (n = 10 times) of a single spermatozoon gave a very small error, as inferred from the coefficient of variation CV between the repeated measures (CV = 0.2% for total length and 1% for head length), suggesting that our method of accessing sperm morphometry is highly repeatable. Besides, we accessed male body size by measuring the maximum head width with a digital calimeter.

We sampled 30 sperm for each male and seven males from each nest, giving a total of 840 sperm from 28 males and four nests.

Data analyses

The morphometric relationship between sperm constituent parts were examined with Linear Mixed Models via penalized quasi-likelihood (LMMpql). This estimation technique was chosen due to its suitability to correlated random effects (Dean *et al.*, 2004), in our study, males and nests. As a result, in this analysis, we used information from each measured sperm cell (n = 840 cells).

We examined the distribution of sperm morphometric within individual males by using one-sample Kolmogorov-Smirnov tests. Also, we combined the probabilities of the Kolmogorov-Smirnov tests using the z-method proposed by Whitlock (2005) to check the overall trend for the total sperm length and for each part of it (head and flagellum). These analyses were performed for each of the 28 available males (30 sperm cells per male).

LMMpql was also used to check whether the sperm morphometric is associated with male body size. In this case, sperm morphometric was averaged from each male (n = 28), so that only nests were included as a random effect.

To test for differences in sperm morphometric across different organizational levels, we used nested ANOVAs, in which male identity and nests were entered as factors while sperm was nested within males and males was nested within colonies. Here too we considered each sperm cell as a replication (n = 840 cells).

Analyzes were performed with the program R version 3.2.4 (R Development Core Team 2016), and with the package MASS version 7.3-45 (available at http://www.stats.ox.ac.uk/pub/MASS4/) and Survcomp (available at http://www.pmgenomics.ca/bhklab/).

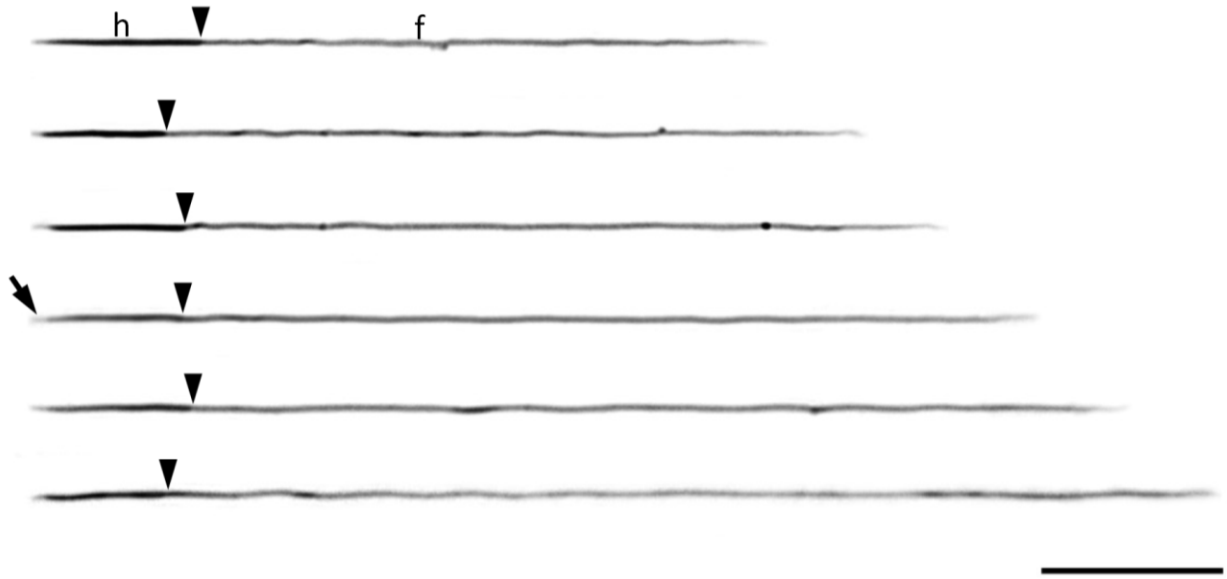

Figure 1. Variation in sperm length in a population of *P. simillimus* males. Cells were observed under a light microscope (magnification 40×), after staining with Harris hematoxylin for 10 min. The arrowheads indicate the transition between head (h) and flagellum (f), and the arrow, the acrosome. Scale bar = 20 µm.

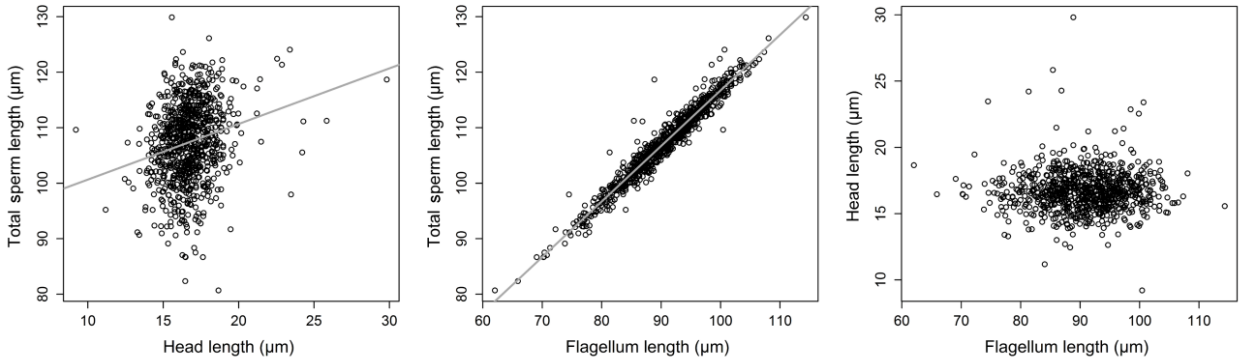

Figure 2. Scatter plots of sperm total length versus sperm head length (a), sperm total length versus sperm flagellum length (b), and sperm head versus sperm flagellum length (c). Each gray dot represents a single sperm cell of *P. simillimus*. Lines represent the regressive models.

Results

Sperm morphology under light microscopy

P. simillimus spermatozoa have a filiform appearance and it is composed of an elongated head and flagellum (figure 1). The overall dimensions of spermatozoa are: total length 107 ± 7 (80-129) µm, coefficient of variation, CV = 8%; flagellum length 91 ± 7 (62-114) µm, CV = 7%; head length 16 ± 2 (9-30) µm, CV = 9%.

Morphometric relationships between sperm constituent parts

There was a weak positive association between the total sperm length and its head length (R^2 = 0.0498, $p < 0.0001$; figure 2). Additionally, there was a strong positive association between the total sperm length and the flagellum length ($R^2 = 0.9502$, $p < 0.0001$; figure 2). However, the head and flagellum length are not associated ($R^2 = 2.4 \times 10^{-7}$, $p = 0.015$; figure 1 and 2). Table 1 summarizes the estimates of all LMMpql's parameters.

Variation in sperm length across different organizational levels

Males produced sperm of only one size class (unimodal distribution), fitting a normal distribution, as illustrated by the non-significant p-values of the Kolmogorov-Smirnov tests in table 2. The combined probability tests indicated that the overall trend also followed a normal distribution, since all p-values were high (total length: $p = 1.000$; head: $p = 1.000$; flagellum: $p = 0.996$).

The multivariate analyses (nested ANOVAs) confirmed that the total sperm length was significantly different between males ($F_{1,24}$ = 8.202, p = 0.0086), but similar between nestmates ($F_{1,24}$ = 1.159, p = 0.2923) and between colonies ($F_{1,24}$ = 2.293, p = 0.1430). In the same way, flagellum length was significantly different between males ($F_{1,24}$ = 7.000, p = 0.0142), but similar between nestmates ($F_{1,24}$ = 0.732, p = 0.4007) and between colonies ($F_{1,24}$ = 1.484, p = 0.2350). The head length, however, was similar across all levels of analysis: between males ($F_{1,24}$ = 2.266, p = 0.1453), between

Table 1. Parameters of the Linear Mixed Models applied to model the sperm morphometric in males of *P. simillimus*. RV = Response Variable; EV = Explanatory Variable; RSE = Residual Standard Error; STL = Sperm Total Length; SHL = Sperm Head Length; SFL = Sperm Flagellum Length; ASTL = Average Sperm Total Length; AHL = Average Sperm Head Length; AFL = Average Sperm Flagellum Length; MBS = Male Body Size.

RV ~ EV	R^2	RSE	Fixed effects		Random Effects		p
			Estimates	Std Error	Intercept	Resiudal	
STL ~ SHL	0.0496	6.850	Intercept: 96.864 SHL: 0.628	Intercept: 2.806 SHL: 0.149	2.286	6.040	<0.001
STL ~ SFL	0.9504	1.566	Intercept: 18.462 SFL: 0.981	Intercept: 0.736 SFL: 0.008	0.736	1.383	<0.001
SHL ~ SFL	2.4×10^{-7}	1.566	Intercept: 18.462 SFL: −0.019	Intercept: 0.736 SFL: 0.008	0.726	1.383	0.015
ASTL ~ MBS	0.0185	3.756	Intercept: 100.121 MBS: 2.124	Intercept: 16.278 MBS: 4.744	2.397	1.044	0.659
AHL ~ MBS	0.000014	0.800	Intercept: 16.801 MBS: −0.024	Intercept: 4.430 MBS: 1.296	0.771	0.007	0.985
AFL ~ MBS	0.000014	0.800	Intercept: 86.247 MBS: 1.292	Intercept: 15.292 MBS: 4.458	2.259	0.973	0.775

Table 2. Summary of variation in sperm total length, as well as the length of its constituent parts in males of *P. simillimus*. The number of sperm measured per male was always 30. Mean ± standard deviation (amplitude) is presented. KS and P values refer to tests for normality.

Nest	Male ID	Total sperm lenght			Head lenght			Flagellum lenght		
		Size±SD (range)	KS	P	Size±SD (range)	KS	P	Size±SD (range)	KS	P
1										
	17	104.7±6.35(89.13-114.2)	0.1280	0.663	15.96±1.5(13.4-18.7)	0.1286	0.704	88.77±6.06(73.81-96.81)	0.1515	0.453
	19	106.3±6.78(92.74-119.6)	0.1081	0.875	16.19±1.11(14.26-18.28)	0.0836	0.985	90.14±7.32(75.26-104.5)	0.0609	0.999
	20	100.5±6.67(80.69-110.9)	0.1634	0.400	16.39±0.80(14.92-18.66)	0.1056	0.891	84.09±6.88(62.03-94.78)	0.1553	0.422
	27	106.7±4.90(97.96-115.8)	0.1065	0.850	16.09±1.75(12.45-23.47)	0.2054	0.159	90.66±5.49(74.49-99.90)	0.0893	0.953
	28	106.9±5.60(94.15-116.2)	0.1141	0.789	16.57±1.36(13.93-20.17)	0.1498	0.511	90.35±5.52(77.90-98.80)	0.0971	0.940
	29	105.9±5.84(93-46-116.8)	0.1182	0.796	16.43±0.91(15.28-19.53)	0.1366	0.631	89.46±6.03(76.34-99.93)	0.1491	0.473
	30	105.9±6.77(86.68-115.8)	0.1415	0.538	16.82±1.65(14.7-24.29)	0.2142	0.110	89.09±6.79(69.05-98.95)	0.1080	0.838
2										
	14	103.4±6.27(90.69-114.1)	0.1192	0.743	16.82±0.91(13.41-18.36)	0.1904	0.200	86.61±6.04(75.54-97.10)	0.0921	0.941
	15	108±4.55(99.23-116-9)	0.0687	0.997	17.21±1.22(14.20-18.97)	0.0921	0.961	90.80±4.76(81.42-99.19)	0.1155	0.776
	21	104.6±5.93(92.78-114.1)	0.1375	0.575	15.54±1.18(12.7-17.56)	0.0739	0.993	89.03±5.65(76.68-98.32)	0.1522	0.490
	23	103±4(93.02-113-2)	0.1307	0.637	15.19±0.89(13.1-18.22)	0.1358	0.638	87.81±3.86(77.53-97.56)	0.1198	0.783
	24	100.2±6.87(88.36-118.7)	0.1502	0.463	17.31±1.21(15.67-21.42)	0.1472	0.534	82.91±6.55(71.31-100.4)	0.1227	0.757
	25	105.1±5.87(92.56-113.6)	0.1212	0.725	17.26±0.97(15.49-19.39)	0.1106	0.856	87.88±5.62(76.05-96.01)	0.0901	0.950
	26	106±5.26(95.20-118.6)	0.1011	0.889	16.33±1.49(11.18-18.45)	0.1265	0.676	89.66±5.01(80.24-101.8)	0.0846	0.970
3										
	31	111.1±5.31(99.10-121)	0.0968	0.941	17±1.15(14.43-19.98)	0.1117	0.848	94.05±5.44(82.83-103.2)	0.1120	0.806
	32	109.8±4.63(95.99-119.3)	0.1450	0.508	17.60±0.08(16.2-19.21)	0.1097	0.863	92.23±4.67(77.16-100.7)	0.0980	0.909
	33	111.4±5.63(93.17-120)	0.1164	0.811	17.88±2.26(9.2-24.2)	0.2089	0.126	93.48±6.24(76.77-101.8)	0.1711	0.307
	41	111.3±5.02(99.91-121)	0.0637	1.000	17.33±2.83(13.44-29.82)	0.2195	0.111	93.97±5.03(83.65-103.9)	0.0835	0.974
	43	112.8±5.08(100.4-122.3)	0.1298	0.646	15.88±0.69(14.57-17.27)	0.0734	0.997	96.91±5.20(83.36-106.5)	0.1068	0.884
	44	107.1±4.5(99.85-119.8)	0.1143	0.787	16.46±0.88(14.83-18.67)	0.0897	0.969	90.66±4.31(83.46-101.1)	0.1099	0.862
	45	114.3±4.51(88.85-119.8)	0.1279	0.663	17.14±1.84(14.15-23.40)	0.2420	0.060	97.21±4.72(86.14-103.8)	0.1774	0.268
4										
	34	113.3±6.57(92.19-122.2)	0.0889	0.972	17.03±1.09(15.07-19.38)	0.1504	0.506	96.29±6.15(76.95-104.7)	0.0931	0.936
	35	112.5±7.30(98.84-126.1)	0.1102	0.821	18.45±1.53(16-22.87)	0.1283	0.707	94.08±7.18(80.4-108.1)	0.1097	0.826
	36	108.6±5.72(96.26-119)	0.1061	0.853	17.72±0.87(15.60-19.87)	0.1567	0.453	90.85±5.69(79.48-103.4)	0.0778	0.993
	37	109.8±6.44(95.98-121.1)	0.0909	0.946	17.45±0.91(15.23-18.97)	0.1188	0.791	92.34±6.4(79.95-104.7)	0.0744	0.996
	38	104.7±6.94(92.26-119.9)	0.1395	0.639	15.34±1.09(12.64-17.63)	0.0736	0.993	89.10±6.98(76.86-101.1)	0.1566	0.411
	39	107.5±9.45(86.69-129.9)	0.1194	0.742	16.25±2.13(13.29-25.84)	0.1994	0.184	91.44±9.16(70.21-114.3)	0.1056	0.857
	40	105.1±8.27(82.36-115.8)	0.1487	0.476	16.41±0.99(14.17-19.03)	0.1091	0.867	88.66±7.98(65.90-98.69)	0.1612	0.417

nestmates ($F_{1,24}$ = 1.303, p = 0.2649), and between colonies ($F_{1,24}$ = 2.381, p = 0.1359).

Sperm morphometric and male body size

Male body size did not predict the mean length of its sperm. This was observed when considering the spermatozoon total length (R^2 = 0.0185, p = 0.491), as well as the length of the flagellum (R^2 = 0.022, p = 0.456) and the length of the head (R^2 = 0.000014, p = 0.941; table 1).

Discussion

P. simillimus mean sperm length (107 μm) is longer than that of other social wasps like *Mischocyttarus cassununga* Von Ihering (97 μm, Moreira *et al*., 2012) but smaller than that reported for *Agelaia vicina* (de Saussure) (218 μm, Mancini *et al*., 2006) as well as for different species of bumble bees (168-213 μm) and honey bees (204-267 μm, Fitzpatrick and Baer, 2011). Despite this

mean value, we found some variation in sperm dimensions across different levels of analyzes. Implications are discussed below.

Across social insect species, polyandry is supposed to reduce variation in sperm total length (Fitzpatrick and Baer, 2011). We found that *P. simillimus* have the highest variation in sperm total length (CV = 8%) reported for a social insect so far, thus suggesting that females are monandric.

The lack of a correlation between sperm constituent parts reported here was also reported in a wide range of animals (Morrow and Gage, 2001; Malo *et al.*, 2006). Contrary to the weakly supported idea of a coevolutionary morphometric relation between sperm constituent parts (Gage, 1998), it seems that variation in flagellum length occur regardless variation in the head length. Even though we found that in *P. simillimus*, longer sperm tend to have both longer head and longer flagellum, the flagellum (rather than the head) is much more important in explaining the total sperm length. It means that males produce sperm of highly variable flagellum length but much less variable head length. The head and flagellum of a spermatozoon are different in many ways. Specifically, the head carries the genetic material, as well as a specialized apical structure, the acrosome, presumably involved in penetration within the ova. In the other hand, the flagellum carries the locomotive machinery of the cell, composed of a pair of mitochondrial derivatives, an axoneme and a connective piece, the centriolar adjunct (e.g. Mancini *et al.*, 2006; 2009; Moreira *et al.*, 2012). From this, it is possible to suppose that differences in the relative contribution of head and flagellum to the variation in spermatozoa length may be a result of differential selection on each part. Broadly, the idea of differential selection implies that measuring only sperm total size when their components are not readily visible (e.g. Morrow and Gage, 2001; Fitzpatrick and Baer, 2011) could potentially obscure correlations resulting from selection acting on specific sperm constituent parts (Lüpold *et al*., 2009). To overcome this issue, we recommend the staining procedure we performed in this study- merging glass slides (with sperm cells) in hemathoxilin for 10 min. This procedure improves the visibility so that the head and flagellum can be readily discriminated (figure 1). The use of this simple technique in future studies might allow researchers to get more detailed information about the morphometric of spermatozoon constituent parts (e.g. Gage, 1998; Ward, 1998).

The variation in sperm length reported here holds some implications for sampling procedures regarding estimation of species typical-sperm length. Thus, because there is a significant between male variation in sperm length, we suggest that a proper sample should measure many different males (see Laskemoen *et al.*, 2007 for a suggestion of sample size). Apart from this, we reserve the judgment about whether or not it is important to include different nests when estimating the typical-sperm length of social insects. This is because the sympatric nest founding behavior of *P. simillimus* (Prezoto, 2001) and the presumably similarity of available resources for male production may ensure comparable genetic and environmental conditions that could potentially obscure higher level differences in sperm morphometric (between nestmates and between colonies). Also, the low number of sampled nests (n = 4) and the low number of sampled wasps per nest (n = 7) limits a conclusive statement. Therefore, these possibilities need further investigation. Of note, it seems that at least for *P. simillimus*, one could sample males for species typical-sperm length analysis regardless variation on its body size, as body size is not associated with sperm morphometric. In contrast, Baer *et al.* (2003) reported for *Bombus hypnorum* (L.), *Bombus lucorum* (L.) and *Bombus terrestris* (L.) (Hymenoptera Apidae) significant differences in sperm length between males, between nestmates as well as between colonies. Furthermore, these authors reported a positive relation between sperm morphometric and male body size in *B. terrestris* and possibly *B. hypnorum*, but not for *B. lucorum*.

Sperm are thought to be selected for a species-specific "optimum" phenotype (Calhim *et al.*, 2007). In this sense, low variance in morphometric sperm traits of a given male may indicate that its gametes are closer to this "optimum", compared to a conspecific male with a higher variance in morphometric sperm traits. Accordingly, Fitzpatrick and Baer (2011) propose that a low variance in sperm traits of a given male may indicate sperm superior quality. These authors compared the intraspecific variation in sperm length across species of social bees and ants with different mating behaviours, and found that increased polyandry is associated with a decreased intraspecific variation in sperm length. Thus, where the risk of sperm competition is high (e.g., highly polyandric species), males produce more homogeneous and, presumably, higher quality sperm. Accordingly, by showing between male variation in sperm morphometric, we highlight for the possibility that males of *P. simillimus* differ in sperm quality. Although *P. simillimus* females are presumably single mated (Strassmann, 2001), the reproductive biology of this species provides a scenario in which the "optimum" sperm phenotype could be under selection. For example, female mate choice via male visual ornamentation has been demonstrated (De Souza *et al*., 2014), even though males provide only the ejaculate, but no other obvious benefits to females (e.g. no parental care by males). Besides, male sperm is stored for many months during female's life and they are progressively used to produce hundreds of workers before the reproductive females. Therefore, the sperm obtained from the single male a female mate with must presumably be of high quality, potentially reflected in sperm morphometry (Fitzpatrick and Baer, 2011). In other words, they should be closer to the "optimum" phenotype. Together, this body of information provides the first step to investigate the relation between female sexual preferences, male sexual ornaments and ejaculate quality (the phenotype-linked fertility hypothesis, Sheldon, 1994), which we now have initiated.

Acknowledgements

This work was supported by FAPESP (2015/05302-0) to A. R. De Souza.

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Received June 2, 2017. Accepted January 15, 2018.