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RESEARCH ARTICLE - BEES

Assessing Sperm Quality in Stingless Bees (Hymenoptera: Apidae)

HM Meneses[1], S Koffler[2], BM Freitas[1], VL Imperatriz-Fonseca[2], R Jaffé[2]

1 - Universidade Federal de Ceará,UFC, Fortaleza, CE, Brazil
2 - Universidade de São Paulo, USP, São Paulo, SP, Brazil

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Corresponding author
Hiara Marques Meneses
Av. Mister Hull 2977, Campus do Picí, Bloco 808, 60021-970, Fortaleza-CE, Brazil
E-Mail: hiarameneses@gmail.com

Abstract

Although stingless bees have a great potential as commercial pollinators, their exploitation depends on the successful reproduction of colonies on a large scale. To do so, it is essential to develop accurate diagnostic tools that enable a better understanding of the reproductive biology of stingless bees. Sperm counts, sperm morphology and sperm viability (the relative proportion of live to dead sperm), are key parameters assessing semen quality and potential fertilization success. Here we present standardized protocols to assess these three parameters. We used *Scaptotrigona* aff. *depilis* (Moure) as a study model. Semen extractions from the seminal vesicles were found to yield better results when performed in mature rather than in younger males. For morphology and viability analyses, the best semen dilution on Hayes solution was adding 120 µl to the contents of the two seminal vesicles. For sperm counts, however, we recommend a higher dilution (1,000 µl). Sperm viability values were higher when Hayes solution was adjusted to pH 8.7, and when samples were analyzed before 24 hours from collection. Based on these results we present standard protocols, hoping they will be useful to future researchers assessing sperm quality in other stingless bee species.

Introduction

In order to exploit the great potential of stingless bees as commercial pollinators, it is of great importance to improve management and optimize practices to produce colonies on a large scale. Therefore, it is essential to develop accurate diagnostic tools that enable a better understanding of the reproductive biology of stingless bees.

Semen quality is known to determine male reproductive success across taxa (Simmons, 2001). In turn, semen quality can be affected by factors such as the number, morphology and viability of sperm (Garófalo, 1980; Simmons, 2001; den Boer et al., 2009). Few studies have looked at sperm characteristics of bees in general, and even less in stingless bees. Cortopassi-Laurino (1979) counted the number of sperm in males of *Plebeia droryana* (Friese) and found 313,000 ± 102,050 sperm per male. Garófalo (1980) evaluated the amount of semen produced by the males and stored in the female's spermathecae in different species of bees, including eight stingless bees, and found that there is no relationship between social levels and the amount of sperm found in females and produced by males. Conte et al. (2005) found that the small spermatids are lost during the early stages of spermiogenesis of *Melipona quadrifasciata anthidioides* Lepeletier. Also addressing the spermatogenesis process, Lino-Neto et al. (2008) found that half of the spermatids formed during the spermatogenesis are not transformed into viable sperm cells in *Scaptotrigona xanthotricha* Moure. Pech-May et al. (2012) found a strong positive relationship between the size of male and semen production in *Melipona beecheii* Bennett. Camillo (1971) noted an increase of 2.35 times in the number of sperm of giant *Friesella schrottkyi* (Friese) males (produced occasionally when an unfertilized egg is raised on a royal cell) compared to what is found in common males. Table 1 summarizes previous contributions to the study of stingless bee sperm.

The viability of spermatozoa, or relative proportion of live to dead sperm, is an important parameter to appreciate reproductive success of males (Simmons, 2005), and it is used to analyze semen quality in honeybees (Collins & Donoghue, 1999; Collins, 2004; den Boer et al., 2009; Gençer et al., 2014). It is also commonly used in other organisms to study various aspects of their reproductive biology (Garner et al., 1994; Ball et al., 2001; Paulenz et al., 2002).

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Recent advances in the use of flow cytometry to assess sperm traits in social insects allowed significant improvements, decreasing processing times and increasing accuracy (Cornault & Aron, 2008; Paynter et al., 2014). However, flow cytometry equipment is not common across the tropics (where stingless bees occur), and the costs involved in their use and maintenance are substantially higher than those involving fluorescence-based methods. We thus provide standard protocols for fluorescence-based methods, which are cheap and ready available across the tropics, aiming at facilitating further research on stingless bees. Although less accurate than flow cytometry methods, fluorescence-based methods have been widely used and they are well accepted as standard methods (Thomas & Simmons., 2007; den Boer et al., 2008, 2010; Cobey et al., 2013). Moreover, sperm viability obtained with fluorescence microscopy and flow cytometry has been found indistinguishable in some cases (Paynter et al., 2014).

To date, no standard protocol is available to assess sperm quality in stingless bees. Here we fill this gap by presenting appropriate protocols for assessing sperm counts, sperm morphology and sperm viability in stingless bees.

Table 1. Works addressing sperm biology in stingless bees.

Species	Topic	Reference
Friesella schrottkyi (Friese)	Giant males	Camilo et al. (1971)
Plebeia droryana (Friese)	Sperm number	Cortopassi-Laurino (1979)
Friesella schrottkyi (Friese) *Lestrimelitta limao* (Smith) *Melipona marginata* Lepeletier *M. quadrifasciata* Lepeletier *M. rufiventris* Lepeletier *Scaptotrigona postica* (Latreille) *Tetragonisca angustula* (Latreille) *Trigona hyalinata* (Lepeletier)	Sperm produced by males and stored in queen's spermathecae	Garófalo (1980)
Melipona quadrifasciata Lepeletier	Spermatogenesis	Conte et al. (2005)
Scaptotrigona xanthotricha Moure	Spermatogenesis	Lino-Neto et al. (2008)
Melipona beecheii Bennett	Sperm number	Pech-May et al. (2012)

Material and Methods

We used established protocols for sperm counts and sperm viability in honeybees (*Apis mellifera* Linnaeus) (Collins & Donoghue, 1999; Cobey et al., 2013), as a guideline to develop protocols to analyze stingless bee sperm. All tests were conducted in the Bee Laboratory, Department of Ecology, University of São Paulo. Our study model was *Scaptotrigona* aff. *depilis* (Moure), a common stingless bee of Southeastern Brazil. Specimens of each colony were collected and identified with the aid of a specialist (Dr. Silvia R. M. Pedro). Voucher specimens can be found in the Coleção Entomológica Paulo Nogueira-Neto (CEPANN), located in the University of São Paulo, São Paulo, Brazil.

Adjustments were implemented to improve the collection of semen from the seminal vesicles (see Supplementary Materials for details). Males were collected from male aggregation and from the interior of colonies. Those found in aggregations (Fig 1) were considered mature and originated from different colonies (Paxton et al., 2000). Males collected inside the colonies were only distinguished as freshly emerged males (usually of a lighter color) or adult (darker) males. However, during dissections, we found marked differences between males in testis size and migration of sperm to the seminal vesicles. We thus used this information as a proxy for male age, where males with bigger testis and incomplete semen migration were characterized as immature males.

Fig 1. Aggregation of *Scaptotrigona* aff. *depilis* males.

Different semen dilutions were tested (40, 100 and 200 µl) to facilitate the counting of sperm cells (5 males per treatment). As pH was found to have a profound effect on sperm viability during our initial trials, we experimentally manipulated pH to identify the pH yielding maximal sperm viability (pH tested was 8.4, 8.7 and 9.0, with 3 males per treatment). Likewise, because sperm cells start to die once the ejaculate has been collected, we also tested the effect of time on sperm viability (fresh, 1, 3, 5 and 24 hours after dissection, with 5 males per treatment).

For the sperm counts and sperm morphology analyses we stained sperm using DAPI (Fig 2, see Supplementary Material for details). In order to assess the morphology of sperm cells, black and white images of the DAPI-stained cells were taken (20x magnification). Images were analyzed with the software Image J, adjusting brightness and contrast for better visualization. For the measurement of the sperm head area, each image was adjusted by changing the threshold parameter, which enhances binary contrast. This modification was done until all head area was selected, excluding the sperm tail and background. After this procedure, head area was selected with the automatic selection tool (wand) and measured. Because threshold adjustments may be subjective, training and standardizing the procedure is suggested before the real measurements are taken. Illustrative results are presented for

10 sperm cells from one male (mean ± SE). Sperm counts were estimated by diluting the total amount of sperm of each male 10,000 times and counting cells in three samples of 1 µl (air dried in microscope slides). The samples were also DAPI-stained. Counting began at the right edge of each sample, and continued until de left side of the sample was reached. The mean value obtained from the three samples was multiplied by 10,000, to estimate the total number of sperm per male. Results for sperm counts are presented for six males (mean ± SE).

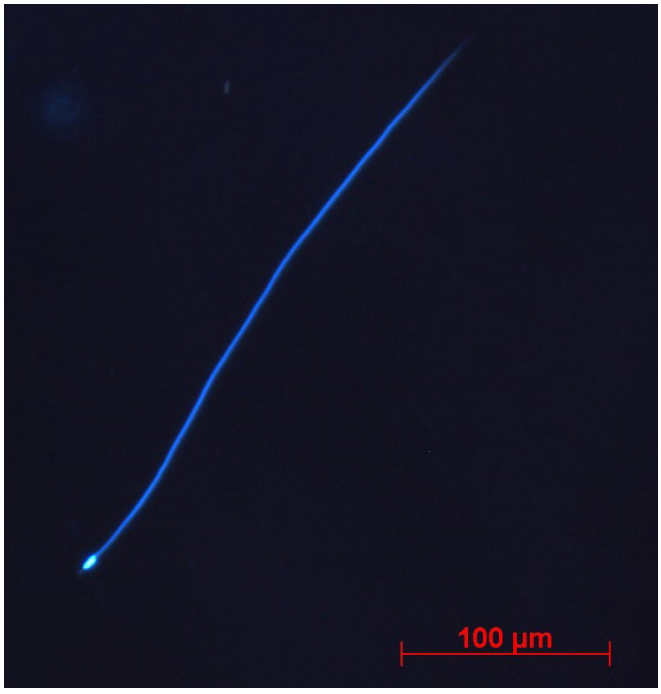

Fig 2. Honeybee (*Apis mellifera*) sperm cell marked with DAPI.

We used the LIVE / DEAD ® Invitrogen Sperm Viability Kit to assess sperm viability. Employing a fluorescence microscope and a cell counter, we proceeded to count the first 400 cells found from the center of the cover slip. Sperm cells were classified as green (live), red (dead) and green / red (dying) (Fig 3). The workflow protocols are summarized in Figs 4 A, B, and C.

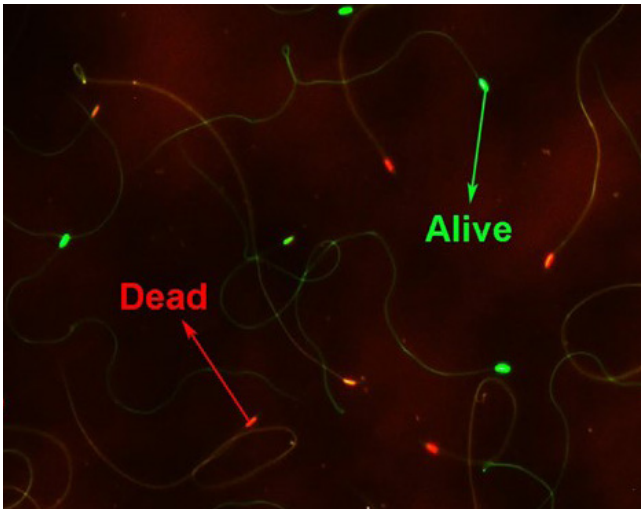

Fig 3. Live (green) and dead (red) honeybee (*Apis mellifera*) sperm.

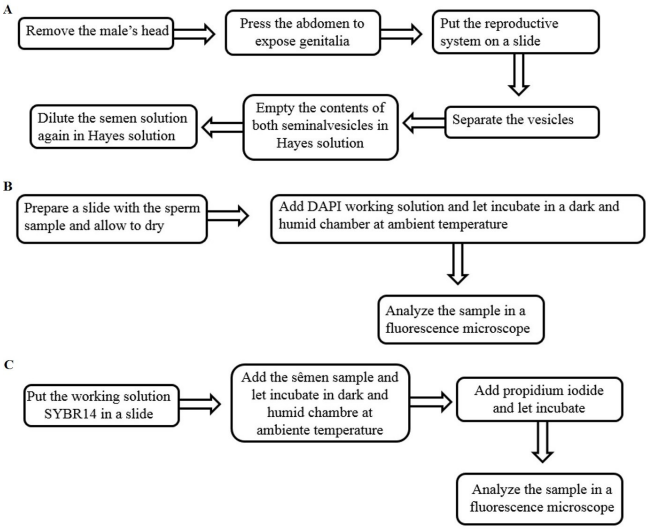

Fig 4 A. Diagram summarizing the workflow of the semen extraction protocol; **B**. Diagram summarizing the workflow of the sperm count and morphology protocol; **C**. Diagram summarizing the workflow of the sperm viability protocol.

Statistical analyses consisted in binomial generalized linear models, as they are the standard method to analyze proportion data (Crawley, 2013), such as sperm viability (dead/alive). Similar analyses are often implemented in studies of sperm viability (den Boer et al., 2009; Stürup et al., 2011). All statistical analyses were implemented in R.

Results

Semen was more easily collected from the seminal vesicles of mature males (Fig 5) rather than younger ones (Fig 6), as semen had already migrated from the testicles to the seminal vesicles in mature males only.

Sperm counts in *S.* aff. *depilis* resulted in an average (± SE) of 1,487,778 ± 36,044 sperm cells per male. When assessing sperm morphology, we found a mean total length of 85.58 ± 1.06 µm, a mean head length of 9.50 ± 0.24 µm, a mean tail length 76.08 ± 0.98 µm and a mean head area of 23.42 ± 0.58 µm^2. Finally, sperm viability ranged from 52 to 87% (Figs 7 and 8).

We found that the best semen dilution for sperm morphology and viability assays was 100 µl Hayes solution to the contents of the two seminal vesicles, in addition to the initial 20 µl Hayes for emptying the vesicles. This was due to the greater ease during the identification and counting of sperm cells. For sperm counts, however, we recommend a higher dilution (10,000 x) in order to identify single isolated sperm cells.

Sperm viability was significantly affected by pH, being highest when Hayes solution was adjusted to a pH of 8.7 (Fig 6; Table 2). We also found a significant effect of incubation time (Table 3). No difference was found in sperm viability for the initial time intervals tested (fresh, 1, 3 and 5 hours after collection), but a marked decrease in viability was found after 24 hours (Fig 7).

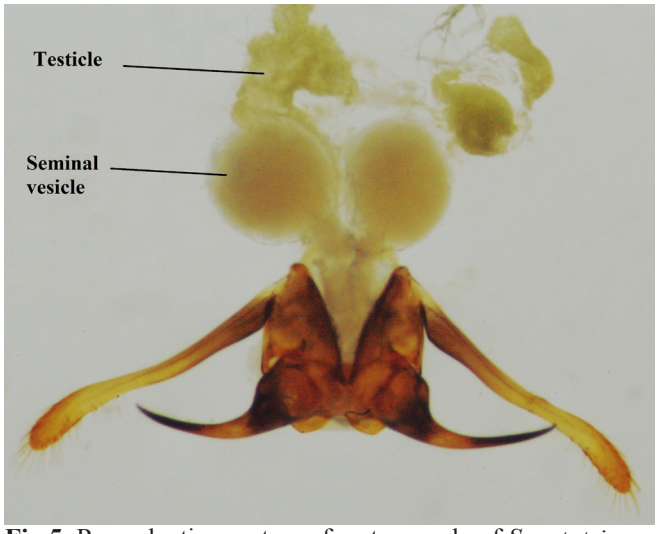

Fig 5. Reproductive system of mature male of *Scaptotrigona* aff. *depilis*.

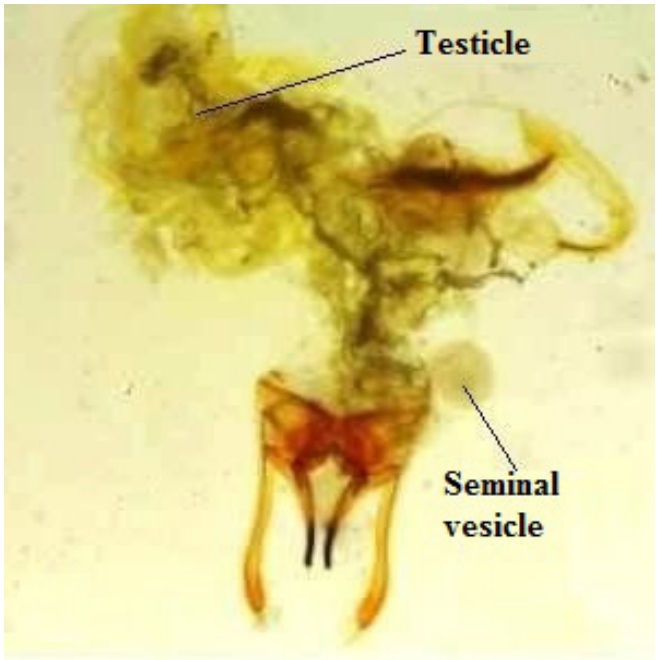

Fig 6. Reproductive system of immature male of *Scaptotrigona* aff. *depilis*.

Table 2. Summary statistics for the effect of pH on sperm viability.

Response	Predictor	Estimate	SE	P-value
Sperm viability	pH 8.4	1.08	0.07	< 0.001
	pH 8.7	0.39	0.10	< 0.001
	pH 9.0	-0.27	0.09	0.003

Discussion

Semen extraction in stingless bees is different than in honeybees. First, stingless bees lack the enormous mucus glands found in honeybees, so the dissection of the reproductive tract is more delicate. Second, the manual collection of the ejaculate from the end phallus is not possible, because the manual eversion of the endophallus fails to stimulate ejaculation. Finally, dissection is more difficult in smaller species, requiring the use of specialized equipment.

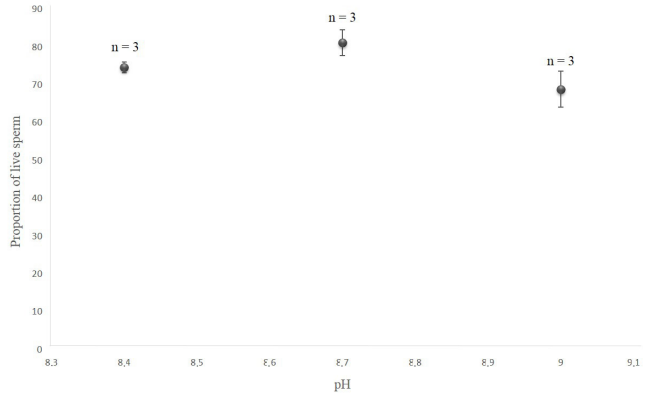

Fig 7. Sperm viability of sperm from *Scaptotrigona* aff. *depilis* diluted in Hayes solution with different pH values.

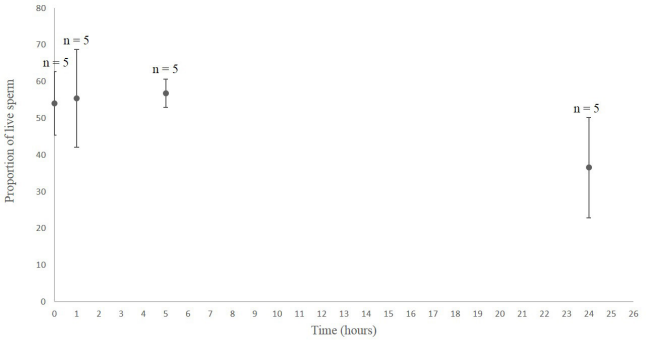

Fig 8. Sperm viability of sperm from *Scaptotrigona* aff. *depilis* assessed on fresh semen, 1, 5 and 24 hours after collection.

Different protocols are available for counting sperm cells. Counting cells using a hemocytometer was described for *A. mellifera* sperm (Cobey et al., 2013; Human et al., 2013), in which a known volume containing unstained cells is analyzed. A drawback of this method is that the counting procedure must be done immediately after dissection. In our protocol, microscope slides are prepared with samples of sperm in a known dilution, and only three samples are counted to estimate the total number of sperm cells. Since samples are dried, the slides can be stored for later analyses, which is an advantage when a high number of males need to be analyzed. Other studies implemented similar techniques successfully (Baer et al., 2006; Stürup et al., 2011).

Our results show that sperm counts, sperm morphology and sperm viability can be effectively assessed

Table 3. Summary statistics for the generalized linear mixed effect model for sperm viability in different intervals between semen collection and viability analysis.

Response	Predictor	Estimate	SE	P-value
Sperm viability	Fresh semen	0.15	0.18	0.41
	1h after collection	0.08	0.26	0.76
	5h after collection	0.12	0.26	0.64
	24h after collection	-0.76	0.26	0.003

in a stingless bee. For sperm morphology and viability, the best semen dilution was found to be 120 µl Hayes solution to the total semen content of a male. For sperm counts, the best dilution factor was 10,000 x. While the pH yielding the highest sperm viability was at 8.7, we found that the best time to assess viability was before 24 h of semen collection. Given the high susceptibility of sperm to manipulation, desiccation, pH and temperature extremes, we recommend that great care must be taken to ensure sperm are analyzed shortly after collection and under the best possible conditions. Based on these results we present standard protocols, hoping they will be useful to future researchers assessing sperm quality in other stingless bee species. Such studies could contribute advance basic knowledge on the reproductive biology of stingless bees, and facilitate their commercial use.

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