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Electroejaculation and semen buffer evaluation in the microbat *Carollia perspicillata*

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ABSTRACT

Scientific interests and conservation needs currently stress the necessity to better understanding bat reproductive biology. In this study, we present the first, safe, inexpensive, and reliable method to obtain sperm from a microbat species (*Carollia perspicillata*) by electroejaculation. This method revealed to be highly efficient (100% success rate). We obtained ejaculates composed of two characteristically different fractions. We compared three buffers and recommend using an Earle's balanced salt solution as a semen extender. Earle's balanced salt solution provided significant repeatable measure of swimming ability (intraclass correlation coefficient: 0.74, $P < 0.01$) and proportion of motile sperms (intraclass correlation coefficient: 0.08, $P = 0.01$) and allowed sperm to maintain optimal swimming capacity over time. None of the buffers could dissolve all the coagulated sperm. Although the trypsin buffer freed a larger fraction of spermatozoa in the ejaculate, it impaired swimming ability without improving motility, viability, and stamina. We thus argue that the sperm population analyzed with Earle's balanced salt solution is a representative of the ejaculate. Finally, we found that the mean sperm velocity of *C perspicillata* (78.8 μm/s) is lower than that predicted by regressing sperm velocity on relative testes mass, a proxy of sperm competition. The question as to whether *C perspicillata* is an outsider for sperm velocity, or whether bats evolved yet another unique mechanism to cope with sperm competition deserves more investigations.

1. Introduction

Although long overlooked, bats are currently a promising research model. Indeed, they represent around a fifth of all mammalian species and are distributed worldwide and reveal a complex and diverse biology [1,2]. The broad ecological services offered by bats range from pest control to forest regeneration and pollination, with a potentially enormous economical value [3,4]. Moreover, conservation strategies are currently urgent, as anthropogenic threats have already caused large reductions of certain populations, driving species toward local or global extinctions [5,6]. Despite those dramatic threats, bat semen preservation for assisted reproduction has seldom been addressed as an appropriate method for species and genetic conservation [7–9]. Such an approach would, however, appear most applicable in species breeding poorly in captivity and for which *ex situ* conservation is not a reasonable option. Besides those conservation perspectives, bat semen studies may be valuable for fundamental research. Indeed, the sperm of some bats species is already known for its extraordinary ability to sustain fertility for periods lasting up to 7 months [10,11]. Additionally, with their rich variety of social systems and ecological niches, bats are ideal models for evolutionary studies of mammalian male reproductive physiology [12–14].

One of the reasons why semen collection methods have not yet been properly undertaken in microbats may be the

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difficulty to sample ejaculates from living wild or captive individuals. However, methods have already been developed for other mammals of similar size and for flying-fox species [15,7]. After the collection, adequate ejaculate processing should provide suitable samples for subsequent analyses or utilization. Semen processing efficiency and measurement reliability depend on species-specific sperm biochemical and physiological characteristics [16]. Consequently, numerous buffers are currently available for mammalian models.

Previously obtained ejaculates from *Carollia perspicillata* were small in volume and heterogeneous, as they comprised coagulated sperm that did not spontaneously dissolve (unpublished data). Semenogelin is a protein that mechanically traps spermatozoa and inhibits sperm motility and capacitation [17]. The addition of trypsin, a serine protease, in the seminal extender can precipitate the dissolution of the sperm coagulum [18–20]. However, although the enzymatic reaction allows the dissolution of coagula and the liberation of bound spermatozoa in some species, it can impair sperm survival and motility in others [21].

With this study, we describe the first method of electroejaculation (EE) for a microbat species, which may permit repeated collection of semen without detrimental effects on the animals. Second, we compare three different buffer solutions as sperm extender to get optimal sperm survival and ejaculate fluidity allowing adequate computer-assisted sperm analysis (CASA).

2. Materials and methods

2.1. Animal welfare and ethics

We monitored animal recovery and welfare as well as possible effects on health during the subsequent days. For the first 20 collections, animals were kept after the manipulation in cages (2.1 m × 0.9 m × 1.4 m for l × l × h) for 5 days with food and water *ad libitum*. We also monitored their post-anesthesia recovery by keeping them for one to three hours in individual cotton bags provided with food *ad libitum* (apple pieces). During this period, we recorded any injury or abnormal behavior that would indicate excessive pain or stress and would require euthanizing the animal in accordance with our guidelines. As no midterm disturbance was detected, the eight bats used for the buffer comparison were released directly after the post-anesthesia monitoring. Experimental setup and detention conditions were authorized by the veterinary office of the Canton of Fribourg after examination by the cantonal ethical committee (FR_2012_15E).

2.2. Study area and model species

Carollia perspicillata (family: phyllostomidae; suborder: yangochiroptera) is a moderate-sized (18.5 g) frugivorous species. This species is common in its natural range (Central and South America) and can easily breed in captivity. Thus, *C perspicillata* is suitable for zoos and research facilities [22]. The present study was performed in the Papiliorama, a tropical zoo (Kerzers, Switzerland) where light cycles are reversed on a 12/12 hour basis. A population of 400 individuals lives in semicaptivity with constant environmental conditions. Bats roost in an artificial cave and can fly freely under a dome, which mimics a tropical environment. A fruit-based mixture is provided twice a day. Males with large scrotal testes are constantly present in captivity and nature, where testes size can vary slightly with reproductive seasons [23]. However, in constant environmental conditions, captive bats do not show any reproductive patterns [22]. Bats were caught with a harp trap (Faunatech Austbat, Australia) or with a hand net, and males with large scrotal testes (approximately 7 mm in length) were detained for semen collection. With a single collection, we tested the efficiency of the method on 20 bats. Then, another eight individuals were used to conduct the sperm buffer comparison on the basis of mobility traits. An insufficient amount of ejaculate (<3 μL) was obtained from one male, reducing the sample size to seven individuals for this analysis.

2.3. Anesthesia

Health status was evaluated before the manipulation by general visual inspection with focus on fur quality, presence of superficial injuries and apathy, and only bats in good condition were kept for the experiment. To avoid hypothermia, animals were laid dorsally on a warming pad. Anesthesia induction was achieved by inhalation of 5% isoflurane (Nicholas Piramal I Ltd, UK) mixed with 0.8 L/min oxygen (Carbagas, Switzerland) through a Rodent Nosecone Non-rebreathing system (Rothacher medical, Switzerland). After the induction phase, anesthesia was maintained during the EE with a lower dose of isoflurane (1.5%–2.5%) mixed with 0.8 L/min oxygen. After manipulation, the bat was provided with pure oxygen until emergence. Throughout the procedure, the respiratory rate was visually monitored.

2.4. Electroejaculation

Any ejaculate containing motile sperm was considered a successful EE. The rectal probe (diameter, 2.5 mm; see Supplementary Fig. 1) was fitted with two 4-mm electrodes situated at 2.5 mm from the probe's distal end (International Canine Semen Bank, USA). After anesthesia induction, the anus and the genitalia were first washed with water-soaked cotton. Then, the probe, coated with an aqueous lubricant (K-Y; Johnson and Johnson), was gently inserted 1-cm deep into the rectum. The electrodes were placed upward to face the prostate and stimulate the nearby nerves, contracting the pelvic muscle. As the nerves passing through this area are responsible for the erection but also activate the leg muscles, we ensured that the probe was correctly positioned by observing the legs' contractions. Three series of stimulations were performed progressively to avoid urinary contamination. Each series consisted of 10 stimulations with increasing intensity (0.3–3 mA, 50 Hz) of 1 second each with a 1-second break in-between. One series ended with a 10-second stimulation at the highest current intensity accompanied with gentle in and out movements of the probe. Two resting periods of 60 seconds were planned between each series. Stimulation series were designed using an audio software (Audacity 2.0.4) and transferred to the probe with an audio amplifier (JVC A-X2). The electrical current was continuously

monitored with a current reader (Fluke 77 multimeter, see Supplementary Fig. 2).

2.5. Ejaculate processing

To find the appropriate semen extender for our model species, we compared three buffered solutions. As a reference solution, we used a TRIS buffer (pH 7.6, 0.05-M Tris, 0.15-M NaCl). TRIS-based extenders are widely used for breeding animals [24–26] and wildlife species [27–29] for their conservation properties. For the second solution, we used 1% trypsin (in TRIS-buffered solution [TBS] buffer). This concentration allows for a rapid liquefaction of the ejaculate and a recovery of mobile sperm in spider monkeys (*Ateles geoffroyi* [20]). Last, the third buffer was a commercial Earle's balanced salt solution (EBSS) semen extender (SpermWash; Cryos, Denmark), which is widely used for human pharmaceutical diagnostics. During the EE procedure, a 0.5-mL tube filled with 20 μL of TBS solution was maintained over the penis. All the ejaculate phases were collected together. After a gentle homogenization, three similar aliquots of 5-μL mix were diluted into 20 μL of the different media to be compared. All the three solutions were then kept at 37 °C on a heating block until sperm mobility analyses (maximum 138 minutes).

2.6. Sperm mobility analyses

Sperm mobility analyses were achieved by loading 3 μL of sperm mix in a 20-μm deep chamber slide (SC 20-01-04-B; Leja, the Netherlands) approximately every 30 minutes, until sperm motility and velocity were substantially reduced (visual inspection by Nicolas J Fasel). As the sperm/buffer solution filled the chamber, sperm cells drifted for a short time. As soon as sperm drift stopped, we started recording. We used a Kappa CF 8/5 camera mounted on an Olympus BX41 microscope with ×400 magnification and a dark field background. For each session, one to 12 2-second films (25 frames/s) were taped, and the mean of several ejaculate traits such as sperm motility, defined as the proportion of motile sperm, the number of sperm tracks (thereafter considered as the number of free cells), curvilinear velocity (μm/s), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), linearity (VSL/VAP), wobble or oscillation of the actual trajectory about its average path (VAP/curvilinear velocity), progression (μm), and beat cross frequency i.e., the number of times the sperm head crossed the average path per second (Hz) were recorded. We performed a principal component analysis and used the first principal component (PC1) as a measure of sperm's swimming ability. Any sperm cells with a VSL lower than 2 μm/s were considered immobile and excluded from the calculation of the trait mean. We further excluded sperm tracks analyzed with less than 10 frames. Sperm mobility analyses were automatically performed with the plugin CASA for ImageJ [30] followed by visual inspection.

2.7. Buffer comparison procedure

The first criterion for the buffer evaluation was the ability to dissolve coagula and consequently release free sperm cells. Practically, as the ejaculate was divided in three aliquots of similar volume, we expected that any significant increase in the number of free sperm cells, compared with observations in TBS, could only result from coagula dissolution. Then, the preservation of motility and sperm swimming ability (PC1) and their maintenance over time (viability and stamina) were further considered of prime importance when comparing buffers. As a final criterion, we tested the accuracy of the various measurements by calculating the repeatabilities as the intraclass correlation coefficients (ICC).

2.8. Statistical analysis

The analyses were performed with R (3.1.0; Development Core Team, 2008), and the significance level for all the tests was set at 5%. The number of free sperm cells, the preservation of swimming ability (PC1), and the motility were analyzed as response variables with linear or generalized linear mixed models (LMM: lmer, package lmerTest [31] and GLMM: glmer package lme4 [32]). For the number of free sperm cells, a negative binomial distribution was used to cope with overdispersion, and a binomial distribution was used for the proportion of motile sperm. The influence of the different buffers, the time since the ejaculation, and their interaction were tested as fixed effects. Male identity was used as the random factor, and individual random slopes were estimated in relation with time. Changes in any of the three response variables within one buffer were calculated with pairwise Tukey *post hoc* comparisons, using the LMM or GLMMs without the time and buffer interaction (function glht, package multcomp [33]). The change of those variables in relation to the fixed effects was evaluated with a type-II ANOVA using Wald chi-square tests on deviance tables (ANOVA, package car [34]).

The repeatability (ICC) of the number of free sperm cells and of the swimming ability measures was then calculated using penalized quasi-likelihood multiplicative generalized linear mixed effect models. We adapted the function rptR (packages rptR [35]) to integrate the time as a covariate. Male identity accounted for the nested structure of the data.

3. Results

3.1. Collection efficiency

Ejaculates were obtained in 100% of the stimulations during both collection events (collection efficiency evaluation and buffers comparison: 20 + 8 attempts). Collected ejaculates were constituted of two phases. Shortly after the beginning of the stimulation and with the onset of erection, a highly viscous fraction was always ejaculated (1–2 μL). A second larger phase (approximately 10 μL) was ejaculated in 64% of the stimulations (11 + 7/28 cases) after the termination of the anesthesia (approximately 10 minutes) accompanied by the first body movements. The entire ejaculate had a viscous and heterogeneous appearance, comprising seminal fluids and free or coagulated spermatozoa. In some cases, dust-like particles soiled the ejaculate.

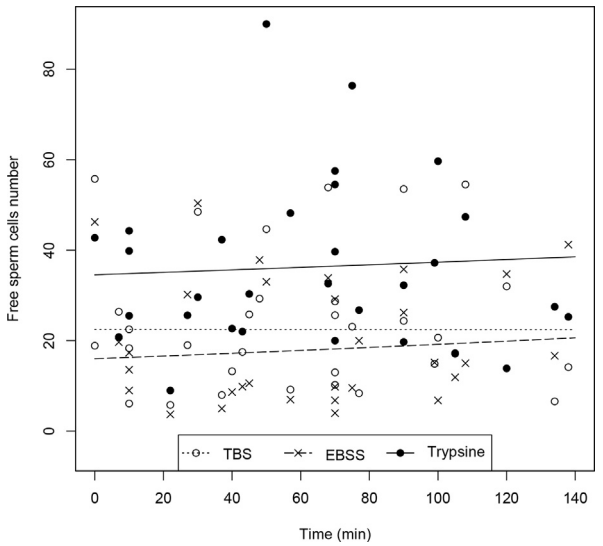

Fig. 1. Number of free sperm cells in relation with time. Free sperm cells are the average number of tracks measured by computer-assisted sperm analysis. EBSS, Earle's balanced salt solution; TBS, TRIS-buffered solution.

3.2. Sperm mobility analysis and buffer evaluation

Sperm mobility measures were taken during a minimal period of 77 minutes and a maximum of 138 minutes (n = 7, median: 105 minutes), approximately every 30 minutes. During that time, the number of free cells remained stable (Fig. 1, time as main effect: type-II Wald chi-square tests: $\chi^2 = 0.28_1$, P = 0.600), with no significant differences across buffers (Fig. 1, time in interaction with buffer: type-II Wald chi-square tests: $\chi^2 = 0.26_2$, P = 0.875). However, the number of free sperm cells differed among buffers at intercept (Fig. 1, buffer as main effect: type-II Wald chi-square tests: $\chi^2 = 25.69_2$, P < 0.001). Sperm dissolution, expressed as a significant increase in the number of free cells, was only observed with the trypsin buffer (Fig. 1, Table 1).

All seven mobility traits were intercorrelated (Table 2). The first two principal components resulting from a principal component analysis of the seven mobility traits extracted by CASA revealed eigenvalues above 1 (Table 3). For the subsequent analyses, we only kept the PC1, explaining 73.2% of the variance, as a measure of swimming ability. Sperm swimming ability decreased significantly with time (Fig. 2, time as main effect: type-II Wald chi-square tests: $\chi^2 = 7.78_1$, P = 0.005), but stamina (i.e., swimming ability decline) was similar in all buffers (Fig. 2, time in interaction with buffer: type-II Wald chi-square tests: $\chi^2 = 3.66_2$, P = 0.161). Swimming ability differed among buffers at intercept (Fig. 2, buffer as main effect: type-II Wald chi-square tests: $\chi^2 = 26.63_2$, P < 0.001). The trypsin buffer significantly reduced sperm swimming ability (Table 1). Finally, a significant reduction of sperm motility was also observed over time (Fig. 3, time as main effect: type-II Wald chi-square tests: $\chi^2 = 8.64_1$, P = 0.003), and no difference in the reduction of motile sperm concentration was detected among buffers (Fig. 3, time in interaction with buffer: type-II Wald chi-square tests: $\chi^2 = 1.68_2$, P = 0.432). Motility was not different among buffers (Fig. 3, buffer as main effect: type-II Wald chi-square tests: $\chi^2 = 3.74_2$, P = 0.154, Table 1). Sperm mobility traits measured within EBSS directly after ejaculation are provided in Table 2.

Table 1
Tukey *post hoc* comparisons.

	Estimate ± standard error	P value
Number of free cells		
EBSS-TBS	−0.23 ± 0.14	ns
Trypsin-TBS	0.48 ± 0.14	**
Trypsin-EBSS	0.70 ± 0.14	***
Sperm swimming ability[a]		
EBSS-TBS	0.69 ± 0.45	ns
Trypsin-TBS	−1.57 ± 0.45	***
Trypsin-EBSS	−2.26 ± 0.45	***
Sperm motility (proportion of motile sperm)		
EBSS-TBS	−1.16 ± 0.59	ns
Trypsin-TBS	−0.23 ± 0.58	ns
Trypsin-EBSS	−0.94 ± 0.59	ns

Abbreviations: EBSS, Earle's balanced salt solution; ns, not significant; TBS, TRIS-buffered solution.
[a] First component of the principal component analysis on the seven parameters.
Statistical differences between buffers indicated by ns (P > 0.05), ** (P ≤ 0.01), or *** (P ≤ 0.001).

3.3. Sperm trait repeatability

The measure of the number of free cells was significantly repeatable in TBS and EBSS but not in the trypsin

Table 2
Mean and standard error of the mean (SEM) of *Carollia perspicillata*'s sperm traits measured within Earle's balanced salt solution extender at the first measure after ejaculation and Pearson's correlation coefficients of the overall different sperm mobility traits.

	Motility[a]	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	LIN (%)	WOB (%)	PROG (μm)	BCF (Hz)
Mean	0.56	78.80	70.01	55.28	79	88	301.00	8.129
SEM	0.27	25.05	24.31	19.06	5	5	112.63	1.81
VAP		0.93***						
VSL		0.91***	0.99***					
LIN		0.41***	0.49***	0.56***				
WOB		0.62***	0.82***	0.84***	0.67***			
PROG		0.62***	0.71***	0.77***	0.76***	0.74***		
BCF		−0.64***	−0.74***	−0.71***	−0.21*	−0.68***	−0.33**	

Abbreviations: BCF, beat cross frequency; LIN, linearity; PROG, progression; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.
[a] Proportion of motile sperm.
Statistical correlation indicated by * (P < 0.05), ** (P ≤ 0.01), or *** (P ≤ 0.001).

Table 3
Eigenvalues and proportion of variance extracted from the two principal components analysis of the sperm mobility traits with correlation coefficients to those various traits.

	PC1[a]	PC2[b]
Eigenvalue	5.11	1.01
Proportion of variance	0.73	0.15
VCL	0.39	0.23
VAP	0.43	0.19
VSL	0.43	0.10
LIN	0.30	−0.64
WOB	0.40	−0.08
PROG	0.37	−0.43
BCF	−0.31	−0.56

Abbreviations: BCF, beat cross frequency; LIN, linearity; PROG, progression; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.
[a] First principal component (PC1) analyzed from the various mobility traits, representing sperm's swimming ability.
[b] Second principal component (PC2).

buffer (Table 4). For the swimming ability, the highest repeatability was found for EBSS buffer and measures appeared significantly reliable in trypsin buffer also, in contrast with those found in TBS (Table 4). Motility measures were reliable within all the buffers with a higher repeatability within trypsin (Table 4).

4. Discussion

Using an EE method, we successfully obtained an ejaculate with motile sperm in all attempts. In the first phase of this study, all males (20/20) emitted a first, small (1–2 μL), and viscous ejaculate with motile sperm. With those males, our concern for animal welfare leads us to keep the handling time short by terminating the procedure shortly after males emerged from narcosis (<10 minutes). However, in 11 cases (55%), we obtained a second, larger (ca. 10 μL), and less viscous ejaculate. This ejaculate was emitted at the very end of the procedure while males were awakening and starting to move. Studies using EE with mammals often report the collection of several ejaculates with distinct characteristics [36,37]. In the second phase with the eight subsequent males, we thus decided to prolong the manipulation time beyond narcosis and electric stimulation, and we obtained a second ejaculate in seven of the eight attempts. Because the second ejaculate was much more abundant than the first one, and to collect significant volumes of semen, we recommend holding the bat in its position with the collection tube over the penis until the male ejaculates a second time.

Among the three buffers tested, EBSS offered the best conditions for sperm mobility analysis. Viability and stamina, as measured by the decline in motility and sperm swimming ability (PC1), respectively, were similar with all buffers. However, the EBSS buffer always yielded reliable,

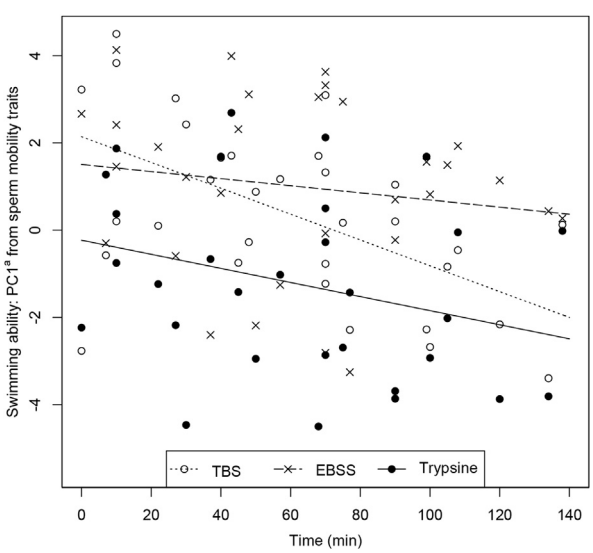

Fig. 2. Sperm swimming ability in relation with time. [a]First axis from principal component (PC1) analysis of sperm mobility traits calculated by computer-assisted sperm analysis. EBSS, Earle's balanced salt solution; TBS, TRIS-buffered solution.

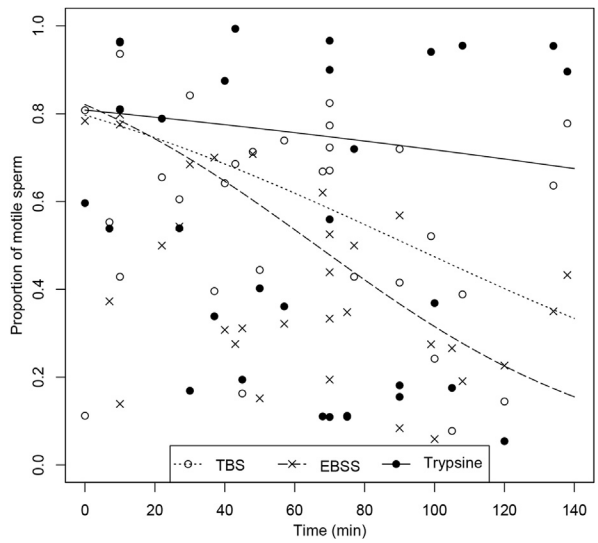

Fig. 3. Motility, proportion of motile sperm, in relation with time. EBSS, Earle's balanced salt solution; TBS, TRIS-buffered solution.

Table 4
Repeatability (intraclass correlation coefficient [ICC]) of the sperm mobility measures in the three different buffers with confidence interval (CI) and P value.

	ICC	CI	P
Number of free cells			
TBS	0.29	0.00–0.66	0.03
Trypsin	0	0.00–0.31	0.95
EBSS	0.75	0.22–0.91	<0.01
Swimming ability[a]			
TBS	0.00	0.00–0.29	0.51
Trypsin	0.59	0.09–0.83	<0.01
EBSS	0.74	0.26–0.90	<0.01
Motility			
TBS	0.06	0.00–0.37	0.02
Trypsin	0.30	0.00–1.00	<0.01
EBSS	0.08	0.00–0.46	0.01

Abbreviations: EBSS, Earle's balanced salt solution; TBS, TRIS-buffered solution.
[a] First component of the principal component analysis on the seven parameters produced by the computer-assisted sperm analysis.

repeatable measures of sperm mobility traits. Although the trypsin buffer enabled the collection of a larger fraction of free spermatozoa, it impaired sperm swimming ability. This was further confirmed by visual inspection of the ejaculates dissolved in the trypsin buffer that contained numerous sperm, which although motile, were spinning around themselves and lacked progression (see videos in online supplementary material). We thus suspect that the trypsin might have triggered a premature acrosomal reaction, hence impeding proper sperm mobility [21].

None of the buffers successfully freed all the sperm in the collected semen, which raises the issue of the representativeness of the free sperm subpopulation. We believe that the EBSS extender does provide a representative sample of the sperm population in the whole ejaculate in terms of sperm quality because sperm motility, viability, and stamina were similar to the measures obtained with the trypsin buffer that dissolved a greater fraction of coagulated sperm.

Our model species, the Seba's short-tailed bat, exhibits a complex mating system where harem and bachelor males compete over access to fertile females. Moreover, females may copulate with more than one male during estrus, as it has been shown in other species with a similar mating system [38]. This generates sperm competition, in which sperm of several males compete to fertilize the ovum of a given female [39,40]. It has been repeatedly shown in several taxa from fish to birds and mammals that sperm competition leads to the evolution of larger testes mass relative to body size and greater sperm swimming speed in species with more intense sperm competition [41–43]. Tourmente et al. [43] illustrated this pattern in a comparative study on mammals, which unfortunately did not include bat species. Interestingly, the average value for VSL that we obtained in *C perspicillata* is lower than the expected value on the basis of interspecific regression. This means that the Seba's short-tailed bat produces slower sperm than their relative testes mass (125 mg for an average body mass of 18.5 g [23]) and level of sperm competition would predict.

In conclusion, we provide, to our knowledge, the first method for collecting sperm from an anesthetized microbat. We also identify EBSS as a suitable sperm extender both to maintain sperm swimming ability and allow repeatable measures of sperm quality as computed by CASA. Last, the fact that sperm velocity was found to be lower than predicted for this species raises the question as to whether bats have evolved yet another specific physiological mechanism to cope with sperm competition, or whether *C perspicillata is* a mammalian outsider.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2014.11.030.

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