

Article

Penis size and sperm quality, are all bats grey in the dark?

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Abstract

Penises play a key role in sperm transport and in stimulating female genitals. This should impact post-copulatory competition, and expose penis characteristics to sexual selective pressures. Studies of male genitalia have repeatedly reported negative static allometries, which mean that, within species, large males have disproportionately small genitals when compared with smaller individuals. Males of some sperm-storing bat species may stand as an exception to such a pattern by arousing from hibernation to copulate with torpid females. The selection for large penises might take place, if a long organ provides advantages during post-copulatory competition and/or if females have evolved mechanisms allowing the choice of sire, relying on characters other than pre-copulatory traits (e.g., penis size). In this study, we measured dimensions of the erected penis in 4 sperm-storing bat species. Furthermore, we collected sperm and evaluated the link between penis dimensions and sperm velocity. Our results revealed steep allometric slopes of the erected penis length in *Barbastella barbastellus* and an inverse allometry of penis head width in *Myotis nattereri*. More detailed studies of copulatory behavior are urgently needed to explain the range of observed scaling relations. Furthermore, penis head width correlates with sperm velocity in *Plecotus auritus*. For this last species, we propose that penis shape might act as a marker of male fertility.

Key words: *Barbastella barbastellus*, genitalia, honest signaling, *Myotis nattereri*, *Plecotus auritus*, sperm competition, sperm quality, sperm velocity, *Vespertilio murinus*.

Naturalists have long recognized the impressive diversity of male genitalia among taxa and used it in taxonomy (Matthews 1937; Hooper and Musser 1964; Dixson 1987; Ryan 1991). Eberhard (1985) emphasized that organs related to copulation have evolved faster than any other traits (Eberhard 1985), and may strengthen reproductive barrier and speciation processes. Current scientific opinions tend to agree that sexual selection drives this diversification (Arnqvist 1998; Hosken and Stockley 2004; Ramm 2007; Eberhard 2010; Simmons 2014; Simmons and Firman 2014). Several non-exclusive and often hardly extricable mechanisms may play a part in shaping male genitalia in species with internal fertilization.

Penis shapes could thus first evolve through selection during post-copulatory competition, to provide some advantages against concurrent suitors (e.g., displace ejaculate from previous males; Gallup et al. 2003; Parga 2003; Kinahan et al. 2006; Waage 2008). The morphology of male genitalia may further become entangled in a genital arms race, when female interests differ from those of males and sexual conflicts arise (Brennan et al. 2010). Large penises could also be selected when female genital tracks have to be reached from distance, as for example during underwater copulation (Miller and Burton 2001). An elaborate penis may then contribute to extending copulation duration by locking the female genitalia (Dixson 1987;

Racey et al. 1987; Ryan 1991; Cryan et al. 2012; Friesen et al. 2016) and reducing female capacity to re-mate with another male within a certain period (Schöfl and Taborsky 2002; Harari et al. 2003). Specific penis shapes or copulatory behaviors could also be selected for by females (Prause et al. 2015), as a way of being honestly informed about the good genes or fertility of their partner. This would enable mechanisms involved in cryptic female choice to generate a collective genital evolution, and might be of special importance when females can only narrowly rely on pre-copulatory traits (Andrés and Cordero Rivera 2000; Miller and Burton 2001; Dixon 2003; Reeder 2003).

It would seem reasonable to assume that penis size and its scaling to the body size should be sexually selected for. Secondary sexual traits mostly display positive allometries (i.e., the slope of the log-log regression between trait and body size is steep and generally greater than 1), meaning that these traits are disproportionately large in large individuals (Voje 2016). However, allometric studies of penis sizes among individuals from similar developmental stages and taxonomic groups (i.e., static allometry) have mostly reported negative allometric scaling (i.e., slopes are smaller than 1; Eberhard et al. 1998; Voje et al. 2014; Voje 2016).

In order to evolve positive static allometry of a given trait, it should be under directional selection and larger individuals should benefit from a higher fitness with similar relative trait sizes (Bonduriansky and Day 2003). The negative static allometry generally observed in male genitals may first be explained by a lack of directional selection on those traits. Indeed, the “one-size-fits-all” hypothesis postulates that male genitalia are under stabilizing selection to match the most common female genitalia present in the population (Eberhard et al. 1998; Eberhard 2008). Furthermore, this organ is not generally used in courtship display or as a weapon against competitors, thus reducing the selection for larger organs. Male genitalia may also evolve independently from body size, leaving allometric intercepts and not slopes to shift. Egset et al. (2012) suggested that intercepts might actually be more evolvable than allometric slopes. Such changes are however not expected to produce positive allometries (Voje et al. 2014).

The allometry of genitalia has been studied far better in insect and spider species than in any other taxonomic group. At first glance, mammal genitalia conform to the general trend, with less than a quarter of the species exhibiting an allometric slope greater than 1 (Voje 2016). It should be stressed, however, that most of the analyses are based on baculum (i.e., penis bone) sizes. This bone may assume crucial functions during penile erection and the subsequent ejaculation (Dixon et al. 2004; Herdina et al. 2015), but there is no evidence that baculum length correlates with penis length (Lüpold et al. 2004). Actual penis length has only been analyzed in a handful of studies (Lüpold et al. 2004; Kinahan et al. 2006, 2008; Manjerovic et al. 2008; Lemaître et al. 2011; Retief et al. 2013), and to our knowledge, the scaling of the functional erected organs has never been investigated.

Females and males from some species of Vespertilionidae and Rhinolophidae (order Chiroptera) have the ability to store sperm for periods extending over several months (Orr and Brennan 2015). This capacity is expected to drastically accentuate sperm competition (Wilkinson and McCracken 2003; Orr and Zuk 2013), as numerous males might engage in copulation up to the occurrence of ovulation (Kleiman and Racey 1969; Gebhard 1995; Mendonça and Hopkins 1997).

In hibernating species, large males are expected to gain more copulation opportunities by arousing from hypothermia longer

(Czenze et al. 2017) and surviving better than lighter ones (Kunz et al. 1998). Furthermore, males with larger penis might have a selective advantage by winning against competitors during post-copulatory competition (e.g., through sperm removal) or by better stimulating female genital tracts. Under these combined conditions, heavier males might benefit from a higher fitness than lighter ones with similar relative penis size. Therefore, prolonged post-copulatory competition in sperm-storing bats could have driven the evolution of steep penis static allometric slopes (Bonduriansky and Day 2003).

Females in torpor may not always have the ability to select mates before copulation, and they are thought to support sperm survival from several mates (Racey 1979). Because of this, they might have evolved mechanisms allowing post-copulatory choices (Lüpold et al. 2004). The extent and mechanisms of such cryptic female choice remain nevertheless to be investigated. It has previously been suggested that penis morphology is used by females as an honest signal of the good quality of their sexual partners (Miller and Burton 2001; Lüpold et al. 2004; Kinahan et al. 2006; Retief et al. 2013). We can thus further hypothesize that the penis phenotype reflects crucial information on male quality, such as its fertility (Sheldon 1994). Assuming that penis size and female choice are heritable traits, female selection for large penises could then be translated into indirect fitness through their male offspring. Penis morphology would then evolve through Fisherian sexy son mechanisms (Fisher 1930; Andersson and Simmons 2006).

In this study, we investigated penis length and penis head width static allometry in 4 species of Vespertilionidae native to temperate regions. In these species, body size should remain stable once adulthood is reached (Wai-Ping and Fenton 1988). With larger males expected to benefit more than smaller ones from an increase in relative penis size, we predicted a positive static allometry of male genitals in these species. Then, assuming that penis morphology has evolved under Fisherian selection, we hypothesized that female cryptic choice relies on male genital traits as an honest signal of fertility. Thus, based on the phenotype-linked fertility hypothesis (Sheldon 1994), we predicted a positive intra-specific correlation between sperm quality and penis length and/or penis head width.

Materials and Methods

Experimental models

Sexually mature males from *Barbastella barbastellus* ($n=17$), *Myotis nattereri* ($n=18$) and *Plecotus auritus* ($n=19$) were captured using mist-nets at swarming sites (buildings and WWII bunkers) in Podlasie (central-eastern Poland). Presence and development of the cauda epididymis were examined and males were kept in individual cotton bags until sperm collection. Temporarily captive males of *Vespertilio murinus* ($n=18$) were additionally included in the study. All measures were performed during the period of the year when mating takes place (August–October 2017).

Sperm quality assessment

Sperm samples were obtained by electro-stimulation under anesthesia following an established protocol (Fasel et al. 2015; Wesseling et al. 2016; Fasel et al. 2017). The probe had a diameter of 1.5 mm and consisted of 2 longitudinal gold-electrodes.

Ejaculates were transferred to a pre-warmed HEPES buffer solution (HBS, for 100 mL: 150 mM NaCl, 5 mM HEPES, pH=7.4) and sperm mobility was measured within swimming chamber

(Leja-Counting Chambers, 20 μm deep) heated to 37°C. Within 10 minutes, 5–10 1-sec-videos were recorded (Basler ACA780-75gc) with 200X magnification using phase-contrast illumination (Nikon E200). Curvilinear sperm velocity (VCL) was measured with the CASA plugin developed for ImageJ (Wilson-Leedy and Ingermann 2011). Only motile sperm (i.e., VCL > 10 $\mu\text{m}/\text{s}$ and straight line velocity > 2 $\mu\text{m}/\text{s}$) were considered for the measurement of sperm velocity.

Morphological measurement

Body mass was measured at a precision of 0.1 g at the time of sperm collection, which took place more than 9 h after the last meal. We thus considered that digestion was almost entirely terminated (Buchler 1975) and consequently that body mass measurements were not affected by stomach or intestinal contents. Moreover, because individuals expressed torpor in the cotton bag, it was assumed that mass loss after digestion was negligible. During anesthesia, after or before stimulation, the erected penis length and the penis head width were measured by the same observer (removed for blinded review). Penis length represents the ventral length between the base of the penis (i.e., sharp transition to a red coloration of the skin) and the tip of the penis. Penis head width represents the maximal length, perpendicular to the penis axis, measured at its extremity. This measure comprises the glans penis and the prepuce (Matthews 1937). Several calipers were used with a precision ranging from 0.1 to 0.01 mm. Some measurements were replicated to estimate their repeatability. Measurements were taken only when males developed a complete erection (i.e., tight skin, sharp red color and rigid structure).

Statistical analysis

All statistics were performed with software R v.3.5.1. The level of significance was set at 0.05.

The adjusted repeatability of penis length and penis head width measures was analyzed with 2 and 3 repetitions on 8 and 17 individuals, respectively. The function “rpt,” package: rpt; (Nakagawa and Schielzeth 2010) was used with morphological values as response variables; the variables “individual” and “species” were treated as random effects. When measurements were repeated, only the pair of penis head width and penis length measurements with maximal penis length were considered.

For the morphological scaling, ordinary least squares regressions (OLS, function “lm”) of body mass on penis measurements (penis length and penis head width) were run. Both explanatory and response variables were log₁₀-transformed. To account for the 3D nature of body mass and to consider a slope equal to one as isometric, we firstly transformed it applying a cube root. Any further mention of body mass will thus refer to the cube-rooted variable. Then, the transformed explanatory variable was centered per species to have the intercepts representing the penis length or penis head width (log₁₀-transformed) size at mean body mass. In addition, the slope and 95% confident interval estimated using a standardized major axis (SMA) regression (function “sma,” package: smatr, (Warton et al. 2012), were provided when estimate from the OLS regression was significantly different than 0.

We finally analyzed the correlation between sperm VCL and penis length and penis head width. The mean VCL value per ejaculate was calculated and further considered. Penis length and penis head width measures were centered per species. For each species, a linear model using the function “lm” was used with the VCL as

response variable. Both penis length and penis head width measures were considered as explanatory variables. In addition, we included body mass to control for any potential mass effect on VCL.

Ethical statements

All experimental procedures were authorized by the general and regional directors for environmental protection (authorizations nb. DZP-Wg.6401.09.2.2014, DZP-Wg.6401.09.1.2015, GDOŚ DZP-Wg.6401.09.5.2016, WPN.6401.102.2015, WPN6401.57.2015, WPN.6401.200.2016) and by the local ethical commissions in Białystok and Olsztyn (authorizations nb. 11/2014, 14/2015, 120/2015, 150/2015, 15/2015, 45/2015, 291/2015, 119/2015, 151/2015, 44/2015, 43/2015, 152/2015, 153/2015). All bats used for this study survived the procedures.

Data sources

Data and script can be downloaded from the Dryad depository.

Results

Statistics for the measured variables can be found in Table 1. Repeatability for the measurements of penis length and penis head width was always significant (Table 2).

The slope estimated by the log₁₀-log₁₀ regression of body mass on penis length was significant in *B. barbastellus* (Table 3, SMA regression estimate: 4.54, 95% CI: 2.81–7.34) and its OLS estimate was greater than one (Figure 1A). No significant relationships were found in *Myotis nattereri*, *P. auritus* and *V. murinus* (Table 3). Penis head width was significantly and negatively related to body mass of *M. nattereri* (Table 3, SMA regression estimate: –4.42, 95% CI: –7.49–2.61), whereas no significant effect of body mass on this measurement was found for the other species (Figure 1B).

Only the slope in *P. auritus* showed a significant positive correlation between penis head width and VCL (Figure 2). VCL was not correlated to penis head width in the other species and was generally not correlated to penis length and body mass (Table 4).

Discussion

In this study, we investigated male genital allometry of 4 species of Vespertilionidae. Sperm competition in this taxonomic group lasts for months and sexual selection is expected to be particularly rigorous (Wilkinson and McCracken 2003; Orr and Zuk 2013). Furthermore, mating behavior of these species could generate the evolution of positive allometry of male genitalia.

Accordingly to this first prediction, we found that penis length exhibited steep allometric slope in *B. barbastellus*. Such positive static allometry of male genitalia is rare and remarkable (Voje 2016). This result may be explained by the mating behaviors of *B. barbastellus*. Indeed, males from this species can copulate throughout winter (Rydell and Bogdanowicz 1997). Thus, individuals in better condition might gain more copulation opportunities by arousing from hibernation longer than those in poorer condition, as shown in *Myotis lucifugus* (Czenze et al. 2017). Heavier males also may survive better than lighter males (Kunz et al. 1998). During their lifetime, heavier males could thus be expected to experience a higher number of mating events than lighter ones. Consequently, out of 2 males having a penis of similar relative size, the larger one could be expected to have a higher fitness.

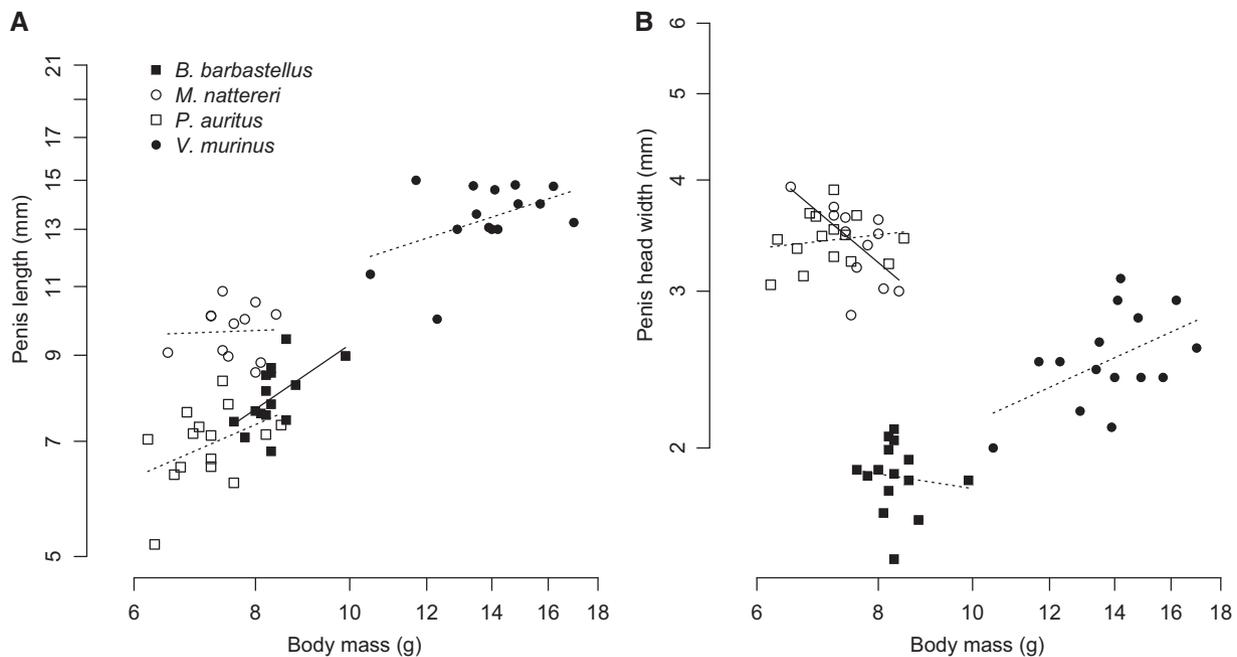
Table 1. Mean, standard error (SEM), minimum (min) and maximum (max) of measures of body mass (untransformed, BM), penis length (PL) and penis head width (PW) from 4 bat species of the Palearctic

	BM (g)				PL (mm)					PW (mm)					
	N	mean	SEM	min	max	N	mean	SEM	min	max	N	mean	SEM	min	max
<i>Barbastella barbastellus</i>	17	8.340	0.503	7.600	9.900	15	8.020	0.734	6.800	9.430	16	1.864	0.160	1.500	2.100
<i>Myotis nattereri</i>	18	7.470	0.603	6.100	8.400	12	9.680	0.748	8.270	10.850	12	3.393	0.332	2.820	3.930
<i>Plecotus auritus</i>	19	7.026	0.627	6.200	8.500	15	6.840	0.793	5.180	8.350	16	3.405	0.239	3.050	3.900
<i>Vespertilio murinus</i>	18	13.100	1.648	10.500	16.500	15	13.480	1.298	10.000	15.000	15	2.540	0.304	2.000	3.100

Table 2. Repeatability (R) of the penis measurements represents the proportion of variation attributed to within-individual and -species variations

	Individual			Species		
	R	95% CI	P	R	95% CI	P
Penis head width	0.051	0.010–0.325	0.008	0.898	0.389–0.968	<0.001
Penis length	0.062	0.016–0.419	<0.001	0.892	0.296–0.966	<0.001

CI, confidence interval.

**Figure 1.** Static allometric relationships of penis length (A) and penis head width (B) with body mass from 4 sperm-storing bat species. Significant relationship represented with solid lines versus non-significant relationships with dotted lines (see Table 3).**Table 3.** Static allometric analyses (OLS regression) between morphological variables and BM of 4 bat species

	Penis length					R ²	Penis head width					R ²
	Intercept ± SEM	P	Slope ± SEM	df	P		Intercept ± SEM	P	Slope ± SEM	df	P	
<i>Barbastella barbastellus</i>	0.901 ± 0.009	<0.001	2.531 ± 1.047	1, 13	0.031	0.310	0.269 ± 0.010	<0.001	−0.509 ± 1.220	1, 13	0.683	0.013
<i>Myotis nattereri</i>	0.984 ± 0.010	<0.001	0.142 ± 1.056	1, 10	0.896	0.002	0.531 ± 0.010	<0.001	−2.755 ± 1.095	1, 10	0.031	0.388
<i>Plecotus auritus</i>	0.835 ± 0.012	<0.001	1.609 ± 0.987	1, 13	0.127	0.170	0.533 ± 0.008	<0.001	0.366 ± 0.624	1, 13	0.585	0.026
<i>Vespertilio murinus</i>	1.130 ± 0.011	<0.001	1.198 ± 0.647	1, 13	0.087	0.209	0.403 ± 0.013	<0.001	1.492 ± 0.709	1, 13	0.055	0.254

In contrast to our prediction, the absence of positive allometric relationships between penis length and body mass observed in the other 3 species could firstly be explained by a size-independent increase in fitness accompanying an increase in relative penis length.

In *M. nattereri*, mating season is suspected to end with the onset of hibernation, as males display empty epididymis in that period (Pfeiffer and Mayer 2012). Consequently, heavier *M. nattereri* males may not benefit from additional rewarding copulations during

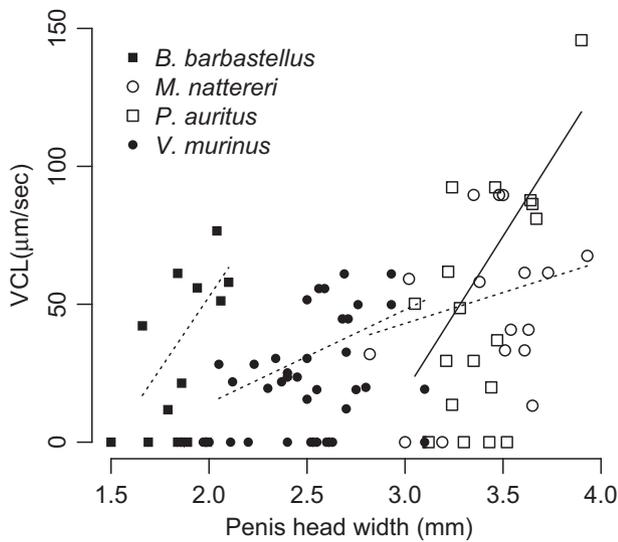


Figure 2. Sperm curvilinear velocity (VCL) in relation to penis head width. Significant relationship represented with solid lines versus non-significant relationships with dotted lines (see Table 4). Points with a VCL of 0 represent samples with only immotile sperm, which were not considered in the analysis.

Table 4. Linear regression relating sperm velocity (VCL, $\mu\text{m/s}$) and species-centered explanatory variables

	df	Estimate	SE	F	P
<i>Barbastella barbastellus</i> 3, 4					
Intercept		38.433	6.378	36.311	0.004
Penis length		0.591	13.313	0.002	0.967
Penis head width		105.299	48.795	4.657	0.097
BM		1317.917	843.937	2.439	0.193
<i>Myotis nattereri</i> 3, 6					
Intercept		53.187	9.596	30.720	0.001
Penis length		-5.859	13.347	0.193	0.676
Penis head width		22.418	38.936	0.332	0.586
BM		528.006	1266.233	0.174	0.691
<i>Plecotus auritus</i> 3, 8					
Intercept		65.398	9.371	48.699	<0.001
Penis length		-13.541	13.003	1.084	0.328
Penis head width		112.694	34.085	10.932	0.011
BM		-623.315	634.700	0.964	0.354
<i>Vespertilio murinus</i> 3, 5					
Intercept		30.849	7.009	19.370	0.007
Penis length		2.791	4.337	0.414	0.548
Penis head width		33.824	31.614	1.145	0.334
BM		116.160	373.515	0.097	0.768

BM, cubic root of body mass; df, degree of freedom; SE, standard errors., F- and P-values are presented.

winter arousals. Moreover, penis head width in that species exhibited a strong inverse allometry with body mass (Figure 1B). Unfortunately, mating behaviors in this species are still poorly described; one can only speculate that lighter males may attempt to force females for copulation or fix them as a post-copulatory guarding strategy. In contrast, heavier males might reduce intromission time to search for future sexual mates. Such divergent reproductive strategies (i.e., a few long copulations versus many short intromissions) may be especially important when copulation events are restricted to a period of male–male competition (i.e., before

hibernation) or when females are still not torpid, and may not favor heavier males only. Nevertheless, size-independent selection for large penis length could still affect trait allometric intercept and explain that *M. nattereri* has on average a large relative penis length (Figure 1A). Second, penis length may not be under directional selection. The copulatory behavior of *V. murinus* is also poorly described, but males are known to produce loud social calls during the mating season (Zagmajster 2003). Pre-copulatory expenditures may thus play a crucial role, reducing the importance of penis length selection.

Interestingly, allometric slopes of penis length differed between *P. auritus* and *B. barbastellus*, despite that mating behaviors of those 2 species show similarities. Indeed, in *P. auritus*, social activity occurs during the entire hibernation period up to spring (Furmankiewicz et al. 2013). Epididymis in *P. auritus* are still swollen at the onset of hibernation, supporting the premise that mating events continue to take place all along winter (Pfeiffer and Mayer 2012). As discussed later, penis may stimulate female genitalia in *P. auritus*. A penis of intermediate “adequate” size could provide a greater pay-off than would a larger one, supporting the “one size fits all” hypothesis (Eberhard et al. 1998, 2018; Eberhard 2008). Instead, the long and thin penis of *B. barbastellus* (Figure 1), whose prepuce is covered with hairs (personal communication) may rather enable sperm displacement and could be considered as “weapon” in male–male post-copulatory competition. Recently, sexually selected traits used in male–male antagonistic interactions (i.e., weapon) have been shown to evolve steep allometric slopes more often than would female courtship traits do (Eberhard et al. 2018). Results from this last study may explain the differences between allometric slopes of penis length in *B. barbastellus*, serving during male–male antagonistic interactions, and in *P. auritus*, serving during female courtship.

Finally, we hypothesized that female cryptic choice relies on male genital traits as an honest signal of fertility. Accordingly, we found that the relationship between penis head width and sperm quality differed among the investigated species. *P. auritus* displayed a significantly positive increase in sperm velocity with increasing penis head width (Figure 1). For this species, females could rely on the genital phenotype to evaluate their partners’ fertility. The relation between sperm velocity and fertility has been repeatedly validated in other species (Gage et al. 2004; Malo et al. 2005; Evans et al. 2013), but remained unproven in *P. auritus* and in bats in general. Our measurement captures this competitive trait in standardized *in vitro* conditions. Admittedly, interactions with ejaculates from competitors and/or the female genital environment are strongly expected to modify sperm competitiveness among various ejaculates. Nevertheless, and bearing in mind the above reservations, our study reveals a possible anchor point for female cryptic choice.

Using the information gained during copulation, females may further decide to hold in or reject ejaculate from their uterus (Guthrie 1933; Pizzari and Birkhead 2000) or may decide whether or not to remain available for additional mating events. Such female-generated selection for larger penis head width may operate independently of the male body mass, as penis head width static allometry was not significant. The average penis head width in *P. auritus* was, in fact, the second largest among the investigated species, even though this species was the lightest on average (Table 1). This suggests an influence of sexual selection pressures on the allometric intercept in that species.

Admittedly, several points of this discussion contain speculative considerations about copulatory behaviors and functional anatomy.

They would greatly benefit from additional studies on bat mating, and we anticipate that the present results will urge bat researchers to report observations and investigations of mating behaviors.

In conclusion, our study reveals positive male genitalia allometry in one sperm-storing bat species. A combination of strong directional sexual selection and particular mating behaviors, characterized by a reduction in pre-copulatory female choice and by extended female sexual receptivity, might have driven the evolution of penis shape in these species. In addition, we showed that penis head width positively correlates with sperm velocity in *P. auritus*. These results provide evidence that a penis trait may honestly express male quality (such as fertility), enabling female cryptic choice.

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Authors' Contributions

N.J.F., E.K., M.K.S., and I.R. contributed to conceptualization of the study; N.J.F. did formal analysis; N.J.F., M.K.S., M.Z., and I.R. contributed to investigations; N.J.F. contributed to writing-original draft; all authors contributed to writing-review and editing; N.J.F. and I.R. contributed to founding acquisition. The authors declare having no conflicting or financial interests. All authors accepted the final version of the manuscript.

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