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 disproportionally 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*. 

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 (Dixon 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 (Scholl 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; Dixson 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 (Dixson et al. 2004; Herdina et al. 2015), but may become disproporionately 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).

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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 penises 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 (Lupold 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; Lupold 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 motility was measured within swimming chamber
(Leja-Counting Chambers, 20 \(\mu m\) 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 \(\mu m/s\) and straight line velocity > 2 \(\mu m/s\)) 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: rptr; (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 consider a slope equal to one as isometric. 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, WPN640157.2015, 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 genitals 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.
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.

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.

| Species                  | BM (g)  | PL (mm) | PW (mm) |
|--------------------------|---------|---------|---------|
|                          | N  | mean | SEM | min | max | N  | mean | SEM | min | max | N  | mean | SEM | min | max |
| Barbastella barbastellus | 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 |
| Myotis nattereri          | 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 |
| Plecotus auritus         | 19 | 7.026 | 0.627 | 6.200 | 8.300 | 15 | 6.840 | 0.793 | 5.180 | 8.350 | 16 | 3.393 | 0.332 | 2.820 | 3.930 |
| Vespertilio murinus      | 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.

| Measure         | 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.

| Species                  | Intercept ± SEM P | Slope ± SEM df P | R² |
|--------------------------|-------------------|------------------|----|
| Barbastella barbastellus | 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 |
| Myotis nattereri          | 0.984 ± 0.010 <0.001 | 0.142 ± 0.556 1, 10 0.896 0.002 | 0.331 ± 0.010 <0.001 | −2.753 ± 1.095 1, 10 0.031 0.388 |
| Plecotus auritus         | 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 |
| Vespertilio murinus      | 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 Myotis 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 Myotis nattereri males may not benefit from additional rewarding copulations during
冬季唤醒。此外，较大的个体在这一物种中表现出强烈的逆所有ometry，体重与体长的比率明显高于其他物种。表2中列出了体重的倒数立方根和体长的相关性。体重为0.001的点代表不育的精子，这些点在分析中未考虑。

Table 4. Linear regression relating sperm velocity (VCL, µm/s) and species-centered explanatory variables

| Species               | df | Estimate | SE  | F    | P   |
|-----------------------|----|----------|-----|------|-----|
| Barbastella barbassellus | 3, 4 | 38.433   | 6.378 | 36.311 | 0.004 |
| Intercepts            |     | -0.591   | 13.313 | 0.002 | 0.967 |
| Penis length          |     | 105.299  | 48.795 | 4.657 | 0.097 |
| Penis head width      |     | 1317.797 | 943.937 | 2.439 | 0.193 |
| BM                    |     | 528.006  | 266.233 | 0.174 | 0.691 |
| Myotis nattereri       | 3, 6 | 53.187   | 9.596 | 30.720 | 0.001 |
| Intercepts            |     | -3.589   | 13.347 | 0.193 | 0.676 |
| Penis length          |     | -22.418  | 38.936 | 0.332 | 0.586 |
| Penis head width      |     | 65.398   | 9.371 | 48.699 | <0.001 |
| BM                    |     | -13.541  | 13.003 | 1.084 | 0.328 |
| Plecotus auritus      | 3, 8 | 112.694  | 34.085 | 10.932 | 0.011 |
| Intercepts            |     | 22.418   | 38.936 | 0.332 | 0.586 |
| Penis length          |     | -110.006 | 266.233 | 0.174 | 0.691 |
| Penis head width      |     | -22.418  | 38.936 | 0.332 | 0.586 |
| BM                    |     | 65.398   | 9.371 | 48.699 | <0.001 |
| Vespertilio murinus   | 3, 5 | 110.610  | 37.515 | 0.097 | 0.768 |

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

**References**

Andersson M, Simmons LW, 2006. Sexual selection and mate choice. *Trends Ecol Evol* 21:296–302.

Andrés JA, Cordero Rivera A, 2000. Copulation duration and fertilization success in a damselfly: an example of cryptic female choice? *Annu Behav 59*:695–703.

Arnqvist G, 1998. Comparative evidence for the evolution of genitalia by sexual selection. *Nature 393*:784–786.

Bonduriansky R, Day T, 2003. The evolution of static allometry in sexually selected traits. *Evolution 57*:2450–2458.

Brennan PLR, Clark CJ, Prum RO, 2010. Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia. *Proc Biol Sci* 277:1309–1314.

Buchler ER, 1973. Food transit time in *Myotis lucifugus* Chiroptera: vesperilionidae. *J Mammal 56*:252–255.

Cryan PM, Jameson JW, Baerwald EF, Willis CKR, Robert RMR et al., 2012. Evidence of late-summer mating readiness and early sexual maturation in migratory tree-roosting bats found dead at wind turbines. *PLoS One 7*:e47586.

Czence ZJ, Jonasson KA, Willis CRK, 2017. Thrifty females, frisky males: winter energetics of hibernating bats from a cold climate. *Physiol Biochem Zool 90*:502–511.

Dixson AF, 2003. Sexual selection by cryptic female choice and the evolution of primate sexuality. *Evol Anthropol 11*:195–199.

Dixson A, Jenna N, Anderson M, 2004. A positive relationship between baculum length and prolonged intromission patterns in mammals. *Acta Zoológica Sinica* 50:490–503.

Dixson AF, 1987. Observations on the evolution of the genitilia and copulatory behaviour in male primates. *J Zool 213*:423–443.
