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Rapid evolution of testis size relative to sperm morphology suggests that post-copulatory selection targets sperm number in *Anolis* lizards

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Abstract

Post-copulatory sexual selection is thought to be responsible for much of the extraordinary diversity in sperm morphology across metazoans. However, the extent to which post-copulatory selection targets sperm morphology versus sperm production is generally unknown. To address this issue, we simultaneously characterized the evolution of sperm morphology (length of the sperm head, midpiece and flagellum) and testis size (a proxy for sperm production) across 26 species of *Anolis* lizards, a group in which sperm competition is likely. We found that the length of the sperm midpiece has evolved 2–3 times faster than that of the sperm head or flagellum, suggesting that midpiece size may be the most important aspect of sperm morphology with respect to post-copulatory sexual selection. However, testis size has evolved faster than any aspect of sperm morphology or body size, supporting the hypothesis that post-copulatory sexual selection acts more strongly upon sperm production than upon sperm morphology. Likewise, evolutionary increases in testis size, which typically indicate increased sperm competition, are not associated with predictable changes in sperm morphology, suggesting that any effects of post-copulatory selection on sperm morphology are either weak or variable in direction across anoles. Collectively, our results suggest that sperm production is the primary target of post-copulatory sexual selection in this lineage.

KEYWORDS
comparative method, midpiece, rate of evolution, sexual selection, sperm competition

1 | INTRODUCTION

Sexually selected traits are predicted to evolve quickly due to strong and diversifying selection arising from intrasexual competition and intersexual mate choice (Andersson, 1994; Gonzalez-Voyer & Kolm, 2011; Seddon et al., 2013; West-Eberhard, 1983). Consistent with this prediction, weapons and ornaments subject to precopulatory sexual selection have accelerated rates of evolution relative to other morphological traits (Fitzpatrick, Almbro, Gonzalez-Voyer, Kolm, &

Simmons, 2012; Friedman & Remeš, 2015; Klaczko, Ingram, & Losos, 2015; Seddon et al., 2013; Simmons & Fitzpatrick, 2016). Sperm is the most morphologically diverse cell type across metazoans (Pitnick, 2009), and may therefore represent an analogous case of accelerated evolution in response to post-copulatory sexual selection, which occurs when sperm from different males compete for fertilization (Birkhead, Hosken, & Pitnick, 2009). However, post-copulatory sexual selection can target sperm number as well as sperm morphology (Bennison, Hemmings, Slate, & Birkhead, 2014; Boschetto, Gasparini,

& Pilastro, 2011; García-González & Simmons, 2007; Laskemoen et al., 2010; Radwan, 1996). Indeed, sperm competition is consistently associated with increased testis size and sperm count across a variety of taxa (Gage, 1994; Møller, 1988, 1989; Simmons, Firman, Rhodes, & Peters, 2004; Stockley, Gage, Parker, & Møller, 1997), whereas evidence for its association with sperm morphology is generally more equivocal (Simmons & Fitzpatrick, 2012). However, no study has directly compared the rates of evolution for these putative targets of post-copulatory sexual selection within the same lineage.

To address this, we characterized sperm morphology and testis size across 26 species in the lizard genus *Anolis*. Several attributes of this genus suggest a high potential for post-copulatory sexual selection and the resulting diversification of reproductive traits, including the rapid evolution of male genitalia (Klaczko et al., 2015), extended sperm storage within the female reproductive tract (Birkhead, 1993; Kahrl & Cox, 2015) and a high frequency of multiple paternity (Calsbeek, Bonneaud, Prabhu, Manoukis, & Smith, 2007). To characterize the evolution of sperm morphology, we examined three parts of the sperm cell: the head (which contains the nuclear material and the acrosome with proteolytic enzymes necessary for egg penetration), the midpiece (which contains the mitochondria) and the flagellum (which aids in propulsion and provides some glycolytic activity). We also measured testis size which, when expressed relative to body size, provides an index of the intensity of sperm competition (Birkhead & Møller, 1998; Simmons, 2001; Simmons & Fitzpatrick, 2012) and the capacity for sperm production (Møller, 1988, 1989).

Using these data, we first tested for differences in the rates of evolution of sperm size (the total length of the cell broken into the head, midpiece, and flagellum), testis length (an index of sperm production and an alternative target of post-copulatory selection) and snout–vent length (SVL, a standard measure of body size that has diverged during the adaptive radiation of this lineage). We predicted that the sperm midpiece would evolve more quickly than other parts

of the cell because midpiece size is associated with reproductive success in anoles (Kahrl & Cox, 2015) and because it evolves more quickly than other parts of the cell in birds (Rowe et al., 2015). Both sperm morphology and sperm count are associated with reproductive success in *A. sagrei* (Kahrl & Cox, 2015), but it is unclear which is the primary target of post-copulatory sexual selection in anoles. If sperm morphology evolves more quickly than testis length, this would suggest that post-copulatory selection primarily targets sperm morphology, whereas if testis length evolves more quickly than sperm morphology, this would suggest that post-copulatory selection primarily targets sperm count. Second, we tested the hypothesis that evolutionary increases in sperm competition, as inferred from increases in relative testis size, are associated with evolutionary changes in sperm morphology (Simmons & Fitzpatrick, 2012). We predicted that evolutionary changes in those components of the sperm cell with high rates of evolution would be most strongly correlated with evolutionary changes in relative testis size.

2 | MATERIALS AND METHODS

2.1 | Specimens and sperm collection

We collected sperm samples and testis measurements from 419 males of 26 *Anolis* species (Figure 1, Supporting information Table S1) from the Bahamas, the Dominican Republic, Puerto Rico and the United States during the middle of their reproductive seasons (May 15–June 30 of 2013–2015). We included species representing all ecomorph groups except crown giants, the largest of the *Anolis* ecomorphs. Crown giants are an order of magnitude larger than other ecomorphs in both body size and testis size, but not in sperm morphology, and would therefore be statistical outliers. We included only adult males and conducted our sampling during the period in when testis size is expected to be near its seasonal
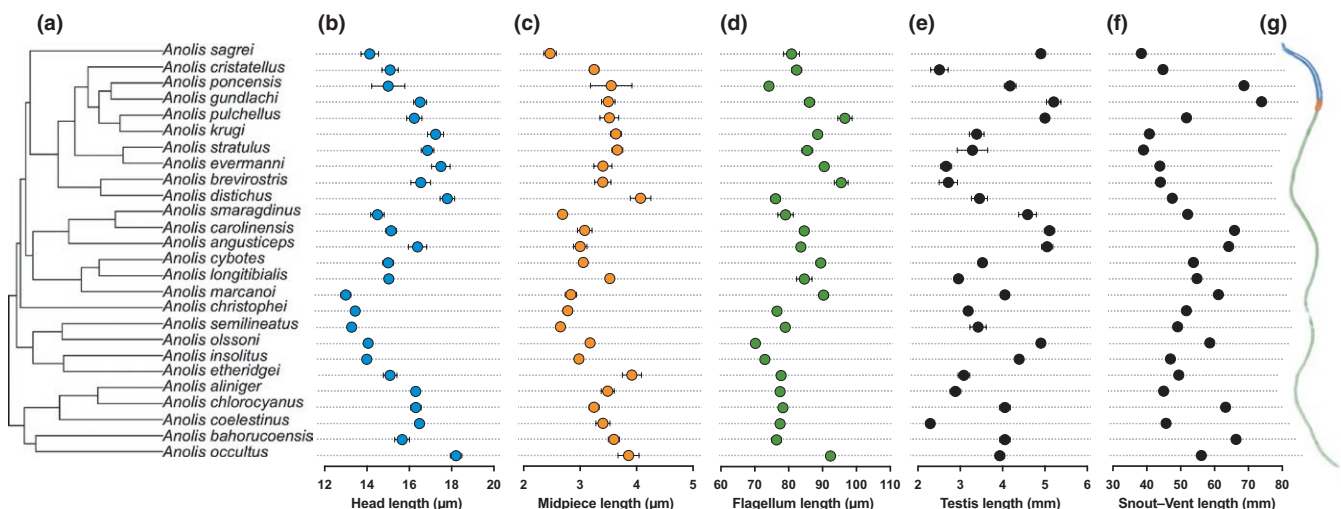

FIGURE 1 Trimmed phylogeny from Pyron and Burbrink (2014) that includes the 26 species used in this study (a) alongside the mean and standard deviation of sperm head (b), midpiece (c), flagellum (d), testis (e) and snout–vent lengths (f) for each species, beside an image of an anole sperm (g)

maximum (Licht & Gorman, 1970). For each species, we collected 4–20 males (mean = 16.0 per species) and recorded SVL (to the nearest 1 mm) for each male (see Supporting information Table S1 for phenotypic data and collection details). We then collected a sperm sample from each male by gently pressing on the pelvic girdle and collecting sperm from the cloaca using a glass capillary tube. We suspended this sample in 500 μl phosphate-buffered saline with 4% paraformaldehyde as a fixative. We transferred the fixed cells to a microscope slide, allowed them to dry and stained them with Sperm Blue™ (Microptic SL, Barcelona, Spain). We imaged the cells using an Olympus Magnafire camera (Olympus America, Melville, NY) at ×100 magnification and measured the length of the sperm head, midpiece and flagellum for 15 cells per male using ImageJ (NIH, Bethesda, MD). From these measurements, we calculated individual means, which we then used to calculate species-level means for each aspect of sperm morphology.

We measured testis size for the same individuals via laparotomy or dissection. For laparotomy, we gave each lizard a 4-μl subcutaneous injection of 0.25% bupivacaine (Auromedics, Dayton, NJ) as a local anaesthetic and analgesic. We then cold-immobilized each animal at −20°C and placed it on a slightly thawed ice pack. We made a 5-mm ventral incision in the abdomen lateral to the midline, located and exposed the right testis using forceps and measured the length and width of the testis using calipers (nearest 0.1 mm). We closed the incision using Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ) and released each animal at its location of capture on the following day. We measured a subset of individuals in the same way immediately following euthanasia. We converted testis length and width into volume using the formula for an ellipsoid:

$$\text{Volume} = \frac{4}{3}\pi a^2 b,$$

where a is the radius of the width of the testis and b is the radius of its length.

2.2 | Phylogenetic analyses

We conducted all statistical analyses in R (v.3.4.3, R Development Core Team 2017). For phylogenetic analyses, we used the ultrametric squamate phylogeny of Pyron and Burbrink (2014), which provides a fully resolved topology and branch lengths for all *Anolis* species in our dataset. We trimmed the original phylogeny to include only those species in our dataset using *ape* (Paradis, Claude, & Strimmer, 2004) and used ln-transformed values of morphological traits in our analyses. For each trait, we estimated phylogenetic signal by calculating Pagel's λ in *phytools* (Pagel, 1999; Revell, 2011) and Blomberg's K in *picante* (Blomberg, Garland, & Ives, 2003; Kembel et al., 2010). Testis size and all aspects of sperm morphology exhibited strong phylogenetic signal, although body size (SVL) did not exhibit significant phylogenetic signal in our dataset (Table 1). For each trait, we compared the fit of three different models of character evolution (Brownian motion, Ornstein–Uhlenbeck and early burst) using the *fitContinuous* function in *geiger*

TABLE 1 Phylogenetic signal (Blomberg's K and Pagel's λ) for each trait examined (all ln-transformed). Significance testing for K indicates $K > 0$, which indicates significant phylogenetic signal. For λ, the maximum-likelihood estimate of λ was tested against models where $\lambda = 0$ using log likelihood-ratio tests

| | Blomberg's K | | Pagel's λ | | |
Trait	K	p	λ	lnL $\lambda = 0$	p
Testis length	0.923	0.029	0.858	0.79	0.076
Body length (SVL)	0.876	0.055	0.668	7.95	0.195
Sperm head length	1.142	0.003	0.999	25.10	0.002
Sperm midpiece length	1.033	0.001	0.999	14.68	0.006
Sperm flagellum length	0.868	0.061	0.771	21.11	0.059

(Harmon, Weir, Brock, Glor, & Challenger, 2008). The early burst model did not converge for any trait when using the default bounds, so we set the maximum bounds to 0 to allow model convergence. Based on AICc model comparison, Brownian motion was the preferred model of character evolution for all traits (Supporting information Table S2), which fits the assumptions of the models used in subsequent analyses of evolutionary rates.

2.3 | Rates of character evolution

To compare rates of evolution among traits, we used a likelihood-based approach developed by Adams (2013) that estimates a Brownian rate parameter (σ^2) describing the speed at which traits have diversified across a phylogenetic tree. Adams' (2013) method simultaneously estimates a separate σ^2 for each trait and then uses likelihood-ratio tests to compare this observed-rate model to a common-rate model in which all traits are constrained to have the same estimate of σ^2. This method also allows for the incorporation of within-species error and covariance for trait values (Adams, 2013). Since evolutionary rates cannot be compared between traits with dissimilar units of measurement, we chose to measure the rate of evolution for testis length rather than testis volume. Because dimensionality can impact trait variance, we used only ln-transformed length measurements for these analyses. We then estimated the rates of evolution of species means for testis length, male SVL, sperm head, midpiece and flagellum length, including measurements of within-species errors and covariances. All model comparisons that did not estimate evolutionary covariances between traits in the observed-rate model, but included measurements of within-species errors and covariances, converged under the L-BFGS-B optimization function (Adams, 2013). We then conducted post hoc tests in which we followed this same model-fitting procedure, using only two traits at a time, to fit all pairwise combinations of the traits.

2.4 | Tests for correlated evolution

To test whether evolutionary increases in the intensity of sperm competition are associated with predictable changes in sperm morphology, we used phylogenetic generalized least squares (PGLS) regression to test for associations between testis volume and sperm morphology, and between all pairs of sperm traits. We used a Brownian motion model of character evolution for all analyses, which we conducted using *ape* (Paradis et al., 2004). In these regressions, ln-transformed species means for the length of the sperm head, midpiece and flagellum were treated as dependent variables in separate analyses for each trait. Species means for testis volume and body size (SVL, included as a covariate) were ln-transformed and included as independent variables in each analysis.

3 | RESULTS

3.1 | Rates of evolution

The "observed" model estimating different rates of evolution for each aspect of sperm morphology, body size and testis size was favoured over the "common" model estimating a single rate for all traits (LRT = 24.3, $p < 0.0001$, $AIC_{observed} = -125.2$, $AIC_{common} = -108.9$, Figure 2). Pairwise post hoc comparisons revealed that rates of evolution for each trait are significantly different from rates of evolution for all other traits (Figure 2, Supporting information Table S3). This result was robust across all models that we tested (i.e., with and without intraspecific error and covariance). Among sperm traits, the length of the sperm midpiece had the highest rate of evolution, followed by the length of the sperm flagellum and then that of the sperm head (Figure 2, Supporting information Table S3). However, testis length had the highest rate of evolution of any trait in our analyses, evolving significantly faster than body size and 3–8 times faster than any aspect of sperm morphology (Figure 2).

3.2 | Tests for correlated evolution

We found no significant relationships between testis size and any measure of sperm morphology (i.e., length of the head, midpiece or flagellum) using PGLS with a Brownian motion model of character evolution (Supporting information Figure S1, Table 2). We found significant positive correlations between sperm head and flagellum length ($\lambda = 1^{0.005, 1}$, $r = 0.45$, $t_{24} = 2.67$, $p = 0.013$) and between sperm head and midpiece length ($\lambda = 0.863^{0.177, 0.423}$, $r = 0.64$, $t_{24} = 4.472$, $p = 0.0001$), but not between sperm flagellum and midpiece length ($\lambda = 1^{0.025, 0.368}$, $r = -0.02$, $t_{24} = -0.121$, $p = 0.905$).

4 | DISCUSSION

Theory suggests, and empirical data show, that sexually selected traits tend to have higher rates of evolution than other traits, likely
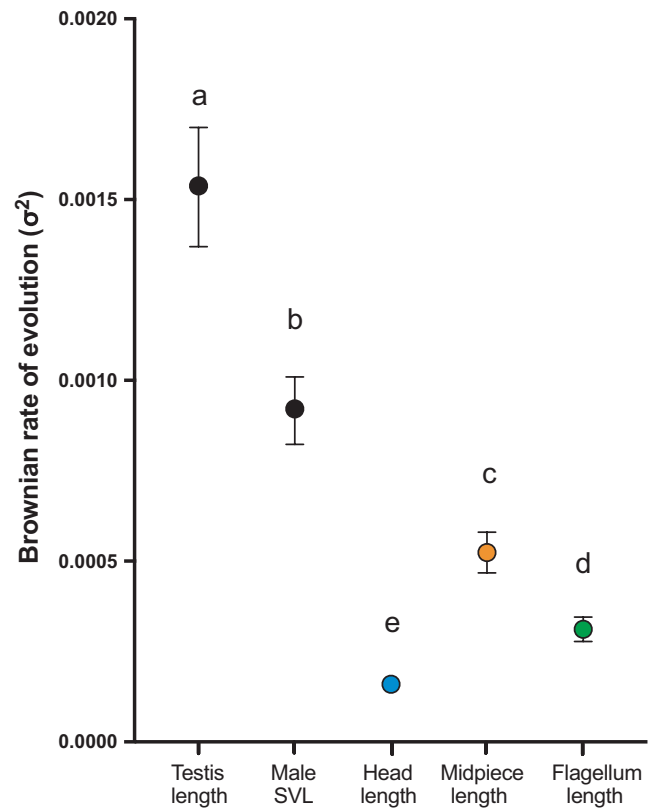

FIGURE 2 Brownian rates of evolution (±95% confidence intervals) for testis length, male snout–vent length (SVL) and the length of the sperm head, midpiece and flagellum across 26 species of *Anolis* lizards. Rates were calculated using ln-transformed means for each species

due to strong diversifying selection (Arnqvist, 1998; Gonzalez-Voyer & Kolm, 2011; Seddon et al., 2013; West-Eberhard, 1983). Although sperm are the most morphologically diverse cells in metazoans, we found that testis size has evolved between 3 and 8 times more quickly than any aspect of sperm morphology in *Anolis* lizards. Previous work has demonstrated that both sperm count and sperm morphology are correlated with fertilization success in one *Anolis* species (Kahrl & Cox, 2015), but our analysis of broad phylogenetic patterns across this genus suggests that post-copulatory sexual selection may generally target testis size (i.e., sperm production and sperm count) to a greater extent than sperm morphology. Despite extensive research characterizing phenotypic diversity and evolution in *Anolis* lizards (Ingram et al., 2016; Klaczko et al., 2015; Mahler, Revell, Glor, & Losos, 2010), our study is the first to use a comparative framework to explore the evolution of sperm morphology and testis size in this group, and also the first in any lineage to test for differences in rates of evolution between these two putative targets of post-copulatory sexual selection. Our results also stand in contrast to the only other study to directly test for different rates of evolution between body size and testis size. In *Onthophagus* beetles, body size evolves significantly faster than testis size, suggesting that precopulatory selection on body size may be stronger than post-copulatory selection on testis size (Simmons & Fitzpatrick, 2016).

TABLE 2 Summary of results from phylogenetic generalized least squares (PGLS) regressions of sperm morphological traits on testis size with male body size (SVL) as a covariate

Sperm morphology	Predictor	Partial r (95% CI)	t, $df = 23$	p	λ
Head length	Testis size	0.24 (−0.18–0.59)	1.28	0.213	$1^{0.0045, 1}$
	SVL	−0.07 (−0.46–0.34)	−0.36	0.718	
Midpiece length	Testis size	0.14 (−0.28–0.51)	0.72	0.474	$1^{0.005, 1}$
	SVL	−0.16 (−0.53–0.26)	−0.83	0.412	
Flagellum length	Testis Size	0.13 (−0.29–0.51)	0.68	0.502	$0.674^{0.074, 0.283}$
	SVL	0.03 (−0.38–0.43)	0.15	0.885	

Notes. All traits were ln-transformed prior to analyses using a Brownian motion model of character evolution. λ (phylogenetic scaling parameter) is listed for each model, followed by superscripts indicating p-values of likelihood-ratio tests against models where $\lambda = 0$ and $\lambda = 1$, respectively.

We interpret the rapid evolution of testis size, relative to sperm morphology, as consistent with the idea that post-copulatory selection may primarily target sperm number, but several other explanations merit consideration. First, differences in the heritability of sperm morphology and testis size could lead to differences in their evolutionary rates even if selection acts similarly on each (Reznick, 1997). Although there are no estimates available for reptiles, most estimates for the heritability of sperm length and testis size in other taxa are fairly high (sperm length mean h^2 = 0.72, range = 0.43–1.14; testis size mean h^2 = 0.53, range = 0.04–0.97; values from Table 10.1 in Simmons & Moore, 2008), suggesting a high potential for evolution in response to selection. Second, the form of selection acting on each trait could differ. Whereas testis size should generally experience positive directional selection in species with high sperm competition, the expectation is less clear for sperm morphology. For example, the female reproductive tract may create stabilizing selection for an intermediate sperm size, as inferred in birds and insects, where species subject to strong post-copulatory selection have reduced variation in sperm morphology (Calhim, Immler, & Birkhead, 2007; Fitzpatrick & Baer, 2011; Immler, Calhim, & Birkhead, 2008). Moreover, because sperm morphology often coevolves with female reproductive anatomy (Higginson, Miller, Segraves, & Pitnick, 2012; Miller & Pitnick, 2002; Presgraves, Baker, & Wilkinson, 1999), the idiosyncrasies of this coevolutionary dynamic could lead to patterns of selection on sperm morphology that are unique to particular species despite similar opportunities for post-copulatory selection. This could explain why we did not find any consistent evolutionary shifts in sperm morphology as testis size (and the inferred strength of sperm competition) increased (Table 2). Finally, because testis size is only a proxy for sperm count, the rate of evolution for testis size may not perfectly correspond to the evolution of sperm count. For example, testis size may also reflect selection for production of androgens, and sperm production can also be influenced by properties other than the size of the testis, such as the density of spermatogenic tissue (Lüpold, Linz, Rivers, Westneat, & Birkhead, 2009) and the spermatogenic efficiency of the testes (Peirce & Breed, 2001; Ramm & Stockley, 2010; Wistuba et al., 2003).

Comparative analyses testing for an evolutionary association between sperm morphology and testis size have spanned most major orders of animals, but their results are fairly equivocal. Whereas some lineages show a strong positive correlation between relative testis size and sperm length, others show a strong negative correlation, and in some, there is no relationship at all (Simmons & Fitzpatrick, 2012). In fact, differences in the evolutionary relationship between sperm morphology and testis size occur even between closely related lineages (Immler & Birkhead, 2007; Simmons & Fitzpatrick, 2012). For example, snakes are the only other reptile lineage in which the evolution of sperm morphology has been examined, and they provide an interesting contrast to anoles. Among species of snakes, the length of the sperm head, midpiece and flagellum is all positively correlated with relative testis size (Tourmente, Gomendio, Roldan, Giojalas, & Chiaraviglio, 2009), suggesting that post-copulatory selection favours large sperm in this lineage. Several species of snakes can store sperm for over five years (Birkhead, 1993), potentially creating selection for sperm cells that can survive extended storage in the female reproductive tract (Friesen, Kerns, & Mason, 2014; Friesen, Mason, Arnold, & Estes, 2014), which significantly exceeds the longest storage times reported for anoles (7 months, Fox, 1963; Licht, 1973). In some species, mitochondria are slowly lost during storage (Vila, Sàbat, Hernandez, & Muñoz, 2007), potentially necessitating increases in midpiece size in species with extended sperm storage. This putative need for greater numbers of mitochondria to offset loss during extended storage may explain why midpiece length averages 60% of total sperm length in snakes, but only 3% of total sperm length in anoles. However, very little is known about the physiological mechanisms of sperm storage, especially in reptiles, so this hypothesis remains speculative.

Our analyses also revealed that the size of the midpiece has a faster rate of evolution than that of the sperm head or flagellum. The midpiece contains mitochondria, and its size is often associated with body condition (Bonanno & Schulte-Hostedde, 2009; Kahrl & Cox, 2015), sperm velocity (Blengini, Sergio, Gabriela, Giojalas, & Margarita, 2014; Firman & Simmons, 2010) and sperm motility (Anderson & Dixson, 2002; Ruiz-Pesini et al., 1998), which are frequently associated with male reproductive success (Simmons & Fitzpatrick, 2012). In passerine birds, the evolutionary trajectory of the midpiece and flagellum differs from that of the sperm head (Immler, Gonzalez-Voyer, & Birkhead, 2012), and evolutionary increases in post-copulatory selection are associated with accelerated phenotypic divergence in midpiece size (Rowe et al., 2015). For

these reasons, it is perhaps not surprising that the midpiece has the highest rate of evolution among sperm morphological traits. In contrast, the sperm head, which contains both the acrosome and the nuclear material, exhibited the lowest rate of evolution, which may be due to functional constraints. For example, longer filiform heads increase drag on the sperm cell, which would then require a larger flagellum to propel them at an equal speed (Humphries, Evans, & Simmons, 2008). Moreover, genome size may prevent the sperm head from attaining a smaller size, though few studies have tested this hypothesis and results are somewhat mixed (Alvarez-Fuster, Juan, & Petitpierre, 1991; Gage, 1998; Gallardo, Mondaca, & Ojeda, 2002). Irrespective of why these three parts of the sperm cell evolve at different rates, our analyses clearly show that testis size evolves several times faster than each, and also significantly faster than body size, which is a key aspect of ecomorphological diversification in the adaptive radiation of this lineage (Losos, 2009). Collectively, our results suggest that post-copulatory sexual selection due to sperm competition acts more strongly upon sperm production than upon sperm morphology, a hypothesis that could be tested further with direct measures of sperm count and/or studies of sexual selection on ejaculate phenotypes.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA ACCESSIBILITY

All data are included in Supporting information.

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