

Review



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Sperm competition in squamate reptiles

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Multiple paternity is ubiquitous within the polyphyletic group called ‘reptiles’, especially within the lizards and snakes. Therefore, the probability of sperm competition occurring, and being intense, is high. Squamates exhibit a diversity of tactics to ensure fertilization success in the face of sperm competition. The duration of female sperm storage, which can be many months and even years in some species, remains an enigma. Here, we emphasize some mechanisms that might affect patterns of paternity, the source and function of ejaculates and features of the female reproductive tract that may aid in long-term sperm storage. In doing so, we present a new analysis of the relationship between sperm size, the strength of sperm competition and the duration of female sperm storage. Lizards and snakes are a diverse group that has provided many excellent models for the study of an array of life-history strategies. However, when it comes to postcopulatory sexual selection, there is much left to discover.

This article is part of the theme issue ‘Fifty years of sperm competition’.

1. Sperm competition in squamates

Over the past 50 years, postcopulatory sexual selection—sperm competition and cryptic female choice—has become a significant area of research [1], which has many outstanding questions at every level of analysis, from proximate to ultimate [2]. Our understanding of postcopulatory mechanisms is notably sparse in taxa other than mammals, birds and insects. The polyphyletic group ‘reptiles’, including turtles, crocodylians, snakes and lizards (but excluding birds), represents nearly 30% of terrestrial vertebrates. Squamates constitute approximately 95% of reptiles and are a monophyletic group sister to Archosaurimorpha, which includes birds, turtles and crocodylians [3]. Here, we review the literature on squamate reptiles—mainly lizards and snakes—the source and functions of ejaculates, and female sperm storage, and present an analysis of the relationship between sperm size and relative testes mass and the duration of female sperm storage.

2. Multiple paternity

Decades before Geoff Parker [1] launched the study of sperm competition, Frieda and Frank Blanchard described long-term sperm storage (approx. eight months) and multiple paternity in garter snakes [4]. Squamates have since been valuable subjects for examining female sperm storage (snakes reviewed in [5], lizards reviewed in [6]).

Although sperm competition also occurs between sperm geno- and phenotypes within a single ejaculate [7], here we define sperm competition as occurring when the ejaculates of more than one male overlap within the reproductive tract of a female, which typically leads to multiple paternity when there is no complete bias towards one rival [8]. Multiple paternity is widespread in squamates [9]; well over 50% of clutches/litters from wild populations exhibit multiple paternity, the degree of which depends on mate encounter rates and mating systems [9]. Snakes, in particular, seem to have higher risk and intensity

of sperm competition than lizards [9]. However, many factors determine whether a particular female mates with more than one male [10] and the distribution of paternity among those males when she does [9,11,12], which may be due, in part, to the duration of female sperm storage.

There are many reasons female sperm storage may evolve under natural selection, for example, delaying fertilization until mates are available or environmental conditions are optimal [13]. However, female sperm storage also extends the overlap of ejaculates within the oviduct—the defining condition for sperm competition [1]. As competition for fertilization generates strong selection on sperm and ejaculate traits, the null hypothesis may be that female sperm storage is simply a consequence of selection on sperm longevity [13]. However, given the high incidence of multiple paternity for most squamates, it seems unlikely that selection on female sperm storage is driven solely to ensure fertilization [11].

3. Female reproductive tract

In squamates, like many amniotes, the vagina and posterior oviduct have concentrations of mast cells [5], which facilitate allergic reactions and inflammation. In mammals, mast cells induce neutrophil migration into the oviducts in response to mating and seminal fluids [14]. Indeed, selective immune responses to particular males, or their sperm, are intriguing mechanisms by which paternity may be biased towards or away from particular males [15]. Male genital or seminal fluid phenotypes could also influence female physiological responses leading to differential fertilization success. For example, the spines adorning male intromittent organs—hemipenes—could stimulate females to resist or facilitate sperm transfer [16,17], damage the vaginal pouch and/or induce immune responses to particular male phenotypes. These processes might influence differential sperm transport and storage or lead to mate-order effects by making the female reproductive tract less hospitable to the next male's sperm [18,19]. Likewise, seminal fluid could elicit some of the same effects as genitalia and sperm themselves could affect female receptivity to mating [20] as they move into female sperm storage tubules (SSTs). Indeed, the muscular vaginal pouch of garter snakes affects sperm and copulatory plug (CP) transfer, indicating sexual conflict and the potential for cryptic female choice during copulation [16,17].

Beyond the vaginal pouch, the squamate oviduct is a tube lacking elaborate sperm-storage organs that might mechanically control sperm entry or usage. Nevertheless, sperm storage in SSTs is conserved across the squamate phylogeny [5,6,21]. The location of SSTs within the oviduct varies considerably across, or sometimes within, squamate families, but the evolutionary significance of these arrangements are only vaguely understood. SSTs are commonly found in the vaginal pouch and infundibulum. Some lineages, like colubrid snakes and phrynosomatid lizards, store sperm in both regions [21], but little is known about the timing of sperm movement and its effect on paternity. The location of secretory cells within SSTs also varies and is correlated with sperm storage duration. Species with secretory cells towards the opening (proximal) of SSTs tend to store sperm for shorter periods than do species with secretory cells toward the end (distal) of SSTs, while species with prolonged sperm storage have secretory cells throughout the

SSTs [21]. But again, more work is needed to address the functional significance of the secretions on sperm longevity, sperm selection and fertilization success among males.

In the few species studied thus far, seasonality of SST secretions coincides with mating (e.g. reviewed in [5,6]), suggesting that females, at minimum, support sperm transport to the SSTs. Secretions at SST openings may attract sperm and then provide mechanical protection to prevent sperm losses [6], but this could be tested by assaying sperm velocity and chemotaxis toward these secretions *in vitro*. Because sperm remain viable beyond the active period of secretory cells, Siegel *et al.* [6] seemed dubious that secretions served a nutritive function that might aid sperm longevity. However, recently, secretory activity of SSTs was demonstrated to continue outside the reproductive season, indicating long-term female support of sperm in two coral snakes (*Micrurus* sp.) [22]. Perhaps changes in secretory composition or embedding of sperm within the epithelium prior to the cessation of secretory activity places sperm in a quiescent, lowered metabolic state until ovulation that would aid sperm longevity. Differences among males in their capacity to endure in the conditions females provide would enhance their competitive fertilization success.

The hypotheses of strong selection on long-term sperm viability within the male reproductive tract (especially in dissociated breeders) may also explain long-term sperm storage within the female [11]. However, it is hard to imagine that SST secretions do not aid sperm viability in some way, but we do not have enough evidence to adjudicate the question. Gene expression of SSTs in mated versus unmated females [23] and the ductus deferens (snakes) or epididymis (lizards), coupled with protein analysis and functional assays, would yield critical data. Devine [24] suggested that SSTs with different probabilities of contributing to fertilization can cause direct (by females) or indirect (by males) bias in paternity among males thus generating mate-order effects on fertilization. One might test differential storage of sperm by spiking males' food using fluorophores and then conducting staged polyandrous matings and histological studies of the oviducts [25].

4. Mate-order effects

Mating order and mating interval effects on paternity are essential components of selection that can set the relative costs and benefits of various offensive and defensive strategies for males (e.g. mate guarding or CPs) and whether females might use these to their evolutionary advantage. Few studies of squamates have used staged or controlled matings to assess sperm competition to study the basic effects of mating order or mating interval.

Presumably, species-specific mate order effects may be determined by the morphology of sperm storage organs and the loading-order of sperm within them; physiological effects of sperm or semen on the female reproductive tract; or effects of semen from the first mating on the performance of the next male to mate's sperm [24]. In the sand lizard (*Lacerta agilis*), there are no mate order or mating interval effects on the probability of paternity, regardless of whether two males mate within an hour or with 24 h between matings (the second category representing an approximate mate guarding interval [26]). In the European common lizard (*Zootoca vivipara*), females take advantage of the second male mate

order advantage to trade up to a male with better body condition or greater heterozygosity [27,28]. Furthermore, the mate order effect on paternity share in *Z. vivipara* depends on both the interval between matings and the period between mating and ovulation: paternity was mixed between males at short intervals, but first male advantage increased drastically with longer delays between matings closer to ovulation [29]. In brown anole lizards (*Anolis sagrei*), the first male to mate sires more offspring [30], but this effect fades over time in the laboratory [31]. In Australian painted dragons (*Ctenophorus pictus*), there is no effect when matings are about 1 hour apart [32]; however, sperm stored from field matings two weeks before ovulation had an advantage over those from subsequent matings in the laboratory, suggesting a weak first male advantage that gradually disappeared with successive clutches, presumably as sperm are used or lost from storage sites during ovulation and egg retention between laying events, as seems likely the case in anoles [11].

There have been only three studies of mate order effects in snakes. In Eurasian vipers (*Vipera berus*), the first male to mate sires an average of 60% of the offspring and all of the offspring in 25% of the litters [33]. In the red-sided garter snake (*Thamnophis sirtalis parietalis*), when females mated with two males in the spring, the first male to mate gained paternity share as the mating interval increased [34]. When the females mated with a single male, 85% of litters nonetheless exhibited multiple paternity, with the single spring sire siring 67% of the offspring [35]. Offspring that could not be attributed to the spring males indicates that the females stored sperm for at least seven months over winter (September to May). Indeed, female garter snakes (*Thamnophis*) have been documented giving birth to live young after isolation from males for well over a year [36].

Paternity success from autumn matings and, thus, female sperm storage (or sperm longevity) can facilitate posthumous male reproductive success, a phenomenon known to occur, for example, in side-blotched lizards (*Uta stansburiana*) [37] and two species of dragon lizards (*Ctenophorus fordi* and *C. pictus*) [32,38]. The potential benefits of posthumous paternity may generate selection for long-term sperm viability or coevolution of sperm and the oviduct's physiological environment. Furthermore, siring offspring posthumously, thus extending the male reproductive lifespan, may provide a payoff for higher investment into current reproductive efforts at the expense of somatic maintenance, especially when conspicuous displays or behaviour increases mortality due to predation [39]. These various mate-order and mating interval effects on patterns of paternity are not yet understood mechanistically. It is not clear whether mating systems or tactics are the results or determinate of the evolution of mate order and intervals; it is likely a dynamic process. Female anatomy and physiology set the stage for sperm competition. However, the ejaculates—sperm and seminal fluid—also evolve in response to selection due to sperm competition and can be a source of sexual conflict [40].

5. Seminal fluid and copulatory plugs

Squamates lack sexual accessory glands, but the sexual segment of the kidney (SSK, or renal sexual segment, RSS) is the principal source of seminal fluid [41]. The SSK, an epithelium lining nephrons' distal tubules, hypertrophies

seasonally under the influence of androgens [42]. Mechanisms for seminal fluid secretion from the SSK and transport through the ureter to the cloaca are debatable [43,44].

The squamate SSK may derive from an ancestral condition shared with fish and amphibians [45], and it may be homologous to the mammalian seminal vesicles or prostate [46]. In snakes, the SSK can represent 60–85% of kidney mass [43, table 11.2]. Thus, the SSK is an attractive, but under-studied, evolutionary model of accessory sex glands.

Seminal fluids may enhance sperm movement and viability, protect paternity or impede rival ejaculates [14]. However, using vasectomized (i.e. SSK secretions only, no sperm) sand lizards (*Lacerta agilis*), Olsson *et al.* [26] showed that seminal fluid itself does not reduce rival males' paternity; in fact, females first mated to a vasectomized male had higher fertility. In the only study to test for effects of reptilian seminal fluid on sperm motility or velocity, Cuellar *et al.* [47] demonstrated that anole lizard seminal fluid sustains and activates sperm. Accessory gland proteins like those in *Drosophila* [48] have not been described in squamates. However, garter snake seminal fluid has prostaglandins [49] that may affect female receptivity, ovulation or egg production [50]. Female garter snakes that mated with vasectomized males' SSK secretions only (no sperm) remated at greater rates than females mated with intact males [20], suggesting CPs do not contain anti-aphrodisiacs but may still affect ovulation and female fecundity.

CPs are often viewed as defences against sperm competition. The focus has been on their role as passive mate guarding devices ensuring fertilization success of the first male to mate and compromising that of subsequent males. CPs also potentially limit female choice and, thus, generate sexual conflict (enforced chastity; [51]). However, in squamates, albeit with limited studies, the defensive utility has not been robustly borne out.

In Iberian rock lizards (*Iberolacerta monticola*), SSK-derived CPs do not prevent subsequent matings or reduce female receptivity [52]. Males that mate when the first male's CP is still intact have higher fertilization success than when the CP has started dissolving [52]. In some species of garter snakes, CPs contain the sperm and thereby prevent sperm leakage or ejection by the female [16,17,41,53]. Dislodging the CP would severely limit a male garter snake's reproductive success, but none of us have observed this dislodgement in real time, and females are often observed with two plugs in their cloaca. Multiple paternity is high in garter snakes [9,34,35], and a number of females remate with CPs intact or after they dissolve [51]. In other species of snake, CPs are less coherent, do not adhere to the female's cloaca and contain few or no sperm [54]. Unfortunately, there are few verified records of CPs in squamates (but see [55]). This deficiency of data, which may represent underreporting of CP *absence* and the inconspicuousness of some CPs *in situ*, makes comparative analyses difficult, but this would be an especially fruitful and useful research project to guide our understanding of the costs and benefits of ejaculate expenditure.

Ejaculates are not cheap [56,57]; for example, CP production can be 5–18% of daily energy expenditure for male garter snakes [58]. Therefore, ejaculate adjustment is likely common. In *Z. vivipara*, it is challenging to assign mate order effects to female physiological processes and reproductive tract morphology because copulation duration, and thus

sperm transfer, also depends on an interaction between mate order and interval. This finding suggests males adjust ejaculates based on sperm competition intensity [29,57]. Indeed, male Mallee dragons (*Ctenophorus fordi*) copulate for 60% longer, and deposit more sperm, when mating after they have observed the female mate with another male [59]. When mating with unmated females, male sand lizards extend copulation duration, and thus ejaculate investment, based on female size [26,60], and a male uses olfactory cues to adjust copulation duration in relation to his rival's relatedness with his female because paternity is biased against related males [60,61]. Such responses to perceived sperm competition or mate quality are not limited to seminal fluid—sperm morphology also responds to environmental and internal cues. In *Anolis sagrei*, males in better body condition have longer sperm and higher sperm counts, changes that also occur in response to the level of competition for mates [62,63].

6. Evolution of sperm morphology

The remarkable diversity in sperm morphology among species has been attributed to variation in the strength of sperm competition and cryptic female choice, and squamate reptiles are no exception to this rule. In general, squamates have a long, filiform sperm cell that varies considerably in size between species (range: 28.5 μm –159.4 μm ; electronic supplementary material, table S1 and figure S1) and can differ between populations and individuals [62–64]. The sperm contains a head, which can range from straight to corkscrew in shape; a midpiece, which contains the mitochondria and surrounds the axoneme; and a flagellum, which contains the axoneme surrounded by a fibrous sheath and dense outer fibres [65]. Though squamate sperm share a similar general shape, there is a substantial difference in midpiece size between lizards and snakes. Snakes have a midpiece that makes up roughly half of the total cell length, while lizard midpiece size is roughly 5% of the cell. The reason for this difference is currently unknown; however, we present data suggesting that it could be due to variation in the strength and targets of sperm competition stemming from differences in reproductive cycles between snakes and lizards.

Currently, only two comparative analyses examining squamate sperm and sperm competition exist in the literature. In snakes, Tourmente *et al.* [65] found that as the strength of sperm competition increased (using increase in relative testis size as a proxy), midpiece and flagellum size increased. However, Kahril *et al.* [66] found no relationship between the strength of sperm competition and sperm morphology in *Anolis* lizards. We have expanded upon these findings by including more species in our analyses to give an enhanced view of the evolution of sperm across squamates. As relative testes size can function as a potential indicator of the strength of postcopulatory selection, we also tested for differences in the rates of evolution of testes size (with body size as a reference) in snakes and lizards (detailed methods and data can be found in electronic supplementary material).

We found no relationship between sperm morphology and sperm competition (relative testes size) in lizards ($p > 0.07$); however, we found a significant positive association between sperm competition and midpiece length in snakes (electronic supplementary material, figure S1). In both

snakes and lizards, our models revealed that the midpiece evolves 2–10 times faster than the head and flagellum (figure 1a,b). In lizards, body mass evolves more than two times faster than testes mass, whereas we found the exact opposite pattern in snakes (figure 1c,d; electronic supplementary material, table S2).

These analyses demonstrate that the strength of sperm competition in snakes and lizards is quite different. Testes mass evolves faster than body mass in snakes, while the reverse is true in lizards, suggesting that sperm competition (or selection for increased sperm production) is stronger in snakes than in lizards. This result supports findings from other studies that show higher rates of multiple paternity in wild-caught snakes than in lizards (see the section on multiple paternity above). While the midpiece evolves quickly in both groups, midpiece length is only evolutionarily correlated with testes mass in snakes. If the midpiece is evolutionarily labile in both groups, but snakes experience more substantial selection for a longer midpiece, this pattern may explain how midpieces have reached their exaggerated state in snakes.

Though sperm competition seems to shape midpiece size in squamates, we have no clear explanation for why this is occurring. One potential option is the need for extended sperm storage in some species of snakes. While most lizards and many snakes have associated reproductive cycles, some snakes have dissociated reproductive cycles where spermatogenesis and fertilization are separated by several months [67,68]. The need for sperm to survive for extended periods in either the female or male reproductive tract could result in selection for sperm with higher energy stores. Reptiles are also the record holders for the maximum duration of sperm storage across vertebrates [69], and snakes top the charts with an impressive seven years of storage reported for the Javan file snake (*Acrochordus javanicus* [70]). Lizards also store sperm for extended periods, but the maximum duration often only spans a single reproductive season [69].

To estimate the shortest interval that sperm need to be stored in the male and female reproductive tracts, we surveyed the literature for the species represented in our sperm dataset and found the month when spermatogenesis was at its peak and noted when copulations were reported to identify these species as associative and dissociative breeders. We then used data from the female reproductive cycle to find the month of ovulation or when follicles were enlarged. The interval between these two time-points was our minimum duration of sperm storage, as it is our best estimate of the shortest interval of time that sperm would need to be retained to ensure fertilization. We found that snakes have longer sperm storage durations than lizards and that snakes have significantly longer sperm midpieces (electronic supplementary material, figure S2A,B; see for details). We attempted to test for a correlation between midpiece size and sperm storage duration in just snakes (as lizards have minimal variance in sperm storage duration), but with very few species in our dataset ($N = 8$) we did not detect a significant correlation when accounting for phylogeny (however, see details in electronic supplementary material, figure S3).

Though these data are a rough approximation of sperm storage duration, they provide potential insight for selection on longer midpieces in snakes. The cell's midpiece contains mitochondria; therefore, a longer midpiece could increase the total amount of ATP the cell produces. In other species, the total amount of ATP correlates with the level of sperm

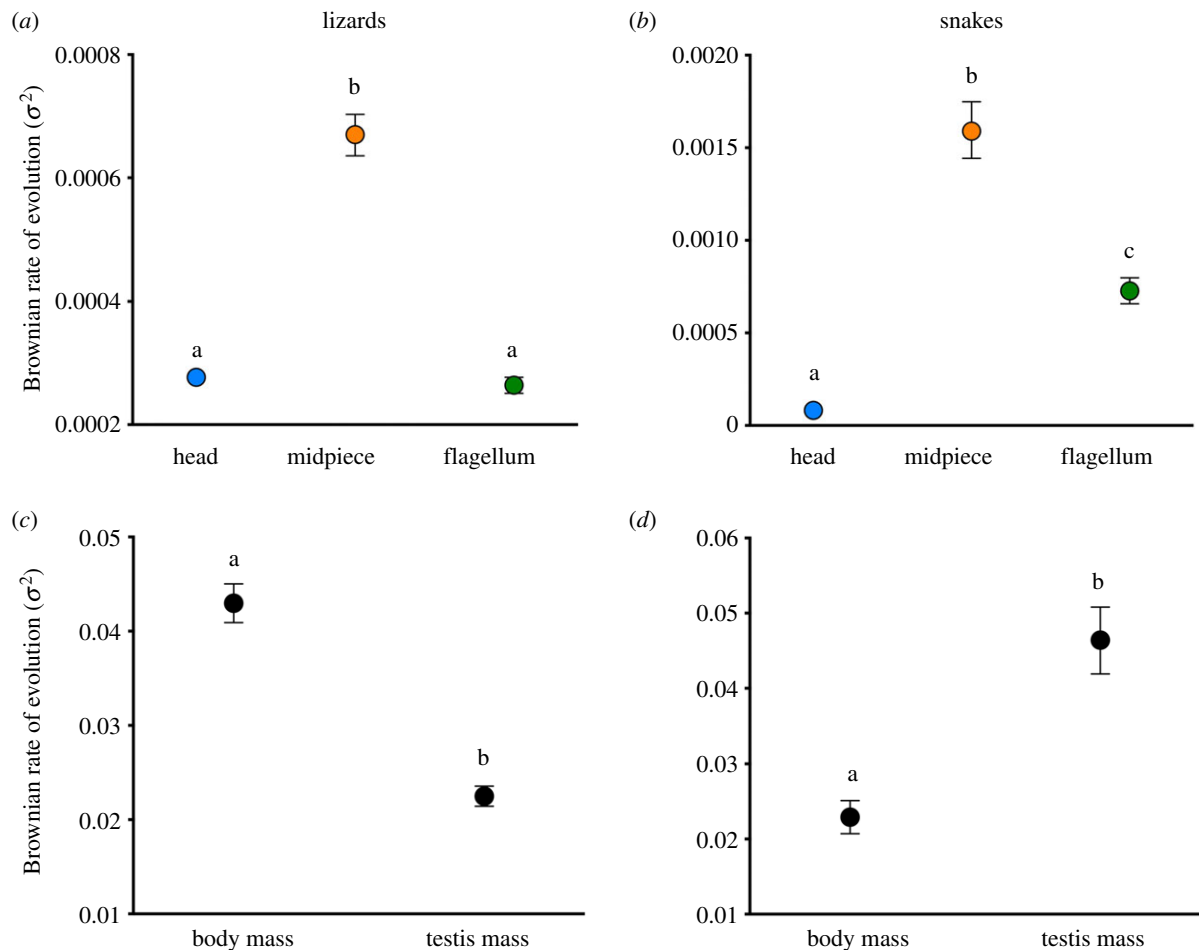


Figure 1. Rates of evolution of the sperm head, midpiece and flagellum and of body mass and testes mass for lizards (*a,c*) and snakes (*b,d*). Significant differences ($P < 0.05$) among means are indicated by different letters. Full methods, all statistical analyses and results are presented in the electronic supplemental material.

competition in rodents [71] and potentially increases cell longevity in birds [72]. Some evidence for this is a relationship between longevity and midpiece size in some birds [73] and fish [74]. However, there is conflicting evidence in other avian species [75], and a study restricted to pheasants found no relationship between the length of sperm storage (less than 20 days), across a clutch, and midpiece length [76]. Our analysis is the first, albeit very limited, test of an association between sperm storage duration (five to eight months) and midpiece length across snakes and one of the very few in vertebrates. Hopefully, it will lead to future studies in squamates and other groups to help unravel the evolution of morphological diversity of sperm.

7. Conclusion and future prospects

Squamate reptiles have extremely variable life histories that may covary with the direction and strength of sexual selection, making them ideal models for comparative studies. Their exceptionally high rates of polyandry, especially in snakes, may suggest that perhaps multiple mating is not so costly for females [9–11], although this may be hard to reconcile with copulations lasting for several hours in many snake species (see also Madsen [77]). Examining not only the benefits but also the costs associated with multiple and prolonged matings (i.e. sperm transfer), such as the risk of injury, predation or disease, for both sexes, is an essential project for squamate biologists [10,78]. Here, we have focused mostly on sperm competition without much attention to

cryptic female choice, which is, of course, essential for a full accounting of postcopulatory selection. To truly understand the importance of sperm competition in squamates, we need real estimates of selection on sperm traits from wild populations, and we also need these studies to partition the variance in reproductive success between pre- and postcopulatory episodes of selection to understand how postcopulatory selection contributes to overall sexual selection. Evolutionary patterns of sperm morphology and seminal fluid composition, CP deposition and reproductive anatomy are helpful, but these need to be complemented with intraspecific data on selection. In addition, with the risk of sexually transmitted infections and sperm being foreign cells entering the female body, interactions between the reproductive and immune systems have the potential to influence postcopulatory sexual selection, yet these interactions remain understudied, particularly in squamates and other non-mammalian vertebrates. The remarkable variation in genital morphology in squamates remains unexplained, with copulatory sexual selection being the most likely mechanism acting on genital traits. Studies on the intraspecific variation of male and female genital traits, a close examination of the form and function of the genitalia of species found in hybrid zones, and further comparative morphological studies using standardized morphological techniques are all needed to discriminate among hypotheses of genital evolution and determine their relative contribution to existing phenotypes. We encourage others to take up this challenge with us.

Ethics. Sperm morphology data were collected under ethical permits from the University of Virginia (IACUC protocol 3896), and with collection permits from The Arizona Game and Fish Department (permit no. SP673841).

Data accessibility. All data are appended as electronic supplementary material.

Competing interest. We declare we have no competing interests.

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