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Sperm form and function: what do we know about the role of sexual selection?

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1 Invited Review

2 **Sperm form and function:**
3 **what do we know about the role of sexual selection?**

4
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16 *Key words:* Sperm morphology, ejaculate evolution, sperm competition, cryptic female choice

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Goals of this review

Sexual selection is widely regarded as the principal agent underlying the astonishing diversification of sperm morphology. Consistent with this perspective, we recently provided evidence that the giant sperm flagella of some *Drosophila* species share attributes with sexual ornaments and in fact, at least using some indices, would qualify as one of the most exaggerated sexual traits in all of nature (Lüpold *et al.*, 2016; Box 1). Whereas we contend that over twenty years of experimental and comparative evolutionary studies of *Drosophila* sperm have conspired to support the claims made by Lüpold *et al.* (2016 and references therein), we note that evidence for a causative role of sexual selection in driving the evolution of sperm traits in the majority of taxa is thin. As such, any objective and rigorous review of the role of sexual selection in driving the evolution of sperm form and function would be anemic. Thus, before reviewing postcopulatory sexual selection theory as it applies to sperm traits, and assessing empirical evidence, we first address why, after over three centuries of interest, our understanding of the adaptive value of sperm form and function is in such a pathetic state.

A brief history of studying sperm diversity, or, why we know so little about the adaptive value of sperm form and function

Sperm are considered one of the most taxonomically diverse and rapidly evolving cell types (Pitnick *et al.*, 2009a; Fig. 1). In 1679, Antonie van Leeuwenhoek noted that sperm varied between taxa, and some 150 years later, Rudolf Wagner illustrated the marked differences in the sperm morphology of mammals, birds, reptiles, amphibians, fish, molluscs and insects (Birkhead and Montgomerie, 2009). Documentation of sperm diversity began in earnest in the late 19th and early 20th century, championed by Emil Ballowitz and Gustaf Retzius, the latter describing in spectacular detail the sperm of over 400 species (Afzelius, 1995; Birkhead and Montgomerie, 2009). The invention of the electron microscope and the development of ultrathin sectioning and staining techniques then sparked an explosion of comparative spermatology in the 1940s, revealing the sperm ultrastructure for thousands of species across several ensuing decades.

59 The fervent interest in sperm structure was predominantly driven by systematists. Retzius
 60 realized that sperm structure increases in similarity with species relatedness and, critically, reflects its
 61 own evolutionary history regardless of that of the overall animal bauplan (Afzelius, 1995; Birkhead
 62 and Montgomerie, 2009). With the advent of transmission electron microscopy came the discovery of
 63 the sperm axoneme and recognition that its structure was highly conserved across taxa (but see Dallai,
 64 1979; Dallai *et al.*, 2006). The opportunity presented by the juxtaposition between this pattern and the
 65 remarkable diversity in sperm size, shape and other aspects of gross morphology (Pitnick *et al.*,
 66 2009a) did not escape systematists. As a prelude to modern molecular phylogenetic approaches,
 67 examining sperm ultrastructure became a quick, easy and robust way of resolving relationships
 68 between species and among higher-order taxa (e.g., Jamieson, 1987, 1991).

69 Although the endeavor of “spermiocladistics” (Jamieson, 1987) accumulated detailed
 70 descriptions of sperm form for countless species across the tree of animal life, it generated shockingly
 71 little knowledge about functional morphology or the selection pressures driving sperm diversification.
 72 Cell biologists and biophysicists also capitalized on the discovery of the sperm axoneme, with myriad
 73 investigations addressing the cellular and molecular mechanisms by which an axoneme drives
 74 flagellar beating (e.g., Katz, 1991; Woolley, 2003). Such investigations have almost universally been
 75 conducted on glass microscope slides with observations of sperm behavior in saline (often of varying
 76 viscosities) beneath a glass coverslip and in planar view. Such a protocol is well-suited for addressing
 77 questions of cellular biomechanics, although more so when capturing the complexity of sperm
 78 motility in all three dimensions (Alvarez, 2017), but it is ill-suited to questions about actual sperm
 79 “behavior” (see below) and the adaptive value of variation in sperm form.

80 In a series of papers published in the 1950s, Åke Franzén was the first to suggest that sperm form
 81 should reflect the mode of fertilization and be shaped by details of the fertilization environment
 82 (Birkhead and Montgomerie, 2009). No formal analyses were ever conducted, but Franzén correctly
 83 recognized that the sperm of externally-fertilizing taxa not only tend to be shorter, but also simpler
 84 and less evolutionarily divergent in form than those of internally-fertilizing taxa (see Fig. 2), in which
 85 environmental selection on sperm is more complex. Sperm released into the water must swim to an

egg (usually involving chemotaxis; Evans and Sherman, 2013) and then fertilize it. In contrast, sperm of internally-fertilizing species must successfully perform numerous functions between insemination and fertilization, including (1) migration/transport to specialized sperm-storage organs (e.g., spermatheca(e) and/or seminal receptacle) or a site of quasi-specialized, short-term storage such as the “sperm reservoir” in mammals (Orr and Brennan, 2015), (2) survive and remain viable in storage for hours to decades (Birkhead and Møller, 1993; Orr and Brennan, 2015), (3) engage in molecular interactions with the female reproductive tract (FRT) epithelium and/or secretome (Pitnick *et al.*, 2009b; Holt and Fazeli, 2016), (4) undergo molecular/structural modifications required for survival, motility and/or fertilization competency (Pitnick *et al.*, 2009b; Stival *et al.*, 2016), (5) exit the storage site and migrate to the site of fertilization at the proper time (Schnakenberg *et al.*, 2012; Sasanami *et al.*, 2013), (6) successfully compete with competitor sperm for a position within the sperm-storage and/or fertilization site (Snook, 2005; Pizzari and Parker, 2009) and then (7) properly fertilize an egg (Karr *et al.*, 2009; Bianchi and Wright, 2016).

Sperm of most internally-fertilizing species are subject to selection at any of these reproductive stages. Nevertheless, the strength and type of selection on sperm adaptations related to these functions is expected to vary dramatically across species given variation in female reproductive ecology, remating behavior and FRT morphology, physiology and biochemistry, in addition to phylogenetic history. In most respects, however, the FRT and the nature of selection underlying its diversification remain largely unexplored despite increasing evidence that FRT morphology is rapidly divergent (e.g., Keller and Reeve, 1995; Eberhard, 1996; Pitnick *et al.*, 2009b; Puniamoorthy *et al.*, 2010; Higginson *et al.*, 2012).

To resolve structure-function relationships for sperm—a necessary first step in addressing the adaptive significance of sperm form—it is important to assay sperm function *in vivo* or under realistic simulated conditions. This is true for two reasons. First, the behavior of sperm may depend critically on the arena in which they are found (in terms of architecture, viscosity and association with other sperm), with misleading conclusions likely to result from studies of adaptation in the absence of the selective forces responsible for the origin and evolutionary maintenance of the traits in question. The

extent to which *in vitro* analyses of the relationship between sperm form and function (i.e., studying motility in planar view between a glass slide and coverslip) inform about the same relationship within the female reproductive tract is an open question (Katz, 1983; Alvarez, 2017). Longer sperm swim faster than shorter sperm in some taxa and more slowly in others (interspecific: e.g., Gomendio and Roldan, 2008; Fitzpatrick *et al.*, 2009; Lüpold *et al.*, 2009a; intraspecific: e.g., Lüpold *et al.*, 2012; Simpson *et al.*, 2014). Whilst relationships between flagellum length and beat frequency may hold across assays conducted in different environments, this is not necessarily true. Also, there is more to sperm behavior than beat frequency, and patterns of sperm motility may differ dramatically between a microscope slide and the more complex, three-dimensional environment of the FRT. For example, the sperm of *Drosophila melanogaster* exhibit sinusoidal flagellar beating when compressed on a glass slide, adopt a tight helical conformation (resembling the spring of a ball-point pen) and spin in place without forward progression in deeper saline, and show high, progressive mobility *in vivo* when making contact with one another and with the walls of the FRT (Manier *et al.*, 2010; S. Pitnick, personal observation). Further, at high density within the elongate seminal receptacle (SR) of females, sperm behave like snakes in a tube, with independent movements between individual sperm; at low-to-moderate density, however, they interact with one another and with the walls of the FRT to form vortexes and other complex, emergent behaviors (S. Pitnick, unpublished data). We contend that sperm form in *Drosophila* has evolved in the context of FRT morphology to execute such behaviors, none of which would be observable on a microscope slide. We are not aware of any studies examining sperm behavior both *in vitro* and *in vivo* to directly compare observed relationships, but such analyses would prove extremely valuable.

The second reason for studying sperm function *in vivo* is the growing recognition of the importance of molecular interactions between sperm and the FRT that are critical to sperm function and survival (Holt and Fazeli, 2016), including sperm behavior. For example, oviductal hormones of eutherian mammals, most notably progesterone, are known to qualitatively influence sperm behavior by mediating hyperactivation, with species-specific changes to flagellar amplitude, beat symmetry and swimming linearity (e.g., Suarez and Ho, 2003; Fujinoki *et al.*, 2016). Hyperactivation is associated

with capacitation, which is frequently posited as a mammalian-specific phenomenon (Gilbert and Barresi, 2016). However, because modifications to sperm within the FRT are taxonomically widespread, having been described for marsupial and prototherian mammals, reptiles, amphibians, tunicates, molluscs and a diversity of arthropods, such modifications likely represent the rule rather than exception for all internally-fertilizing species (Yoshida *et al.*, 2008; Pitnick *et al.*, 2009b). In fact, even in the diverse and widespread taxa with external fertilization (e.g., many species of cnidarians, echinoderms, molluscs, ascidians and fishes), ovarian (or egg-derived) fluid surrounding eggs has been shown to induce species-specific modifications to sperm motility and behavior, in addition to the chemotactic response (Evans and Sherman, 2013; Yeates *et al.*, 2013; Yoshida *et al.*, 2013), suggesting an ancient origin of molecular sperm \times female interactions.

On a related note, some of the post-insemination sperm modifications referred to above include dramatic alterations to gross sperm morphology in addition to beat frequency. For example, the sperm of the fungus gnat, *Sciara coprophila*, lose approximately one-half of their volume within the female spermathecae (Phillips, 1966). The sperm of spiders and most other chelicerates are quiescent, rolled into balls and encapsulated at insemination, with the capsules later lysed by the female to release motile, flagellated sperm (Alberti, 1990). In some ticks, immature “prospermia” are transferred to females, which, once inside the FRT, essentially turn inside-out and can nearly double in length (Oliver, 1982). Moreover, within the FRT of the Chinese soft-shelled turtle, *Pelodiscus sinensis*, the sperm midpiece loses its large cytoplasmic droplet containing lipid droplets and modifies its mitochondria, which is thought to be linked to endogenous energy production during several months of sperm storage (Zhang *et al.*, 2015). Finally, for all of the taxonomically diverse species with conjugated sperm, the sperm obviously must disassociate from one another within the FRT before fertilization (Higginson and Pitnick, 2011). Caution is thus warranted when assaying sperm form using sperm obtained from males. Whenever possible and biologically relevant, sperm should also be examined following protracted storage within the female.

Given the importance of examining sperm structure-function relationships within their selective environment, why have so few studies managed to do so? First, it is technically challenging, limiting

successful attempts primarily to transparent organisms (LaMunyon and Ward, 1998, 1999; Temkin and Bortolami, 2004; Schärer *et al.*, 2011; Ting *et al.*, 2014) and those for which sperm bearing fluorescent tags could be genetically engineered (see below; Civetta, 1999; Manier *et al.*, 2010, 2013b; Marie-Orleach *et al.*, 2014; Droge-Young *et al.*, 2016; but note that such investigations are also becoming increasingly tractable for other study organisms: e.g., Kim *et al.*, 2017). Second, evolutionary biologists interested in adaptation (as opposed to phylogenesis, see above) took little interest in sperm biology prior to the formal development of postmating sexual selection (post-MSS) theory by Parker (1970). Until then, investigators of sexual selection restricted their studies to ornaments, armaments and female preferences functioning in the competition for mates, overlooking sperm, seminal fluid, genitalia and FRTs as targets of sexual selection (Andersson, 1994). Third, throughout the first few decades of research into post-MSS, there was an overemphasis on pattern at the cost of investigating process, similar to the entire field of behavioral ecology (Birkhead and Monaghan, 2010). A plethora of investigations of diverse taxa quantified patterns of sperm precedence (e.g., the proportion of progeny sired by the last male to mate with a female). Although these investigations, importantly, served to stimulate hypotheses about mechanism giving rise to the observed patterns (e.g., Lessells and Birkhead, 1990), they tended to be uninformative about both the strength and targets of post-MSS. Meanwhile, relatively few studies directly investigated the mechanisms underlying variation in competitive fertilization success, such as genital, sperm and FRT traits and their interactions (e.g., Waage, 1979; Birkhead and Hunter, 1990; Gomendio and Roldan, 1993; Birkhead and Biggins, 1998; Simmons and Siva-Jothy, 1998; Hotzy *et al.*, 2012). Also, for a number of complex, interacting reasons (detailed in the following paragraphs), any variation in competitive fertilization success tended to be attributed to males (i.e., sperm competition *sensu stricto*; Parker, 1970) rather than to females (i.e., cryptic female choice [CFC]; Thornhill, 1983; Eberhard, 1996) and/or male \times female interactions. Further, male-mediated variation was largely ascribed exclusively to variation in sperm quantity rather than quality (i.e., sperm form).

In a series of influential theoretical papers, Geoff Parker developed post-MSS theory (Parker, 1970), widely-held models for the origin (Parker *et al.*, 1972) and maintenance of anisogamy (Parker,

1982), and sexual conflict theory (Parker, 1979). These topics interrelate, in that competition for gamete fusions underlies the origin of sexes, with the sex differences in gamete investment (and hence investment per zygote) also representing the “primordial sexual conflict” (Parker, 1979). Together with the related development of parental investment theory (Trivers, 1972), these contributions provide the foundations of modern sex difference theory (Parker, 2014). As with the genesis of any new field of thought, initial priorities were to explain the most prevalent patterns. In this case, the primary goal was to explain the causes and consequences of males typically producing vast numbers of tiny sperm. Over the intervening years, numerous theoretical models (mostly by Parker and colleagues) addressed conditions affecting relative investment by males in sperm production (reviewed by Parker and Pizzari, 2010), comparative studies examined the relationship between relative testis mass and the intensity of post-MSS (reviewed in Parker and Pizzari, 2010; Simmons and Fitzpatrick, 2012), and experimental studies explored relationships between sperm number and sperm competition success (e.g., Martin *et al.*, 1974). Given the conceptual goals and the amenability of “ejaculate investment” to game theory modeling, sperm size and other aspects of sperm form were not (until recently) considered (e.g., Parker *et al.*, 2010). Males were presumed to produce the smallest sperm possible, as competition was perceived as functioning in a manner equivalent to a raffle—either fair or loaded—with sperm equating to tickets (Parker and Pizzari, 2010). Because increases in sperm size were assumed to only be adaptive to the extent that they served as paternal investment enhancing zygote viability, one highly influential model found that even low levels of sperm competition would maintain the state of males producing the smallest size sperm possible. Simply put, even doubling the size of sperm would make only a negligible nutritional contribution to the zygote despite highly jeopardizing numbers-based sperm competition by halving the number of sperm produced (Parker, 1982).

These theoretical and empirical studies offered powerful explanations for the most widespread patterns and typical sex roles. Meanwhile, most of the astonishing variation in sperm size and other axes of sperm form were ignored (but see, e.g., Sivinski, 1984). This problem was compounded because, with few exceptions (e.g., Hellriegel and Ward, 1998; Ball and Parker, 2003), theoretical

models generally omitted any female role beyond providing the arena for competition, probably due to mathematical complexity. Modeling reproductive outcomes between varying numbers of sperm contributed by competing males is relatively straightforward. By contrast, female-mediated processes, such as biases in sperm storage or utilization, are inherently challenging to predict theoretically. The formulation of clear assumptions about possible trade-offs and constraints is often hampered in particular by missing information on the costs and benefits of any sperm-use biases, and on the mechanisms themselves (Parker, 2006).

An important shift in emphasis came in the 1990's that we credit to the coupled recognition that studies had been collectively too male-biased and insufficiently mechanistic. Birkhead *et al.* (1993a), Keller and Reeve (1995) and Eberhard (1996) all elegantly and convincingly made the case for FRT morphology, physiology and biochemistry as the wellspring of intense selection on male copulatory and ejaculatory traits, including sperm form. Snook (2005) considered the role of post-MSS in driving sperm form, and many subsequent reviews addressed sperm diversity, ejaculate biochemistry and ejaculate-female interactions (e.g., Poiani, 2006; Ravi Ram and Wolfner, 2007; Pitnick *et al.*, 2009a, b; Wolfner, 2011; Ah-King *et al.*, 2014; Fitzpatrick and Lüpold, 2014).

Some skepticism remains about the importance of CFC, possibly due to male bias (Ah-King *et al.*, 2014) and unwarranted incredulity reminiscent of criticisms of Darwin's (1871) consideration of female choice/epigamic selection in his (premating) sexual selection theory. An unjustified empirical primacy has also been afforded to male-male sperm competition over CFC, with the perspective that all variation in competitive fertilization success should be presumed attributable to sperm competition until female mediation has been demonstrated (see Birkhead, 1998; Eberhard, 2000; Pitnick and Brown, 2000). In addition to the methodological challenges of undeniably showing CFC (Pitnick and Brown, 2000), the role of female-imposed selection has also been deemed limited on theoretical grounds, based on the logic of asymmetric benefits. The contention is that selection on males to influence paternity be more intense than that on females, given that males risk losing offspring and females only risk producing offspring of lower quality (Parker, 1984). A larger consideration may be the extent of power asymmetry between the sexes. As discussed by Eberhard (1996) and McLeod and

Day (2017), females far outstrip males in diversity of mechanisms to influence sperm usage and in the likelihood that they will arise. Because the outcome is determined within their bodies, females also may physiologically and evolutionarily “get in the last word.” Indeed, Parker (1984, p. 25) agrees “it may be relatively easier for females, morphologically and behaviorally, to prevent males from achieving their objective.” Some of this logic, however, applies primarily to traits in conflict, and the extent of net sexual conflict over sperm traits influencing paternity is not apparent (McLeod and Day, 2017).

Having addressed the general methodological challenges and knowledge gaps in the study of sperm evolution, we will explore in the following sections the specific attributes of, and selection on, sperm and FRTs. We will adopt the traditional definitions of sexually selected traits as used in the context of premating sexual selection (pre-MSS), in order to draw attention to the interplay between male and female mediation in determining reproductive success and, ultimately, driving sexual trait evolution.

Pre- and postcopulatory sexual ornaments, armaments and preferences

In addition to gonads and gametes, which are essential for reproduction, reproductive characters also include the so-called ‘secondary’ sexual traits that mediate the likelihood of individuals *competitively* reproducing and propagating their genes (Darwin, 1871; Andersson, 1994; Leonard and Córdoba-Aguilar, 2010). As described by Darwin (1871), two mechanisms mediate pre-MSS: intrasexual (typically male) competition, and intersexual (typically female) choice. By definition, sexual armaments (e.g., horns or antlers) influence the outcome of (typically male–male) contest competition over mates, and ornaments provide information on their bearer’s breeding value that triggers nonrandom fitness biases resulting from ‘preferences’ of the opposite sex (usually females). For example, female preferences for brighter, more elaborate or otherwise exaggerated ornaments mediate male mating success (Darwin, 1871; Andersson, 1994). It is important to note, however, that many sexual traits (e.g., sexual calls or body size) may be under both intra- and intersexual selection, simultaneously or sequentially (Berglund *et al.*, 1996; Hunt *et al.*, 2009).

Due to the direct fitness effects of ornament or armament expression, males should increase their investment in these traits as sexual selection intensifies (Wade and Arnold, 1980), but this requires resources that could otherwise be allocated to somatic maintenance and survival, or even to paternal investment (Trivers, 1972). Consequently, sexually selected traits tend to be costly for males to grow and maintain (Emlen, 2001; Allen and Levinton, 2007). Any among-individual variation in energy reserves used to grow and maintain sexual traits can arise from underlying variation in somatic, genetic or epigenetic condition (i.e., “the capacity to withstand environmental challenges”: Hill, 2011). If so, males of superior condition should be able to invest more in such costly traits than others, rendering the phenotypic expression of sexual traits condition-dependent (Cotton *et al.*, 2004). Sexual selection will promote those female preferences that precisely target those condition-dependent male traits that honestly signal male genetic condition, as females would benefit by producing offspring that inherit those qualities (Andersson, 1994; Rowe and Houle, 1996). Condition-dependent sexual traits are therefore a central component of sexual selection theory, including ‘good genes’ models (Grafen, 1990; Iwasa *et al.*, 1991) and models of the maintenance of genetic variation in sexually selected traits (Rowe and Houle, 1996; Houle, 1998; Tomkins *et al.*, 2004). Sexual selection would further favor these female preferences to the extent that male ornaments reliably indicate any direct benefits accrued by females as a result of mating with well-ornamented males, such as better breeding territories, oviposition substrate, paternal care of young or even male fertility (Kirkpatrick and Ryan, 1991; Sheldon, 1994).

With pre-MSS, intrasexual competition and intersexual choice tend to be operationally discrete (but see Wiley and Poston, 1996), and hence so are designations of specific traits as either armaments and ornaments (but see Berglund *et al.*, 1996; Hunt *et al.*, 2009). In contrast, as described below, mechanisms of sperm competition and CFC tend to be far less discrete, blurring the lines between intra- and intersexual selection acting on post-MSS traits and, therefore, their function as armaments and ornaments following traditional terminology and definitions *sensu* pre-MSS traits. There are other conspicuous differences between pre- and postmating sexual traits, with important consequences for the evolvability and the evolutionary trajectory of traits under sexual selection. For example, pre-MSS

traits tend to be complex somatic traits controlled by multiple genes (Lande, 1980), whereas at least some ejaculate traits that are putative targets of post-MSS, such as seminal fluid proteins, are single active molecules expressed by single genes (Dorus and Karr, 2009; Pitnick *et al.*, 2009a; Avila *et al.*, 2011; but see Findlay *et al.*, 2014). Unfortunately, the genetic basis of flagellum length and of other putative sperm ornaments and/or armaments (e.g., hooks on the sperm heads of some rodents) is unknown. Another important difference is that female preferences for pre-MSS ornaments tend to be mediated by female sensory biology (e.g., vision, hearing, smell, touch) followed by cognitive processing (Ryan, 1990; Jennions and Petrie, 1997; Kirkpatrick *et al.*, 2006). In contrast, nonrandom paternity shares based on postmating female biases tend to result from interactions of ejaculates with the morphology, biochemistry and neurophysiology of the FRT (Pitnick *et al.*, 2009b) that do not require sensory organs and cognitive processing. Despite these differences, sperm, ejaculate, FRT and other sex-specific traits subject to post-MSS meet the definitional criteria of “ornament,” “armament” and “preference” as well as traditionally considered pre-MSS traits (Darwin, 1871; Andersson, 1994). Primary sexual traits therefore can bear secondary sexual traits (see *Theory of postcopulatory sexual selection* below).

To be clear, ejaculates may also share some of the fundamental attributes of pre-MSS traits, in that there can be heritable variation in their expression within populations (reviewed in Simmons and Moore, 2009) and such variation may influence competitive fertilization success (reviewed in Simmons and Fitzpatrick, 2012; Fitzpatrick and Lüpold, 2014). Additionally, there are significant costs associated with the production of ejaculates (Dewsbury, 1982; Pitnick, 1996; Olsson *et al.*, 1997; Thomsen *et al.*, 2006), and ejaculate quantity and/or quality can vary with the male’s nutritional state (Gage and Cook, 1994; Perry and Rowe, 2010; Rahman *et al.*, 2013; Kahrl and Cox, 2015; Kaldun and Otti, 2016). Ejaculate quality has further been linked to male quality as reflected by positive associations with well-characterized pre-MSS traits, although such links are usually weak (reviewed in Mautz *et al.*, 2013).

Keeping these similarities and differences between pre- and postcopulatory sexual traits in mind, we now review evidence of cryptic female choice and intermale sperm competition exerting selection

on ejaculate traits, as well as the extent to which such selection shares attributes with that on male premating ornaments and armaments.

Theory of postcopulatory sexual selection

As described above, early theoretical models analogized sperm competition to a lottery, with selection favoring males that produce the smallest possible sperm (Parker, 1982, 1993). The production of vast numbers of sperm as an adaptation to postcopulatory, intrasexual competition meets the definitional criteria of an armament. However, with growing empirical evidence from comparative studies for positive selection on sperm size in diverse taxa (Gage, 1994; Briskie *et al.*, 1997; Byrne *et al.*, 2003; Fitzpatrick *et al.*, 2009; Lüpold *et al.*, 2009b; Tourmente *et al.*, 2011), more recent sperm competition models have sought to predict when selection should indeed favor sperm number over sperm size and *vice versa* (Parker *et al.*, 2010). These models uniformly predicted that the overall gamete investment should increase with the intensity of post-MSS, which can arise from greater investments in both sperm size and number. However, when the overall investment in sperm production is maximized, the two traits necessarily trade off with one another (Pitnick, 1996; Lüpold *et al.*, 2009c, 2016). At this point, Parker *et al.* (2010) predicted selection to be stronger on sperm number than sperm size when sperm competition follows the principles of a raffle and the density of sperm at the fertilization site is relatively low (e.g., through sperm dilution within the FRT in relatively large-bodied species; Immler *et al.*, 2011; Lüpold and Fitzpatrick, 2015). If, however, sperm end up being densely packed within a small FRT (e.g., in insects and other invertebrates), physical interactions among sperm may be inevitable, in which case the quality of individual sperm may confer a competitive advantage. For example, sperm may compete to occupy limited space within the female's specialized sperm-storage organs through physical displacement, with excess sperm discarded by the female (Miller and Pitnick, 2002; Pattarini *et al.*, 2006; Manier *et al.*, 2010, 2013b; Lüpold *et al.*, 2012). Under such conditions, selection can favor sperm quality over quantity (Parker *et al.*, 2010; Immler *et al.*, 2011), with the quality trait (e.g., larger size) also meeting the definitional criteria of a sexually selected armament.

Yet, there is accumulating evidence for an active role of females in fertilization events (Firman *et al.*, 2017), with the most conspicuous example being females that eject sperm from their reproductive tract after copulation (e.g., Pizzari and Birkhead, 2000; Snook and Hosken, 2004; Wagner *et al.*, 2004; Peretti and Eberhard, 2010; Lüpold *et al.*, 2013; also see Schärer *et al.*, 2004 for active sperm removal in hermaphroditic flatworms). Further, when ejaculates compete within the FRT, variation in the morphology or biochemistry of this competitive environment is likely to influence the outcome through differential ejaculate–female compatibilities. For example, in both birds (Birkhead *et al.*, 1993b) and mammals (Suarez and Pacey, 2006), the selective environment of the FRT prevents the vast majority of inseminated sperm from reaching the egg(s). Even if such female selective processes may have initially evolved to fight pathogens invading the FRT during copulation, avoid polyspermy or reject fertilization-incompetent sperm rather than sexual selection (Birkhead *et al.*, 1993b; Eberhard, 1996; Pitnick *et al.*, 2009b), the same mechanisms will inevitably bias competitive fertilization whenever females mate with two or more males whose sperm differ in their ability to overcome the female barriers, thus giving rise to post-MSS (Curtsinger, 1991; Keller and Reeve, 1995; Yasui, 1997). Any variation in the FRT environment may change the conditions under which sperm compete, and therefore shift the relative competitive advantage between the same males (e.g., Lüpold *et al.*, 2013). Consequently, similar to premating sexual traits under both intra- and intersexual selection, post-MSS is largely mediated by male \times male \times female interactions (Bjork *et al.*, 2007), which do not necessarily favor the best sperm based purely on some intrinsic quality(ies), but rather those sperm that are best able to operate within the specific conditions set by the female and given the specific competitor male(s). Any sperm traits arising through selection generated by FRT-imposed fertilization biases meet the definitional criteria of sexually selected ornaments.

Postcopulatory male \times female interactions influencing reproductive outcomes, supportive of some female mediation in the process, have been documented in species with both internal fertilization (e.g., Lewis and Austad, 1990; Wilson *et al.*, 1997; Clark *et al.*, 1999; Miller and Pitnick, 2002; Nilsson *et al.*, 2003; Birkhead *et al.*, 2004) and external fertilization (e.g., Turner and Montgomerie, 2002; Evans and Marshall, 2005; Rosengrave *et al.*, 2008; Simmons *et al.*, 2009;

Alonzo *et al.*, 2016), thereby suggesting that the competitive fertilization process may rarely be independent of female effects. Experimental evidence suggests that sperm quantity and quality can both independently operate and interact to influence competitive fertilization success (Pattarini *et al.*, 2006; Parker *et al.*, 2010). Consequently, in a proximate sense, sperm competition and CFC represent a false dichotomy (Eberhard, 1996; Lüpold *et al.*, 2016), and any sperm traits arising through post-MSS are simultaneously ornament and armament (similar to certain traits under pre-MSS; Berglund *et al.*, 1996; Hunt *et al.*, 2009). Moreover, there may be developmental integration of sperm quantity and quality traits, given that they compete locally for resources within the testes, which in an ultimate sense may constrain the degree to which sperm competition and CFC operate as discrete processes of post-MSS (Parker *et al.*, 2010; Lüpold *et al.*, 2016; see below).

Despite the ubiquitous potential (Eberhard, 1996) and accumulating evidence (Firman *et al.*, 2017) for an active female role in reproductive outcomes, very little is known about the extent to which CFC ultimately influences the trajectory of ejaculate evolution. Current evidence is largely restricted to comparative studies showing co-diversification of sperm length and either female postmating behavior (Schärer *et al.*, 2011) or the length of some critical dimension of female sperm-storage structures, such as in birds (Briskie and Montgomerie, 1992; Briskie *et al.*, 1997), moths (Morrow and Gage, 2000), diopsid stalk-eyed flies (Presgraves *et al.*, 1999), *Drosophila* fruit flies (Pitnick *et al.*, 1999), *Scathophaga* dung flies (Minder *et al.*, 2005), *Bambara* featherwing beetles (Dybas and Dybas, 1981), bruchid seed beetles (Rugman-Jones and Eady, 2008), and dytiscid diving beetles (Higginson *et al.*, 2012). Higginson *et al.* (2012) importantly moved the paradigm of sperm-FRT co-diversification beyond the single axis of length (i.e., sperm flagellum and sperm-storage organ duct/capsule) with a multivariate, comparative analysis of 42 species of diving beetles. Evolutionary remodeling of several different FRT organs and structures were significantly associated with changes in sperm length, head shape, gains and losses of sperm conjugation and conjugation size. Moreover, analyses suggest that changes to FRTs occur first and then elicit changes in sperm form (Higginson *et al.*, 2012). Another interesting, and altogether different, example comes from hermaphroditic flatworms of the genus *Macrostomum*, in which sperm bear stiff, lateral bristles that

appear to have coevolved with thickened epithelium of the female’s sperm-receiving organ (Schärer *et al.*, 2011). Nevertheless, interspecific correlations themselves are no proof of causation. To date, Miller and Pitnick (2002) provide the only experimental evidence for the FRT as an agent of selection capable of driving the evolution of sperm form generally, and for the pattern of sperm and female sperm-storage organ length co-diversification in particular (also see Miller and Pitnick, 2003; Bjork and Pitnick, 2006; Pattarini *et al.*, 2006).

To more convincingly show a contribution of intersexual selection to the diversification of ejaculate traits, some understanding of the underlying mechanisms is necessary, thereby highlighting the critical need for investigations of post-MSS in the selective environment (Pitnick and Brown, 2000). Understanding the processes of post-MSS, particularly in internal fertilizers, requires overcoming important methodological challenges, such as reliably discriminating among sperm of different males and visualizing them within the FRT to follow their fate through the sequence of postmating reproductive events. Distinction of competing sperm itself has been achieved by use of discrete sperm sizes among males (LaMunyon and Ward, 1998; Hellriegel and Bernasconi, 2000; Pattarini *et al.*, 2006; Bennison *et al.*, 2015), application of fluorescent dyes (King *et al.*, 2002; Ting *et al.*, 2014; Lymbery *et al.*, 2016) or genetic engineering of males so their sperm express fluorescent protein (Civetta, 1999; Manier *et al.*, 2010; Marie-Orleach *et al.*, 2014; Droge-Young *et al.*, 2016). Fluorescently tagged sperm, so far successfully applied in several *Drosophila* species (Manier *et al.*, 2010, 2013a), the flatworm *Macrostomum lignano* (Marie-Orleach *et al.*, 2014) and the red flour beetle *Tribolium castaneum* (Droge-Young *et al.*, 2016), are particularly promising as, once transgenic populations with distinct sperm-tag colors are established, competing ejaculates are unambiguously identifiable and sperm behavior is visible in the selective environment following natural inseminations.

Such tools enable experimental studies addressing the functional significance of ejaculate traits in response to processes of female sperm selection, thereby filling an important gap by establishing the extent to which female biases contribute to variation in, and selection on, male ejaculate traits. Combining detailed examination of postcopulatory processes using transgenic flies with experimental

evolution and comparative studies has revealed a relatively good understanding of possible mechanisms through which female postcopulatory biases or ‘preferences’, interacting with male–male competition, may influence the evolution of sperm length in *Drosophila*, as outlined in Box 1. Yet, although these results strongly suggest that both sperm competition and CFC have been instrumental in driving the dramatic diversification in sperm length throughout this lineage, at least four important questions currently remain unanswered. First, it is unclear whether the parallel elongation of the female seminal receptacle and sperm, sharing characteristics of female preference and male preferred traits, is a case of true coevolution or whether sperm length simply tracks independent evolution of SR length (e.g., due in part to a genetic link between the two traits; Lüpold *et al.*, 2016). Second, we do not know what triggered the diversification of SR length in the first place, given the associated costs for females of developing and/or maintaining longer organs (Miller and Pitnick, 2003). Third, the extent to which different models of sexual selection (e.g., ‘good genes,’ Fisherian runaway, sensory exploitation, antagonistic coevolution) drive the evolution of female preferences (e.g., sperm choice) and hence selection on sperm is largely unknown (but see Lüpold *et al.*, 2016). Fourth, it also remains to be determined how the empirically-supported selective processes attributed to male- and female-mediated sperm evolution in drosophilid fruit flies apply to other organisms, for which co-diversification of sperm length and female sperm-storage structures has been reported (see above).

Where to go from here?

Recent theoretical and methodological advances have made substantial progress in uncovering the multifaceted patterns of ejaculate evolution. Yet, we would like to draw attention to several important gaps in our understanding for future investigation, pertaining in particular to (1) the role of different selective agents in ejaculate evolution, (2) the non-independence of different ejaculate traits and their phenotypic and genetic variation, and (3) the relative importance of intra- and intersexual selection, which we discuss in turn.

First, although there is evidence that sexual selection plays a pivotal role in ejaculate evolution, its importance relative to non-sexual selection generally remains unclear. Specifically, sexual

selection is a subset of natural selection, which shapes sperm form and function to maximize fertility in general (Tobias *et al.*, 2012). Thus, any change in the broad fertilization environment can select for modifications of ejaculate traits to ensure successful fertilization even in the absence of sexual selection. For post-MSS to operate, sperm of different males (at least potentially) must coincide within the FRT or the site of fertilization, and so its impact revolves around traits mediating *competitive* fertilization success. Thus, studies incorporating information on the overall intensity of selection, as well as on the relative importance of sexual and non-sexual selection, will provide a far more complete understanding of ejaculate evolution than those with an exclusive focus on post-MSS.

The relative importance of sexual and non-sexual selection may vary greatly with the mode of fertilization, duration of sperm storage, mating system (including the relative importance of pre-MSS and post-MSS), phylogenetic constraints, or general susceptibility of male fertility to environmental effects. For example, sperm length varies in response to female sperm storage duration (related to clutch size and egg laying frequency) but not to sperm competition levels across pheasant species (Immler *et al.*, 2007), whereas the opposite pattern seems to hold across a range of songbirds (Kleven *et al.*, 2009). Further, across fishes, sperm length differs between buccal and substrate spawners (Balshine *et al.*, 2001) and between internal and external fertilizers (Stockley *et al.*, 1996; also see Fig. 2). Sperm quantity and quality of externally-fertilizing species show interspecific associations with egg numbers and the risk of gamete dispersal by water currents (Stockley *et al.*, 1997; Liao *et al.*, 2018). Despite being associated with proxies of sperm competition in isolation (Balshine *et al.*, 2001; Fitzpatrick *et al.*, 2009; Liao *et al.*, 2018), simultaneous examination of different selective agents rendered non-sexual selection a more important predictor of macroevolutionary ejaculate variation than sexual selection (Liao *et al.*, 2018). Consequently, by focusing exclusively on sperm competition, we risk overlooking other, potentially important factors that can greatly influence the evolution of ejaculate traits either directly or by modifying the conditions under which sperm compete. It is thus critical to consider how the selective environment (e.g., spawning conditions or the FRT), as well as nutritional, health or environmental effects on the males themselves, influences

competitive fertilization success and selection on sperm form and function (also see Reinhardt *et al.*, 2015).

Second, individual ejaculate traits do not evolve in a univariate fashion. For example, according to Parker *et al.*'s (2010) models, evolutionarily stable patterns of investment between sperm size and number are expected to vary with the size of the fertilization site and associated mechanisms of post-MSS. Since the covariation between the two ejaculate traits is predicted to change from positive to negative as post-MSS intensifies and selection should be stronger on one or the other trait depending on the taxon (Parker *et al.*, 2010; Immler *et al.*, 2011), knowledge of different ejaculate traits is necessary to understand the evolutionary trajectory of each. In other words, examining multivariate selection on ejaculates and the resulting fitness landscape can be substantially more informative than single-trait studies (Pizzari and Parker, 2009; Fitzpatrick and Lüpold, 2014). This is particularly true if sperm quality and quantity exhibit trade-offs in time, space and resources during spermatogenesis. Such covariation exposes a false dichotomy between sperm competition and cryptic female choice, both of which may underlie the evolution of sperm number, size, shape and behavior (Lüpold *et al.*, 2016). In addition, to better predict the response to selection and the evolvability of ejaculate traits, we need detailed investigations of within-population relationships between sperm structures and measures of sperm performance, ideally even in a quantitative genetic framework (Birkhead *et al.*, 2005; Lüpold *et al.*, 2012). It would be particularly fruitful to conduct such studies with other putative sperm phenotypes believed to be subject to sexual selection, including but not limited to the hooked sperm heads of some murine rodents (in terms of their ability to form trains), the size and performance of sperm conjugates, the proportion of non-fertilizing sperm in polymorphic species, or of undulating membranes (Pitnick *et al.*, 2009a). Such intraspecific examination should then be integrated with broad macroevolutionary studies (including major changes in the general sperm bauplan) in response to differences in mating system and reproductive modes, or in reproductive physiology (e.g., Schärer *et al.*, 2011).

Third, processes of sperm competition and CFC are also challenging (if not impossible) to clearly delineate, particularly in internal fertilizers due to the intimate association between sperm and the

FRT (Pitnick *et al.*, 2009b). Whatever the primary agent of ejaculate evolution, changes in ejaculates may impact how they are processed by the female. Likewise, any modifications of the female reproductive tract or mode of sperm storage and utilization may select for adjustments in ejaculates to maximize their fertilization efficiency and competitiveness. When males and females differ in their optimal trait investment and each sex benefits by differentially influencing fertilization, a conflict between the sexes is likely to arise (Parker, 1979, 2006; Arnqvist and Rowe, 2005). Therefore, detailed information on the sex-specific costs and benefits, in addition to understanding the mechanisms of post-MSS, is necessary to disentangle the relative importance of intra- and intersexual selection and the potential conflict between the sexes in driving the evolution the sexual traits of interest. Until we achieve a more-than-cursory understanding of the functional design of the FRT and of the structure-function relationships underlying sperm behavior within the FRT, our understanding of the adaptive value of the staggering diversity in sperm form will be limited.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Box 1: Evolution of sperm length in *Drosophila* fruit flies

An example of how the interplay between sperm competition and cryptic female choice may drive the evolution of exaggerated sperm phenotypes comes from *Drosophila* fruit flies (Fig. 3), with *D. bifurca* producing by far the longest sperm in any species examined to date (58.3 mm or approximately 20-fold body length; Pitnick *et al.*, 1995; Figs. 2 and 3). In *D. melanogaster*, the length of the primary female sperm-storage organ, the seminal receptacle (SR), has been shown to play an important role in biasing competitive fertilization: longer SRs enhance the advantage for relatively long sperm in the process of sperm displacement, when sperm of the last male enter the SR and displace resident sperm from it (Miller and Pitnick, 2002; Lüpold *et al.*, 2012). The female effect on sperm storage is mediated primarily by variation in the timing of ejecting a mass containing displaced resident sperm and excess last-male sperm, a process that itself is genetically correlated with SR length (Lüpold *et al.*, 2013, 2016). Comparative studies, however, have revealed that selection for longer sperm enhances the evolutionary trade-off between sperm size and number (Pitnick, 1996; Immler *et al.*, 2011), thereby reducing the number of sperm available to fertilize eggs (Bjork and Pitnick, 2006). Consequently, as sperm length evolutionarily increases, females remate faster to replenish sperm reserves. In fact, SR length again is genetically correlated with both sperm length and faster female remating (Lüpold *et al.*, 2016). Each mating event creates an opportunity for sexual selection both before and after mating. Larger males have a higher mating success (e.g., Partridge *et al.*, 1987), and females gain genetic benefits for their offspring by mating with larger, healthier males. Since body size but not sperm length is condition-dependent, larger males also pay a relatively lower price per sperm, which allows them to produce more sperm and become less susceptible to sperm depletion than smaller males (Lüpold *et al.*, 2016). Consequently, these larger males are best able to capitalize on the heightened mating opportunities, and the genes associated with their longer sperm are more likely to spread in the population, thus feeding the cycle of gradual sperm length exaggeration.

(Insert Figure 3 here)

Figure legends

Figure 1: Sperm morphological diversity. Scanning electron micrographs of: A *Macrobiotus cf. hufelandi* (Tardigrada: Macrobiotidae; L. Rebecchi, U. of Modena e Reggio Emilia), B *Caenorhabditis elegans* (Nematoda: Rhabditida; T. Roberts, Florida State U.), C *Mytilocypris mytiloides* (Crustacea: Ostracoda – posterior end of long, filiform sperm; R. Matzke-Karasz, Ludwig Maximilian U. of Munich), D *Drosophila bifurca* (Insecta: Drosophilidae; R. Dallai, U. of Siena), E *Patinopecten yessoensis* (Mollusca: Ostreoida; from Li *et al.*, 2000), F *Iporangaia pustulosa* (Arachnida: Opiliones; from Moya *et al.*, 2007), G *Trialeurodes vaporariorum* (Insecta: Aleyrodidae; R. Dallai), H *Allacma fusca* (Hexapoda: Sminthuridae; from Dallai *et al.*, 2009), I *Colostethus marchesianus* (Anura: Aromobatidae; from Veiga-Menoncello *et al.*, 2007), J *Paralichthys olivaceus* (Actinopterygii: Paralichthyidae; from Zhang *et al.*, 2003), K *Gopherus agassizii* (Reptilia: Testudinata; L. Liaw, Beckman Laser Institute at U. California Irvine), L *Passer domesticus* (Aves: Passeridae; R. Dallai), M *Phataginus tricuspi* (Pholidota: Manidae; L. Liaw), N *Uromys caudimaculatus* (Rodentia: Muridae; W. Breed, U. of Adelaide). All published photos reprinted with permission from Elsevier; all unpublished photos courtesy of authors in parentheses.

Figure 2: Variation in sperm length across the animal kingdom (in μm on a logarithmic scale). Each horizontal line spans the range of sperm lengths reported in the literature for each taxon. Line colors depict different fertilization modes (light gray: external fertilization, from broadcast spawning in marine invertebrates to female-directed sperm release in frogs; dark gray: spermcasting; black: internal fertilization). Dotted lines indicate considerable extension of the sperm length range by inclusion of an extreme outlier (e.g., *Neoceratodus forsteri* in the externally fertilizing bony fishes and *Discoglossus pictus* in the frogs). Data were retrieved from Pitnick *et al.* (2009a).

Figure 3: Schematic illustration of a likely process of postcopulatory sexual selection on sperm length in *Drosophila*, involving aspect so of both sperm competition and cryptic female choice. SS = sexual selection at both pre- and postmating stages.





