Sperm form evolves rapidly and dramatically, particularly in taxa with internal fertilization. Post-copulatory sexual selection at the level of individual sperm has been suggested to explain the evolution of two enigmatic sperm phenotypes: sperm heteromorphism, where more than one type of sperm is produced by a male, and sperm conjugation, where multiple sperm join together for motility and transport through the female reproductive tract before dissociation prior to fertilization. I explore the taxonomic distribution of sperm heteromorphism and conjugation, speculate on the potential developmental origins and discuss functional hypotheses for evolutionary maintenance of these remarkable traits. I subsequently focus on the patterns of sperm morphological evolution in diving beetles (Dytiscidae), an excellent model to examine the evolution of sperm heteromorphism and conjugation. I use phylogenetically controlled regression and Bayesian estimation to infer ancestral sperm traits, identify both the rate and directionality of probable evolutionary transitions and test if the evolution of female reproductive tract design might have driven the evolution of complex, multivariate sperm form. I found sperm conjugation to be the ancestral condition in diving beetles, with subsequent diversification into three qualitatively unique forms (i.e., aggregates, pairs and rouleaux), each exhibiting varying degrees of evolutionary loss and convergence. Evolution of sperm head shape was correlated with conjugation, consistent with statistical support for non-random patterns of evolutionary transitions between the different forms of conjugation. The results suggest that both sperm length and sperm heteromorphism have independent evolutionary trajectories from conjugation. Results of Bayesian analyses suggest that the evolution of sperm morphology tracks changes in female reproductive structures. Data from both behavioral studies and transmission electron microscopic images of sperm conjugates stored in the female tract support the interpretation that sperm conjugation is an adaptation for maintaining favored positions for fertilization in the female reproductive tract. The results show that although sperm often have complex and varied sperm morphology, diversification of sperm form might
also be constrained along particular evolutionary pathways. Moreover, the results highlight the importance of sperm-female interactions as an agent of diversification.
EVOLUTION OF COMPLEX SPERM MORPHOLOGY

by

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DISSERTATION

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# Table of Contents

Chapter 1: Evolution of intra-ejaculate sperm interactions: do sperm cooperate? ........1


I. Introduction..................................................................................................................4

II. A brief overview of spermatogenesis........................................................................7

III. Haploid expression...................................................................................................8

IV. Types of putative sperm cooperation.......................................................................10

   1. Sperm heteromorphism.........................................................................................10

      a. Mechanisms......................................................................................................12

      b. Function...........................................................................................................12

   2. Sperm conjugation.................................................................................................13

      a. Types of conjugation.......................................................................................14

         i. Primary conjugation.....................................................................................15

         ii. Secondary conjugation...............................................................................16

      b. Mechanisms......................................................................................................21

         i. Primary conjugation.....................................................................................21

         ii. Secondary conjugation...............................................................................23

      c. Function...........................................................................................................24

         i. Motility...........................................................................................................25

         ii. Alternative hypotheses...............................................................................28

V. Evolutionary patterns................................................................................................33

   1. Origins...................................................................................................................33

      a. Sperm heteromorphism.....................................................................................33
Chapter 2: Convergence, recurrence and diversification of complex sperm traits in a family of aquatic beetles

I. Introduction ............................................................................................................. 86

II. Results .................................................................................................................. 88

III. Discussion .......................................................................................................... 91

IV. Materials and methods ...................................................................................... 94

V. Acknowledgements ............................................................................................ 96

VI. References ......................................................................................................... 102

VII. Supplemental material ..................................................................................... 103
Chapter 3: Female reproductive tract form drives the evolution of complex sperm morphology……………………………………………………………………………………133

I. Body of paper…………………………………………………………………...134

II. Methods summary……………………………………………………………..139

III. Acknowledgements…………………………………………………………141

IV. References……………………………………………………………………142

V. Supplemental Material ………………………………………………………152
LIST OF ILLUSTRATIVE MATERIAL

Chapter 1

Table 1.1. Summary of the different types of conjugation.........................79

Fig. 1.1. Primary conjugation..................................................................83

Fig. 1.2. Secondary conjugation...............................................................84

Fig. 1.3. Heterospermatozeugmata.............................................................85

Chapter 2

Fig. 2.1. Head shape and conjugation in diving beetles.........................113

Fig. 2.2. Transitions in sperm conjugation in diving beetles..................114

Fig. 2.3. Evolutionary transitions in sperm head shape and conjugation....115

Table S2.1. Sperm traits in Dytiscidae, Amphizoidae and Paelobiidae.......117

Table S2.2. Mean transition rate coefficients between the different forms of sperm conjugation.................................................................122

Table S2.3. Mean transition rate coefficients of correlated evolution between conjugation and sperm head shape..................................................123

Fig. S2.1. Majority consensus tree............................................................128

Fig. S2.2. Distribution of sperm length.....................................................129

Movie S2.1. Heterospermatozeugmata of *Ilybius larsoni*.......................130

Movie S2.2. Heterospermatozeugmata of *Ilybius* sp................................131
Chapter 3

Table 3.1. Results from GLS stepwise multiple regression.........................145

Figure 3.1. Types of sperm conjugation..............................................149

Figure 3.2. Phylogeny and representatives of three basic designs of diving beetle
female reproductive tracts.................................................................150

Figure 3.3. Conjugate-female interactions.............................................151

Table S3.1. Mean trait values of female reproductive tract and sperm
characters............................................................................................157

Table S3.2. Mean evolutionary transition rate coefficients estimated by reversible
jump-MCMC........................................................................................158

Table S3.3. Genbank accession numbers for DNA sequence data..........159

Table S3.4. Results of phylogenetically controlled GLS analyses..........160

Figure S3.1. Majority consensus tree used for phylogenetically controlled GLS
and logistic regression analyses.............................................................163

Movie S3.1 Conjugates in Neopours female reproductive tract................164
Evolution of intra-ejaculate sperm interactions: do sperm cooperate?

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ABSTRACT

Sperm are often considered to be individuals, in part because of their unique genetic identities produced as a result of synapsis during meiosis, and in part due to their unique ecology, being ejected away from the soma to continue their existence in a foreign environment. Selection at the level of individual sperm has been suggested to explain the evolution of two enigmatic sperm phenotypes: sperm heteromorphism, where more than one type of sperm is produced by a male, and sperm conjugation, where multiple sperm join together for motility and transport through the female reproductive tract before dissociation prior to fertilization. In sperm heteromorphic species, only one of the sperm morphs typically participates in fertilization, with the non-fertilizing “parasperm” being interpreted as reproductive altruists. Likewise, in species with sperm conjugation, high levels of sperm mortality have been suggested to be required for conjugate break-up and this has been considered evidence of kin-selected altruism. However, it is unclear if sperm possess the heritable variation in fitness (i.e., are individuals) required for the evolution of cooperation. We investigate the question of sperm individuality by focusing on how sperm morphology is determined and how sperm conjugates are formed.

Concentrating on sperm conjugation, we discuss functional hypotheses for the evolutionary maintenance of this remarkable trait. Additionally, we speculate on the potential origins of sperm heteromorphism and conjugation, and explore the diversification and losses of these traits once they have arisen in a lineage. We find that current evidence is insufficient to support the concept of sperm control over their form or function. Thus, without additional evidence of haploid selection (i.e., sperm phenotypes that reflect their haploid genome and result in heritable differences in fitness), sperm
heteromorphism and conjugation should be interpreted not as cooperation but rather as traits selected at the level of the male, much like other ejaculatory traits such as accessory gland proteins and ejaculate size.

*Key words:* sperm heteromorphism, sperm conjugation, haploid expression, sperm morphological evolution, motility, sperm competition.
I. INTRODUCTION

Sperm are exceedingly unusual cells due to their dual nature (Sivinski, 1984). On one hand, they are highly differentiated, and seemingly simple, haploid cells of a male’s body that transmit his genes from one generation to the next. On the other hand, after ejaculation, sperm are free-living organisms with unique genetic identities that perform a host of functions independent of the male that produced them, including (i) maturation, transformation or capacitation after ejaculation, (ii) location of an egg in a water column or navigation of a female reproductive tract, (iii) prolonged storage within the female, (iv) a variety of biochemical and cellular interactions between sperm, seminal plasma and the female, and often, (v) competing with the sperm of other males to fertilize a female’s eggs (Pitnick, Hosken & Birkhead, 2009b; Pitnick, Wolfner & Suarez, 2009c; Pizzari & Parker, 2009). Thus, it is little wonder that sperm are frequently interpreted as individuals, replete with their own evolutionary interests (i.e., fitness costs and benefits that might differ from the male) and reproductive fitness (e.g., Moore et al., 2002; Pizzari & Foster, 2008; Sivinski, 1984).

The concept of sperm individuality has been broadly applied to explain the evolution of two unusual spermatozoic forms: heteromorphism and conjugation. Sperm heteromorphism occurs when more than one type of sperm is produced in a single testis, with typically only one morph participating in fertilization (Till-Bottraud et al., 2005). Sperm conjugation involves the joining together of two or more sperm for motility or transport before dissociating at the site of fertilization or storage (Pitnick et al., 2009b). Remarkably, these seemingly disparate phenomena sometimes co-occur with two distinct sperm types joining together to form complex conjugates (e.g., Afzelius & Dallai, 1983;
Ferraguti, Grassi & Erséus, 1989; Healy & Jamieson, 1993), thereby suggesting that the selective function of these two phenotypes may be complementary. Both sperm heteromorphism and conjugation have been interpreted as sperm cooperation, including reproductive altruism, that enhances fertilization success in competitive environments (Baker & Bellis, 1987; Buckland-Nicks, 1998; Holman & Snook, 2006; Immler, 2008; Kura & Nakashima, 2000; Pizzari & Foster, 2008; Pizzari & Parker, 2009; Silberglied, Shepherd & Dickinson, 1984; Sivinski, 1984), akin to the sterile worker castes of eusocial insects. This conceptual interpretation implies that these sperm morphologies evolved through selection acting at the level of individual sperm. Traditionally, however, it is the diploid genome of the male that has been thought to control the phenotypes of the sperm they produce (Eddy, 2002; Joseph & Kirkpatrick, 2004). Thus, any variation in the reproductive fitness of sperm would be attributable to males and not to the genotypes of individual sperm. It is unclear if spermatozoa themselves possess the heritable variation in fitness required for the evolution of cooperation, or any other trait.

Discussion of sperm heteromorphism and conjugation has often been confused by unstated assumptions about the meaning of cooperation. Evolutionary biologists use the term cooperation to describe social interactions, where the actions of one individual benefit another individual, that have evolved through fitness benefits accrued via kin selection (Hamilton, 1964), reciprocal interactions between group members (Trivers, 1971), and higher-order selection among interacting groups (e.g., for reviews see Michod, 1999; Okasha, 2006). In general usage, however, cooperation simply means to work together, irrespective of evolutionary process (we term this “functional cooperation”). Further complicating matters, cooperation can shift from “evolutionary” to “functional”
during transitions in the unit of selection. For example, evolutionary cooperation among interacting unicellular organisms becomes functional cooperation if the organisms merge to form a multicellular entity (Michod, 1999). In a recent review, Immler (2008) defined sperm cooperation as “the partitioning of function and/or the mutual interaction between sperm of one male (i.e., sibling sperm) to increase a male’s fertilisation success”.

Unfortunately, Immler’s (2008) definition of sperm cooperation is not explicit about origins and could include both functional and evolutionary cooperation. For clarity, we recommend that the term sperm cooperation be applied only to instances where the traits of interest have evolved by sperm-level selection.

To all appearances, heteromorphic sperm or members of a sperm conjugate function in concert with other sperm, satisfying the criteria of functional cooperation, but the selective environments that have resulted in the evolution of these unusual phenomena are unknown. If males control sperm phenotype, then sperm may be viewed as captives of male evolutionary interests, and variation in sperm phenotype should be interpreted similarly to variation in other ejaculatory traits such as seminal proteins or ejaculate volume. However, if sperm can influence their fate (i.e., haploid gene expression underlies relevant variation in sperm form), then it is both appropriate and necessary to consider the evolutionary interests of sperm and potential conflict with the male that produced them (Immler, 2008; Pizzari & Foster, 2008). To establish if sperm heteromorphism or conjugation represents evolutionary or merely functional cooperation it must be determined if selection acts at the level of sperm, males or both. Simply put: are sperm individuals?
The biological processes that control sperm phenotype and conjugate formation have been largely overlooked in discussions of sperm cooperation but provide clues to the question of sperm individuality. Here, we review the spermatogenic mechanisms determining sperm form and discuss the functional hypotheses for the maintenance of sperm heteromorphism and conjugation. Additionally, we speculate on the potential origins and diversification of these remarkable traits. Lastly, we critically examine the evidence for haploid selection of sperm and thus, the potential for evolutionary cooperation among sperm.

II. A BRIEF OVERVIEW OF SPERMATOGENESIS

Spermatogenesis in the majority of metazoa involves, as an early step, the proliferation of the germ cell population by a species-specific number of amplifications (i.e., mitotic divisions; Kurokawa & Hihara, 1976; Schiff, Flemming & Quicke, 2001; Schärer, Da Lage & Joly, 2008; Virkki, 1969, 1973). Development is syncytial with each of the resulting groups of “sister” primary spermatocytes remaining connected to one another by relatively large intercellular bridges through which materials are shared (e.g., Braun et al., 1989). Each spermatocyte then goes through two meiotic divisions to become four haploid spermatids. It is not until late in spermiogenesis (i.e., following elongation of the flagellum in the case of flagellated sperm) that spermatids become fully cellularized. One of the final events in the production of spermatozoa, even after “individualization” of the flagella, is for the sperm heads to separate from one another, these being embedded up to this point in somatic support cells or an extracellular matrix (for reviews of animal
spermatogenesis see, e.g., Fuller, 1993; L'Hernault, 2006; Rouse, 2006; Scheltinga & Jamieson, 2003; White-Cooper, Doggett & Ellis, 2009).

III. HAPLOID EXPRESSION

Sperm possess unique haploid genomes resulting from chromosomal segregation and crossing over during meiosis that would seemingly provide the necessary heritable variance on which selection might act. However, the extent to which within-ejaculate variation in sperm form and function is a consequence of haploid gene expression, and the target of sperm-level selection, is an open question (Joseph & Kirkpatrick, 2004). Sperm phenotypes are predominantly determined by testicular gene expression, and hence, the diploid genome of the male (Eddy, 2002). Although the general consensus is that mature spermatozoa are transcriptionally inert (Hecht, 1998), it is now clear that post-meiotic gene expression occurs (reviewed by Dadoune, Siffroi & Alfonsi, 2004; Erickson, 1990; Joseph & Kirkpatrick, 2004). The precise timing of expression has not, however, been determined for most of the genes required for spermatogenesis and for sperm function. Such determination is complicated by the fact that, due to DNA condensation and repackaging during spermatogenesis, some genes required for spermatocyte or spermatid function will be transcribed in primary spermatocytes, with RNA persisting to be translated later in sperm development (reviewed by Dorus & Karr, 2009; Hecht, 1998; Kleene, 2001).

Even with post-meiotic gene expression, it is altogether unclear how much of the phenotypic variation among sperm within an ejaculate is attributable to allelic variation among haplotypes, and hence amenable to sperm-level selection (Joseph &
Kirkpatrick, 2004). As described above, syncytial development with sister spermatids connected by large intercellular bridges through which cytoplasm can be shared is widespread, occurring even in diploblastic basal metazoan lineages (Gaino et al., 1984). This arrangement has been experimentally demonstrated with mice to result in phenotypically diploid spermatids, despite haploid expression of genetic differences between developing sperm cells (Braun et al., 1989; Erickson, 1990). We are aware of only two characteristics of sperm form or function that have been demonstrated to exhibit natural variation resulting from haploid gene expression. First, the sperm adhesion molecule (Spam1) in mice is an antigen that enables sperm to penetrate the cumulus and is involved in sperm-egg binding. The gene is expressed post-meiotically and the mRNA is compartmentalized within a developing spermatid. The resulting protein does not diffuse in the cytoplasm but rather is immediately inserted into the plasma membrane (Zheng, Deng & Martin-DeLeon, 2001a). Reduced expression of Spam1 results in reduced ability of sperm to penetrate the cumulus and females mated to males with Spam1-deficient sperm produce smaller than average litters (Zheng et al., 2001b). The second example involves the well-studied t-locus meiotic drive system in mice. Sperm from males hemizygous for the t-locus (t/+) have poor motility and egg penetration, presumably of wild-type sperm, resulting in non-Mendelian inheritance of the t-locus, with the t-haplotype being transmitted to up to 99% of their offspring (reviewed in Lyon, 2003). The t complex responder (Tcr) gene and its wild-type counterpart sperm motility kinase-1 (Smok1) are expressed post-meiotically. The gene products are retained within haploid sperm cells, resulting in phenotypic differences between t and wild-type sperm (Véron et al., 2009). It is unlikely coincidental that both examples of heritable variation
in fitness attributable to sperm haplotype occur in mice, well-studied model organisms for gene expression. Increasingly refined molecular techniques might reveal additional haploid expressed and compartmentalized gene products in other taxa.

IV. TYPES OF PUTATIVE SPERM COOPERATION

(1) Sperm heteromorphism

There is always some, and often considerable, within-male variation in sperm morphology (reviewed by Calhim, Immler & Birkhead, 2007; Cohen, 1973, 1975; Immler, Calhim & Birkhead, 2008; Morrow & Gage, 2001; Pitnick et al., 2009b; Ward, 1998). Baker & Bellis (1987) proposed that such variation might be an adaptation to sperm competition, with different sperm morphologies fulfilling different roles in an ejaculate (the “kamikaze sperm” hypothesis). This hypothesis, however, has not been supported by either comparative (Harcourt, 1988, 1991; Møller, 1989) or experimental analyses (Moore, Martin & Birkhead, 1999), and such variation is generally attributed to poor quality control by the male and errors in spermatogenesis (e.g., Cohen, 1967; Hunter & Birkhead, 2002). Nevertheless, it is clear that there is a separate, distinct phenomenon of sperm heteromorphism, in which different types of sperm perform discrete functions. Here we refer to cases where there are distinct morphological classes of sperm whose production is tightly regulated (Friedländer, 1997; Schrader, 1960b). Because this phenomenon has been the subject of numerous reviews (Baccetti, 1972; Baccetti & Afzelius, 1976; Buckland-Nicks, 1998; Dallai, Lupetti & Mencarelli, 2006; Fain-Maurel, 1966; Friedländer, 1997; Hayakawa, 2007; Hodgson, 1997; Jamieson, 1987; Jamieson, Dallai & Afzelius, 1999; Silberglied et al., 1984; Swallow & Wilkinson, 2002; Till-
Bottraud et al., 2005), we restrict ourselves here to addressing briefly a few of the most salient features and conceptual implications.

Sperm heteromorphism has numerous, independent origins throughout Metazoa. First discovered by von Siebold (1836) in a gastropod mollusc, sperm heteromorphism has since been described for various species of rotifers, gastrotrichs, platyhelminths, nematodes, pogonophorans, molluscs, annelids, tardigrades, centipedes, spiders, insects, echinoderms, priapulids, hemichordates, cephalocordates, urochordates and chordates (Till-Bottraud et al., 2005). In nearly all known cases, two distinct sperm morphologies are produced (Swallow & Wilkinson, 2002; Till-Bottraud et al., 2005; but see e.g., Buckland-Nicks et al., 1982; Chawanji, Hodgson & Villet, 2005). The two sperm types may appear very different from one another, or they may be of generally similar appearance, differing only in total length or the proportional dimensions of their parts (e.g., head:tail ratio). Although not confirmed in all cases, it is probably true that only one of the sperm types (often referred to as “eusperm” or “eupyrene” sperm) is ever genetically functional in egg fertilization (Till-Bottraud et al., 2005; but see Au, Reunov & Wu, 1998). The non-fertilizing sperm type (or “parasperm”) may lack DNA (“apyrene” sperm) or contain only a partial chromosome complement (“oligopyrene” sperm; reviewed by Buckland-Nicks, 1998; Hayakawa, 2007). In other cases, the chromosomal complement of parasperm may be normal. Nevertheless, these sperm may not be functional in fertilization, as demonstrated for numerous sperm-heteromorphic Drosophila species (Snook & Karr, 1998; Snook, Markow & Karr, 1994).
(a) Mechanisms

Irrespective of the degree of morphological divergence between heteromorphic sperm, all are made in the same testes and derived from similar spermatogonia or spermatocytes (Healy & Jamieson, 1981; Lucas, 1971). Physiological aspects of the responsible spermatogenic mechanisms, however, may differ substantially among taxa. Production of the different sperm types may be spatially separated into different compartments within each testis, such as occurs with the “harlequin lobes” of hemipteran insects (Schrader, 1960b). Production may be temporally separated within the same region of the testis, as occurs in Lepidoptera, with eupyrene sperm produced essentially prior to pupation and apyrene sperm produced thereafter, yet both sperm types derived from the same bipotential spermatocytes (Friedländer, 1997). Both sperm types may be produced contemporaneously and side-by-side, albeit in separate spermatogenic cysts, as occurs in some Drosophila species (Beatty & Burgoyne, 1971; Beatty & Sidhu, 1969). Finally, as with the sculpin (fish) Hemilepidotus gilberti, paraspermatids and euspermatids may co-occur within the same cyst with intercellular bridges between them (Hayakawa, 2007; Hayakawa, Komaru & Munehara, 2002). Experimental work with moths has demonstrated an activational role of hormones, with glucose and ecdysteroid interacting in the induction of apyrene sperm production (e.g., Jans, Benz & Friedländer, 1984; Kawamura, Sahara & Fugo, 2003).

(b) Function

Decades of research has resulted in six non-mutually exclusive functional hypotheses for sperm heteromorphism including (1) non-adaptive errors in spermatogenesis, (2)
transport or capacitation of eusperm aided by parasperm, (3) enhanced fertilization success in the presence of sperm competition, (4) protection from spermicidal environments, (5) nutrient provisioning of females or other sperm via senescence of parasperm, and (6) control of sex ratio. However, most of the current functional hypotheses lack convincing support in any species (for reviews see Dallai et al., 2006; Friedländer, 1997; Hodgson, 1997; Holman & Snook, 2006; Jamieson, Dallai & Afzelius, 1999; Swallow & Wilkinson, 2002; Till-Bottraud et al., 2005). Given the diversity of forms and numerous independent origins, parasperm have likely evolved for different (or multiple) reasons in different lineages. Perhaps the one clear conclusion to be drawn from sperm heteromorphism is that sperm may serve a variety of functions in addition to, or before they are able to participate in egg fertilization. Selection can either shape a single, complex sperm phenotype to perform all of these functions, or there can be a division of labor, with discrete sperm types each specialized to perform different reproductive functions.

(2) Sperm conjugation

Sperm conjugation occurs when two or more sperm physically unite for motility or transport through the female reproductive tract. The sperm typically disassociate from one another only after reaching the site of sperm storage or fertilization (but see Buckland-Nicks et al., 1999; Hayashi & Kamimura, 2002a,b for a description of sperm conjugates disassociating in the female’s bursa prior to movement to the spermatheca). Similar to sperm heteromorphism, conjugation has numerous evolutionary origins among a diversity of taxa, including annelid and polychaete worms, gastropod molluscs,
myriapods, spiders, insects and both marsupial and eutherian mammals (Pitnick et al., 2009b).

(a) Types of conjugation

Here, we briefly review the usage of terminology in the literature to describe sperm conjugates and we recommend stricter criteria for the application of terms. Our goal is to facilitate future evolutionary investigations of sperm conjugation, including evolutionary origins, transitions, trajectories, constraints, and morphogenetic convergence. Synthesis of sperm conjugation has been inhibited by a lack of cohesion in the literature due to the phenomenon being referred to by many different, often taxon-specific, terms [i.e., pairs, couples, conjugates, rouleaux, bundles, trains, aggregates (with spermatostyles), spermatodesm (-a, -ta), spermatozeugma (-ta) and spermatophores (Table 1.1). Apart from “pairs” and “rouleaux” (meaning a stack of disc-shaped objects), the terms that have been applied are too general to ascribe to variation in biological phenomenology. The Greek root word “zeugma” means yoke, and “desma” means bond, fetter, or head-band. Hence, “spermatozeugma” and “spermatodesma” are synonymous with one another and with all other terms used to describe sperm conjugates. For example, “spermatozeugma” has been used to refer to the complex conjugates formed when numerous, tiny eusperm attach to a giant, highly modified parasperm in some prosobranch snails (see Section IV.2a.ii), as well as to the conjugates of some internally fertilizing fish that involve highly variable numbers of monomorphic sperm embedded within an extracellular matrix (Downing & Burns, 1995).
When considering developmental mechanisms underlying different forms of sperm conjugation, it is useful to discriminate conjugates derived from spermatogenic processes (“primary conjugation”) from those forms arising through post-spermatogenic mechanisms (“secondary conjugation”), as this dichotomy has important implications for variation within species (and within ejaculates) and as well as for evolutionary diversification among species in conjugate size (see Section V.2a). Primary conjugation is achieved through postponement of the disassociation of the sperm heads of each cyst from the material they are embedded in. Instead of individualizing at the end of spermatogenesis, sister sperm remain attached to one another until they reach the site of sperm storage or fertilization within the female reproductive tract. Such heterochronic evolution is common with sperm. For example, numerous taxa have evolved to delay sperm maturation, transformation (i.e., capacitation), and/or activation until they are within the female (reviewed by Pitnick et al., 2009c). With secondary conjugation, sister sperm within cysts undergo typical disassociation from one another during spermatogenesis and then later, “downstream” of the testes (e.g., within the seminal vesicles), conjugate with not necessarily sister spermatozoa.

(i) Primary conjugation

The term “spermatodesm(-a, -ta)” has most frequently been used to describe conjugates formed by the products of a single spermatogonium remaining grouped together following spermiogenesis, with their heads embedded in a cyst cell or cap of gelatinous material and the tails free (Fig. 1.1B, G). Conjugation is primary, and we encourage future use of this term only when referring specifically to this mechanism. Such
spermatodesms have been described from many species of insects, including orthopterans (Fig. 1.1C, D; Baccetti, 1986), a grylloblattid (Dallai et al., 2005), a mantophasmid (Dallai et al., 2003), some hemipterans (Chawanji et al., 2005; Jamieson et al., 1999; Nur, 1962), several species of beetles (Fig. 1.1A, E, F, G; Breland & Lino-Neto et al., 2008; Simmons, 1970; Sasakawa, 2007; Takami & Sota, 2007), members of the hymenopteran suborder Symphyta (Fig. 1.1B; Quicke et al., 1992; Schiff et al., 2001) and several species of dragonflies (Abro, 1998). Although referred to as spermatozeugmata, the conjugates of the gymnolaemate bryozoan Membranipora membranacea are also probably spermatodesms, as the syncytial sperm occur in aggregates of 32 or 64 (Temkin & Bortolami, 2004), and thus are likely generated by a spermatogenic mechanism (see Section V.2a). What appear to be spermatodesms have also recently been described in a monotreme: the short-beaked echidna Tachyglossus acelatus, although it has not been established that the sperm comprising each bundle are the product of a single cyst (Johnston et al., 2007).

(ii) Secondary conjugation

All other forms of sperm conjugation are secondary. Given the many independent evolutionary origins of secondary conjugation and dramatic subsequent diversification, it is no surprise that the mechanisms by which sperm become conjoined are diverse. Any categorization of this variation will be somewhat contrived. Nevertheless, some broad categories do emerge based on variation in mechanisms, which may include various attributes of sperm per se and/or extracellular materials, and in the size and organization of conjugates, both of which co-vary with the mechanisms.
Sperm “pairing” or “coupling” has referred to cases where spermatozoa form pairs in the seminal vesicles. Sperm of such species typically exhibit asymmetrical, flat-sided heads that facilitate pairing and result in a shape apparently conducive to locomotion of the bi-flagellate product (e.g., Fig. 1.2B, C, I). In the relic silverfish, Tricholepidion gertschi, cell membranes of mature, individualized sperm fuse to form a common syncytium, creating a bi-nucleate and bi-flagellate spermatozoan (Alberti, 2000; Alberti & Weinmann, 1985; Dallai et al., 2001, 2004). Sperm pairing in the firebrat Thermobia domestica uniquely involves the intertwining and cell adhesion of the anterior third of the length of the paired sperm (Bawa, 1964). Other insects with sperm pairing include some species of burrowing water beetles (D.M. Higginson, unpublished data) and diving beetles (Auerbach, 1893; Ballowitz, 1895; Dallai & Afzelius, 1985; Mackie & Walker, 1974; Werner, 1983). Pairing has been reported in marine snails of the genus Turritella (Afzelius & Dallai, 1983), millipedes (Reger & Cooper, 1968), and the new world opossums (Biggers & Creed, 1962; Moore, 1996; Phillips, 1970; Selenka, 1887).

The term “rouleaux” has been used to describe the orderly stacks of sperm that form in the lumen of the cauda epididymides of guinea pigs and naked-tail armadillos (Fawcett & Hollenberg, 1963; Heath, Meritt & Jeyendran, 1987; Shepherd & Martan, 1979). These conjugates are organized with the convex surface of one paddle-shaped sperm head fitting into the concave surface of another, followed by adhesion of their plasma membranes (Fawcett, 1975; Fawcett & Hollenberg, 1963; Shepherd & Martan, 1979). We suggest that this term should be extended to other taxa for which sperm bear adaptations for orderly stacking, including some solifuges (Fig. 1.2L; Alberti, 2000) and dytiscid beetles (Fig. 1.2G, K; D.M. Higginson & S. Pitnick, unpublished data). In the
case of dytiscids with rouleaux, sperm heads are cone-shaped with a concave, hooded base. The rouleaux form within the seminal vesicles when the apical point of one sperm head slips into the hood of another sperm’s head, in the manner of stacking cups (Fig. 1.2G).

Sperm “bundles” are conjugates in which head binding is largely achieved by extracellular material (reviewed by Hayashi, 1997) and does not necessarily involve any morphological attributes of sperm. The sperm bundles of some megalopteran fishflies involve both mechanisms; the hooked tips of sperm heads embed in an extracellular hyaline material (Fig. 1.2A; Hayashi, 1996, 1997). In the majority of cases, the heads of sperm within bundles are tightly clustered with bundle shape slender and elongate in some species and nearly spherical in others (Hayashi, 1996, 1998). The bundles of some homopteran cicadas, leaf hoppers, and spittlebugs are uniquely organized with the sperm heads aligned along a rope- or rod-like structure composed of hyaline material (Fig. 1.2H, J; Hayashi & Kamimura, 2002a) that are similar in appearance to the spermatostyles of whirligig beetle spermatodesms (Fig. 1.1E).

Sperm “trains” form when sperm cling or adhere to one another in an imprecise location by a “grappling” structure (and may not involve any extracellular material), resulting in conjugates that are highly variable in size and exhibiting a relatively disordered arrangement (apart from all heads pointing in roughly the same direction). Sperm trains are only known to occur in several species of muroid rodents (Fisher & Hoekstra, 2010; Immler et al., 2007; Moore et al., 2002), where the associations between sperm form by a conspicuous apical hook (Fig. 1.2D, E).
Sperm heteromorphism and conjugation sometimes co-occur. In the simplest case, *Turritella* spp. snails produce single apyrene sperm and paired eusperm (Afzelius & Dallai, 1983). Similarly, the ground beetle *Scarites terricola* produces short sperm that form large “sperm bundles” and long sperm that remain single (Sasakawa, 2009). In other species, eusperm and parasperm interact to form complex conjugates, typically referred to as “spermatozeugma(-ta).” Often, the parasperm are specialized to aid in the transport of the eusperm (Healy & Jamieson, 1981). In some gastropods, eusperm attach to the surface of parasperm that effectively act as transport vessels (Fig. 1.3C). These parasperm may be gigantic vermiform cells or possess multiple tails (ranging from seven to hundreds; e.g., Fretter, 1953; Healy & Jamieson, 1993). Perhaps even more unusual are the conjugates of some annelids, where hundreds of apyrene sperm join together to form hollow cylinders that fill with loose eusperm (Fig. 1.3D; Braidotti, Ferraguti & Fleming, 1980; Ferraguti et al., 1989). “Spermatozeugma” has also been applied to the monomorphic conjugates of some internally fertilizing fish where the heads of large numbers of sperm are embedded in an extracellular matrix (Downing & Burns, 1995; Fishelson et al., 2007). The extracellular matrix appears to be secreted by the Sertoli cells and coheres the sperm of a single cyst (Fishelson et al., 2007, but see Grier, 1984); thus, we suggest referring to these conjugates as spermatodesms. To mitigate ongoing confusion between researchers working on different taxa, Pitnick et al. (2009b) proposed a new term, “heterospermatozeugma” to describe conjugates composed of heteromorphic sperm or sperm which adopt discrete, alternative roles. Heterospermatozeugma have been described in prosobranch snails (Fretter, 1953; Hanson, Randall & Bayley, 1952; Nishiwaki, 1964; Woodard, 1940), bivalves (Jespersen, Kosuge & Lützen, 2001), some
polychaete and oligochaete worms (Braidotti et al., 1980; Ferraguti, 2000; Ferraguti et al., 1989; von Nordheim, 1989) and some dytiscid beetles (Fig. 1.3A, B, E, F; D.M. Higginson, unpublished data).

Finally, an unusual form of sperm conjugation is observed in some arachnids. A sheath, probably secreted by epithelial cells of the male vas deferentia, surrounds mature, coiled spermatozoa such that sperm take the form of small, immotile spheres (Alberti, 1990, 2000). Sperm may be individually encapsulated (“cleistospermia” - most Araneomorphae: Alberti, 1990; Alberti & Colye, 1991; Alberti & Weinmann, 1985; Boissin, 1973; Wu, Song & Chen, 1997) or they may be conjugated into various-sized groups (“coenospermia” - Theraphosidae and Filistatidae: Alberti, 1990; Alberti, Afzelius & Lucas, 1986; Alberti & Weinmann, 1985; Bertkau, 1877). For example, individual coenospermia of Liphistius cf. phuketensis can include more than 30 sperm (Michalik, 2007). Coenospermia may also include parasperm in addition to fertilizing eusperm (Alberti, 2005). In some taxa, at the end of spermiogenesis, spermatids fuse to form syncytial spermatozoa that are then encapsulated (“synspermia” - Scytodiade, Sicariidae, Segestriidae, Dysderidae: Alberti, 2000; Alberti & Weinmann, 1985; Michalik et al., 2004). Synspermia are thus a kind of spermatodesm. Ensheathed spermatodesms are not unique to spiders, but also have been described for certain insects: mealy bugs (Nur, 1962) and silverfish in the family Ateluridae (Dallai et al., 2002; Wygodzinsk, 1958).

Although the term “spermatophore” has been used in some cases when referring to sperm conjugates (e.g., spermatodesms of the grasshopper Conocephalus saltator; Cruz-Landim & Ferreira, 1977), we consider such usage incorrect and a source of confusion worth avoiding. Conjugates differ from spermatophores, the latter being
chitinous or cellular capsules surrounding sperm aggregates and seminal fluid (typically an entire ejaculate; Davey, 1960; Jamieson, 1987). The capsules are derived in part from male accessory gland secretions (Chapman, 1998) and, in the case of internally inseminating species, may be formed into characteristic shapes within the female reproductive tract. Whereas it is true that ensheathed spermatodesms of some species may similarly enclose secretions from the male genital tract (Alberti, 2000; Dallai et al., 2002), ensheathed or encapsulated sperm are often further packaged within a larger spermatophore (Alberti, 2000), as may be other forms of conjugated sperm (e.g., Hayashi, 1996). We therefore see no justification for equating the phenomena of sperm conjugation and spermatophore production. There are myriad species where males produce spermatophores but do not exhibit sperm conjugation.

(b) Mechanisms

(i) Primary conjugation

Spermatodesms are characterized by a mass of extracellular material that binds together the products of a single cyst. In vestimentiferan worms, spermatodesms are formed by transversal rows of microfilaments that encircle the spermatozoa and hold them together (Marotta et al., 2005). In orthopteran insects, conjugates are formed by a “muff” of polysaccharides secreted by the glandular walls of the deferent ducts (Jamieson et al., 1999). Spermatodesms may be further stabilized by protein interactions. In Mantophasmatodea, adherens junctions and a thin layer of extracellular material (Dallai et al., 2003) act in concert to bind the spermatozoa together. However, adherens junctions were not observed in the very similar spermatodesms of Galloisiana yuasai.
(Grylloblattodea), although cross striations in the intercellular space between sperm suggest that septate junctions may be present (Dallai et al., 2005). As described above, the bound sperm of spermatodesms may additionally be surrounded by a sheath arising in the testes (Nur, 1962) or from the vasa deferentia (Alberti, 2000; Dallai et al., 2002).

We have described spermatodesms as the product of normal spermatogenic mechanisms, with sperm bundles essentially delaying the last step of individualization. Spermatodesms may, however, bear additional conjugation-specific cellular adaptations in response to selection for coordinated group motility (although we are not aware of any studies that compare the ultrastructure of the bound heads of spermatodesms in one species with the ultrastructure of sperm heads within a sperm cyst just prior to individualization in a closely related species lacking sperm conjugation). For example, in whirligig beetles of the genus *Dineutus*, upon leaving the testes the sperm bundles pass through a long series of slender ducts, within which the gelatinous material into which the heads are embedded becomes lengthened and hardened into a long, stiff rod (a “spermatostyle”) along which the heads are attached (Fig. 1.1E, F; Breland & Simmons, 1970). In other cases, such as the spermatodesms of some katydids, the sperm display a very precise, crystalline arrangement (see Fig. 1.1C). Spermatodesms may also undergo profound reorganization after ejaculation. For example, the spermatodesms of several katydid species lose their polysaccharide cap but the sperm do not dissociate. Instead, the spermatozoa become tightly linked by their acrosomes (Viscuso, Brundo & Sottile, 2002).
(ii) Secondary conjugation

With secondary conjugation as well, there is the appearance of adaptations to selection for design conducive to efficient, coordinated motility. There have been detailed morphological studies of sperm pairs for several species of opossum (Moore, 1996; Phillips, 1970; Temple-Smith & Bedford, 1980), for example, and all describe intimate association of the plasma membrane that overlies the acrosome and peripheral regions that result in what Moore (1996, p 606) describes as “coordinated alignment of exquisite precision that enables spermatozoa to behave as a single biflagellate unit”.

Secondarily-conjugated sperm often have numerous intermembranous particles at the site of conjugation. In the complex heterospermatozeugmata formed by *Tubifex tubifex*, large numbers of parasperm are joined together along their flagella by adherens-like and septate-like junctions to form a cortex around a loose core of eupyrene sperm (Braidotti et al., 1980; Ferraguti et al., 1989). Intermembranous particles may form specialized regions (Bawa, 1964; Dallai & Afzelius, 1985; Dallai et al., 2004) or be more generally distributed (Afzelius & Dallai, 1983; Dallai & Afzelius, 1987). Often electron-dense or granular material is associated with the regions of adhesion (Bawa, 1964; Dallai & Afzelius, 1987; Mackie & Walker, 1974; Werner, 1976, 1983) and the intermembrane particles may represent anchor sites for a specialized glycocalyx (Dallai & Afzelius, 1985).

Additionally, extracellular material may be associated with secondary conjugation. For example, sperm of the silverfish *Tricholepisma aurea* are loosely bundled into small groups by a granular substance (Dallai et al., 2004). Similarly, the sperm of the dytiscid beetle subfamily Colymbetinae are cemented together by an
electron-dense substance that is composed of carbohydrate and protein (Dallai & Afzelius, 1985, 1987; Mackie & Walker, 1974; Werner, 1983). The unusual intertwined sperm pairs of the firebrat *T. domestica* also show an electron-opaque substance filling the gaps between the distinct opposing cell membranes. Additionally, there appear to be some regions in which the membrane surrounding both spermatozoa is continuous (Bawa, 1964).

Conjugation achieved through purely biomechanical interactions between sperm appear to be a relatively rare phenomenon, with muroid rodents presenting the most obvious example. In the wood mouse, *Apodemus sylvaticus*, the apical hook of epididymal spermatozoa is “closed” and attached to a peri-nuclear process (Fig. 1.2D; Moore et al., 2002). Upon release into the fertilization medium, the apical hook undergoes morphological remodelling such that the apical hook “opens” and is released from the peri-nuclear process. The hooks then become entangled with other sperm and the inner surface of the hooks adheres to sperm they contact (Fig. 1.2E; Moore et al., 2002). In other muroid rodents, however, cell-cell adhesion appears to be absent and the apical hooks seem only to stabilize sperm groups (Immler et al., 2007). Biomechanical interactions between sperm are also evident in the formation of rouleaux in some dytiscid beetles (Fig. 1.2G, K; D.M. Higginson, unpublished data). However, given that these rouleaux can comprise thousands of sperm, it is likely that some cell-cell adhesion also is at play.

*(c) Function*
Similar to sperm heteromorphism, conjugation takes many forms, has multiple independent origins and has likely evolved for more than one reason. Several of the (non-mutually exclusive) proposed functions of sperm conjugation overlap with sperm heteromorphism; sperm conjugation has been hypothesized to (1) facilitate sperm transport (Afzelius & Dallai, 1987; Breland & Simmons, 1970; Dallai & Afzelius, 1985), (2) enhance competitive fertilization success (Moore et al., 2002), and (3) provide physical protection from spermicidal environments (Phillips, 1970). Additionally, sperm conjugation has been proposed to (4) permit molecular exchange between spermatozoa (Auerbach, 1893; Bawa, 1975) and to (5) enhance egg penetration (Mackie & Walker, 1974). We review the limited information pertaining to the function of sperm conjugation. However, in no case is the critical biology understood sufficiently to evaluate alternative hypotheses, resulting in a universal lack of knowledge regarding the adaptive significance of sperm conjugation.

(i) Motility

It is generally regarded that sperm conjugation enhances sperm motility, an expectation grounded on sound hydrodynamic and biomechanic principles. In brief, maximum sperm velocity is proportional to the wavelength generated, and wavelength depends in part on the length of the flagellum (Lighthill, 1976). Longer sperm are predicted to swim faster because they generate increased force with proportionately less drag (Dresdner & Katz, 1981; Dusenbery, 2000; Hoekstra, 1984; Turner, 2006). A comparative analysis of 29 species of African cichlids (Fitzpatrick et al., 2009) and a quantitative genetic analysis of intraspecific variation (Mossman et al., 2009) provide strong support for this predicted
relationship. Nevertheless, our understanding of the relationship between sperm size and motility is far from complete (Humphries, Evans & Simmons, 2008). Most studies of variation in sperm size within populations have failed to find any relationship with motility (e.g., Birkhead et al., 2005; Gage et al., 2002; reviewed by Montgomerie & Fitzpatrick, 2008; Pizzari & Parker, 2009). Also, recent manipulative experiments have found that social factors (i.e., relative dominance of males) relevant to the competitive fertilization environment can significantly impact motility without affecting sperm morphology (Cornwallis & Birkhead, 2007; Rudolfsen et al., 2006). Hence, within species, non-morphological aspects of sperm energetics [e.g., levels of adenosine triphosphate (ATP)] appear to predict motility better than does sperm size (e.g., Burness et al., 2004). Moreover, sperm performance will in large part be determined by external conditions, and the selective environment in which sperm migrate and compete to fertilize eggs tends to be complex and in general is poorly understood in all species with internal fertilization (Pitnick et al., 2009c).

Some forms of sperm conjugation will lengthen each motile entity (e.g., sperm trains of mice, the spermatodesms of whirligig beetles and the rouleaux of some diving beetles; see Figs 1E, F and 2E, G, K), whereas with some other forms all of the tails will essentially be side by side (e.g., Figs 1A, B, G and 2B, C, F, I). Either way, the number of sperm per conjugate may correlate with swimming velocity due to enhanced force generation without substantially greater drag. This theoretical expectation should hold best if the beating of sperm flagella within a conjugate is synchronous, thus minimizing interference between flagella. Indeed, studies of diverse taxa have noted that the multiple flagella within conjugates do beat in a coordinated manner (e.g., Ferraguti et al., 1988;
Hayashi, 1996, 1998; Moore & Taggart, 1995; D.M. Higginson, personal observations). Such coordinated motility, in the case of spermatodesms of the bryozoan *Membranipora membranacea*, has been observed to include spontaneous shifts between small-amplitude, large-amplitude and reverse waveforms (Temkin & Bortolami, 2004).

Although not well understood, synchronization between flagella in contact with one another may be a natural hydrodynamic property of cilia and flagella, as consequence of reduced drag (Gray, 1930; Machin, 1963; Taylor, 1951). Recent investigations of the behavior of bull sperm *in vitro* have confirmed that, when two sperm contact one another, they synchronize their beat phase and frequency (Woolley *et al.*, 2009). The velocity of synchronized sperm “pairs” was found to be significantly greater than that of either single sperm prior to or subsequent to their contact and synchronization. This effect was attributed to an increase in wave velocity of the flagella – presumed to be a consequence of combined force generation with less than additive drag (which is the equivalent of sperm swimming in lower viscosity fluid; Woolley *et al.*, 2009).

Unfortunately, relatively few studies have quantified motility of conjugated sperm, and those that have done so collectively provide inconclusive or mixed results. Sperm of the firebrat, *T. domestica* are only motile when in conjugated pairs, not as single spermatozoa (Bawa, 1964). Swimming speed of paired sperm of the marine snail *Turritella communis* was examined under a range of viscosity conditions, with no differences from that of single sperm detected (Ishijima, Ishijima & Afzelius, 1999). By contrast, sperm pairs were found to swim faster than unpaired sperm in the opossum *M. domestica*, with the difference in motility increasing with increasing viscosity of the
medium (note: fluid within the isthmic region of the oviduct of *M. domestica* is highly viscous; Moore, 1996; Moore & Taggart, 1995). However, because opossum sperm are morphologically adapted for pairing with asymmetrical heads (Fig. 1.2B, C), the significance of this comparison is not altogether clear. Unpaired sperm tend to swim in circles and the thrust produced by the flagellum is largely dissipated by lateral movements of the head. Head displacement is dampened in paired sperm, presumably due to the acquired head symmetry, the angle of insertion of the flagella (Fig. 1.2C), and the reported (but not quantified) observation that “the flagellum of each paired spermatozoon always beats in equal but opposite synchrony with its partner” (Moore & Taggart, 1995, p. 951). If true, the mechanism underlying such coordinated, 180° out-of-phase synchrony remains a mystery. Sperm trains of European wood mice (Fig. 1.2E; Moore *et al.*, 2002), deer mice *Peromyscus maniculatus* (Fisher & Hoekstra, 2010), and Norway rats *Rattus norvegicus* (Immler *et al.*, 2007) swim faster than their respective single sperm, but sperm trains of the house mouse *Mus musculus* do not (Immler *et al.*, 2007). Hayashi (1998) found a significant positive relationship between the size of sperm bundles and swimming speed in the fishfly *Parachauliodes japonicus*.

(ii) *Alternative hypotheses*

*Sperm transfer* - Sperm conjugation may facilitate the transfer of large numbers of spermatozoa to females and has been proposed to be a precursor to more efficient mechanisms of transfer, such as spermatophores (Afzelius & Dallai, 1987; Breland & Simmons, 1970; Dallai & Afzelius, 1985). Reducing sperm loss may be important in *Bryozoa* where conjugates are released into the water column before entering the
intertentacular organ of a maternal individual, dissociating and migrating to the surface of the ovary (Temkin, 1994). However, this is unlikely to be a common function of conjugation; we are unaware of conjugation occurring in externally fertilizing species and, in contrast to Bryozoa, the vast majority of species deposit conjugates in the female reproductive tract. Moreover, although the hypothesis has not been subject to evolutionary analysis, the concomitance of conjugation and spermatophores as mentioned above (Alberti, 2000; Hayashi, 1998; Sasakawa, 2007; Takami & Sota, 2007) suggests that conjugation serves a function beyond merely grouping sperm.

*Sperm competition* - The female reproductive tract provides the arena of competition and intimately links female preference with sperm competition (Eberhard, 1996; Pitnick *et al.*, 2009c). In addition to the potential role of conjugate motility in sperm competitiveness, we propose that sperm conjugation may produce sperm morphologies that are “preferred” by females. Sperm size is positively correlated to competitive fertilization success in the snail *Viviparus ater* (Oppliger, Ribi & Hosken, 2003), the nematode *Caenorhabditis elegans* (LaMunyon & Ward, 1999), the bulb mite *Rhizoglyphus robini* (Radwan, 1996), and the fly *Drosophila melanogaster* (Miller & Pitnick, 2002; Pattarini *et al.*, 2006). In the only study to examine conjugate size and female reproductive tract morphology, spermatodesm length was found to be positively correlated with spermathecal length in 30 species of the ground beetle tribe Pterostichini (Sasakawa, 2007), but remains to be tested using rigorous statistical methods accounting for phylogeny. Sperm conjugation may be a mechanism for increasing size in an energetically inexpensive way. Sperm size has been proposed to trade-off against sperm numbers (Parker, 1982), although empirical studies have often failed to find a
relationship (reviewed by Parker et al., 2010; Pizzari & Parker, 2009; Snook, 2005; but see Pitnick, 1996). Sperm conjugation may avoid this potential trade-off and maintain high sperm numbers without the increased metabolic costs of producing large sperm [e.g., large testes (Pitnick, 1996) or delayed maturation (Pitnick, Markow & Spicer, 1995)].

**Protection from spermicidal environments** - Conjugation may also preserve sperm viability, to the extent that the female reproductive tract presents an environment that is damaging to sperm (Birkhead, Møller & Sutherland, 1993; Holman & Snook, 2006). The rouleaux of guinea pigs nominally partitions the acrosomes of all but the uppermost spermatozoa in the stack from the environment (Fawcett, 1975; Friend & Fawcett, 1974; Phillips, 1970) and may thereby protect the acrosomes from degradation. A similar protective role has been proposed for sperm pairing in American marsupials, where sperm are tightly apposed along the acrosomal surface (Bedford, Rodger & Breed, 1984). In fertilization medium, paired sperm of opossums maintained motility for longer periods than did single sperm (Moore & Taggart, 1995). While intriguing, this result does not specifically address acrosomal integrity and should be interpreted with caution as the sperm heads of opossums are morphologically adapted for conjugation and sperm that fail to pair may be defective in some way. In many cases, however, conjugation leaves the acrosomes exposed (e.g., some diving beetles: Dallai & Afzelius, 1985; rodents: Breed, 2004; Immler et al., 2007). Moreover, acrosomes are a nearly universal feature of animal spermatozoa, whereas conjugation is rare (Pitnick et al., 2009b). In species with sperm conjugation, there is no evidence that environmental degradation of acrosomes is commonplace and the role of conjugation in maintenance of acrosomal integrity has not
been subjected to experimental investigation. It is also plausible for some conjugate forms that the chemical environment in the interior of conjugates may be significantly different from the surrounding environment. In the complex heterospermatozeugma of oligochaete worms, small molecules can pass through the cortex of parasperrn into the central, eusperm-containing core and has been proposed to increase sperm survival during the long storage period before fertilization (Ferraguti et al., 1988). However, it remains to be demonstrated that the female reproductive tract contains nutrient molecules and that these are of the appropriate size to permeate the conjugates.

Conversely, dissociation of conjugates has been proposed to result in the death or damage of some of the participating sperm. In the wood mouse, sperm-train break-up is suggested to be concomitant with premature acrosome reaction by approximately half of the participating spermatozoa (Moore et al., 2002). Likewise, it has been argued that the dissociation of sperm pairs in the opossum tends to result in loss of motility of one of the participants, possibly due to disruption of the cell membrane (Moore & Moore, 2002). Where cell-cell adhesion is important for conjugate formation and stability it seems inevitable that sperm degradation or death will result in conjugate break-up. Additionally, theoretical models suggest that hydrodynamically synchronized sperm clusters fall apart when there is substantial disparity in sperm beat frequencies as would be the case with weakly motile, dying sperm (Yang, Elgeti & Gompper, 2008). Nonetheless, it does not necessarily follow that conjugate break-up results in sperm death or that sperm mortality is a normal mechanism of conjugate dissociation. In the wood mouse, for example, it is difficult to assess the significance of a high rate of acrosome reaction in conjugated sperm without also knowing the ‘normal’ rate of premature
acrosome reaction for single sperm in this species under the experimental conditions examined. Unfortunately, in most cases, the appropriate experimental tests have not been undertaken to substantiate the potential association between conjugate break-up and sperm mortality. At least in some species, however, conjugate break-up is not associated with sperm mortality; the paired sperm of the diving beetle *Graphoderus liberus* undergo natural or mechanically induced separation without associated sperm mortality (D.M. Higginson, K.R. Henn & S. Pitnick, in preparation).

**Molecular exchange** - Molecular exchange to limit variability between spermatozoa was the earliest proposed function of conjugation (Auerbach, 1893; Bawa, 1975). As described above, cytoplasmic bridges between developing spermatids effectively reduce phenotypic variation between spermatozoa (e.g., Braun *et al.*, 1989). Thus, molecular exchange among members of a conjugate would only be important if there was post-individualization haploid expression. To date, there is no evidence of exo- or endocytosis in conjugated sperm (e.g., Dallai & Afzelius, 1987; Dallai *et al.*, 2003; Friend & Fawcett, 1974; but see Werner, 1976). Additionally, any hypothetical molecular exchange between spermatozoa would be limited to cases of intimate association between sperm heads, such as is seen in most, but not all, forms of secondary conjugation; the distance between primarily conjugated sperm is typically occupied by gel-like or solid extracellular material that would present a barrier to efficient diffusion of molecules (Amsden, 1998).

**Egg penetration** - Lastly, sperm conjugation has been proposed as a mechanism to improve sperm penetration of the egg membrane by the joint action of the multiple acrosomes present in a conjugate or by increasing thrust force (Mackie & Walker, 1974).
This hypothesis minimally requires that sperm remain conjugated until contacting an egg. We can find no evidence supporting a role of sperm conjugation in fertilization. Where the timing of conjugate dissociation is known, it invariably occurs before contact with an egg (opossum: Taggart et al., 1993; guinea pigs: Martan & Shepherd, 1973; fishflies: Hayashi, 1997; snails: Buckland-Nicks et al., 1999). Furthermore, polyspermy has not been reported in any species with sperm conjugation.

V. EVOLUTIONARY PATTERNS

Discussion of the evolution of sperm heteromorphism and conjugation has often focused on theoretical analysis of the role of haploid expression and the origins of cooperation (Haig & Bergstrom, 1995; Immler, 2008; Kura & Nakashima, 2000; Parker & Begon, 1993; Pizzari & Foster, 2008; Swallow & Wilkinson, 2002). Given the current lack of empirical support for haploid control (see Section VI.1), we advocate a more pragmatic approach combining phylogenetic hypothesis testing to identify selective environments (e.g., mating system, female reproductive tract morphology) associated with the origin, maintenance, or loss of sperm heteromorphism or conjugation. We further advocate renewed effort to identify the physiological and molecular mechanisms that produce these remarkable variations in sperm morphology. Below, we consider possible physiological origins, modifications and losses of sperm heteromorphism and conjugation.

(1) Origins

(a) Sperm heteromorphism
It seems probable that heteromorphic sperm with aberrant chromosome complements originate from mutations disrupting the normal course of spermatogenesis resulting in nondisjunction, nuclear elimination, or asymmetric cytokinesis. In the sculpin *Hemilepidotus gilberti*, eusperm and parasperm develop synchronously within a common cyst (Hayakawa *et al.*, 2002). Spermatogenesis proceeds typically until the second meiotic division, when asymmetrical cytokinesis results in two haploid eusperm and one diploid parasperm (Hayakawa, 2007). In some cases, the asymmetrical cytokinesis that gives rise to parasperm is reminiscent of that seen during oogenesis (Fain-Maurel, 1966). Spatial or temporal changes in the chemical environment in which sperm develop might also result in abnormal chromosome complements. The testes of hemipteran insects are partitioned into several lobes, with one morphologically and presumably chemically distinct “harlequin” lobe (Schrader, 1960a,b; Schrader & Leuchtenberger, 1951). During meiosis in the harlequin lobes, autosomes typically form a chain or become an indistinguishable mass that is laterally displaced from the normal mitotic axis and fails to segregate appropriately during cell division (Schrader, 1945, 1946a, b, 1960a). Likewise, in Lepidoptera, changes in circulating hormones with the onset of pupation result in a shortened prophase and the elimination of nuclei from developing spermatids (Friedländer, 1997; Kawamura *et al.*, 2003; Sahara & Kawamura, 2004).

The origins of size dimorphism in contemporaneously developing spermatids with normal chromosomal complements are enigmatic; spermatogenesis is similar to closely related sperm monomorphic species and cysts seemingly share a common developmental environment. Additionally, the paucity of studies of spermatogenesis in
sperm-length-heteromorphic species provides few clues to the developmental mechanisms that result in alternative morphs. In the handful of studies addressing spermatogenesis, there were no clear differences in development between cysts that could explain the resultant long and short sperm in cicadas or fruit flies (Chawanji et al., 2005, 2006, 2007; Hauschteck-Jungen & Maurer, 1976; Kubo-Irie et al., 2003). Comparative genomic analysis between sperm hetero- and monomorphic *Drosophila* species targeting spermatogenesis-related genes and regulatory sequences might reveal prospective variants associated with sperm heteromorphism. Candidate genes could be subjected to functional analysis to characterize their role in spermatogenesis.

(b) Conjugation

By definition, primary conjugation is the result of delayed sperm individualization and thus has most likely resulted from mutations delaying or suppressing expression of genes involved in the final stages of spermatogenesis. By contrast, secondary conjugation is achieved by several, distinctly different means (i.e., biomechanical interactions, cell-surface interactions, membrane fusion, or a combination thereof). Accordingly, there may be more evolutionary avenues that result in secondary sperm conjugation than primary conjugation.

Some sperm structures that have evolved in the context of selection for enhanced fertility or competitiveness might preadapt sperm for conjugation. The sperm heads of most species of muroid rodents have large apical hooks primarily composed of acrosomal material (Breed, 2004, 2005; Roldan, Gomendio & Vitullo, 1992), suggesting that the hooks evolved in the context of sperm-egg interactions. Alternatively, sperm-
female interactions might have selected for sperm morphologies that improve sperm retention and movement through the female reproductive tract; apical hooks act like barbs, allowing sperm to attach to the female epithelium periodically and to maintain their position within the tract, potentially reducing energy expenditure (Firman & Simmons, 2009). Nonetheless, the hooks of some species interact to form sperm conjugates (see Section IV.2a.ii; Immler et al., 2007; Moore et al., 2002). Although it is not clear if all sperm with apical hooks conjugate, at least in rodents, hooks appear to be a prerequisite for conjugation. Likewise, hooded sperm heads in diving beetles (Jamieson et al., 1999) might facilitate the formation of rouleaux in the deferent ducts of males where sperm are densely packed, oriented in a single direction, and motile. Hooded sperm heads are found in at least three subfamilies, collectively accounting for more than half of all diving beetle species (Dallai & Afzelius, 1985, 1987; Mackie & Walker, 1974; Werner, 1976, 1983). Currently, phylogenetic relationships within Dytiscidae are insufficiently resolved (Miller, 2001; Ribera, Vogler & Balke, 2008) to infer if morphological features of sperm heads, such as the depth of the hood, are correlated with conjugate evolution.

Sperm pairing might have originated by cooption of cell surface proteins involved in other cell-cell interactions. “Green-beard” selection has been proposed to explain sperm pairing in opossums (Moore & Moore, 2002). Green-beard selection for cooperation occurs when a gene, or group of linked genes, produces a recognizable phenotype (e.g., a green beard) and cooperates with other individuals that share that phenotype (Dawkins, 1976; Hamilton, 1964). Green-beard-mediated cooperation is generally considered to be evolutionarily unstable because of the breakdown of linkage
disequilibrium between the genes encoding the phenotype and those controlling the cooperative behavior (Lehmann & Keller, 2006). However, green-beard effects may be important in the evolution of secondary sperm conjugation. Moore & Moore (2002) propose that cell-adhesion molecules mediate sperm conjugation in the opossum. In this case, a single gene might control both the green-beard and cooperation. Here, linkage disequilibrium would be absolute and could not decay over time. As pointed out by Keller (2002), more information is required to determine if green-beard selection was important in the evolution of sperm pairing. First, the molecular mechanism of conjugation should be confirmed and it be demonstrated that only sperm with the green-beard conjugate. Second, the fitness benefits (if any) must be quantified. Neither the molecular mechanism nor reproductive fitness benefits have been studied in opossums or any other species with sperm pairing.

(2) Modifications

(a) Conjugate size

The mechanisms by which sperm conjugate have important selective consequences, as they may determine the extent of among-male variation and otherwise constrain the evolvability of conjugate size. With sperm pairing, for example, the morphological and cellular mechanisms of conjugation (see Section IV.2b.ii) are not conducive to the formation of conjugates of more than two sperm. In the grey short-tailed opossum *Monodelphis domestica*, for instance, presumably all males in the population have paired sperm, and within males, 80-90% of spermatozoa in the caudal region of the epididymis were found to be (correctly) paired, with remaining sperm being either single or members
of misaligned pairs (Moore, 1996). Because single sperm are either immotile or exhibit impaired or limited motility (see below), they tend not to be among the sperm population competing to fertilize eggs (e.g., Moore 1996); hence, there is effectively no additive genetic variation in conjugate size. Where sperm pairing has arisen, it has presumably gone to fixation or been lost.

There will similarly be limited variation in conjugate size when conjugates form by primary, spermatogenic mechanisms (i.e., spermatodesms). The number of sperm per conjugate ($N$) will be determined by the formula, $N = 2^n \times 4$, with $n$ equal to the number of pre-meiotic, mitotic divisions (also referred to as amplifications). That is, each germ cell giving rise to a sperm cyst first divides binomially $n$ times, with the resulting primary spermatocytes then entering meiosis to each give rise to four spermatids. [Note that some species are known to show intermediate or slight deviations from the $N = 2^n \times 4$ formula (e.g., Nur, 1962; reviewed by Schärer et al., 2008)]. The number of amplifications tends to be species-specific and exhibits considerable phylogenetic inertia (Kurokawa & Hihara, 1976; Schiff et al., 2001; Schärer et al., 2008; Virkki, 1969, 1973). As a consequence, irrespective of the species-specific number of sperm per conjugate (e.g., 16, 32, 64, 128, 256, etc.), minimal variation within and between males in conjugate size is expected (e.g., Nur, 1962). There is known to be some variation in the number of sperm per cyst, and hence theoretically in the number of sperm per spermatodesm, although the extent of such variation and its consequences have not been well explored (see Schärer et al., 2008). With primary conjugation, therefore, any evolutionary response to selection for increased or decreased conjugation size requires the gain or loss of an amplification step (and the respective doubling or halving of the number of sperm per conjugate),
which does not appear to be an evolutionarily labile trait. Moreover, given that a sperm conjugate has a complex, functional phenotype (e.g., architecture, flagellum length, beat frequency) that is presumably subject to multivariate selection, it may be that hypothetical alternative discrete character states (e.g., an increase from 256 to 512 sperm per conjugate) will not reside on adaptive peaks.

Other forms of conjugation are more conducive to extensive and continuous variation in conjugate size (e.g., see Fig. 1.2F, G). However, no thorough qualitative or quantitative genetics studies of variation in sperm conjugate size have been conducted with any species. The rouleaux of guinea pigs are reported typically to include from 2 to 14 sperm (Fawcett & Hollenberg, 1963), and those of the solifugid *Eusimonia mirabilis* (see Fig. 1.2L for a closely related species) vary from 4 to more than 50 sperm (A. Klann & G. Alberti, personal communication). Among rouleaux-producing species of dytiscid beetles, some produce rouleaux of relatively invariant size (6—8 in *Neoporus dimidiatus*), whereas others, such as species of *Hydroporus*, produce rouleaux that vary in size from only a few to thousands of sperm (D.M. Higginson, unpublished data). Similarly, coenospermia of the spider *Liphistius cf. phuketensis* were observed to contain anywhere from only a few to over 30 spermatozoa (Michalik, 2007). The mechanism of sperm train formation also inherently results in conjugates of variable size. Sperm from the vas deferens and caudal epididymis were found to be in groups of 5—50 in the Norway rat and from 3 to 30 in the house mouse (Immler *et al.*, 2007). *In vitro*, trains can grow to include thousands of sperm (Moore *et al.*, 2002). Studies of conjugate size heritability, or even repeatability across ejaculates within males would prove valuable, as
clearly would investigations of the relationship between among-male variation in conjugate size and (non-competitive and competitive) fertilization success.

(b) Division of labor

Understanding of the evolution of heterospermatozeugmata may be enriched by theory developed to explain the origins of multicellularity. Heterospermatozeugmata are typically (perhaps always) composed of fertile and non-fertile sperm (see Section IV.2.a.ii) and can be viewed as analogous to differentiated cell clusters with reproductive and somatic cells. Formation of simple cell aggregates is postulated to be the first step towards multicellularity, with specialization of cell function occurring secondarily (Mable & Otto, 1998; Michod, 2007; Michod & Roze, 2001; Pfeiffer & Bonhoeffer, 2003); thus, one might expect conjugation to evolve before sperm heteromorphism in lineages leading to heterospermatozeugmata. However, models of the evolution of multicellularity are based on the assumption that cells behave as individuals and the applicability of these models to the evolution of heterospermatozeugmata will depend on the extent that sperm demonstrate evolutionary interests independent of the male that produced them. As both conjugation and sperm heteromorphism have evolved independently in several lineages (see Section IV.2.a.ii), it seems equally probable that parasperm might evolve prior to conjugation in lineages with heterospermatozeugmata. Currently, incomplete knowledge of sperm characters in basal taxa does not permit inference about the sequence of evolution of heterospermatozeugmata.
(3) Losses

The developmental process of sperm morphogenesis will influence the evolution of sperm traits. Sperm heteromorphism and primary conjugation that likely arose through spermatogenic mechanisms may be more resistant to loss than secondary conjugation. The overwhelming majority of mutations disrupting the normal course of spermatogenesis are expected to have severe, negative fitness consequences (e.g., Cooke & Saunders, 2002; Hackstein et al., 1990; Wakimoto, Lindsley & Herrera, 2004).

Once sperm heteromorphism arises, it typically persists in a lineage. Sperm heteromorphism appears to have originated in the basal lineages of Lepidoptera and is ubiquitous in the derived clades (Jamieson et al., 1999). Interestingly, both monomorphic and heteromorphic sperm have been reported in members of Micropterygidae, the sister group to the rest of Lepidoptera (Jamieson et al., 1999). It has been suggested that sperm heteromorphism might have two independent origins in Lepidoptera (Sonnenschein & Häuser, 1990; Swallow & Wilkinson, 2002). However, the hypothesis of multiple origins of sperm heteromorphism in Lepidoptera has not been tested and may not be supported by current phylogenetic hypotheses (Weigmann, Regier & Mitter, 2002). In flies, sperm heteromorphism occurs in some Drosophila spp. (Drosophilidae) and in stalk-eyed flies (Diopsidae). Sperm heteromorphism in Drosophila appears to have a single origin in the obscura subgroup and is maintained in all examined members of the clade (N = 17 of 41 species; Gao et al., 2007; Snook et al., 1994; Swallow & Wilkinson, 2002). Outside of the obscura subgroup, sperm polymorphism has only been reported in some (but not all) populations of D. tessieri (Joly, Cariou & Lachaise, 1991). Although sperm heteromorphism appears to be an
evolutionarily stable trait, stalk-eyed flies provide an example of loss of sperm heteromorphism. Sperm dimorphism is the ancestral condition of Diopsidae, but only monomorphic sperm are found in the derived genus *Diasemopsis* (Presgraves, Baker & Wilkinson, 1999).

Conversely, traits that mediate secondary conjugation such as sperm gross morphology (Pitnick *et al.*, 2009b) and cell surface proteins (Vacquier, 1998) are remarkably labile making this type of conjugation susceptible to loss or modification. Phylogenetic analyses examining sperm morphology in rodents suggest that apical hooks that participate in conjugate formation (see Section IV.2.b.ii) are the ancestral condition of the superfamily Muroidea and have been secondarily lost numerous times resulting in paddle-shaped sperm heads (Breed, 2004, 2005). (Note: earlier analyses using previous phylogenetic hypotheses for generic and subfamily relationships proposed paddle-shaped sperm as the ancestral trait; Roldan *et al.*, 1992). Although it has not been verified, we can infer that the loss of apical hooks also results in a loss of conjugation. Unfortunately, no studies have examined the evolutionary trajectory of sperm conjugation for any lineage. Future studies examining the evolution of conjugation through a lineage would be informative for identifying mating systems and selective environments that result in the loss or maintenance of conjugation.

**VI. EVIDENCE FOR COOPERATION**

Two conditions are required for the evolution of sperm cooperation (Nowak, 2005). First, sperm must possess heritable variation in reproductive fitness (i.e., be individuals). Second, individual sperm must be subject to natural selection. In light of these conditions,
we consider if sperm heteromorphism or conjugation fulfill the requirements for evolution of cooperation.

(1) Are sperm individuals?
Sperm have unique haploid genomes resulting from segregation and crossing over during meiosis that would seemingly provide the necessary heritable variance. The weight of empirical evidence, however, suggests that sperm genomic diversity is rarely reflected in sperm phenotypes due to male control of sperm form and sharing of post-meiotically expressed gene products (see Section IV.1 and 2). This conclusion may in part be due to a general lack of knowledge of haploid gene expression and technical hurdles required to demonstrate compartmentalization of transcripts and gene products within developing spermatids. Nonetheless, until evidence to the contrary exists, we contend that in general sperm are not individuals and sperm phenotype should be considered an expression of the male phenotype, similar to other ejaculatory traits such as seminal proteins.

(2) Is there haploid selection?
The meiotic drive genes and alleles are notable examples of haploid selection (reviewed in Immler, 2008; Lyon, 2003; Lyttle, 1991; Taylor & Ingvarsson, 2003). However, outside of meiotic drive, there is little evidence of sperm-level selection (Clark, Dermitzakis & Civetta, 2000; Pitnick, Dobler & Hosken, 2009a), the second requirement for the evolution of sperm cooperation. Sperm morphology generally exhibits rapid and dramatic evolutionary diversification (reviewed by Pitnick et al., 2009b). Selection on sperm form and function is known to be intense, largely as a consequence of
postcopulatory sexual selection (i.e., sperm competition and cryptic female choice; reviewed by Birkhead & Møller, 1998; Eberhard, 1996; Keller & Reeve, 1995; Pitnick et al., 2009b,c; Pizzari & Parker, 2009). The most widely investigated sperm attribute known to be subject to postcopulatory sexual selection is flagellum length (reviewed by Snook, 2005; Pitnick et al., 2009b; Pizzari & Parker, 2009). Using crosses between discrete laboratory populations that had been subjected to divergent artificial selection for sperm length (i.e., long-sperm and short-sperm populations: Dobler & Hosken, 2009; Miller & Pitnick, 2002), Pitnick et al. (2009a) demonstrated with both Drosophila melanogaster and Scathophaga stercoraria that haploid gene expression does not contribute to sperm length for these species. More generally, by analyzing segregation ratios of offspring of heterozygous males derived from chromosome-extracted lines of D. melanogaster representing a range of sperm-precedence phenotypes, Clark et al. (2000) demonstrated that sperm competition success depends on the diploid male genome and is not a property of the haploid sperm.

Moore et al. (2002) postulated that the pronounced apical hook on the sperm head of mice, which appears to have diversified in response to postcopulatory sexual selection (Immler et al., 2007; but see Firman & Simmons, 2009) and is critical to the formation of sperm trains (Moore et al., 2002), is a result of haploid gene expression. In support of this claim, some genes involved in the patterning of the hooked heads of mice have been shown to exhibit post-meiotic expression (Kim et al., 1989; Kleene, 2001; Xu et al., 1999). However, to establish if this trait might be subject to haploid selection it is necessary to demonstrate that post-meiotic gene products responsible for apical hooks are
not shared between syncytial spermatids and that hook morphology influences fertilization success.

(3) Are sperm heteromorphism and conjugation cooperation?

Three lines of evidence indicate that, in the majority of cases, sperm heteromorphism is male controlled. First, the influence of haploid gene expression on parasperm morphogenesis is precluded in those species in which parasperm lack nuclear DNA (e.g., apyrene sperm of Lepidoptera). Second, sperm heteromorphism occurs in the haploid males of *Dahlbominus fuscipennis* (Hymenoptera; Lee & Wilkes, 1965) where sperm are genetically identical to one another. Third, if sperm morphology were controlled by the haploid genotype of the sperm, morphological variation would be expected to occur within cysts rather than among cysts or testicular lobes. Instead, most of the observed intraspecific variation in sperm form is observed among males with relatively little within-male variation (reviewed by Pitnick *et al.*, 2009b). Production of alternative sperm morphs (i.e., parasperm) is controlled by circulating hormones (Lepidoptera, Friedländer, 1997; Jans *et al*., 1984; Kawamura *et al*., 2003) or is spatially isolated from that of eusperm (i.e., harlequin lobes in Hemiptera: Schrader, 1960b; separate cysts in Diptera: Beatty & Burgoyne, 1971).

Evidence similarly supports male control of primary conjugation. Males produce the extracellular material or sheaths that bind sperm together (Alberti, 2000; Dallai *et al*., 2002; Nur, 1962). Thus it seems likely that these remarkable traits have evolved through male-level selection and do not represent evolutionary cooperation, but rather the theoretically unproblematic, functional cooperation seen in multicellular organisms.
below the level of the individual. Sub-individual cooperation among cells is maintained by selection for organismal integrity via germ line sequestration (Buss, 1987), high levels of relatedness among cells (Maynard Smith & Szathmáry, 1995) and policing of selfish tendencies (Boyd & Richerson, 1992; Frank, 2003).

It is less clear whether the male or the sperm control secondary conjugation. It is conceivable that mature sperm might exert considerable influence over the “decision” to conjugate and the duration of the association. Whereas the cementing materials that bind conjugates together in the diving beetle *Dytiscus marginalis* are derived from cells surrounding the developing spermatids, in the related genus *Rhantus*, the cementing material appears to be derived from the sperm themselves (Werner, 1976, 1983). It is unknown, however, whether production of the material is controlled by the sperm haploid genome or if it results from shared or male-derived transcripts. Ejaculates from different males frequently overlap in female reproductive tracts of a promiscuous deer mouse, *Peromyscus maniculatus*, where copulations with different males can occur less than one minute apart (Dewsbury, 1985). Sperm conjugate after ejaculation and, *in vitro*, conjugated more frequently with sibling sperm than with the sperm of full-sibling littermates or of heterospecific males (Fisher & Hoekstra, 2010). This capacity for discrimination was not found in the monogamous sister species *P. polionotus*; their sperm conjugated indiscriminately with regard to the degree of relatedness (Fisher & Hoekstra, 2010). The mechanism that permits discrimination among sperm is unknown, but it seems probable that it is mediated through macromolecules on the sperm surface. If this is the case, the ‘self recognition’ may be sperm-specific or male-specific. Knowledge of the timing of gene expression and whether or not gene products are shared between
developing spermatids would determine the relative influence of the diploid male genotype or haploid sperm genotype on conjugation in *P. maniculatus*.

Sperm influence over secondary conjugation could result in conflict between male and sperm evolutionary interests (Immler, 2008; Pizzari & Foster, 2008). We expect that the level of male-sperm conflict, if it exists, will be inversely related to the intensity of sperm competition (Parker & Begon, 1993); when sperm competition is high, both male and individual sperm fitness may be maximized by cooperation. However from the sperm-level view, when there are few or no sperm from rival males, competition between sibling sperm becomes an increasingly important selective force and cooperation may become unfavourable. Competition between sibling sperm may reduce male reproductive fitness, even in monogamous systems, by reducing the number of fertilization-competent sperm per ejaculate (e.g., killing of Y-chromosome-bearing sperm in the case of sex chromosome meiotic drive) or by displacing sibling sperm from the site of storage or fertilization. Male-level selection for adaptations that reduce intra-ejaculate competition in favour of improved whole-ejaculate success aligns the interests of males and the sperm they produce. When competition between sibling sperm is restricted or prevented, individual sperm fitness can only be maximized by enhancing inter-ejaculate competitive success (Frank, 2003). We refer interested readers to two recent reviews for a more detailed discussion of sperm-level selection and male-sperm conflict (Immler, 2008; Pizzari & Foster, 2008).

Nonetheless, to date, no studies have investigated whether the haploid genotype of sperm influences the likelihood, or any other aspect, of conjugation. It is possible that ever-increasingly sophisticated molecular techniques will reveal a role of individual
sperm in controlling conjugation. However, in accordance with the preponderance of current evidence suggesting that sperm do not possess heritable variation in fitness, it is prudent to interpret secondary conjugation as a trait that has evolved via male-level selection and not an example of evolutionary cooperation.

VII. CONCLUSIONS

(1) Although rare, sperm heteromorphism and conjugation are both taxonomically widespread and have multiple, independent origins throughout Bilateria. Sperm heteromorphism and conjugation are largely restricted to internally fertilizing species, with sculpin fish and echinoderms providing exceptions. Additionally, heterospermatozeugmata - complex sperm conjugates composed of more than one sperm type - have evolved in multiple, phylogenetically distinct lineages, suggesting that sperm conjugation and heteromorphism might have complementary functions.

(2) Sperm conjugation may arise from spermatogenic mechanisms (primary) or from post-spermatogenic mechanisms (secondary). Secondary conjugation is expected to have greater evolutionary lability that permits formation of more complex conjugates (i.e., heterospermatozeugmata).

(3) A multitude of ultrastructure studies have provided detailed understanding of conjugate morphology, yet our understanding of the functional or evolutionary significance of this morphology is rudimentary. Many hypotheses for sperm conjugation have been advanced but few have been tested and none have convincing empirical support.
(4) Sperm heteromorphism and primary conjugation appear to be male controlled and, in the absence of evidence of sperm individuality, sperm heteromorphism and primary conjugation should not be interpreted as evolutionary cooperation but rather as male-selected traits. Secondary conjugation might be influenced by the sperm haploid genome, but no studies have examined haploid contribution to the conjugation phenotype. Barring evidence of sperm-level selection for conjugation, we suggest the use of the moniker ‘sperm cooperation’ to be restricted to discussions of functional interactions among sperm and be avoided when discussing the evolution of sperm phenotype.
VIII. ACKNOWLEDGEMENTS

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Table 1.1. **Summary of the different types of conjugation.** See text for references and more detailed explanations of terms.

<table>
<thead>
<tr>
<th>Type of conjugation</th>
<th>Examples</th>
<th>Description</th>
<th>Taxonomic distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>spermatodesm(-a, -ta)</td>
<td>sperm produced by a single spermatogonium remain grouped together, with the heads embedded in gelatinous material</td>
<td>fish, gymnolaemate bryozoan, insects, monotremes</td>
</tr>
<tr>
<td></td>
<td>synspermia</td>
<td>encapsulated, syncytial sperm</td>
<td>spiders</td>
</tr>
<tr>
<td>Secondary</td>
<td>pairs</td>
<td>spermatozoa form pairs in the seminal vesicles</td>
<td>American marsupials, insects, marine snails, millipedes</td>
</tr>
<tr>
<td></td>
<td>rouleaux</td>
<td>spermatozoa form orderly stacks</td>
<td>guinea pigs, insects, naked-tail armadillos, solifuges</td>
</tr>
<tr>
<td></td>
<td>bundles</td>
<td>mature spermatozoa are bound together by extracellular material</td>
<td>insects</td>
</tr>
<tr>
<td></td>
<td>trains</td>
<td>sperm cling or adhere to one another in a disorganized manner</td>
<td>muroid rodents</td>
</tr>
<tr>
<td></td>
<td>heterospermatozeugmata</td>
<td>heteromorphic sperm form conjugates</td>
<td>annelids, bivalves, gastropods, insects, polychaete and oligochaete worms, prosobranch snails</td>
</tr>
<tr>
<td></td>
<td>coenspermia</td>
<td>mature, coiled spermatozoa are encapsulated</td>
<td>spiders</td>
</tr>
</tbody>
</table>
Fig. 1.1. Primary conjugation. (A) Spermatodesm of the leaf beetle *Xanthogaleruca* sp. Merged interference contrast and fluorescence image of Hoechst-stained nuclei provided by R. Dallai. (B) Scanning electron micrograph (SEM) of a spermatodesm from the sawfly *Arge pagana*. After Lino-Neto *et al.* (2008). (C) Transmission electron micrograph (TEM) and (D) darkfield microscopy of spermatodesm of the katydid *Platycleis intermedia*. From Viscuso *et al.* (1998). (E) Interference contrast and (F) SEM of spermatostyles of the whirligig beetle *Dineutus assimilis*. Images by D. M. Higginson and B. A. Byrnes. (G) Interference contrast image of the spermatodesm of the crawling water beetle *Haliplus immaculollis*. Image by D. M. Higginson. Note: images are not to scale. a = acrosome; f = flagella; m = median axis; n = nuclei.

Fig. 1.2. Secondary conjugation. (A) Sperm bundles of the fishfly *Parachauliodes japonicus*. Note the extreme variation in conjugate size. Image by F. Hayashi. (B) Phase contrast image of the paired spermatozoa of the opossum *Monodelphis domestica*. Image provided by H. D. M. Moore. A longitudinal transmission electron micrograph (TEM) section through the paired heads (C) highlights the asymmetry of heads and the angle of flagellum attachment. After Moore (1996). (D) Scanning electron micrograph (SEM) of the sperm head of the wood mouse *Apodemus sylvaticus* showing the large apical hook. Upon ejaculation, the apical hook opens facilitating formation of large sperm trains (E). After Moore *et al.* (2002). (F) Interference contrast image showing tight association of sperm heads a conjugate of the diving beetle *Rhantus consimilis*. Image by D. M. Higginson. (G) Fluorescence image of Hoechst-stained nuclei of a large rouleaux in the diving beetle *Hydroporus* sp. Each annulation represents the transition between
one sperm head and the next. Sperm tails are faintly visible in the background. Image by D. M. Higginson. (H) Interference contrast image of sperm attached to a hyaline rope in the leafhopper *Ledra auditura*. Image by F. Hayashi. (I) Darkfield microscopy of the paired sperm of the diving beetle *Graphoderus liberus*. Image by D. M. Higginson. (J) Interference contrast image of sperm attached to a hyaline rod in the spittlebug *Aphrophora major*. Image by F. Hayashi. (K) Darkfield microscopy of two rouleaux in the diving beetle *Neoporus dimidiatus*. Each conjugate is composed of eight sperm. Image by D. M. Higginson. (L) TEM longitudinal section through a rouleaux in the solifugid *Eusimonia* sp. nov. Sperm are plate-shaped and arranged as stacked pairs that can be found in both the testes and vas deferens. While the presence of rouleaux in the testes casts doubt on whether conjugation is secondary, we have classified it as such based on three lines of evidence. First, no stages of spermatogenesis have been observed in adults, suggesting that sperm maturation occurs before males reach reproductive maturity. Second, the pairing of sperm within the stack and third, the highly variable number of sperm conjugates is consistent with secondary conjugation. Image by A. E. Klann. Note: images are not to scale.

**Fig. 1.3. Heterospermatozeugmata.** (A, B) Fluorescence image of Hoechst-stained sperm nuclei of the diving beetle *Hygrotus sayi*. (A) Mature, individualized sperm as they exit the testis. Two sperm morphs are clearly visible, one with filamentous-shaped nuclei and a second with cone-shaped nuclei. (B) After passage through the deferent ducts, both sperm morphs interact to form a large conjugate composed of thousands of sperm. The tip of a cone-shaped sperm head slips into the concave portion of another creating the
“backbone” of the conjugate to which the filamentous-headed sperm attach. No flagella are visible. Images by D. M. Higginson. (C) Interference contrast image of a heterospermatozeugmata of the marine snail *Fusitriton oregonensis*. Approximately fifty eusperm attach to a worm-shaped parasperm. This species also produces a second, lancet-shaped parasperm (not shown). Neither of the parasperm morphs contain chromatin. After Buckland-Nicks *et al.* (1982). (D) Scanning electron micrograph (SEM) of the heterospermatozeugmata of the oligochaete worm *Tubifex tubifex*. Parasperm are helically arranged to form a hollow cylinder that is filled with loose eusperm. The pictured conjugate has been broken to reveal the inner cortex of eusperm. After Ferraguti *et al.* (1988). (E) Fluorescence image of Hoechst-stained nuclei of a partially dissociated conjugate recovered from the sperm storage organ of a female diving beetle, *Hygrotus picatus*. (F) Intact conjugate of *H. picatus*. Highly motile short sperm conjugate with a second, weakly motile and much longer sperm type. Images by D. M. Higginson. Note: images not to scale. eu = eusperm; p = parasperm; s = short morph sperm; * = long morph sperm.
Convergence, recurrence and diversification of complex sperm traits in a family of aquatic beetles

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\textbf{Author contributions:} D.M.H. and S.P. conceived the project. D.M.H gathered morphological data and performed the analyses. K.B.M. collected and provided many specimens. D.M.H. conducted the phylogenetic inference with assistance from K.A.S. D.M.H and S.P. wrote the paper.
Sperm form is predicted to influence male reproductive success when competing with the sperm of rival males and female reproductive tract architecture may bias fertilization in favor of particular sperm morphologies, yet patterns of sperm diversification have been scarcely examined. Here, we investigate sperm evolution in diving beetles (Dytiscidae), revealing dramatic diversification in flagellum length, head shape, presence of sperm heteromorphism in both length and head shape, and the presence and type of sperm conjugation, an unusual trait where two or more sperm unite for motility or transport through the female reproductive tract. We use Bayesian estimation to infer ancestral sperm traits and identify both the rate and directionality of probable evolutionary transitions in sperm form while accounting for phylogenetic uncertainty. Sperm conjugation was found to be the ancestral condition in diving beetles, with subsequent diversification into three qualitatively unique forms (aggregates, pairs and rouleaux), each exhibiting varying degrees of evolutionary loss and convergence. Sperm head shape was found to evolve in a significantly correlated manner with conjugation, consistent with statistical support for non-random patterns of evolutionary transition between the different forms of conjugation, which entail different mechanisms for precise head alignment and binding. Analyses suggest that both sperm length and sperm heteromorphism have independent evolutionary trajectories from sperm conjugation. Our study reveals that sperm morphological evolution is channelled along particular evolutionary pathways (i.e., conjugate form) yet subject to considerable diversification within those pathways through modification in sperm length, head shape and heteromorphism.
The description of the sperm from thousands of species have been driven by biologists’ fascination with their unusual ecology of sperm, being cast from the soma into a foreign environment to function independently of the males that produced them, as well as their central role in sexual reproduction (1-12). Comparative studies of sperm form have focused on one of two goals: (i) resolution of phylogenetic relationships or (ii) testing correlations between sperm form (i.e., total sperm length (13) or head shape (14)) and the intensity of selection (i.e., risk of sperm competition or some critical dimension of the female reproductive tract (13, 15, 16)). Collectively, these studies have generated three well-supported, conclusions about sperm evolution. First, sperm are the most diverse cell type, exhibiting dramatic morphological modifications in nearly all taxa, yet are subject to evolutionary constraints (13). Specifically, some aspects of sperm morphology evolve slowly enough to provide useful phylogenetic characters for deep branches of ancient radiations (e.g., axoneme structure of insects (8)), whereas other aspects often diverge rapidly and differ among closely related species (13). Second, postcopulatory sexual selection is a potent force underlying sperm diversification with both sperm competition and cryptic female choice shaping unique aspects of sperm morphology (13, 16). Third, for internally fertilizing species, sperm length exhibits a pattern of correlated evolution with features of female reproductive tract design (15).

Despite the massive research effort describing sperm ultrastructure (1-12), we have limited understanding of why and how sperm evolve specific morphologies. For example, although correlated evolution of sperm length and female reproductive tract architecture is found in a wide variety of taxa (15), there have been few investigations of how sperm function within the female reproductive tract (e.g., (17-20)), resulting in the
adaptive significance of variation in sperm form being largely unknown. Also, evolution of sperm form *per se* has scarcely been investigated (21, 22), resulting in a meager understanding of the origins of novel sperm forms, subsequent diversification, and evolutionary loss of derived traits. Here, we amend this latter deficiency by conducting detailed analyses to infer patterns of sperm evolution within a large radiation of aquatic beetles (ca. 4000 species worldwide (23, 24)) exhibiting dramatic, multivariate diversity in sperm form.

The sperm of diving beetles (Dytiscidae) attracted attention over a century ago due to an unusual variant in morphology: conjugation, where two or more spermatozoa join together at the head for motility or transport through the female reproductive tract (25, 26). Sperm conjugation is rare but has several independent origins across Metazoa, occurring in relatively few species of marsupials, eutherian mammals, gastropods, annelids, myriapods and some insects (27, 28). This remarkable variation in sperm form has been of considerable interest to evolutionary biologists due to its potential role in sperm competition (14, 29), implications for cooperation and cheating (29, 30) and the possibility of haploid-diploid conflict between males and the sperm they produce (27, 28, 31). Additionally, some species of diving beetles produce two distinct sperm morphs that vary in total length or head shape. Such sperm heteromorphism sometimes co-occurs with conjugation resulting in some of the most diverse and extravagant sperm forms observed in nature.

Here, we use Bayesian analyses that deploy reversible-jump Markov Chain Monte Carlo (rj-MCMC), which explores models of evolution that vary in parameter
number and precise values of the parameters, to infer the sequence of diversification in conjugate form and examine patterns of convergence, recurrence and loss of conjugation and sperm heteromorphism. We also test hypotheses of correlated evolution among conjugation, head shape, sperm length and heteromorphism. Head shape might influence the propensity of sperm to conjugate. Flattened sperm heads might contribute to the structural stability of a conjugate by providing increased surface area for hydrophobic, surface-protein or glycocalyx interactions. Similarly, a reduction in sperm head width may contribute to an evolutionary loss of conjugation. If this were true, sperm conjugation and head shape would be predicted to evolve in a correlated manner. Total sperm length might also be expected to show correlated evolution with conjugation. Conjugation might provide a mechanism for increased sperm velocity by combining the force generated by multiple flagella (32) without the energetic costs of producing long sperm (i.e., delayed maturation (33) and/or increased investment in testes (34)). Both theoretical and empirical studies indicate a positive relationship between sperm length and swimming speed (35, 36). Thus, if selection favored increased sperm velocity, short sperm would be predicted to evolve in the presence of conjugation and vice versa. Finally, although not proven in diving beetles, in sperm heteromorphic species, typically only one sperm type is ever genetically functional in egg fertilization (37). If conjugates are composed of fertile and non-fertile sperm they can be viewed as analogous to differentiated cell clusters with somatic and reproductive cells. Theory developed to explain the origins of multicellularity postulates that formation of simple cell aggregates occurs prior to the specialization of cell function (38-41); thus, one might predict that
conjugation to evolve before sperm heteromorphism in lineages where the two traits co-occur.

Results

General patterns of sperm morphological diversity. Diving beetles were found to have undergone extensive and multivariate diversification in sperm form, including variation in sperm length (128 to approximately 4450 µm), head shape (Fig. 2.1A – F) and the presence of sperm heteromorphism (production of two distinct sperm morphs that differ in total length and/or head shape; Table S2.1; Fig. 2.1A, E, F). In addition, sperm were found to be either single or conjugated in one of three forms: i) sperm aggregates composed of variable numbers of sperm with their heads aligned in register (Fig. 2.1A, 2A), ii) pairs with two sperm aligned anti-parallel to each other (Fig. 2.1D, 2.2B), or iii) rouleaux, where the tip of one sperm head slips into the hollow, hooded portion of another to form orderly stacks that may be composed of a just a few to several hundred sperm (Fig. 2.1B – C, F, 2.2D). Sperm heteromorphism and conjugation were found to sometimes co-occur, resulting in complex conjugates called heterospermatozeugmata (e.g., Fig. 2.1A, E, F (13, 27)).

Transitions among types of conjugation. The reconstructions of ancestral character states suggest sperm conjugation was present in the ancestor of diving beetles and there has been a minimum of four subsequent losses distributed across the tree (Fig. 2.2). Sperm aggregates are strongly supported as the ancestral condition for Dytiscidae (models where the ancestral state is constrained to aggregates have greater likelihood than those constrained to have single sperm, paired conjugates, or sperm rouleaux (Bayes
Factor > 5). Additionally, both sperm pairing and rouleaux are identified as derived forms of conjugation originating from sperm aggregates (Fig. 2.2; Table S2.2). The distribution of sperm pairing in diving beetles suggests that it has evolved twice. To test this hypothesis, we used the most recent common ancestor to reconstruct an internal node that minimally contained all of the species showing sperm pairing. We then compared the harmonic means of the likelihood of our models of evolution when this internal node was constrained to sperm pairing, indicating a single origin, or to aggregates, permitting multiple origins of pairing. Two origins of pairing was strongly supported (Bayes Factor > 5). Additionally, reversions from rouleaux to the ancestral aggregates were (i) identified as probable evolutionary transitions by our rj-MCMC and (ii) observed nested well within the main lineage exhibiting rouleaux indicating that the apparent reversions are not likely to be the result of incomplete lineage sorting. Loss of conjugation was observed both from lineages with paired sperm and from those with rouleaux, but only the transition from rouleaux to single sperm was identified as probable in our rj-MCMC analysis (see Fig. 2.2; Table S2.2).

**Correlated evolution between conjugation and head shape.** We used rj-MCMC to test whether the rates of (i) gains or losses in conjugation and (ii) changes in head shape differed depending on the character state of the other trait. Sperm heads were classified as elongate (approximately the same width as the flagellum) or broad (substantively wider than the flagellum). Conjugation was characterized as present or absent. We found very strong support for correlated evolution between head shape and presence of conjugation (Bayes Factor > 10). Ancestral state reconstructions support the presence of conjugation and broad heads in the ancestor of diving beetles (Bayes Factor > 5). Examination of the transition rates indicates that evolution away from the ancestral state is likely to occur either as the loss of conjugation or the elongation of the sperm
heads (Fig. 2.3; Table S2.3). However, elongation of sperm heads was unlikely to result in a subsequent loss of conjugation. Interestingly, the presence of broad-head sperm in the absence of conjugation was suggested to be an evolutionary unstable condition, indicated by the very high transition rates away from this condition, a conclusion that is supported by there only being a single observation of this character state combination among all 141 species examined (see Fig. 2.2 lower left panel; Table S2.1).

**Relationship between conjugation and other sperm traits.** To test for correlated evolution between sperm length and conjugation, we classified sperm as long or short based on the bimodal distribution of the sperm length data (Fig 2.2; Table S2.1) and determined if transition rates from long to short sperm were different depending on whether conjugation was present or vice versa. We found that the dependent and independent models of trait evolution fit the data equally well (i.e., three runs of each model type resulted in overlapping harmonic means).

The co-occurrence of heteromorphism and conjugation in species (e.g., 2.1A, E – F) prompted testing for correlated evolution between heteromorphism and conjugation. Ancestral state reconstruction supports a minimum of five origins of heterospermatozeugmata within Dytiscidae. We compared the likelihood of competing models of evolution (i.e., independent or correlated) for conjugation and heteromorphism and found that despite multiple co-occurrences, heteromorphism and conjugation most likely evolve independently from each other (Bayes Factor = 2).
Discussion

Despite long standing recognition that sperm vary not only in total length but also in the presence and organization of their constituent parts (acrosome, nucleus, mitochondria and flagellum), we have only a meagre insight in the evolution of sperm characters. Particularly poorly understood is how sperm morphology can transition between discrete, alternative, character states and how selection on one aspect of sperm morphology may results in correlated changes in other sperm traits. Our case study sperm evolution in diving beetles revealed that sperm morphology readily switches between three discrete forms of conjugation (aggregates, pairs and rouleaux), although not all transitions are equally likely. Additionally, we found that head shape, but not dimorphism or sperm length, showed correlated evolution with conjugation. None-the-less, functional interactions between the presence of conjugation, sperm length and dimorphism are expected to impact how sperm move through their environment and are perceived by females.

Within the diving beetle lineage there has been dramatic and multivariate diversification of sperm morphology. Sperm conjugation was found to be the ancestral condition in diving beetles that has subsequently been lost (i.e., reverting to single sperm) a minimum of four times throughout the lineage. We were able to differentiate between three qualitatively different forms of sperm conjugation: aggregates, pairs and rouleaux. Adding to the complexity of sperm phenotypes, among species exhibiting aggregate- or rouleaux-type conjugates, we found substantive variation in sperm phenotypes including the number of sperm per conjugate, flagellum length, head shape, and the presence of sperm heteromorphism.

We found strong support for convergent evolution of sperm pairing, but only a single origin of rouleaux formation among diving beetles (with a recurrence event within
Hygrotini). There were also two independent recurrences of the aggregate sperm conjugate state within the clade of rouleaux-producing species. Convergence and recurrence implicate similar selective environments and evolution via regulatory changes in developmental pathways (42). Given that similar phenotypes do not necessarily result from similar genetic mechanisms, we do not attempt to distinguish convergence from parallel evolution (43, 44). Notably, convergence of sperm form is also seen among related families of beetles. Conjugation is found in whirligig beetles (Gyrinidae), crawling water beetles (Haliplidae), burrowing water beetles (Noteridae) and the more distantly related ground beetles (Carabidae) (27). Sperm morphology of burrowing water beetles has scarcely been examined, but both sperm pairing and rouleaux have been found (D.M.H., unpublished data). With the exception of ground beetles, female reproductive tracts in these families characteristically form conduits, with sperm entering and exiting storage through separate ducts (as opposed to cul-de-sac type reproductive tracts where sperm enter and exit through a common duct) (45). Perhaps the similar selective environments experienced by sperm in these families might explain the repeated evolution of such an unusual variation in sperm morphology as conjugation (46).

Sperm head shape was found to evolve in a significantly correlated manner with conjugation, as predicted, given that conjugation involves the conjoining of sperm heads in a manner that must accommodate functionality of the resulting multiflagellated sperm unit (27). Consistent with this result, our analysis of evolutionary transition rates between different forms of conjugation revealed non-random evolutionary trajectories (Fig. 2.2; Table S2.2). Specifically, paired and rouleaux conjugates, which require different mechanisms for precise head alignment and binding (Fig. 2.1B,D), appear never to derive from one another or from single sperm, but rather only from the aggregate condition, where variable numbers of sperm conjugate with their heads less precisely aligned. Heterochronic evolution of the timing of conjugate formation provides one possible
explanation for the observed pattern of rouleaux derived from sperm aggregates. Post-
insemination sperm transformations (e.g., capacitation) are widespread in other internally
fertilizing animals (15). Examination of conjugates collected from males and the sperm
storage organs of females indicates that the conjugates of some species undergo
morphological transformation during or after transfer to females. For example, the sperm
of *Rhantus* spp. (Colymbetini) form typical aggregates, with the heads aligned, within the
seminal vesicles of males, but after transfer to females the conjugates elongate and appear
reminiscent of rouleaux (Fig. 2.1E). Broadly, changes in the timing of ontogenetic
processes leading to conjugation might give rise to the different forms of conjugation,
with the post-ejaculation transformations observed in *Rhantus* occurring within the
seminal vesicles of rouleaux-producing species.

Given motility-related functional relationships between conjugated sperm (see
Movies S2.1 – 3 (14, 27, 29, 36, 47)), the lack of significant correlated evolution between
total sperm length and conjugation (i.e., models of independent and dependent evolution
were equally well supported) was surprising. It is possible that our simplistic
categorization of sperm as long or short may have obscured more subtle relationships.
On the other hand, results of recent analyses of co-evolution of sperm and female
reproductive tract morphology in diving beetles suggest that conjugation is selectively
advantageous because it enhances the probability of occupying a location favorable for
(competitive) fertilization rather than due to any motility advantage (46). Sperm length
and/or head shape heteromorphism, which is also taxonomically widespread but
relatively uncommon among metazoans (14, 49, 65), was also discovered to have evolved
numerous times within the Dytiscidae. Although our results suggest that heteromorphism
and conjugation have independent evolutionary trajectories in diving beetles, their
frequent (and numerous independently derived) co-occurrence has given rise to some of
the most diverse and extravagant sperm forms observed. These heterospermatozeugmata
all exhibit uniquely organized structures (Fig. 2.1A, E – F) that appear to dramatically alter the manner of sperm movement (Movies S2.1 – 3).

Because phylogenetic analyses of sperm trait evolution are rare (e.g., (20, 21)), it is not possible to assess whether the dynamic nature of sperm evolution in the Dytiscidae is unusual. Recognizing, however, that the female reproductive tract is the selective environment for sperm of internally fertilizing species (15), we note that female reproductive tracts are remarkably variable among diving beetles (45). With the exception of the fertilization duct, any of the main features (e.g., the spermathecal duct, spermatheca or receptacle) may be absent or highly elaborated, with dimensions of every component of the female tract varying substantively among species (45, 46). Moreover, as is the case for most species, these beetles store sperm after copulation for prolonged periods (15), and polyandry with ensuing postcopulatory sexual selection (i.e., sperm competition and cryptic female choice) is believed to be widespread (48). Hence, sperm form in this lineage appears to be subject to intense, diversifying selection. Within Dytiscidae, dimensions of the female sperm-storage organ and fertilization duct are correlated with the presence of conjugation, and changes in female morphology appear to drive the evolution of numerous aspects of sperm form (46). What is now sorely needed, both in diving beetles and other taxa, are in vivo investigations of sperm behavior and fate relative to variation in sperm form.

**Materials and Methods**

**Sperm characters.** Freshly euthanized, Kahle’s medium- or alcohol-preserved male beetles were dissected in phosphate-buffered saline and their sperm harvested from the seminal vesicles. The sperm were dried on a subbed microscope slide, fixed, and DNA stained (Hoechst’s or DAPI). To confirm the presence or absence of conjugation, sperm
found in the female sperm-storage organs were also examined when female specimens were available (the majority of species). Sperm were visualized and imaged using darkfield and epifluorescence microscopy.

A species was classified as lacking sperm conjugation if there was no evidence of physical association among sperm in the samples. Species were considered to have aggregate-type conjugation when variable numbers of sperm per unit were aligned with their heads in register within the seminal vesicles of males. Sperm pairing was classified as when two sperm aligned with their heads oriented anti-parallel to each other. Rouleaux were classified as the orderly stacking of sperm, where the tip of one sperm head slips into the hooded portion of another (27), regardless of the number of sperm involved (e.g., two in Bidessonotus inconspicuous, dozens in Neoporus undulatus and hundreds in Hydroporus sp.). Typically, some single sperm were also present in the seminal vesicles of males with aggregated or paired sperm. In contrast, single sperm were never observed within the seminal vesicles of males that produced rouleaux.

Sperm length was measured from digitized images using Image J (49). In some instances, mature individualized sperm could not be obtained from a specimen. To obtain a minimum estimate of sperm length from these specimens, we measured the total length of mature sperm bundles (i.e., sperm had taken on their mature head shape but had not yet individualized). A species was considered to be sperm heteromorphic when two distinct (i.e., non-overlapping) sperm lengths or head shapes were produced by a single male (see Fig. 2.1A, E, F). One to nine individuals per species (mean = 3) were examined (Table S2.1). Sperm traits (i.e., presence and type of conjugation, head shape, presence of heteromorphism, and sperm length) were largely consistent within genera (see Table S2.1 and SOM for additional information). Where species did not overlap between available sequence data and morphological data (most cases), sperm characters were mapped to the phylogenetic tree by assigning values to genera or subgenera (e.g., subgenera for Agabus
and *Ilybius* where there has been recent and considerable taxonomic flux (23, 50); data matrix available on TreeBASE). When there was observed variation in these characters within a genus, the data were coded to reflect all observed character types (e.g., both elongate and broad sperm heads present).

**Phylogenetic trees.** To provide trees upon which to test models of character evolution, we used the large sequence data set compiled by Ribera et al. (accession numbers listed in Appendix 1 of (51)). The data set is composed of two mitochondrial genes (COI and 16s) and two nuclear genes (H3 and 18s) with excellent taxonomic sampling that includes 222 diving beetle species, 25 of 26 tribes and 116 of 174 known genera. We truncated the 18s sequences at the 5’ end to reduce the amount of missing sequence data. Sequences were aligned with the PRANK$_{+F}$ algorithm (52), which avoids over-penalization of insertion and deletion events common among distantly related sequences such as those in this study. To assess the quality of our alignments, we used the heads-or-tails (HoT) methodology that compares alignments that differ in the directionality that the sequences entered the alignment algorithm (i.e., a “heads” sequences is entered 5’ to 3’ whereas “tails” represents the identical sequence entered in the 3’ to 5’ direction) (53). If sequence alignments are unambiguous, both heads and tails alignments provide identical results (53). PRANK$_{+F}$ produced heads and tails alignments with 93.6 to 99.9% (16s and H3 respectively) identical residues within the aligned genes and resulted in phylogenetic trees that did not differ substantively (alignments available upon request). Appropriate models of sequence evolution for each of the four genes were determined using DT-ModSel (54). We performed Bayesian analyses on 2696 aligned base pairs, partitioned by gene, using MrBAYES (55). To encourage convergence of the MCMC chains, we provided a starting tree produced using the neighbour-joining method in PAUP (56). The starting tree was randomly perturbated four times prior to the starting of the chains. Four
separate MCMC runs of $4 \times 10^7$ generations were performed using uninformative priors (i.e., MrBAYES default prior values (55)), six chains per run with 0.15 heating. Convergence of the runs was assessed using AWTY (57) and the first $3 \times 10^7$ generations were discarded as burnin. After the burnin period, the MCMC chains visit alternative phylogenetic trees in proportion to their probability of being true given the model, priors and data. Despite using different alignment procedures and MCMC conditions, the phylogenetic analyses produced a majority consensus tree highly similar to that of Ribera et al. (51).

**Comparative analyses.** The strength of conclusions that can be drawn from comparative analyses is influenced by both the confidence in the evolutionary relationships among the species and taxonomic sampling. Diving beetles are part of an ancient radiation of aquatic beetles (58) and resolving phylogenetic relationships among tribes and subfamilies has been problematic (45, 51, 59). Although we used the best available data for phylogenetic inference, relationships among the basal branches of the tree remain uncertain (51). We deployed Bayesian MCMC methods to account for uncertainty in both the phylogenetic relationships and ancestral states (60, 61).

All analyses were conducted with BayesTraits using reversible-jump MCMC (60-62) analyses and 1000 post-burnin trees (described above). To explore transitions between different states of conjugation (absent, aggregate, paired or rouleaux) and to infer probable evolutionary transitions, we used the program MULTISTATE in BayesTraits (62, 63), a hyperprior with a uniform distribution of 0 – 100 to seed the mean of our exponential rate prior. At each generation, a new model of character evolution is proposed by altering the pre-existing transition rate values by a rate deviation value of 0.5 (resulting in the recommended value of approximately 20% of proposals being accepted (60)). The rj-MCMC chain was run for 5,050,000 iterations with the first 50,000 iterations discarded as burnin. We reconstructed ancestral character states using the most
recent common ancestor approach, which accounts for uncertainty associated both with the phylogeny and the character reconstruction (60). To test support for a given character state, the model of evolution was constrained to the state of interest at the node and the resulting marginal likelihood of the model (as approximated by the harmonic mean) compared to that of when the model is constrained to take alternative character states using Bayes Factors. Each run was repeated three times to check stability of the harmonic means. To test for correlated evolution between conjugation and sperm head shape, presence of heteromorphism or sperm length we used the program DISCRETE (61, 62, 64) and the conditions described above (with the exception of the rate deviation that was increased or decreased to obtain appropriate acceptance rates). Using Bayes Factors we compared the likelihoods of models where the traits were free to evolve independently to those where they were constrained to evolve in a correlated manner (61).
Acknowledgements. We are grateful to Ignacio Ribera for making available the remarkable set of DNA sequence data set for Dytiscidae and the IBEST Bioinformatics Core at the University of Idaho for providing the computing resources for phylogenetic tree inference (NIH/NCRR P20RR16448 and P20RR-16454). This research was supported by the National Science Foundation (DDIG-0910049 to D.M.H. and S.P.; DEB-0743101 to K.A.S; DEB-0814732 and DEB-6990357 to S.P.) and the Natural Sciences and Engineering Research Council (PGS-D 331458 to D.M.H.).


46. Higginson DM, Miller KB, Segraves KA, & Pitnick S (Female reproductive tract form drives the evolution of complex sperm morphology. *submitted.*


**Fig. 1. Head shape and conjugation in diving beetles.** (A) Elongate heads of *Ilybius oblitus* form sperm aggregates and surround second sperm morph with broad heads (faintly visible as region of higher intensity fluorescence). (B) The cone-shaped heads of *Neoporus undulatus* stack together with the tip of one sperm head slips into the pocket at the base of another to form orderly stacks. In cross-section, rouleaux have an onion-like appearance with the sperm heads forming concentric circles (seven sperm heads are visible in each of the conjugates). (C) Fluorescent image of a *N. undulatus* rouleau. Large basal spurs of each sperm head are clearly visible projecting along one edge of the conjugate. (D) Composite darkfield and fluorescence image of the broad, flat sperm heads of *Hydaticus bimarginatus*. In the seminal vesicles of males, the sperm heads align anti-parallel to each other and conjugate to form pairs (far right panel). (E) Heterospermatozeugmata in *Ilybius larsoni*. Within the seminal vesicles of males elongate headed sperm (indicated by *) slip into the pocket of an individual broad headed sperm to form sperm aggregates. During or after transfer to the female, some of the broad headed sperm associate with each other becoming highly structurally and functionally (see Movie S1) reminiscent of rouleaux. (F) A heterospermatozeugmata of *Derovatellus peruanus*. Broad heads form a rouleaux with an overall helical shape. A second sperm morph, with very elongate heads, is attached to the tip of the rouleaux (indicated by *). Broadly triangular and elongate head shapes are common in diving beetles. Fluorescent images of DAPI or Hoechst’s stained sperm heads (A, C – F); transmission
electron micrograph (B) and darkfield image in the left panel of (D). Flagella not visible in the fluorescent images. An asterisk indicates the elongate sperm morph in sperm heteromorphic species. Scale bars A, C – F = 10 µm, B = 1 µm.

Fig. 2. Transitions in sperm conjugation in diving beetles. (A) Aggregate-type conjugation in Platambus semivittatus. Aggregates form the ancestral condition in Dytiscidae. (B) Paired sperm of Thermonectus marmoratus. (C) Single sperm of Pachydrus princeps. (D) A rouleaux of Neoporus undulatus. Arrows indicate probable evolutionary transitions (i.e., rate is rarely assigned to zero; Z% < 5). All other possible transitions among conjugate types and single sperm were much less probable than those indicated by arrows (i.e., they were absent from 30 to 84% of the models produced by the MCMC chain; see Table S2 for mean transition rates). Sperm characters are mapped to the majority consensus tree with corresponding colors (grey indicates missing data). Two-color dashed lines indicate that both character states are present. Pie charts indicate the probability of the character states of the basal nodes. Stars indicate origins of sperm heteromorphism. For taxa and node posterior probabilities, see Fig. S1. Darkfield images; scale bars = 10 µm.

Fig. 3. Evolutionary transitions in sperm head shape and conjugation.
Transition rates (q = changes per unit branch length) that are rarely assigned to zero (z < 5% of models of trait evolution) are considered probable events (shown
in green; unlikely transitions in grey). The upper left panel represents the ancestral state of broad heads and conjugation (illustrated by *Rhantus consimilis*). Transitions away from the ancestral state through a change in head shape or loss of conjugation occur at similar rates. The upper right panel is an example of the co-occurrence of elongate heads and conjugation (*Pachydrus* sp.). Single sperm may have broad (lower left; *Porhydrus* sp.) or elongate heads (lower right; *Desmopachria convexa*). Transition rates away from broad heads in the absence of conjugation are very high, suggesting that this is an evolutionarily unstable state. Upper panels and lower left: fluorescent images of DNA-stained heads, no flagella visible. Lower right panel: darkfield image, head not visually distinguishable from flagellum. Scale bars = 10 μm.
Supplementary Methods

Sperm characters. There is some uncertainty to the type of conjugation present in *Hygrobia*. We were only able to obtain sperm from females and whereas conjugation was unambiguously present, we could not distinguish between aggregates and rouleaux (see Discussion). Given the position of *Hygrobia* in the phylogeny, we chose to conservatively interpret the conjugation as aggregates. However, we explored the impact of *Hygrobia*’s type of conjugation on our models of trait evolution and found no substantive difference in transition rates or likelihood when *Hygrobia* was considered to have aggregates or rouleaux.

Although no losses of conjugation were observed in the main lineage with sperm aggregates, we would be unsurprised if additional sampling within Agabini revealed species with single sperm. Large numbers of single sperm were often observed within the seminal vesicles, potentially providing phenotypic variation that could be subject to direction selection in some populations. By comparison, single sperm are rarely observed in species with sperm pairs or rouleaux. Additionally, Ballowitz (1) examined the sperm of *Agabus* sp. and *Ilybius* sp. and did not observe conjugation but was unwilling to exclude the possibility of conjugation being present. Despite considerable collecting effort, we were unable to obtain mature sperm from Cybistrini and Laccornini. Future sampling of these tribes would be of interest as they occupy a region of the tree that is poorly sampled for sperm characters.
Table S2.1. Sperm traits in Dytiscidae, Amphizoidae and Paelobiidae. Whenever specimens were available, we examined sperm from both the seminal vesicles of males and the female sperm storage organs. Conjugation was classified as i) aggregates, where variable numbers of sperm group with their heads aligned, ii) pairing, where strictly two sperm align in an anti-parallel manner, and iii) rouleaux, where the tip of one sperm head slips into the pocket at the base of another to form orderly stacks that vary from just a few to several hundred of sperm. Head shape was categorized as broad or elongate (i.e., substantially wider than the flagellum or of similar width as the flagellum respectively; long morph only, see below). To determine sperm length, we measured five sperm per individual. We present species mean length (mean of n male means, long morph if sperm dimorphic). Sperm were classified as long or short (see Fig. S2.2). Males of some species produce two distinct types of sperm that differ in total length and/or head shape. Both sperm morphs are transferred to females, but based on findings in other taxa (2-4), only the long morph is expected to participate in fertilization. When dimorphism is present, we additionally provide mean sperm length of the short morph sperm. Given the sparsity of species with dimorphism we made no attempt to characterize trait evolution of short morph sperm. In some instances, mature intact sperm were unavailable. To provide a minimum sperm length measure for the species, we measured mature sperm bundles (i.e., DNA had condensed and the nuclei elongated to take on their mature form; indicated by *) found within the testes. All sperm lengths are in millimeters and “?” indicates uncertainty if conjugation is present in a species.
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<th>head shape</th>
<th>mean length (SE)</th>
<th>Long sperm?</th>
<th>Dimorphism?</th>
<th>Short morph mean length (SE)</th>
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<td>N Y</td>
<td>0.1286</td>
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<td>0.378 (0.009)</td>
<td>N Y</td>
<td>0.184 (0.0001)</td>
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<td>broad</td>
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<td>Y N</td>
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<td>1.159*</td>
<td>Y N</td>
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<td>broad</td>
<td>0.853*</td>
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<td></td>
<td>N</td>
<td>(7)</td>
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<td>Necterosoma sp. 1</td>
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<td>rouleaux</td>
<td>elongate</td>
<td>0.822*</td>
<td>Y N</td>
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<td>rouleaux</td>
<td>elongate</td>
<td>0.704*</td>
<td>Y N</td>
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<td>Y N</td>
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<td>broad</td>
<td>1.665*</td>
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<td>broad</td>
<td>1.352 (0.038)</td>
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<td>rouleaux</td>
<td>broad</td>
<td>1.561*</td>
<td>Y N</td>
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<td>Neoporus superioris</td>
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<td>broad</td>
<td>1.164 (0.059)</td>
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<td>1.342 (0.020)</td>
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<td>broad</td>
<td>1.556*</td>
<td>Y N</td>
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<td>Neoporus dimidiatus</td>
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<td>broad</td>
<td>2.099 (0.023)</td>
<td>Y N</td>
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<tr>
<td>Oreodytes congrus</td>
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<td>broad</td>
<td>0.215 (0.005)</td>
<td>N N</td>
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<tr>
<td>Oreodytes obsesus</td>
<td>5</td>
<td>rouleaux</td>
<td>broad</td>
<td>0.252 (0.005)</td>
<td>N N</td>
<td></td>
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<tr>
<td>Oreodytes scitulus</td>
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<td>broad</td>
<td>0.406</td>
<td>N N</td>
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<td>broad</td>
<td>0.379 (0.011)</td>
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<td>aggregate</td>
<td>elongate</td>
<td></td>
<td>N</td>
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<td>elongate</td>
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<td>2.088*</td>
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<td>elongate</td>
<td>1.626*</td>
<td>Y N</td>
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<td>paired</td>
<td>broad</td>
<td>0.314</td>
<td>N N</td>
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<td>N</td>
<td>Sex</td>
<td>Body Shape</td>
<td>Postabdominal Gland</td>
<td>Tail Length</td>
<td>Measurement</td>
<td>Standard Deviation</td>
<td>Width</td>
<td>Comments</td>
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<td>Y</td>
<td>aggregate</td>
<td>broad</td>
<td>N</td>
<td>0.168 (0.002)</td>
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<td>aggregate</td>
<td>broad</td>
<td>N</td>
<td>0.149 (0.002)</td>
<td>N</td>
<td></td>
<td>this study</td>
</tr>
<tr>
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<td>N</td>
<td>0.317</td>
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<td>aggregate</td>
<td>broad</td>
<td>N</td>
<td>0.182 (0.003)</td>
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<td>aggregate</td>
<td>broad</td>
<td>N</td>
<td>0.155 (0.004)</td>
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<td></td>
<td>this study</td>
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<td>aggregate</td>
<td>broad</td>
<td>N</td>
<td>0.227 (0.001)</td>
<td>N</td>
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<td>Y</td>
<td>0.642</td>
<td>N</td>
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<td>this study</td>
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<td>rouleaux</td>
<td>broad</td>
<td>N</td>
<td></td>
<td>N</td>
<td></td>
<td>this study</td>
</tr>
<tr>
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<td>Y</td>
<td>rouleaux</td>
<td>broad</td>
<td>Y</td>
<td>0.539*</td>
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<td>broad</td>
<td>N</td>
<td></td>
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<td></td>
<td>this study</td>
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<tr>
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<td>N</td>
<td>0.521*</td>
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<td>N</td>
<td>0.472</td>
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<td>elongate</td>
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<td>0.202 (0.002)</td>
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<td>paired</td>
<td>broad</td>
<td>N</td>
<td>0.251 (0.001)</td>
<td>N</td>
<td></td>
<td>this study</td>
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<td>paired</td>
<td>elongate</td>
<td>N</td>
<td>0.288</td>
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<td>rouleaux</td>
<td>elongate</td>
<td>Y</td>
<td>1.716</td>
<td>N</td>
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</tr>
<tr>
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<td>rouleaux</td>
<td>broad</td>
<td>Y</td>
<td>1.091*</td>
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</table>
**Table S2.2.** Mean transition rate coefficients between the different forms of sperm conjugation. Transition rates (per unit branch length) and Z-values are based on 50,000 observations from 500,000 iterations from each of three independent reversible jump-MCMC runs. Z-values indicate the percentage of times that the transition rate was assigned to zero, removing the pathway from the model of trait evolution. Evolutionary transitions are considered probable when rarely assigned to zero (e.g., the transition from rouleaux to single sperm are much more probable than those to paired sperm). If the outgroup taxa of Amphizoidae and Asidytidae are included in the analysis, a transition from single sperm to aggregates becomes probable (0.31 ± 0.23, Z = 7.7%) but other transitions are only slightly affected.

<table>
<thead>
<tr>
<th>Evolutionary transition</th>
<th>mean rate</th>
<th>SD</th>
<th>Z%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single to paired</td>
<td>0.11</td>
<td>0.14</td>
<td>53.8</td>
</tr>
<tr>
<td>Single to aggregated</td>
<td>0.12</td>
<td>0.15</td>
<td>45.8</td>
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<td>Single to rouleaux</td>
<td>0.16</td>
<td>0.16</td>
<td>35.9</td>
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<tr>
<td>Paired to single</td>
<td>0.11</td>
<td>0.14</td>
<td>50.4</td>
</tr>
<tr>
<td>Paired to aggregated</td>
<td>0.14</td>
<td>0.15</td>
<td>42.6</td>
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<td>Paired to rouleaux</td>
<td>0.10</td>
<td>0.14</td>
<td>56.2</td>
</tr>
<tr>
<td>Aggregated to single</td>
<td>0.16</td>
<td>0.14</td>
<td>29.9</td>
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<tr>
<td>Aggregated to paired</td>
<td>0.26</td>
<td>0.12</td>
<td>0.1</td>
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<tr>
<td>Aggregated to rouleaux</td>
<td>0.25</td>
<td>0.12</td>
<td>2.0</td>
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<tr>
<td>Rouleaux to single</td>
<td>0.24</td>
<td>0.10</td>
<td>0.4</td>
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<tr>
<td>Rouleaux to paired</td>
<td>0.02</td>
<td>0.06</td>
<td>84.1</td>
</tr>
<tr>
<td>Rouleaux to aggregated</td>
<td>0.21</td>
<td>0.10</td>
<td>3.4</td>
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</table>
Table S2.3. Mean transition rate coefficients of correlated evolution between conjugation and sperm head shape. Despite the gain of conjugation in the presence of broad heads or the loss of broad heads in the presence of single sperm rarely being removed from the models of evolution (i.e., Z < 5%) the rj-MCMC chain gave a broad range of transition rate values (indicated by large standard deviations). Maximum likelihood estimation of these rate parameters (mean ± SD: gain of conjugation = 14.79 ± 4.57, loss of broad heads = 28.28 ± 4.60) results in more precise values, but the mean is of a similar magnitude as those produced by rj-MCMC.

<table>
<thead>
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<th>Evolutionary transition</th>
<th>mean rate</th>
<th>SD</th>
<th>Z%</th>
</tr>
</thead>
<tbody>
<tr>
<td>gain of broad heads in presence of single sperm</td>
<td>0.56</td>
<td>0.81</td>
<td>29.0</td>
</tr>
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<td>gain of conjugation in the presence of elongate heads</td>
<td>0.26</td>
<td>0.38</td>
<td>64.5</td>
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<td>gain of conjugation in presence of broad heads</td>
<td>34.79</td>
<td>28.29</td>
<td>1.9</td>
</tr>
<tr>
<td>gain of broad heads in presence of conjugation</td>
<td>0.30</td>
<td>0.38</td>
<td>59.0</td>
</tr>
<tr>
<td>loss of broad heads in presence of single sperm</td>
<td>36.78</td>
<td>27.62</td>
<td>0.4</td>
</tr>
<tr>
<td>loss of conjugation in presence of elongate heads</td>
<td>0.06</td>
<td>0.21</td>
<td>92.3</td>
</tr>
<tr>
<td>loss of conjugation in presence of broad heads</td>
<td>0.74</td>
<td>0.22</td>
<td>2.0</td>
</tr>
<tr>
<td>loss of broad heads in presence of conjugation</td>
<td>0.77</td>
<td>0.20</td>
<td>&lt;0.1</td>
</tr>
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</table>


**Fig. S2.1.** Majority consensus tree derived from 40,000 post-burnin trees from four independent MCMC runs (see main text for details). Numbers indicate the posterior probability of a given node.

**Fig. S2.2.** Distribution of sperm length. Dark grey bars indicate mean species sperm length that was categorized as short (<0.65. Light grey bars indicate long sperm (≥ -0.65).
Movie S2.1. Heterospermatozeugmata of *Ilybius larsoni*. Within the seminal vesicle of males multiple elongate-headed sperm slip within the pocket of a single broad-headed sperm, essentially forming an aggregate-rouleau combination. During or after transfer to females, multiple conjugates stack together to form variable sized structures highly reminiscent of the rouleaux (see Fig. 2.1E). Sperm were harvested from the female reproductive tract and visualized using differential interference contrast microscopy.

Movie S2.2. Heterospermatozeugmata of *Ilybius sp.* Aggregate-type conjugates were harvested from the female reproductive tract and visualized using differential interference contrast microscopy.

Movie S2.3. Heterospermatozeugmata of *Hygrotus sayi*. A single conjugate obtained from a female storage organ (visible on the right). The rouleau is composed of hundreds of broad-headed sperm that form the scaffolding of the conjugate to which elongate headed sperm attach. Visualized using darkfield microscopy.
Female reproductive tract form drives the evolution of complex sperm morphology

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Sperm form evolves rapidly and dramatically, particularly in taxa with internal fertilization\(^1\). Despite its importance to our understanding of reproduction, sexual selection and speciation\(^2\), the adaptive significance of variation in sperm form is poorly understood\(^1\). Here, we use phylogenetically controlled generalized least squares\(^3\) and logistic regression\(^4\) to test whether the evolution of female reproductive tract design might have driven the evolution of complex, multivariate sperm form (including sperm length, head shape, conjugation, conjugate size, and sperm dimorphism) in a family of aquatic beetles. The results reveal that female reproductive tracts have undergone extensive diversification in diving beetles\(^5\), with remodelling of size and shape of several organs and structures being significantly associated with changes in sperm size, head shape, gains and losses of conjugation, and the evolution of conjugate size. Further, results of Bayesian analyses\(^6\) suggest sperm morphological evolution tracks changes in female reproductive structures. Behavioral and ultrastructural examination of sperm conjugates stored in the female tract support the interpretation that sperm conjugation is an adaptation for maintaining optimal positions in the female reproductive tract relevant to competitive fertilization. The results underscore the importance of sperm-female interactions\(^7\) as an agent of diversification.

The structure-function relationships\(^1\) and the selective basis of diversification in sperm morphology\(^7\) are poorly understood. Comparative analyses of diverse taxa (e.g., beetles\(^8,9\), birds\(^10\), flies\(^11-13\), mammals\(^14\), moths\(^15\) and snails\(^16\)) have revealed a widespread pattern of correlated evolution between sperm morphology and dimensions of some critical region of the female tract (but see\(^17,18\)). These investigations, and the resultant emerging paradigm of sperm-female coevolution are primarily based on a single axis of variation: sperm length and the length of the female sperm-storage organ(s) or its duct,
whereas sperm and female reproductive tracts can differ among species in a multitude of ways\textsuperscript{1,7}.

Our comparative investigations of sperm form in diving beetles (Dytiscidae) have revealed an astonishing diversity (see Chapter 2) of (i) total length (128 to 4493 µm), (ii) head shape, (iii) flagellum length (iv) head shape dimorphism (e.g., Fig. 3.1b and f), and (v) both single and conjugated sperm. Conjugation is an unusual, yet taxonomically widespread, phenomenon in which two or more sperm physically unite for motility or transport through the female reproductive tract\textsuperscript{19}. Among diving beetle species with conjugation, the size and organization of conjugates varies greatly and includes at least three distinct forms: (1) pairs, (2) aggregates, and (3) orderly stacks of sperm called rouleaux (Fig. 3.1). Finally, although the evolutionary origins of sperm dimorphism and conjugation are independent across the diving beetle lineage (Fig. 3.2; Chapter 2), the two character states sometimes co-occur (Fig. 3.1b and e).

To investigate whether the evolution of female reproductive tract design might have driven the evolution of such complex sperm forms, we quantified female tract morphology for 42 species of diving beetles, then used phylogenetically controlled generalized-least squares\textsuperscript{3} and logistic regression\textsuperscript{4} to explore potential coevolutionary relationships with sperm form, and Bayesian methods\textsuperscript{6} to infer the probable sequence of sperm and female character transitions. With the exception of the fertilization duct, any of the main features of the female tract (i.e., the spermathecal duct, spermatheca or receptacle; Fig. 3.2) may be absent or highly elaborated, with dimensions of every component varying substantially among species (e.g., spermathecal ducts; Fig. 3.2 and Table S3.1).
Results of logistic regression revealed that sperm conjugation was significantly explained by the presence of relatively short fertilization ducts (standardized mean coefficient -1.55, bootstrapped 95% CI: -3.70 to -0.20, \( p = 0.03 \)) and round spermathecae (i.e., negatively associated with spermathecal length, -2.20, 95% CI: -5.86 to -0.15, \( p = 0.04 \), but positively associated with spermathecal area, 3.29, 95% CI: 0.29 to 8.20, \( p = 0.04 \)). Bayesian inference of character evolution supported the regression-based results, showing strong support for correlated evolution of sperm conjugation, short fertilization ducts, and relatively round spermathecae (i.e., models of correlated evolution have a greater likelihood than models of independent evolution, Bayes Factor (BF) > 7). Ancestral trait reconstruction indicates the presence of sperm aggregates and compact spermathecae with relatively short fertilization ducts as the basal condition in diving beetles (this study BF > 2 and Fig. 3.3a). Based on evolutionary transition rates, the female reproductive tracts appear to change in advance of sperm form (reproductive tract 5.52 ± 0.0065 > sperm 0.03 ± 0.0004 changes per unit branch length) such that reproductive tract evolution elicits corresponding modification in sperm morphology (Figure 3a and Table S3.2 for additional transition rates).

Variation in female reproductive tract form explained a significant amount of the interspecific variation in sperm length, conjugate length, and head length. In two of the three major clades in our phylogeny, dimensions of the spermatheca and/or fertilization duct explained 92% (clade 2) and 54% (clade 3) of the variation in sperm length. In clade 1, sperm length was only associated with body size. Clade 1 shows comparatively little variation in sperm and reproductive tract dimensions relative to clades 2 and 3 (e.g., sperm length ranges from 177 – 283 \( \mu m \) in clade 1 versus 298 – 1,965 and 241 – 3,581 \( \mu m \) in clades 2 and 3 respectively; see Table S3.1). Because sperm forming rouleaux-type conjugates are longer than individual sperm, we additionally examined sperm unit length (the distance from the tip of the
Conjugation has been proposed to evolve through kin and multilevel selection at the level of individual sperm to enhance competitive ability through increased swimming speed^20-23. The paucity of correlations between sperm morphology and the presence or dimensions of the spermathecal duct suggests that selection for enhanced speed of sperm arrival in storage or at the site of fertilization has not been the primary factor influencing sperm conjugation in diving beetles. Instead, we propose that conjugation in these beetles is an adaptation for positional advantage in the displacement-based system of sperm competition observed in many insects^24 (see Supplemental Material 3.1 for further discussion). The association of sperm conjugation with short fertilization ducts and round spermathecae would be explained if the physical structure of conjugated heads enhances anchoring within the fertilization duct. If true, conjugates are predicted to remain aggregated upon arrival in the spermatheca until occupying the distal end of the fertilization duct (the source of sperm for fertilization). Here, rouleaux would provide a further selective advantage: those with anterior ends anchored in the fertilization duct could maintain a queue for fertilization despite a voluminous spermatheca.
We tested these predictions in species with sperm pairs and rouleaux by examining the extent of disassociation of sperm conjugates in wild caught females and by ultrastructural examination of sperm-female interactions within the fertilization duct. Intact, motile conjugates with their tips positioned in fertilization ducts were found in the spermatheca in 34 of 35 females among four species (*Hygrotus sayi*, 15/15; *Nebrioporus rotundatus*, 3/3; *Neoporus dimidiatus*, 5/5; and *N. undulatus*, 16/17; Fig. 3.3b–c; Supplementary Movie 1). Furthermore, the sperm of *Acilius mediatus* remained paired in the spermatheca but were primarily single within the fertilization duct and tightly associated with the duct walls (Fig. 3.3e), whereas sperm remained associated in rouleaux within the fertilization duct of *N. undulatus* (Fig. 3.3d). In all species examined, individual sperm detached from conjugates only when positioned for fertilization (but see25 for an example of paired sperm dissociating within the spermatheca). Combined, the results support the hypothesis that conjugation provides sperm with a positional advantage for fertilization.

Across the metazoa, sperm have diverse and often complex morphology. Our results show that understanding the evolutionary origin and maintenance of this variation requires consideration of the largely neglected selective environment of the female reproductive tract. Additionally, our results suggest that conjugation in diving beetles helps sperm maintain prime positions for fertilization within the reproductive tract. Selection to increase the likelihood of sperm being present in an appropriate location for fertilization might be a generalizable principle of sperm morphological evolution, equally applicable to internally and externally fertilizing species.
Methods Summary

Morphological characters. Sperm were harvested from the seminal vesicles of field collected or alcohol preserved specimens, DAPI or Hoechst’s stained, and imaged using darkfield microscopy. Female reproductive tracts were dissected from preserved specimens, processed as described by Miller⁵, and imaged using differential interference microscopy. Sperm length and female reproductive tract dimensions were measured from digital images using Image J²⁶. To permit inference of probable evolutionary pathways of sperm and female reproductive tract coevolution, female multivariate morphology was categorized as a binary trait by examining the predicted values produced by our logistic regression equation and assigning species falling above or below the mean a value of one and zero, respectively. Total body length was used as a measure of body size. See Supplemental Material Table S3.1 for species mean values and sample sizes.

Transmission electron microscopy. Reproductive tracts were dissected from wild-caught females, fixed in 2.5% glutaraldehyde and 1% tannic acid, post-fixed with 1% osmium tetroxide, embedded in plastic, and sectioned with a Leica EM UC6 microtome. Sections were observed with a JEOL JSM-2000EX transmission electron microscope at 100 kV.

Phylogenetic inference. Evolutionary relationships were inferred from partial DNA sequences of two mitochondrial (COI and 16s) and three nuclear (H3, Wnt1 and 18s) genes (see Table S2.3 for accession numbers). Ribosomal genes were aligned using PRANK+_F²⁷ and hypervariable regions removed using Gblocks²⁸; the remaining genes were aligned by eye. Models of sequence evolution were determined using DT-ModSel²⁹ and phylogenetic trees sampled in proportion to their probability using MRBAYES³⁰ (see Supplemental Material 3.1 for MCMC conditions and TreeBASE for alignments).
**Statistical analyses.** A majority consensus tree (Fig. S3.1), derived from 20,800 post burn-in trees (assessed using AWTY\(^3\)), was used to create a variance-covariance matrix to account for correlation resulting from evolutionary relationships among species. Separate analyses were performed on the three major sub-clades in the phylogeny (Fig. 3.2; see Supplemental Material 3.1 for justification). Forward and backward stepwise factor selection was used for both GLS\(^3\) and logistic regression\(^4\), with only significant explanatory variables retained in the final models. The results were robust to the assumed model of evolution (e.g., Brownian motion, stabilizing or accelerating/decelerating evolution) and produced qualitatively or quantitatively similar results regardless of the method used to generate the variance-covariance matrix from the consensus tree (data not shown). To explore rates of evolutionary transitions among correlated traits and infer probable evolutionary pathways (among all three clades) we used reversible-jump Markov Chain Monte Carlo\(^6\) analyses and 1000 post burn-in trees (available on TreeBASE). We used a beta-distributed prior with its parameters seeded from uniform hyperpriors (distributions: 0 – 30, 0 – 5) and a rate deviation of 6 that resulted in mean acceptance of 24% of the rate parameter proposals. The chain was run for 10,050,000 iterations with the first 50,000 discarded as burn-in. Each run was repeated three times to check stability of the harmonic means.

**Supplementary Information** accompanies the paper.
Acknowledgements. We thank M. Pagel and A. Meade for assistance with reversible-jump Markov Chain Monte Carlo analyses of probable evolutionary pathways. A. Ives and W.T. Starmer provided helpful discussion of phylogenetic logistic regression and other statistical advice. The IBEST Bioinformatics Core at the University of Idaho supported the phylogenetic tree inference (NIH/NCRR P20RR16448 and P20RR-16454). We also thank R.P. Smith for technical assistance with sectioning and TEM images. This research was supported by the National Science Foundation (DDIG-0910049 to D.M.H. and S.P.; DEB-0743101 to K.A.S; DEB-0814732 and DEB-6990357 to S.P.), the Natural Sciences and Engineering Research Council (PGS-D 331458 to D.M.H.) and the Systematics Research Fund (to D.M.H.).

Author Contributions. D.M.H. and S.P. conceived the project and gathered morphological data. D.M.H. and K.B.M. contributed specimens and DNA sequences. D.M.H. conducted the phylogenetic inference with assistance from K.A.S and performed the statistical analyses. D.M.H and S.P. wrote the manuscript.

Author Information Authors declare no competing financial interests. Correspondence and requests for materials should be addressed to D.M.H. (e-mail: dmhiggin@syr.edu).


Table 3.1. Results from GLS stepwise multiple regression. Body size and dimensions of fourteen measures of female reproductive tract morphology were considered: presence/absence of a spermathecal duct; length, minimum and maximum width of the spermathecal duct; presence/absence of a receptacle; length, area, minimum and maximum width of the receptacle when present; spermathecal length, area, minimum and maximum width; and fertilization duct length. All variables were log transformed.

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Figure 3.1. Types of sperm conjugation. Diving beetles exhibit three general forms of conjugation: (1) pairing with heads tightly binding along corresponding sides, (2) aggregations of varying size (typically less than 25) with heads in register and (3) sperm stacked into structures called rouleaux, where the tip of one head slips into a hollow pocket at the base of the head of another sperm cell and results in conjugates that have greater total length than the sperm they contain (up to three times longer, Table S3.1). a, Sperm pairing of Graphoderus liberus. b, Aggregate, sperm dimorphic (sperm with broad, flattened heads on the interior, surrounded by filamentous head sperm) conjugate of Ilybius oblitus. c, Sperm rouleaux of Uvarus lacustris. d, Composite image of a single sperm head and a rouleau of Neoporus undulatus. Sperm heads stack tightly together with basal spurs exposed. e, Rouleau-type sperm dimorphic conjugate of Hygrotus sayi (sperm with broad, heads with basal spurs stack to form the scaffolding to which sperm with filamentous heads attach). f, Dimorphic sperm heads of Hygrotus sayi. a, Darkfield microscopy; heads and flagella visible. b – f, Epifluorescence microscopy with only DNA-stained heads visible. Scale bars = 20 µm.

Figure 3.2. Phylogeny and representatives of three basic designs of diving beetle female reproductive tracts. Female diving beetles have “conduit” type reproductive tracts, in which sperm enter and exit storage through different ducts. a, Large sock-shaped spermatheca without a distinct spermathecal duct; Graphoderus liberus. b, Clearly defined spermathecal duct, spermatheca and fertilization duct; Rhantus binotatus. c, Typically narrowed and lengthened spermathecal ducts and, in some species, the addition of a receptacle, that
might act as a secondary site of sperm storage; *Nebrioporus rotundatus*. b = bursa, c = common oviduct, fd = fertilization duct, g = gland, r = receptacle, s = spermatheca, sd = spermathecal duct. Coloured branches indicate ingroup taxa (see Fig. S3.1 for branch support). Clade 1 (red) is characterized by species with paired sperm and large sperm-storage organs (type a). Clade 2 (blue) contains species with paired sperm or larger side-by-side type conjugates and type a or b female tracts. Clade 3 (orange) is characterized by sperm that conjugate in rouleaux and type c tracts. Dashed lines indicate species where sperm do not conjugate and stars show species with sperm dimorphism. Outgroup taxa are shown in black or grey. Grey is used where sperm data is missing.

**Figure 3.3. Conjugate-female interactions.** a, Diagram showing evolutionary transitions in sperm and reproductive tract form. Histograms show the posterior distribution of evolutionary transition rates per unit of branch length (y-axis: percentage of models). Transition rates that are rarely assigned to zero (Z < 5% of models of trait evolution) are considered probable events (shown in dark red, y-axis % of models; marginal events, Z ~ 10%, are shown in light red). The bold upper-left text indicates the ancestral condition for sperm and reproductive tract form in diving beetles; italicized text indicates character transitions. Female reproductive tract evolution away from the ancestral state is more probable than changes in sperm form (Z: 0.36% << 97.4%). Change in reproductive tract design results in a correlated loss of sperm conjugation (far right histogram, Z < 5%). Transition rates and Z-values are based on 100,000 observations from 10,000,000 iterations from each of three independent runs of the Markov chain.
b, Sperm storage organ of *Hydrovatus pustulatus* stained with chlorazol black. c, Orcein stained rouleau-type conjugates within the fertilization duct of *H. pustulatus*. d, Sagital section showing two conjugates of *Neoporus undulatus* occupying the fertilization duct and oriented towards the site of fertilization. Vertical lines are the margins of the stacked heads (see Fig. 3.1d for explanation of rouleaux formation). Flagella can be seen between the two rouleaux and extend into the spermatheca. e, Sperm of *Acilius mediatus* are paired within the spermatheca but are mostly single within the fertilization duct and tightly associated with the duct walls. Similar to *N. undulatus*, the sperm heads are oriented towards the exit of the fertilization duct. Differential interference micrographs and scale bars in b–c = 50 µm. Transmission electron micrographs and scale bars in d–e = 2 µm. f = flagellum, fd = fertilization duct, s = spermatheca, arrow = sperm head(s).
conjugated sperm
short fertilization duct
compact spermatheca

Z = 0.36%

conjugated sperm
long fertilization duct
elongate spermatheca

Z = 97.4%
Z = 1.74%

single sperm
short fertilization duct
compact spermatheca

Z = 10.7%

single sperm
long fertilization duct
elongate spermatheca

Z = 9.09%
Z = 12.1%

Z = 0.13%
Z = 97.4%
Z = 10.7%
Supplemental Material 3.1

Justification for clade-level analyses. We performed separate analyses on each of the three major sub-clades in our phylogeny because (1) lineage-wide analyses of correlated trait evolution can obscure important relationships when these differ in direction and/or magnitude among sub-lineages\(^1\), (2) there were qualitative among-clade differences in female tract and sperm design (Fig. 3.2), and (3) because of uncertainty of evolutionary relationships in the basal branches of the diving beetle lineage (Fig. S3.1).

Supplemental results. Across the entire diving beetle lineage, the length of individual sperm (using the longer sperm morph in the case of sperm heteromorphic species; see below for justification) was only associated with the presence of a female receptacle (Table S3.4; Fig. 3c; an organ of unknown function that sometimes contains sperm and thus might act as a secondary sperm-storage organ). Of the species possessing receptacles (n = 11), sperm length was positively correlated with the smallest dimension of the organ and negatively correlated with the largest dimension (Table S3.4).

Males of some species of diving beetles produce two distinct types of sperm that differ in total length and head morphology (Fig. 3.1 and 3.2). Both sperm morphs are transferred to females, but based on findings in other insect species\(^2\)-\(^4\), only the long morph is expected to participate in fertilization. Neither the presence of dimorphism, nor the length of the short sperm morph was correlated with any aspect of female morphology (\(p > 0.05\)).

Displacement hypothesis. To explain the putative sperm-female interactions giving rise to this hypothesis, we note that male crickets from populations experimentally evolved to have longer sperm have no competitive fertilization advantage over males with shorter sperm within the short, round spermathecae of females\(^5\). By contrast, in *Drosophila*
studies have shown that (1) physical displacement by competitor sperm is a critical determinant of competitive fertilization success in the long, narrow female sperm-storage organ\textsuperscript{6}, (2) longer sperm are better at displacing, and resisting being displaced by, shorter sperm from the proximal end of the organ closest to the site of egg fertilization\textsuperscript{7}, (3) that sperm and female tract morphology interact such that the fitness advantage to males of producing relatively long sperm increases with increasing length of narrow sperm-storage organ\textsuperscript{8}, and as a consequence, (4) the evolution of longer sperm-storage organs drives the evolution of longer sperm\textsuperscript{8}.

**Supplemental methods.** For phylogenetic inference of the evolutionary relationships among diving beetles, aligned DNA sequences (available from TreeBASE) were partitioned by gene and an appropriate model of sequence evolution (as determined by DT-ModSel\textsuperscript{9}) was specified for each gene. We used uninformative priors for all of the model parameters (i.e., MrBayes default priors). Four independent runs of Markov chain Monte Carlo (MCMC) of 100,000,000 generations, consisting of six chains each, were used to sample phylogenetic tree space. After a burn-in period (assessed using AWTY\textsuperscript{10}), trees are visited in proportion to their probability of being true given the model, priors and data, and can be used to determine the posterior probability of a branching event and branch lengths. Below is the MrBayes block used to specify data partitions, models of evolution, and MCMC conditions including heating of chains (temp; facilitates chain exploration of tree space) and number of swaps attempted at each generation (nswaps; attempts to swap states between two randomly selected chains).

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begin mrbayes;
  charset COI = 1-700;
  charset H3 = 701-1010;
  charset Wnt1 = 1011-1455;
  charset 16s = 1456-1914;
  charset 18s = 1915-2497;
  partition genes = 5: COI, H3, Wnt1, 16s, 18s;
```
set partition = genes;
\text{lnset applyto}=(1, 3, 4) \text{nst}=6 \text{rates}=\text{invgamma};
\text{lnset applyto}=(2, 5) \text{nst}=2 \text{rates}=\text{invgamma};
\text{unlink statefreq}=(\text{all}) \text{revmat}=(\text{all}) \text{shape}=(\text{all}) \text{pinvar}=(\text{all});

\text{mcmc}
\begin{align*}
n\text{runs} &= 4 
n\text{ngen} &= 100000000 
n\text{printfreq} &= 1000000 
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n\text{nchains} &= 6 
n\text{savebrlens} &= \text{yes} 
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n\text{nswaps} &= 2 
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\end{align*}

\text{sump burnin} = 19800;
\text{sumt burnin} = 19800;

\text{end};


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Table S3.2. Mean evolutionary transition rate coefficients estimated by reversible jump-MCMC. Transition rates that are rarely assigned to zero (Z-value, i.e., <5% of models of trait evolution) are considered probable transitions.

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### Table S3.3. Genbank accession numbers for DNA sequence data.

*Indicates sequence was from a closely related species to the species of interest.

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Table S3.4. Results of phylogenetically controlled GLS analyses that found significant relationships between sperm traits and female reproductive tracts.

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Figure S3.1. Majority consensus tree used for phylogenetically controlled GLS and logistic regression analyses. The tree was derived from 20,800 post-burnin trees from four independent MCMC runs. Node values indicate the posterior probability of a branching event. Images to the right of the tree illustrate some of the observed diversity of female reproductive tract design and sperm form (top to bottom row: *Laccophilus maculosus, Acilius semisulcatus, Rhantus binotatus, Uvarus lacustris, Hydrovatus pustulatus,* and *Neoporus dimidiatus*). *Acilius* have paired sperm. *Rhantus* produce variable sized sperm aggregates. *Neoporus, Uvarus* and *Hydrovatus* have rouleaux-type conjugation. Uniquely, sperm are single within the seminal vesicles of *Laccophilus* males but are similar in appearance to rouleaux within the fertilization ducts of females. Female reproductive tract were imaged using differential interference microscopy. Sperm, with the exception of *Laccophilus* (fluorescence microscopy of DNA stained heads; flagella not visible), were visualized with darkfield microscopy. Scale bars = 50 μm.
**Movie S3.1.** Motile conjugates within the female reproductive tract of *Neoporus.*

The reproductive tract was dissected in supplemented Grace’s insect cell culture medium (Invirtorgen) and visualized with differential interference microscopy. The spermatheca, fertilization duct and sperm conjugates are clearly visible. Conjugates are oriented with their tips within the fertilization duct.
VITA

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Natural Sciences and Engineering Research Council (NSERC) of Canada postgraduate scholarship

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