CONVERGENCE, RECURRENCE AND DIVERSIFICATION OF COMPLEX SPERM TRAITS IN DIVING BEETLES (DYTISCIDAE)

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Sperm display remarkable morphological diversity among even closely related species, a pattern that is widely attributed to postcopulatory sexual selection. Surprisingly few studies have used phylogenetic analyses to discern the details of evolutionary diversification in ornaments and armaments subject to sexual selection, and the origins of novel sperm traits and their subsequent modification are particularly poorly understood. Here we investigate sperm evolution in diving beetles (Dytiscidae), revealing dramatic diversification in flagellum length, head shape, presence of sperm heteromorphism, and the presence/type of sperm conjugation, an unusual trait where two or more sperm unite for motility or transport. Sperm conjugation was found to be the ancestral condition in diving beetles, with subsequent diversification into three forms, each exhibiting varying degrees of evolutionary loss, convergence, and recurrence. Sperm head shape, but not length or heteromorphism, was found to evolve in a significantly correlated manner with conjugation, consistent with the different mechanisms of head alignment and binding required for the different forms of conjugation. Our study reveals that sperm morphological evolution is channeled 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.

KEY WORDS: Ornament evolution, postcopulatory reproductive traits, sexual selection, sperm conjugation, sperm heteromorphism.
and Brown 2000). Mechanistically, the interaction between sperm length and seminal receptacle morphology results in long sperm being better able to displace and resist displacement by competitor sperm (Pattarini et al. 2006). In this instance, a long sperm tail acts both as an ornament that females use to discriminate among sires and as an armament when in competition with rival sperm. Alternatively, sperm morphology can also act as a weapon in evolutionary arms races. Many species of the hermaphroditic flatworm of the genus Macrostomum participate in reciprocal matings that terminate with a sucking behavior to remove sperm and seminal fluids deposited by the mating partner (Schärer et al. 2004). Stiff lateral bristles on sperm, a trait that is hypothesized to impede sperm removal by sucking, exhibits an evolutionary association with reciprocal mating, with sperm bristles being absent in species that circumvent the sucking behavior by hypodermically inseminating sperm into the body of the mating partner (Schärer et al. 2011).

Motivated by the central role of sperm in sexual reproduction, their unusual ecology (being cast from the soma into a foreign environment to function essentially as free-living organisms), and their usefulness in systematics (Jamieson et al. 1999), biologists have described the sperm of thousands of species (Pitnick et al. 2009a). Collectively, these studies indicate that sperm are the most diverse cell type, exhibiting dramatic morphological modifications in nearly all taxa (Pitnick et al. 2009a). Despite the massive research effort describing sperm structure in a myriad of species, the origins of novel sperm forms, their subsequent diversification, and evolutionary loss of derived sperm traits have scarcely been investigated (Roldan et al. 1992; Breed 2005), resulting in a limited understanding of sperm phenotypic evolution. Here, we amend this deficiency by conducting detailed analyses to infer patterns of sperm evolution within a large radiation of aquatic beetles (Dytiscidae with ca. 4000 species worldwide, Nilsson 2001; Nilsson and Ferry 2006) exhibiting dramatic, multivariate diversity in sperm form.

The sperm of diving beetles (Dytiscidae) first 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 (Auerbach 1893; Ballowitz 1895). Sperm conjugation is rare, but has several independent origins throughout the Metazoa, occurring in relatively few species of marsupials, eutherian mammals, gastropods, annelids, myriapods, arachnids, and insects (Immler 2008; Higginson and Pitnick 2011). This remarkable modification in sperm form has been of considerable interest to evolutionary biologists due to its potential role in sperm competition, implications for evolutionary cooperation, and the possibility of haploid–diploid conflict between males and the sperm they produce (Immler 2008; Pizzari and Foster 2008; Higginson and Pitnick 2011). Additionally, some species of diving beetles produce two distinct sperm morphs that vary in total length or head shape (Higginson and Pitnick 2011). Such sperm heteromorphism sometimes co-occurs with conjugation, resulting in complex conjugates constructed of both sperm morphs.

Although female mating frequency has not been determined for any species of diving beetle, several lines of evidence suggest that sexual selection has been important in shaping their behavior and morphology. First, male investment in sperm production is among the highest recorded for any taxa (up to 13% of their body mass in Dytiscus sharp, Inoda et al. 2007). Relative testis mass is positively correlated with polyandry and is a robust indicator of the intensity of sexual selection in mammals (Harcourt et al. 1981; Gomendio and Roldan 1991), fish (Stockley et al. 1997), birds (Møller and Briskie 1995), and insects (Gage 1994). Second, sperm competition avoidance tactics of mate-guarding and copulatory plugs are observed in diving beetles (Smith 1973; Aiken and Wilkinson 1985; Aiken 1992; Simmons 2001; Inoda 2003; Cleavall 2009). Third, in some diving beetle lineages there is evidence of sexual arms races, where evolution of structures that improve females’ ability to resist mating is followed by the evolution of improved male structures used to grasp females during copulation (Miller 2003; Bergsten and Miller 2007). Finally, sperm morphology in diving beetles is correlated with dimensions of the female reproductive tract and evolves in response to changes in the architecture of female sperm-storage organs (Higginson et al. unpubl. ms.).

Here we use Bayesian analyses deploying reversible-jump Markov Chain Monte Carlo (rj-MCMC) to (1) explore different models of evolution while accounting for uncertainty in phylogenetic relationships, (2) infer the sequence of diversification in conjugate form, and (3) examine patterns of convergence, recurrence and loss of conjugation, and sperm heteromorphism. We also test hypotheses of correlated evolution among sperm traits including conjugation, head shape, sperm length, and heteromorphism. Head shape might affect the propensity of sperm to conjugate by modifying hydrophobic, surface-protein, or glycolcalyx interactions. Additionally, both theoretical and empirical studies indicate a positive relationship between sperm length and swimming speed (Lighthill 1976; Fitzpatrick et al. 2009). Conjugation might provide a mechanism for increased sperm velocity by combining the force generated by multiple flagella (Woolley et al. 2002; Immler et al. 2007) without the energetic costs of producing long sperm (e.g., delayed maturation and increased investment in testes; Pitnick et al. 1995; Pitnick 1996). Thus, if selection favored increased sperm velocity, long sperm would be predicted to evolve in the absence of conjugation and vice versa. Finally, conjugation might facilitate the evolution of heteromorphism by lessening the selective constraints on sperm morphology imposed
by individual sperm having to affect their own transport to the site of fertilization (i.e., through functional specialization).

**Methods**

**SPERM CHARACTERS**

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 dark-field and epifluorescence microscopy (100 ×–400 × as appropriate for sperm length; 1000 × for head shape).

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 antiparallel 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 (Higginson and Pitnick 2011), regardless of the number of sperm involved (e.g., two in Bidessonotus inconspicuous, dozens in Neoporus undulatus and hundreds in Hydromorus 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 (Rashband 1997–2008). In some instances, mature individuated sperm could not be obtained from a species. In these cases we measured the total length of mature sperm bundles (i.e., sperm had taken on their mature head shape but had not yet individualized), providing a minimum length for the species. A species was considered to be sperm heteromorphic when two distinct (i.e., nonoverlapping) sperm lengths or head shapes were produced by a single male (see Fig. 1A, E, F). One to nine individuals per species (mean = 3) were examined (Table S1). Sperm characters were largely consistent within genera. However, 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 and Table S1). 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. 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 (i.e., subgenera for Agabus and Ilybius where there has been recent and considerable taxonomic flux, Nilsson 2000, 2001). 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). In total, we examined 141 species of diving beetles that provided sperm morphological data for 138 tips of our phylogeny.

**PHYLOGENETIC TREES**

To provide trees upon which to test models of character evolution, we used the large sequence dataset compiled by Ribera et al. (2008; see Appendix 1 therein for accession numbers). The dataset 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,1,F algorithm (Löytynoja and Goldman 2008) that avoids overpenalization 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, Landan and Graur 2007). If sequence alignments are unambiguous, both heads and tails alignments provide identical results. PRANK,1,F produced heads and tails alignments with 93.6–99.9% (16s and H3 respectively) identical residues within the aligned genes and resulted in phylogenetic trees that did not differ substantively (alignments available from TreeBASE, http://purl.org/phylo/treebase/phylows/study/TB2:S12131). Appropriate models of sequence evolution for each of the four genes were determined using DT-ModSel (Minin et al. 2003). We used MrBAYES (Ronquist and Huelsenbeck 2003) to infer phylogenetic relationships based on our partitioned four gene, 2956 base pair dataset. To encourage convergence of the MCMC chains, we provided a starting tree produced using the neighbor-joining method in PAUP (Swofford 2002). The starting tree was randomly perturbated four times prior to the starting of the chains. Four separate MCMC runs of 4 × 10⁷ generations were performed using uninformative priors (i.e., MrBAYES default prior values, Ronquist and Huelsenbeck 2003), six chains per run, with 0.15 heating. Convergence of the runs was assessed using AWTY (Nylander et al. 2008) and the first 3 × 10⁷ generations were discarded as burn-in. After the burn-in period, the MCMC chains
Figure 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 dark-field and fluorescence image of the broad, flat sperm heads of *Hydaticus bimarginatus*. In the seminal vesicles of males, the sperm heads align antiparallel 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 Video clip 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 *). Both 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.
program MULTISTATE (Pagel 2002; Pagel and Meade 2006b), a hyperprior with a uniform distribution of 0–50 or 0–100 to seed the mean of our exponential rate prior and a rate deviation value resulting in the recommended value of approximately 20% of proposals being accepted (Pagel et al. 2004). The rj-MCMC chain was run for 10,050,000 iterations with the first 50,000 iterations discarded as burn-in. Mean rate, standard deviation (SD), and the percentage of times a transition rate was assigned a value of zero (Z%) were calculated from posterior rate distributions. Transition rates that were rarely assigned to zero (i.e., Z% < 10) were considered probable evolutionary events.

To test for correlated evolution between conjugation and sperm head shape, presence of heteromorphism, or sperm length, we used the program DISCRETE (Pagel 2000; Pagel and Meade 2006a, 2006b) with the conditions described above. Bayes Factors (BFs) were used to evaluate alternative models, where the traits were allowed to evolve independently or constrained to evolve in a dependent manner. BFs are two times the difference in the marginal likelihoods of the best-fit and worse-fit models, as approximated by the harmonic means from the final iteration of the MCMC runs. We follow the convention that a BF > 2 is supportive of the best-fit model and that BF > 5 or 10 is considered strong or very strong support, respectively (Pagel et al. 2004; Pagel and Meade 2006a).

We used the most recent common ancestor (MRCA) approach to infer ancestral states. This method identifies a node that represents the MRCA to the group species of interest for each tree the MCMC chain visits. For some trees this node will only contain the species of interest, whereas for other trees the node will additionally include other taxa. Thus, for every tree the ancestral state at this internal node can be estimated and across trees, the posterior distribution of the ancestral state can be examined (Pagel et al. 2004). This method has the advantage of combining uncertainty about the existence of a node and that of its character state (Pagel et al. 2004). To test if one ancestral state was more likely than another, we compared the likelihoods of models using BFs, where the ancestor of the species of interest was constrained to take alternative character states (i.e., the fossil command in Bayes/Traits, Pagel et al. 2004).

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 μm–4493 μm), head shape (Fig. 1A–F), and the presence of sperm heteromorphism (production of two distinct sperm morphs that differ in total length and/or head shape; Table S1: Figure 1A, E, F). In addition, sperm were found to be either single or conjugated in one of three forms: (1) sperm aggregates composed of variable numbers of sperm with their heads aligned in register (Figs. 1A, 2A), (2) pairs with two sperm aligned antiparallel to each other (Figs. 1D, 2B), or (3) rouleaux, where the tip of one sperm head slips into the hollow, hooded portion of another sperm’s head to form orderly stacks that may be composed of a few to several hundred sperm (Figs. 1B–C, F, 2D). Sperm heteromorphism and conjugation were found to sometimes co-occur, resulting in complex conjugates called heterospermatozeugmata (e.g., Figs. 1A, E, F; Pitnick et al. 2009a; Higginson and Pitnick 2011).

**TRANSITIONS AMONG TYPES OF CONJUGATION**

The reconstructions of ancestral character states suggest sperm conjugation was present in the ancestor of diving beetles, with multiple subsequent losses distributed across the tree (Fig. 2). Sperm aggregates are supported as the ancestral form of conjugation (models where the ancestral state is constrained to aggregates have greater likelihood than those constrained to have single sperm, paired conjugates, or sperm rouleaux (aggregates vs. single sperm BF > 7, aggregates vs. pairs > 3). Additionally, both sperm pairing and rouleaux are identified as derived forms of conjugation originating from sperm aggregates (Fig. 2; Table 1). The distribution of sperm pairing in diving beetles suggests that it has evolved three times. To test this hypothesis, we used the MRCA 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); multiple origins of pairing were supported (BF > 2). Moreover, species with sperm pairing failed to form a monophyletic clade in any of the 40,000 postburn-in trees produced by MrBAYES (127 trees were consistent with only two origins of pairing). Reversions from rouleaux to the ancestral aggregates were (1) identified as probable evolutionary transitions by our rj-MCMC and (2) observed nested well within the main lineage exhibiting rouleaux, indicating that the apparent reversions are not likely the result of incomplete lineage sorting. Loss of conjugation was observed both from lineages with paired sperm (a single event) and from those with rouleaux (multiple events), but only the transition from rouleaux to single sperm was identified as probable in our rj-MCMC analysis (see Fig. 2; Table 1).

**CORRELATED EVOLUTION BETWEEN CONJUGATION AND HEAD SHAPE**

We used rj-MCMC to test whether the rates of (1) gains or losses in conjugation and (2) 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)
Figure 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% < 10). 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 31% to 76% of the models produced by the MCMC chain; see Table 1 for mean transition rates). Sperm characters are mapped to the majority consensus tree with corresponding colors (gray 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 Figure S1. Darkfield images; scale bars, 10 μm.

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 (BF > 13). Ancestral state reconstructions support the presence of conjugation and broad heads in the ancestor of diving beetles (BF > 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. 3; Table 2). However, elongation of sperm heads was unlikely to result in a subsequent loss of conjugation. Interestingly, broad-head sperm in the absence of conjugation is very uncommon, with only a single observation of this character
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. 4; Table S1) 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 were equally likely). Sperm conjugation was found to be the ancestral condition in diving beetles, which has subsequently been lost (i.e., reverting to single sperm) at multiple times throughout the lineage. Additionally, we found that head shape, but not heteromorphism or sperm length, showed correlated evolution with conjugation. Nonetheless, the frequent co-occurrence of heteromorphism and conjugation has given rise to some of the most diverse and extravagant sperm forms observed in nature. These heterospermatozeugmata exhibit uniquely organized structures (Figs. 1A, E–F) that appear to dramatically alter the manner of sperm movement (Video clips S1–3).

**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, Pitnick et al. 2009a), we have only limited insight into 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 result in correlated changes in other sperm traits. Our study of 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. Sperm conjugation was found to be the ancestral condition in diving beetles, which has subsequently been lost (i.e., reverting to single sperm) at multiple times throughout the lineage. Additionally, we found that head shape, but not heteromorphism or sperm length, showed correlated evolution with conjugation. Nonetheless, the frequent co-occurrence of heteromorphism and conjugation has given rise to some of the most diverse and extravagant sperm forms observed in nature. These heterospermatozeugmata exhibit uniquely organized structures (Figs. 1A, E–F) that appear to dramatically alter the manner of sperm movement (Video clips S1–3).

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 (Higginson and Pitnick 2011). Consistent with this result, our analysis of evolutionary transition rates between
Figure 3. Evolutionary transitions in sperm head shape and conjugation. Transition rates (q = changes per unit branch length) that are rarely assigned to zero (Z% < 10 of models of trait evolution) are considered probable events. 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; *Porrhydrus* sp.) or elongate heads (tip and terminus of head indicated by *; 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. Color online.

Probable transitions shown in green; unlikely transitions in gray.

different forms of conjugation revealed nonrandom evolutionary trajectories (Fig. 2; Table 1). Specifically, paired and rouleaux conjugates that require different mechanisms for precise head alignment and binding (Figs. 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 of the observed transitions between aggregates and rouleaux. Within-species comparisons of conjugates collected from males and from the sperm-storage organs of females revealed 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. 1E). Changes in the timing of ontogenetic processes leading to conjugation might thus give rise to the different forms of conjugation, with the postejaculation transformations observed in *Rhantus* occurring within the seminal vesicles of rouleaux-producing species.

We found strong support for multiple origins of both sperm pairing and heteromorphism, but only a single origin of rouleaux formation among diving beetles (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/or evolution via regulatory changes.
Evolutionary transition | Mean rate | SD   | Z%   
--- | --- | --- | --- 
Gain of broad heads in presence of single sperm | 0.56 | 0.81 | 29.0 
Gain of conjugation in the presence of elongate heads | 0.26 | 0.38 | 64.5 
Gain of conjugation in presence of broad heads | 34.79 | 28.29 | 1.9 
Gain of broad heads in presence of conjugation | 0.30 | 0.38 | 59.0 
Loss of broad heads in presence of single sperm | 36.78 | 27.62 | 0.4 
Loss of conjugation in presence of broad heads | 0.06 | 0.21 | 92.3 
Loss of conjugation in presence of elongate heads | 0.74 | 0.22 | 2.0 
Loss of broad heads in presence of conjugation | 0.77 | 0.20 | <0.1 

Figure 4. Distribution of sperm length. Dark gray bars indicate mean species sperm length that was categorized as short (≤-0.65). Light gray bars indicate long sperm (≥-0.65).

In the present study and most previous investigations of the evolution of sperm form (reviewed by, e.g., Keller and Reeve 1995; Pizzari and Birkhead 2002; Snook 2005; Pitnick et al. 2009a,b; Pizzari and Parker 2009), there is an underlying assumption that postcopulatory sexual selection is the principal agent of diversification. In the case of diving beetles, the demonstrated coevolution of sperm form and female reproductive tract design (Higginson et al. unpubl. ms.) supports this contention. The present analyses of macroevolutionary patterns sets the stage for future microevolutionary investigations of the relationship between variation in sperm form and fitness within species exhibiting a diversity of sperm forms across this evolutionarily dynamic lineage.
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Supporting Information

The following supporting information is available for this article:

**Table S1.** Sperm traits species in Dytiscidae, Amphizoidae and Paelobiidae.

**Figure S1.** Majority consensus tree.

**Video clip S1.** Heterospermatozeugmata of *Ilybius larsoni.*

**Video clip S2.** Heterospermatozeugmata of *Ilybius* sp.

**Video clip S3.** Heterospermatozeugmata of *Hygrotus sayi.*

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