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RELATIONSHIPS BETWEEN SPERM LENGTH AND SPEED DIFFER AMONG THREE INTERNALLY AND THREE EXTERNALLY FERTILIZING SPECIES

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It is often assumed that longer sperm, by virtue of their increased swimming speed, have a fertilization advantage over shorter sperm when in competition to fertilize eggs. However, there is surprisingly little evidence for a positive correlation between sperm length and speed. Here we use an approach that accounts for within-male variation in sperm traits to examine the relationships between sperm length and sperm speed across a broad range of species, including three internally fertilizing species and three externally fertilizing species. Our results reveal that correlations between sperm size and speed are indeed present and possibly more common than currently thought. However, the direction of the correlations between sperm length and speed, which are more prevalent within a male's ejaculate than among males, were influenced by fertilization mode in contrasting and unexpected ways. Broadly, the patterns revealed that in externally fertilizing species sperm with longer flagellum and shorter heads relative to their flagellum swam faster, whereas in internally fertilizing species sperm with shorter flagellum and longer heads relative to their flagellum swam faster. We discuss these results in light of sperm competition theory and contrast the intraspecific patterns observed in this study with macroevolutionary patterns of sperm evolution reported elsewhere.

KEY WORDS: **Cryptic female choice, sperm competition, sperm length components.**

Sperm competition, where sperm from two or more males compete to fertilize a female's eggs (Parker 1970), is a powerful evolutionary force that selects for ejaculate traits that enhance a male's competitive fertilization success (Birkhead and Møller 1998). Over the past 20 years substantial effort has been devoted to explaining sperm morphological diversity, including research on how sperm competition influences the evolution of typical sperm morphologies (i.e., sperm with a flagellum, head, and midpiece, reviewed by Pitnick et al. 2009) and the evolution of atypical sperm morphologies (e.g., aflagellate and multiflagellate sperm; Morrow 2004; Pitnick et al. 2009). In species with typical sperm morphologies, the sperm flagellum, perhaps the most extensively studied sperm morphological trait, generates the thrusting force necessary to propel sperm (Katz et al. 1989). As faster sperm are expected to be superior sperm competitors (an idea with ample empirical support, reviewed by Simmons and Fitzpatrick 2012),

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a longstanding and influential idea in the literature is Gomendio and Roldan's (1991) prediction that sperm competition will select for longer and faster swimming sperm and that a relationship exists between these sperm traits. However, sperm head morphology should also be influenced by sperm competition because the thrust generated by the flagellum is countered by the drag produced by the sperm head (Humphries et al. 2008). In addition, sperm competition is also expected to influence the size of the sperm midpiece, which contains the mitochondria and is the primary site of energy production required to power the flagellum (Cardullo and Baltz 1991). Yet despite the pervasive expectation that sperm morphology is under selection due to its link with sperm performance, there remains equivocal evidence of a relationship between sperm morphology and swimming speed.

Although a functional link between sperm flagellum, head, and midpiece length and swimming speed has been demonstrated in recent comparative studies of mammals (Gomendio and Roldan 1991, 2008; Montoto et al. 2011; Tourmente et al. 2011), fish (Fitzpatrick et al. 2009), and birds (Lüpold et al. 2009), detecting similar correlations between sperm size and speed at the intraspecific level has proven problematic. Humphries et al. (2008) highlighted this issue when reporting that sperm length–speed correlations were only present in two of nine species examined at the time (sperm with longer heads and smaller midpieces swam faster in red deer, *Cervus elaphus hispanicus* [Malo et al. 2006] and sperm with longer heads swam faster in guppies, *Poecilia reticulata* [Pitcher et al. 2007]). Although several intraspecific studies have subsequently demonstrated positive (flagellum length: Mossman et al. 2009; Fitzpatrick et al. 2010; midpiece length: Firman and Simmons 2010) and negative (e.g., flagellum: Lüpold et al. 2012) sperm length–speed correlations, the overall relationship between sperm size and speed remains far from clear. This ambiguity in the link between sperm size and speed is starkly illustrated by three recent studies of zebra finches *Taeniopygia guttata* (Mossman et al. 2009), New World blackbirds (Icteridae; Lüpold et al. 2009), and mussels *Mytilus galloprovincialis* (Fitzpatrick et al. 2012), where correlations between sperm flagellum length and speed were either weakly positive or nonexistent despite extensive sampling efforts (i.e., $n > 100$ individuals). Thus, the primary issue highlighted by Humphries et al. (2008) remains; sperm morphology is either not, or is weakly, linked with sperm swimming speed in the majority of species examined to date, in contrast with the pattern observed across species (Simmons and Fitzpatrick 2012).

The lack of a clear association between sperm length components and speed may stem from a variety of sources. First, the contrasting environments in which fertilization takes place, broadly categorized as internal and external to the female's body, can dramatically influence how selective pressures will influence the functional morphology of sperm (Parker 1993; Ball and Parker 1996). Consequently, it is questionable whether sperm length–speed correlations will be present and in the same direction in internally fertilizing species, where sperm swimming speed near surfaces of the female's reproductive tract are influenced by hydrodynamic "wall effects" (Winet 1973; Cosson et al. 2003; Denissenko et al. 2012), and externally fertilizing species, where physical constraints imposed by females are absent. Second, the complexities surrounding sperm hydrodynamics have important, but underappreciated, implications for sperm evolution as movement in the sperm microenvironment is dominated by viscosity and hydrodynamic forces that are very different to those experienced by larger organisms (Humphries et al. 2008). At scales relevant to sperm, streamlining is irrelevant and so the adaptive reasons for a particular morphology are much less clear than for large organisms, leading to the suggestion that a ratio of head length to flagellum length could be a more accurate predictor of sperm speed (Humphries et al. 2008). Finally, intramale variation in sperm traits could also mask length–speed relationships, as within-male variation is hidden when assigning average values to sperm length and speed (Fitzpatrick et al. 2010; Gadelha et al. 2010). By measuring multiple morphological traits for individual sperm cells and accounting for intramale variation, length–speed relationships might become apparent.

Here we use six species—three internally fertilizing and three externally fertilizing—to assess relationships between sperm length and speed. We use a recently developed approach that assesses length and speed of individual sperm cells within a single ejaculate (Fitzpatrick et al. 2010), measuring various sperm components including ratios of head length to flagellum length (Humphries et al. 2008). Our analyses of length–speed relationships for internal and externally fertilizing species span a broad taxonomic range (a mollusk, two fishes, an amphibian, a bird, and a mammal). They reveal that sperm length–speed relationships are more common than current evidence suggests, but also that the fertilization environment has opposing effects on the direction of correlations between sperm length and speed.

Methods

The methods employed to collect samples for this analysis reflect the best available practice for the individual species concerned. Digital video recordings of activated sperm samples were collected from three internally fertilizing (humans *Homo sapiens*, guppies *Poecilia reticulata*, and emu *Dromaius novaehollandiae*) and three externally fertilizing species (mussels *Mytilus galloprovincialis*, rainbowfish *Melanotaenia australis*, and frogs *Crinia georgiana*). Videos were recorded before this study for three of the species examined (humans: Kilgallon and Simmons 2005; frogs: Dziminski et al. 2009; emu: Sood et al. 2011a, 2011b). From the videos, sperm swimming speed and the different components of sperm length were measured from the same

individual sperm cells using the species-specific methods outlined later. Videos were considered of sufficient quality when they showed clearly intact, motile sperm swimming with no evidence of bulk fluid flow in the sample (Wilson-Leedy and Ingermann 2006). Any videos not meeting these requirements were not analyzed.

SPERM COLLECTION

Internally fertilizing species

Human (Homo sapiens): Male volunteers from the University of Western Australia were recruited to donate semen samples (for details, see Kilgallon and Simmons 2005). Samples were collected and analyzed in accordance with protocols from the World Health Organization (1999). Briefly, 10 μL of semen were mounted on a microscope slide under a coverslip, and viewed at 400× magnification at a temperature of 23°C. Video recordings were made on a Sony videocassette recorder via a camera mounted onto a compound microscope. Of 52 male subjects, the quality of video recording for 29 individuals met the above criteria for analysis with between three and six individual sperm measured per male.

Emu (Dromaius novaehollandiae): Digital video recordings of motile emu sperm were taken using fresh emu semen collected using an artificial cloaca (for details, see Sood et al. 2011a). Videos were captured at 200× magnification under phase contrast using an Olympus DP 71/25 digital camera attached to an Olympus BX60 microscope (Olympus Australia Pty, Ltd, Mt Waverley, Vic, Australia). For video recording, the concentration of spermatozoa was adjusted to 17–20 × 10^6 sperm/mL with Dulbecco's Modified Eagle's Medium containing 0.03% bovine serum albumin (BSA) at 37°C. For each sample, videos were recorded for 10 sec each under a phase contrast objective. The videos for nine males had sufficient resolution to measure length and speed. Ten individual sperm were measured per male.

Guppy (Poecilia reticulata): Captive-bred guppies used in this study were descendants of fish caught in 2006 from a feral population in the Alligator Creek River in Queensland, Australia. Sexually mature males were anesthetized and placed on a glass slide under a dissecting microscope (see Evans 2009). The ventral side of each male was gently dried before 60 μL of an extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, and 10 mM Tris with pH 7.5) was added to the base of the male's gonopodium. This extender solution is designed to ensure sperm bundles stay intact and the sperm within remain dormant (Gardiner 1978). Light pressure was then applied to the male's abdomen to release the sperm bundles into the extender medium (Matthews et al. 1997). The sperm were activated with 60 μL of a 150 mM KCL solution (Billard et al. 1990) containing 2 mg/L BSA to prevent sperm from sticking to the glass slide (Pitcher et al. 2007). From each activated sample, individual sperm bundles were taken up in 3 μL of solution and placed into individual wells of a 12-cell multitest slide (MP Biomedicals, Aurora, OH). Slides and cover slips had been coated with 1% polyvinyl alcohol (PVA) to further prevent sperm from sticking to the slides (Wilson-Leedy and Ingermann 2007). Recordings of motile sperm were taken using a phase contrast microscope (Olympus BX41) at 97 frames per second (fps) under 400× magnification for 1 sec using a Prosilica EC-650 digital camera (resolution 640 × 480 px) and Norpix StreamPix 3.4 image capture software. Several 1-sec clips were taken in quick succession. Data were gathered and analyzed from 18 males, with 3–10 individual spermatozoa measured for speed and length per male.

Externally fertilizing species

Rainbowfish (Melanotaenia australis): Rainbowfish used in this study were captured in 2006 from a wild population in the Fortescue River near Wittenoom, Western Australia. Fish were returned to the lab and maintained in mixed-sex aquaria. Sexually mature males were taken from stock aquaria, given a lethal dose of anesthetic (clove oil), and the testes were removed. Dissected testes were used in this analysis as sperm could not be manually stripped from mature males. Testes were macerated and a ∼1 μL sample of milt (i.e., sperm and seminal plasma) was activated through dilution with 0.5 mL distilled water (for similar methods applied to other fish, see Fitzpatrick et al. 2007). A 2 μL sample of the activated sperm was immediately placed on a 12-cell multitest slide for recording motility, which was assessed using the video collection methods described earlier for guppies. From the 31 males sampled, motility and length data could be gathered from 14 males, and from these samples between three and 10 individual sperm were measured per male.

Mussel (Mytilus galloprovincialis): Mussels were collected from the Claremont Jetty, Western Australia, in July 2010, returned to the laboratory and maintained in seawater at 18–20°C. After collection, approximately 100 mussels were given a heat shock by placing them in a warm water bath (30°C) to stimulate gamete release. Following the onset of gamete release, males were removed from the warm water bath, washed in clean seawater to prevent contamination from other gametes, and placed in individual containers with 250 mL of sea water (following methods in Fitzpatrick et al. 2012). Males continued to release sperm in their individual containers, and fresh sperm was collected from individual males. A 2 μL sample from each male was placed on a 12-cell multitest slide for recording motility, which was assessed using the video collection methods described earlier for guppies. Data were collected from 20 individual males. For each male, length components and speed of between seven and 21 individual spermatozoa were recorded.

Quacking frog (Crinia georgiana): Frogs were collected during the winter breeding season from populations in Western Australia. Sperm extractions were carried out following the methods of Dziminski et al. (2010). Briefly, male frogs were killed (double pithing) and their testes were removed and crushed in Petri dishes containing chilled simplified amphibian ringer (SAR) to prevent activation of the sperm (Dziminski et al. 2010). Sperm were activated by the addition of fresh pond water, and motility was recorded using a Leica DICOMAR 3CCD digital video recorder mounted on a Leica DME compound microscope, using 100× magnification at 13°C (Dziminski et al. 2010). Sperm from 16 males could be measured adequately from the videos, with between three and 10 individual spermatozoa measured per male.

SPERM MORPHOLOGY

Three components of sperm length were measured: head length (HL), head width (HW), and flagellum length (FL). From these measurements it was possible to estimate total sperm length (TL = HL + FL) and head to flagellum ratio (HL:FL = HL/FL). Head morphology can also be an important determinant of swimming trajectory (Gillies et al. 2009; Gadelha et al. 2010), so we therefore calculated head volume (HV) for all species based on the assumption that the head was an ellipsoid (Humphries et al. 2008, see Table S1 for additional details). Head volume was not calculated for mussels as limitations in video resolution meant it was not possible to accurately measure HW for this species. We were unable to include sperm midpiece morphology (i.e., width, length, or volume) in our analyses as this morphological feature was impossible to clearly distinguish from the sperm head for the majority of species examined given the magnification level required to capture sperm motility videos of sufficient quality for analyses. We refrained from using principal component analyses (PCA) to reduce sperm morphology traits into noncorrelated predictor variables for two primary reasons: first, we were interested in comparing the direction and strength of the effect of each sperm morphology trait on sperm swimming speed; and second, we were concerned that a PCA approach would obscure meaningful results in cases when potentially correlated sperm morphology traits exerted opposing effects on sperm swimming speed (indeed, this was often the case; see Table 1).

SPERM MOTILITY ANALYSIS

Where appropriate, digital videos of motile sperm were edited into 1–2 sec clips using QuickTime Pro (version 10.0), then converted into image stacks so that sperm motility could be analyzed using NIH ImageJ (version 1.42o) with the computer-assisted sperm analysis (CASA) plugin (Wilson-Leedy and Ingermann 2007). Once imported into ImageJ, image stacks were prepared by applying the "find edges" and "threshold" functions before isolating the focal cell and running CASA. Only one cell was analyzed at a time and all other sperm were cleared from the clip by isolating the focal cell and using the "clear outside" function to remove all other cells from the clip. Once all other cells and any background noise had been removed from around the focal spermatozoan, the speed of the isolated cell could be recorded using CASA. Species-specific CASA software settings were determined for optimal sperm tracking (see Table S1). Three highly colinear sperm speed measurements were recorded: average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL). We used PCA to incorporate as much information as possible in our measure of sperm velocity. Each species-specific PCA returned a single principal component (PC1) with an eigenvalue >1, which explained 70.9–95.1% of the variation in sperm velocity measures (see Table S2) [Correction added on November 13, 2013, after first online publication: In the preceding sentence, 71.9–96.1% was corrected to 70.9–95.1%.]. Thus, PC1 values were used as composite sperm velocity measures in all analyses. Analyzing data using VCL, VAP, or VSL as measures of sperm swimming speed produced qualitatively similar results as PC1 values (see Tables S3–S5 for full details of all speed measures). To measure sperm length, the zoom function was used to magnify the focal cell (up to 200%) with the "brightness" and "contrast" altered as necessary to allow the head and flagellum to be seen clearly (see Supporting Information for details of technique used to measure morphology). A minimum of three spermatozoa per male were measured at three points along their path (at the start, middle, and toward the end). An average of the three measures was used for each cell in all analyses.

Statistical Analyses

We explored the relationships between sperm length (individual components, composite measures, and ratios) and sperm swimming speed (PC1) using within-subject centering (Van de Pol and Wright 2009), a technique that enables the separation of within-male from between-male effects when assessing sperm length–speed relationships (see Fitzpatrick et al. 2010). Following van de Pol and Wright (2009) we used mixed-effects models with intra- and inter-male sperm length components fitted as fixed effects and use their terminology of "within" and "between" male effects. We entered male identity as a random effect and fitted both (i) random intercept and (ii) random intercept and slope models to all data sets. The random intercept model (eq. 2 of Van de Pol and Wright 2009) allowed the magnitude of speed measures to vary between males, and in the simplest case this model accounts for the possibility that two males could have sperm that are of similar length, but that one of the males might have generally faster sperm. In contrast, the random slopes and intercepts model (eq. 4 in Van de Pol and Wright 2009) allows both the magnitude of the speed measure and its relationship to length

Table 1. Results for sperm length–single principal component (PC1) relationships from mixed model centering allowing random effect intercepts for between- and within-male analysis. HW = head width, HL = head length, HV = head volume (no HV measure for mussel data), FL = flagella length, TL = total length, HL:FL = ratio of head length to flagellum length, n_{total} = total number of males df_{bw} = degrees of freedom between-male ($df_{w/in}$ = degrees of freedom within-male), t = effect estimate from linear model. Significant correlations ($P \leq 0.05$) are presented in bold with 95% confidence intervals (95% CI) calculated for each effect size (r). [Correction added on November 13, 2013, after first online publication: Table 1 was corrected to reflect reanalysis of our data revealed, which revealed some differences in the results from sperm length-swimming speed relationships from mixed model centering analyses.].

			Between-male effects					Within-male effects				
Species	n_{total}	Sperm trait	df_{bw}	t	P	r	95% CI	$df_{w/in}$	t	P	r	95% CI
(a) internally fertilizing species												
Human	29	HW	27	0.13	0.90	0.02	– 1.83, 2.09	78	– 2.05	**0.04**	**– 0.27**	**– 4.02, –0.05**
		HL	27	– 0.39	0.70	– 0.05	– 2.35, 1.58	78	0.80	0.43	0.11	– 1.17, 2.76
		HV	27	0.00	1.00	0.00	– 1.96, 1.96	78	– 1.32	0.19	– 0.18	– 3.29, 0.65
		FL	27	0.16	0.87	0.02	– 1.80, 2.12	78	– 0.80	0.43	– 0.11	– 2.76, 1.17
		TL	27	0.08	0.94	0.01	– 1.88, 2.04	78	– 0.60	0.55	– 0.08	– 2.56, 1.36
		H.F	27	– 0.47	0.64	– 0.06	– 2.43, 1.50	78	1.08	0.28	0.15	– 0.89, 3.05
Emu	9	HW	7	2.57	**0.04**	**0.28**	**0.15, 4.88**	80	2.68	**0.01**	**0.29**	**0.67, 4.67**
		HL	7	2.19	0.06	0.23	– 0.13, 4.39	80	7.55	**<0.01**	**0.61**	**5.25, 9.81**
		HV	7	3.42	**0.01**	**0.36**	**0.73, 5.99**	80	4.85	**<0.01**	**0.48**	**2.73, 6.93**
		FL	7	– 0.91	0.39	– 0.10	– 2.89, 1.14	80	– 3.32	**<0.01**	**– 0.33**	**– 5.34, –1.29**
		TL	7	0.23	0.83	0.02	– 1.74, 2.18	80	0.88	0.38	0.09	– 1.09, 2.84
		H.F	7	2.12	0.07	0.21	– 0.18, 4.30	80	8.43	**<0.01**	**0.63**	**6.06, 10.76**
Guppy	18	HW	6	– 0.46	0.65	– 0.06	– 2.42, 1.51	46	– 0.36	0.72	– 0.05	– 2.31, 1.61
		HL	15	0.12	0.90	0.01	– 1.84, 2.08	79	0.35	0.73	0.04	– 1.61, 2.31
		HV	6	0.85	0.40	0.11	– 1.12, 2.82	46	1.05	0.30	0.13	– 0.93, 3.01
		FL	16	– 0.56	0.58	– 0.06	– 2.52, 1.40	94	– 0.06	0.95	– 0.01	– 2.02, 1.90
		TL	16	– 1.09	0.28	– 0.11	– 3.05, 0.88	94	– 0.38	0.70	– 0.04	– 2.34, 1.58
		H.F	15	0.21	0.84	0.02	– 1.76, 2.16	79	0.40	0.69	0.04	– 1.56, 2.36
(b) externally fertilizing species												
Mussel	20	HL	18	– 0.54	0.60	– 0.03	– 2.50, 1.44	255	– 0.57	0.57	– 0.03	– 2.53, 1.39
		FL	18	2.74	**0.01**	**0.16**	**0.55, 4.86**	255	2.08	**0.04**	**0.12**	**0.11, 4.04**
		TL	18	2.73	**0.01**	**0.16**	**0.55, 4.85**	255	1.99	**0.047**	**0.12**	**0.03, 3.96**
		H.F	18	– 2.05	**0.05**	**– 0.12**	**– 4.10, 0.04**	255	– 1.77	0.08	– 0.10	– 3.74, 0.20
Rainbow fish	14	HW	12	0.05	0.96	0.00	– 1.91, 2.01	69	– 2.66	**0.01**	**– 0.25**	**– 4.66, –0.64**
		HL	12	– 0.96	0.34	– 0.09	– 2.92, 1.01	69	– 1.48	0.14	– 0.14	– 3.45, 0.50
		HV	12	– 0.41	0.68	– 0.04	– 2.37, 1.55	69	– 2.43	**0.02**	**– 0.22**	**– 4.42, –0.42**
		FL	12	1.12	0.27	0.11	– 0.86, 3.08	69	2.06	**0.04**	**0.20**	**0.06, 4.04**
		TL	12	0.98	0.33	0.10	– 0.99, 2.94	69	1.84	0.07	0.18	– 0.15, 3.82
		H.F	12	– 1.25	0.22	– 0.12	– 3.21, 0.73	69	– 2.41	**0.02**	**– 0.22**	**– 4.40, –0.40**
Frog	16	HW	14	– 0.57	0.58	– 0.06	– 2.53, 1.41	68	0.69	0.50	0.07	– 1.28, 2.65
		HL	14	– 0.18	0.86	– 0.02	– 2.14, 1.78	68	1.23	0.22	0.13	– 0.74, 3.20
		HV	14	– 0.39	0.70	– 0.04	– 2.35, 1.58	68	0.95	0.35	0.10	– 1.02, 2.91
		FL	14	0.60	0.56	0.07	– 1.38, 2.56	68	0.83	0.41	0.09	– 1.14, 2.79
		TL	14	0.24	0.81	0.03	– 1.73, 2.20	68	1.50	0.14	0.16	– 0.48, 3.47
		H.F	14	– 0.46	0.65	– 0.05	– 2.42, 1.52	68	0.62	0.54	0.07	– 1.35, 2.58

components to vary between males. Data were log transformed (natural log) to help account for positive correlations between means and variances. Mixed-effects models were fitted to log-transformed data using the nlme package (Pinheiro et al. 2013) in R version 2.13.0 (R Development Core Team 2011). We used analysis of variance to distinguish between the two models for each data set, choosing the one that explained the most variance, and the model with the lowest AIC score if the amount of variance explained did not differ significantly. The random intercept model explained the most variance in 95.6% of the models (260 of 272 models among all sperm components and species) [Correction added on November 13, 2013, after first online publication: In the

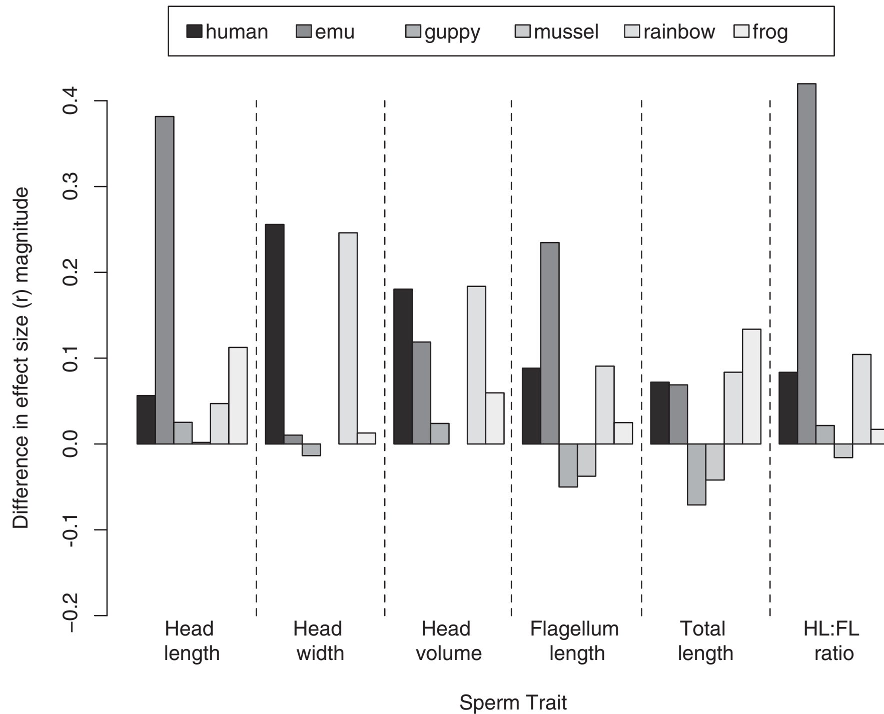

Figure 1. Differences in absolute effect size (within-male − between-male) for the relationship between sperm length and speed (single principal component, PC1) for each sperm trait analyzed in each of the species examined. Positive values indicate that the effect size for the relationship between sperm length and speed was greater in the within-male analyses and negative values indicate larger effect sizes in the between-male analyses. These data are for illustrative purposes only. Effect sizes and statistical models are presented in full in Table 1 [Correction added on November 13, 2013, after first online publication: Figure 1 was corrected to reflect reanalysis of our data].

preceding sentence, 97.4% of the models (226 of 232 was corrected to 95.6% of the models (260 of 272]. This analysis indicated that the random intercept model explained most of the variance for most of the data comparing sperm length to sperm velocity (PC1). In recognition of the small sample size, we did not use Bonferroni corrections when analyzing subsets of the data as this can increase type II errors (Nakagawa 2004). Instead, we calculated effect size (r) and 95% confidence intervals (CIs) for the mixed-effects models using the methods given in Nakagawa and Cuthill (2007) allowing us to quantify the effect size for both fixed effect components of the model (within-male and between-male variation) [Correction added on November 13, 2013, after first online publication: We discovered a conversion error in our raw data file following the on-line publication of this paper. We have reanalyzed our data and indicate differences between our original and revised results. While some of the specific results are altered the broad patterns reported in this paper remain intact].

Results

The mixed-effects models revealed significant correlations between sperm length components and swimming speed (PC1) in both internally and externally fertilizing species in four of the six species examined in this study (Table 1, also see Tables S3–S5 for all results). However, the strength and direction of these sperm length–speed correlations depended on which sperm morphology trait was examined, whether correlations were assessed at the between-male level versus within-male level, and whether a species exhibited internal versus external fertilization. The mixed-effects models also revealed that within-male effects were stronger and more prevalent than between-male effects (Fig. 1 and Table 1). For example, in humans significant within-male correlations between sperm size and speed were evident but no such correlations were apparent between-males. Similarly, in emu and rainbowfish various sperm components were significantly correlated with sperm swimming speed at the within-male, but not between-male, level. These results suggest that sperm length–speed correlations are more readily apparent within a male's ejaculate rather than when comparing these variables among males. Neither guppies nor frogs exhibited significant correlations between any measure of sperm length and sperm swimming speed (Fig. 2, Table 1, Tables S3–S5, and Fig. S5). Therefore, we focus our discussion of the results primarily on those species where significant correlations were detected.

SPERM HEAD WIDTH, LENGTH, AND VOLUME

Although sperm traits–speed correlations were present in three species, these correlations did not exhibit clear patterns across

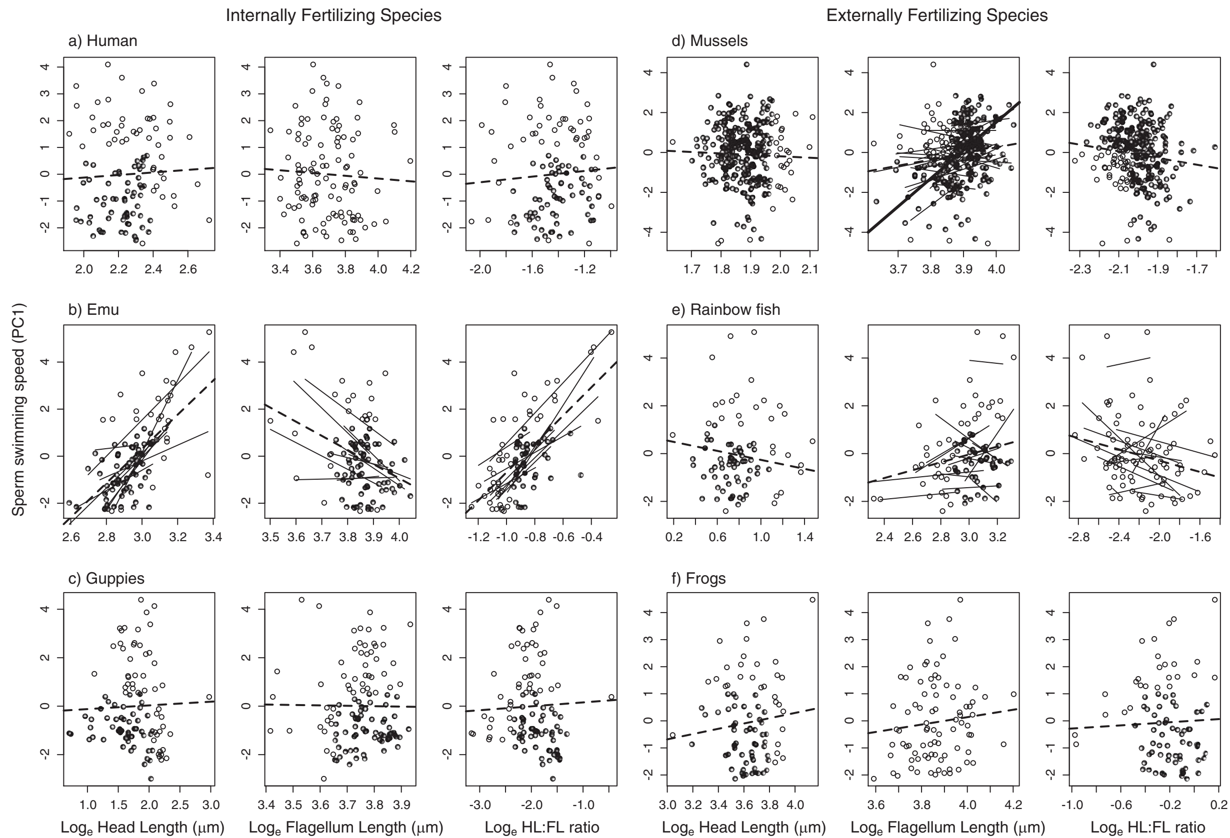

Figure 2. Relationships between sperm head length, flagellum length, ratio of head length-to-flagellum length (HL:FL), and sperm swimming speed (single principal component, PC1) within- and between-males across internally (left three columns) and externally (right three columns) fertilizing species. Thick solid black lines describe significant between-male effects from the mixed-effect model, whereas thin solid lines depict individual within-male slopes from linear regressions as a visual guide to significant within-male effect (marginal significance within-male effects for human and rainbow fish are included $P = 0.06$). The dashed black line depicts the estimate from a standard mixed model of the effect of the length measure on the speed measure (as a guide only) [Correction added on November 13, 2013, after first online publication: Figure 2 was corrected to reflect reanalysis of our data].

species or fertilization modes. At the within-male level, human and rainbowfish sperm with wider and more voluminous heads swam slower than sperm with thin heads and smaller volumes (Table 1). However, there were no significant between-male correlations detected for humans or rainbowfish. In contrast, both within- and between-males, emu sperm with wider, longer, and more voluminous heads swam faster than sperm with heads that were thin, short, and with smaller volumes. In mussels, head length (head width and volume were not measured for this species) was not significantly related to sperm swimming speed either within- or between-males [Correction added on November 13, 2013, after first online publication: The preceding paragraph was corrected to reflect changes in the data following reanalysis. Sperm head length was not significantly correlated with swimming speed in rainbowfish].

SPERM FLAGELLUM AND TOTAL LENGTH

Sperm flagellum and total length exhibited significant correlations with swimming speed (PC1) in emus, rainbowfish, and mussels (Figs. 2, S5, and Table 1). However, correlations between flagellum or total length and swimming speed appeared to differ between internal and external fertilizers, with negative correlations being detected in internally fertilizing species and positive correlations in externally fertilizing species. Specifically, in internally fertilizing emu, sperm with longer flagella swam slower at the within-male level (Figs. 2, S5, and Table 1). No correlations between flagellum or total length and sperm speed were detected for humans or emus at the between-male level. In contrast, sperm with a longer flagellum and total length swam faster at the between-male level in externally fertilizing mussels. Similarly, both flagellum and total length were positively correlated with swimming speed at the within-male level for mussels, whereas a positive correlation between flagellum length and sperm swimming speed was also detected for rainbowfish at the within-male level (Fig. 2 and Table 1) [Correction added on November 13, 2013, after first online publication: The preceding paragraph was corrected to reflect changes in the data following reanalysis. There was no significant correlations between sperm flagellum or total

length and swimming speed at the between-male level in rainbowfish or the within-male level in humans. At the within-male level, sperm flagellum length was positively correlated with swimming speed in rainbowfish].

SPERM HEAD LENGTH:FLAGELLUM LENGTH RATIO

The sperm head length:flagellum length ratio (HL:FL) was significantly correlated with swimming speed (PC1) at the within-male level for emus and rainbowfish, and there was a negative trend ($P = 0.08$) at the within-male level for mussels (Fig. 1 and Table 1) [Correction added on November 13, 2013, after first online publication: The preceding sentence was corrected to reflect changes in the data following reanalysis. HL:FL was significantly correlated with swimming speed at the within-male level for emus and rainbowfish and weakly correlated in mussels. HL:FL was not correlated with swimming speed in humans]. The HL:FL ratio was also significantly correlated with sperm swimming speed between-males for mussels (Table 1). Unexpectedly, the direction of the HL:FL ratio–speed correlations also differed between internal and external fertilizers. In externally fertilizing mussels and rainbowfish, there was a negative relationship between HL:FL and swimming speed at the within-male level, and a negative relationship between HL:FL and swimming speed at the between-male level for mussels. This indicates that sperm with shorter heads relative to their flagellum (or with a longer flagellum relative to their heads) swam faster. In contrast, for internally fertilizing emu, sperm swam faster when the head was large in relation to the flagellum (or the flagellum was small in relation to the head) at the within-male, but not between-male level [Correction added on November 13, 2013, after first online publication: In the preceding sentence emu and human was corrected to emu].

Discussion

More than two decades ago, Gomendio and Roldan (1991) put forward the influential hypothesis that sperm competition should lead to the production of longer sperm, basing their argument on the assumption that there is an underlying relationship between sperm size and performance. While there is now compelling evidence that sperm competition promotes the evolution of longer sperm in a variety of taxa (reviewed by Gomendio and Roldan 2008; Pitnick et al. 2009; Simmons and Fitzpatrick 2012), intraspecific evidence of a relationship between sperm size and speed remains ambiguous (Humphries et al. 2008). Here, by using a method to examine sperm morphology and performance at the individual cell level, our results suggest that correlations between sperm size and speed are indeed present. Correlations between various sperm morphological components and sperm swimming speed were present in four of the six species examined in this study—a ratio that far surpasses the two of nine species where correlations were present in Humphries et al.'s (2008) review. Although our results remain suggestive and require further validation, particularly given the limited number of species examined in this study and the fact that no clear pattern emerged between sperm size and speed in two of the species assessed, they nevertheless suggest that sperm length–speed correlations are likely to be more prevalent than current evidence suggests. Our results also demonstrate that relationships between sperm morphology and performance are not straightforward. Specifically, although our estimates of sperm flagellum/total length, head length/width/volume and the sperm head:flagellum length ratio were often associated with sperm swimming speed, the strength and direction of these correlations differ within- and between-males, among species, and between fertilization modes.

The idea that sperm with longer flagella are capable of achieving greater swimming speeds is generally applied to both internally and externally fertilizing species. However, our findings raise some questions about the generality of this assumption. In this study, where significant correlations were detected between sperm size and speed, the direction of correlations between sperm flagellum/total length and sperm swimming speed differed between the internally and externally fertilizing species. This suggests that these relationships may be influenced by the microenvironment in which sperm operate. In externally fertilizing mussels and rainbowfish, the positive correlations detected between flagellum/total sperm length and sperm swimming speed when assessed between-males (in mussels) and within-males (in mussels and rainbowfish) is in keeping with the traditional view that longer sperm swim faster (sensu Gomendio and Roldan 1991) [Correction added on November 13, 2013, after first online publication: In the preceding sentence between-males (in mussels and rainbowfish) and within-males (in rainbowfish) was corrected to between-males (in mussels) and within-males (in mussels and rainbowfish)]. However, in internally fertilizing emus, within-male effects revealed that sperm with longer flagella swam more slowly than shorter sperm produced in the same ejaculate [Correction added on November 13, 2013, after first online publication: The preceding sentence was corrected to indicate that the negative relationship between sperm flagellum length and speed was only detected in emus]. These unexpected results provide some of the first evidence that sperm flagellum/total length and speed are *negatively* correlated in some species and to our knowledge there is no theoretical basis for predicting such relationships. The only other evidence of a negative relationship between sperm flagellum length and speed that we are aware of is from a recent study of *Drosophila melanogaster*, another internally fertilizing species, where Lüpold et al. (2012) demonstrated both that longer sperm swim more slowly and that males with long and slow sperm experience a fertilization advantage in competitive matings. Reconciling these intraspecific negative relationships

between sperm flagellum length and swimming speed with the emerging macroevolutionary patterns of increasing sperm flagellum length in response to sperm competition represents a major challenge for future studies.

Using the ratio between the sperm head and flagellum length as a measure of sperm morphology in our correlations provided further suggestive evidence that the phenotypic relationships between sperm components and swimming speed are influenced by fertilization mode. In externally fertilizing mussels and rainbowfish faster swimming sperm had shorter heads relative to their flagellum, whereas in internally fertilizing emu sperm with longer heads relative to their flagellum swam faster [Correction added on November 13, 2013, after first online publication: The preceding sentence was corrected to indicate that no relationship between HL:FL and sperm speed was observed in humans]. When proposing that the sperm head:flagellum ratio should, in theory, predict sperm swimming speed, Humphries et al. (2008) suggested that this measure may be more applicable to externally fertilizing species. Indeed, the negative relationship detected in externally fertilizing species here is in keeping with the direction of the effect in another externally fertilizing species, the sea urchin *Heliocidaris erythrogramma* (Fitzpatrick et al. 2010). However, the positive relationships between the sperm head:flagellum ratio and swimming speed in internally fertilizing species are at odds with previous findings. In the house sparrow *Passer domesticus* (Helfenstein et al. 2010), zebra finch *Taeniopygia guttata* (Mossman et al. 2009), and across mammals (Tourmente et al. 2011), sperm with shorter heads relative to their flagellum (i.e., larger flagellum in relation to head length) swam faster—a pattern that contrasts with our findings for internally fertilizing species but is consistent with our findings for externally fertilizing species. Humphries et al. (2008) predicted such inconsistent relationships between head:flagellum ratios and speed in internal fertilizers because in these species female effects are likely to play an important role in influencing sperm performance.

Although the inconsistent relationships between sperm flagellum/total length or head:flagellum ratios and speed in internally and externally fertilizing species were unanticipated, it seems probable that these contradictory patterns could be linked to how sperm operate in the microenvironment of the female reproductive tract of internal fertilizers compared with the relatively less viscous environment experienced by sperm of external fertilizers. In particular, the physics of motion in viscoelastic fluids, such as those found in the female's reproductive tract, can influence sperm performance because the elasticity in the medium (not present in water) introduces new forces acting on a moving head and flagellum by pushing back on the sperm in a way that other liquids do not (Lauga 2007). The complex microstructure of mucus in the female reproductive tract has been shown to influence flagellar waveform and sperm swimming trajectory (Lauga 2007). The relationship between sperm flagellar beat frequency, swimming speed, and reproductive tract mucus remains to be fully understood, but it is clear that relationships are complex and may be species specific. Although there is ample evidence that the female's reproductive tract influences the evolution of sperm morphology (Pitnick et al. 2009), our results suggest that the underlying relationship between sperm morphology and performance might also be influenced by the females' reproductive tract. Although this awaits further validation (ideally *in vivo*) in other internally fertilizing species, it is interesting to note that recent work has established how the ability of sperm to orient and swim against the flow of oviductal fluid is a major taxic factor in both mouse and human sperm (Miki and Clapham 2013). Thus, the influence of the female's reproductive tract on sperm may help to explain the short sperm advantage observed in competitive matings in some internally fertilizing species (including mice, dung beetles, crickets, and fruit flies; reviewed by Simmons and Fitzpatrick 2012).

The importance of the sperm head in governing sperm fluid dynamics, and thus sperm performance, has received increased attention recently (e.g., Humphries et al. 2008; Gillies et al. 2009; Kirkman-Brown and Smith 2011). In this study, the three measures of sperm head morphology examined (width, length, and volume), produced almost identical results when correlated with sperm swimming speed. Thus, any of these head measures may be appropriate for quantifying sperm head morphology in future studies. However, we found both positive and negative relationships between sperm head traits and sperm swimming speed in three of the species examined (humans, emu, and rainbowfish). Contrasting the pattern observed for flagellum/total length and for head:flagellum ratios, there were no consistent differences in the direction of the sperm head–speed correlations between internally and externally fertilizing species. Instead, sperm head morphologies were significantly negatively related to speed when assessed within-males in human and rainbowfish, and positively related to speed both within- and between-males in emu. While assessing sperm head morphology–speed correlations in a greater number of species may help to resolve the present inconsistent pattern, these results could also support Humphries et al.'s (2008) argument that the ratio of head:flagellum length captures more meaningful information than assessing a single sperm component.

The suggestion that variation in the microenvironment in which sperm operate generates differences in the relationship between sperm morphology and performance must be tempered by considering some of the limits of our study. An obvious difference between the internally and externally fertilizing species examined in this study lies in our ability to experimentally examine sperm performance in a biologically realistic environment. For internal fertilizers, *in vitro* measurements of sperm velocity are largely divorced from the conditions in which sperm operate, as it is

currently impossible to assess sperm swimming speed inside the female's reproductive tract for most species (for an exception see Manier et al. 2010; Lüpold et al. 2012). In contrast, *in vitro* measurements of sperm velocity in external fertilizers more readily mimic natural fertilization conditions, although here too important female effects (e.g., sperm–ovarian fluid interactions, Turner and Montgomerie 2002) are not always incorporated into analyses. Despite this important caveat, which is common to all studies assessing sperm performance *in vitro*, there are reasons to think that the results presented here are biologically valid. For example, the negative relationship between sperm flagellum length and speed, we detected *in vitro* for an internal fertilizing species matches the relationship recently reported *in vivo* in the internally fertilizing fruit fly *D. melanogaster* (Lüpold et al. 2012) [Correction added on November 13, 2013, after first online publication: In the preceding sentence two was corrected to an]. In addition, several previous studies of internally fertilizing species have demonstrated that *in vitro* measures of sperm performance, imperfect as they may be, predict fertilization success and are therefore providing biologically meaningful information (see Table 2 in Simmons and Fitzpatrick 2012).

An important limitation of our study is that we were unable to assess how the sperm midpiece—the primary site of energy production—influences sperm swimming speed. However, incorporating sperm energetics into an assessment of sperm performance is far from straightforward. The few studies that have investigated the midpiece have produced contradictory results, with reports of positive (Gil et al. 2009; Firman and Simmons 2010), negative (Malo et al. 2006), and no relationships (Birkhead et al. 2005; Mossman et al. 2009) between midpiece size and sperm swimming speed. In addition, sperm are nourished by oxidative phosphorylation, glycolysis, lipid metabolism, and/or a combination of these energy sources, and how sperm derive energy remains a hotly debated topic that may differ greatly among species or taxa (Ford and Rees 1990; Mansour et al. 2003; Turner 2006; Ford 2006; Cosson et al. 2008; Storey 2008; Cummins 2009). Consequently, a robust examination of the sperm midpiece, including approaches that incorporate the biochemical underpinnings of sperm motility into studies assessing sperm swimming speed, represent an important challenge for the future (but see Rowe et al. 2013 and Tourmente et al. 2013, for recent studies examining how selection acts on sperm energetics). However, such an approach would necessitate an examination of species where all sperm components (i.e., head, midpiece and flagellum) can be distinguished clearly, which was not the case in the species examined here.

In addition, although we advocate the use of our methodology in future studies assessing the link between sperm size and speed, it is worth noting some of the methodological considerations that arose from our examination of within- and between-male correlations. Because our within-male measure of sperm morphology and swimming speed were sampled from the same video sequence, this may have inadvertently introduced bias, whereby variation in sampling procedures could have led to inflated variation between-males that was minimized within-males. Such a sampling effect may explain why sperm length–speed correlations are more evident when assessed using within-male effects rather than between-male effects (see Fig. 1). Future studies assessing within- and between-male effects may consider measuring sperm motility and swimming speed for within-male effects from multiple sperm video sequences (each prepared independently) to make the level of potential experimentally induced variation in within-male samples more closely match those from between-male samples.

A final caveat to our argument that sperm size–speed correlations are influenced by fertilization mode is that no such correlations were detected in internally fertilizing guppies and externally fertilizing frogs. It is unclear why sperm components were unrelated to sperm motility in these two species. In the myobatrachid frog studied here, as with most frogs, sperm are shed onto the egg clutch and must penetrate and swim through the jelly coat of the egg (Reinhart et al. 1998), a very different "external" environment to that experienced by rainbow fish and mussels. Moreover, sperm differ dramatically from the "typical" sperm morphology and swimming mechanism, as sperm are propelled by an undulating membrane that is supported by a longitudinal axial fiber that stretches from the base of the head to the tip of the "tail" (Jamieson et al. 1993). However, this is not the case in guppy sperm, which conform to the "typical" sperm morphology, making it unclear why sperm length–speed correlations were absent in this species (although we note that correlations between sperm head length and swimming speed have been detected previously in guppies: Pitcher et al. 2007). Further examinations of sperm length–speed correlations using the approaches employed here across an increasing taxonomic breadth may help to clarify how differences in fertilization modes influences the underlying relationship between sperm size and speed.

The difficulties in detecting sperm length–speed correlations have been a vexing problem for evolutionary biologists, as explaining the macroevolutionary pattern of increasing sperm size in response to sperm competition has been difficult without evidence of a clear relationship between sperm size and speed at the intraspecific level where selection acts. Consequently, the positive and negative relationships observed between sperm components and swimming speed detected here serve to both assuage and complicate our understanding of how sperm competition shapes sperm evolution. Among external fertilizers, the positive correlation between sperm flagellum length and swimming

speed is consistent at the intraspecific level with the pattern typically observed at the macroevolutionary scale (e.g., in fish see Fitzpatrick et al. 2009). However, the negative relationship between sperm flagellum length and swimming speed observed in emu directly contradicts macroevolutionary patterns observed in mammals and birds (reviewed by Simmons and Fitzpatrick 2012) [Correction added on November 13, 2013, after first online publication: In the preceding sentence humans and emu was corrected to emu]. It is unclear what is causing this discrepancy in internally fertilizing species but one possibility that deserves further attention is that females are exerting cryptic female choice on sperm morphology that selects for larger sperm, which possibly live longer, rather than selecting for shorter but faster swimming sperm. This scenario may explain the selective forces that drive the observed macroevolutionary patterns in internally fertilizing species, irrespective of the functional relationship between sperm size and speed. A further complication arises from the fact that sperm length–speed correlations are more prevalent within, rather than between, males. Because selection acts on ejaculate traits between-males, rather than within-males, the reduced prevalence of sperm length–speed correlations between-males suggests that males may experience weak or no selection to produce larger sperm than rival males. This would once again be at odds with the macroevolutionary pattern commonly observed but may explain why selection lines consistently fail to find changes in sperm morphology in response to selection histories that alter the risk of sperm competition (e.g., Pitnick et al. 2001; Firman and Simmons 2010).

Although the implications of our results for internal fertilizers may cast doubt on the role of sperm size in sperm competition, it is also clear from our results that the relationships among sperm components are often contradictory. One way to resolve these contradictions may be to examine a group of closely related species that experience different risks of sperm competition to see how mating systems influence the within- and between-male sperm length–speed correlations. Specifically, reconciling intraspecific and macroevolutionary patterns would be easier if highly promiscuous species exhibit within- and between-male sperm length–speed correlations whereas monogamous species only exhibit within-male correlations. In addition, future studies that assess the factors that influence trade-offs between sperm size and number (sensu Immler et al. 2011) when examining sperm size and speed correlations will help to clarify whether and how the oftentimes opposing selective pressures on the production of larger and/or numerous sperm effects the functional relationship between sperm morphology and performance. Although the full implications for our results to the wider field of sperm evolution remain ambiguous, reconciling the contradictory relationships between sperm morphology and speed represents a major research challenge for future studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website . [Correction added on November 13, 2013, after first online publication: The Supporting Information was corrected to reflect reanalysis of our data.]:

Figure S1. Representative frames from video recordings for each species analyzed.
Figure S2. Representative images for each species analyzed once zoomed in on focal sperm cell.
Figure S3. Application of threshold function: (a) alter threshold calibration until sperm heads become red and (b) apply the threshold function to remove background noise from the original video, leaving only sperm heads, which are now black.
Figure S4. Isolating focal sperm: (a) only the focal sperm remains once all other sperm have been removed from every frame and (b) visualization of the actual path of the focal sperm.
Figure S5. Relationships between sperm head width, head volume, total sperm length, and sperm swimming speed (PC1) within- and between-males across internally (left two columns) and externally (right two columns) fertilizing species.
Table S1. Summary of species-specific computer-assisted sperm analysis (CASA) software settings (CASA—http://rbs.info.nih.gov/ij/plugins/casa.html) used to record individual cell velocity using the ImageJ version 1.44o CASA plugin (Wilson-Leedy and Ingermann 2007).
Table S2. Principal component analysis (PCA) output with PC1 eigenvectors, eigenvalues, and percentage of variance explained by PC1 presented for each species.
Table S3. Results for sperm length–curvilinear velocity (VCL) relationships from mixed model centering allowing random effect intercepts for between- and within-male analysis.
Table S4. Results for sperm length–average path velocity (VAP) relationships from mixed model centering allowing random effect intercepts for between- and within-male analysis.
Table S5. Results for sperm length–straight-line velocity (VSL) relationships from mixed model centering allowing random effect intercepts for between- and within-male analysis.