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Sperm competitiveness in frogs: slow and steady wins the race

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When sperm compete to fertilize available ova, selection is expected to favour ejaculate traits that contribute to a male's fertilization success. While there is much evidence to show that selection favours increased numbers of sperm, only a handful of empirical studies have examined how variation in sperm form and function contributes to competitive fertilization success. Here, we examine selection acting on sperm form and function in the externally fertilizing myobatrachid frog, *Crinia georgiana*. Using *in vitro* fertilization techniques and controlling for variation in the number of sperm contributed by males in competitive situations, we show that males with a greater proportion of motile sperm, and motile sperm with slower swimming velocities, have an advantage when competing for fertilizations. Sperm morphology and the degree of genetic similarity between putative sires and the female had no influence on competitive fertilization success. These unusual patterns of selection might explain why frog sperm typically exhibit relatively slow swimming speeds and sustained longevity.

Keywords: sperm competition; polyandry; genetic compatibility; sperm velocity; sperm length; frogs

1. INTRODUCTION

Whenever the sperm of two or more males compete to fertilize available ova, selection is expected to favour ejaculate traits that maximize an individual's fertilization success (Parker 1970). Theory predicts that selection from sperm competition should favour increased sperm production (Parker 1998; Parker & Ball 2005), a prediction for which there is now considerable support (Birkhead & Møller 1998; Byrne *et al.* 2002). However, there is every reason to suspect that selection should also act on sperm quality (Snook 2005). Indeed, comparative studies from a variety of taxa suggest that variation among species in the strength of selection from sperm competition can be associated with variation in sperm morphology (Gomendio & Roldan 1991; Byrne *et al.* 2003; Gomendio & Roldan 2008; Immler *et al.* 2008), swimming speed (Fitzpatrick *et al.* 2009), fertilization capacity (Gomendio *et al.* 2006) and viability (Hunter & Birkhead 2002).

Surprisingly, few studies have actually examined directly the influence of sperm quality on competitive fertilization success. Perhaps not surprisingly, relative sperm motility (Birkhead *et al.* 1999; Denk *et al.* 2005; Pizzari *et al.* 2008) and viability (García-González & Simmons 2005) are major determinants of fertilization success when sperm from two males compete in fowl and crickets, respectively. In *Drosophila*, males with longer sperm out-compete their shorter sperm rivals for fertilizations (Miller & Pitnick 2002), whereas in crickets (Gage & Morrow 2003), dung beetles (García-González & Simmons 2007) and mice (Firman & Simmons 2008), males with shorter sperm are more successful. With internal fertilizers, it is not always possible to attribute selection to sperm competition *per se* because of potential female effects. Indeed, in *Drosophila* and dung beetles, it seems that spermathecal dimensions mediate the advantages associated with long and short sperm, respectively (Pattarini *et al.* 2006; García-González & Simmons 2007). External fertilizers are ideal for studying sperm competition acting directly on sperm characteristics because *in vitro* fertilization (IVF) techniques can be used to control for variation in sperm numbers, the influences of potential positional advantages males might have during spawning and for female, though not ova, effects. Such an approach using externally fertilizing fishes has documented a competitive advantage for faster swimming sperm in salmon (Gage *et al.* 2004), Arctic charr (Liljedahl *et al.* 2008) and walleye (Casselman *et al.* 2006).

Here, we use IVF techniques to examine the influence of sperm morphology and motility on the competitive fertilization success of the externally fertilizing myobatrachid frog, *Crinia georgiana*. Sperm competition is particularly intense in *C. georgiana*, where up to 64 per cent of the females that arrive at a spawning site can be amplexed by two to seven males (Byrne & Roberts 2004). There is considerable among male variation in the morphology of sperm (Hettyey & Roberts 2006) and in sperm motility (Simmons *et al.* 2009). We show that for *C. georgiana*, both the relative proportion of sperm cells that are motile and the relative swimming velocity of motile sperm are significant predictors of a male's paternity success when in competition for fertilizations.

2. MATERIAL AND METHODS

(a) *Frog collection and sperm extraction*

On wet nights during the winter breeding season (July and early August), haphazardly located, non-amplexed, gravid

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female and calling male *C. georgiana* were collected from the same populations, two near Perth and three near Nornalup, in the southwest of Australia (there is no evidence of genetic differentiation over this geographical range; Edwards *et al.* 2007). In the laboratory, male frogs were killed by double-pithing, and their testes removed and weighed to the nearest 0.1 mg. Testes were crushed in 50 mm Petri dishes in 5–15 ml of chilled (13°C) simplified amphibian ringer (SAR), which does not activate sperm (Rugh 1962). Testes crushes have long been used to efficiently obtain high numbers of mature sperm in amphibian embryology (Rugh 1948), and this method of sperm collection has been widely used in amphibian fertilization and sperm quality studies (e.g. Browne *et al.* 1998; Edwards *et al.* 2004; Hettyey & Roberts 2006). Studies of frogs in the genus *Rana* have found no differences in the ultrastructure (Poirier & Spink 1971) or motility (Sliwa 1983) of sperm derived from testes crushes and those awaiting ejaculation in the seminal vesicles. In *C. georgiana*, normal rates of fertilization success and embryo development are achieved using sperm from testes crushes (Byrne & Roberts 2000; Dziminski *et al.* 2008), indicating that sperm stored in the testes of frogs are functionally mature. Petri dishes containing sperm suspension were placed on ice. The sperm concentration of each suspension was measured using an improved Neubauer haemocytometer and standardized by dilution with SAR to a concentration of 8×10^6 sperm ml^{-1}.

We injected 20 µl of sperm suspension into a 5 µm deep chamber on a slide kept at 13°C (ambient for breeding pairs in the field) on a stage cooler. To activate sperm, 20 µl of 13°C pond water was injected from the same side of the slide. Sperm motility was measured using the CEROS Sperm Analysis System v. 12 (Hamilton Thorne, Beverley, MA, USA). We recorded the proportion of cells that were motile and the average path velocity (VAP). We have shown elsewhere that there are consistent and significant differences between males in these parameters (Hettyey & Roberts 2007; Simmons *et al.* 2009). Slides were then left to air dry on the laboratory bench.

At the same time, the proportion of live sperm (sperm viability) was assessed using a live/dead assay (Molecular Probes). We mixed 5 µl of sperm suspension with 5 µl of 1 : 50 diluted 1 mM SYBR-14 with SAR on a slide. This was left to incubate in the dark for 10 min at room temperature, then 2 µl of 2.4 mM propidium iodide was added and incubated in the dark for a further 10 min. The slide with a coverslip was observed under a fluorescence microscope, and 500 sperm were scored as dead (red) or live (green). Later, using the air-dried samples, the head and tail lengths of 10 sperm from each male were measured to the nearest 0.01 µm using the software AxioVision v. 4.6 (Zeiss), with images taken under a Zeiss Axio-Imager microscope. Preliminary analysis of sperm derived from 10 males revealed significantly greater variance between than within individuals (head length: $F_{9,90} = 10.06$, $p < 0.001$; tail length $F_{9,90} = 9.54$, $p < 0.001$) (see also Hettyey & Roberts 2007). We calculated a mean value of sperm head and tail length for each male.

(b) *Sperm competition trials*

The sperm from two males and the eggs of a single female were used in each of the 40 sperm competition trials. Within trials, all frogs were derived from the same population, and trials conducted on the night frogs were collected. Eggs were squeezed gently from gravid females, and 20–50 eggs were placed on one side of a white plastic weighing dish (base diameter 50 mm). From each of the two males, a volume of sperm suspension that contained 1×10^6 sperm was transferred to the opposite side of the dish and mixed using the pipette tip. Stream water at 13°C was then injected into the dish, first over the eggs and then to a total volume of 5 ml. This resulted in a sperm concentration of 0.2×10^6 sperm ml^{-1}, which was determined from a pilot study to result in asymptotic rates of fertilization. The dish was agitated vigorously for 10 s and left on the laboratory bench for 15 min.

Eggs were transferred to rectangular clear plastic dishes ($145 \times 90 \times 55$ mm) and covered to a depth of 12 mm with stream water. Dishes were allocated at random to a position on a shelf in a controlled temperature room at 18°C with a 12 L : 12 D photoperiod provided by fluorescent overhead lighting. Grow lights (Gro-lux Sylvania, Danvers, MA, USA) provided additional UV light for 2 h centred on mid-day. All viable embryos hatched within 10 days of fertilization. Hatched tadpoles and a toe clip from the female and both males from each trial were preserved in 100 per cent ethanol.

(c) *Microsatellite analyses*

Genomic DNA was extracted from tail tips of preserved tadpoles and adult toe clips, using the EDNA HiSpEx tissue kit (Fisher Biotec). Tadpole DNA was screened using three microsatellite markers: Cg2Ca24, Cg3Ca8 and Cg1Ca9 (table 1). These were multiplexed into one polymerase chain reaction (PCR) containing 1× PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl) (Invitrogen), 3 mM $MgCl_2$ (Invitrogen), 200 µM of each dNTP (Invitrogen), 250 nM of each forward primer, Cg2Ca24 labelled with NED (Applied Biosystems), Cg3Ca8 labelled with VIC (Applied Biosystems), Cg1Ca9 labelled with 6-FAM (Geneworks) (each labelled primer was diluted with unlabelled primer 1 : 10, except Cg1Ca9 which was diluted 1 : 1), 250 nM of each reverse primer, 0.5 U of Platinum Taq polymerase (Invitrogen) and 1–10 ng DNA. PCR amplification was performed with the following cycling conditions: 94°C for 3 min, then 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min and finally 72°C for 30 min. The PCR product (1.5 µl) was then analysed on an ABI3730 Sequencer, sized using Genescan-500 LIZ internal size standard and genotyped using Genemapper software (v. 3.7).

Paternity was assigned using CERVUS 3.0.3 (Kalinowski *et al.* 2007), all assignments being made at the strict 95 per cent confidence level. The presence of null alleles at locus Cg1Ca9 meant that this locus was used for paternity assignment in only 12 of 40 sperm competition trials. For two trials, the three microsatellite loci did not provide sufficient variation for paternity assignment, so an additional two loci were used: Cg1Ca2 labelled with NED (Applied Biosystems) and Cg1Ca5 labelled with VIC (Applied Biosystems), multiplexed together in a PCR and analysed as described earlier (table 1). All parents were screened with the five loci, as well as with a sixth: Cg2Ca6 (table 1).

We also used the DNA from 106 adult females and males sampled in this study to estimate allele frequencies. The observed and expected heterozygosities and frequency of null alleles are shown in table 1. Each locus was tested for Hardy–Weinberg equilibrium using GENEPOP v. 3.4 (Raymond & Rousset 1995). Four loci showed a significant

Table 1. Characterization of microsatellite loci in *C. georgiana*. The number of individuals that gave an amplification product (n) out of 106 individuals tested, the number of alleles observed (N_A), allele size range in base pairs (bp), observed heterozygosity (H_O), expected heterozygosity (H_E) and frequency of null alleles (F_{Null}) are listed for each locus.

locus	repeat motif in clone	GenBank accession no.	primer sequence (5′–3′)	n	N_A	bp	H_O	H_E	$F_{(null)}$
Cg2Ca6	$(CA)_{36}$	EF101689	F: CAGTATTCATATACCAATAAGGAAACC; R: TCATCTCTCTCCCCACTGC	81	34	68–224	0.457	0.943	0.338
Cg3Ca8	$(AC)_{18}GC(AC)_6$	EF101690	F: TCGACAGTTAGCTCTATACCTTCC; R: CGTTTCACTGTTTTGAACTTGC	106	37	72–152	0.906	0.962	0.027
Cg2Ca24	$(TG)_2(CG)_2(TG)_7(CG)_2(TG)_6(CG)_2(TG)_7CG(TG)_6(CG)_2(TG)_5$	EF101691	F: TGACCTACACACCTTGTTAATGG; R: TGCTCAATGAAGCAGTAAGCAC	106	30	73–164	0.953	0.927	−0.017
Cg1Ca9	$(GT)_8AT(GT)_{12}$	EF101692	F: AAAGAATAGATATTTAGACAACGTC; R: TTCCTCAAGGTAGTTTGTATG	100	38	94–178	0.460	0.941	0.340
Cg1Ca5	$(CA)_{14}AA(CA)_5$	EF101693	F: GGGGCCTCACTTTGCTATG; R: AAGACCTGCGGTACCTCTCC	99	11	69–94	0.414	0.813	0.321
Cg1Ca2	$(GC)_3(GT)_{28}GC(GT)_6GC(GT)_{15}$	EF101694	F: TTTCATCACACCTAGGATAGGG; R: CCACTTGTCTATACTGGAAGTAGGC	96	41	97–209	0.573	0.909	0.227

heterozygote deficit, possibly because of null alleles or because of between-population variation in allele frequencies.

There is evidence from a number of taxa (Olsson *et al.* 1996; Birkhead *et al.* 2004; Simmons *et al.* 2006), including frogs (Sherman *et al.* 2008*b*), that genetic compatibility or relatedness between a female and competing males can sometimes influence a male's fertilization success. To control for potential compatibility effects, we therefore calculated the relatedness (r) between the female and each male in each trial using Relatedness v. 5.0.8 (Queller & Goodnight 1989). We used the six microsatellite loci described earlier and population origin as the deme variable. We also calculated shared alleles (number of shared alleles/number of possible shared alleles) between the female and each male in each trial. We estimated the power of our microsatellite loci for detecting genetic similarity between two individuals using the probability of identity (p_{ID}) statistic (Waits *et al.* 2001). This statistic provides an estimate of the probability by chance alone that two individuals would share a multilocus fingerprint and was calculated using GIMLET v. 1.3.3 (Valiere 2002). Across all six loci, p_{ID} was extremely low (2.76×10^{-13}), giving us high power to detect relatedness between individuals. Four of our loci were not in Hardy–Weinberg equilibrium, so that errors in detecting identity could arise. However, given their considerable variability, even using only the two loci that were in Hardy–Weinberg equilibrium (cg2Ca24 and cg3Ca8) gave high power to detect relatedness ($p_{ID} = 1.62 \times 10^{-5}$), and using r calculated from only these two loci in our statistical analyses of paternity returned qualitatively and quantitatively similar results.

(d) *Statistical analyses*

For each fertilization trial, we selected a focal male (male A) at random and calculated the difference (male A–male B) in proportion of live sperm, sperm head length, tail length, VAP, proportion of motile sperm and relatedness to the female. We used a generalized linear model with a logit-link function. The dependent variable was the number of eggs fertilized by male A with the total number of eggs as the binomial denominator. The differences in proportion of live sperm, sperm head length, tail length, VAP, proportion of motile sperm and relatedness were entered as independent variables. The proportions of motile cells and live cells were arcsine transformed to satisfy normality of distribution. Because of over dispersion, we report the estimated F ratios instead of χ^2 as recommended by Crawley (1993). We duplicated the above analysis using the proportion of alleles shared instead of r. We conducted a preliminary analysis in which we also entered into the model quadratic terms for each sperm trait. None was significant (see table S1 in the electronic supplementary material) and they were removed from our final analysis.

3. RESULTS

Summary statistics for sperm variables across all 80 males are provided in table 2. There was a strong and significant positive correlation between the proportion of sperm that were motile and the average swimming velocity of motile sperm (table 3). Males that had sperm with longer tails tended to also have more viable sperm, but the significance of this correlation did not survive Bonferroni correction. There were no significant correlations between sperm motility and sperm morphology.

Table 2. Summary statistics for dependent variables from the 80 males involved in sperm competition trials.

variable	minimum	maximum	mean	s.d.
proportion of live sperm	0.20	0.86	0.56	0.14
head length (μm)	29.46	37.13	33.19	1.44
tail length (μm)	50.63	58.42	53.63	1.92
VAP (μm s^{-1})	8.00	34.20	20.61	4.94
proportion of motile sperm	0.06	0.97	0.65	0.18
relatedness to female	−0.17	0.36	0.05	0.13
proportion alleles shared with female	0.00	0.50	0.15	0.11

Table 3. Correlations between sperm morphology and performance variables. $n = 80$; table-wise Bonferroni critical value for significance, $p = 0.005$.

	proportion motile	proportion live	head length	tail length
VAP	0.581**	0.094	0.176	−0.101
proportion motile		0.071	−0.022	−0.084
proportion live			0.097	−0.246*
head length				0.042

*$p < 0.05$, **$p < 0.001$.

Table 4. Genaralized linear model of the effects of relative variables (male A−male B) on the relative number of offspring sired by the focal male (male A) in 40 competitive fertilization trials.

source	deviance	d.f.	*F*-value	*p*-value
difference in proportion of live sperm	131.0	1	2.054	0.161
difference in head length	22.6	1	0.355	0.555
difference in tail length	34.0	1	0.533	0.471
difference in VAP	587.9	1	9.218	0.005
difference in proportion of motile sperm	363.4	1	5.699	0.023
difference in relatedness	3.2	1	5.047×10^{-2}	0.824
error		33		

The mean (±1 s.d.) paternity for the focal male was 0.514 ± 0.247 (range 0.059–1.00) and did not differ significantly from 0.5 (one sample *t*-test: $t_{39} = 0.361$, $p = 0.640$). There were no effects of relative sperm viability (proportion of live sperm) or relative sperm morphology on fertilization success (table 4). There was a significant effect of relative sperm swimming speed (VAP), and the relative proportion of sperm cells that were motile, on fertilization success (table 4). Fertilization success for the focal male increased as relative sperm swimming speed decreased and relatively more sperm were motile (figure 1).

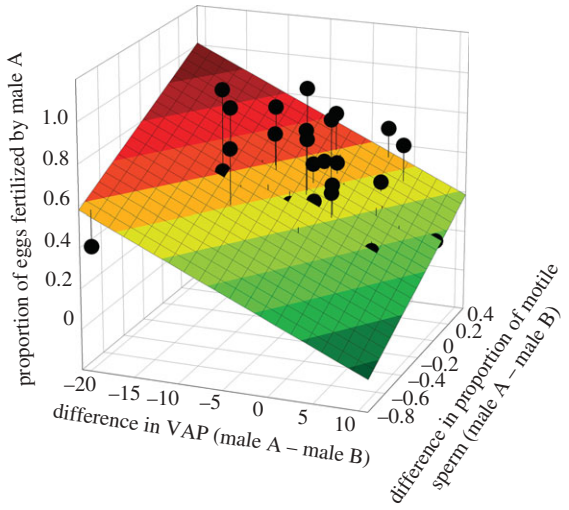

Figure 1. Response surface showing the effects of relative sperm swimming velocity (VAP) and the relative proportion of motile sperm on fertilization success in sperm competition trials involving two males. Green to red represents increasing proportion of eggs fertilized by male A.

Our two measures of genetic similarity, relatedness and number of shared alleles, were highly correlated (Pearson's $r_{79} = 0.876$, $p < 0.001$). There was no effect of relative relatedness to the female on the focal male's relative fertilization success (table 3). Using shared alleles rather than relatedness in our analysis returned quantitatively similar results for all variables, so we present the analysis using relatedness only.

4. DISCUSSION

Using IVF techniques by which we controlled the number of sperm contributed by each of two males, we found a competitive fertilization advantage for male frogs that produce a relatively higher proportion of sperm that are motile and motile sperm that have a relatively slower swimming velocity. We found no significant influence of sperm morphology on a male's competitive fertilization success and no influence of a male's genetic similarity to the female contributing eggs.

In the only other study of competitive fertilization success in frogs, Sherman *et al.* (2009) reported consistent male effects on the outcome of sperm competition in the tree frog *Litoria peronii*, no effect of the proportion of live sperm (Sherman *et al.* 2008*b*) and relatively weak and inconsistent effects (present in one dataset but not a second) of genetic similarity between competing males and the female (Sherman *et al.* 2009). They concluded that male effects on competitive fertilization success were stronger than female effects. Our findings for *C. georgiana* are broadly similar to those for *L. peronii*, but our study is the first to identify the ejaculate traits that contribute to a male frog fertilization success. Previously, we found consistent differences between males in both the proportion of sperm that are motile and the swimming speed of motile sperm (Simmons *et al.* 2009), and we find here that both ejaculate features contribute to a male's competitive fertilization

success. In our previous study, we also found significant independent egg jelly effects on these sperm performance variables (Simmons *et al.* 2009), suggesting that female effects on sperm performance might compound a male's competitive fertilization success. Previously, we found no significant male by female interaction effects on the proportion of motile sperm or the swimming velocity of motile sperm (Simmons *et al.* 2009), and here we found no effect of genetic similarity between male and female on a male's competitive fertilization success. The variation in allele sharing between males and females across the six loci screened (0–0.50) exceeded that in Sherman *et al.*'s (2008*b*) study of *L. peronii* (0–0.30), so that we had enough variation to detect a similar effect of relative genetic similarity on competitive fertilization success if one were present. Arguably, a more powerful approach to detect these types of compatibility effects might be to conduct sperm competition trials between each pair of males across a number of different females (e.g. Birkhead *et al.* 2004). Such an approach might yet reveal significant compatibility effects in *C. georgiana*. However, this approach was adopted by Sherman *et al.* (2009) in their second study of *L. peronii*, and contrary to expectation, this failed to find significant variation in the outcome of sperm competition that was owing to female identity. Combined our results suggest that, at least in these two frog species, male effects on sperm competitiveness are likely to be greater than interaction effects between male and female genotypes.

The effects of sperm performance on competitive fertilization success we have observed are in contrast to those seen in monogamous fertilization trials, in which the proportion of motile sperm and sperm swimming speed has no influence on the proportion of eggs fertilized (Dziminski *et al.* 2009, unpublished data). Rather, there are strong male by female interaction effects on fertilization success and on offspring performance (Dziminski *et al.* 2008). Our competitive fertilization trials suggest that competition among sperm might override subtle gametic interactions that would otherwise favour fertilizations by genetically compatible sperm that enhance female fitness. Multiple male amplexus has also been found to reduce the total proportion of a female's clutch that is fertilized in natural spawnings of *C. georgiana* (Byrne & Roberts 1999), so that sperm competition between males in this species is expected to generate significant sexual conflict (Stockley 1997).

We found no influence of either sperm head or tail length on competitive fertilization success or on the swimming speed of sperm. Such a finding is inconsistent with macro-evolutionary patterns. Across 114 species of myobatrachid frogs, the strength of selection from sperm competition is positively associated with sperm head and tail length, implying that selection from sperm competition should favour longer sperm components (Byrne *et al.* 2003). This paradox is not unique to frogs. A recent comparative analysis of 29 species of cichlid fishes revealed positive macro-evolutionary associations between the strength of selection from sperm competition and both sperm length and swimming speed and a positive macro-evolutionary covariation between sperm length and swimming speed (Fitzpatrick *et al.* 2009). However, within species of cichlids, longer sperm did not swim faster than shorter sperm, suggesting different responses to selection at macro- and micro-evolutionary scales. Fitzpatrick *et al.* (2009) argue that at micro-evolutionary scales, sperm competition may act first on sperm performance, as shown for *C. georgiana* in our study, with responses in gross sperm morphology occurring over much larger evolutionary scales.

Our finding that relatively slower swimming sperm had a fertilization advantage is somewhat counterintuitive, though not unprecedented (Rudolfsen *et al.* 2008). *In vitro* studies of externally fertilizing fishes have generally found positive associations between sperm swimming velocity and competitive fertilization success (Gage *et al.* 2004; Casselman *et al.* 2006; Liljedahl *et al.* 2008), and selection from sperm competition does appear to have favoured rapidly swimming and short-lived sperm in these taxa (Cosson *et al.* 2008; Fitzpatrick *et al.* 2009). However, frog sperm are structurally and behaviourally very different from the archetypal sperm of fishes; frog sperm can remain motile for several hours (Hettyey & Roberts 2006; Sherman *et al.* 2008*a*) and swim very slowly (see also Reyer *et al.* 2003; Edwards *et al.* 2004; Muto & Kubota 2009). In myobatrachids, sperm are propelled by an undulating membrane that is supported by a longitudinal axial fibre that stretches from the base of the head to the tip of the 'tail' (Lee & Jamieson 1992). Furthermore, unlike fishes, fertilization is not instantaneous in frogs. Rather, sperm must traverse several viscous jelly coats before they come into contact with the egg. In *Xenopus*, it was shown that up to 50 per cent of the sperm take indirect, energy-wasting routes and become trapped within the jelly layers (Reinhart *et al.* 1998). Those that are successful in reaching the egg appear to exhibit fewer instances of stopping and starting, suggesting that successful sperm are those that exhibit sustained levels of motility (Reinhart *et al.* 1998). Our competitive fertilization trials suggest that in frogs, selection acts for slow and steady swimming performance, a selection pressure that may underlie the evolution of the unusual sperm form and function that is characteristic of frogs. The phenotypic correlation between the proportion of sperm that were motile and sperm swimming speed was positive, yet these traits had opposite effects on a male's competitive fertilization success. If the genetic correlation between these traits is also positive, antagonistic selection on these ejaculate features has the potential to maintain additive genetic variance for ejaculate competitiveness.

All animals were collected and maintained according to the standards of the Animal Ethics Committee of the University of Western Australia (approval numbers RA/3/100/467 and 05/100/467) and the Department of Environment and Conservation, Western Australia (permit numbers SF005477 and CE001156).

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