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 char (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 1942). 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, Beverly, 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 of 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 × 10^8 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 × 10^8 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 × 90 × 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 X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl) (Invitrogen), 3 mM MgCl2 (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 (H) observed (N), allele size range in base pairs (bp), observed heterozygosity (H), expected heterozygosity (E) and frequency of null alleles (null) are listed for each locus.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5'-3')</th>
<th>GenBank accession no.</th>
<th>n</th>
<th>H</th>
<th>bp</th>
<th>N</th>
<th>H</th>
<th>E</th>
<th>null</th>
</tr>
</thead>
<tbody>
<tr>
<td>cg2Ca6</td>
<td>F: CAGTCTCTCTCCGTCAGA; R: TCACCTCTGTCAGCCTCC</td>
<td>EF101689</td>
<td>81</td>
<td>34</td>
<td>68–224</td>
<td>0.457</td>
<td>0.943</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>cg3Ca8</td>
<td>F: TCGACAGTTAGCTCTATACCTTCC; R: CGTTTCACTGTTTTGAACTTGC</td>
<td>EF101690</td>
<td>106</td>
<td>37</td>
<td>72–152</td>
<td>0.906</td>
<td>0.817</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>cg2Ca24</td>
<td>F: TGCTCAATGAAGCAGTAAGCAC; R: TGCTCAATGAAGCAGTAAGCAC</td>
<td>EF101691</td>
<td>106</td>
<td>38</td>
<td>73–164</td>
<td>0.953</td>
<td>0.943</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>cg1Ca9</td>
<td>F: AAAGAATAGATATTTAGACAACGTC; R: TTCCTCAAGGTAGTTTGTATG</td>
<td>EF101692</td>
<td>100</td>
<td>38</td>
<td>94–178</td>
<td>0.460</td>
<td>0.813</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>cg1Ca5</td>
<td>F: GGGGCCTCACTTTGCTATG; R: AAGACCTGCGGTACCTCTCC</td>
<td>EF101693</td>
<td>99</td>
<td>11</td>
<td>69–94</td>
<td>0.414</td>
<td>0.730</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>cg1Ca2</td>
<td>F: TTTCATCACACCTAGGATAGGG; R: CCACTTGTCTATACTGGAAGTAGGC</td>
<td>EF101694</td>
<td>96</td>
<td>41</td>
<td>73–164</td>
<td>0.573</td>
<td>0.817</td>
<td>0.027</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>variable</th>
<th>minimum</th>
<th>maximum</th>
<th>mean</th>
<th>s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>proportion of live sperm</td>
<td>0.20</td>
<td>0.86</td>
<td>0.56</td>
<td>0.14</td>
</tr>
<tr>
<td>head length (µm)</td>
<td>29.46</td>
<td>37.13</td>
<td>33.19</td>
<td>1.44</td>
</tr>
<tr>
<td>tail length (µm)</td>
<td>50.63</td>
<td>58.42</td>
<td>53.63</td>
<td>1.92</td>
</tr>
<tr>
<td>VAP (µm s⁻¹)</td>
<td>8.00</td>
<td>34.20</td>
<td>20.61</td>
<td>4.94</td>
</tr>
<tr>
<td>proportion of motile sperm relatedness to female</td>
<td>0.17</td>
<td>0.36</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>proportion alleles shared with female</td>
<td>0.00</td>
<td>0.50</td>
<td>0.15</td>
<td>0.11</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>proportion motile</th>
<th>proportion live</th>
<th>head length</th>
<th>tail length</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAP</td>
<td>0.581**</td>
<td>0.094</td>
<td>0.176</td>
</tr>
<tr>
<td>proportion motile</td>
<td>0.071</td>
<td>−0.022</td>
<td>−0.084</td>
</tr>
<tr>
<td>proportion live</td>
<td>0.097</td>
<td>−0.246*</td>
<td></td>
</tr>
<tr>
<td>head length</td>
<td>0.042</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.001.

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.

sperm swimming speed decreased and relatively more sperm were motile (figure 1).

Our two measures of genetic similarity, relatedness and number of shared alleles, were highly correlated (Pearson’s rs = 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. 2008a) 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) 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 (Cossor 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. 2008a) 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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