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ORIGINAL ARTICLE

Gary Burness · Stephen J. Casselman · Albrecht I. Schulte-Hostedde · Christopher D. Moyes · Robert Montgomerie

Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*)

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Abstract Under sperm competition, a male's fertilization success depends largely on the ejaculate characteristics of competing males. Theoretical models predict that, in external fertilizers, increased risk of sperm competition should result in selection for increased sperm swimming speed. To test this prediction, we studied the behavior of sperm from parental and sneaker male bluegill (*Lepomis macrochirus*), a fish species characterized by high levels of cuckoldry due to alternative reproductive tactics of males (parentals and cuckolders). Because cuckolders (sneakers and satellites) always spawn in the presence of a parental male, but the reverse is not true, cuckolders experience the greater risk of sperm competition. We show here that the spermatozoa of sneakers have faster initial swimming speeds but shorter periods of motility than the sperm of parental males. Moreover, we show that sperm swimming speeds shortly after activation (when most fertilization occurs) are correlated with starting ATP levels in spermatozoa, suggesting that sperm competition has selected for higher energetic capacity in the sperm of sneakers. Thus, the higher energetic capacity and initial swimming speed of sneaker sperm may explain why, despite having fewer sperm per ejaculate than parentals, sneakers fertilize more eggs than parental males when they compete to fertilize a clutch of eggs.

Keywords Alternative mating tactic · Motility · Energetics · ATP · Sperm competition

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G. Burness (✉) · S. J. Casselman · A. I. Schulte-Hostedde · C. D. Moyes · R. Montgomerie
Department of Biology,
Queen's University,
Kingston, Ontario, K7L 3N6, Canada
e-mail: garyburness@trentu.ca
Tel.: +1-705-7481011 ext 5288
Fax: +1-705-7481205

Present address:
G. Burness, Department of Biology,
Trent University,
Peterborough, Ontario, K9J 7B8, Canada

Present address:
A. I. Schulte-Hostedde, Department of Biology,
Laurentian University,
Sudbury, Ontario, P3E 2C6, Canada

Introduction

Fish species with alternative male reproductive tactics (cuckolder vs parental or territorial males; Gross 1982) provide excellent models to test predictions of sperm competition theory, because the risk of sperm competition differs markedly between tactics. Cuckolders always spawn when a parental male is spawning, so their sperm always competes with those of parentals to fertilize a female's eggs. Thus, because cuckolders are in a disfavored role with respect to sperm competition, they generally invest more in sperm production than do parentals (e.g., Gage et al. 1995; Leach and Montgomerie 2000), in accordance with theory (Parker 1990).

At the level of individual gametes, theory predicts that sperm swimming speed will increase with increasing risk of sperm competition, at the expense of the duration of motility (Ball and Parker 1996). The rationale for this prediction is that fertilization success is positively related to sperm swimming speed (Kime et al. 2001), and that swimming speed is dependent upon the amount of energy available (Jeulin and Soufir 1992). Because both swimming fast and for long periods of time require energy, a trade-off between these sperm traits is expected (Ball and Parker 1996; Levitan 2000). Sperm swimming speed is also believed to be positively related to sperm length (Gomendio and Roldan 1991), with the increased energy required to overcome the hydrostatic drag of a long flagellum further compromising sperm longevity (Stockley et al. 1997). While these mechanisms seem plausible to explain the theoretical predictions of Ball and Parker (1996), few empirical data exist on the relationships among sperm size, swimming speed energetics, and risk of sperm competition.

Whether the physiological quality of sperm varies with the risk of sperm competition within species has been studied in detail only in Atlantic salmon (*Salmo salar*; Vladic and Järvi 2001; Vladic et al. 2002). In this species, sperm from parr (cuckolders) had more than twice the percent motility and energy stores of the sperm of anadromous (territorial) males. Because these two sperm traits also predicted fertilization success, Vladic and Järvi (2001) concluded that sperm quality differed between alternative mating tactics, as predicted by sperm competition theory. No differences in sperm swimming speed, longevity (Vladic and Järvi 2001), or sperm morphology (Vladic et al. 2002) were detected between parr and anadromous tactics.

In the current study, we report on physiological and morphological variation in the sperm of the male bluegill (*Lepomis macrochirus*), a species exhibiting alternative male reproductive tactics, with parentals and both satellite and sneaker types of cuckolders (Gross 1982). Parental male bluegill delay reproduction until ca. 7 years of age, when they compete for nesting sites and actively court females. Cuckolder males mature precociously (usually beginning at ca. 2 years old), and parasitize the nests of parental males, either by darting into a parental male's nest from nearby vegetation (sneakers) or, when older and larger, by mimicking a female (satellites). In each case, the cuckolder releases sperm beneath a spawning pair. Because parental males usually spawn without rivals, they experience lower levels of sperm competition than do parasitic males, who almost always spawn at the same time as a parental male (Fu et al. 2001).Thus, we tested the prediction that sneaker male bluegill produce sperm that swim faster and are of higher physiological quality than the sperm of parental males.

Methods

Collection of milt

Using a dip net, we captured eight parental and 14 sneaker male bluegill from an active colony in Lake Opinicon, Ontario, Canada in late June 2002. Following capture, males were transported to the laboratory and transferred to a tank with water (15–20°C) provided continuously from the lake. Within 1–5 h of capture, each male was removed from the tank, anesthetized using clove oil, and stripped of milt by applying gentle pressure to its abdomen. Milt was collected into microcapillary tubes, and used immediately for analysis of sperm motility. Milt contaminated with either urine or feces was discarded.

Measuring sperm motility

To dilute the milt without activating the sperm, we added 5 µl of freshly collected milt to 750 µl of a high osmolarity extender (20 mM Tris, 2 mM KCl, 200 mM NaCl, pH 9.0, 400 mosmol; Cosson et al. 1999). This dilution technique results in the simultaneous activation of sperm when the solution is added to a small amount of water and makes it easier to track individual spermatozoa with our video analysis system (see below), because of the lower sperm density. We then placed ~1 μl of diluted, but inactive, sperm on an Improved Neubaur hemocytometer, and added a cover slip. Motility was initiated by flooding the hemocytometer with lake water at the temperature of the spawning sites (20°C). Sperm activity was recorded on videotape using a high-resolution monochrome CCD camera (model XCST50, Sony, Tokyo) mounted on a negative phase contrast CH30 microscope (Olympus, Tokyo) at 100×.

We analyzed the videotapes using a CEROS (v.12) video sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA). For each male, at 5, 10, 15, 30, 45, 60, and 90 s following activation of sperm motility, we quantified the swimming paths of all spermatozoa for 0.5 s each. We deleted sperm with incomplete paths (i.e., those that went in or out of the field of view), and calculated the average sperm swimming velocity (VAP; average smoothed path velocity) of up to 25 spermatozoa per time interval. The straightness (STR) with which the sperm of each male swam was calculated as STR=VSL/VAP for each sperm track, where VSL is the speed over the straight-line distance between the start and end of the sperm's path. At each time interval, we also calculated the percentage of cells in the field of view that were motile (percent motility). We considered sperm to be motile if their progressive motility was >25 μm/s (see Lahnsteiner et al. 1998 for similar criterion).

As an index of the duration of sperm motility (longevity), we determined the percentage of sperm from each male showing progressive motility (>25 μm/s) at 60 s following activation. Most other studies have measured sperm longevity as the duration of forward motility of spermatozoa (or a proportion thereof, usually 95%) in a field of view under the microscope (Stockley et al. 1997; Gage et al. 2002). We chose to measure this index at 60 s post-activation because thereafter the mean percent motility of both sneakers and parentals was <25%, and we assume that they no longer have any capacity to fertilize an egg. Our index reduces the potential for statistical artifacts that result from more dense ejaculates having a greater probability of containing a few motile sperm than do less dense ejaculates. A similar approach has been used as an index of longevity in other species (Uglem et al. 2001). These techniques provide repeatable measures of swimming speed and motility (Leach and Montgomerie 2000).

Sperm morphometry

To prepare samples for measurement, we diluted 5 μl fresh milt from each male in 750 μl fixative (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0). The diluted milt sample was spread thinly across a microscope slide and air-dried. Under 400× magnification, we photographed ten haphazardly chosen sperm from each male, using a microscope-mounted camera. We measured tail length to the nearest 0.1 μm on digitized images of each sperm using NIH image (v.1.69; available at http://rsb.info.nih.gov/nih-image) and an Intuos graphic tablet (Wacom Co. Ltd., Japan). We calculated the median sperm tail length for each male for use in further analyses.

ATP measurement

In the field, 2 μl milt from each male was diluted in 300 μl sperm extender described above. Then a known volume (typically 100 μl) was transferred to a microcentrifuge tube and immediately frozen in a liquid N_2-charged dry shipper. To extract ATP, we placed the frozen milt samples on dry ice and added 900 μl ice-cold 3.3% perchloric acid to each sample. We sonicated each sample three times, each time in 5- to 10-s bursts, separated by 10-s pauses. Each sample was centrifuged for 2 min at 15,000 rpm. We then neutralized 180 μl supernatant by adding it to 10.3 μl 2 M KCl, 56.5 μl 1 M KOH, 10.3 μl saturated Tris, and ~1 μg phenol red (phenolsulfonphthalein).

To measure ATP concentration in each neutralized sample, we added purified luciferin-luciferase (dissolved in 100 mM glycine, 20 mM $MgSO_4$, pH 7.4). Measurement of bioluminescence was performed using a 96-well Lmax luminometer (Molecular Devices

Corp., Sunnyvale, Calif., USA). The ATP content of each sample was calculated from a series of ATP standards run in duplicate with the milt samples. We calculated sperm density in the milt of each male following Leach and Montgomerie (2000), and expressed ATP concentration as nmol ATP per 10^8 spermatozoa.

Data analysis

To improve normality and homogeneity of variances, ATP concentrations were $\log_{10}$ transformed; percent motilities and straightness were arcsine transformed. If data failed to meet assumptions for parametric statistical tests, we used non-parametric analyses. We used contrast analysis for post hoc tests following ANOVA, and corrected the *P* values for multiple comparisons using the sequential Bonferroni method (Rice 1989). Because sample sizes were small and statistical power therefore low, we report the 95% confidence limits (CL; calculated by bootstrapping with 1,000 iterations; Manly 1997) for effect sizes, to give some indication of the magnitude of the biological effect, even for results that are not statistically significant (see also Colgreave and Ruxton 2003).

Results

In males of both mating tactics, VAP declined significantly over time (repeated-measures ANOVA, $F_{6,113}$= 426.7, P<0.0001; Fig. 1A). This decline in swimming speed occurred at tactic-specific rates, as indicated by a significant interaction between time since activation and tactic (parental or sneaker; $F_{6,113}$=10.5, P<0.0001). At 5 and 10 s post-activation, sneakers had significantly faster-swimming sperm than did parental males (contrast analyses, 5 s: $F_{1,113}$=21.4, P<0.001; 10 s: $F_{1,113}$=7.4, P=0.045). By 60 s post-activation, the sperm of sneakers swam significantly slower than the sperm of parentals ($F_{1,113}$=7.2, P=0.04). At no other time post-activation did the sperm of sneaker and parental males differ significantly in swimming speed, although it is clear that sneaker sperm started faster and finished slower, swimming at the same speed as parental sperm at about 25 s post-activation (Fig. 1A).

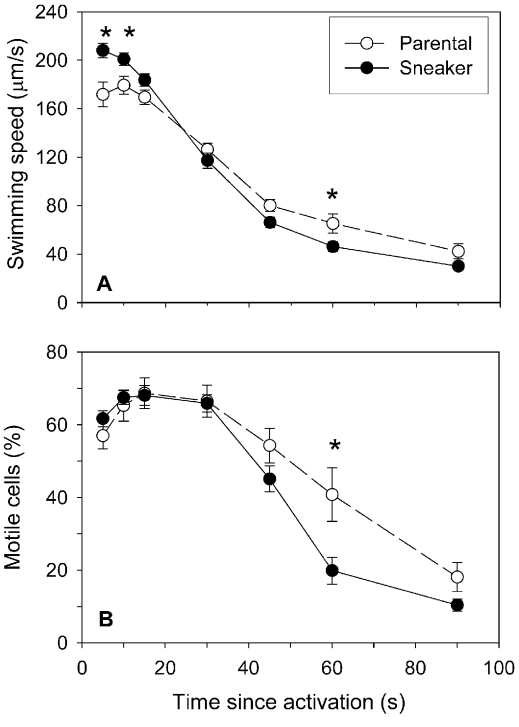

Fig. 1 **A** Mean sperm swimming speed (VAP), and **B** percentage of sperm showing motility at different times post-activation for sneaker (n=14) and parental (n=8) male bluegill (±1 SE). * P<0.05, for contrast analysis after sequential Bonferroni correction

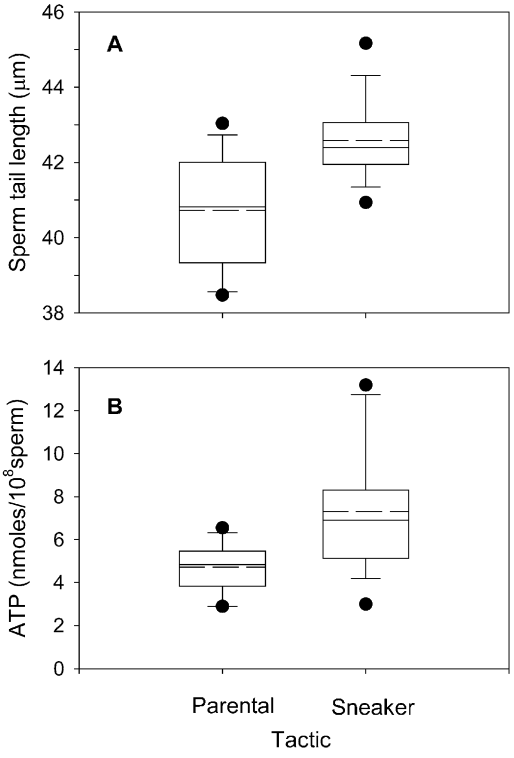

Fig. 2 **A** Length of flagellum, and **B** pre-activation ATP levels from sneaker (n=14) and parental (n=8) male bluegill. Box plots show horizontal lines at 10th, 25th, 50th (median), 75th, and 90th percentiles and all data points falling outside this range. *Dashed line* is the mean

Sperm from males of both tactics swam with increasing straightness throughout their active period (repeated-measures ANOVA, $F_{6,113}$=93.3, P<0.0001). The interaction between time and tactic was also significant ($F_{6,113}$=2.2, P=0.05), suggesting that the sperm of sneakers and parentals changed the straightness of their swimming path at different rates. For example, at 5 s post-activation, mean STR for sneaker sperm was 58.9 (±4.4 95% CL, n=14) whereas that of parental sperm was 47.6 (±6.4 95% CL, n= 8) though the difference was not significant (contrast analysis, P=0.11). By 45 s post-activation, STR was virtually the same for both tactics.

The percentage of sperm showing forward motility decreased significantly over time in both tactics (repeated-measures ANOVA, $F_{6,116}$=83.7, P<0.0001; Fig. 1B). As with swimming speed, the rates of decline differed between tactics ($F_{6,116}$=4.0, P=0.001). Although at 5–45 s

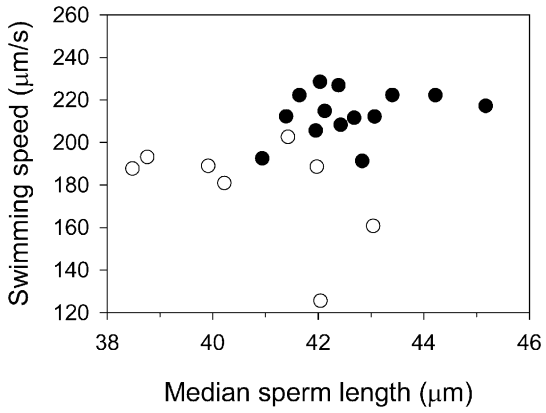

Fig. 3 Relationship between sperm swimming speed at 5 s post-activation and median sperm tail length for each male bluegill. Parentals are shown with *open* and sneakers with *closed circles*

post-activation there was no difference between tactics (P>0.90 at each time interval), by 60 s post-activation sneaker males had significantly lower sperm motility than did parental males (contrast analysis, $F_{1,116}$=12.0, P=0.005). At both 60 and 90 s post-activation, the motility of sneaker sperm was only half that of parental sperm. Thus, the sperm of sneakers have a reduced longevity compared to parental males.

The sperm of sneakers had slightly, but significantly, longer tails than the sperm of parentals (t=3.2, P=0.005, n=8 parentals, n=14 sneakers; Fig. 2A). There was also a positive correlation between tail length and sperm swimming speed at 5 s (r_S=0.41, 95% CL=–0.04, 0.73, P=0.055, n=22; Fig. 3) and 10 s post-activation (r_S=0.19, 95% CL=–0.23, 0.59, P=0.40) but neither of these was statistically significant. There was a negative relationship between sperm length and the duration of forward motility (r_S=–0.21, 95% CL=–0.55, 0.23, P=0.35, n=22) but this too was not significant.

Consistent with sneaker males having faster swimming sperm at 5 and 10 s post-activation, sneaker males produced sperm that contained 1.5 times more ATP than the sperm of parental males (t=2.4, P=0.02, n=8 parentals, n=14 sneakers; Fig. 2B). Sperm with greater ATP stores swam faster at 5 s (r_S=0.60, P=0.003, n=22), and 10 s post-activation (r_S=0.39, 95% CL=–0.01, 0.70, P=0.08, n=22) but the latter is not statistically significant. Interestingly, the correlation between sperm ATP stores and the duration of forward motility was negative (r_S=–0.62, P=0.002, n=22) and significant.

Discussion

This is the first study to demonstrate, empirically, that the spermatozoa of disadvantaged males have faster initial swimming speeds but shorter periods of motility than the sperm of favored males. Most importantly, we provide a physiological mechanism to explain this difference, with sneakers having about 50% more ATP before sperm activation than parentals, and slightly longer flagella. This is the first evidence that alternative reproductive tactics have different sperm size and swimming speeds, and is consistent with sperm competition theory that predicts selection for more competitive sperm in sneakers, the tactic experiencing the highest level of sperm competition (Ball and Parker 1996).

We found that the sperm of sneaker bluegill had higher intra-cellular ATP stores than the sperm of parental males. ATP stored in spermatozoa prior to ejaculation provides the necessary chemical energy to sustain flagellar beating and sperm motility (Cosson et al. 1999). High initial ATP levels in sneaker sperm seem to be directly responsible for their higher swimming speeds as there was a significant correlation between average sperm swimming speed at 5 s post-activation and initial ATP content across tactics. Jeulin and Soufir (1992) reported a similar correlation between sperm ATP content and swimming speed in mammals. The high initial swimming speed of sneaker sperm apparently traded-off with sperm longevity, as is often assumed in theoretical models (e.g., Ball and Parker 1996). Although long-lived sperm may be favored when not all eggs are fertilized immediately (Ball and Parker 1996), fertilization is rapid in bluegill, with the majority of a female's eggs fertilized within 5–10 s of ejaculation (Schulte-Hostedde, Burness and Montgomerie, unpublished data). Such rapid fertilization probably places a premium on high initial sperm swimming speeds, rather than longevity. The mechanism for this trade-off is unknown. Our results from this study, and for other species (Burness and Montgomerie, unpublished data), suggest that ATP stores must be rapidly depleted shortly after activation in order to achieve rapid acceleration and a concomitantly high swimming speed. Nonetheless, the ATP concentration at the end of the period of forward motility is likely still in excess of that required by the dynein ATPases for flagellar beating (Cosson et al. 1999).

The only previous study to look at the relationship between sperm energetics and sperm competition risk found a difference between tactics in percent motility rather than sperm swimming speed (Vladic and Järvi 2001). In that study, parr and anadromous male Atlantic salmon sperm showed 90% and 41% motility, respectively, at 10 s after activation; a difference consistent with differences in ATP stores. In contrast, we could not detect any differences between tactics in percent motility in bluegill. The factors that favor differences in percent motility between tactics in salmon, but not bluegill, remain unknown.

In externally fertilizing fishes, a positive relationship between fertilization success and sperm swimming speed has been predicted theoretically (Ball and Parker 1996) and demonstrated empirically (e.g., Lahnsteiner et al. 1998; Jobling et al. 2003). Thus, the differences that we describe in the swimming speeds of sperm from sneaker and parental males explain why, in sperm competition, sneakers reportedly fertilize more eggs than do parental males (Fu et al. 2001). We suggest that the higher

swimming speeds of sneaker sperm immediately after ejaculation may offset the numerical disadvantage of producing absolutely fewer spermatozoa per ejaculate (Leach and Montgomerie 2000).

The sperm of sneaker bluegill had significantly longer flagella than sperm from parental bluegill, though the difference was slight (<2 μm, or about 4% of flagellar length). This finding supports theoretical models predicting that, in external fertilizers, sperm size should increase with sperm competition intensity (Ball and Parker 1996). Theoretically, a longer flagellum generates greater forces and concomitantly higher swimming speeds than a short flagellum (Katz and Drobnis 1990; Gomendio and Roldan 1991). The increased hydrostatic drag of a long flagellum may, however, result in decreased sperm longevity (Stockley et al. 1997), presumably due to the depletion of energy stores. A recent study of Atlantic salmon has found that sperm with long tails had decreased longevity, but no relationship between sperm tail length and swimming speed could be detected (Gage et al. 2002). In contrast, in bluegill there was a positive relationship between sperm length and swimming speed at 5 and 10 s post-activation, and a negative relationship between sperm length and the duration of forward motility, although none of these trends was statistically significant. These results do suggest, however, that longer sperm swim faster at the expense of a shorter period of progressive motility. Further work, with much larger samples will be needed to confirm these relationships.

Leach and Montgomerie (2000) have previously reported that the sperm of sneaker bluegill swam *slower* than those of parentals and that there was no difference in sperm length. In that study, however, sperm swimming speed was not measured until 45 s post-activation. In the present study, we also found that sneaker males had slower swimming sperm than parentals at 45 s post-activation; significantly so by 60 s. Most importantly, we found that sneakers had *faster* swimming sperm during the initial few seconds following activation, the time period during which most eggs are fertilized (Schulte-Hostedde, Burness and Montgomerie, unpublished data). Why Leach and Montgomerie (2000) could not detect morphological differences between the sperm of each tactic is not clear, although in the current study we used slightly different techniques, which may have improved our ability to detect small differences in sperm length. Thus, in the present study, we used a pen-controlled graphics tablet rather than a mouse to trace and measure sperm tail length. It is also possible that the difference between these studies could be accounted for by variation in male sperm traits between years and spawning bouts (Casselman and Montgomerie 2004), even though both studies were conducted at the same locality.

If parental males lose fertilizations to sneaker males because parentals have slower swimming sperm, it is not clear why selection does not favor parental males with faster swimming sperm. One possibility is that the optimal swimming speed required to maximize fertilization success differs between sneakers and parental males. Sneakers are always spawning in the presence of another male; consequently, the optimal solution may be to produce fast-swimming sperm that are active for only brief periods. In contrast, because parental males usually spawn in the absence of a cuckolder, increased sperm longevity, at the expense of initial swimming speed, may ensure that a parental male fertilizes all of a female's eggs.

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