Fertilization dynamics of sperm from different male mating tactics in bluegill (*Lepomis macrochirus*)

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Abstract: Sperm competition results in the evolution of ejaculate characteristics such as high sperm density, high motility, and fast sperm swimming speed. A fundamental assumption of sperm competition theory is that ejaculates with high motility and fast-swimming sperm have an advantage with respect to fertilization success. We tested this assumption by studying the fertilization dynamics of alternative mating tactics (cuckolders and parentals) of male bluegill (*Lepomis macrochirus* Rafinesque, 1819). Sneakers (cuckolders) have faster swimming sperm and a higher proportion of motile sperm immediately following sperm activation than do parentals; however, these variables decline more quickly over time in sneaker sperm than in the sperm of parental males. We used a controlled fertilization experiment to test the prediction that parental males will have higher fertilization success than sneakers late in the sperm activation cycle because of the reduced rate of decline in ejaculate quality over time. We found that as the time from sperm activation increases parental sperm fertilizes more eggs than the sperm of sneakers. Our results support the idea that fertilization success is higher when ejaculates contain a higher proportion of either motile sperm or faster swimming sperm, all else being equal. In addition, after controlling for time from sperm activation, we found a significant bias in fertilization success toward parental males, suggesting that cryptic female choice might play a role in fertilization dynamics.

Résumé : La compétition spermatique entraîne l'évolution des caractéristiques de l'éjaculat, telles que la forte densité, la grande motilité et la vitesse de nage élevée des spermatozoïdes. Une présupposition fondamentale de la théorie de la compétition spermatique est que les éjaculats contenant des spermatozoïdes à forte motilité et à vitesse de nage élevée sont avantagés dans le succès de la fécondation. Nous avons testé cette présupposition en étudiant la dynamique de la fécondation chez des crapets arlequins (*Lepomis macrochirus* Rafinesque, 1819) mâles en fonction de deux tactiques d'accouplement, celles de l'intrus trompeur et du parent fidèle. Par rapport aux pères fidèles, les intrus ont des spermatozoïdes à nage plus rapide et une proportion plus importante de spermatozoïdes mobiles juste après l'activation; cependant, ces caractéristiques décroissent plus rapidement dans le temps chez les spermatozoïdes des intrus que chez ceux des fidèles. Nous avons monté une expérience contrôlée pour vérifier la prédiction que les pères fidèles ont un succès de fécondation plus élevé que les trompeurs tard dans le cycle d'activation des spermatozoïdes à cause du taux plus faible de déclin de la qualité de leur éjaculat en fonction du temps. Avec le temps qui passe depuis l'activation, les spermatozoïdes des pères fidèles fécondent plus d'oeufs que ceux des intrus. Nos résultats appuient l'hypothèse selon laquelle le succès de la fécondation est plus élevé lorsque l'éjaculat contient ou bien une plus forte proportion de spermatozoïdes mobiles, ou alors des spermatozoïdes à nage plus rapide, toutes autres choses étant égales. De plus, une fois qu'on a tenu compte du temps passé depuis l'activation des spermatozoïdes, il y a un décalage significatif du succès de la fécondation en faveur des pères fidèles, ce qui laisse croire que le choix caché des femelles peut jouer un rôle dans la dynamique de la fécondation.

[Traduit par la Rédaction]

Introduction

Sperm competition occurs when the sperm from two or more males compete to fertilize a female's egg(s). One of the predicted consequences of sperm competition is that males will evolve ejaculate traits that will maximize the probability of successfully fertilizing the eggs (Parker 1998). Ejaculate characteristics that are expected to be associated with success in sperm competition include sperm density, motility, swimming speed, morphology, and the longevity of active sperm (Parker 1990; Gomendio and Roldan 1991; Gage et al. 1995; Ball and Parker 1996; Stockley et al. 1997), but empirical evidence in support of these expectations remains limited.

Many fish species exhibit alternative male reproductive tactics (cuckolder or sneaker vs. parental or territorial males; Taborsky 1998) that provide an excellent opportunity to test sperm competition theory empirically, because males adopting divergent reproductive tactics often experience different risks of sperm competition. Because cuckolders always spawn in the presence of a parental male, cuckolders are always engaged in sperm competition with parentals (but the reverse is not always true). The theory predicts that cuckolders should therefore invest more heavily in ejaculates than parentals (Parker 1990, 1993), and they do (e.g., Gage et al. 1995; Leach and Montgomerie 2000).

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Here we test predictions of sperm competition theory using bluegill sunfish (*Lepomis macrochirus* Rafinesque, 1819), a species with two distinct life histories (sneakers and parentals) that result in different male mating tactics (Gross 1982). Because sneakers always spawn at the same time as parental males, they always experience sperm competition and are thus predicted to have higher quality ejaculates than parentals. Indeed, sneaker males have relatively larger testes (Ehlinger et al. 1997; Neff et al. 2003), produce ejaculates with higher sperm densities (Leach and Montgomerie 2000; Neff et al. 2003), and produce individual sperm with faster initial swimming speed and greater ATP (adenosine triphosphate) stores than parental males (Burness et al. 2004).

A fundamental assumption of sperm competition theory is that ejaculates with high motility and fast-swimming sperm have an advantage with respect to fertilization success (Ball and Parker 1996; Kime et al. 2001; Gage et al. 2004). When spawning, sneaker males evidently fertilize more eggs than parental males under sperm competition (Fu et al. 2001), yet parental male sperm has been shown to achieve higher fertilization success in sperm competition under controlled conditions (Neff et al. 2003). Recently, we have found that both the percentage of sperm that are progressively motile and the mean swimming speed of individual sperm decline more quickly over time from activation in sneakers than in parentals (Fig. 1 in Burness et al. 2004). Parentals engage in sperm competition far less often than sneakers and increased sperm longevity may ensure that a parental male fertilizes all of a female's eggs.

Here we use a controlled fertilization experiment to test the prediction, arising from Burness et al. (2004), that parental males will have higher fertilization success than sneakers late in the sperm activation cycle because of the reduced rate of decline in ejaculate quality over time. We suggest that this tactic-specific decline in ejaculate quality may help explain the discrepancy in fertilization success reported between field and laboratory studies (Fu et al. 2001; Neff et al. 2003).

Methods

Female, and parental and sneaker male, bluegill were captured from a single spawning colony in Lake Opinicon, Ontario, Canada, over 2 days in late June 2003. We used a dip net to capture males near nests, and transported them to a nearby laboratory (Queen's University Biological Station) where they were kept in tanks at lake temperature (ca. 20 °C). Shortly after capture (2–6 h), we stripped each male of milt by applying gentle pressure to the abdomen. Milt was collected from the gonopore in microcapillary tubes for use in the fertilization experiment and for the determination of sperm density; milt contaminated by urine or faeces was discarded. We collected eggs from ripe females by gently applying pressure to the abdomen and extruding the eggs into a plastic weighing boat. All gametes that we collected were immediately used in fertilization experiments. To minimize the effects of holding the eggs outside of the body and in the open air, the eggs were kept in the weighing boat a maximum of 90 s after the clutch was stripped from the female.

To ensure that we controlled the number of sperm used in the fertilization experiments, we used a two-step process to calculate sperm density for each of the males used in the experiment. First, we added 5 μL of freshly collected milt to 750 μL of a high osmolarity extender (20 mmol/L of Tris, 2 mmol/L of KCl, 200 mmol/L of NaCl, pH 9.0, 400 mosmol) that diluted the milt without activating the sperm. We then placed a drop of diluted, but inactive, sperm on an Improved Neubaur hemocytometer, added a cover slip, and counted the number of sperm in 10 haemocytometer cells. The density was determined by multiplying by the appropriate dilution factor. From this estimate of density, we calculated the total volume of milt required for each experimental trial to maintain sperm density at 2×10^6 sperm/mL of lake water.

We conducted a fertilization experiment to manipulate the time elapsed between sperm activation and fertilization (referred to here as "treatment"). We paired a female's eggs with sperm from a sneaker and a parental male and used these gametes for one series of treatments for each male phenotype (ie. 0 s post activation, 10 s post activation, 30 s post activation, 60 s post activation). This procedure was replicated 5 times, each with a unique female and unique sneaker and parental males. In total, the gametes from five females, five sneaker males, and five parental males were used.

To conduct a fertilization trial we placed the calculated volume of milt in the bottom of two 500 mL transparent plastic cups. One cup contained the sperm of a sneaker male, and one cup contained the sperm of a parental male. We then poured 100 mL of lake water at lake temperature directly onto the sperm in each cup, briefly and gently swirling the water in the cup to promote simultaneous activation of the sperm.

In the first treatment, 50–75 eggs were placed in each cup and activated at the same time as the sperm (time from sperm activation = 0 s). For the subsequent three treatments we activated the sperm by pouring water into the cup, then waited 10, 30, or 60 s before adding 50–75 eggs to the activated sperm in lake water. Because the sperm of bluegill swim for less than 2 min (Leach and Montgomerie 2000; Burness et al. 2004), the water was drained, and fresh lake water was poured onto the eggs 2 min post activation. The eggs were then incubated for 12 h at room temperature (ca. 20 °C) to allow the fertilized embryos to develop. We then examined 50 eggs per cup at 30× magnification to determine fertilization success. Fertilized eggs had distinct developmental characteristics, including eye spots and somites, that were absent from unfertilized eggs. Only clear, undamaged eggs were counted and categorized with respect to fertilization success.

Statistical analysis

We used a repeated-measures ANOVA with male identity as a repeated measure, fertilization success (arcsine transformed) as the dependent variable, and time from sperm activation and tactic (both fixed effects) as factors. Because we were also interested in whether individual females may be able to bias fertilization of their eggs toward a sneaker or parental male, we conducted a three-factor ANOVA using female identity, male tactic, and time from sperm activation as factors. Because of constraints associated with sample size in the three-factor ANOVA, we were unable to test the three-way interaction of time from activation × female identity ×

Fig. 1. Mean (±SE) fertilization success declines more quickly over time in sneaker male relative to parental male bluegill (*Lepomis macrochirus*). A quadratic curve is fitted to both sneakers and parentals. *, $P = 0.058$; **, $P < 0.001$.

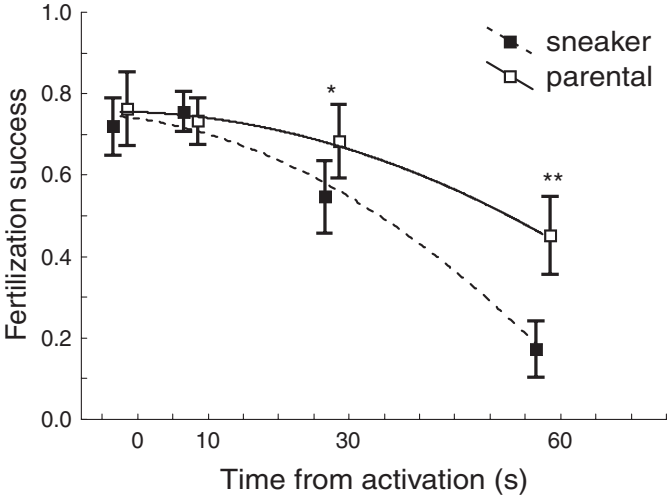
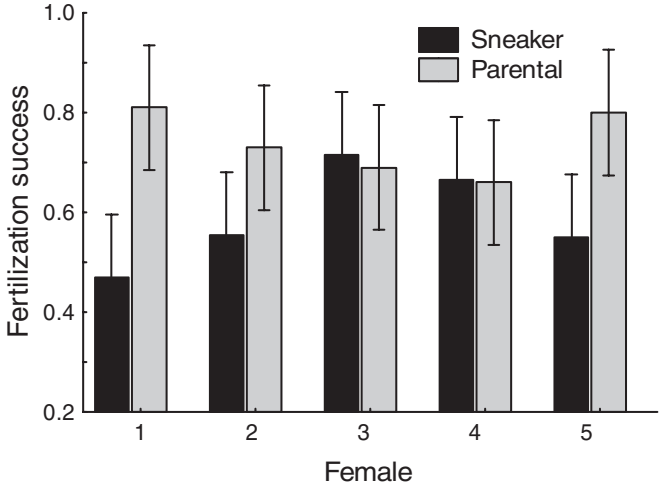


Fig. 2. Least squares means (LSM) of fertilization success for sneaker and parental males for each of five female bluegill. Bars represent 95% confidence intervals. LSM control for time from sperm activation.



male tactic, but we were able to test for all two-way interactions

Results

Our results indicated a significant difference between tactics in fertilization success (repeated-measures ANOVA: $F_{[1,23]} = 6.31$, $P = 0.020$), and in males of both mating tactics, fertilization success declined with time from sperm activation (time, $F_{[3,23]} = 1.66$, $P < 0.001$). Interestingly, there was a tactic × time interaction ($F_{[3,23]} = 4.22$, $P = 0.016$), indicating a tactic-specific decline in fertilization success over time. Both tactics had similar fertilization success during the first 10 s post activation, but by 30 and 60 s post activation, post hoc contrasts indicated differences between the tactics. At 30 s post activation, the two tactics showed a nonsignificant difference in fertilization success ($F_{[1,23]} = 3.98$, $P = 0.058$; Fig. 1), and at 60 s post activation there was a significant difference in fertilization success between parental and sneaker males ($F_{[1,23]} = 15.78$, $P < 0.001$; Fig. 1). Parental males had higher fertilization success than sneakers, consistent with parental sperm's higher motility and swimming speed at those times from activation (Burness et al. 2004).

A three-factor ANOVA ($F_{[12,27]} = 5.66$, $P = 0.002$) was used to test whether females biased fertilization toward either male tactic. Although our sample size prohibited us from (*i*) using male identity as a repeated measure and (*ii*) examining the three-way interaction among time from sperm activation and female identity and male tactic, our results describe some interesting patterns (Fig. 2). First, as was the case in the repeated-measures ANOVA, time from activation ($F_{[3,12]} = 32.24$, $P < 0.001$), male tactic ($F_{[1,12]} = 16.31$, $P = 0.002$), and the time × tactic interaction ($F_{[3,12]} = 4.21$, $P = 0.03$) were each significant. Second, although total fertilization success did not vary among individual females ($F_{[4,12]} = 0.4$, $P = 0.81$), there was a significant interaction between female identity and male tactic ($F_{[4,12]} = 3.82$, $P = 0.032$).

Discussion

Our results indicate a clear relation between tactic-specific sperm traits and fertilization success. We show for the first time that the sperm of parental and sneaker males have different fertilization capacities as time from activation increases. Although sneaker males apparently fertilize more eggs per ejaculate than parentals under field conditions (Fu et al. 2001), our results indicate that, when controlling for sperm density, parentals have an increasing advantage over time from ejaculation. This is due to the steep decline in motility and sperm swimming speed over time that sneakers experience, relative to sperm from parentals (Burness et al. 2004). Our results are entirely consistent with the prediction that, in externally fertilizing fishes, ejaculates with a high proportion of motile sperm and with fast swimming sperm will have relatively high fertilization success (Parker 1998; Gage et al. 2004). In addition, we provide evidence that the eggs of individual females may have a fertilization bias with respect to male tactic. In this case, the sperm of three parental males had higher fertilization success with the eggs of three of five females. This supports the intriguing hypothesis that females may individually bias the fertilization of their eggs toward one male tactic (sensu Evans et al. 2003).

The superior fertilizing capacity of parental sperm relative to sneaker sperm was recently noted by Neff et al. (2003). They conducted a sperm-mixing experiment and found that it took 1.7 times more sneaker sperm than parental sperm to obtain equal paternity between the two mating tactics. While it is unclear whether the protocol of Neff et al. (2003) resulted in immediate fertilization following activation of the sperm, our data show that any delay in fertilization would provide an unintended but clear advantage for parental males. This demonstrates the importance of taking the elapsed time from sperm activation into account when performing fertilization experiments in the laboratory.

If parental males produce larger ejaculates (Leach and Montgomerie 2000), as well as sperm with superior fertiliz-

ing capacity relative to sneakers (Neff et al. 2003; this study), how do sneakers sire more offspring during spawning under field conditions (Fu et al. 2001)? We suggest two testable hypotheses. (1) Laboratory studies, as a consequence of allowing sneaker and parental sperm extended contact with eggs, may unintentionally favour parental fertilization success. (2) A key component of fertilization success in sneakers in the field may be the physical position of the female when she spawns (Fu et al. 2001). Sneakers may thus achieve higher fertilization than parentals by positioning themselves closer to the female, by timing their ejaculations more precisely (i.e., exactly when a female releases her eggs), and by having initially faster swimming sperm than parental males. Sneaker bluegill may also release more sperm per ejaculate than parentals in the field, although currently this remains unknown.

A further hypothesis that may explain the relative success of parental males in sperm competition in the laboratory may be the presence of a fertilization bias toward one male over another. This type of bias in fertilization success with respect to male phenotype has been shown in the guppy (*Poecilia reticulata* Peters, 1859), where an artificial insemination experiment that controlled sperm numbers revealed that small males and heavily ornamented males had an advantage in sperm competition (Evans et al. 2003). Although our experiments were not performed in sperm competition, our results also reveal a bias in fertilization success toward one male tactic over another within a single female. This could represent some form of cryptic female choice (Birkhead and Pizzari 2002). Recent studies of Arctic char (*Salvelinus alpinus* (L., 1758)) suggest that sperm may differentially interact with the ovarian fluid produced by the female, and thus the sperm may swim more quickly in the ovarian fluid of some females compared with others (Urbach et al. 2005).

Although our results indicate that sperm from parentals that have been active for a relatively long period of time will have an advantage with respect to fertilization success over sneakers, our design prevented us from testing the reciprocal prediction that the sperm of sneakers have an advantage over parentals shortly post activation. To test this would have required the separation of sperm from the eggs after brief contact (e.g., 10 s), a technique that results in damage to the eggs (A.I. Schulte-Hostedde, S.J. Casselman, and R. Montgomerie, unpublished data). Indeed, although sneakers may have an early advantage when fertilizing eggs, our results indicate that sneakers and parentals fertilize the same number of eggs, and that a larger proportion of fertilizations in parentals take place late post sperm activation relative to sneakers. Biologically, this is relevant because bluegill are protracted spawners and females may release thousands of eggs over a period of time (Neff et al. 2003). Because the sperm of parental males is capable of fertilizing eggs for a longer period of time than the sperm of sneakers, their sperm may have an advantage over the sperm of sneakers when females continually release eggs. Under these conditions, sneakers will be at a disadvantage because they are producing small volumes (Leach and Montgomerie 2000) of fast swimming sperm that lose motility and speed rapidly (Burness et al. 2004).

Interestingly, greater longevity of sperm appears to occur in the cuckolder (sneaker) tactic rather than the parental tactic in other species of fish. For example, in the rose bitterling (*Rhodeus ocellatus* (Kner, 1866)), sneakers ejaculate before the female spawns, and therefore the sperm must swim for a lengthy period of time to fertilize the eggs (Kanoh 1996). In Atlantic salmon (*Salmo salar* L., 1758), parr (sneakers) have a longer duration of motility than anadromous (parental) males (Gage et al. 1995), and higher fertilization success (Vladic and Järvi 2001). Sneaker bluegill may have sperm with lower longevity, owing to selection favouring alternative ways in which to maximize fertilization success.

Our study has shown that ejaculates with high motility and high sperm swimming speed late in the sperm activation cycle are advantageous with respect to fertilization in a situation without sperm competition. This has potential implication for interpreting laboratory studies that demonstrate higher fertilization success in parental than sneaker males (e.g., Neff et al. 2003). We suggest two complimentary lines of enquiry for future studies. (1) In the laboratory, using a two-step sperm dilution process to test whether sneaker sperm have higher fertilization success than parental sperm immediately following activation. Currently, separating sperm and eggs at different times post activation without damage is challenging. (2) In the field, identifying whether sneaker and parental males time their ejaculations differently with respect to female egg release. We suggest that these two complimentary studies can together reconcile the high fertilizing capacity of the sperm from parental males in the laboratory (Neff et al. 2003; this study) with the high fertilization success of sneakers during spawning in the field (Fu et al. 2001).

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