Sperm traits in relation to male quality in colonial spawning bluegill

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Sperm traits (morphology, motility and concentration within ejaculates) and various correlates of male quality (age, body condition, spawning location and timing) were studied in bluegill *Lepomis macrochirus*, breeding in both the interior and periphery of six colonies in Lake Opinicon, Ontario, Canada. Sperm traits varied significantly more among than within males suggesting that some aspect of male phenotype might influence sperm morphology and behaviour. No measures of male body condition or size were correlated with any sperm or ejaculate traits, when controlling statistically for confounding variables. Sperm swimming speed increased significantly with male age and varied significantly among spawning bouts (controlling for sperm tail length) suggesting that some unknown aspects of male quality might influence the fertilization capacity of spermatozoa. Sperm concentration in ejaculates was significantly higher in males nesting in the interior rather than the periphery of a colony suggesting that those males might also have higher fertilization capacity correlated with their superior dominance status or the lower risk of sperm competition. Thus, older males nesting in the interior of a colony during the first spawning bout of the season are expected to be the best sperm competitors in this population, but the physiological reasons for this increased fertilization capacity remain unknown.

Key words: age; bluegill; colonial spawning; condition; sperm competition; sperm motility.

INTRODUCTION

In externally fertilizing fishes, male fertilization success might be expected to be influenced by stage of the breeding season (Methven & Crim, 1991), distance between the sexes (Gross, 1985), sperm competition with other males (Stockley *et al*., 1997), variation in ejaculate and sperm traits (Marconato & Shapiro, 1996; Lahnsteiner *et al*., 1998), male condition (Rakitin *et al*., 1999) and interactions between sperm and eggs (Rakitin *et al*., 1999; Turner & Montgomerie, 2002). Despite recent work, and considerable speculation, on the influence of these factors on fertilization success, little is known about how and why they might differ among males in a population. For example, sperm quality of Atlantic halibut *Hippoglossus hippoglossus* (L.) declines during the breeding season (Methven & Crim, 1991), but the reasons for this decline and for differences among males have yet to be determined. Similarly, ejaculate and sperm traits are often found to be highly
variable among male fishes (Gage & Morrow, 2001) but relatively little is known about how these traits are influenced by male phenotypic quality with respect to factors like age, body condition and experience. Thus, while there is clearly considerable variation in male fertilization success in natural (and hatchery) populations (Pennell & Barton, 1996), the genetic and phenotypic basis for such variation has rarely been studied (Rakitin et al., 1999; Simmons & Kotiaho, 2002). An understanding of the causes of variation in sperm quality can thus provide important insights into the factors that affect male reproductive success in fishes.

This study examined several ejaculate and sperm traits of male bluegill Lepomis macrochirus Rafinesque in relation to a variety of measures of male phenotypic quality and sperm competition risk. Based on previous work in externally fertilizing fishes (Stockley et al., 1997; Rakitin et al., 1999) in general, and bluegill in particular (Gross, 1982), it was expected that male age (Babushkin, 1972), body condition (Rakitin et al., 1999), dominance (Gross, 1982) and the timing of spawning (Rakitin et al., 1999) might all influence fertilization success. Thus, one goal of this research was to determine the potential influence of sperm quality on the reproductive success of male bluegill that varied in their phenotypic quality.

Bluegill are a common centrarchid in central and eastern North America, nesting in colonies of up to 100 males or more in the littoral zone of freshwater lakes. Bluegill males adopt either parental or cuckolder breeding tactics (Gross, 1982) that have very different spawning behaviours, ejaculate investments and sperm competition risks but no apparent difference in sperm traits (Leach & Montgomerie, 2000). Parental males build nests on small contiguous territories that they defend until the offspring disperse, about a week after the male spawns with one or more females. Cuckolder males are either sneakers or satellites who do not build or defend nests and always spawn in the nest of a parental male (Gross, 1982). The present study focuses only on parental males so that analyses would not be confounded by differences among the different male tactics.

This study was also designed to look at the relation between nest location within a colony and sperm traits, because previous studies had shown that interior nesting males were more likely to be larger, dominant and less often threatened by egg predators than males nesting on the periphery (Gross, 1980; Gross & MacMillan, 1981). Additionally, there may be variation in sperm competition within a colony, with males nesting near the periphery of a colony experiencing higher levels of sperm competition from cuckolders than males in the interior (Neff et al., 2003). Thus, males nesting at the periphery of a colony were expected to have sperm traits that would make them better sperm competitors, for traits (e.g. sperm concentration) that males might be able to adjust in the short-term based on their location within a colony.

**MATERIALS AND METHODS**

**SAMPLING**

Sampling was conducted at the Queen’s University Biology Station on Lake Opinicon, Ontario (44°33'N; 76°19'W) during the bluegill spawning season (12 June to 5 July) in 2000. From late May to early July, the littoral zone adjacent to 2 km of shoreline was
monitored daily for spawning activity. The study population and breeding habitat in this lake are described in Gross (1982) and Gross & MacMillan (1981).

Nesting males were collected on the first day after a spawning event in six relatively large colonies (>50 nesting males) where nest locations within colonies could be clearly characterized. Bluegill spawn during up to five bouts, a few days apart, in each breeding season (Gross, 1982). These spawning bouts last up to 3 days and are synchronous both within and among colonies (Gross & MacMillan, 1981), though the synchrony among colonies tends to decline as the season progresses (Gross, 1980). Two colonies were sampled during the first spawning bout (12–13 June), two in the second bout (17–18 June) and one in each of the third (22–23 June) and fourth bouts (26–27 June).

Males were taken from both the interior and periphery of each colony: 12 males from the interior such that there were at least three other males nesting in each direction to the edge of the colony, and 10 males from nests at the very periphery of each colony. Sampled fish were transported back to the laboratory where they were kept alive in large tanks with flowing lake water until processing, <4 h after capture. Approximately 40 μl of milt was collected in a capillary tube placed at the gonopore while gently applying pressure along the abdomen of the fish. Milt contaminated with urine or water was discarded. After milt collection, fish were humanely killed by transecting the vertebral column.

The standard length (L_s) of each male was measured to the nearest mm and its mass (M) weighed to the nearest 0.1 g. To determine male age, the sagittae otoliths were removed and embedded in epoxy. Transverse sections (400 μm) were then removed from the nucleus using an Isomet saw. The otoliths were ground, polished and acid-etched, and the etched surface was replicated with cellulose acetate dissolved in acetone. Age was determined by viewing these replicas with transmitted light (Casselman & Gunn, 1992).

**Sperm Morphology**

To measure sperm tail lengths, 5 μl of milt from each male was diluted in 750 μl of water, then a drop of this solution was placed on a microscope slide and allowed to dry. The slide was then viewed under a microscope at ×600 magnification and images of individual sperm were digitized on a computer. Object Image (version 2.07, available at http://simon.bio.uva.nl/object-image.html) was used to measure the length (μm) of 10 haphazardly chosen sperm from each fish, measuring from the tip of the tail to the base of the sperm head.

**Sperm Concentration**

Sperm concentration was measured using a haemacytometer (improved Neubauer counting chamber). Freshly stripped milt was diluted 150-fold in water and a sample of this solution placed in the haemacytometer chamber. When sperm ceased forward motility, the number of sperm in 10 haphazardly chosen haemacytometer cells (50 × 50 μm) were counted under ×400 magnification. The average number of sperm per ml of semen was then determined for each fish using the number of sperm counted, the dilution and the volume of liquid in each cell counted.

**Sperm Motility**

Milt samples were prepared for measuring sperm motility by first adding 5 μl of milt to 750 μl of high osmolarity solution (c. 400 mOsM; 20 mM Tris, 200 mM NaCl, 2 mM KCl). This dilution does not activate the sperm but improves estimates of sperm motility because sperm in such diluted samples are more likely to be activated simultaneously when an activation medium is added to the sample (Billard & Cosson, 1992). Then, 1-0 μl of this diluted but inactive milt was placed in the haemacytometer chamber and flooded with lake water at 20°C (ambient water temperature at bluegill nests) to activate the sperm. The swimming sperm were video-taped at 30 frames s⁻¹ under ×400 magnification for a minimum of 60 s after activation.
The video-tapes of swimming sperm were then digitized by importing clips from the video-tapes into a computer using Avid Cinema (v. 1.3.1) and edited to produce clips of an appropriate length for analysis using QuickTime (v. 4.0). These edited clips were viewed using Object Image which allows each clip to be viewed frame by frame and x–y co-ordinates to be determined for the sperm heads in each frame. Movement variables were determined by following 10 sperm from each male for 10 frames at 10, 30 and 60 s post-activation and recording their location at frames 1, 4, 7 and 10. Swimming speed was calculated from the distance travelled between frames 1, 4, 7 and 10, straight speed from the straight line distance travelled between frames 1 and 10, and a linearity index as the swimming speed divided by the straight speed. For some analyses, the median of each of these measures per male was used. Though most studies that measure sperm swimming speeds use mean speeds per male (Leach & Montgomerie, 2000; Gage et al., 2002), the distributions of swimming speeds were significantly negatively skewed within some males. For example, for swimming speed at 10 s post-activation, 16 of 23 males had negatively skewed distributions and this is significantly more than expected by chance (Binomial test, \( P = 0.05 \)). Thus, mean values somewhat underestimate the central tendency of motility variables. Given that a tiny proportion of sperm from an ejaculate actually fertilizes eggs, median sperm speeds may more accurately reflect sperm quality relevant to fertilization success if the fastest sperm are most likely to be successful (unpubl. data). Mean swimming speed was highly correlated with median swimming speed among males (\( r = 0.987, P < 0.0001, n = 22 \)) and analyses of mean rather than median speeds showed the same patterns. Sperm swimming speeds at 10 s post-activation are used here because recent work has shown that the majority of fertilization probably occurs within a few seconds after ejaculation in externally fertilizing fishes (Vladic & Järvi, 1997; Hoysak & Liley, 2001).

BODY CONDITION

Four different measures of body condition were calculated. Fulton’s condition factor (\( K \)) was calculated for each fish as \( K = ML^{-3/4} \times 10^5 \) (Sutton et al., 2000). Second, to estimate liver glucose concentration, the liver of each freshly killed fish was removed and stored at \(-60^\circ C\) until it could be analysed. Glycogen was later purified from a sample of liver using the procedure outlined in Arthur et al. (1992). The sample of purified glycogen was then hydrolysed using amyloglucosidase and the resulting glucose measured using spectrophotometric enzyme-linked assays (Arthur et al., 1992). Third, per cent dry muscle mass was determined by dividing the dried mass of a muscle sample by its fresh mass. To do this a strip of white muscle was removed from the dorsal side along the vertebrae of each partially thawed fish, and weighed to the nearest 0.001 g. Each muscle sample was then placed in an oven at 60 \(^{\circ} \) C and weighed daily until its mass remained constant. Fourth, residual body mass was calculated as the residuals from the regression of \( \log_{10} M \) and \( \log_{10} L_S \).

ANALYSES

In all analyses involving sperm swimming speed, residual sperm swimming speed from the regression of sperm swimming speed on sperm length is used because a positive relation has been found between these two variables in this and some other fish populations (unpubl. data). All analyses were performed on mean (or median) sperm traits per male. Descriptive statistics are reported as mean ± s.e.

RESULTS

COMPARING SPAWNING BOUTS

To determine whether male phenotype, as well as sperm and ejaculate quality, varied among the four consecutive spawning bouts, these variables were
compared among spawning bouts with ANOVA, including nest location (inter-
ior \textit{v.} periphery) as a factor to account for potential effects of nest location. In no case was nest location or the interaction term significant so statistics are reported here only for the effect of spawning bout (with interaction term removed). Male \( L_S \), \( M \), age and the three body condition indices did not differ significantly among the four spawning bouts sampled (ANOVA, \( F_{3,15} \), all \( P > 0.31 \)) nor did sperm length (\( F_{3,14}, P = 0.29 \)) or sperm concentration (\( F_{3,15}, P = 0.08 \)). Despite this, all of the sperm movement variables measured at 10 s after activation differed significantly among spawning bouts (Table I), with males spawning during the first bout of the season having the fastest sperm and males spawning during the second bout having the slowest (Fig. 1). Straight speed and linearity index showed the same general pattern (Table I). Because swimming speed varied among spawning bouts, spawning bout is controlled for statistically in subsequent analyses of this variable; because none of the other variables showed significant variation among spawning bouts, spawning bouts were pooled for analyses of those variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>( F_{3,19} )</th>
<th>Rank</th>
</tr>
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<tbody>
<tr>
<td>Swimming speed</td>
<td>7.9 ( (P = 0.001))</td>
<td>1–4–3–2</td>
</tr>
<tr>
<td>Straight speed</td>
<td>9.4 ( (P = 0.0005))</td>
<td>1–4–3–2</td>
</tr>
<tr>
<td>Linearity index</td>
<td>3.5 ( (P = 0.04))</td>
<td>1–4–2–3</td>
</tr>
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Table I. Variation in sperm swimming speed, straight speed and a swimming linearity index of male bluegill spermatozoa at 10 s post-activation among consecutive spawning bouts tested by ANOVA. The ranks of spawning bouts are shown from highest to lowest within each variable, where 1 is the first bout in the season and 4 is the fourth and probably last (see Fig. 1).

Fig. 1. Sperm swimming speed at 10 s post-activation for male bluegill in four consecutive spawning bouts. Data plotted are the median sperm swimming speed for each male. Box plots show 10th, 25th, 50th, 75th and 90th percentiles with horizontal lines and all data points outside that range; sample sizes (number of males) are shown on top of each box. All data points are shown for 3rd and 4th sawning bouts. \rightarrow, median for each colony.

COMPARING COLONIES

To compare male phenotypes and sperm traits among colonies, data for the first two spawning bouts were analysed, where two colonies were sampled within each bout. There was no significant variation in any of the male phenotype (all $P > 0.06$) or sperm traits (all $P > 0.25$) between colonies, within spawning bouts. Although sample sizes are small, this analysis suggests that it is reasonable to pool males across colonies, within spawning bouts, for further analysis.

COMPARING NEST LOCATIONS

Males nesting in the interior of a colony had higher sperm concentration ($9.0 \pm 0.6$ million sperm $\mu l^{-1}$, $n = 11$) and slightly shorter sperm ($44.1 \pm 0.7 \mu m$, $n = 11$) than males nesting on the periphery of a colony ($7.5 \pm 0.6$ million sperm $\mu l^{-1}$, $n = 12$; $45.1 \pm 0.6 \mu m$, $n = 11$) but these differences were not significant ($t$-test, $P = 0.08$ and 0.26; Fig. 2). The males that nested in the interior of a colony had, however, on average, 1.5 million more sperm $\mu l^{-1}$ in their milt than those on the periphery, but this analysis had relatively low power (0.41) to detect a significant difference of this magnitude with this small sample size. Comparing mean sperm concentrations of males in the interior and periphery of colonies, by colony, revealed higher values for interior-nesting males in all but one colony and the difference between these means is significant (paired $t$-test, $P = 0.05$, $n = 6$ colonies). In the one colony where mean sperm concentrations were higher for periphery-nesting males, the difference was slight (inner = 7.23, outer = 7.85 million sperm $\mu l^{-1}$) and the sample sizes were very small (one outer, two inner males). Males nesting in the interior ($n = 12$) and periphery ($n = 11$) of a colony also did not differ in age ($t$-test, $P = 0.61$), $L_S$ ($t = 0.4$, $P = 0.97$), any of the condition indices that were measured ($P > 0.35$ in each case), nor any measures of sperm swimming speed ($P > 0.50$).

COMPARING MALES

Both mean sperm tail length (ANOVA, $F_{21,198}$, $P < 0.0001$) and median sperm swimming speed at 10 s post-activation (Kruskal–Wallis test, $\chi^2 = 124$, d.f. = 22,
$P < 0.0001$) varied significantly among all males sampled (Fig. 3). Because median sperm swimming speeds varied significantly among spawning bouts, this variable was also analysed among males within both spawning bouts and colonies. Sperm swimming speed varied significantly among males within three of the six colonies ($\chi^2 \geq 12.2$, d.f. > 2, $P \leq 0.01$, in each case), and two of the four spawning bouts ($\chi^2 \geq 12.2$, d.f. > 2, $P \leq 0.002$, in each case). Mean sperm tail length also varied significantly among males within four of the six colonies sampled ($F$-test, d.f. > 1, 18, $P \leq 0.02$, in each case). Median sperm swimming and straight speeds and the linearity index varied significantly among males for the majority of sampling times post-activation within spawning bouts (Kruskal–Wallis tests, $P \leq 0.05$ in 37 of 54 analyses).

![Graph showing variation in sperm tail lengths and swimming speeds among males](image)

**Fig. 3.** Variation in (a) sperm tail lengths and (b) sperm swimming speeds (at 10 s post-activation) within and among male bluegill. Box plots as in Fig. 1. Ten sperm were measured and tracked for each male. Colonies and spawning bouts are indicated at the top of the figure.
Some of the variation in sperm swimming speeds among males can be explained by variation in sperm size as there was a significant positive correlation between median swimming speed at 10 s post-activation and mean sperm tail length among males ($r = 0.67$, $P = 0.0007$, $n = 22$). Because of this correlation, sperm tail length was controlled statistically in the following analyses.

Sperm concentration and sperm tail length were not significantly correlated among males ($r = -0.21$, $P = 0.35$, $n = 22$ males). Nor was median sperm swimming speed, at any post-activation time, correlated with sperm concentration among males (all $P > 0.23$, controlling for sperm length).

Sperm tail length was not correlated with male age ($r = -0.08$, $P = 0.73$, $n = 22$). Sperm swimming speed at 10 s post-activation, however, was significantly related to male age (partial $r = 0.44$, $P = 0.047$, $n = 22$, controlling for sperm length). Since there were only two 6 year-old males in this sample, the (residual) median sperm swimming speeds of 7 and 9 year-olds were also compared, and again an age effect was found ($t$-test with unequal variances, $P = 0.03$, $n = 15$, 5). Thus, the spermatozoa of older males swam faster than those of younger males, on average, when controlling for variation in sperm tail length among males (Fig. 4).

Sperm swimming speed at 10 s post-activation was negatively correlated with per cent muscle dry matter (partial $r = -0.47$, controlling for sperm tail length), $K$ (partial $r = -0.34$), liver glucose (partial $r = -0.14$) and residual body mass (partial $r = -0.18$); but none of these correlations is significant after Bonferroni correction for multiple analyses ($n = 22$, $P > 0.10$, in each case). The large effect size for per cent dry matter suggests that there may be a biologically interesting relation between sperm swimming speed and that measure of condition but per cent dry matter also declined significantly with age ($r = -0.80$, $P < 0.0001$, $n = 23$). If condition has an important effect on sperm swimming speed, a relation would be expected between condition and swimming speed within age classes. There was, however, no significant relation between sperm swimming speed at 10 s post-activation and per cent dry matter when age and sperm tail length were controlled (partial $r = -0.22$, $P = 0.87$, $n = 22$), nor was there a significant

![Fig. 4](image_url)
relation within the 7 year-old age class (partial $r = -0.20$, $P = 0.49$, $n = 16$), where the sample size was largest, though both correlations remain negative and are worth further investigation. Results of this study, however, indicate that sperm swimming speed in bluegill appears not to have been influenced by the body condition measures that were assayed, and sperm swimming speed increased with age (controlling for sperm length) independent of body condition.

**DISCUSSION**

Despite considerable variation among males in different indices of body condition, there was no evidence whatsoever of a positive relation between sperm swimming speed and male body condition, independent of male age. Such a relation between sperm quality and male body condition might not be remarkable, for the following reasons. First, there may simply be no causal relation between male condition and those characteristics of ejaculate quality that would influence sperm swimming speed. Spermatogenic tissue, for example, might be designed to produce spermatozoa whose structure and energetic capacity are not affected by variation in male body condition. Second, the indices of body condition that were used (Fulton's index, liver glycogen, percent dry matter, residual body mass) might not be correlated with aspects of condition that impinge on sperm quality. Though the condition indices that were used are commonly employed in studies of fish ecology and behaviour, they are sometimes only weakly correlated with one another (Sutton et al., 2000), and thus probably measure different aspects of physiological condition. Third, the body condition of males at spawning might well be a consequence rather than a cause of variation in sperm and ejaculate quality. Thus, males in good condition before spawning might be able to allocate more resources into ejaculates, resulting in larger sperm or sperm with higher energetic capacity, thereby reducing condition at spawning (when they were measured in the present study). This situation would result in a negative correlation between sperm swimming speed and male condition at spawning. Indeed, all of the measures of condition were negatively correlated with sperm swimming speed (controlling for sperm length), though none of these relations was statistically significant. Clearly, some experimental work is needed to determine how varying the body condition of individual males influences their sperm and ejaculate quality.

The significant variation in sperm swimming speed with male age, and among males spawning in different bouts, however, does suggest that sperm swimming speed might vary with some aspect of male quality. Though the sample sizes were small, it was clear that the swimming speed of sperm from males spawning in the first bout of the season was significantly faster than the sperm of males spawning in the second bout (Fig. 1). Because these first two bouts began only 6 days apart, the second spawning bout of the season could not have included males who were successful at raising a brood during the first bout (since the period from fertilization to independence of fry is 8–9 days in bluegill; Gross, 1982). Thus males spawning in the second bout were either failed breeders from the first bout, or males that delayed until the second spawning bout before attempting to defend a territory. Under both situations, males spawning in the
second bout would be expected to be in poorer condition or otherwise of lower quality. Sperm swimming speed seemed to increase through the third and fourth spawning bouts (Fig. 1) as might be expected if those bouts included successful males from the first bout, but the sample sizes from these last two bouts was too small to be certain that this apparent trend was real. It is also possible that social conditions and pheromonal environment at the time of spawning might vary between spawning bouts and that this might influence sperm swimming speeds of the males in each bout (Zheng et al., 1997). More work will be needed to determine if these factors influence sperm traits in bluegill.

The positive relation between sperm swimming speed and the age of male bluegill (Fig. 4) is more difficult to explain. Unfortunately, most of the males sampled were 7 year-olds so it was not possible to be certain of the shape of the relation between these two variables. Nonetheless, it seems clear that this positive relation is not simply due to the differential mortality of males with slower sperm as all of the 6 year-olds that were sampled had slower sperm than all of the 9 year-olds (Fig. 4). Since only 1 year was sampled, it is possible that this speed and age relation reflects variation in male sperm quality among cohorts and is not related to age per se. A study that samples the same cohorts, or even the same males, in different years will be needed to resolve this. It is also possible that sperm quality simply improves as males age such that the sperm of older males is morphologically superior (e.g. more streamlined) or has higher energetic capacity.

The only difference found between interior- and periphery-nesting males was a higher concentration of spermatozoa in the ejaculates of interior nesters. While this might reflect differences in male quality, it could also be a simple consequence of differences in spawning rates; as interior males have higher clutch sizes (Gross, 1980), possibly due to more frequent visits by spawning females, they may simply have higher sperm concentration in their ejaculates. More work will be needed to assess how ejaculate size and sperm concentration within ejaculates changes with the number and frequency of ejaculations.

These findings are particularly interesting in the context of sperm competition. Selection to maximize reproductive success should favour the evolution of sperm and ejaculate traits that enhance a male’s capacity to fertilize eggs during spawning (Parker, 1984; Ball & Parker, 1996). Thus males with the highest phenotypic quality in a population are expected to produce the highest quality ejaculates. Only limited evidence, however, was found for this, though, as noted above, more research will be needed to determine whether male condition at spawning is a consequence rather than a cause of sperm quality. Older males nesting in the interior of colonies during the first spawning bout of the season are thus expected to have the highest fertilization capacity, and to be most successful under sperm competition within this study population, because they have the fastest swimming sperm (Figs 1, 2 and 4) and the highest sperm concentration in their milt (Fig. 3). It remains to be determined whether these males actually have the highest reproductive success.

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