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Reproductive-Tactic-Specific Variation in Sperm Swimming Speeds in a Shell-Brooding Cichlid[1]

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ABSTRACT

Theory predicts that males experiencing elevated levels of sperm competition will invest more in gonads and produce faster-swimming sperm. Although there is ample evidence in support of the first prediction, few studies have examined sperm swimming speed in relation to sperm competition. In this study, we tested these predictions from sperm competition theory by examining sperm characteristics in *Telmatochromis vittatus*, a small shell-brooding cichlid fish endemic to Lake Tanganyika. Males exhibit four different reproductive tactics: pirate, territorial, satellite, and sneaker. Pirate males temporarily displace all other competing males from a shell nest, whereas sneaker males always release sperm in the presence of territorial and satellite males. Due to the fact that sneakers spawn in the presence of another male, sneakers face the highest levels of sperm competition and pirates the lowest, whereas satellites and territorials experience intermediate levels. In accordance with predictions, sperm from sneakers swam faster than sperm from males adopting the other reproductive tactics, whereas sperm from pirates was slowest. Interestingly, we were unable to detect any variation in sperm tail length among these reproductive tactics. Thus, sperm competition appears to have influenced sperm energetics in this species without having any influence on sperm size.

alternative reproductive tactics, behavior, Cichlidae, ejaculates, fishes, gamete biology, sperm, sperm competition, sperm motility and transport, teleost

INTRODUCTION

Sperm competition is recognized as a powerful evolutionary force shaping male reproductive behaviors and physiology [1]. Males can mate in a favored role (i.e., gaining primary access to females) or in a disfavored role (i.e., mating in the presence of another male), and the risk of sperm competition is contingent upon male reproductive roles. Males mating in favored roles sequester and guard females, experiencing sperm competition only when other males are present during mating. Yet disfavored males, who often perform sneak fertilizations, experience sperm competition during every mating, as they always release sperm in the presence of a competing male. Theory predicts that disfavored males should invest more heavily in ejaculates and allocate greater numbers of sperm during mating [2, 3], as sperm quality [4, 5] and number [6] influence the probability of successfully fertilizing eggs. As predicted from theory, males in a variety of species performing sneak fertilizations (disfavored males) have larger relative testis mass than males in favored roles [7–21 but see 22]. Yet male reproductive roles are often dependent upon environmental and social conditions, and males can often rapidly adjust ejaculate characteristics; for example, ejaculating greater sperm numbers when mating in a disfavored role [23–27]. Alternatively, in species with alternative male reproductive tactics, some males will consistently mate in either the favored (conventional, parental, or territorial males) or the disfavored (sneaker males) role, providing a convenient natural system for the study of sperm competition.

In addition to its influence on ejaculate size and allocation, sperm competition is also expected to affect sperm morphology and behavior, particularly swimming speed [28]. Sperm from males mating in the disfavored role (i.e., sneaker males), which are experiencing greater levels of sperm competition, are expected to have longer tails [28] that displace a greater volume of water with each undulation, and are thus capable of swimming faster [29]. If sperm swimming speed is related to fertilization success, as demonstrated in a number of taxa (fishes [4, 5, 30], birds [31–33], invertebrates [34], and mammals [35]), then longer and faster sperm may increase the fertilization success of sneaker males relative to conventional males. Hence, in externally fertilizing fishes, sperm swimming speed may be particularly strongly influenced by sperm competition, as the first sperm cell to reach the micropyle (a narrow opening on the egg surface) of an ovum is most likely to successfully fertilize the egg [36, 37]. Consequently, an appropriate analogy for fertilization dynamics in externally fertilizing fishes is that of a race among sperm to reach the micropyle, with the fastest sperm winning the race.

Despite these predictions, the influence of sperm competition on sperm morphology, swimming speed, and longevity remains unclear, with recent studies presenting contradictory results, even within a single species. In support of theoretical predictions, sperm from sneaker males were longer in bluegill (*Lepomis macrochirus* [38]) and dung beetles (*Onthophagus binodis* [21]) and swam faster in bluegill [38] and arctic char (*Salvelinus alpinus* [23, 26]) than sperm from conventional males. However, contrary to predictions, no differences in sperm morphology [19, 39, 40] or swimming speed [19, 39, 41, 42] were detected between conventional males and sneakers in salmon (*Salmo salar*), roach (*Rutilus rutilus*), or a different study of bluegill. Furthermore, because both sperm swimming speed and the duration of sperm motility (i.e., sperm longevity) represent an energetic demand, a tradeoff is expected between sperm swimming speed and longevity [28]. Therefore, everything else being equal, sperm from sneakers are expected

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to swim faster but for shorter time periods than sperm from conventional males, a prediction that has also received mixed empirical support. Some studies have demonstrated that sneaker sperm are shorter lived (bluegill [19, 43] and roach [42]), some have failed to detect a difference between male tactics (salmon [39]), and still others showed that sneaker males had longer-lived sperm (salmon [7]; corkwing wrasse and *Symphodus melops* [20]; and stickleback and *Gasterosteus aculeatus* [44]). The inconsistency of these findings suggests that further exploration of sperm characteristics in species characterized by alterative reproductive tactics is warranted.

In this study, we examine sperm characteristics in *Telmatochromis vittatus*, a small, shell-brooding cichlid endemic to Lake Tanganyika, with four alternative male reproductive tactics: territorial, pirate, satellite, and sneaker [17]. In most species with alternative reproductive tactics, there are only two male morphs (territorial-parental-conventional males versus sneaker males), so our study species provides an unusual opportunity to assess differences among four distinct male tactics. Territorial males (50–62 mm standard length) control a nest made of many shells occupied by several females (density: 1.6 ± 0.8 females/m^2 [17]). These males court females by performing vigorous body quivers, then lead receptive females to a shell to spawn. Once the female has entered a shell, the male positions his genital papilla directly over the shell opening for 2–3 sec, apparently ejaculating sperm into the shell while the female lays her eggs [17]. Territorial males continue this ejaculatory posture for several hours. Pirate males (61–67 mm, the largest males in the population and more than 1.3 times the size of territorial males), patrol several shell nests that they frequently enter, displacing territorial males and fertilizing the ova of any female that the territorial male has attracted. Pirate males are nonterritorial, but they exclude other males from the vicinity of the shell while they are spawning. Satellite males (38–56 mm) are submissive to territorial and pirate males and, unlike territorial males, control only a single shell on or near the periphery of territorial males' shell nests. Satellite males guard their single shell and use it to court and spawn with a female. Satellite males do not attempt to spawn in the territorial male's shell nest. Sneaker males (22–34 mm) do not court females, but instead have small home ranges around a territorial male's shell nest. Sneakers parasitize territorial and satellite male spawnings either by entering a shell nest with the female and releasing sperm, or by ejaculating into a shell nest when the territorial or satellite male is absent. The reproductive tactics and body sizes of 148 males were studied in detail by Ota and Kohda [17, 45].

As both pirate and sneaker males engage in parasitic fertilizations, one might expect sperm characteristics to be enhanced in both of these tactics. However, while sneaker males likely perform parasitic spawnings involving simultaneous sperm release with other males, sperm release by pirate males is temporally and spatially separated from that of other males, because they exclude all other males from a nest during spawning [17]. As sneaker males release sperm simultaneously with other males during every spawning, they face higher levels of sperm competition than males adopting the other tactics. Therefore, based on both sperm competition theory [28] and the carefully studied behavioral and physiologic differences among males adopting the different reproductive tactics in *T. vittatus* [17], we predicted that selection would favor the production of faster-swimming sperm with longer tails in sneaker males (who experience higher levels of sperm competition) compared with all males using other reproductive tactics, particularly compared with pirate males (who likely experience the lowest level of sperm competition).

MATERIALS AND METHODS

Between 26 March 2005 and 25 April 2005, 38 male *T. vittatus* were collected using SCUBA from depths of 2 to 7 m at the southern end of Lake Tanganyika, primarily from Kasakalawe Bay, west of Mpulungu, Zambia. This research was conducted with the permission and cooperation of the Zambian Department of Fisheries, and all procedures described were reviewed and approved by the Animal Research Ethics Board of McMaster University and the Canadian Council for Animal Care guidelines. We located shell nests and collected males using a 1-mm-mesh fence net (1 m × 5 m) and handnets. In total we collected 5 pirates (standard length [SL]: 61.7–67.1 mm), 7 territorial males (SL: 50.5–61.1 mm), 15 satellites (SL: 38.0–47.6 mm), and 11 sneakers (SL: 25.6–37.5 mm). The male reproductive tactics were assigned based on the distributions of body sizes described by Ota and Khoda [17, 45]. According to Ota and Kohda [17], there is a 6-mm (50–56 mm) overlap in the size range of territorial and satellite males, so we classified these tactics with caution. However, only two males fell within this overlap, and collapsing territorial and satellite males into a single category or excluding these two males in this overlap category from our analysis did not change any of our conclusions.

At the surface we measured each fish's SL to the nearest 0.1 mm and body mass to the nearest 0.001 g. We then anesthetized the fish in benzocaine, quickly killed them via cervical severance, and removed their testes, which we then weighed to the nearest 0.001 g. Following Ota and Kohda [17], we defined mature males as individuals with testes mass >0.002 g.

Sperm Analysis

After the testes were weighed, one testis from each male was put on a glass slide, split open with a scalpel, and a drop of milt was collected and placed in a 2.0-ml microcentrifuge tube. Sperm were activated by adding 0.25 ml lake water (previously boiled and cooled to lake temperature) and gently shaking the tube for 1–2 sec [46]. We then placed 60 µl of the sperm/water mixture in a 1-mm-deep well on a slide, with a cover slip covering half of the depression so that the sperm/water sample could easily be added to the well. This sample was viewed on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, NY) mounted with a PixeLINK Megapixel PL-A662 digital video camera (PixeLINK, Ottawa, ON, Canada) to record sperm motility. Video recordings began as soon as the water was added to the microcentrifuge tube so that the period since activation could be determined accurately. Videos of active sperm were captured at 60 frames/sec at 200× magnification. Images were recorded using PixeLINK PL-A600 Series Camera Software (v3.1).

Sperm velocity was measured for 1 sec at 0.5, 1, 2, 3, 4, 5, 6, and 7 min after sperm activation using a CEROS (v12) video sperm analysis system (Hamilton-Thorne Research, Beverly, ME). The researcher was blind to male size and reproductive tactic when measuring sperm velocity. From some males, sperm continued to swim beyond 7 min after activation, but because the proportion of a male's sperm that was active past 7 min fell dramatically, we confined our analyses of sperm velocity to the first 7 min after activation. Due to water movement and variation in video quality, 0.5 min was the earliest time after activation that we were able to reliably measure sperm swimming speed of all males. We analyzed only those spermatozoa whose forward movement was traced for at least 0.33 sec (i.e., ≥20 frames, see [30, 38, 46] for a similar measurement criterion). The median sperm velocity (VAP; median smooth path velocity) and median curvilinear track velocity (VCL) were calculated for all spermatozoa recorded at each time period after activation (mean number of sperm recorded ± SEM: 76.7 ± 4.5, n =261, range: 5–488). Although VAP and VCL are strongly correlated (see Results), we analyzed and reported both of these measures of sperm swimming speed, as they measure slightly different aspects of sperm motion and currently there is no consensus in the literature as to which is more useful. We also measured sperm path straightness (STR), an estimate of the spermatozoon's departure from a straight line while swimming, as STR = VSL / VAP (where VSL is the velocity along a straight line connecting the start and end of the spermatozoon's path during the 1-sec period of each measurement).

Following a period of declining swimming speed, spermatozoa cease forward movement and begin to vibrate, providing a clear end point to measure sperm longevity. The longevity of sperm movement was measured as the time since activation at which 95% of the sperm no longer exhibited forward movement [46–49].

To measure sperm lengths, the remaining testis of each male was split open, and the free-flowing milt was diluted with lake water, spread over the glass slide, and allowed to air dry. The first 10 clear sperm images detected from each male were magnified to 1000× on the computer monitor, photographed, and measured using the National Institutes of Health's ImageJ software (v. 1.36, available at http://rsb.info.nih.gov/ij/). We traced a freehand line over the tail, measuring tail lengths (to the nearest 0.1 µm) from the center of the sperm's head to the end of its tail [50]. To avoid observer bias, all samples were measured blind to male size or reproductive tactic.

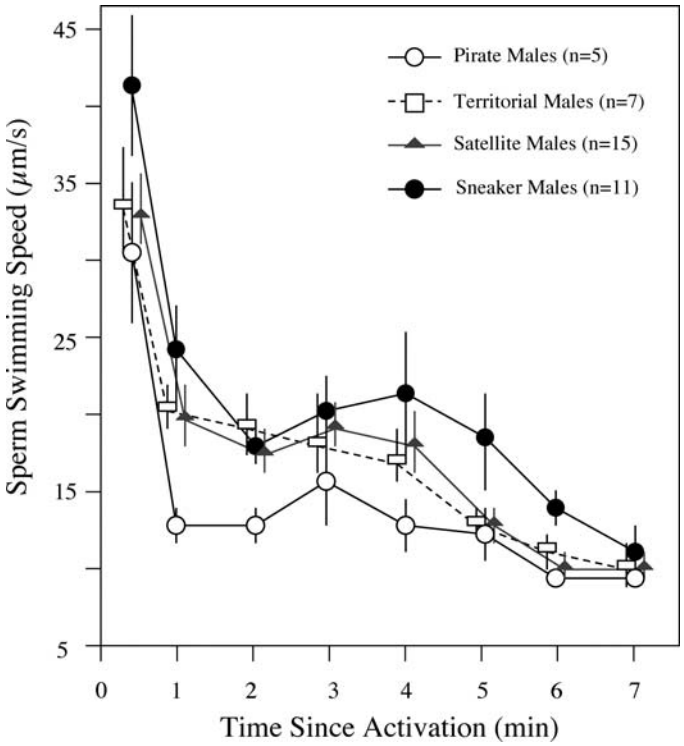

FIG. 1. Mean ± SEM sperm swimming speed (VAP) at different times after activation for males adopting different mating tactics. Points at each time period are staggered for clarity.

Statistical Analysis

Statistical analyses were performed with JMP (version 6.0.3, 2006; SAS Institute Inc., Cary, NC). Data and residuals were tested for normality and transformed when necessary to improve the fit to normality. Sperm swimming speed measured by VAP, VCL, and STR were $\log_{10}$ transformed for every analysis and analyzed using repeated-measures ANOVAs. We used Tukey HSD tests for post-hoc comparisons. To avoid pseudoreplication and to minimize the influence of outliers, all statistical analyses involving sperm length were performed using a single median value from each male; we reached the same conclusions using mean sperm length (data not shown). As we were not able to measure sperm length and longevity from one satellite male, the sample size was reduced to 37 when analyzing those variables.

RESULTS

The two measures of sperm swimming speed (VAP and VCL) were significantly positively correlated at all time periods after activation ($r > 0.69$, $P < 0.0001$, n = 27 to 37 males per time period), pooling male tactics. Pooling all of the data across all time periods after activation, the correlation between VAP and VCL is high ($r = 0.93$, $P < 0.0001$, n = 276 mean values). Although these measures are highly correlated and yielded similar results, we present both VAP and VCL results to facilitate comparison with studies on other species.

VAP and VCL of sperm varied significantly among males that adopted different tactics (Fig. 1 and Table 1). There was a significant and progressive decline in sperm swimming speed within tactics, with both VAP and VCL at 0.5 min after activation significantly higher than at all other times after activation (Tukey tests following separate ANOVAs for each tactic, $P < 0.05$; Fig. 1 and Table 1). In general, sperm from pirate males swam slower than sperm from males adopting all other tactics, whereas sperm from sneaker males was, on average, faster than those of all other tactics at all times after activation (Fig. 1). Post-hoc tests revealed that sperm from sneakers swam significantly faster (VAP and VCL) than sperm from pirates, but that none of the other pairwise comparisons between tactics were significant. There was no significant interaction between tactic and time since activation in either model ($P > 0.71$ in both cases), so this term was removed from the final models. Thus, there was no indication that the pattern of decline in VAP or VCL with time, since activation differed among the male tactics.

TABLE 1. Repeated measures ANOVAs examining the effect of male reproductive tactic and time since sperm activation on the VAP and VCL of sperm recorded at different time periods postactivation.

Velocity measure	Effect	Test statistic	P	R^2_{adjusted}
VAP	Tactic	$F_{3,33} = 3.2$	0.04	0.79
	Time	$F_{7,232} = 97.6$	< 0.0001	
VCL	Tactic	$F_{3,33} = 3.2$	0.04	0.76
	Time	$F_{7,233} = 74.4$	<0.0001	

Adding median sperm length to the models shown in Table 1 did not change our conclusion that there was significant variation in sperm swimming speed both among male tactics (VAP: $F_{3,31} = 3.5$, $P = 0.03$; VCL: $F_{3,31} = 3.7$, $P = 0.02$) and with time since activation (VAP: $F_{7,226} = 97.0$, $P < 0.0001$; VCL: $F_{7,227} = 75.4$, $P < 0.0001$). The effect of sperm length in both models was not significant (VAP: $F_{1,31} = 1.2$, $P = 0.28$; VCL: $F_{1,31} = 2.4$, $P = 0.13$).

STR also differed significantly among reproductive tactics (indicated by a significant interaction between time since activation and reproductive tactic, repeated measures ANOVA, interaction effect: $F_{21,212} = 2.7$, $P = 0.0002$). At 30 sec after activation, STR was not significantly different among tactics ($F_{3,29} = 0.3$, $P = 0.82$), with sperm from males of all tactics swimming in a relatively straight path (mean STR: 0.75–0.82). By 6 min after activation, however, the sperm of sneakers were still swimming in a relatively straight path (mean STR: 0.67, n = 10 males), whereas those of males adopting the other tactics had a more curvilinear path (mean STR: 0.44–0.55, n = 5 to 14 males per tactic), and the variation among tactics was significant ($F_{3,31} = 5.1$, $P = 0.006$). At 7 min after activation, the same pattern of variation in STR according to tactic was apparent and significant.

There were no significant differences in sperm tail length among tactics ($F_{3,33} = 0.81$, $P = 0.50$, Table 2). Similarly, although sperm swimming longevity varied across individual males, ranging from 287 to 728 sec in duration, it did not differ significantly among reproductive tactics ($F_{3,33} = 1.45$, $P = 0.25$, Table 2).

There were no relationships between VAP or VCL and median sperm tail length ($r = -0.01$ to 0.34, $P \geq 0.09$, n = 27 to 36 males) at all times after activation, pooling across tactics. Sperm tail length was also not significantly related to sperm longevity ($r = -0.04$, n = 36, $P = 0.83$) or male body size (SL: $r = 0.23$, n = 37, $P = 0.18$).

DISCUSSION

Consistent with theoretical predictions, sperm from males encountering elevated levels of sperm competition (sneakers) had the fastest swimming sperm, whereas males experiencing the lowest levels of sperm competition (pirates) had the slowest (Fig. 1). The faster-swimming sperm of sneakers may be advantageous, as fertilization success can be positively correlated with sperm velocity in fishes [4, 5]. Interestingly, our results show that in *T. vittatus*, sperm from sneakers swam faster initially than sperm from pirate males, but the sperm continued to swim at faster speeds than all other tactics

TABLE 2. No significant differences in median sperm length (measured from 10 sperm per male) or sperm longevity were detected among male reproductive tactics in *T. vittatus*.

Tactic	Median sperm length (μm)[a]	Sperm longevity (sec)[a]
Pirate (n = 5)	33.7 ± 1.5	406.8 ± 40.7
Territorial (n = 7)	32.3 ± 1.1	456.9 ± 51.9
Satellite (n = 14)	31.6 ± 0.8	393.1 ± 14.1
Sneaker (n = 11)	32.0 ± 0.4	447.3 ± 17.3

[a] Values are mean ± SEM.

throughout the duration of its forward motility. Therefore, the predicted tradeoff between sperm swimming speed and longevity [28] observed in other fishes [38] was not detected in *T. vittatus*.

Longer sperm tails are believed to provide greater propelling force than shorter ones [29]. However, as in previous studies of fish spermatozoa [7, 19, 39, 49], sperm lengths were not correlated with sperm swimming speed, nor were there any differences in sperm length among reproductive tactics in *T. vittatus*. In fishes, differences in sperm lengths between tactics within a species may be small. For example, in bluegill, in one study sneakers had slightly, but signficantly, longer (>2 μm) sperm than parental males [38]. Thus, our results suggest that sperm from different tactics may differ energetically (i.e., in ATP stores) while not differing in length and, intriguingly, indicate that selection has acted on sperm energetics rather than on sperm size in *T. vittatus*. In bluegill and salmon, ATP levels are greater in sperm from sneaker males [8, 38, 41], suggesting that in order to ensure faster swimming speeds, selection has favored increased energy stores in sperm from males performing sneak fertilizations. As ATP levels are correlated with initial sperm swimming speed [38], the faster swimming of sperm observed in *T. vittatus* sneakers may indeed be due to greater energy stores. While the mechanism underlying the tactic-specific sperm swimming speeds in *T. vittatus* remains unknown, further studies using more refined techniques (e.g., electron microscopy) addressing differences in spermatozoa energy stores and larger sample sizes will improve our understanding of tactic-specific sperm investment. Such studies investigating the tradeoff between sperm energetics and other sperm traits will further reveal how sperm competition has shaped the evolution of sperm phenotypes.

Both life history traits and the fertilization dynamics may influence the evolution of sperm characteristics in *T. vittatus*, selecting for faster-swimming sperm as well as sperm that continue to swim faster in sneakers. Prolonged periods of sperm swimming may be particularly advantageous if sneaker males engage in preoviposition ejaculation, as has been reported in other teleosts [51], where sperm are shed before ova are released. Preoviposition ejaculation can be an effective way for sneaker males to successfully fertilize eggs, particularly when territorial males aggressively guard females. In the rose bitterling (*Rhodeus ocellatus*), fertilization occurs within the gill cavity of a freshwater mussel that is defended by territorial males [52]. Territorial males chase away competing males, leaving their mussel nest vulnerable to sperm release by other males. Sneaker males capitalize on temporary periods of relaxed mussel guarding by intruding into the territory and releasing sperm [51]. As the number and timing of female visits and spawning events is unpredictable, sneaker males release sperm whether or not a female is spawning, and are able to successfully fertilize eggs laid for a period of several minutes after the ejaculation [51, 52]. Similarly, *T. vittatus* sneaker males are likely constrained by the number and timing of spawning opportunities. Indeed, Ota and Kohda [17] observed *T. vittatus* sneakers ejaculating into spawning shells while territorial males were absent, suggesting that *T. vittatus* sneakers may also be taking advantage of periods of relaxed shell guarding to gain a measure of reproductive success. In many cichlid species, gametes remain viable for extended periods. For example, in Nile tilapia *Oreochromis niloticus,* eggs remain fertilizable for approximately 15 min after release [53], whereas in the Tanganyikan cichlid *Opthalmotilapia ventralis,* sperm remain viable for 10–15 min after release from a male (M. Haesler, personal communication). Therefore, the prolonged increase in sperm swimming speed observed in sneakers relative to all other tactics may confer a reproductive advantage to sneakers and should be examined in future studies in relation to the relative timing of ejaculates in relation to egg laying among the male tactics.

Asymmetries in the risk of sperm competition can select for higher-quality ejaculates in males performing sneak fertilizations [28]. This study, together with a handful of other studies in fishes [23, 26, 41], demonstrates that males mating in the disfavored role produce faster-swimming sperm than males in the favored role. These results suggest that differences in sperm swimming speeds among male tactics in *T. vittatus* are an adaptation to sperm competition. We suggest that changes in ATP production or allocation among sperm over an individual's lifetime may underlie differences in sperm swimming speed among reproductive tactics. As it is currently unclear whether male reproductive tactics in *T. vittatus* are fixed (genetically determined) or plastic (socially determined), we are unable to assess whether differences in sperm swimming speed remain static or change over the course of an individual's lifetime. If tactics are plastic and dependent upon body size relative to conspecifics, as is the case in other fishes [54], then the sperm swimming speed of *T. vittatus* males would decrease as they grow from sneakers to pirates, largely as a consequence of changes in sperm energetics. In conclusion, our study demonstrates fine-tuned selection on sperm swimming speed due to sperm competition in a species characterized by an unusual number of male reproductive tactics, and it sets the stage for further examinations of the factors underlying the phenotypic variation in sperm among male tactics.

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