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Evolution of sperm quality but not quantity in the internally fertilized fish *Xiphophorus nigrensis*

C. C. SMITH & M. J. RYAN

University of Texas at Austin, Section of Integrative Biology, Austin, TX, USA

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Abstract

Species with alternative reproductive strategies are characterized by discrete differences among males in suites of traits related to competition for fertilizations. Models predict sneaker males should allocate more resources to their ejaculates because they experience sperm competition more frequently and often occupy a disfavoured 'role' owing to subordinance in intramale competition and female preferences for larger males. We examined whether sperm number and quality differed between male strategies in the internally fertilized fish *Xiphophorus nigrensis* and explored the relationship between sperm morphology and performance. We found sneaker males had similar testes sizes compared to courting males but ejaculates with both more viable and longer lived sperm. Sneaker sperm also had longer midpieces, which was positively correlated with both velocity and longevity. Our study suggests that the evolution of sperm quantity and quality can be decoupled and that the sperm morphology is likely to play an important role in mediating sperm competition through its effects on sperm performance.

Sperm competition, or competition between sperm of two or more males for a set of ova, has widespread effects on the evolution of animals (Birkhead & Møller, 1998). Sperm competition's role in evolution may be particularly prominent in species with alternative reproductive strategies, where suites of physiological, morphological and behavioural traits have diversified among males as a result of competition for fertilizations (Oliveira *et al.*, 2008). Male mating strategies commonly take two forms, a 'bourgeois' strategy that attempts to monopolize females or the resources they require to mate and a 'parasitic' strategy that exploits the bourgeois strategy by sneaking copulations with the females they attract (Taborsky, 1998). Given a trade-off between investment in ejaculates and other traits necessary for obtaining mates (e.g. courtship, territory defence), models predict parasitic males should allocate more resources to their ejaculates because (i) parasitic males are typically at a disadvantage (occupy a disfavoured 'role') in mating because of subordinance in male–male competition and female preferences for bourgeois males (Parker, 1990a) and (ii) parasitic males often have a higher risk of sperm competition, that is they experience sperm competition more frequently than bourgeois males (Parker, 1990b).

A universal expectation of theory is that investment in spermatogenesis should increase with sperm competition risk (Parker, 1998). As predicted, ejaculate sizes are typically larger in parasitic males (Taborsky, 1998). Studies using phylogenetic approaches, experimental evolution and analyses of phenotypic plasticity have found similar responses of traits influencing sperm number. Testes size often increases in species where females mate multiply (Birkhead & Møller, 1998), increases when polyandry is experimentally enforced in evolving populations (Hosken & Ward, 2001; Pitnick *et al.*, 2001; LaMunyon & Ward, 2002; Simmons & García-González, 2008; but see Crudgington *et al.*, 2009), and males facultatively increase their ejaculate size when the perception of sperm competition risk is heightened (Wedell *et al.*, 2002; Pizzari *et al.*, 2003, Rudolfsen *et al.*, 2006, delBarco-Trillo & Ferkin, 2006). A few notable exceptions, however, exist where species with alternative male strategies show no differences in testes or ejaculate size between male morphs (Simmons *et al.*, 1999; Neat, 2001; Byrne, 2004; Kelly, 2008). The

Correspondence: Chad C. Smith, University of Texas at Austin, Section of Integrative Biology, Austin, TX 78712, USA.
Tel.: 512 475 6164; fax: 512 471 3878;
e-mail: chadsmith@mail.utexas.edu

discrepancy between theory and data is most commonly ascribed to a violation of the model's assumptions (Parker, 1990a). For example, if the frequency of the parasitic males in the population is high enough, the risk of sperm competition might be equivalent for each tactic, resulting in no difference in ejaculate investment.

In addition to variation in the number of sperm available for mating, sperm competition may affect the quality of the sperm males produce. Sperm size, for example, has been hypothesized to increase fertilization success by increasing longevity, swimming speed, or ability to displace rival males' sperm and varies markedly within and among species (Pitnick, 1996; Gage, 1998; Ward, 1998; Snook, 2005; Malo *et al.*, 2006; Sherman *et al.*, 2008). Sperm size, however, covaries inconsistently with sperm competition risk among species (Gomendio & Roldan, 1991; Snook, 2005; Fitzpatrick *et al.*, 2009, Lüpold *et al.*, 2009). The lack of a consistent relationship between the theory and data is likely because of differences in the metric chosen to quantify sperm competition risk among studies (Snook, 2005), violations of the assumed relationships between sperm size, swimming speed and longevity (Parker, 1998; Snook, 2005; Humphries *et al.*, 2008) or effects of male–female coevolution on gamete morphology rather than sperm competition risk *per se* (Eberhard, 1996; Snook, 2005).

Species with alternative reproductive strategies are an important foil for the comparative method because confounds such as differences in female anatomy between species are controlled, and fertilization success between males that differ in ejaculate characteristics can be measured experimentally. In the few species with alternative reproductive strategies that have been examined, sperm size typically does not differ between tactics (Gage *et al.*, 1995; Hettyey & Roberts, 2006; Stoltz & Neff, 2006; Fitzpatrick *et al.*, 2007; Locatello *et al.*, 2007; but see Burness *et al.*, 2004, Simmons *et al.*, 1999). Parasitic sperm, however, have been found to be of higher quality when metrics of sperm performance rather than morphology alone are considered (Vladic & Jarvi, 2001; Locatello *et al.*, 2007). Parasitic male Alantic salmon (*Salmo salar*), for example, have higher sperm motility, velocity, ATP concentration and sire more offspring when mixed with an equal number of bourgeois sperm (Vladic & Jarvi, 2001). Similarly, parasitic male bluegill sunfish (*Lepomis macrochirus*) have faster sperm, higher concentrations of ATP (Burness *et al.*, 2004; but see Stoltz & Neff, 2006), and sire twice as many offspring *in vitro* when differences between males in sperm number are statistically controlled (Stoltz & Neff, 2006). Finally, sperm quality and number covary with tactic in species where male tactic depends on social status. Experimental manipulations of social status in some species have shown that subordinate males (i.e. the disfavoured role) produce more, higher quality sperm compared to their dominant counterparts (Rudolfsen *et al.*, 2006; Cornwallis & Birkhead, 2007, Pizzari *et al.*, 2007; but see Fitzpatrick *et al.*, 2008; Montrose *et al.*, 2008; Kruczek & Styrna, 2009; Thomas & Simmons, 2009).

Information gained from these systems is invaluable because they provide a unique data set for comparison to studies using the comparative method and experimental evolution. Although studies of species with alternative reproductive strategies and external fertilization have produced substantial insights into the role of sperm competition in evolution, species with alternative reproductive strategies and internal fertilization have been less well characterized. Interactions between the sexes and the physical environment in which sperm compete are vastly different between internal and external reproductive modes, and as a consequence we might expect the selective forces on ejaculate traits to also differ. In external fertilizers, sperm swimming speed rather than longevity is thought to be optimized in response to sperm competition, because reproductive success is dependent on sperm–egg collision rate over a short period of time (Parker, 1993). In contrast, mating and fertilization are often decoupled in internal fertilizers, particularly in those species with sperm storage. As a consequence, traits that increase sperm survival (Taborsky, 1998; Birkhead *et al.*, 1999; Pizzari *et al.*, 2008) or facilitate the displacement of rival sperm within the female reproductive tract, such as sperm size (LaMunyon & Ward, 1998), may confer a selective advantage.

In this study, we characterize variation in sperm quantity and quality between alternative reproductive strategies in the internally fertilized swordtail *Xiphophorus nigrensis*. Regardless of reproductive mode, the canonical expectation is that investment in sperm number should be greater for males that face either a higher risk of sperm competition or occupy a disfavoured 'role' in mating (Parker, 1990a,b), and as a consequence we expect investment in spermatogenesis should be greater for parasitic males (Taborsky, 1998). How sperm quality should vary with sperm competition risk, however, is less well understood, and model expectations depend on the assumed relationships between sperm size, velocity and longevity (Parker, 1993). Models clearly predict larger, faster sperm should evolve with increased sperm competition risk at the expense of longevity when reproductive success is determined by a race to the egg (c.f. external fertilization, Parker, 1993). If reproductive success is primarily mediated by surviving a period of sperm storage, however, sperm survival (and sperm size) only varies with risk if more strict assumptions are met; for example, if the evolution of sperm number is constrained or the benefits of sperm size increase with sperm density in competition (Parker, 1993). Although many studies have considered the relationship between sperm size and risk, debate continues over the expected relationships between morphology and performance (Malo *et al.*, 2006, Humphries *et al.*, 2008) central to model predictions. Here, we also examine the covariation between sperm morphology and performance in the

context of the evolution of alternative reproductive strategies.

Methods

Study system

Swordtails (genus *Xiphophorus*) are small livebearing fish, endemic to the freshwater drainages of eastern Mexico and Central America (Kallman & Kazianis, 2006). Fertilization occurs via a modified anal fin (the gonopodium) that passes bundles of sperm (spermatozeugmata) to the female reproductive tract. After insemination, sperm dissociate from the bundles and can be stored within the folds of the ovary for several months (Constantz, 1989). Offspring gestate for approximately 30 days after which they are born with no further parental investment.

In the swordtail *X. nigrensis,* male body size at sexual maturity is determined by genetic variation at a Y-linked locus (heritability = 91%) that is thought to modulate the activation of the pituitary-gonadal axis during development (Kallman, 1989). Males that mature small obtain fertilizations by rapidly chasing females and thrusting the gonopodium into the female gonopore, whereas males that mature at intermediate and large sizes defend and court females between copulations (Ryan & Causey, 1989). Reproductive tactics are fixed over an individual's lifetime because of the rapid decline in growth at sexual maturity. Females prefer larger males (Ryan *et al.*, 1990; Cummings & Mollaghan, 2006), who in addition to exhibiting courtship possess enlarged dorsal fins and an elongated caudal fin (the sword), although previous work has found that the female preference for the sword has been lost in *X. nigrensis* (Rosenthal *et al.*, 2002). In the wild, large males actively chase smaller males away from females foraging along the stream bottom (Morris *et al.*, 1992) and sire more offspring than expected given their frequency (Ryan *et al.*, 1990). Females often move between mixed sex foraging groups and are subject to approximately four mating attempts per hour by multiple males (C.C. Smith, unpublished data). The incidence of multiply sired broods among female *X. nigrensis* is currently unknown but is high (33–64%) in other species of swordtails (Luo *et al.*, 2005; Simmons *et al.*, 2008; Tatarenkov *et al.*, 2008).

Testes mass

Male *X. nigrensis* were collected from the headwaters of the Rio Choy, Mexico (21°59′18″N 98°53″W) in December 2006, euthanized in a lethal dose of clove oil, fixed in 10% formalin and then transferred to 70% ethanol. Before dissection, standard length (SL) (tip of the mouth to the end of the caudal peduncle) was measured with dial calipers to the nearest 0.1 mm. The threshold body size for the expression of courtship behaviour in *X. nigrensis* is 26 mm SL (Ryan & Causey, 1989); males smaller than 26 mm were therefore designated as 'sneaker' males (mean SL ± SD: 21.4 ± 2.0 mm, $n = 12$), and those larger than 26 mm were designated as 'courting' males (mean SL ± SD: 31.0 ± 3.2 mm, $n = 25$). Testes and soma were dissected and dried separately overnight in a 55 °C oven, allowed to cool in a desiccator and weighed on a Mettler Toledo AT21 Comparator balance to the nearest 0.01 mg.

Sperm number and velocity

Males were collected in May 2007 from the headwaters of the Rio Choy, Mexico, transported to the University of Texas at Austin, and housed in single sex groups with visual access to females on a 14L:10D light cycle for at least 3 months prior to data collection. They were fed on a diet of Tetramin© fish flakes twice daily and brine shrimp nauplii (San Francisco Bay Brand©) once daily. Sperm were stripped by placing the male ventral side up against a Petri dish lined with cotton. The gonopodium was swung forward, and gentle pressure was applied to the testes by massaging the body anteriorly toward the gonopore with the forefinger to expel the spermatozeugmata. These were collected with a mouth aspirator and put on ice in a solution of 20–100 μL sperm extender (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM, $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5) (Gardiner, 1978) depending on the size of the ejaculate. Sperm remained quiescent within this solution until activation (within 2 h of collection).

We quantified the amount of sperm stripped and sperm velocity from samples of sneaking (mean SL ± SD: 22.0 ± 0.93 mm, $n = 18$) and courting (mean SL ± SD: 32.8 ± 2.56 mm, $n = 20$) males. We resuspended sperm with a micropipette and gently vortexed for 1 min to break apart the spermatozeugmata and homogenize the solution. Sperm were activated by adding 4 μL of sample to 12 μ 150 mM KCl (Morisawa & Suzuki, 1980). Five minutes post-activation, sperm were pipetted into a disposable Microcell© (MC-20-4; Conception Technologies, San Diego, CA, USA) fixed depth (20 μL) counting chamber and videotaped with a Canon XLS camcorder mounted to a Zeiss Axiovert 25 microscope using dark field microscopy. The first 2 s of video were digitized and analysed with the computer-assisted sperm analysis plugin for ImageJ (Wilson-Leedy & Ingermann, 2007) to obtain measures of sperm concentration and velocity. Sperm concentration was estimated by multiplying the number of sperm tracked (mean ± SD: 173 ± 91, range 28–400) by the dilution of the solution. Assays were performed at room temperature (23–24 °C), two to three degrees cooler than water temperature at the field site (and presumably the internal temperature of females).

Sperm velocity was calculated using three metrics: straight line velocity (VSL, the straight line distance

between the start and end of the sperm path), curvilinear velocity (VCL, the velocity along the sperm path) and average path velocity (VAP, the velocity over a smoothed sperm path) (Rurangwa *et al.*, 2004). Sperm motion was curvilinear in *X. nigrensis,* and all measures of velocity were correlated (VCL ~ VAP: $R^2 = 0.96$, $t_{36} = 29.21$, $P < 0.001$; VCL ~ VSL: $R^2 = 0.80$, $t_{36} = 11.94$, $P < 0.001$; VAP ~ VSL: $R^2 = 0.90$, $t_{36} = 17.47$, $P < 0.001$). Two subsamples of the stripped ejaculate were measured for each male. The average of these two measurements weighted by the number of motile sperm in each subsample was used for analysis.

Sperm viability and longevity

We evaluated sperm viability and longevity using a fluorescence-based assay (Molecular Probes LIVE/DEAD® Sperm Viability Kit, Carlsbad, CA, USA); sperm with intact cell membranes stain green (SYBR®14), and dead sperm with compromised cell membranes stain red (propidium iodine). The proportion of viable sperm in sneaking (mean SL ± SD: 22.5 ± 0.99 mm, $n = 17$) and courting male (mean SL ± SD: 32.8 ± 2.62 mm, $n = 19$) ejaculates was revealed by adding 0.5 μL SYBR®14 nucleic acid stain to 9.5 μL unactivated sperm solution (final concentration of SYBR®14 = 200 nM), incubating sperm for 10 min at room temperature, and then adding 1 μL propidium iodine (final concentration of propidium iodine = 12 μM) followed by a second 10-min incubation period. Sperm were photographed with a Zeiss Axiocam Mrc camera mounted on a Zeiss AX10 microscope at 100× magnification with Rhod FS15 and GFP FS17 prisms to visualize fluorescent cells. Images were manually thresholded in ImageJ, sperm counted with the analyse particles function (mean ± SD: 2,294 ± 1500, range 287–5656), and the output compared against bright field images to verify sperm were correctly identified. To measure sperm longevity, 18 μL of KCl was then added to remaining 6 μL of unactivated sperm, and viability re-measured 3 h later. The proportion of viable sperm before and 3 h after activation was subtracted for each male to measure the proportion of sperm surviving over the time interval.

The relationship between sperm performance *in vivo* is likely to be influenced by the properties of both the sperm and the physiological characteristics of the female reproductive tract. As a consequence, the *in vitro* measures of sperm performance in this study attempt to capture intrinsic differences between males in sperm performance without variation because of female effects (e.g. variation in sperm provisioning or anatomy). Three hours was chosen to facilitate comparison to a previous study in the guppy Poecilia *reticulata* that found more ornamented males had greater sperm survival using similar methodology to that used here (Locatello *et al.*, 2006). Sample sizes differ between the two analyses owing to an equipment malfunction that did not allow us to measure longevity for six males (five sneakers and one courting male).

Sperm morphology

Xiphophorus spermatozoa are composed of three structures: an elliptical head that stores the genetic information, a midpiece housing the mitochondria that provide energy via oxidative phosphorylation for motility, and a tail generating its momentum. To evaluate differences in morphology between strategies (sneaking males, mean SL ± SD: 23.7 ± 1.0 mm, $n = 16$; courting males, 33.3 ± 2.5 mm, $n = 24$), ten sperm were photographed from the inactivated ejaculate using a Leica DMLB phase contrast microscope and Leica DF320 camera under 400× magnification. Head shape (head length/head width), midpiece length and tail length were measured for each sperm using ImageJ and the average values taken for statistical analysis. A subsample of 57 sperm from six males was measured twice to determine the repeatability of our measurements. Repeatability (intraclass correlation, Zar, 1984) was moderate to high (r_i: head shape, 0.64, midpiece size, 0.94, tail length, 0.93; total length, 0.86). Repeatability is lower for head shape because the glow under phase contrast made the borders of the head less defined than for the other structures, increasing measurement error.

Statistical analysis

Statistical analyses were conducted using R v.2.8.7 (R Core Development Team, 2009). For analyses of variance and regression, departures from normality and homogeneity of variances were checked by visual inspection of quantile plots and Levene's test. Differences in testes investment between strategies were evaluated using ANCOVA with log gonad mass as the dependent variable and log soma mass, strategy and their interaction as independent variables. The interaction was not significant in our study (see Results), indicating that the allometric relationship between testes and soma mass is equivalent between strategies in *X. nigrensis* (Tomkins & Simmons, 2002). Proportion data were analysed using a generalized linear model with a logit link and scaled dispersion parameter (glm function, family = quasibinomial) to correct for overdispersion (Collett, 2003; Faraway, 2006). Models initially included all two-way interactions between predictors and then were simplified by removing the least significant predictors in a stepwise fashion. Effect sizes and their 95% confidence intervals (Nakagawa & Cuthill, 2007) were calculated to provide a standardized measure of differences between tactics and interpret coefficients in the binomial GLMs (Cohen's D: MBESS package; odds ratios: MASS package). Cohen's D (the difference in group means standardized by their pooled standard deviation) values of 0.8, 0.5 and 0.2 are considered large, medium and small differences between

groups, respectively (Cohen, 1988). Reported descriptive statistics are mean ± standard error unless indicated otherwise. All statistical tests were two-tailed.

Results

Sperm number

Testes size was equivalent between strategies after controlling for the positive allometric relationship between testes mass and soma mass using ANCOVA (strategy: $F_{1,34} = 0.69$, $P = 0.41$; log soma mass: $F_{1,34} = 14.85$, $P \leq 0.001$, Fig. 1). The interaction between soma mass and strategy was not significant ($F_{1,33} = 1.16$, $P = 0.29$) and removed from the final model (Tomkins & Simmons, 2002). The amount of stripped ejaculate was also not significantly different between strategies after controlling for the positive relationship between the number of sperm stripped and the body size (measured as SL), with a trend toward courting males having larger numbers of sperm stripped for their size (strategy: $F_{1,34} = 3.41$, $P = 0.07$; log SL: $F_{1,34} = 0.78$, $P = 0.384$). Again, the interaction was not significant ($F_{1,33} = 0.96$, $P = 0.34$) and removed from the final model.

Sperm velocity

Sperm velocity was statistically equivalent between strategies with a trend toward higher straight line velocity (VSL) in sneakers (Table 1, Fig. 2). Sperm velocity varied substantially across all males (mean ± SD: VAP, 29.1 ± 6.3, range 27.2 μm s^{-1}; VSL: 24.7 ± 4.9, range 23.0 μm s^{-1}; VCL: 31.5 ± 6.9, range 30.9 μm s^{-1}) as has been found in studies of other taxa (Gage *et al.*, 2002; Locatello *et al.*, 2006; Stoltz & Neff, 2006).

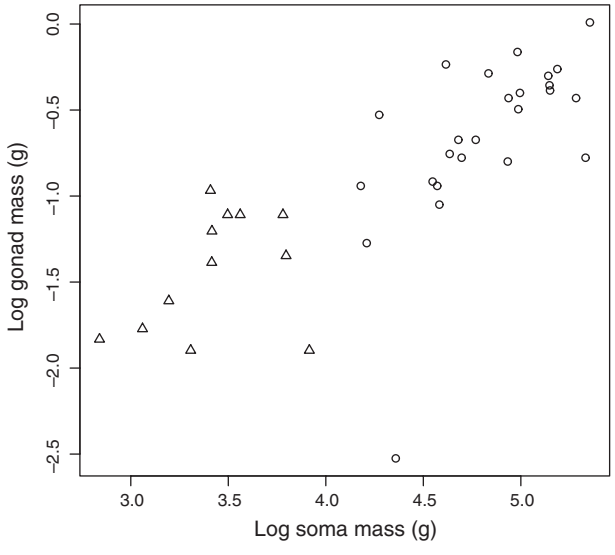

Fig. 1 Equivalent testes investment of sneaking (Δ) and courting (○) males. Testes mass does not deviate from the expected allometric relationship with soma mass for either strategy.

Sperm morphology

Sneaker males had longer midpieces but equivalent head shape and tail lengths compared to courting males (Table 1, Fig. 3). Sneaker male midpieces were one standard deviation (Cohen's D = 0.97) larger than courting males, a large difference using the standard criteria for interpreting Cohen's D in the social sciences (Cohen, 1988). There was no difference in the total length of the sperm between strategies (Table 1). As has been reported for other species (Ward, 1998), variation in sperm morphology was greater among than within males (head shape: $F_{40,361} = 3.58$, $P < 0.001$, midpiece length: $F_{40,361} = 8.86$, $P < 0.001$, tail length: $F_{40,361} = 5.84$, $P < 0.001$, total length: $F_{40,361} = 10.2$, $P < 0.001$).

Sperm viability and longevity

Unactivated ejaculates from sneaker males had a greater proportion of viable sperm than courting males (Table 1, Fig. 4). Sneaker sperm was 2.7 times more likely to be alive before activation compared to courting males (Table 1). Once activated, sneaker sperm was 3.5 times more likely to survive until viability was resampled 180 min later (Table 1, Fig. 4).

Sperm morphology and performance

We used multiple regression to analyse the relationships between sperm performance and morphology across all males ($n = 18$) for which we had a complete data set. Average path velocity increased with longer midpieces and shorter tails (full model: $R^2 = 0.51$, $F_{3,14} = 6.93$, $P = 0.004$, Table 2, Fig. 5) but did not vary with the total sperm length ($R^2 = 0.00$, $F_{1,16} = 0.065$, $P = 0.80$). Interactions in these models were not significant and removed in a backwards stepwise procedure. Humphries *et al.* (2008) recommended explicitly evaluating the relative effects of force created by the flagellum (tail + midpiece) and drag produced by the head by regressing the ratio of their lengths on velocity. We found no relationship between the flagellum/head length ratio and sperm velocity (VAP: $R^2 = 0.07$, $F_{1,16} = 2.31$, $P = 0.15$). Similar results were observed for VCL and VSL for all analysis.

Sperm viability was not related to morphology before the sperm were activated (GLM: head shape: $\beta = -0.44$, $F_{1,14} = 0.06$, $P = 0.82$; midpiece length: $\beta = 0.93$, $F_{1,14} = 2.46$, $P = 0.14$; tail length: $\beta = -0.24$, $F_{1,14} = 0.60$, $P = 0.45$). None of these predictors were significant using backwards stepwise elimination. Sperm survival after activation, however, increased with midpiece size (GLM: $\beta = 1.41$, $F_{1,14} = 6.03$, $P = 0.03$, Fig. 6) after the nonsignificant effects of head shape and tail length were

Table 1 ANOVA comparing sperm quantity and quality between sneaking and courting males. Group means ± SE and 95% confidence intervals for effect sizes [Cohen's D: sperm morphology (μm), velocity (μs^{-1}); odds ratios: sperm viability] are given. Significant differences between strategies at the 5% level are in bold.

	Sneaker	Courter	Effect size	*F*	*P*
Sperm velocity*					
VAP	30.2 ± 1.48	28.1 ± 1.40	0.35 (–0.30, 0.99)	1.25	0.27
VCL	32.5 ± 1.51	30.5 ± 1.65	0.30 (–0.34, 0.94)	1.11	0.30
VSL	26.2 ± 1.19	23.3 ± 1.01	0.62 (–0.04, 1.27)	3.61	0.07
Sperm morphology†					
Head shape	2.68 ± 0.05	2.77 ± 0.33	0.51 (–0.15, 1.16)	2.29	0.14
Midpiece length	**8.94 ± 0.15**	**8.41 ± 0.11**	**0.97 (0.29, 1.65)**	**8.28**	**0.007**
Tail length	45.4 ± 0.24	45.3 ± 0.25	0.07 (–0.57, 0.72)	0.05	0.83
Total length	57.7 ± 0.33	57.2 ± 0.30	0.50 (–0.16, 1.15)	2.21	0.15
Sperm viability‡					
Proportion alive	**86.8 ± 2.5**	**71.3 ± 4.6**	**2.7 (1.4, 5.5)**	**9.52**	**0.004**
Proportion surviving	**89.2 ± 4.7**	**70.4 ± 6.6**	**3.5 (1.1, 13.9)**	**4.62**	**0.04**

*d.f. = 1,39.
†d.f. = 1,38.
‡Proportion alive: df = 1,36; Proportion surviving: df = 1,29.
VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

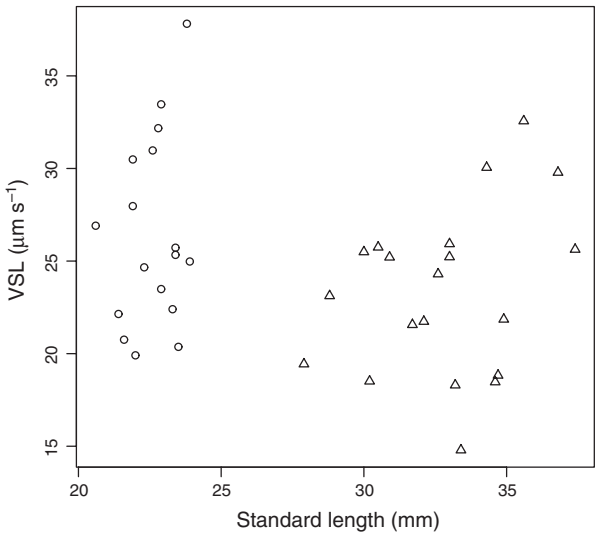

Fig. 2 Equivalent straight line velocities (VSL) for sneaking (Δ) and courting (○) males measured 5 min post-activation. Plots of curvilinear velocity (VCL) and average path velocity (VAP) are qualitatively similar and not shown.

iteratively removed from the final model. Sperm were 2.5 times more likely to be alive with every standard deviation increase in the midpiece.

Discussion

Species with alternative reproductive strategies are pronounced examples of intraspecific diversification because of competition for fertilizations (Oliveira *et al.*, 2008). As a consequence, they provide a unique opportunity to investigate the evolution of ejaculates in response to sperm competition. Our results show that sneaker males in the poeciliid fish *X. nigrensis* produce ejaculates with a greater proportion of viable and longer-lived sperm compared to that of courting males. The strategies do not, however, differ in investment in sperm number despite theoretical and empirical support for the evolution of traits that increase spermatogenesis when males differ in sperm competition risk or occupy different 'roles' in mating (Parker, 1998). We also report, to our knowledge, the first case of the evolution of larger midpieces in sneaker males. Larger midpieces were associated with both faster- and longer-lived sperm, suggesting that the action of mitochondria within the midpiece is important in sperm motility and that sperm morphology can evolve in response to sperm competition within species.

Sperm viability and spermatogenesis

Sperm viability contributes to reproductive success by increasing the number of sperm capable of fertilization within the ejaculate. In insects, sperm viability is greater in polyandrous compared to monandrous species (Hunter & Birkhead, 2002) and increases fertilization success when two males compete in the cricket *Teleogryllus oceanicus* (García-González & Simmons, 2005). Viability has also been shown to vary with sperm competition risk in species with alternative reproductive strategies. In the black goby *Gobius niger,* sneaker males have higher sperm viability compared to bourgeois males, whereas in the grass goby *Zosterisessor ophiocephalus,* sperm viability is equivalent across tactics (Locatello *et al.*, 2007). Species differences in nest construction are thought to underlie the discrepancies in ejaculate quality; black gobies build single-entrance nests that are easier to defend from

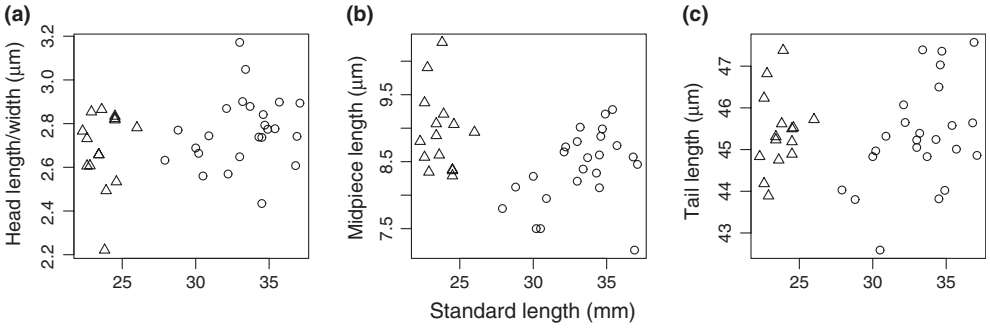

Fig. 3 Differences in sperm morphology between sneaking (Δ) and courting (○) males. Sneaker males had significantly longer midpieces (b) whereas head shape (a) and tail length (c) were similar between strategies.

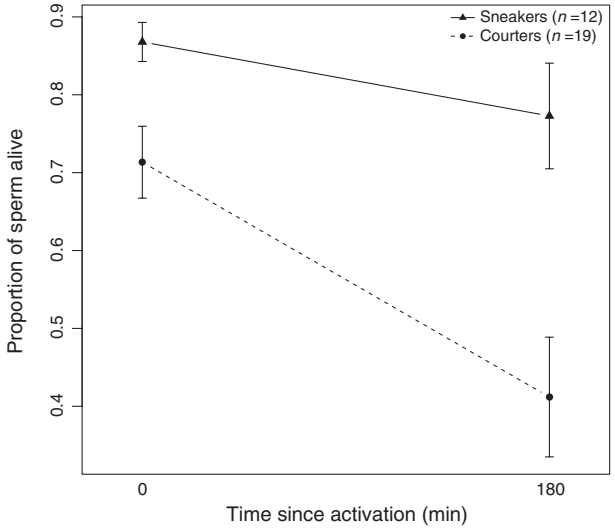

Fig. 4 Sneaker males have more viable sperm before activation (time 0) and sperm that are longer-lived (difference in viability before and 180 min after activation).

sneakers, resulting in asymmetries in sperm competition risk between tactics and greater ejaculate investment by sneaker males. In contrast, grass gobies build muti-entrance nests that are likely more difficult to monopolize (Locatello *et al.*, 2007). In *X. nigrensis*, small males may also benefit from increased sperm viability because their mating opportunities are restricted by larger males (Morris *et al.*, 1992) and females do not prefer them (Ryan *et al.*, 1990; Cummings & Mollaghan, 2006). Despite its importance to fertility in humans and other animals (World Health Organization, 1999, Katila, 2001; Rijsselaere *et al.*, 2005), the mechanisms resulting in differences in sperm viability between males are not well understood. Males with greater sperm viability might be either more efficient during the process of spermatogenesis or better able to maintain viable sperm within the testes after it is produced, for example by enhanced protection of sperm from oxidative damage within the testes (Bernasconi *et al.*, 2004; Helfenstein *et al.*, 2010).

Larger testes should also be favoured by selection as this can increase sperm number and, we assume, the probability of achieving fertilization. Here, however, we found no difference in testes size between alternative strategies in *X. nigrensis*. Testes size often covaries positively with sperm competition risk both within and between species (Birkhead & Møller, 1998), but this pattern is not universal among studies of species with alternative reproductive tactics (Simmons *et al.*, 1999; Byrne, 2004; Kelly, 2008). The absence of differences in testes size between tactics is often attributed to equal levels of sperm competition, which according to models would remove selection on sneaker males to increase ejaculate investment (Parker, 1998). Our study illustrates, however, that sperm quantity and quality need not evolve in concert.

Sperm number and quality may evolve independently if they are polygenic and unlinked. Quantitative genetic studies have found the relationship between sperm

Table 2 Multiple regression examining the relationship between sperm velocity (VSL: straight line velocity; VAP: average path velocity; VCL: curvilinear velocity) and sperm morphology. Significant relationships at the 5% level are in bold. Velocity is log transformed for all analyses.

	VSL				VAP				VCL			
	β	SE	*t*	*P*	*β*	SE	*t*	*P*	*β*	SE	*t*	*P*
Head shape	−0.26	0.23	−1.14	0.27	−0.43	0.21	−2.05	0.06	−0.36	0.23	−1.75	0.10
Midpiece length	0.19	0.07	2.65	**0.02**	0.14	0.06	2.13	**0.05**	0.14	0.07	2.24	**0.04**
Tail length	−0.08	0.04	−2.05	0.06	−0.10	0.04	−2.67	**0.02**	−0.09	0.04	−2.71	**0.02**

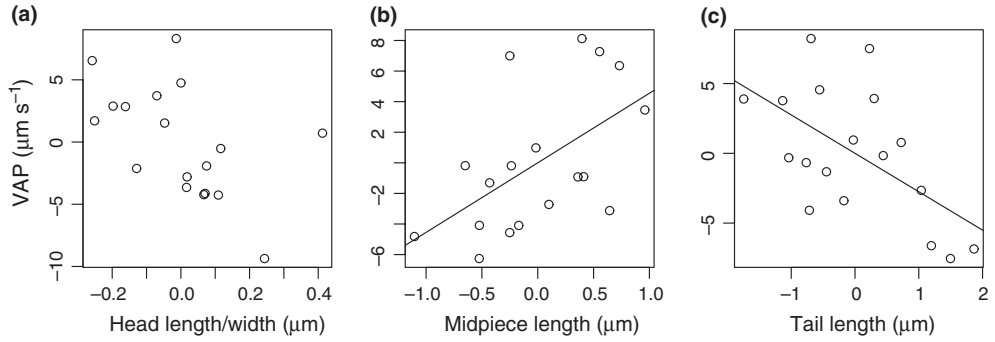

Fig. 5 Partial regression plot of average path velocity (VAP) and sperm morphology from the multiple regression analysis. Residuals of velocity on residuals of (a) head shape, (b) midpiece length and (c) tail length are shown to depict the relationships between VAP and each predictor after the effect of the other predictors are removed. Plots of straight line velocity (VSL) and curvilinear velocity (VCL) are qualitatively similar and not shown.

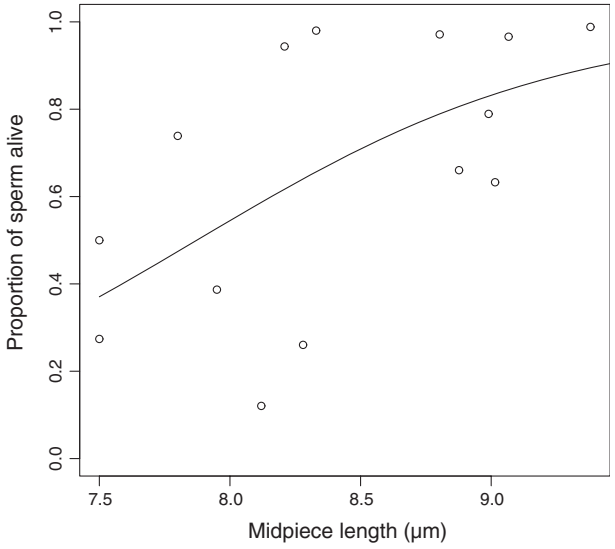

Fig. 6 Sperm longevity increases with midpiece size. Longevity was calculated as the difference in the proportion of living sperm before and 3 h after activation for each male. The curve represents the slope of the relationship as estimated by the generalized linear model.

number and quality varies substantially among traits. Moore *et al.* (2004) reported a strong negative genetic correlation between sperm viability and testes mass in the cockroach *Nauphoeta cinerea,* whereas genetic variation in sperm length was independent of testes size in the beetle *Onthophagus taurus* and *Drosophila hydei* (Pitnick & Miller, 2000; Simmons & Kotiaho, 2002). Ejaculate evolution therefore appears complex, with pleiotropy and/or linkage disequilibrium affecting the evolution of some traits but not others. Although *Xiphophorus* is a model system for genetics (Kallman, 1989; Walter *et al.*, 2006), the relationship among genes influencing ejaculate traits is presently unknown. Quantitative genetic analysis is needed, because phenotypic correlations are not always indicative of the underlying genetic relationships (Moore *et al.*, 2004).

The ultimate cause for increased sperm quality in sneakers but lack of differences in testes size between strategies in *X. nigrensis* might be because of the mechanics of fertilization in poecliid fish. Copulation in poeciliids is rapid (< 1 s) and does not involve male mounting or clasping that may increase male control over sperm transfer (Birkhead & Møller, 1998). Pilastro *et al.* (2004) found male guppies (*P. reticulata)*, utilizing a sneak tactic similar to that in *Xiphophorus*, have shorter copulation durations and transfer 100-fold fewer sperm than copulations preceded by courtship. Those results suggest that female behaviour in poeciliids is often effective in limiting the size of the ejaculate transferred by terminating copulation early. Traits that increase sperm quality, such as viability, rather than ejaculate size or the number of sperm produced might evolve more readily in species in which males have little control over the amount of sperm inseminated. Although some poecillids have been shown to elevate the sperm production and expenditure in response to variation in sperm competition risk (Evans *et al.*, 2003, Aspbury, 2007; but see Evans, 2009), the influence of female behaviour could potentially explain the lack of testes size evolution in *X. nigrensis*.

Sperm longevity

In addition to a greater proportion of living sperm in the ejaculate, small *X. nigrensis* also produced longer-lived sperm. Although we might expect sperm longevity to be under strong selection when mating and fertilization are temporally decoupled, sperm longevity only increases with sperm competition risk under a fairly restrictive set of assumptions (Parker, 1993). Interspecific comparisons have found a positive relationship in mammals between sperm longevity and the interval between female

receptivity and ovulation (Gomendio & Roldan, 1993), and in birds a similar correlation between sperm longevity and the duration of egg laying (Birkhead & Møller, 1992). Studies of species with external fertilization and alternative reproductive strategies have found that sperm from sneakers are typically not longer-lived (Vladic & Jarvi, 2001; Burness *et al.*, 2004; Hettyey & Roberts, 2006; Fitzpatrick *et al.*, 2007), although egg-sperm collision rate is more likely determined by sperm velocity rather than longevity *per se* in these species. A notable exception is in the black goby, *Gobius niger*, in which sperm is slowly released from mucus trails deposited along the nest surface and sneakers have higher sperm longevity and ATP content (Locatello *et al.*, 2007).

Evidence that sperm longevity affects the outcome of sperm competition in internal fertilizers is rare, because most studies have not measured sperm longevity or assessed paternity from sperm stored over multiple reproductive cycles. Indirect evidence is available from a field study of the side-blotched lizard *Uta stansburiana*, where yellow-throated sneakers were more likely than aggressive orange-throated or mate guarding blue-throated males to sire offspring after disappearing (likely owing to mortality) from the population census (Zamudio & Sinervo, 2000). Although the evidence is indirect, the siring of more offspring by sneaker males without providing fresh sperm to females suggests enhanced sperm longevity could be responsible for their elevated reproductive success. More direct evidence comes from an experiment in domestic fowl, where males inseminating fewer but higher quality sperm had an advantage in sperm competition in later clutches (Pizzari *et al.*, 2008).

In poeciliid fish, sperm can be stored for months within the oviduct and paternity is biased toward the last male to mate (Constantz, 1984, 1989; Pitcher *et al.*, 2003). Although the precise mechanism for last-male advantage is unknown, sperm survival among competing males is likely to be important because female poecilids lack spermatheca, sperm storage tubules, or other structures that result in sperm stratification or displacement following successive inseminations (Birkhead & Møller, 1998). Our results suggest sneaker sperm is intrinsically more capable of surviving for a longer period of time after activation. How intrinsic differences between males in sperm longevity interacts with the physiological environment within the female reproductive tract, and female roles in manipulating that environment, is a major gap in our understanding of sperm competition that deserves further investigation.

Sperm morphology and performance

Sperm morphology is also thought to affect sperm competitive ability through its effects on sperm velocity and longevity. Although variation in sperm velocity was not associated with reproductive strategy in *X. nigrensis*, velocity ranged widely among males and was negatively correlated with tail length. Longer tails are thought to produce greater thrust and therefore higher velocities but at the expense of greater energy expenditure (Snook, 2005). Because our assays were taken 5 min after the sperm had been activated, it is possible that energy depletion occurred at a greater rate in longer-tailed sperm, resulting in slower velocities at the time of measurement. Likewise, head shape might affect velocity if more elongate heads reduce drag by streamlining sperm movement (Malo *et al.*, 2006; Gomendio *et al.*, 2007). In our study, head shape was not significantly associated with velocity (controlling for midpiece and tail length), suggesting that drag by head shape does not have a strong effect on sperm velocity. Humphries *et al.* (2008) argued that viscosity, not streamlining, is the dominant force influencing sperm movement, and therefore the flagellum size (force) to head surface area (drag) is more likely to determine velocity. No such relationships were found in our study, however, reinforcing that the sperm head appears to have little influence on sperm velocity in *X. nigrensis*.

Despite recent suggestions that glycolysis rather than oxidative phosphorylation is the main driver of sperm motility (Miki, 2008), our data suggests that sperm with large midpieces swim faster in swordtails. Perhaps this is because of increased loading of mitochondria within the midpiece, as suggested by comparative studies in primates (Anderson & Dixson, 2002), some bird taxa (Immler & Birkhead, 2007) and an intraspecific study of guppies where sperm with longer midpieces also swam faster (Skinner & Watt, 2007). Oxidative phosphorylation is thought to be an important contributor to sperm motility in fish (Ingermann, 2008), and velocity is a significant predictor of fertilization success in both external and internal fertilizers (Birkhead *et al.*, 1999; Burness *et al.*, 2004; Gage *et al.*, 2004; Malo *et al.*, 2005; Liljedal *et al.*, 2008). Although the mechanisms in internal fertilizers are not well understood, studies in domestic fowl have shown that more mobile ejaculates (which covaries with velocity and mitochondrial function) are more successful in sperm competition and less likely to be lost from the site of sperm storage (Birkhead *et al.*, 1999; Froman *et al.*, 2002; Froman, 2008; Pizzari *et al.*, 2008). Similar dynamics may occur in swordtails, where sperm can be stored within the ovarian follicles for months (Constantz, 1989).

Sperm morphology and alternative reproductive strategies

Although evidence that total sperm length and midpiece size increase with sperm competition between species is mixed (Gage & Freckleton, 2003; Anderson *et al.*, 2005; Snook, 2005; Immler & Birkhead, 2007), studies examining sperm morphology in species with alternative reproductive strategies have typically not found differences

between tactics (Gage *et al.*, 1995; Hettyey & Roberts, 2006; Stoltz & Neff, 2006; Fitzpatrick *et al.*, 2007; Locatello *et al.*, 2007; but see Burness *et al.*, 2004). An exception in an internally fertilized species is the dung beetle *Onthophagus binodis,* where hornless sneaker males have longer sperm than horned guarding males (Simmons *et al.*, 1999). In competitive matings, however, hornless males do not sire more offspring than horned males suggesting that females might bias fertilization success toward horned males in post-copulatory sexual selection (Tomkins & Simmons, 2000). Evidence from a variety studies has suggested that sperm size is driven by the interaction between male sperm and female reproductive anatomy rather than differences between the sperm of competing males alone *per se* (Miller & Pitnick, 2002; Snook, 2005; García-González & Simmons, 2007). Whether female reproductive tract morphology and other aspects of cryptic female choice affect the evolution of sperm size in species without specialized organs for sperm storage (e.g. swordtails) remains to be investigated.

Conclusion

Internal fertilization is expected to have a pronounced effect on ejaculate evolution because mating and fertilization are temporally decoupled. Our results suggest that selection on alternative tactics in *X. nigrensis* has resulted in a lengthening of the midpiece and correlated increase in sperm longevity in sneaker males. Furthermore, sperm number and quality were decoupled, with differences in sperm number between strategies apparent only in the proportion of viable sperm produced rather than through the evolution of larger testes size as is often found in other systems. Whether the disassociation between traits that increase sperm number and quality is a common phenomena remains to be seen, as only recently have researchers began to examine sperm number and multiple dimensions of quality (i.e. viability, morphology and performance).

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