The Dissertation Committee for Chad Christopher Smith certifies that this is the approved version of the following dissertation:

Sperm competition and the evolution of alternative reproductive tactics in the swordtail *Xiphophorus nigrensis* (Poeciliidae)

Committee:

_____

Michael Ryan, Supervisor

_____

Thomas Juenger

_____

Dan Bolnick

_____

Gil Rosenthal

_____

Ulrich Mueller

Sperm competition and the evolution of alternative reproductive tactics in the swordtail *Xiphophorus nigrensis* (Poeciliidae)

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Chad Christopher Smith, B.S.; M.S.

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Sperm competition and the evolution of alternative reproductive tactics in the swordtail *Xiphophorus nigrensis* (Poeciliidae)

Chad Christopher Smith, Ph.D.

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Supervisor: Michael Ryan

Darwin identified sexual selection as an important evolutionary process resulting from differences among males in their ability to secure mates. In the latter half of the 20th century, it became apparent that females often mate with multiple partners within the same reproductive cycle, leading to the overlap of ejaculates from multiple males and sperm competition for the fertilization of the eggs. Here, I examine how sperm competition has influenced the evolution of *Xiphophorus nigrensis,* an internally fertilized, livebearing fish with alternative male mating tactics that are dependent upon male size. I find that variation in male tactic is correlated with variation in traits relevant to sperm competition: small males that sneak copulations produce ejaculates with a greater proportion of fertilization-capable sperm (sperm viability) and sperm that is longer-lived following activation compared to large males that court females. Sperm morphology is also divergent between tactics and correlated with sperm performance: smaller males have larger midpieces and midpiece size is positively correlated with sperm velocity and longevity. Social environment also affects ejaculate quality, with sperm velocity rapidly increasing when a small male is exposed to another small male compared to when he is exposed to a large male. Large male ejaculates were invariant across social environments. Next, I demonstrate experimentally that the observed variation in sperm quality has important consequences for the outcome of sperm competition. Males with superior sperm viability sire more offspring, while sperm velocity is negatively associated with sperm competitive ability when sperm are stored

within the female prior to fertilization. Finally, I show that sperm competition is likely to have important effects on male reproductive success in the wild by characterizing the genetic mating system of *X. nigrensis*. I find that 61% of females collected from the Nacimiento Río Choy produce offspring sired by 2-4 males. Paternity is strongly skewed among sires, with an average of 70% of offspring sired by one of the males represented in the brood. These studies illustrate sperm competition can have potent effects on the evolution of animals.

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Introduction

A ubiquitous feature of animal mating systems is that males compete for females in order to reproduce. One of Darwin's (1871) great insights was that traits leading to variation among males in their ability to secure mates, either because of their attractiveness to females or their efficacy in dispatching rivals males, could evolve by sexual selection. With the advent of molecular tools for assessing paternity a century later, the study of sexual selection was revolutionized when it became apparent that even among birds, long considered a bastion of monogamy among animals, females often produce offspring sired by males other than their social mate (Griffith et al. 2002). Male mating success thus only explains portion of their reproductive output when females are promiscuous because of the overlap of ejaculates from more the one male at the site of fertilization. Parker (1970) coined the term "sperm competition" to refer to the process by which sperm of multiple males compete for the fertilization of a set of ova, now recognized as a major force in the evolution animals (Birkhead & Møller 1998). Sperm competition constitutes a second episode of sexual selection because, like intra- and intersexual interactions prior to mating, it results in variance among males in the number of offspring produced.

The evolutionary responses of males to sperm competition have been well documented (Birkhead & Møller 1998; Birkhead et al. 2009) due in large part to the extensive theoretical framework laid down by Geoff Parker (reviewed in Parker & Pizzari 2010). Parker (1970) first envisaged sperm competition as a raffle in which the male with the most "tickets" (i.e. sperm) in competition would sire the most offspring, leading to selection to increase the number of sperm inseminated. Given a trade-off between the allocation of resources to the acquisition of mates and investment into the ejaculate, Parker predicted that investment in sperm number should increase with the probability females remate. These models are predicated on the assumption that ejaculate production has a cost. While historically sperm was often thought be "cheap" (Dewsbury 1982), several lines of evidence now show that the ejaculate is a finite resource that is adaptively partitioned among females (Wedell et al. 2002) with measurable costs (Pitnick 1996; Olsson et al. 1997; LaMunyon & Ward 1998).

A major accomplishment of sperm competition research is empirical validation that sperm competition often selects for traits that increase capacity for sperm production and the number of sperm males ejaculate (Birkhead & Møller 1998). Sperm number, however, only explains a portion of the observed variance in fertilization success when two or more males compete (Snook 2005). A second source of variation among males is the quality of the ejaculates they produce. Sperm quality is a multidimensional trait that includes features of the ejaculate that enhance siring success beyond sperm number, such as sperm swimming speed, the proportion of living sperm in the ejaculate (sperm viability), the longevity of sperm once it is ejaculated, and compounds of the seminal fluid that affect sperm-female or sperm-sperm interactions. Sperm morphology is also considered a component of sperm quality, either because it directly affects sperm performance (e.g. sperm with longer flagella may swim faster) or because sperm morphology itself affects the outcome of sperm competition (e.g. if longer sperm physically displace shorter sperm in the female reproductive tract). Unlike sperm number, the evolutionary response of sperm quality to sperm competition appears far more complex (Snook 2005). The most parsimonious explanation for the variety of responses or lack of response, of sperm quality to sperm competition is that the importance of different components of sperm quality depends on the reproductive biology of a given taxon (e.g. internal vs. external fertilization). Earlier studies also relied on sperm morphology as their metric of quality without knowing the relationship between morphology and performance (Humphries et al. 2008). With the development of computer-assisted semen analysis (CASA) and molecular technologies for visualizing sperm, however, the technical limitations hampering previous studies have given way to new developments in our understanding of how ejaculates evolve.

In Chapter 1, I employ these techniques to examine the evolution of sperm number and quality in the swordtail *Xiphophorus nigrensis,* an internally fertilized fish with male alternative reproductive tactics (ART). Alternative male reproductive tactics evolve in response to strong sexual selection, which results when a subset of males in the population is able to monopolize females or the resources they require to mate (Shuster & Wade 2003; Brockmann & Taborsky 2008). Traits that allow otherwise unsuccessful

males to obtain fertilizations can take many forms, but usually involve the evolution of characteristics that allow subordinate males to subvert the efforts of dominant males to monopolize females or mating behaviors that enable unpreferred males to fertilize a female's eggs without their cooperation (Oliveira et al. 2008). Alternative reproductive tactics are a striking example of how sexual selection can generate diversity in anatomical, behavioral, and physiological traits within one sex, and because ART evolve in response to competition for fertilizations, such species have served as powerful model systems for the study of sperm competition (Simmons et al. 1999; Pizzari et al. 2003; Taborsky 2008).

Game-theoretical models predict that males that "sneak" matings should have higher levels of ejaculate investment than male that "guard" reproductive resources because (1) sneaker males almost always experience sperm competition, while guarding males experience sperm competition a proportion of the time (Parker 1990a) and (2) sneaker males are at a disadvantage in precopulatory sexual selection (Parker 1990b), which affects their ability to successfully transfer sperm or mate when the eggs are most likely to be fertilized. Using this predictive framework, I compare the number of sperm available for mating and sperm quality between *X. nigrensis* males that sneak copulations and those that court females, which depends on male body size (Ryan & Causey 1989). I also explore how sperm form and function are related by comparing sperm morphology (head shape, midpiece length, and flagellum length) to my measures of sperm performance. I find that small males that sneak copulations have higher sperm quality (viability and longevity) than large males that court females, but that traits influencing sperm number are invariant between tactics. Sperm morphology is also correlated with sperm velocity and longevity, providing evidence that morphology can evolve in response to sperm competition through its effect on performance. The study is a rare examination of the interrelationships between ART, sperm morphology and sperm performance in a species with internal fertilization. Chapter 1 was published in the *Journal of Evolutionary Biology* in 2010.

In Chapter 2, I explore whether variation in a male's social environment affects both the number and quality of sperm produced. Studies of species with ART often

compare sperm quantity and quality among tactics without considering plasticity as an important feature of the phenotype. Ecological and demographic factors are known to lead to fluctuations in the frequency of ART in the wild (Sinervo & Lively 1996), and as a consequence ejaculate traits may be plastic depending upon the identity of the males in competition. I measure sperm quantity and quality after exposing male *X. nigrensis* to large, courting males or small, sneaker males in a repeated measures design. In one of the few demonstrations that the phenotype of a male's competitor can affect sperm quality, I find that sperm velocity rapidly increases when a male's competitor was small compared to large. Interestingly, the plastic response is only exhibited by small males, suggesting that the costs and benefits of plasticity differ between tactics. This study is currently in press in *Biology Letters.*

The first two chapters provide evidence differences in sperm viability and velocity between tactics may evolve in response to sperm competition in *X. nigrensis*. If this is the case, variation in sperm quality between males should affect siring success when two or more males compete for the fertilization of the eggs. Chapter 3 describes a study where I artificially inseminated sperm from two males into a female and assigned paternity to the resulting offspring with molecular markers. As predicted, males with superior sperm viability sire more offspring, which is expected because sperm viability increases the number of fertilization-capable sperm in the ejaculate. In contrast, males with faster swimming sperm sire fewer offspring, which is surprising because sperm velocity is thought to provide a competitive advantage by increasing the probability sperm to reach the eggs first. I discuss how tradeoffs between sperm velocity and longevity might account for this result due to the decoupling of mating and fertilization in species where females store sperm.

In the final chapter, I use molecular markers to describe the genetic mating system of a wild population of *X. nigrensis*. Two parameters of interest in the context of sperm competition are the frequency of females mated by more than one male and the distribution of paternity among offspring. I find that 61% of the females produce offspring sired by 2-4 males. The distribution of paternity is also strongly skewed, with one male garnering an average of 70% of the reproductive success within broods. Due to

the strong correlation between the body size of fathers and sons in *X. nigrensis* (Dries et al. 2001), I also examine the relationship between body size and siring success by phenotyping male offspring produced by the females collected. Male body size is not correlated with their reproductive success, which was surprising because larger males are more likely to displace smaller males in agonistic interactions (Morris et al. 1992) and are preferred by females (Ryan et al. 1990). Sperm competition is thus a common occurrence in *X. nigrensis*, however traits important in precopulatory sexual selection (i.e. male body size) do not appear to affect how many offspring a male sires within a given brood. Finally, I find that female fecundity is higher when more males are represented in the brood, suggesting polyandry may be adaptive in *X. nigrensis*. I discuss some potential benefits of polyandry in species where males provide no resources to females other than sperm.

These studies offer insight into how sperm competition can influence the evolution of animals. Sexual selection was long thought to result from variance among males in mating success, leading to the evolution of traits that increase attractiveness to females or success in male-male competition. As I demonstrate, the reach of sexual selection can extend beyond the acquisition of mates when females mate with more than one male. Incorporating postcopulatory processes into our worldview will thus enrich our understanding of the role of sexual selection in evolution.

Chapter 1 Sperm quantity, quality, and the evolution of alternative reproductive tactics in the swordtail *Xiphophorus nigrensis*

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 disfavored "role" due 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 the 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 sperm morphology is likely to play an important role in mediating sperm competition through its effects on sperm performance. This chapter was published[1] with my supervisor, Michael Ryan, as a coauthor. I was responsible for all phases of the project, including the formulation of the question, execution of the study, analysis and interpretation of the data, and manuscript preparation.

[1]Smith C.C., Ryan .M.J.. 2010. Evolution of sperm quality but not quantity in the internally fertilized fish *Xiphophorus nigrensis*. *Journal of Evoluationary Biology* 17, 1759-1771.

INTRODUCTION

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 discrete alternative reproductive strategies, where suites of physiological, morphological, and behavioral traits have diversified among males due to 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 tradeoff between investment in ejaculates and other traits necessary for obtaining mates (e.g. courtship, territory defense), models predict parasitic males should allocate more resources to their ejaculates because (1) parasitic males are typically at a disadvantage (occupy a disfavored "role") in mating because of subordinance in male-male competition and female preferences for bourgeois males (Parker 1990a) and (2) 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 & Pizzari 2010). As predicted, ejaculate sizes are typically larger in parasitic males (Taborsky 1998). Studies using phylogenetic approaches, experimental evolution, and analysis of phenotypic plasticity in ejaculate allocation 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 & Garcia-Gonzalez 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; delBarco-Trillo & Ferkin 2004; Urbach et al. 2005). A few notable exceptions, however, exist where species with discrete 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 discrepancy between theory and data are 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 risk 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). 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 due to 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 and Pizzari 2010, 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 parasitic/bourgeois 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 2004, Tomkins & Simmons 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 within dominance hierarchies. Experimental manipulations of social status in some species have shown that subordinate males (i.e. the disfavored role) produce more, higher quality sperm compared to their dominant counterparts (Rudolfsen et al. 2006; Cornwallis & Birkhead 2007; Pizzari et al. 2007; but see Montrose et al. 2008; Fitzpatrick et al. 2008; Thomas & Simmons 2009; Kruczek & Styrna 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. While 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 the present 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 disfavored "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). While 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, while 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 due to 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 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 (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 Río Choy, Mexico (21° 59' 18" N 98° 53' 2" 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 (tip of the mouth to the end of the caudal peduncle) was measured with dial calipers to the nearest 0.1mm. The threshold body size for the expression of courtship behavior in *X. nigrensis* is 26 mm standard length (SL) (Ryan & Causey 1989); males smaller than 26 mm were therefore designated as "sneaker" males (mean SL $\pm$ s.d.: 21.4 $\pm$ 2.0 mm, n=12) and those larger than 26 mm were designated as "courting" males (mean SL $\pm$ s.d.: 31.0 $\pm$ 3.2 mm, n=25). Testes and soma were dissected and dried separately overnight in a 55^{o}C oven, allowed to cool in a desiccator and weighed on a Mettler Toledo AT21 Comparator balance to the nearest 0.1mg.

Sperm number and velocity

We determined the number of sperm within ejaculates and sperm swimming speed from fish collected in May 2007 from the headwaters of the Río Choy, Mexico. Males

were transported to the University of Texas at Austin, housed in single sex groups with visual access to females on a 14L:10D light cycle for at least three 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 (http://xiphophorus.org/ai.htm) and put on ice in a solution of 20-100µl sperm extender (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM, MgCl2, 0.41 mM MgSO4, 10 mM Tris, pH 7.5) (Gardiner 1978) depending on the size of the ejaculate. Sperm remained quiescent within this solution until activation (within two hours of collection).

We quantified the amount of sperm stripped and sperm velocity from samples of sneaking (mean SL ± s.d.: 22.0 ± 0.93 mm, n=18) and courting (mean SL ± s.d.: 32.8 ± 2.56 mm, n=20) males. We resuspended sperm with a micropipette and gently vortexed for one minute to break apart the spermatozeugmata and homogenize the solution. Sperm were activated by adding 4µl of sample to 12µ 150mM KCl (Morisawa & Suzuki 1980). Five minutes post-activation, sperm were pipetted into a disposable Microcell© (MC-20-4, Conception Technologies, San Diego) fixed depth (20µl) counting chamber and videotaped with a Canon XLS camcorder mounted to a Zeiss Axiovert 25 microscope using dark field microscopy. The first two seconds of video were digitized and analyzed with the computer assisted sperm analysis (CASA) plugin for ImageJ (Wilson-Leedy et al. 2007) to obtain measures of sperm concentration and velocity. Sperm concentration was estimated by multiplying the number of sperm tracked (mean ± s.d.*:* 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). Sperm with intact cell membranes are stained green (SYBR®14) and dead sperm with compromised cell membranes are stained red (propidium iodine). The proportion of viable sperm in sneaking (mean SL ± s.d.: 22.5 ± 0.99 mm, n=17) and courting male (mean SL ± s.d.: 32.8 ± 2.62 mm, n=19) ejaculates was revealed by adding 0.5µl SYBR®14 nucleic acid stain to 9.5 µl unactivated sperm solution (final concentration of SYBR®14 = 200nm), incubating sperm for ten minutes at room temperature, and then adding 1 µl propidium iodine (final concentration of propidium iodine = 12 µM) followed by a second ten minute incubation period. Sperm were photographed with a Zeiss Axiocam Mrc camera mounted on a Zeiss AX10 microscope at 100x magnification with Rhod FS15 and GFP FS17 prisms to visualize fluorescent cells. Images were manually thresholded in ImageJ, sperm counted with the analyze particles function (mean ± s.d.: 2,294 ± 1500, range 287-5,656) 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 three hours later. The proportion of viable sperm before and three hours 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 attempts to capture intrinsic differences between males in sperm performance

without variation due to 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 due to an equipment malfunction that did not allow us to measure longevity for six males (5 sneakers and 1 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 $\pm$ sd: 23.7 $\pm$ 1.0 mm, n=16; courting males, 33.3 $\pm$ 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 400x magnification. Head shape (head length/head width), midpiece length, and tail length were measured for each sperm using ImageJ and the average taken for statistical analysis. A subsample of 57 sperm from six males were 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* (Simmons & Tomkins 2002). Proportion data were analyzed 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 2007) were calculated to provide a standardized measure of differences between tactics and interpret coefficients in the binomal 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 $\pm$ 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=<0.001, Fig. 1.1). 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 body size (measured as standard length), 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 standard length: $F_{1,34}$=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 1.1, Fig. 1.2). Sperm velocity varied substantially across all males (mean $\pm$ s.d.: VAP, 29.1 $\pm$ 6.3, range 27.2 µm/s; VSL: 24.7 $\pm$ 4.9, range 23.0 µm/s; VCL: 31.5 $\pm$ 6.9, range 30.9 µm/s) as has been found in studies of other taxa (Gage et al. 2002; Stoltz & Neff 2006a; Locatello et al. 2007).

Sperm morphology

Sneaker males had longer midpieces but equivalent head shape and tail lengths compared to courting males (Table 1.1, Fig. 1.3). 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 1.1). 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 1.1, Fig. 1.4). Sneaker sperm was 2.7 times more likely to be alive before activation compared to courting males (Table 1.1). Once activated, sneaker sperm was 3.5 times more likely to survive until viability was resampled 180 minutes later (Table 1.1, Fig. 1.4).

Sperm morphology and performance

We used multiple regression to analyze the relationships between sperm performance and morphology across all males (n=18) for which we had a complete data set. Average path velocity (VAP) increased with longer midpieces and shorter tails (full model: R^2=0.51, $F_{3,14}$=6.93, p=0.004, Table 1.2, Fig. 1.5) 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 their ratio 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 curvilinear and straight line velocity for all analysis.

Sperm viability was not related to morphology before the sperm were activated (GLM: head shape: β=-0.44, $F_{1,14}$=0.06, p=0.82; midpiece length: β=0.93, $F_{1,14}$=2.46, p=0.14; tail length: β=-0.24, $F_{1,14}$=0.60, p=0.45). Sperm survival after activation, however, increased with midpiece size (GLM: β=1.41, $F_{1,14}$=6.03, p=0.03, Fig. 1.6) after the nonsignificant effects of head shape and tail length were iteratively removed from the final model. Sperm were 2.5 times more likely to be alive with every standard deviation increase in the midpiece.

Table 1.1 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, velocity; 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 (μ/s)	30.2 ± 1.48	28.1 ± 1.40	0.35 (-0.30, 0.99)	1.25	0.27
VCL (μ/s)	32.5 ± 1.51	30.5 ± 1.65	0.30 (-0.34, 0.94)	1.11	0.30
VSL (μ/s)	26.2 ± 1.19	23.3 ±1.01	0.62 (-0.05, 1.27)	3.61	0.07
Sperm morphology ‡					
Head shape (μm)	2.68 ± 0.05	2.77 ± 0.33	0.51 (-0.15, 1.16)	2.29	0.14
Midpiece length (μm)	**8.94 ± 0.15**	**8.41 ± 0.11**	**0.97 (0.29, 1.65)**	**8.28**	**0.007**
Tail length (μm)	45.4 ± 0.24	45.3 ± 0.25	0.07 (-0.57,0.72)	0.05	0.83
Total length (μm)	57.7 ± 0.33	57.2 ± 0.30	0.50 (-0.16, 1.15)	2.21	0.15
Sperm viability *					
Proportion alive (%)	**82.8 ± 3.2**	**56.7 ± 5.1**	**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**

† df = 1,39

‡ df = 1,38

* Proportion alive: df = 1,36;
Proportion surviving: df = 1,29

Table 1.2 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	0.27
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**

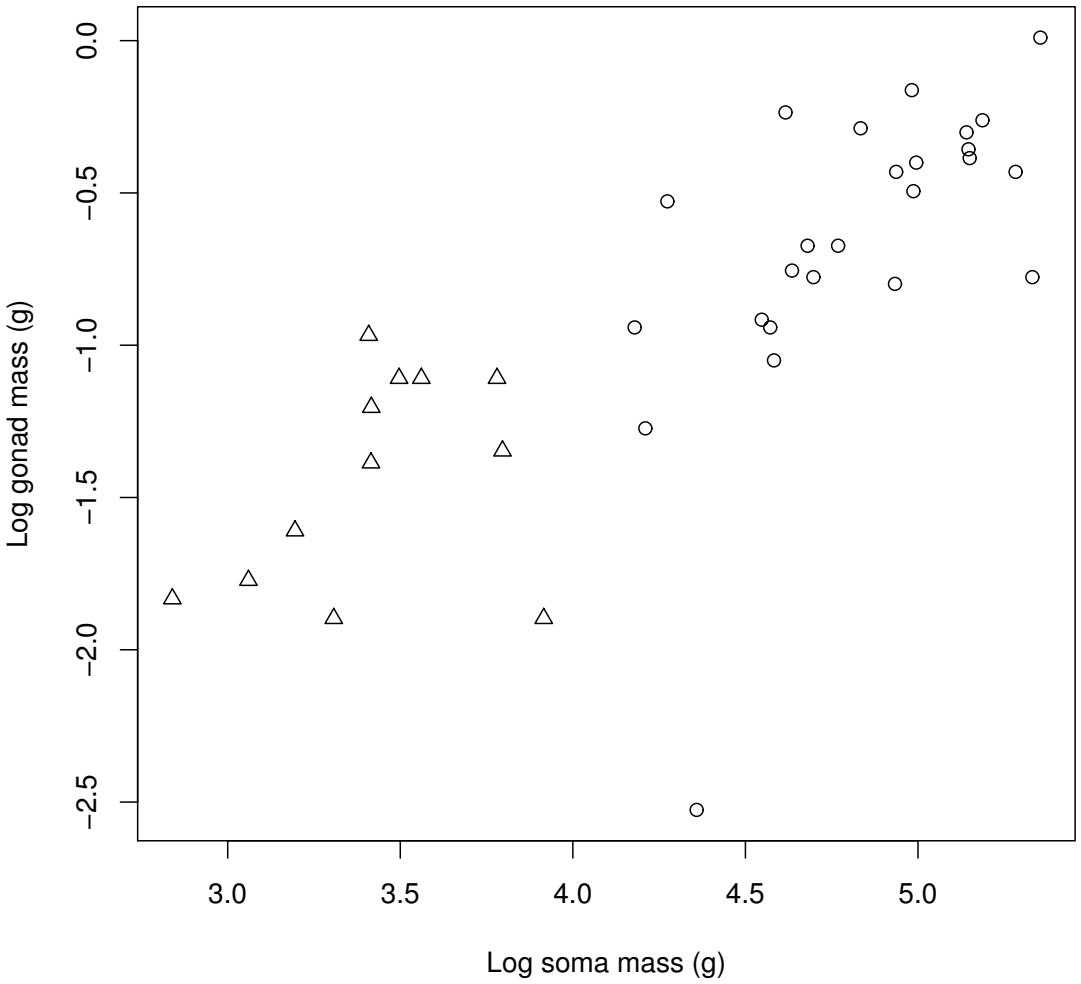

Figure 1.1 Equivalent testes investment of sneaking (Δ) and courting (○) males. Testes mass does not deviate from the expected allometric relationship with soma mass for either strategy.

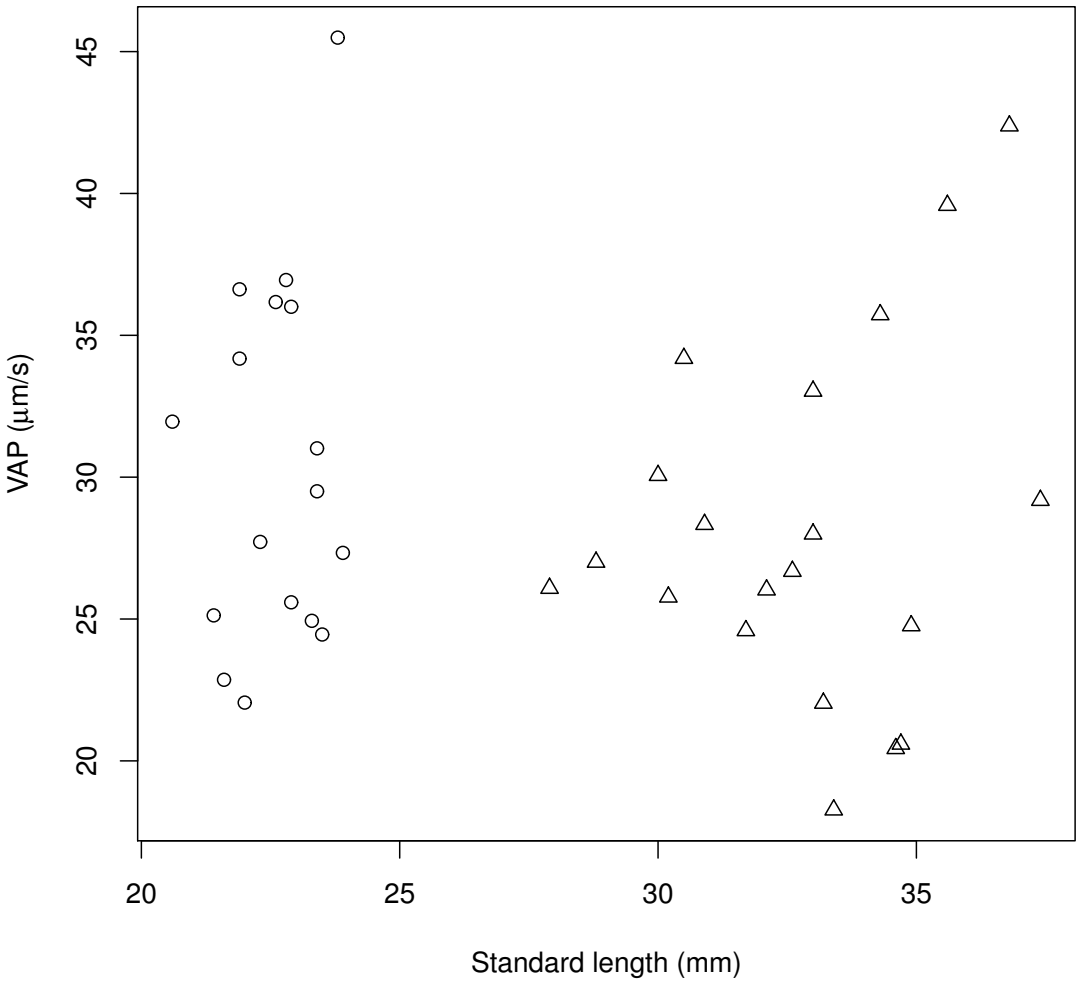

Figure 1.2 Equivalent average path velocities (VAP) for sneaking (Δ) and courting (○) males measured five minutes post-activation. Plots of curvilinear velocity (VCL) and straight line velocity (VSL) are qualitatively similar and not shown.

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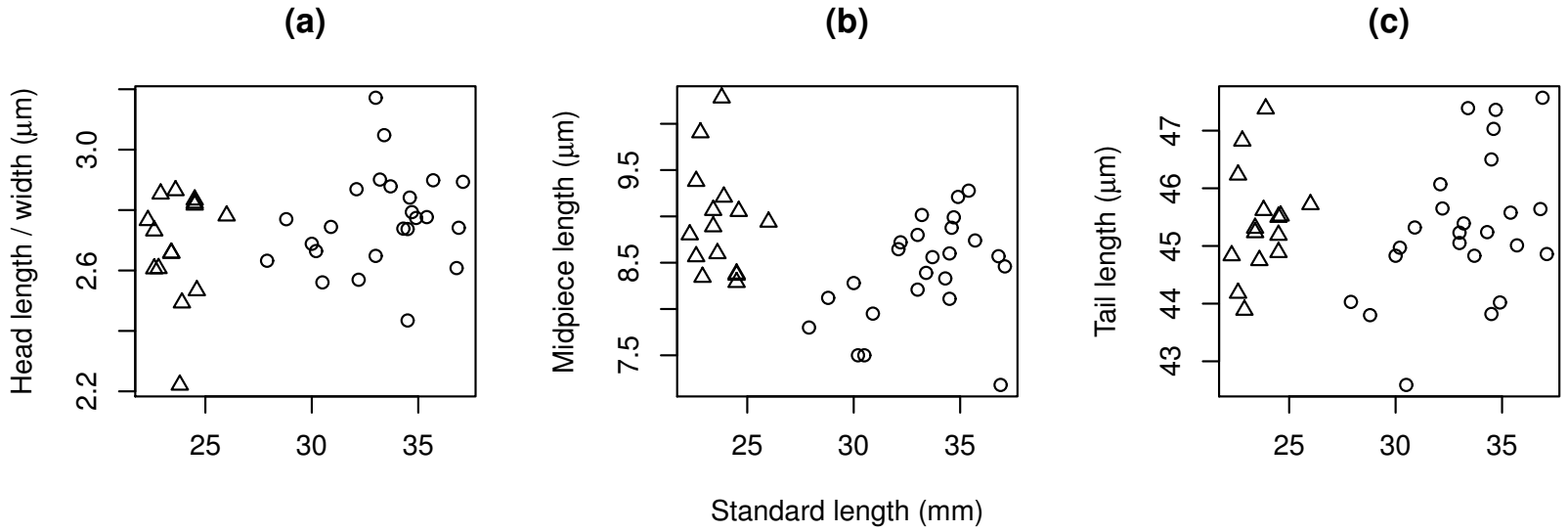

Figure 1.3 Differences in sperm morphology between sneaking (Δ) and courting (○) mles. Sneaker males had significantly longer midpieces (b) while head shape (a) and tail length (c) were similar between strategies.

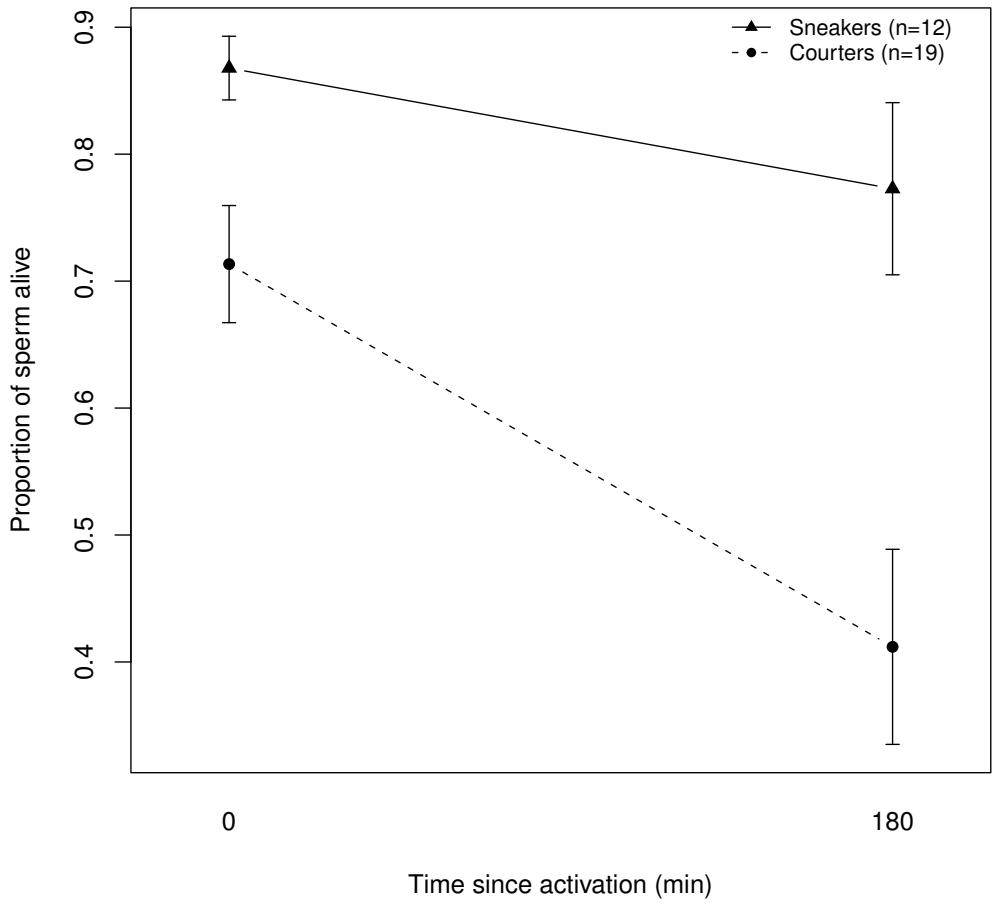

Figure 1.4 Sneaker males have more viable sperm before activation (time 0) and sperm that are longer lived (difference in viability before and 180 minutes after activation).

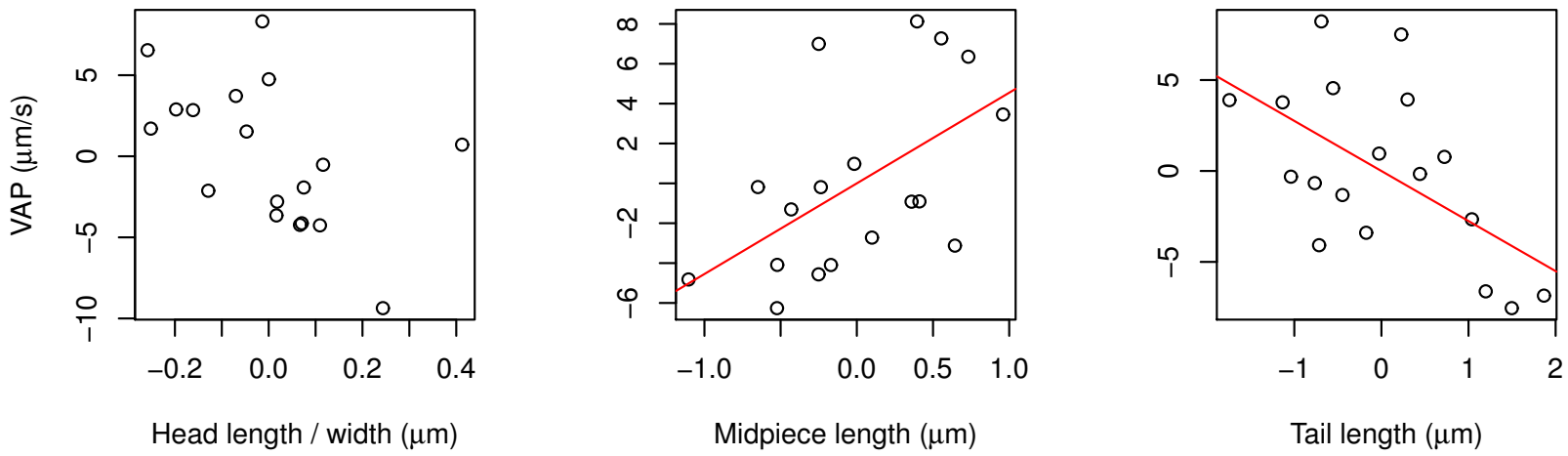

Figure 1.5 Added-variable plot of average path velocity (VAP) and sperm morphology from the multiple regression analysis. Residuals of velocity on residuals of (a) head shape, (b) midpiece length and (c) tail length are shown to depict the relationships VAP and each predictor after the effect of the other predictors is removed. Plots of straight line velocity (VSL) and curvilinear velocity (VCL) are qualitatively similar and not shown.

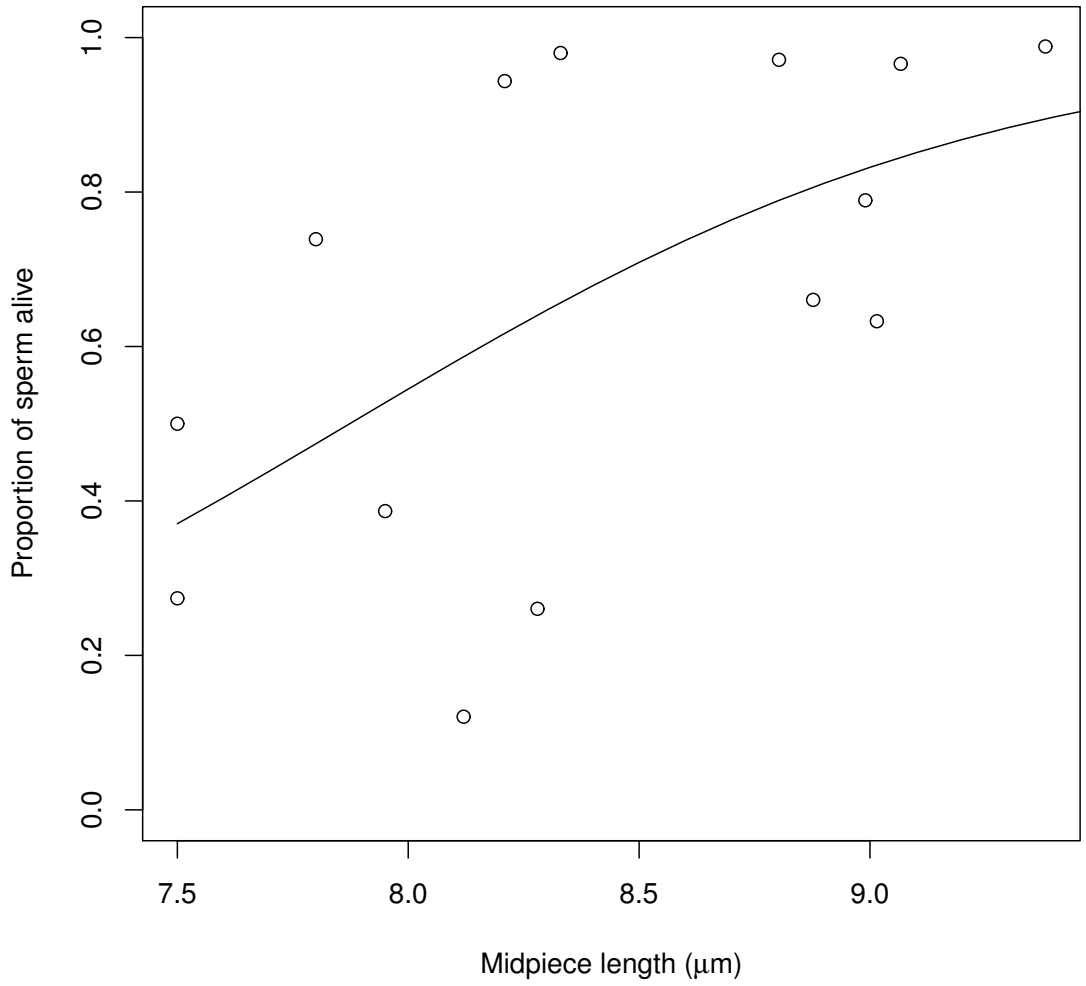

Figure 1.6 Sperm longevity increases with midpiece size. Longevity was calculated as the difference in the proportion of living sperm before and 3 hours after activation for each male. The curve represents the slope of the relationship as estimated by the generalized model.

DISCUSSION

Species with alternative reproductive strategies are pronounced examples of intraspecific diversification due to 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 1990b). 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 are 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 both greater in polyandrous compared to monandrous species (Hunter & Birkhead 2002) and increases fertilization success when two males compete in the cricket *Teleogryllus oceanicus* (Simmons & García-González 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 while 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 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 either be 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 favored by selection as this can increase sperm number and, we assume, the probability of achieving insemination. 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 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,* while genetic variation in sperm length was independent of testes size in the beetle *Onthophagus taurus* (Simmons & Kotiaho 2002) and *Drosophila hydei* (Pitnick & Miller 2000). 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)(Kallman 1989, Walter 2006), the relationship among genes influencing ejaculate traits are 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 due to the mechanics of fertilization in poecliid fish. Copulation in poeciliids is rapid (less than 1 second) and does not involve male mounting or clasping that may increase male control over sperm transfer (Birkhead & Møller 1998). Pilastro (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 behavior 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. While some poecillids have been shown to elevate the sperm production and expenditure in response to variation in sperm competition risk (Evans et al. 1993, Aspbury 2007, but see Evans 2009), the influence of female behavior could potentially explain the lack of testes size evolution in *X. nigrensis*. While the capacity for females to influence post-copulatory sexual selection has been well described (Eberhard 1996), the role of cryptic female choice in the evolution of sperm quantity and quality is only now becoming elucidated (Snook 2005).

Sperm longevity

In addition to a greater proportion of living sperm in the ejaculate, small *X. nigrensis* also produced longer-lived sperm. While 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*. 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 due 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 domestic fowl, where sperm quality had a greater effect on the outcome of sperm competition than sperm number over multiple 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, Constantz 1989; Pitcher et al. 2003). While 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. While 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 five minutes 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 flagellum length) suggesting that drag due to 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 head surface area (drag) to flagellum size (force) ratio is more likely to determine velocity. No such relationship was found in our study, however, reinforcing that the sperm head has 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 due to 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; 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; Pizzari et al. 2008b; Froman 2008). Similar dynamics may occur in swordtails where sperm can be stored within the ovarian follicles for months (Constanz 1989).

Sperm morphology and alternative reproductive strategies

While 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 2006a; 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 typically 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). Natural variation in sperm quality between tactics in *X. nigrensis* will lend itself to future

work examining the contribution of cryptic female choice and sperm quality to the outcome of sperm competition in internally fertilized species.

ACKNOWLEDGEMENTS

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Chapter 2 Plasticity in ejaculate traits across socio-sexual environments in the swordtail *Xiphophorus nigrensis*

ABSTRACT

In species with alternative reproductive tactics, males that sneak copulations often have larger, higher quality ejaculates relative to males that defend females or nest sites. Ejaculate traits can, however, exhibit substantial phenotypic plasticity depending on a male's mating role in sperm competition, which may depend on the tactic of his competitor. We tested whether exposure to males of different tactics affected sperm number and quality in the swordtail *Xipophorus nigrensis*, a species with small males that sneak copulations and large males that court females. Sperm swimming speed was higher when the perceived competitor was small than when the competitor was large. Plasticity, however, was only exhibited by small males. Sperm number and viability were invariant between social environments. Our results suggest sperm quality is role-dependent and that plastic responses to the social environment can differ between male reproductive tactics. This chapter was published[1] with my supervisor, Michael Ryan, as a coauthor. I was responsible for all phases of the project, including the formulation of the question, execution of the study, analysis and interpretation of the data, and manuscript preparation.

[1]Smith CC, Ryan MJ. (in press). Tactic-dependent plasticity in ejaculate traits in the swordtail *Xiphophorus nigrensis*. Biology Letters.

INTRODUCTION

Parker (1990a) predicted that, given a trade-off between investment in the ejaculate and other expenditures important in obtaining mates, "parasitic" males that sneak fertilizations should exhibit greater ejaculate investment relative to "bourgeois" males that guard females. As predicted, parasitic males often do have larger testes, ejaculate more sperm of higher quality, and have higher success in sperm competition (Taborsky 2008). Few studies, however, have examined the extent to which ejaculate traits are phenotypically plastic in response to the phenotype of a male's competitor (Rudolfsen et al. 2006; Pizzari et al. 2007). Ecological and demographic factors are known to lead to temporal and spatial fluctuations in the frequency of male reproductive tactics in the wild (Brockmann & Taborsky 2008). Consequently, plasticity in ejaculate traits may be favoured if changes in the phenotype of a male's competitor(s) affects the optimal ejaculate allocation strategy.

Males are predicted to alter ejaculate investment if they consistently experience an advantage (or disadvantage) in sperm competition (Parker 1990b). Dominant males, for example, are often in the favoured mating "role" in sperm competition because they can exclude subordinates from the best physical and temporal mating positions (Stoltz & Neff 2006b). Females can also influence mating roles by lengthening or shortening copulation (Pilastro et al. 2007), ejecting sperm (Pizzari & Birkhead 2000) or biasing sperm use inseminated by preferred males (Edvardsson & Göran 2000), which is typically the bourgeois phenotype in species with alternative tactics (Taborsky 2008). Theory predicts males should increase ejaculate investment when they consistently occupy the disfavoured role and decrease it when in the favoured role (Parker 1990b). Experimental manipulations of social status in birds and fish have shown some support for the theory, with sperm quantity and quality quickly declining when males become dominant (the favoured role) (Rudolfsen et al. 2006; Pizzari et al. 2007).

We examined whether phenotypic plasticity in ejaculate traits were manifest in the swordtail *Xiphophorus nigrensis,* an internally fertilized fish with size-dependent alternative male reproductive tactics. Male body size depends on a Y-linked polymorphism that induces sexual maturation and dramatically slows growth (Lampert et al. 2010). Males that mature at a small size obtain fertilizations by coercively sneaking

copulations while males of large and intermediate size court females but rarely sneak (Ryan & Causey 1989). Sperm competition has also played a role in the evolution of the tactics; sneaker males produce more viable sperm and sperm that is longer lived (Smith & Ryan 2010). Large males, however, are superior in male-male competition (Morris et al. 1992) and preferred by females (Ryan et al. 1990). Here, we manipulated the tactic of a male's perceived competitor and measured the number and quality of sperm produced. We predicted that small males would elevate ejaculate investment when their competitor was large (sneaker males in the disfavoured role), while large males would reduce investment when their perceived competitor was small (courting males in the favoured role).

METHODS

X. nigrensis were collected from the Nacimiento Río Choy, Mexico in May 2008 and housed with visual access to the other sex for at least four weeks prior the study. On the first day of the 14 day trial, sperm were stripped from a large [35.1 $\pm$ 1.73 mm standard length (SL), n=11] or small (25 $\pm$ 0.83 mm SL, n=17) focal male. The male was then placed in the center of a 23 L aquarium divided evenly into thirds with two translucent, water-permeable barriers. A stimulus female (34.1 $\pm$ 1.5 mm SL) was placed in one compartment and either a large (35.4 $\pm$ 1.8 mm SL) or small (24.4 $\pm$ 1.1 mm SL) stimulus male in the other. The barrier allowed passage of visual and chemical stimuli but prevented physical contact. On day seven the focal male's ejaculate was stripped for analysis. A stimulus male of the opposite tactic was then swapped into the aquarium after a complete water change while the same female remained in her compartment. On day 14 the focal male's ejaculate was stripped again for the second assay.

Ejaculate traits were assessed as in (2010). Briefly, the number of sperm stripped and swimming velocity were determined by activating 4 µl of ejaculate with 12 µl 150 mM KCl, pipetting the solution in a Microcell© (Conception Technologies, San Diego) fixed depth (20 µl) slide and analyzing the first two seconds of video one minute post-activation (178 $\pm$ 70 sperm tracked, range 44-341). Two subsamples of the stripped ejaculate were measured for each male. Sperm viability was assessed by photographing sperm stained with the Molecular Probes LIVE/DEAD® fluorescence assay (3,227 $\pm$

1,600 sperm, range=1,100-6,750) and calculating the proportion alive with ImageJ. An angular transformation was used to normalize the proportions. Data were analyzed using repeated-measures ANOVA in Systat 11. All statistical tests were two-tailed.

RESULTS

The tactic of the stimulus male had no significant effect on the number of sperm stripped (stimulus male tactic: $F_{1,26}$=2.06, p=0.16; stimulus x focal male tactic: $F_{1,26}$=1.31, p=0.26) or sperm viability (stimulus male tactic: $F_{1,26}$=0.96, p=0.34; stimulus x focal male tactic: $F_{1,26}$=0.49, p=0.49) of focal males. In contrast, sperm velocity was higher overall in the presence of sneaker males compared to courting males (stimulus male tactic: $F_{1,26}$=4.53, p=0.043) but a male's response did not depend on his own tactic (stimulus x focal male tactic: $F_{1,26}$=2.46, p=0.13, Fig. 1). Our power to detect the interaction, however, was low (1-β=0.33), so we decomposed the interaction to increase power. We detected a 13% increase in sperm swimming speed when sneakers were exposed to other sneaker compared to when they were exposed to courting males (paired t-test: t_{16}=3.04, p=0.008, Bonferroni-corrected α=0.025, Cohen's D=0.76, Fig. 1). In contrast, the velocity of sperm produced by courting males was not different when the tactic of their competitor was altered (paired t-test: t_{10}=0.34, p=0.74, Cohen's D=0.12, Fig. 1). These results suggest the significant overall increase in velocity in the ANOVA, which we had more power to detect (1-β=0.54), was driven by the response of sneaker males to the treatments.

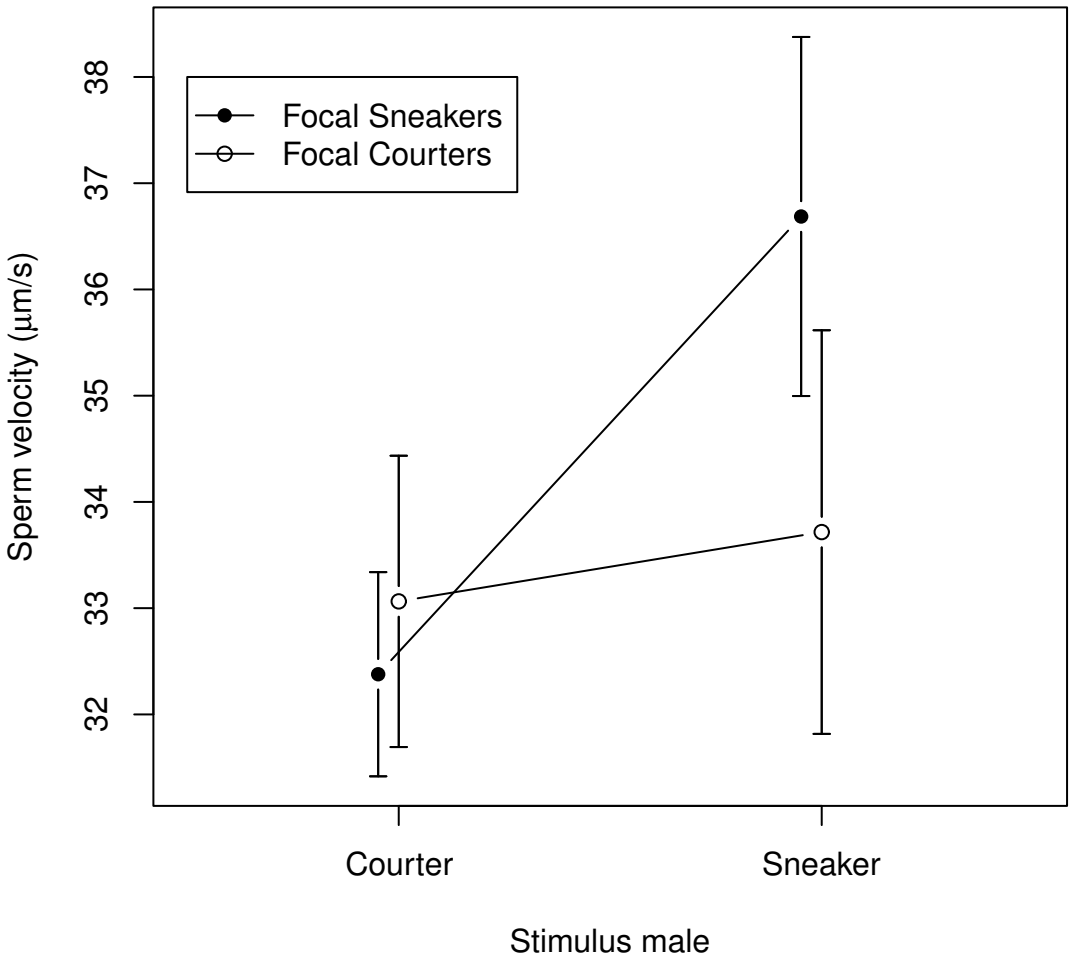

Figure 2.1 Average path velocity of sperm from courting and sneaking males when exposed to stimulus males of each tactic. Depicted are the means + 1 SE.

DISCUSSION

Several studies have demonstrated male ejaculate traits are phenotypically plastic given variation in the number of male rivals or female mating status (Wedell et al. 2002). Our study shows that changes in the phenotype of a male's perceived competitor can result in rapid alterations in sperm quality, but that plasticity can be tactic-dependent. Only small sneaker males responded to variation in the tactic of their competitor, producing faster swimming sperm in the presence of other small males. Sperm velocity increases sperm competitive ability in swordtails (Gasparini et al. 2010) and other internal fertilizers (Denk et al. 2005), thus plasticity in velocity may be have important consequences for a male's success in sperm competition.

Temporal and spatial heterogeneity in the frequency of alternative male tactics could lead to phenotypic plasticity in ejaculate traits if such shifts also alter the optimal ejaculate allocation strategy. The frequency of sneaker males varies over space and time in swordtails (Rios-Cardenas et al. 2007) and has well documented effects on sperm competition risk in other species (Alonzo & Warner 2000; Neff et al. 2008). Mating roles may fluctuate with tactic frequency by altering male-male dominance interactions and female preferences, both of which can depend on the frequency of male phenotypes in the population (Farr et al. 1986; Gibson & Langen 1996).

Ejaculate traits in large male *X. nigrensis,* however, did not depend on their competitor's phenotype in our study. One explanation is plasticity may not confer a selective advantage for these males. Tradeoffs between the ejaculate and other activities that increase fertilization success are at the heart of sperm competition theory, and it is possible that bourgeois males may gain higher fitness returns by allocating energy to resource defense and attracting females rather than investing in sperm competition (Alonzo & Warner 2000). Alternatively, competitor phenotype may simply not provide information about the mating roles in *X. nigrensis*. This is unlikely, however, as male mating tactics can have large effects on mating success (Ryan et al. 1990; Pilastro et al. 2007) and small males responded to the treatments in our experiment.

Considering their disadvantaged mating role, we expected small males to have higher ejaculate investment in the presence of large, not the small, male competitors.

Other studies have found sperm quality is lower in subordinate males when dominance interactions prevent access to females and the energetic costs of aggression are steep, resulting in a "wait to mate" until higher social status can be obtained (Fitzpatrick et al. 2007). We think this explanation is unlikely here because (1) males do not live in stable social groups where females are well defended (2) male growth declines rapidly at sexual maturity preventing predictable transitions in social status with age and (3) aggressive interactions between individuals that often mediate these effects were precluded by a physical barrier (although fish could see and smell each other).

Alternatively, small males might be responding to variation in their perception of sperm competitive ability rather than asymmetries in precopulatory sexual selection. A previous study found sneaker male *X. nigrensis* have more viable and longer lived sperm than courting males (Smith & Ryan 2010), both of which are known to contribute to sperm competitive ability in internal fertilizers (García-González & Simmons 2005; Pizzari et al. 2008b). Differences in sperm competitive ability might have stronger effects on mating roles than male-male competition and female preferences, all of which are known to influence reproductive success in swordtails (Zimmerer & Kallman 1989; Ryan et al. 1990; Gasparini et al. 2010). Disentangling the relative importance of precopulatory and postcopulatory interactions on reproductive success is a major aim of research in sexual selection (Hunt et al. 2009). Our study suggests these episodes of selection might shape plasticity in ejaculate traits, and that the costs and benefits of plasticity differ between male tactics.

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Chapter 3 Opposing effects of sperm viability and velocity on the outcome of sperm competition in the swordtail *Xiphophorus nigrensis*

ABSTRACT

Sperm competition occurs when sperm from more than one male compete for the fertilization of the eggs. Sperm competition is often envisaged as a raffle where the relative contribution of sperm from each male influences the number of fertilizations obtained. When the quality of sperm from competing males differs, however, the raffle is thought to be "loaded" in favor of the male with the higher quality sperm. Here, I use artificial insemination to assess how success in sperm competition in the swordtail *Xiphophorus nigrensis* (Teleostei:Poeciliidae) is predicted by two metrics of sperm quality, sperm viability and sperm velocity. I found males with a greater proportion of living sperm in the ejaculate (sperm viability) sired more offspring in sperm competition. In contrast, sperm velocity was negatively correlated with siring success. The negative relationship between velocity and siring success was only observed in broods that were born weeks after the expected date of parturition, suggesting sperm from competing males were stored prior to fertilization in these females. Sperm velocity thus might increase siring success in a "race to the egg" when eggs are available for fertilization, but as reported here, decrease success siring success when sperm are stored. The evolutionary implications for the evolution of alternative tactics in *X. nigrensis* and tradeoffs between components of ejaculate quality are discussed.

INTRODUCTION

Darwin (1871) first recognized sexual selection was a potent evolutionary force arising from variation among males in their ability to secure mates. It is now widely recognized, however, that sexual selection can continue after mating has terminated because females often mate multiply, resulting in competition among sperm from more than one male for the fertilization of the eggs (Birkhead & Møller 1998). Parker (1970) envisaged sperm competition as being equivalent to a raffle wherein the male possessing the most tickets (e.g. sperm) would outcompete their rivals, and several lines of evidence suggest that the number of sperm produced and ejaculated do evolve in response to sperm competition. Testes size typically increases with the risk of sperm competition among species (reviewed in Birkhead & Møller 1998), increases when polyandry is experimentally enforced in evolving populations (Hosken & Ward 2001; Pitnick et al. 2001; Simmons & Garcia-Gonzalez 2008; but see Crudgington et al. 2009), and the number of sperm inseminated is facultatively increased when a male's perception of sperm competition risk is manipulated (reviewed in Wedell et al. 2002).

Sperm number, however, only explains a portion of the observed variation in paternity (Gage & Morrow 2003; reviewed in Snook 2005; Boschetto et al. 2010) and its importance varies widely across species (Simmons et al. 2003; Gage et al. 2004). An additional factor thought to contribute to the outcome of sperm competition is the quality of the ejaculate. Sperm swimming speed, longevity, and sperm viability (the proportion of living sperm in the ejaculate) may all potentially affect the probability of fertilization by “loading” the raffle toward males with higher quality sperm (reviewed in Snook 2005). Given the enormous diversity in reproductive systems across animals, we might expect the relative importance of different components of sperm quality to vary with the environment under which fertilization occurs. Models for external fertilizers, for example, have proposed the outcome of sperm competition is likely to be influenced by the rate of sperm-egg collisions over a short period of time, and thus predict that sperm velocity should be optimized at the expense of sperm longevity (Parker 1993). In contrast, ejaculate-female interactions (Parker et al. 2010) and sperm survival (Parker 1993) have been modeled with internal fertilization in mind because mating and

fertilization can be spatially and temporally decoupled, particularly in species with sperm storage.

Evidence for this dichotomy is mixed. In fishes with external fertilization, velocity increases with the risk of sperm competition across species (Fitzpatrick et al. 2009) and fast, shorter lived sperm sire more offspring in sperm competition (Gage et al. 2004) as theory predicts. Male x female interactions, however, can also influence sperm velocity via the ovarian fluid released during spawning in external fertilizers (Urbach et al. 2005; Simmons et al. 2009) and not all studies have found the expected relationship between velocity and sperm competitive ability (Stoltz & Neff 2006a; Dziminski et al. 2009). As a consequence, the same metrics of sperm quality do not appear to be equally important across species, even within the same reproductive mode. In internal fertilizers, sperm viability (the proportion of sperm alive in the ejaculate) has been shown to depend on male genotype, interactions with seminal fluid from competing males, and the time elapsed in storage within the female (reviewed in Holman 2009; den Boer et al. 2010). Comparative studies have shown sperm viability is higher in polyandrous compared to monogamous insects (Hunter & Birkhead 2002), suggesting sperm viability might evolve in response to sperm competition in internal fertilizers. Sperm velocity, however, also increases with rates of extra-pair paternity in birds (Kleven et al. 2009). In mammals, sperm size is correlated with velocity (Gomendio & Roldan 2008) and covaries with sperm competition risk across species (Gomendio & Roldan 1991; Anderson & Dixson 2002; Anderson et al. 2005; but see Gage & Freckleton 2003). Although the mechanisms are not well understood, sperm velocity could affect sperm competition in internal fertilizers if (a) higher velocity enables sperm to overcome physical barriers within the female reproductive tract, (b) faster sperm reach the site of sperm storage quickly before being flushed out or destroyed by the female immune system or (c) stored sperm are rarely used to fertilize eggs (Birkhead et al. 1993; Froman 2003; Suarez & Pacey 2006).

The comparative method, experimental evolution, and analyses of plastic responses of the ejaculate across social environments have all been successfully employed to identify associations between variation in sperm quality and the level of sperm competition (Birkhead & Møller 1998; Birkhead et al. 2009). Fewer studies, however, have directly assessed how sperm quality affects siring success. Experiments

are necessary because genetic correlations with other traits important in sperm competition may be responsible for their evolution (reviewed in Simmons & Moore 2009) or the trait may have evolved for other reasons rather than as a consequence of sperm competition *per se*.

Here, I examine the relationship between ejaculate quality and sperm competitive ability in *Xiphophorus nigrensis*, an internally fertilized freshwater fish with alternative male reproductive tactics that differ in sperm quality. Male body size is correlated with mating tactics in *X. nigrensis* such that small males obtain fertilizations by chasing females and forcing the gonopodium, a modified anal fin used to transfer sperm, into the female, whereas intermediate and large males perform courtship displays between copulations (Ryan & Causey 1989). Theory predicts small males that sneak matings should have greater investment in their ejaculates because they have a higher risk of sperm competition (Parker 1990a) and are at a disadvantage in male-male competition and female mate choice (Parker 1990b). In line with these predictions, small male ejaculates contain a higher proportion of living sperm (i.e. they have higher sperm viability) and sperm that is longer lived *in vitro* once activated (Smith & Ryan 2010). In addition, differences in sperm velocity between tactics are revealed when males are exposed to different social environments. Small males exhibit an increase in velocity in the presence of other small males while large males lack this plastic response (Smith & Ryan in press). If sperm competition is the selective force responsible for differences in sperm quality between tactics, variation between males in viability and sperm velocity should affect the outcome of sperm competition.

METHODS

Study population

In the summer of 2008 and 2009, I examined the effects of sperm viability and velocity on siring success using artificial insemination to control sperm number. Males were paired for the sperm competition experiments from three locations: the Nacimiento of the Río Choy (hereafter Nacimiento), Mexico (n=11 pairs, 21 59' 18" N 98 53' 2" W), outdoor stock tanks housing descendents from the Las Palmas population (n=1 pair, 22 0' 51" N 98 52' 17" W) at Brackenridge Field Lab (Austin, TX) and offspring birthed by

females collected from the Nacimiento and raised to sexual maturity in the lab (n=4 pairs). Pairs of males were constructed so they were always competing with a rival from the same source. Males were housed in single-sex groups on a 14L:10H light cycle at the University of Texas at Austin with visual access to females. Fish were fed Tetramin fish flake twice daily and brine shrimp once daily except on the weekends, when they were fed flake and brine shrimp once daily.

Virgin females were obtained by collecting juveniles from Nacimiento populations housed in stock tanks at Brackenridge Field Lab. Fish were raised on the same feeding regime as above except in 2009 fish were given black worms daily starting four weeks prior to the beginning of the experiment to increase fecundity. Female virginity was ensured by removing developing males (identifiable by the maturing gonopodium) from the tanks prior to sexual maturation. Of the 27 artificial inseminations performed in 2008, ten (37%) resulted in offspring. To increase the sample size, seven wild females that had been individually isolated following their collection from the Nacimiento in May 2008 were added to the experiment. Female *Xiphophorus* can store sperm for months and are lecithotrophic, meaning a clutch of eggs is yolked prior to fertilization with no additional nutrient transfer to the offspring during gestation. Parturition of the entire brood occurs approximately 30 days following fertilization in *X. nigrensis*. To maximize the odds that sperm from experimental males fertilized the eggs, wild-caught females were inseminated within three days of the production of their third brood (n=4 females) or fourth brood (n=3 females), the period during which the next clutch is usually fertilized (Siciliano 1972). Offspring from sires other than those used in the experiment were identified using a panel of seven microsatellite loci (see *Paternity analysis*). The multi-locus exclusion probability (the probability of excluding a random individual from the population) of these loci is 99.9% (see Chapter 4), allowing sufficient power to identify offspring sired from stored sperm. Four of the seven wild-caught females produced broods entirely from previous inseminations (38/38 offspring) and three wild caught females produced one or two offspring assigned to males from previous inseminations (4/27 offspring). Removing wild-caught females from the analysis did not alter the outcome of the statistical tests, but they were retained to increase the accuracy of the parameter estimates.

In 2009, all females were virgins raised in the lab and 8/28 (28.5%) produced broods, resulting in a total of 21 successful artificial inseminations from both years. Females producing less than three offspring (2008: n=4 females, 2009: n=1 female) were removed from the analysis, resulting in a final sample of 16 females and 155 offspring (mean ± s.d. offspring per female: 9.6 ± 6.6, range=3-20). Seven of these females produced two broods from which paternity could be analyzed, while the other nine females produced only one brood.

Artificial insemination

I determined the relationship between sperm quality and sperm competitive ability by artificially inseminating the same number of sperm from a small (mean standard length ± s.d.: 24.4 ± 1.5 mm) and a large (mean SL ± s.d.: 34.7 ± 1.7 mm) male into a female (see *Study population*). *Xiphophorus* transfer sperm in bundles (spermatozeugmata) via the gonopodium during copulation. Sperm number in poeciliids can therefore be controlled if the number of sperm per spermatozeugmata is similar between males (Evans et al. 2003b; Evans & Rutstein 2008; Gasparini et al. 2010). In this study, the number of sperm per spermatozeugmata did not differ significantly between male pairs (paired t-test: t_{16}=0.41, p=0.69) and did not predict siring success in the statistical analysis (see *Results*) suggesting it was successfully controlled.

Sperm were collected by placing the male ventral side up in a Petri dish lined with wet cotton. The gonopodium was swung forward and gentle pressure was applied to the testes by massaging the body anterior to posterior with the forefinger to expel the ejaculate. Spermatozeugmata were transferred with a mouth aspirator to a microscope slide containing a drop of sperm extender (248 mM NaCl, 6.5 mM KCl, 1.6 mM CaCl2, 0.59 mM, MgCl2, 0.49 mM MgSO4, 12 mM Tris, pH 7.5, modified from Gardiner 1978) wherein sperm remain dormant until activation.

Ejaculates from each male were partitioned into three aliquots. Two microliters containing ten spermatozeugmata were added to 2µl of sperm extender for artificial insemination, two microliters containing 24-80 spermatozeugmata were added to 12 µl of sperm extender to measure sperm velocity and the number of sperm per bundle, and the rest of the ejaculate was transferred to a third tube for assaying sperm viability. Following

collection of sperm, samples were put on ice until artificial insemination (within 10 minutes of collection) or the sperm quality assays (within 4 hours of collection). Small and large males were processed in alternating order to control for order effects on insemination success. Whether a male was stripped first or second had no significant relationship on siring success (see *Results*).

Females were anesthetized in clove oil (1 drop per 100 ml water) and placed ventral side up on a piece of wet cotton set into a foam cradle. The aliquot of sperm from the two males was activated with 6 µl of 150 mM KCL (Morisawa & Suzuki 1980), gently mixed and inseminated using a mouth aspirator with either a trimmed gel loading pipette tip or a machine-pulled glass pipette at approximately 2 mm penetration depth. Each female was revived and individually isolated in 5 liter aquarium containing a ball of yarn (2009 only) and a sponge filter (2008 and 2009) to provide cover for the female and her offspring. Tanks were checked daily for broods and offspring individually stored in 95% ethanol at -20 degrees C after euthanizing them with an overdose of clove oil

DNA amplification and visualization

DNA was extracted from fish using a standard salt extraction protocol. The PCR cocktail (final volume 10 µL) consisted of 5 µl of 2× Multiplex PCR Master Mix (Qiagen, Valencia, CA) 1ul of the 10x primer set (see *Paternity analysis*) and 1ul of genomic DNA (5-20 ng/µl) for each sample. PCR products were amplified using a 15 minute hot-start activation step followed by 30s at 94°C for denaturation, 35 cycles of 90 s at 57°C (multiplex group 1) or 51°C (multiplex group 2) for annealing and 90s at 72°C for extension and finally 10 minutes at 72°C for the final extension. One µl of multiplexed PCR product was then added to 9 µL of deionized formamide and 0.3 µL GS500 ROX size standard (Applied Biosystems, Carlsbad, CA) and analyzed on an ABI 3730 sequencer. Alleles were scored using GeneMarker 1.7 (Softgenetics, State College, PA).

Paternity analysis

Paternity analysis in 2008 was conducted using Colony 2.0 (Wang 2004; Wang & Santure 2009), a program that uses maximum likelihood to infer parentage using the multi-locus genotypes of the offspring and candidate parents (see appendix for the

program settings used). Population allele frequencies from fish sampled from the Nacimiento (see Chapter 4) were provided to Colony to accurately exclude offspring assigned to males other than those used to artificially inseminate the female. In 2009, all putative sire genotypes were known. Paternity was unambiguously assigned by identifying the maternally inherited allele at each locus and matching the offsprings' paternally inherited allele to the sire.

Sperm number and quality assay

All assays were conducted as in Smith and Ryan (2010) except as noted below. Sperm number and velocity were assessed by gently vortexing the aliquot for two minutes to break apart the spermatozeugmata and adding 12 µl 150 mM KCl (Morisawa & Suzuki 1980) to 4 µl of sample to activate the sperm. One minute post-activation, sperm were pipetted into a disposable Microcell© (MC-20-4, Conception Technologies, San Diego) fixed depth (20µl) counting chamber and videotaped with a Canon XLS camcorder mounted to a Zeiss Axiovert 25 microscope using dark field microscopy. The first two seconds of video were digitized and analyzed with the computer assisted sperm analysis (CASA) plugin for ImageJ (Wilson-Leedy et al. 2007). Sperm number per spermatozeugmata was estimated by multiplying the number of sperm tracked (mean $\pm$ s.d.: 175 $\pm$ 111, range=36-377) by the dilution of the solution and dividing by the number of bundles collected. Average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) are highly correlated in *X. nigerensis* (Smith & Ryan 2010) and gave equivalent results so only VAP is presented here for brevity. Two subsamples of the stripped ejaculate were measured for each male. The average velocity weighted by the number of motile sperm in each subsample was used for analysis.

Sperm viability was evaluated by using the Molecular Probes LIVE/DEAD® Sperm Viability Kit. The proportion of viable sperm was determined by adding 1 µl SYBR®14 nucleic acid stain to 9 µl unactivated sperm solution (final concentration of SYBR®14 = 200nm), incubating sperm for ten minutes at room temperature, and then adding 1 µl propidium iodine (final concentration of propidium iodine = 12 µM) followed by a second ten minute incubation period. Sperm were photographed with a Zeiss Axiocam Mrc camera mounted on a Zeiss AX10 microscope at 100x magnification with Rhod FS15 and GFP FS17 prisms to visualize fluorescent cells. Sperm were counted

with the analyze particles function in ImageJ (mean ± s.d.: 671 ± 458, range=185-1841) and the output compared against bright field images to verify sperm were correctly identified.

Statistics

Statistical analyses were conducted in R v2.12.0 (R Development Core Team 2010). Generalized linear models with binomial errors and a logit-link were used to assess the relationship between siring success and (1) the differences in sperm quality between males (small male – large male) and (2) male tactic. Data were arbitrarily coded from the small males' perspective, with offspring sired by the small male as the number of successes over the total number of offspring in the brood. Diagnostic plots were used to ensure the deviance residuals were randomly distributed over the fitted linear predictors and overdispersion was accounted for by specifying the quasibinomial family, which scales up the standard errors by a dispersion parameter (Faraway 2006). Analysis of deviance was used to assess the significance of predictors and their interactions. Non-significant predictors were sequentially dropped in a backwards stepwise procedure until the final model was reached. To obtain effect sizes, odds ratios and their confidence intervals were estimated from the GLMs (Venables & Ripley 2002; Nakagawa & Cuthill 2007). Generalized estimating equations (Højsgaard et al. 2005) were used to assess whether the siring success of small males varied over successive broods produced by females. An auto-regressive correlation structure and binomial errors were specified with female ID coded as the clustering factor to account for the repeated measures.

RESULTS

Sperm quality and siring success

Males with higher sperm viability compared to their rivals sired a greater proportion of offspring (Figure 3.1, Table 3.1). Differences in sperm number between males did not predict siring success, suggesting sperm number was adequately controlled for by inseminating the same number of spermatozeugmata from each male (Table 3.1).

In contrast to sperm viability, velocity did not predict siring success (Table 3.1) despite up to two-fold differences in velocity between male pairs (mean difference in

VAP (μm/s) ± sd: 4.84 ± 4.67, range = 0.34 - 15.8). The absence of an effect of sperm velocity is therefore unlikely to be due to a lack of variability between males. Most females, however, did not give birth within the ~30 day gestation period expected if mature eggs were present at the time of insemination. Sperm survival within the female rather than a "race to the egg" could thus have determined the outcome of sperm competition if sperm were storage prior to fertilization. Production of the first brood was bimodal (Figure 3.2), with five females producing offspring at 35 days (range=31-42 days), while the other females produced offspring around 60 days (range=49-98 days) following insemination (Fig 3.2a). A bimodal distribution of births following artificial insemination was also reported by Siciliano (1972), who attributed the pattern to variation among females in their position in the oogenesis/egg resorption cycle rather than to differences in gestation time. Environmental conditions known to affect gestation time (e.g. temperature) were controlled in the current study and thus cannot explain the differences in birth dates among females. Nutritional needs of embryos are provided prior to fertilization when the eggs are yolked in *Xiphophorus* (Reznick & Miles 1989), and thus differences among females in the amount of nutrients transferred during gestation are unlikely to explain the bimodal distribution.

If sperm were stored prior to fertilization in some females, the relationship between velocity and siring success might differ depending upon how much time had elapsed between insemination and fertilization. To explore this hypothesis, I tested the interaction between sperm velocity and the time elapsed between insemination and production of the first brood on siring success. The interaction was significant (Binomial GLM: VAP β=0.32, $F_{1,12}$=0.30, p=0.60; day since insemination, β=0.01, $F_{1,12}$=1.23, p=0.29; VAP x day since insemination, β=-0.01, $F_{1,12}$=10.37, p=0.007), indicating the effect of velocity did depend on the time between insemination and fertilization. I decomposed the interaction by partitioning the data into whether the first brood was born early (31-42 days) and late (49-98) using the natural breaks in the frequency distribution (Fig 2) and found sperm velocity was not related to siring success in early broods (GLM: β=0.02, $F_{1,3}$=0.49, p=0.54, Figure 3.2b), although my power to detect an effect was low as few females gave birth early (n=5). In contrast, sperm velocity was negatively related to siring success in broods born late (GLM: β=-0.22, $F_{1,9}$=7.35, p=0.024, n=11, Figure

3.2c), suggesting males with slower swimming sperm had a disadvantage in sperm competition when fertilization was delayed following insemination.

Male tactics and siring success

While differences in sperm quality predicted siring success, sneaker males did not sire a greater proportion of offspring compared to their courting male competitors (mean proportion of offspring sired by sneakers $\pm$ s.d.: 0.53 $\pm$ 0.27; Binomial GLM: intercept z=1.16, p=0.25). This result suggests that sperm quality did not covary with male tactic in this study. Indeed, sperm velocity (ANOVA: tactic $F_{1,30}$=0.48, p=0.64) and sperm viability (Binomial GLM: tactic $F_{1,30}$=0.29, p=0.60) were not significantly higher in sneakers compared to courting males compared to previous studies that reported (1) sperm viability was higher in sneaker males (Smith and Ryan 2010) and (2) velocity was plastic in sneaker males depending upon their social environment.

Smith and Ryan (2010) also found that in addition to higher viability, sneaker sperm was longer-lived *in vitro* compared to courting males. The proportion of offspring sired by small males should therefore increase as more time elapses from insemination to parturition if sperm longevity affects siring success. The siring success of sneaker males, however, did not vary with the time from insemination to parturition (Binomial GEE: Day since insemination, Wald=0.17, p=0.68).

Table 3.1 Effects of sperm number and quality on the proportion of offspring sired by one of the two males. Parameter estimates from the GLM, their odds-ratios, and the analysis of deviance for each term in the model are given. Order refers to whether a male's sperm was stripped first or second prior to artificial insemination.

Generalized Linear Models					
Full model†	β	s.e.	odds ratio (95% CI)	F	p
Sperm number	0.16	0.16	1.17 (0.86,1.62)	0.49	0.50
Sperm velocity (VAP)	0.03	0.02	1.03 (0.98,1.08)	0.79	0.39
Sperm viability	1.99	1.20	7.34 (0.72, 85.4)	1.42	0.26
Order	0.46	0.36	1.58 (0.79, 3.19)	0.84	0.38
Sperm quality only‡					
Sperm velocity (VAP)	0.02	0.02	1.02 (0.98,1.07)	0.39	0.54
Sperm viability	2.93	1.08	18.7 (2.43,173)	4.34	0.058
*Final model**					
Sperm viability	**2.97**	**3.17**	**19.5 (2.56,176)**	**4.78**	**0.046**

† d.f.=1,11 for each term

‡ d.f.=1,13 for each term

* d.f.=1,15 for each term

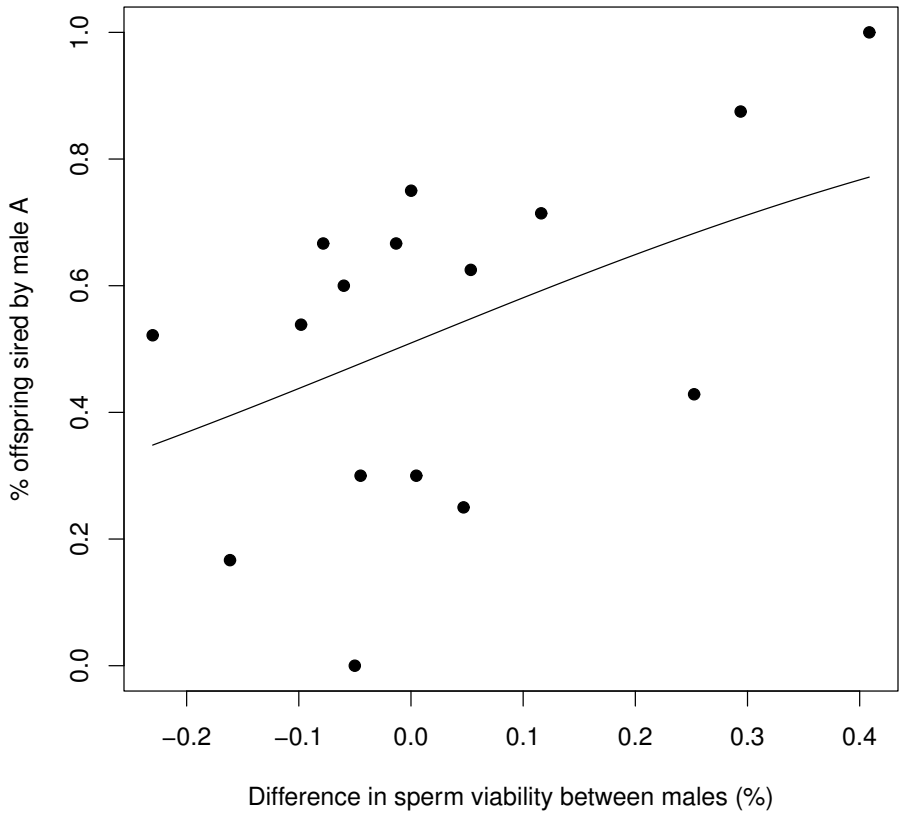

Figure 3.1 Males with superior sperm viability (the proportion of sperm alive in the ejaculate) sire more offspring than their competitor.

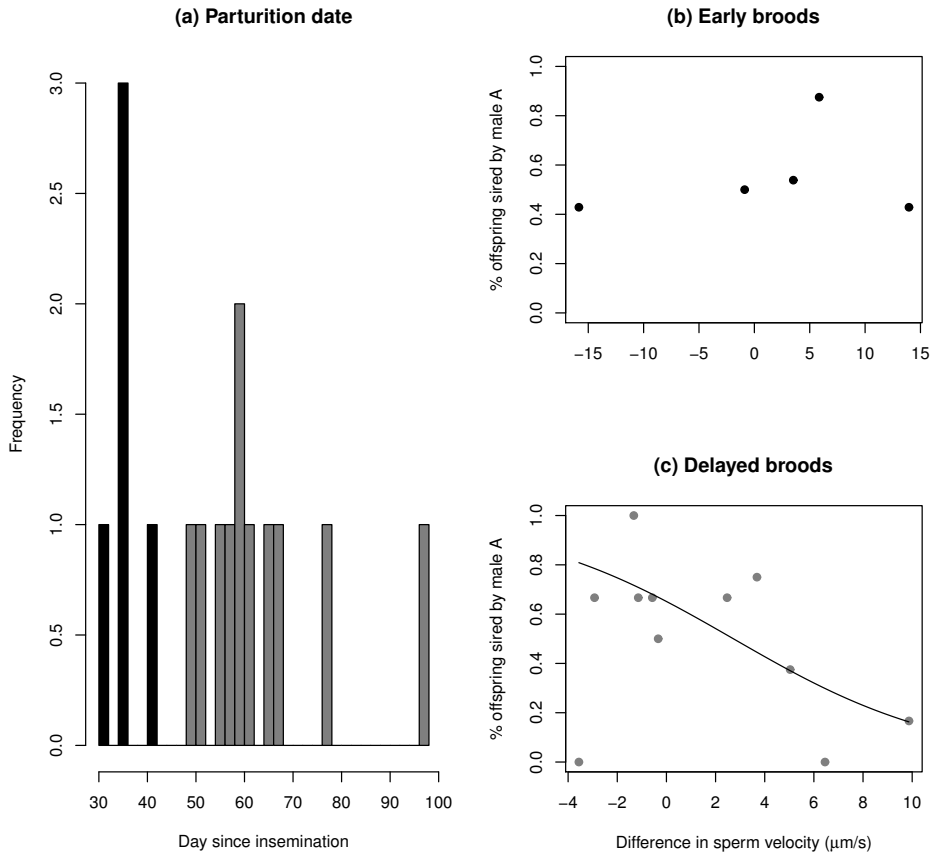

Figure 3.2 The effect of sperm velocity on siring success depends on when females produced their first brood. (a) The date of brood production was bimodal. Broods produced < 42 days ("early") are colored in black, broods produced > 49 days from insemination ("delayed") are in grey. (b) Sperm velocity was unrelated to siring success when females produced their first brood early. (c) Sperm velocity was negatively related to siring success when the production of the first brood was delayed. Sperm in (c) were stored within the female prior to fertilization (see *Results*).

DISCUSSION

Using the comparative method, experimental evolution, and manipulations of the social environment, studies have shown that ejaculate quality is associated with the level of sperm competition (Snook 2005). These approaches are important in identifying putative traits that have evolved in response to sperm competition, but experiments are required to provide definitive evidence that sperm quality influences siring success when two or more males compete. Sperm quality is also a multidimensional trait whose components may have varying effects on the outcome of sperm competition depending upon the species under study. Experiments allow for the relative effects of these components to be directly quantified. Here, I show that males with superior sperm viability sire more offspring in sperm competition when the number of sperm is controlled. In contrast, sperm velocity had a negative effect on siring success when parturition was delayed, suggesting faster sperm are at a disadvantage when they are stored prior to fertilization. The effect of sperm velocity in sperm competition may therefore depend critically on the timing of insemination relative to the reproductive state of the female.

Sperm viability and sperm competition

Sperm viability is a measure of the number of fertilization-capable sperm in the ejaculate. Males with more viable sperm in their ejaculates are therefore likely to have a higher probability of fertilizing the eggs if sperm competition is a numerical raffle. Several lines of evidence suggest sperm viability can evolve in response to sperm competition. Sperm viability is higher in polygamous compared to monogamous insects (Hunter & Birkhead 2002), higher in parasitic males in species with alternative reproductive tactics (Locatello et al. 2007; Smith & Ryan 2010), and is plastic within male crickets depending on the female's mating history (Thomas & Simmons 2007; Simmons et al. 2007b), the number of male competitors (Simmons et al. 2007b) and dominance relationships with other males (Thomas & Simmons 2009). Quantitative genetic studies have also found sperm viability has significant amounts of additive genetic variation (Simmons & Moore 2009) and is genetically correlated with other traits important in sexual selection, including male mating behavior (Evans 2010), visual

(Evans 2010) and acoustic (Simmons et al. 2010) signal characteristics and other components of ejaculate quality (reviewed in Simmons & Moore 2009). These studies show sperm viability has the prerequisites to evolve in response to sperm competition and that the evolutionary dynamics are likely to be complex.

Although sperm viability has been an important metric in assessing fertility for decades (World Health Organization 1999), its effect on the outcome of sperm competition has only been examined in a few species. Sperm viability increases siring success in the field cricket *Teleogryllus oceanus* (García-González & Simmons 2005) and the stalk-eyed fly *Cyrtodiopsis whitei* (Fry & Wilkinson 2004) whereas no effect was reported in Peron's tree frog *Litoria peronii* (Sherman et al. 2008; Sherman et al. 2009). In the current study, the average difference in sperm viability between male pairs was 12%, which translates into a 43% increase in the odds of siring offspring in sperm competition (Table 3.1).

A positive relationship between viability and siring success is of interest because a previous study in *X. nigrensis* found that small males that sneak copulations have higher sperm viability than males that delay maturation and court females (Smith & Ryan 2010). Differences in viability between tactics are therefore much more likely to have arisen in response to sperm competition than as a consequence of selection on other traits with which viability is genetically correlated. Interestingly, I did not find the same difference in viability between tactics in this experiment, and as a consequence there was no effect of tactic *per se* on siring success. Courting males had higher sperm viability in the current study compared to Smith and Ryan (2010), which reduced the difference between tactics in this trait. The most likely explanation for the discrepancy is that environmental differences between studies had an effect on their ejaculate quality. Males collected from the field were maintained in the lab for several months longer than in Smith and Ryan (2010) and males sourced from captive populations are likely to have experienced more benign environments than their field counterparts. Nevertheless, enough variation in viability between males was available to detect a significant effect on siring success. Quantitative genetic studies in *X. nigrensis* are now needed to determine the contribution of genetic and environmental variation to sperm viability, and identify genetic correlations with other traits important in pre- and post-copulatory sexual selection to

predict how these traits evolve under selection.

Sperm velocity and sperm competition

Sperm velocity can positively affect sperm competitive ability in at least two ways. First, sperm competition might be a "race to the egg" if females are frequently inseminated at the time when eggs are mature. In *X. nigrensis*, females receive an average of four mating attempts an hour in the field (Smith unpublished), and therefore it is conceivable that sperm from multiple males could overlap within the oviduct at the time eggs are fertilized. Female cues in poeciliid fish produced during the most fertile period of the reproductive cycle, such as olfactory cues (Crow & Liley 1979; Brett & Grosse 1982), the enlargement of the ventral brood spot as parturition approaches (Peden 1973; Benson & Fox 2007), and increases in female receptive behaviors (Liley 1966; Houde 1997; but see Ramsey et al. 2011), may also promote a race to the egg by inciting male mating behavior. For the race to the egg have important consequences for sperm competition, previously stored sperm must also be at a disadvantage compared to recently inseminated sperm. While sperm senescence within the female is generally expected to result in a decline in the probability of fertilization (reviewed in Pizzari et al. 2008a), sperm from prior matings do have an advantage in sperm competition in some taxa (Birkhead & Møller 1998; Olsson et al. 2009). In another poeciliid, the guppy *Poecilia reticulata*, the second male to mate sires more offspring as long as the delay between matings is sufficiently short (Evans & Magurran 2001; Pitcher et al. 2003), suggesting fresh sperm have an advantage over stored sperm in poeciliids. Second male advantage, however, could also be mediated by female control over the number of sperm transferred in these studies (Pilastro et al. 2002, Pilastro et al. 2004) rather than an effect of storage *per se* on sperm competitive ability. Artificial insemination bypasses female effects on the number of sperm transfer, and studies in *X. helleri* (Gasparini et al. 2010) and guppies (Boschetto et al. 2010) have shown sperm velocity increases siring success when sperm from two males are artificially inseminated. A race to the egg thus may be an important mechanism of sperm competition in poeciliid fish when females are inseminated during the receptive period of their reproductive cycle.

It is difficult to assess the race to the egg mechanism in *X. nigrensis* with the data at hand because 69% of females did not produce offspring within the first gestation

window. The bimodal distribution of births found here and in other studies using similar methods (Siciliano 1972) may result from the cyclical production and reabsorption of eggs or stimulatory effects of sperm on oogenesis following insemination. Other mechanisms, however, do not require a race to the egg for velocity to impact sperm competition. Higher velocity could confer an advantage if it enables sperm to overcome physical and chemical obstacles in the female reproductive tract that reduce the proportion of sperm that reach the site of fertilization (Birkhead et al. 1993; Suarez & Pacey 2006). Froman (2003) proposed fluid movement in the females flushes sperm from the sperm storage organs unless sperm maintain a threshold velocity, resulting in the observed paternity biases between males that vary in sperm quality (Birkhead et al. 1999). Similarly, studies in humans have found that sperm swimming below a threshold velocity do not generate the required force to penetrate mucus residing in the os of the cervix (Mortimer et al. 1986). Finally, sperm unable to quickly reach the site of sperm storage may also be destroyed by non-specific and specific components of the female immune system (reviewed in Suarez & Pacey 2006).

X*iphophorus* sperm are deposited in the female gonoduct and then ascend the female reproductive tract into the ovaries where fertilization takes place. In *X. maculatus*, sperm are stored in association with specialized epithelial cells (SACs), either in deep pockets along ovarian epithelium or after being fully incorporated into the cytoplasm of the SACs where they are presumably sheltered from the female immune system and/or provided nourishment (Potter & Kramer 2000). Higher sperm velocities may confer an advantage in sperm competition if it improves sperm passage through the gonoduct, incorporation into the SACs, or penetration of the membrane separating the ovarian lumen from the matured ova at the time of fertilization (Nagahama 1983). I found a negative relationship between sperm velocity and siring success following sperm storage, suggesting sperm velocity is unlikely to affect sperm competitive ability via the latter two mechanisms. In contrast, these results suggest that any advantage sperm velocity might confer in a race to the egg might trade-off with siring success following sperm storage, for example if faster swimming sperm are short lived.

Studies examining the relationship between sperm velocity and longevity have been primarily conducted in external fertilizers with mixed results, with positive,

negative, and no correlation between these traits depending upon the species (Snook 2005; Rudolfsen et al. 2006). Longer sperm are shorter lived across species of fish (Stockley et al. 1997), which could be taken as evidence for a trade-off if sperm size results in an increase in speed but at the cost of longevity (Ball & Parker 1996; but see Humphries et al. 2008). Studies of trade-offs in sperm performance in internal fertilizers are scarce because of the difficulty in measuring these quantities *in vivo.* Some evidence of a trade-off comes from a comparative study in birds, which found velocity declines with increases in the inter-clutch interval (Kleven et al. 2009), a proxy for the duration of sperm storage. In contrast, *in vitro* measures of sperm mobility (which is correlated with velocity) in domestic fowl are correlated with increased fertilization success in broods before (Birkhead et al. 1999) and after sperm are stored (Pizzari et al. 2008b), indicating velocity and longevity are positively related. In *X. nigrensis*, velocity was unrelated to longevity *in vitro* (Smith, unpublished), however whether this is the case *in vivo* has yet to be determined. The variable relationship between velocity and longevity across studies is perhaps not that surprising considering these are only two traits among several between which trade-offs can potentially occur. Individual condition may also affect the correlation between ejaculate traits, for example males in higher condition may be able to simultaneously invest in multiple traits simultaneously. Comparative studies that assay multiple sperm phenotypes or within-species studies that can measure condition are needed to further our understanding on how tradeoffs affect sperm performance.

In conclusion, I found that sperm viability and sperm velocity have opposing effects on the outcome of sperm competition. Because sperm viability increases success in sperm competition, selection should favor adaptations that increase sperm survival before and after ejaculation, for example by increasing the efficiency of spermatogenesis, enhancing protection of sperm from oxidative damage and producing compounds in the seminal fluid that protect sperm within the female reproductive tract (Pizzari et al. 2008a). Selection on sperm velocity may be more complicated if faster swimming sperm are beneficial in some contexts but not others. Species where the duration of sperm storage is short, for example, are more likely to evolve faster swimming sperm if velocity and longevity trade-off (Kleven et al. 2009). A comparative study examining sperm velocity in poeciliids with superfetation, where multiple clutches of different stages of

development overlap, and those without superfetation (e.g. *Xiphophorus*) would be an interesting test of this hypothesis. Plasticity in sperm velocity may also evolve if environmental cues provide information about the likelihood a male's sperm will be stored. In *X. nigrensis,* small sneaker males produce faster swimming sperm in the presence of other small males compared to large males (Smith & Ryan in press). Female *X. nigrensis* prefer large males (Ryan et al. 1990) and large males restrict mating opportunities for small males (Morris et al. 1992), thus it is possible that differences in the competitive environment affect the probability sperm are stored. Female reproductive state may also be crucial to whether a race to the egg or sperm storage is more likely and affects spermatogenesis (reviewed in Wedell et al. 2002) and sperm quality (Thomas & Simmons 2007) in other taxa. Similar studies in poeciliid fish have yet to be conducted.

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Chapter 4 Polyandry and paternity in a wild population of the swordtail *Xiphophorus nigrensis*

ABSTRACT

Genetic analyses of parentage in wild populations have revealed that polyandry is a common feature of mating systems with important consequences for sexual selection. I used a panel of seven microsatellite loci to determine the frequency of multiply sired broods, the number of sires, and reproductive skew within broods collected from female *Xipophorus nigrensis,* a livebearing poeciliid fish. A subset of broods was also raised to sexual maturity to estimate the size of each sire from their sons. This is possible in *Xiphophorus* because Y-linked genetic variation has strong effects on male size at sexual maturity and male growth dramatically slows after puberty. I found 61% of broods were sired by 2-4 males and exhibited high reproductive skew among males, with an average of 70% of the offspring sired by one male in the brood. Female size was not related to the number of sires, but sire number was positively related to fecundity, suggesting polyandry may be beneficial to females. While previous studies have shown large male body size is favored by precopulatory sexual selection, larger males did not garner a greater share of paternity within broods as would be expected if they transfer more sperm during copulation. Larger males were also just as likely to sire offspring in singly compared to multiply sired broods, suggesting male size does not affect the number of males that successfully inseminate females. Implications for postcopulatory sexual selection and the evolution of polyandry are discussed.

INTRODUCTION

Polyandry, when females mate with more than one male within the same reproductive cycle, has profound effects on the evolution of animals. An immediate consequence of polyandry is the opportunity for postcopulatory sexual selection, which results from the overlap of ejaculates from more than one male and competition among sperm for the fertilization of the eggs. Postcopulatory sexual selection has potent effects on the evolution of behavioral, anatomical, and physiological traits that enhance male fertilization success (Birkhead & Møller 1998) and female traits that bias the success of competing ejaculates (Eberhard 1996). Polyandry also can result in sexual conflict because sperm competition can only decrease a male's share of paternity. Male traits that increase paternity share but exact costs on the females with which they mate can therefore be favored, resulting in a co-evolutionary arms race of adaptations and counter-adaptations between the sexes (Arnqvist & Rowe 2005). Our understanding of the extent to which postcopulatory sexual selection, sexual conflict, and a host of other evolutionary phenomena related to polyandry (Westneat & Sherman 1993; Zeh & Zeh 1996; Hughes et al. 2008; Price et al. 2010) affect evolution in any particular system, however, is dependent on assessing its prevalence in the wild. The advent of molecular tools for assessing relatedness has only recently made this information available, revolutionizing the study of mating systems and sexual selection (e.g. Griffith et al. 2002; Avise et al. 2002).

A major finding from studies of parentage in the wild is the occurrence of mismatches between male mating success and reproductive success, indicating that postcopulatory sexual selection can have important fitness consequences in natural populations. For example, field studies in insects using molecular markers have found the number of males represented in female sperm stores and the number of males that actually sire offspring are decoupled in some species but not others (Bretman & Tregenza 2005; Simmons et al. 2007a; Simmons & Beveridge 2010). Similarly, dominant male Soay sheep obtain more matings than their subordinate counterparts but become sperm-depleted late in the season, equalizing reproductive success among males (Preston et al. 2001). Parentage analyses also provide information on reproductive skew among males that successfully mate. When sperm are equally mixed within the female reproductive

tract, sperm competition can be a “fair raffle” wherein males sire offspring in proportion to the number of sperm in competition (Parker 1990b; Colegrave et al. 1995). In many species, however, the raffle is “loaded” because of variation among males in the quality of the sperm inseminated and how sperm are processed and stored in the female (Birkhead & Møller 1998; Snook 2005). Second male sperm precedence, for example, can result when sperm from previous males are displaced or ejected (Pattarini et al. 2006; Manier et al. 2010), generating a skew in paternity toward the second male. Skew thus can result from variance among males in the number of sperm they inseminate and/or a strong effect of sperm precedence on siring success, while low skew indicates postcopulatory sexual selection is relatively weak, with paternity equally distributed among males.

Why females mate with multiple males has been a puzzle in evolutionary biology because in theory all the eggs could be fertilized by sperm from one male. While ecological factors such as population density, sex ratio, or the distribution of resources may affect the opportunity for polyandry to occur (Emlen & Oring 1977; Shuster & Wade 2003), empirical and theoretical work has suggested polyandry may evolve because it is adaptive for females (Jennions et al. 2000; Zeh & Zeh 2003; Simmons 2005). This hypothesis is a direct challenge to a long-held assertion that, unlike males, female fitness depends on the number of gametes they produce rather than the number of mates they obtain (Bateman 1948; Trivers 1972; Shuster & Wade 2003). Jones' (2002) analysis of parentage in rough-skinned newts provided the first direct evidence that Bateman's principles held in wild populations (Birkhead & Møller 1998), but the extent to which they apply equally to all taxa is controversial (Dewsbury 2005; Tang-Martinez & Ryder 2005; Snyder & Gowaty 2007). Females in many species, for example, actively solicit copulations from multiple males and some studies have shown polyandrous females have higher reproductive success compared to when females are monogamous (Newcomer et al. 1999; Evans & Magurran 2000; Worden & Parker 2001; reviewed in Tang-Martinez & Ryder 2005). At the other extreme, sexual conflict can result in a decline in female fitness when the number of partners increases due to the cost incurred from superfluous mating (Rice 1996; Wigby & Chapman 2004; Arnqvist & Rowe 2005). The benefits of polyandry are thus likely to vary among species depending upon ecological and evolutionary forces

that influence the costs and benefits of mating.

Poeciliid fishes are well suited to studies of parentage in wild populations because they are live-bearing, allowing for entire broods to be collected from gravid females. Polyandry is expected to be common in poeciliid fish because males provide no additional reproductive investment besides sperm and ecological conditions typically favor scramble competition for females, resulting in a promiscuous mating system. The frequency of multiply sired broods in poeciliid fish ranges widely between species and populations (Evans & Pilastro 2011), but can exceed 90% with up to 9 fathers reported in some cases (Zane et al. 1999; Neff et al. 2008). The role females play in facilitating polyandry and its fitness consequences are a topic of debate. Studies in guppies show females prefer novel males (Eakley & Houde 2004) and re-mate more often with males that are more attractive than their previous mates (Pitcher et al. 2003). Multiply mated females also appear to benefit directly from polyandry, producing larger broods at shorter intervals than monogamous females (Evans & Magurran 2000). In contrast, high mating rates in poeciliid fish have also been suggested to result in lower female fitness due to reduced female foraging efficiency (Magurran & Seghers 1994; Schlupp et al. 2001; Pilastro et al. 2003), greater conspicuousness to predators (Pocklington & Dill 1995) or damage to the female reproductive tract sustained from male genitalia during copulation (Deaton, unpublished).

The poeciliid swordtail *Xiphophorus nigrensis* is an internally fertilized, livebearing fish endemic to the Río Choy in the Huasteca region of San Luis Potosí state of Mexico. Males and females forage in unstable social assemblages along the stream bottom where females receive frequent copulation attempts from multiple males (4 copulation attempts per hour, Smith unpublished). Like other poeciliid fish, females can store sperm for months and thus the opportunity for ejaculates from multiple males to overlap within the reproductive tract is high. *X. nigrensis* has been a model system for the study of sexual selection due to a unique genetic system that influences body size in males, an important trait in male-male competition and female mate choice in animals (Andersson 1994; Ryan & Rosenthal 2001). Kallman (1989) first reported the strong influence of a Y-linked factor on male size at sexual maturity in *X. nigrensis*, which has recently been attributed to copy number variation in a gene for the Mc4r melanocortin

receptor (Lampert et al. 2010). Following the onset of sexual maturity, male growth rapidly declines in *X. nigrensis*, leading to fixed differences in male size in natural populations (Ryan et al. 1990). This attracted the attention of Ryan and Causey (1989), who observed a correlation between male size and male mating tactics. Small males chase females and rapidly thrust the male intromittent organ (the gonopodium) into the female gonopore to obtain fertilizations while intermediate and large-sized males copulate with females between courtship bouts, which increase in frequency and duration with male size. Recently, variation between tactics in traits important in postcopulatory sexual selection have also been shown to correlate with male body size: small male ejaculates have a greater proportion of living sperm, sperm that is longer lived following activation (Smith & Ryan 2010), and plasticity in sperm velocity across different socio-sexual environments (Smith & Ryan in press)

Here, I present an analysis of parentage in a wild population of *X. nigrensis.* The aims of the study are four-fold. First, the frequency of multiply sired broods and the number sires per brood provide an estimate of the opportunity for postcopulatory sexual selection. This information is useful because theory predicts that male responses to sperm competition are sensitive to these parameters (Parker & Pizzari 2010). Second, previous research has shown that larger males win more agonistic encounters (Morris et al. 1992), are preferred by females in dichotomous choice tests (Ryan et al. 1990), and sire more offspring in the field than expected given their frequency (Ryan et al. 1990). If sexual selection is strong, unequal distributions of paternity within female broods should be common, particularly in broods produced by large male sires. Finally, female fecundity and the number of sires will be positively related if polyandry is beneficial to females. In poeciliid fish, males only provide sperm to females and thus any fecundity benefit of polyandry is likely to result from an increase in the probability the eggs are fertilized or from the increased likelihood of genetic compatibilities that enhance fertilization success or embryo survival (reviewed in Ridley 1988; Jennions et al. 2000; Tregenza & Wedell 2000). Alternatively, the relationship between sire number and fecundity will be negative if sexual conflict over mating negatively impacts female fitness (Arnqvist & Rowe 2005). The lack of a relationship, in contrast, would provide support for Bateman's (1948) assertion that variance in female fitness is unrelated to the number of mates.

METHODS

Study population

In May 2008, 40 females were collected with a seine from the Nacimiento Río Choy, Mexico (21° 59' 18" N 98° 53' 2" W), transported to the University of Texas at Austin and individually isolated in 5L containers. Containers were checked daily over 24 weeks for offspring, which were euthanized in an overdose of clove oil and individually preserved in 95% ethanol until DNA extraction. A sponge filter in the container provided filtration and a refuge for offspring to reduce cannibalism. One female died before producing a brood, five females did not produce any offspring and three females produced less than three offspring and were excluded from the analysis, resulting in a total of 453 offspring from 31 females (mean $\pm$ s.d.: 14.3 $\pm$ 7.3 offspring per female, range 3-31). At the conclusion of the experiment, female standard length was measured with dial calipers (mean $\pm$ s.d: 31.3 $\pm$ 1.6 mm, range=28.5-34.8) and a fin clip taken for genotyping.

The first brood produced by 25 females was raised to sexual maturity in indoor aquaria at Brackenridge Field Laboratory (Austin, TX) to estimate the body size of the sires. In total, 103 males and 109 females were raised over a 16 month period. Mortality in the rearing tanks was low for most families (mean proportion surviving per brood $\pm$ s.d: 0.91 $\pm$ 0.11, range=0.62-1.0). Twenty eight males from ten broods did not complete sexual maturity before the fish had to be harvested and were excluded from the calculation of sire size. Standard length (tip of the mouth to the end of the caudal peduncle) of male offspring was measured from digital photographs using ImageJ. Standard lengths from males identified as descendents from the same father by parentage analysis (see below) were then averaged to obtain an estimate of their father's body size. Sword length was not measured because sword development was still in its early stages for many males at the time the fish were collected.

A complication in inferring sire size from offspring size in *X. nigrensis* is the presence of an autosomal sex determination factor in the population that produces small homogametic (XX) males (Kallman 1989; Ryan et al. 1990; Lampert et al. 2010). Sire body size cannot be inferred from XX sons because XX males lack a Y chromosome, and thus will mature small regardless of their father's Y-linked P-locus genotype. XX males were identified in two ways. First, the caudal fin of small males (< 26 mm) was examined

for yellow caudal fin pigmentation, which should be absent in XX males because this trait is determined by a Y-linked factor in XY males (Kallman 1984). Second, each paternal sibship was plotted and small male outliers in families with otherwise large males were identified. XX males identified by these criteria were removed when calculating the average standard length of the sire for each patriline. In practice, only three families had small males, two of which contained XX males using the above criteria (see *Results*).

All fish were kept on a 14L:10D light cycle and fed Tetramin fish flake twice daily and fresh brine shrimp once daily, except for weekends when they were fed flake and brine shrimp once daily.

DNA amplification

Genomic DNA was extracted by boiling ground tissue in 100ul of lysis solution (100 mM NaCl and 0.5% sarcosyl) for 15 minutes, adding 100 ul of 20% Chelex and boiling for 15 minutes more and then centrifuging for 10 minutes. Eight microsatellite loci from Seckinger et al. (2002) and four loci previously screen by the *Xipophorus* Genetic Stock Center (Texas State University, San Marcos) were examined to assess their suitability for parentage analysis. Of the twelve loci, five were not used due to low levels of genetic variation (KonD6, KonD8, KonD26, and KonD29) or inconsistent amplification (KonT30). The remaining seven microsatellite loci were combined into two multiplex groups (Table 1) using the program "multiplx" (Kaplinski et al. 2005) after designing optimal primers with Primer3 (Rosen & Skaletsky 2000). The PCR cocktail (final volume 10 µL) consisted of 5 µl of 2× Multiplex PCR Master Mix (Qiagen, Valencia, CA), 1 µl of the 10x primer set, 3 µl of DNAase free water and 1 µl of genomic DNA (5-20 ng/µl) for each sample. PCR products were amplified using a 15 minute hot-start activation step followed by 30 s at 94°C for denaturation, 35 cycles of 90 s at 57°C (multiplex group 1) or 51°C (multiplex group 2) for annealing and 90 s at 72°C for extension. A final extension of 10 minutes at 72°C terminated the program. To visualize the PCR products, one µl of multiplexed PCR reaction was added to 9 µL of deionized formamide and 0.3 µL GS500 ROX size standard (Applied Biosystems, Carlsbad, CA) and analyzed on an ABI 3730 sequencer. Alleles were scored using GeneMarker 1.7 (Softgenetics, State College, PA).

Paternity analysis

Colony 2.0 (Wang 2004; Wang & Santure 2009) uses maximum likelihood to infer parentage from the multi-locus genotypes of offspring and candidate parents. Colony also incorporates the error rate due to mutation, allelic dropout, and mistyping into the maximum likelihood calculation. Wang (2004) showed that parentage assignment was robust to a large range of user-defined error rates, and thus the error rate was set to 0.01 using Wang (2004) as a guideline. Siblings and their known mothers were specified to provide additional power for identifying the number and genotypes of putative fathers. Males were permitted to sire offspring with multiple females in the sample. Exclusion probabilities and the frequency of null alleles were calculated with Cervus 2.0 (Kalinowski et al. 2007), while heterozygosity and deviations of loci from H-W equilibrium were calculated using GENEPOP 4.0 (Rousset 2008). The probability of detecting multiple mating was calculated using PrDM (Neff & Pitcher 2002), which incorporates information about the allele frequencies, the number of offspring analyzed, and the paternity skew among sires. Two males and 0.67/0.33 split in paternity were used as parameters for skew as these were representative of the dataset.

Statistics

Statistical analyses were performed in R v.2.12.2 (R Development Core Team 2010). Reproductive skew for each brood was calculated using the skew index (*B*) (Nonacs 2000), here defined as the observed binomial variance in siring success minus the expected binomial variance due to chance sampling of males in the fertilization set. Negative values of *B* indicate paternity was more evenly distributed than expected by chance and positive values indicate paternity was unequally distributed among males. A one-sample Wilcoxon signed-rank test was used to determine whether *B* is significantly different from zero, while Spearman's rank correlation was used to examine relationships between *B,* sire size, and female size. Changes in *B* over sequential broods produced by individual females were assessed using a linear mixed model with brood number as the fixed factor and female identity as a random effect to account for repeated measurements.

Count data were analyzed by fitting the data to a generalized linear model (GLM) with a Poisson error distribution and a log-link. Model assumptions were checked by examining plots of the deviance residuals against the fitted values. Overdispersion was

corrected where necessary by scaling the standard errors of the regression coefficients by a dispersion factor (family=quasipoisson). Hypothesis tests for overdispersed data were conducting by comparing the deviance of full and reduced models using *F*-tests (Faraway 2006). Otherwise, a χ^2 test was used to test for significant reductions in the deviance between models (Faraway 2006). Binary data were similarly analyzed with a binomial GLM and a logit-link.

Some males sired offspring in the broods of more than one female. As a consequence, generalized linear mixed models (GLMM) were used for analyses examining the relationship between male reproductive success or the number of sires per brood (dependent variables) and sire size (the independent variable). Male ID was coded as a random effect to account for the repeated measures. For male reproductive success, only the first brood females produced was analyzed.

If polyandry is adaptive for females, a positive relationship between the number of mates and fecundity is expected. Because the number of mates is rarely known in studies of wild populations, however, the number of sires is often used as a proxy. Unfortunately, a positive correlation between the number of sires and fecundity can be generated by chance simply because more males in the fertilization set are likely to be sampled in larger broods. I examined this null expectation by simulating sperm competition under a fair raffle, where males sire offspring in proportion to the number of sperm they have in competition. I took the observed number of offspring that females produced and randomly assigned them to a set of sires from the original dataset, simulating matings between females and between 1 and 4 males. The number of males producing offspring in each brood was then determined probabilistically by drawing from the binomial distribution based on their reproductive skew in the original data set. To illustrate, suppose a female producing five offspring is randomly assigned to a set of two males that sired 80% and 20% of the offspring in the original data set. The number of "coin flips" is five and the probability of "success" is 80% and 20% for male 1 and male 2. Females with smaller broods thus may produce offspring from only one male by chance, while females with larger broods will almost always sample all the males in the fertilization set because there are more opportunities for the sperm of all males to fertilize the eggs.

The simulated data were then fit to a generalized linear model and the χ^2 statistic for brood size on sire number was calculated, including female size as a covariate to control for female size effects on fecundity as done in the analysis of the actual data. This was repeated 1000 times to generate a sampling distribution against which the χ^2 from the observed data could be compared.

All statistical tests were two-tailed. When multiple comparisons were made, Type I error was controlled using the False Discovery Rate (FDR) (Benjamini & Yekutieli 2001). Effect sizes for GLMs were calculated by taking the exponential of the slope to get the odds-ratio (Faraway 2006). Confidence intervals for effect sizes were obtained using the confint function in the MASS package (Venables & Ripley 2002).

RESULTS

Microsatellites

Full descriptions of population genetic parameters for the microsatellites are presented in Table 1. Mean heterozygosity was high (0.784) and no significant deviations from H-W equilibrium were detected for any of the seven loci. The high degree of polymorphism and low frequency of null alleles resulted in high power for assessing paternity: the combined probability of excluding a random male from parentage was 0.999, with only four pairs of offspring in the sample having the same multilocus genotypes. Three of these pairs were full siblings from the same mother while the fourth pair were half siblings from different mothers but inferred as originating from the same father. The average probability of detecting multiple mating in the sample was 0.996, ranging from 0.58 – 1.0 upon the number of offspring females produced.

Sixteen offspring were identified by Colony as potentially having typing errors. Nine of these cases were identified as scoring errors, seven occurring in loci with dinucleotide repeats (msa066, konD21, konD15) where distinguishing heterozygotes with alleles of similar size can lead to mistyping. The two remaining scoring errors were due to dropout of large alleles at locus msc036. In three cases, offspring mismatched the most likely father at one locus (msd020). Colony assigned these offspring to a father shared by other siblings rather than to a unique father as this was the most likely configuration given consistency in parentage at the other loci. The remaining four cases appear to be

due to *de novo* mutations as maternal and offspring genotypes differed by one tandem repeat (two instances in msc036, one instance in msd042 and one instance in msd020).

Paternity analysis

Of the 31 females analyzed, 61.3% produced broods fathered by 2-4 sires (mean number of sires ± sd: 1.87 ± 0.85) and 17 females produced additional broods following the first (mean ± s.d. number of broods: 3.47 ± 0.64, range=2-4), which could only be fertilized by stored sperm as females were sexually isolated. Paternity was significantly skewed within females [median *B* index (95% CI), 0.11 (0.025,0.22); Wilcoxon signed rank test, p=0.008), Figure 4.1], with the majority male siring an average of 70% (range=44-96%), the second male siring 26% (range=5-50%), the third male siring 8% (range 4-14%) and the fourth male siring 9% (one brood only) of the offspring. Skew did not vary over the successive broods produced by each female (GLMM: β=0.04 ± 0.025, χ^2=2.89, p=0.09), suggesting that the proportion of offspring sired by each male did not change as the duration of time sperm were in storage increased. Skew, however, is calculated without respect to the identity of the male obtaining the largest paternity share. Although it is unlikely, similar values of skew from one brood to the next could be due a complete reversal of paternity between males rather than each male siring the same proportion of offspring from brood to brood. To examine this, deviations in the number of offspring sired by the male with the highest paternity share was examined using a χ^2 test. The proportion of offspring sired by the majority male did not significantly deviate from brood to brood for any of the females examined ($p > 0.05$), indicating that consistent skew resulted from males siring a similar proportion of offspring from one brood to the next as would be expected in a fair raffle. Only broods that had at least four offspring (mean number of offspring per brood ± s.d.= 7.8 ± 3.8, n=10 females) were considered in all analyses to minimize unreliable estimates of skew due to stochastic sampling of male sperm. Nevertheless, these results should be interpreted cautiously as brood sizes declined after the first brood was produced (mean size of the first brood ± s.d.: 11.4 ± 3.4, mean size of subsequent broods ± s.d.: 5.4 ± 1.35) and skew was already high, making further increases more difficult to detect.

Colony reconstructed 43 sires, nine of which sired offspring in more than one female (range: 1-4 females). Of the broods where males were raised to sexual maturity,

26 of the 31 sires inferred by Colony were represented in male offspring. The remaining five sires produced only females and thus estimates of sire size were unavailable, however these five sires contributed a small number of offspring (10/216 or 4.6%) to the sample and thus are unlikely to affect the results. Estimates of sire size were calculated from 2.84 $\pm$ 1.68 (range: 1-7) offspring per sire (Fig. 4.2). XX males were identified in two families and removed from the estimate of sire size (see *Methods,* Fig. 4.2). A third family had four small males that were clearly XY based on the presence of yellow coloration on the caudal fin but were assigned to a paternal sib group with a much larger male from the same brood (Fig. 4.2). This sib group was sufficiently large (n=5) such that removing the larger male had little effect on the estimated mean sire size. Using the median sire size rather than the mean did not change the outcome of the statistical tests.

Male reproductive success

There was no relationship between male size and whether offspring occurred in multiply or singly sired broods (binomial GLMM: β=0.04 $\pm$ 0.15, χ^2=0.88, p=0.99), the number of co-sires per brood (Poisson GLMM: β=0.02 $\pm$ 0.05, χ^2=0.21, p=0.65) or the total number of offspring he sired (Poisson GLMM: β=0.06 $\pm$ 0.05, χ^2=1.33, p=0.25). Reproductive skew was uncorrelated with the size of the largest male in the brood (Spearman's r=0.37, p=0.14) nor with the variance in male size in the brood (Spearman's r=0.29, p=0.41). There was no evidence of assortative mating by body size: the average sire size and the female size were uncorrelated (Pearson's r=0.04, p=0.87). For offspring who were mated multiple, female size was uncorrelated with the size of the sire who produced the most offspring (Pearson's r=0.06, p=0.93).

Female reproductive success

Female fecundity increased 19.7% (95% CI: 2.0%, 40.2%) for each additional sire when examining the first brood (Poisson GLM: number of sires, β=0.18 $\pm$ 0.08, χ^2=4.84, p=0.02; covariates: female standard length, β=0.19 $\pm$ 0.04, χ^2=24.6, p<0.001, Figure 3) or when all broods were considered (GLM: number of sires, β=0.16 $\pm$ 0.07, χ^2=5.1, p=0.02; covariates: female standard length, β=0.15 $\pm$ 0.03, χ^2=20.4, p<0.001, number of broods produced, β=0.26 $\pm$ 0.04, χ^2=41.8, p<0.001). The positive relationship between fecundity and sire number was not due to chance sampling of fewer sires in smaller

broods and more sires in larger broods, as the χ^2 for sire number was highly unlikely to be observed by chance when compared against the simulation's sampling distribution (p=0.001, see *Statistical analysis*). Female size was unrelated to the number of sires (Poisson GLM: β=0.04 $\pm$ 0.08, χ^2=0.35, p=0.55), and females with more sires did not produce more broods (Poisson GLM: β=0.01 $\pm$ 0.016, χ^2=0.008, p=0.93).

Table 4.1 Description of microsatellite loci. bp=range of allele lengths, mplex=multiplex group, n=number of fish genotyped, $N_{alleles}$ = number of alleles, H_{obs} = observed heterozygosity, H_{exp} = expected heterozygosity, P_{excl} = exclusion probability, F_{null} = frequency of null alleles.

Locus	GenBank	Repeat	Primers	bp	mplex	n	$N_{alleles}$	H_{obs}	H_{exp}	P_{excl}	F_{null}
Msa066	AY258678	(gt)	CACCTGTTGCTGTACCCTCT	185-199	1	50	6	0.72	0.72	0.46	-0.002
			AGTCTCTCACAGCAATGCCTGA								
KonD15	AF368429	(ac)	CATCCAGCCTGCTTAGTGAG	247-255	1	48	3	0.38	0.42	0.22	0.049
			TGTTCGTCATTAATTTGCAG								
Msd042	AY258752	(ctac)	CTGCCCTACTGTGGTGAGTTACC	267-356	1	50	20	0.94	0.90	0.78	-0.031
			TGAAAACAGTCAACCTTGTGCTAAT								
KonT38	AF368434	(tta)	CGACGTGTAGAAACTGAGTA	151-191	1	50	14	0.94	0.90	0.79	-0.025
			CTCTATTCCTGGTTTGACAT								
msd020	AY258886	(gata)	AGTCGATCACAGAGCAAAATGA	185-187	2	66	21	0.92	0.94	0.86	0.003
			CAGCTAAGTTTCCTGGCAGA								
konD21	AF368430	(ac)	TCATCTGGAGCAGGCACATG	119-140	2	67	6	0.75	0.68	0.46	-0.046
			AGTCCTGTCACCCTGCGT								
msc036	N/A	(tgat-ctat)	TTTGGTAATTGCCGTGACATC	236-400	2	67	29	0.96	0.92	0.84	-0.021
			CAGGCGCCGATGACAT								

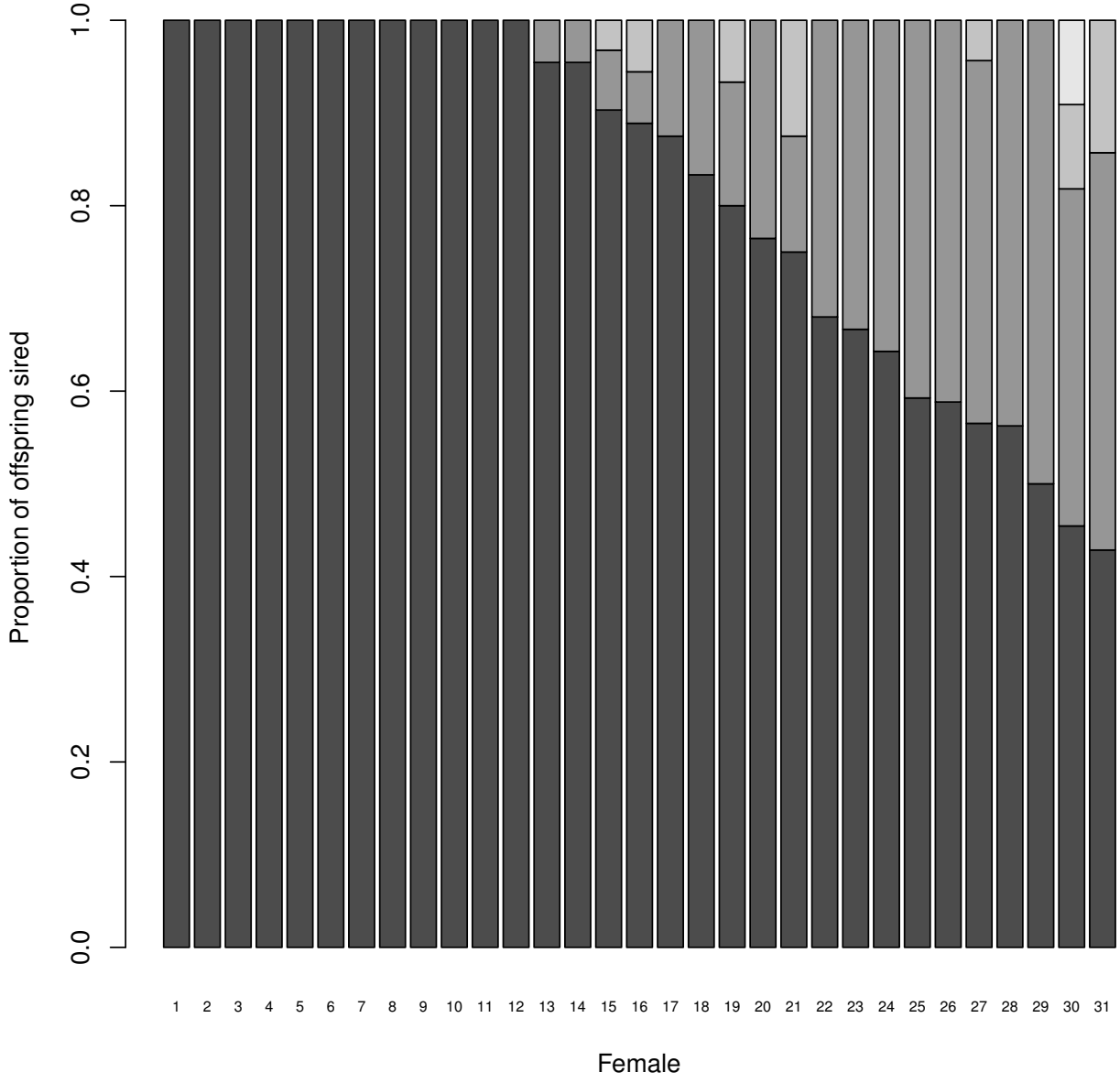

Figure 4.1 Distribution of paternity within female broods. Different shadings represent unique sires within a brood. The probability of detecting multiple sires in females where only one sire was detected ranged from 0.584 – 0.997 (mean + s.d. = 0.90 $\pm$ 0.12).

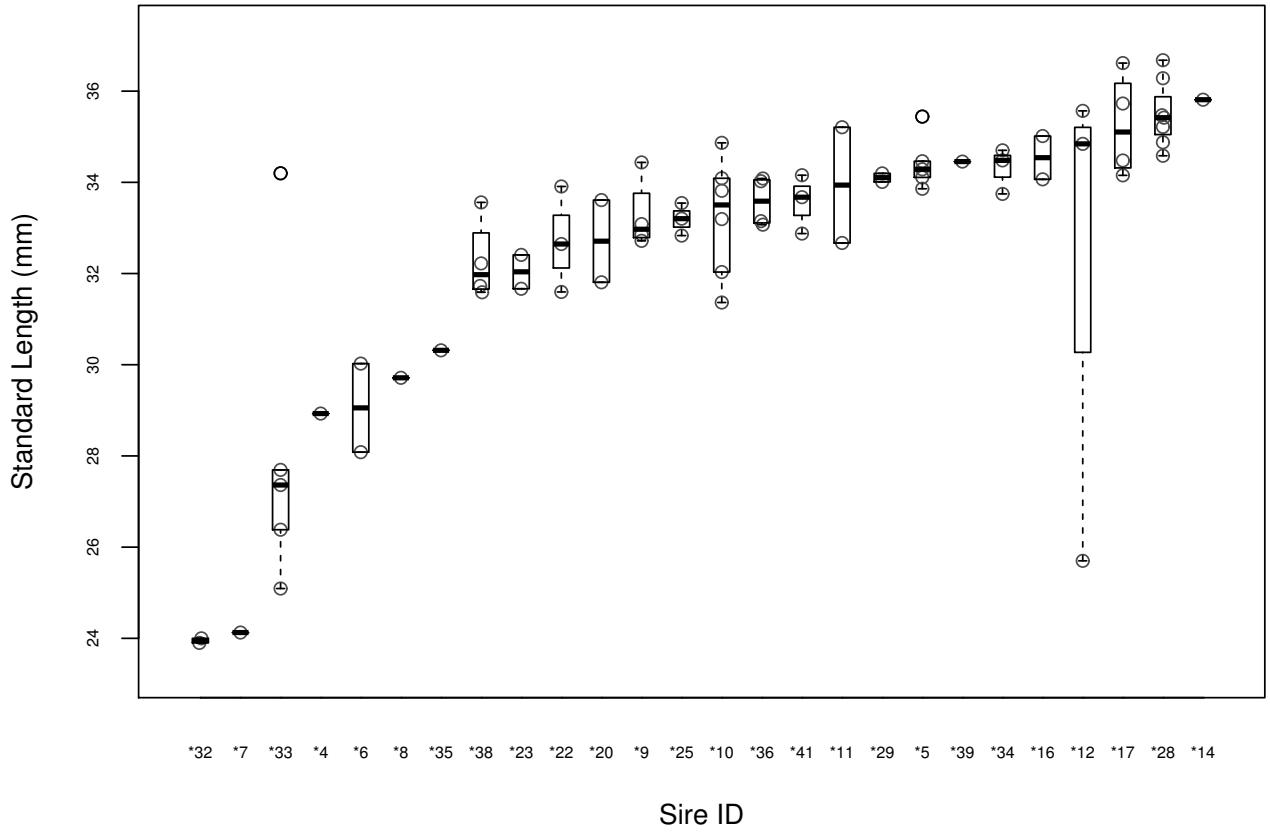

Figure 4.2 Box plots of the standard length of all males raised to sexual maturity for each patriline. Male offspring sired by *32 and the outlier for sire *12 were determined to be XX and excluded when average sire size was calculated. The outlier for sire *33 was assigned as a full sib to the small XY males in that family despite their drastic differences in size. All offspring from sire *7 were classified as XY. Sires *5, *8, *9, *10, *11, and *20 sired offspring from more than one female.

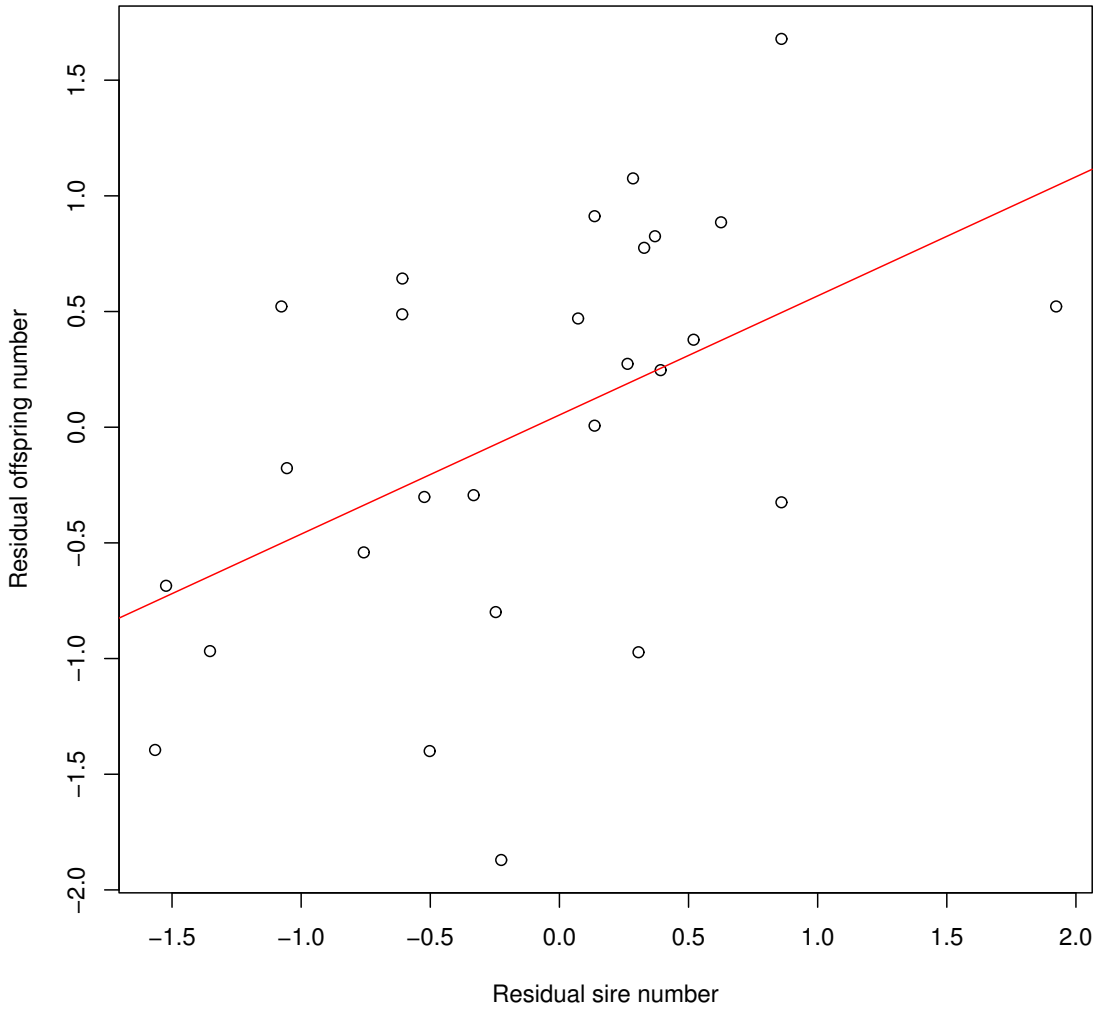

Figure 4.3 Added-variable plot showing the size of the first brood increases with the number of sires. Residuals of fecundity are plotted on residuals of sire number after the effect of female size on fecundity was removed.

DISCUSSION

Using a panel of seven microsatellite loci, I found 61% of female *X. nigrensis* produced offspring from more than one male, with up to four sires contributing to the progeny produced. This is a minimum estimate of the number of mates because some males may successfully inseminate females but sire no offspring. Reproductive skew was also high within broods, with one male siring an average of 70% of the offspring. Although previous studies have shown sexual selection favors large male *X. nigrensis* (Ryan et al. 1990; Morris et al. 1992), larger males were not more likely to sire offspring in singly-sired broods, were not associated with higher reproductive skew within broods, and did not produce more offspring overall. Females with more sires, however, were more fecund, suggesting polyandry may increase female fitness in *X. nigrensis*.

Factors influencing the rate of multiple paternity

The frequency of multiple paternity reported here in *X. nigrensis* is similar to that found in other poeciliid fish, which is typically 50-80% but can range from a low as 15% to up to 100% depending upon the species and population (Evans & Pilastro 2011). The high levels of multiple paternity and the observed variation within and among poeciliid species is intimately linked to both ecological factors and features of their reproductive biology. Population density, for example, is positively correlated with the frequency of multiply sired broods in *Heterandria formosa* (Soucy et al. 2003), most likely due to the effect of density on encounter rates between the sexes. Sex ratios in poeciliid fish are also typically female-biased (2:1 at the Nacimiento Choy in May 2008), which facilitates promiscuity by increasing the opportunity for males to inseminate multiple females rather than incur the energetic and opportunity costs of defending female harems (Emlen & Oring 1977). Larger male *X. nigrensis* are more likely to win male-male agonistic interactions in the field (Morris et al. 1992), but as the paternity data suggests, males do not appear capable of monopolizing females. Indeed, fish forage in mixed sex foraging flocks that frequently change membership and females often receive mating attempts from multiple males (Smith, *pers. obs*). Female poeciliids can also store sperm for months (Constantz 1989), which may further increase the frequency of multiply-sired

broods by providing a genetic repository of sperm from previous copulations. Sperm storage is also likely to reduce paternity certainty, further devaluing the benefits of mate guarding and facilitating promiscuity. Four males in the current study, for example, were unrepresented in the first brood but produced offspring in subsequent broods.

Variation among females in the number of sires was also high, which can result if particular female phenotypes are more likely to be multiply mated. In poeciliid fish, females grow throughout their lives and size is correlated with fecundity (Constantz 1989). Larger females are thus expected to have a higher reproductive value to males, and behavioral studies in some species of have shown males prefer large females (reviewed in Ala-Honkola et al. 2010). Although larger female *X. nigrensis* were more fecund as expected, the number of sires was not related to female size. Other studies in poeciliid fish have found female size and sire number are related in some, but not all species: a weak relationship was reported in *H. formosa* (Soucy et al. 2003) and *X. maculatus* (Borowsky & Kallman 1976), a positive relationship was reported in some *Poecilia latipinna* populations (Trexler et al. 1997) while no relationship was found in guppies (Neff et al. 2008; but see Herdman et al. 2004).

Female size and sire number may not be correlated for at least two reasons. Males may not exhibit a preference if the benefit of increased mating effort with more fecund females is discounted by the decrease in paternity share due to higher levels of sperm competition (Schwagmeyer & Parker 1990; Wedell et al. 2002). Male preferences in guppies, for example, disappear when the female is observed mating with other males (Dosen & Montgomerie 2004) and depend on female reproductive state (i.e. virgin vs. non-virgin, Guevara-Fiore et al. 2009). The number of sperm ejaculated is also predicted to decrease as the number of potential competitors increases due to the reduction in reproductive returns (Parker et al. 1996), which could result in a smaller number of sires producing offspring if sperm senescence reduces the likelihood sperm from all males survive to fertilize the eggs. Evidence that males adjust sperm expenditure in relation to the number of male competitors in their social environment, however, is lacking in the two species examined thus far (Evans et al. 2003a; Aspbury 2007; Evans 2009). Male mollies do increase sperm production in the presence of larger females (Aspbury &

Gabor 2004), but it is unknown whether they also transfer larger ejaculates as a consequence.

Alternatively, male mating activity may not be correlated with insemination success if females have control over sperm transfer. In guppies, for example, the number of sperm inseminated increases if a male is relatively more attractive than a rival (Pilastro et al. 2004) and males that court prior to copulating transfer far more sperm than males that sneak matings (Pilastro et al. 2007). Similar dynamics may occur in *X. nigrensis* and other swordtails, where female preferences for large males strengthen with female size (Wong, unpublished; Morris et al. 2003). While this does not necessarily preclude choosy females from mating with several males of the preferred phenotype, the number of copulation attempts and the number of sires per brood may be uncorrelated if unattractive males transfer little or no sperm. Studies examining the interaction between male preferences, sperm allocation, and female roles in sperm transfer in *X. nigrensis* are clearly needed to test these hypotheses.

Reproductive Skew

Reproductive skew within female broods was pronounced, with an average of 70% of the offspring allocated to the most successful sire. Other studies in swordtails have reported similar estimates (Luo et al. 2005; Tatarenkov et al. 2008), suggesting high skew is a common feature in *Xiphophorus*. In the most comprehensive study of parentage to date in poeciilds, Tatarenkov (2008) collected all male and female *X. helleri* from isolated pools and reported that the majority of males (73%) mated with at least one female, as deduced from genetic analysis of the broods. As in the current study, reproductive skew within broods was high, suggesting that while most males sire at least some offspring, paternity was unequally distributed within individual females. Given that females store sperm for extended periods of time and are frequently subject to mating attempts by multiple males, it is remarkable that all broods are not multiply sired and paternity more evenly distributed. Low power to detect multiple sires cannot explain the presence of singly sired broods in the current study; the average probability of detecting multiple mating in the 12 singly sired females was 90% (see legend of Figure 1).

Postcopulatory sexual selection is thus likely an important contributor to the

observed reproductive skew. An important factor affecting paternity share in many animals is the order in which inseminations are received, which can "load" the raffle in favor of the first or last male that mates depending on how sperm are processed within the female (Eberhard 1996; Birkhead & Møller 1998). Precopulatory preferences in guppies appear to have an important role in determining paternity in guppies, where the second male to mate sires more offspring when two males are mated sequentially to a virgin female (Evans & Magurran 2001; Pitcher et al. 2003). When sperm from two males are mixed and artificially inseminated, however, paternity is uniformly distributed (Evans et al. 2003b; Evans & Pilastro 2011) and siring success is determined by the relative velocity and the number of sperm inseminated (Boschetto et al. 2010). Similar experiments have shown sperm quality is an important predictor of siring success in *X. helleri* (Gasparini et al. 2010) and *X. nigrensis* (Chapter 3). Reproductive skew in poeciliids, then, is likely a consequence of both variation in the quality of competing ejaculates and the number of sperm transferred, which can depend on female cooperation during copulation (Pilastro et al. 2007).

Female preferences prior to copulation in *X. nigrensis*, however, do not appear to affect male success in sperm competition because larger males did not garner a larger share of the paternity. The lack of a relationship between skew and sire size could result from weak discrimination or non-linear female preferences, such that males close in size aren't distinguished or those over a threshold size are equally preferred. In dichotomous choice tests, Ryan (1990) found a large (4-5mm) difference in male size was required to observe a female preference. Preferences were also weaker when given a choice between two males that court versus a sneaker male and a courting male, even if the size difference between fish was equivalent. Low within-female repeatability in preference (r=0.32, Cummings & Mollaghan 2006) may also reduce the likelihood of detecting an effect of sire size on paternity share, and while male size is positively correlated with courtship frequency (Ryan & Causey 1989), substantial variation among male effort could increase variation in the number of sperm transferred.

Finally, the correlation between the size of male offspring and their fathers may not be strong enough to use offspring size as a proxy for sire size. Dries et al. (2001)

crossed four small and three large male *X. nigrensis* with virgin females and found 93% of the variation in male offspring size was explained by sire size, suggesting the size of the father is predictable from the size of their sons. A larger quantitative genetic study using the full range of male sizes and more families would be useful to obtain a more precise estimate of the heritability of male size.

The high skew observed in the current study thus is likely to be due to last male sperm precedence (Evans & Magurran 2001; Pitcher et al. 2003), but larger differences in male size and information about male mating behavior prior to copulation may be required to observe an effect of female preferences on paternity share. An examination of the relationship between male size, mating tactic, and sperm transfer in controlled experiments is required to further evaluate this hypothesis. These studies are difficult in swordtails because successful copulations are hard to identify.

Fecundity and polyandry

Fecundity was positively related to sire number, suggesting polyandry confers a direct benefit to females. The benefits of multiple mating in species in non-resourced based mating systems are unclear because males do not provide obvious contributions to females that may enhance fitness, such as nuptial gifts or parental care. Nevertheless, positive relationships between the number of sires and female fecundity have also been observed in other poeciliids (Travis et al. 1990; Trexler et al. 1997; but see Tatarenkov et al. 2008) and females appear to solicit matings from multiple males (Houde 1997; Barbosa & Magurran 2010) despite the suggestion that polyandry entails fitness costs (Magurran & Seghers 1994; Schlupp et al. 2001; Pilastro et al. 2003; but see Smith & Sargent 2005). Sire number, however, is unlikely to be an accurate proxy for mating rate in poeciliids, which is thought to be the primarily driver of sexual conflict by reducing female foraging efficiency. In practice, female reproductive success is probably optimized at an intermediate number of mates (Arnqvist & Nilsson 2000), accruing until the costs of mating with an additional male exceeds the benefit.

Females with more sires may produce larger broods in non-resource based mating systems if additional inseminations reduce the likelihood of sperm limitation or reduce the likelihood of genetic incompatibilities between male and female genomes. Although

sperm have previously been perceived as abundant and cheap, spermatogenesis can entail significant time and energetic costs (Dewsbury 1982; Pitnick 1996; Olsson et al. 1997; LaMunyon & Ward 1998). Female-biased sex ratios in poeciliids may facilitate sperm limitation if males have low sperm reserves due to high mating frequency or if partitioning smaller ejaculates among multiple females increases overall fitness, even if it reduces the number of eggs that are fertilized per female (Warner et al. 1995). Field studies in mollies have found the proportion of females with sperm in the genital tract (but not necessarily in storage) declines as sex ratios become more female-biased (Riesch et al. 2008), although whether fecundity declines as a consequence was not examined. Field studies of male ejaculate stores are urgently needed, as sperm depletion has significant effects on the outcome of sexual selection in other taxa (reviewed in Tang-Martinez & Ryder 2005).

Polyandry may also be beneficial if it reduces the probability of male x female genetic incompatibilities (Zeh & Zeh 1997; Tregenza & Wedell 2000). The best evidence polyandry reduces the incidence of incompatibilities comes from experiments showing reductions in fecundity due to embryo abortion when females mate with close relatives (Newcomer et al. 1999) and from studies that manipulate relatedness but control the number of matings females receive (e.g. Worden & Parker 2001). In guppies, females produce fewer offspring when mated to one male compared to four males (Evans & Magurran 2000), however this experiment was not able to distinguish between sperm limitation or an increase in the likelihood of genetic incompatibilities as possible causes. Relatedness also reduces siring success in sperm competition in guppies (Gasparini, unpublished), which might arise from genetic incompatibilities between the gametes of siblings.

At the population level, inbreeding does not appear to be a persistent phenomenon at the Nacimiento Choy population of *X. nigrensis*. Heterozygosity was high and an excess of homozygotes due to inbreeding would have resulted in a deviation in H-W equilibrium. Inbreeding, however, is only one class of male x female interactions that may affect fecundity. Genetic incompatibilities at a small number of loci can result in abnormal development of embryos, and sperm-egg (Palumbi 1999) or ejaculate-female

interactions (Wolfner et al. 2009) may also influence the likelihood of fertilization. Whether a potential mate is genetically compatible is difficult to discern prior to copulation, thus polyandry may be an important mechanism for females to hedge against embryo failure and the production of unfit offspring (Zeh & Zeh 2003). Given that fecundity does appear to increase with the number if sires in *X. nigrensis*, controlled experiments evaluating the significance of male x female interactions would be useful for evaluating the mechanisms.

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