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**SEXUAL SELECTION AND SPERM COMPETITION IN THE GUPPY (*POECILIA RETICULATA*)**

By

**KAREN ELIZABETH ELGEE**

A Thesis  
Submitted to the Faculty of Graduate Studies  
through Biological Sciences  
in Partial Fulfillment of the Requirements for  
the Degree of Master of Science at the  
University of Windsor

Windsor, Ontario, Canada

2009

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## DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: Both data chapters were co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Indar Ramnarine. In each case, my collaborators provided valuable feedback, helped with the project design and statistical analysis, and provided editorial input during the writing of each manuscript; however, in both cases the primary contributions have all been by the author. Chapter 2 has been prepared as a manuscript for publication in *Molecular Ecology* and Chapter 3 has been prepared for submission to *Journal of Evolutionary Biology*.

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## ABSTRACT

When differences among individuals in the ability to acquire mates result in evolutionary change, this is known as sexual selection. My goal was to investigate sexual selection on male traits in the guppy (*Poecilia reticulata*). I assessed reproductive skew and correlates of male reproductive success in a wild population and found that male reproductive success was strongly skewed and correlated with gonopodium length, but not with the relative area of coloured spots, body length, or sperm velocity. I then determined the role of sperm competition in shaping sperm form and function by comparing sperm traits across populations. I found that males in high predation populations, which presumably experience more intense sperm competition, had significantly faster sperm with longer midpieces than males in low predation populations, which experience less intense sperm competition. These results suggest that gonopodium length is a sexually selected trait and that sperm competition selects for sperm velocity.

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## CHAPTER I: GENERAL INTRODUCTION

One of the assumptions of Darwin's (1859) theory of natural selection is that there is variation among individuals in fitness, and that this variation leads to certain individuals contributing more offspring than others to the next generation. Fitness can be defined as lifetime reproductive success (LRS), which is described as follows:  $LRS = \sum l_x \times m_x$ , where  $l_x$  is survivorship to age  $x$  and  $m_x$  is the number of offspring produced at age  $x$  (Stearns 1992). A primary objective among evolutionary biologists has been to document survivorship in populations in relation to evolutionary change. A prime example is found in long-term studies of Darwin's finches in the Galapagos islands, (Grant & Grant 2003). When rainfall is low, larger seeds predominate, resulting in differential survival of larger individuals of a population of Darwin's medium ground finch (*Geospiza fortis*). Individuals with slightly larger beaks therefore contribute disproportionately to the next generation, resulting in evolutionary change in beak size (Boag & Grant, 1981). A year of heavy rainfall, on the other hand, favours smaller seeds, leading to increased survival of individuals with smaller beaks and a shift towards smaller body size in the population (Gibbs & Grant, 1987). The effect of reproduction, the other component in the fitness equation, on evolutionary change generally receives less attention. When differences among individuals in the ability to acquire mates result in evolutionary change, this process is known as sexual selection (reviewed in Anderson, 1994).

## **Sexual selection**

Reproductive skew refers to how evenly reproductive success is distributed among individuals. The effect of the strength of reproductive skew on sexual selection can often be seen in comparisons between males and females. In most species, females invest heavily in each offspring, thereby limiting the number of offspring that can be produced, while males are capable of siring a large number of offspring (Bateman 1948; Trivers 1972). The higher limit on male reproductive potential often results in intense competition among males and a large degree of reproductive skew, leading to strong sexual selection on particular traits (Bateman 1948; Trivers 1972; Shuster 2009). The most extreme examples of sexual selection are found in highly polygynous species, where reproductive success is limited to a small number of males (Clutton-Brock & Vincent, 1991; Shuster 2009).

### *Mechanisms of sexual selection*

In his initial definition of sexual selection, Darwin (1871) proposed two types, each resulting in a different kind of phenotypic trait. The first is direct competition between males for access to mating opportunities and usually results in traits that maximize male fighting ability, such as the enlarged mandibles of male stag beetles (*Lucanus* sp.) (Otte & Stayman 1979), or the extreme sexual size dimorphism in the highly polygynous elephant seal (*Mirounga*), where fights over females favour large male body size (eg. Bartholomew 1970). The second form of sexual selection is female choice that results in elaboration of ornamental traits such as the colourful dewlap of male *Anolis* lizards (eg. Crews 1975) or the complex vocalizations of male songbirds (Catchpole 1987). These are all examples of pre-copulatory sexual selection; however, it is now

recognized that females of most species mate with more than one male, allowing for competition among males to continue after mate selection has taken place (i.e. post-copulatory competition) (Birkhead & Moller 1998).

The most obvious form of post-copulatory competition is sperm competition, which is the competition between the ejaculates of different males for a single set of ova (Parker, 1970, Birkhead & Moller 1998; Simmons 2001; Birkhead et al. 2009). Sperm competition can vary in risk, which is the probability of facing sperm competition (Parker et al. 1997) or intensity, which is the average number of males competing for each set of eggs (Parker et al. 1996; Parker et al. 1997). The classic model of sperm competition follows a fair raffle principle, whereby the more sperm (or tickets) a male enters, the higher his odds are of achieving fertilization (winning the prize) (Parker et al. 1990). The fair raffle model therefore predicts that sperm competition will select for males that are capable of producing large ejaculates and overall greater sperm production (Parker 1998). An association between ejaculate size and paternity success has been demonstrated in several species from diverse taxa, including the bluegill sunfish (*Lepomis macrochirus*) (Stoltz & Neff 2006), the scorpionfly (*Panorpa cognata*) (Engqvist et al. 2007) and the snail (*Viviparus ater*) (Oppliger et al. 2003). However, success at sperm competition can also be determined by various traits collectively referred to as sperm quality, an effect known as the 'loaded raffle' (Parker et al. 1990; Snook, 2005). Velocity has been shown to affect paternity in the common carp (*Cyprinus carpio*) (Linhart et al. 2005) and in the domestic fowl (*Gallus gallus*) (Birkhead et al. 1999). Viability (proportion live sperm in ejaculate) has been shown to be positively related to paternity success in the Australian field cricket (*Teleogryllus oceanicus*) (Garcia-Gonzalez & Simmons 2005). Sperm size and morphology has been



implicated in a number of studies of sperm competition, although there is no general pattern across species (Snook 2005). For example, larger sperm have a fertilization advantage over smaller sperm in the nematode (*Caenorhabditis elegans*) (LaMunyon & Ward 1998) and in the snail (*Viviparus ater*) (Oppliger et al., 2003), whereas Garcia-Gonzalez and Simmons (2007) found that shorter sperm had a fertilization advantage in the dung beetle (*Onthophagus taurus*). One possible reason for this variation among species is evident in studies that examine female reproductive tract in relation to sperm size. Many studies have found a relationship between sperm size and the size and shape of the female reproductive tract, particularly in insects (Snook 2005; Garcia-Gonzalez & Simmons 2007). Aspects of female post-copulatory behaviour or physiology that affect the outcome of sperm competition are known as cryptic female choice (Eberhard 1996).

In animals with internal fertilization, sperm competition and cryptic female choice can also favour changes in genital morphology that allow males to gain paternity over previous mates or guard against subsequent mates (Eberhard 1985). For example, a comparative analysis in rodents revealed that higher levels of sperm competition are associated with longer male genitalia (Ramm 2007). In the damselfly (*Calopteryx haemorrhoidalis asturica*), the width of the aedagus (the male intromittent organ) is positively related to the ability of the male to remove stored sperm in the female sperm storage organs prior to sperm transfer (Cordoba-Aguilar 1999) and in the dung beetle *O. taurus*, male fertilization success is related to the size of chitinous sclerites on the endophallus (House and Simmons 2003).

## Overview of thesis

My general research goal was to investigate sexual selection on male traits in the guppy (*Poecilia reticulata*), and to identify traits subject to sexual selection. My first objective was to determine which traits contribute to male reproductive success in a wild population. I considered traits that have been posited as being subject to female choice such as male colouration and body size, as well as traits thought to enhance post-copulatory success, such as sperm quality and genitalia size. My second objective was to determine the role of sperm competition in shaping sperm form and function by comparing sperm traits across several populations experiencing different levels of sperm competition.

### *The guppy*

The guppy occurs naturally in Venezuela and the adjacent islands of Trinidad and Tobago (Houde 1997). It has been particularly well studied in Trinidad's Northern Mountain Range, where it occupies a series of streams that vary dramatically in ecological conditions, making this system an ideal natural experiment for examining how ecological variables shape various traits (Lily & Seghers 1975; Houde 1997; Magurran 2005). Another reason for the wealth of research on guppies is their sexual dimorphism in colouration, which makes it a frequent subject of research on sexual selection, particularly regarding female choice for male colouration (Houde 1997; Magurran 2005). Males exhibit highly polymorphic and complex colour patterns composed of carotenoids (yellow, orange, red) melanins (black) and guanine crystals (iridescent green, blue and purple) (Endler 1980, 1983). The size and placement of coloured spots is highly heritable (Houde 1992), although the chroma of carotenoid-based spots

is affected by overall condition and carotenoid intake (e.g. Houde & Torio 1992, Kodric-Brown 1989). The most consistent finding regarding female choice has been a preference for orange colouration, usually defined as the relative area of carotenoid-based pigmentation (Houde 1987; Endler & Houde 1995; Brooks & Caithness 1995a). Female preference for iridescent (Kodric-Brown 1985, 1993) and black colouration (Brooks and Caithness 1995b) and larger body size (Reynolds & Gross 1992; Magellan et al. 2005) have also been demonstrated, although there is considerable variation in female preferences (Endler & Houde 1995; Brooks & Endler 2001).

Guppies are ovoviparous (eggs develop inside the female, prior to live birth) and fertilization is internal (Constanz 1989). Developing embryos are carried by the female for 3 to 4 weeks in non-overlapping broods, and females are only sexually receptive and responsive to males for a few days following parturition (Houde 1997). Unlike males, females continue to grow after maturation, resulting in female-biased sexual size dimorphism. Male courtship of females consists of the 'sigmoid display', during which the male will twist his body into an 'S' shape. If the female is responsive, she will orient towards the male and glide towards him (Houde 1997). Males also employ an alternative mating tactic known as the gonopodial thrust, during which they will approach the female from behind and attempt to insert the gonopodium (the anal fin modified for sperm transfer) without any prior courtship (Constanz 1989; Houde 1997). Females typically attempt to avoid gonopodial thrusts by swimming away (Houde 1997). The rate of gonopodial thrusts in the wild is unknown, although there is evidence that it is higher in locations where predation intensity is high and the conspicuous sigmoid display carries a high risk of predation (Luyton & Liley 1985; Magurran & Seghers 1990).

Multiple mating in guppies is relatively high (Kelley et al. 1999; Neff et al. 2008). Neff et al. (2008) found that 70% to 100% of broods were sired by more than one male and that the mean number of sires per brood was more than four in some populations. Sperm competition is therefore likely to be an important evolutionary force in this species. Artificial insemination studies have revealed an important role of sperm velocity in fertilization success, when controlling for all other factors (Evans et al. 2003; Locatello et al. 2006; Pitcher et al. 2007; but see Skinner & Watt 2007; Boschetto, Evans & Pilastro, personal communication). The amount of sperm transferred to the female is also an important predictor of fertilization success (Boschetto, Evans & Pilastro, personal communication). In practice, ejaculate size is primarily determined by copulation duration, which is under female control (Pilastro et al. 2007), although features of the gonopodium may also play a role. The gonopodium has a hook on the dorsal side that appears to facilitate sperm transfer, as does the width of the apical tip (Cheng 2004; Evans et al. 2009), and overall length of the gonopodium is related to successful sperm transfer during gonopodial thrusts (Evans et al. 2009).

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## CHAPTER 2: REPRODUCTIVE SKEW AND CORRELATES OF MALE REPRODUCTIVE SUCCESS IN A WILD POPULATION OF THE TRINIDADIAN GUPPY (*POECILIA RETICULATA*)<sup>1</sup>

### SYNOPSIS

In most species, males have a higher reproductive potential than females, leading to severely skewed reproductive success, particularly in mating systems where pre- or post-copulatory sexual selection reinforces this inequality in male mating success. Pre-copulatory sexual selection can lead to ornaments that increase attractiveness to females, while post-copulatory sexual selection can result in the elaboration of male genitalia or increased investment in sperm quality. We investigated multiple paternity, reproductive skew and correlates of male reproductive success in a wild population of the Trinidadian guppy (*Poecilia reticulata*). We used nine microsatellite loci to assess the frequency of multiple paternity, number of sires per brood, reproductive skew and standardized variance in reproductive success. Next, we examined correlations between male reproductive success and sexual colouration, sperm velocity and gonopodium length. We found that male reproductive success was highly skewed and correlated with gonopodium length, suggesting that gonopodium length is a sexually selected trait. Area of orange, black and iridescent colouration, and sperm velocity were not related to reproductive success.

<sup>1</sup>This chapter is the product of joint research with Dr. Trevor Pitcher and Dr. Indar Ramnarine (University of West Indies)

## Introduction

When males and females differ in the total number of offspring they can produce, it can result in intense competition for mating opportunities among members of the sex with a higher reproductive potential (reviewed in Clutton-Brock & Vincent 1991; Shuster 2009). In most species, females are more limited than males in the number of offspring they can produce because of a greater initial investment in each gamete and subsequent parental investment, whereas males have the potential to sire numerous progeny but are limited primarily by access to females (Bateman 1948; Trivers 1972; Clutton-Brock & Vincent 1991). Offspring production by males is likely to be more severely skewed than that of females in mating systems where pre- or post-copulatory sexual selection reinforces inequality in male mating success. Pre-copulatory sexual selection can lead to exaggerated male traits, such as weapons for direct competition with rival males, or ornaments that increase their attractiveness to females (reviewed in Anderson 1994). Post-copulatory sexual selection acts on male traits that increase the likelihood of fertilization success when females mate with multiple males, and can result in the elaboration of male genitalia (Hosken & Stockley 2004; Arnqvist & Rowe 2005) or increased investment in sperm quality (Birkhead & Moller 1998; Birkhead *et al.* 2009).

In the present study we investigate multiple paternity, reproductive skew and correlates of male reproductive success in a wild population of the guppy (*Poecilia reticulata*). The guppy can be found throughout numerous freshwater habitats in Trinidad and is a small, live-bearing fish with internal fertilization and a non-resource based, promiscuous mating system (reviewed in Houde 1997; Magurran 2005). A number of male traits have been examined with regard to female choice in guppies, the most common finding being a preference for males with a larger

area of orange carotenoid colouration (Houde 1987; Endler & Houde 1995; Brooks & Caithness 1995a). For example, females have been shown to 'trade-up' and mate with a second male that possesses more area of orange colouration, and is presumably of higher genetic quality, than a previous mate (Pitcher *et al.* 2003). Other findings regarding female mate choice in guppies include a preference for the area of black melanin colouration (Brooks & Caithness 1995b), area of iridescent colouration (Kodric-Brown 1985; 1993), or larger body size (Reynolds & Gross 1992; Magellan *et al.* 2005). However, there is variation in female preference for these traits across populations (Endler & Houde 1995) and among individuals (Brooks & Endler 2001).

Male guppies attempt to initiate copulation by employing a form of courtship known as the sigmoid display (Houde 1997). If females are unresponsive to this display, males will often attempt a form of sneaky mating, called the gonopodial thrust, in which the male approaches a female and forcefully attempts to insert his gonopodium, the modified anal fin used for sperm transfer, into her gonopore (Constanz 1989, Houde 1997). Females typically attempt to avoid gonopodial thrusts by swimming away, and thrusts appear to result in successful insemination on occasion, although determining the frequency of successful gonopodial thrusts in the wild is problematic. However, a study of eight wild populations of Trinidadian guppies found that nearly half of the females examined contained sperm presumed to be from unsolicited copulations (Evans *et al.* 2003, also see Matthews & Magurran 2000). Finally, male guppies with longer gonopodia achieve higher contact success (the number of thrusts that resulted in contact between the males' and females' genitalia) than those with shorter gonopodia (Evans *et al.* 2009).

Frequent unsolicited copulations and the potential benefits of polyandry (Evans & Magurran 2000; Pitcher *et al.* 2003; Ojanguren *et al.* 2005) are likely responsible for the high levels of multiple paternity observed in wild populations of guppies (Kelly *et al.* 1999, Neff *et al.* 2008), which results in a high risk of sperm competition. Experimental studies that artificially inseminate a female, which removes any pre-copulatory cues, with sperm from two competing males suggest that sperm competition success in guppies appears to be mediated, in large part, by sperm velocity. Evans *et al.* (2003) used artificial insemination to demonstrate that sperm competition success is correlated with area of orange colouration, which in turn is correlated with sperm velocity (see Locatello *et al.* 2006; Pitcher *et al.* 2007; but see Skinner & Watt 2007). A more recent artificial insemination experiment has confirmed a positive relationship between competitive fertilization success and sperm velocity (Boschetto, Evans & Pilastro, personal communication).

Despite the long history of the guppy as a model system in the study of sexual selection (reviewed in Houde 1997; Magurran 2005), no study to date has examined correlates of male reproductive success in a wild population. Houde (1988) allowed guppies to freely mate in aquaria and examined male reproductive success by scoring paternity of sons using Y linked colour patterns, revealing no relationship between the amount or type of ornamental colouration and reproductive success. Becher and Magurran (2004) also allowed guppies to mate freely in aquaria, then collected and genotyped all the resulting offspring using four polymorphic microsatellite loci for parentage analysis and related male reproductive success to a variety of morphological and behavioural traits. They found that smaller males sired significantly more offspring, but no other male trait was related to reproductive success, including the amount of



orange and black colouration, number of courtship displays and sneaky mating attempts, gonopodium length and total sperm stores (Becher and Magurran 2004).

Here, we used nine microsatellite loci to assess the frequency of multiple paternity, number of sires per brood, reproductive skew and standardized variance in reproductive success in a wild guppy population. Next, we examined correlations between male reproductive success and a variety of traits, including sexual colouration, sperm velocity and gonopodium length, that may allow us to specify particular features that are under sexual selection in our study population of this species. We discuss our results in terms of potential selective pressures on male and female guppies, as they relate to their standardized variance in reproductive success (Wade & Arnold 1980), and also compare our results to those of other studies that examine reproductive skew in other wild populations.

## **Materials and methods**

### *Study system*

We isolated a section of the Tunapuna stream (N10°42'38.22" W061°21'20.36"), a low predation (i.e. predation is restricted to juvenile guppies by a gape limited fish, *Rivulus hartii* (Houde 1997)) guppy population located in Trinidad's Northern Mountain Range, by setting up two barriers in the stream approximately 35 m apart. The isolated study area contained 10 pools that varied in depth (approximately 25 cm to 90 cm), separated by small riffles. The barriers were permeable to water and did not significantly disrupt stream flow, but were sufficient to

prevent migration (or emigration) of adult guppies from (or to) the study area. The barriers were constructed by stretching netting across the stream and securing it in place using rocks. The barriers were left in place for 65 days, which is enough time for females that had mated prior to the study to give birth and mate with available males within the study area (Houde 1997). The study area was inspected periodically to ensure that the barriers remained intact. After 65 days, we seined the study area, taking care to collect all adult guppies between the barriers, ensuring that no adult individuals remained. All females were immediately euthanized and preserved in ethanol in order to collect offspring (see below). Males were brought to the lab and assessed for sexual maturity, and traits related to reproductive success (see below).

### *Male trait assessment*

Field collected males were first assessed for sexual maturation; males were considered to be adult (reproductively mature) if the hood was extended beyond the tip of the gonopodium (Houde 1997). Sperm bundles were extracted to assess sperm velocity (see below) and a digital photograph (Nikon Coolpix 950) was taken of the left side of each adult male. From these photographs, we used ImageJ software (available at <http://rsbweb.nih.gov/ij/>) to measure total body length (from the tip of the mouth to the base of the caudal fin, along the central axis), gonopodium length (from the base to the distal tip, following Kelly *et al.* (2000)), total body area (excluding all fins except the caudal fin) and total area of orange, black and iridescent colouration (see Pitcher *et al.* 2007) (Table 2). Because the amount of orange and black colouration was related to body area (orange:  $R^2 = 0.48$ ,  $df = 80$ ,  $p < 0.001$ ; black:  $R^2 = 0.10$ ,  $df = 80$ ,  $p = 0.004$ ), we used the residuals of the regression of body area on each colour area

(hereafter relative orange colouration and relative black colouration) in all analyses. Total iridescent area was not correlated with body area ( $R^2 = 0.03$ ,  $df = 80$ ,  $p = 0.14$ ), so the absolute value was used in all analyses. For gonopodium length, the residuals of the regression of gonopodium length on body length ( $R^2 = 0.17$ ,  $df = 80$ ,  $p < 0.001$ ), hereafter relative gonopodium length, were used in subsequent analyses (see Kelly *et al.* 2000; Evans *et al.* 2009).

Sperm was extracted following Matthews *et al.* (1997). Individuals were anaesthetized, placed under a dissecting microscope, and sperm was then extracted by swinging the gonopodium forwards and applying pressure to the side of the abdomen with a blunt probe until all spermatozeugmata (i.e. sperm bundles) were released. A fixed number of sperm (25 bundles, to control for sperm density) were drawn up in a pipette and added to 250  $\mu$ L Courtland's saline, which contained bovine serum albumin at 1% v/v (hereafter saline solution) (Pitcher *et al.* 2007; Evans 2009). The resulting solution was drawn repeatedly into the pipette to break the sperm bundles and activate the sperm for velocity analysis. Video recordings for sperm velocity analyses were made using a CCD B/W video camera module at 50Hz vertical frequency, mounted on a digital compound microscope (magnification 400 X, Olympus BX60). We used an 8  $\mu$ l sample of semen in saline solution on a haemocytometer, covered with a cover slip. To maximize the time until the sperm stuck to the glass, the glass slide and cover slip were pre-coated by immersion in 1% bovine serum albumin followed by a rinse in distilled water (see Billard *et al.* 1995). Video-recordings were analyzed using the HTM-CEROS sperm tracking packing (CEROS version 12, Hamilton Thorne research, Beverly, MA, USA), an objective tool for studying sperm motility in fish (see Kime *et al.* 2001; Rurangwa *et al.* 2004), set at the following recording parameters: number of frames = 25; minimum contrast = 15; minimum cell

size = 5 pixels. The variables we assessed for each male's sperm were: average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell) at five seconds post-activation (i.e. breaking of the bundle) (Table 2). Because the variables describing sperm velocity (VAP, VSL and VCL) were highly correlated, we performed a Principle Component Analysis on these variables, which yielded one PC axis (hereafter referred to as sperm velocity) that explained 69.4% of the variation. Overall results were consistent even if we used any of the individual sperm velocity metrics in analyses (data not shown). The sperm velocity estimates used in the final analyses corresponds to the mean velocity of all motile cells analyzed for each male. Sperm that were stuck to one another or the glass slide and those whose movement beneath the cover slip was caused by convection currents were excluded from analyses. Following sperm collection, adult males were preserved in ethanol for genetic analyses.

### *Microsatellite genotyping*

All adult females (n = 118) were dissected and 67 females had developing embryos. Only broods consisting of a minimum of 4 offspring that were large enough for DNA extraction were genotyped (n = 32). DNA was extracted from individual whole embryos (mean +/- s.d.: 7.19 +/- 2.15 offspring per brood (range 4 – 12)) and from muscle tissue of the caudal peduncle of all females that contained embryos as well as all adult males (n = 81) by salt precipitation using the Wizard Genomic DNA Purification Kit (Promega). Samples were screened using up to 9 microsatellite markers, 6 developed for guppies: Pr39, Pr92 (Becher *et al.* 2002), Pre9, Pre15

(Paterson *et al.* 2005), AGAT-11 (Olendorf *et al.* 2004) and 9-1 (van Oosterhout *et al.* 2006) and 3 developed for *Poecilia parae*: PP\_GATG\_F4, PP\_GATA\_H2 and PP\_GATA\_5 (Nater *et al.* 2008) (see Table 1). Each 12.5  $\mu$ L PCR reaction contained 10x PCR buffer (100mM Tris-HCl, 500mM KCl, Applied Biosystems), 2 mM MgCl<sub>2</sub> (for Pr39 and 9-1) or 2.5 mM MgCl<sub>2</sub> (for all primers excluding Pr39 and 9-1) (Applied Biosystems), 0.8 mM of each dNTP (Fermentas), 0.02  $\mu$ M forward dye-labelled primer (IR-700, IR-800, LiCor), 4  $\mu$ M reverse primer (Sigma-Genosys), 0.125 units Taq DNA polymerase (Applied Biosystems) and ~75 ng genomic DNA. Cycle parameters for Pr92, Pr39, 9-1 and PP\_GATG\_F4 were as follows: 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 54°C (for Pr92 and 9-1) or 52°C (for Pr39) or 60°C (for PP\_GATG\_F4) for 20 sec, 72°C for 20 sec, and a final elongation at 72°C for 5 min. Cycle parameters for Pre15 and Pre9 were as follows: 94°C for 3 min, then 5 cycles of 94°C for 20 sec, 58°C (for Pre15) or 65°C (for Pre9) for 20 sec, 72°C for 20 sec, then 25 cycles of 94°C for 20 sec, 53°C (for Pre15) or 60°C (for Pre9) for 20 sec, 72°C for 20 sec and a final elongation at 72°C for 5 min. Cycle parameters for PP\_GATA\_H2 and PP\_GATA\_5 were as follows: 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 56°C for 90 sec, 72°C for 60 sec, and a final elongation at 72°C for 5 min. Cycle parameters for AGAT-11 were as follows: 94°C for 3 min, then 30 cycles of 94°C for 20 sec, 56°C for 20 sec, 72°C for 20 sec, and a final elongation at 72°C for 5 min. All PCR reactions were carried out on a PTC-100 Programmable Thermal Controller (MJ Research Inc.) or a MyCycler Thermal Cycler (Biorad). PCR product was loaded on a LiCor 4300 DNA analyzer and fragment sizes scored using GENE IMAGIR 4.05 software (Scanalytics, Inc.).

### *Reproductive skew*

Because we were not able to assign paternity to all of the offspring (see below), we used Colony (version 1.2; Wang 2004) to estimate the number of sires per broods and reproductive skew for each brood. This program uses a maximum likelihood method to estimate full-sib relationships within half-sib broods (i.e. number of sires per brood, see Neff *et al.* 2008). We assumed a genotyping error rate of 0.02. Skew was estimated by first calculating the effective number of sires from  $1/\sum(rs_i / brood\ size)^2$ ; where  $rs_i$  is the number of offspring assigned to sire  $i$ , and the summation is over all sires contributing to a brood. Skew was then expressed as  $1 - (\text{effective number of sires} / \text{actual number of sires})$  (see Neff *et al.* 2008). Linear regressions were used to assess the relationships between female body length and brood size, female body length and number of sires, and brood size and reproductive skew. Finally, we calculated the standardized variance in male and female reproductive success ( $I_m$  and  $I_f$ , respectively) as the variance in reproductive success (total number of offspring) divided by the square of the mean reproductive success (Wade & Arnold 1980).

### *Correlates of male reproductive success*

Paternity was assigned to the 81 candidate sires using Cervus ver. 3.0 software (available at <http://www.fieldgenetics.com>), which uses a likelihood-based method to assign paternity (Marshall *et al.* 1998; Kalinowski *et al.* 2007). We assumed a typing error rate of 0.01. To

account for sperm storage by females and possible male mortality during the study period, we assumed that 90% of all potential sires were included among the 81 males sampled (see Tatarenkov *et al.* 2008). Of the 233 offspring genotyped, Cervus was able to assign paternity to 197 individuals with at least 80% certainty. From this, we obtained a total number of offspring for each male, which we divided by 197 to obtain an estimate of relative reproductive success (RRS) for each male. In order to determine how equally mates and reproductive success were shared among males and among females, we created frequency distributions of the number of offspring and number of mates for each sex. Male number of mates represents the number of females with which each male successfully produced offspring. Female number of mates represents the number of sires contributing to a single brood. We then compared these distributions between males and females using Kolmogorov-Smirnov tests.

Relative reproductive success (RRS) was then related to the male traits described above (relative orange colouration, relative black colouration, iridescent colouration, body length, relative gonopodium length and sperm velocity) using linear regressions. The distribution of RRS was highly skewed (Kolmogorov-Smirnov test for normality,  $Z = 2.0$ ,  $p = 0.001$ ) and could not be normalized through any transformation. For this reason, we estimated statistical significance for the linear regressions relating RRS to male traits, using a randomization test (Manly 1997) executed in PopTools, a Microsoft Excel add-in (available at <http://www.cse.csiro.au/poptools>). Briefly, the x and y variables were shuffled (with replacement) to create randomized x and y pairings, from which a new regression coefficient was calculated. This was repeated 10,000 times, creating a distribution of regression coefficients and their associated t (the constant being excluded from the model). Regression coefficients that fell

outside the 95% CI of this distribution were considered statistically significant. Exact p values were estimated by calculating the proportion of randomized datasets that had r square values greater than or equal to the r square value calculated from the actual data.

## Results

### *Reproductive skew*

Nearly all broods (94%) were multiply sired, with a mean ( $\pm$  s.d.) of 2.9  $\pm$  0.98 (range 1 – 5) sires per brood and a mean reproductive skew of 0.14  $\pm$  0.12 (range: 0 – 0.48). There was variation in female body length (mean  $\pm$  s.d.: 24.9 mm  $\pm$  1.8; range: 21.6 – 28.76) and bigger females had larger broods ( $R^2 = 0.20$ ,  $df = 31$ ,  $p = 0.01$ ). There was no significant relationship between female body length and number of sires ( $R^2 = 0.001$ ,  $df = 31$ ,  $p = 0.84$ ) or between brood size and reproductive skew ( $R^2 = 0.07$ ,  $df = 31$ ,  $p = 0.15$ ).

The frequency distribution of number of offspring ( $Z = 4.0$ ,  $P < 0.001$ ; Fig. 2.1) and the number of mates ( $Z = 2.4$ ,  $P < 0.001$ ; Fig. 2.2) differed significantly between males and females. For females both offspring number and mate number did not deviate significantly from normality (offspring:  $Z = 1.1$ ,  $p = 0.2$ ; mates:  $Z = 1.3$ ,  $p = 0.07$ , Figs. 2.1 & 2.2), whereas for males they both deviated significantly from normality (offspring:  $Z = 2.0$ ,  $p = 0.001$ ; mates:  $Z = 2.3$ ,  $p < 0.001$ ) and were instead highly skewed to the right (Figs. 2.1 & 2.2).

Individual reproductive success was compiled as the total number of offspring assigned to each adult in the population. For females, this number ranged from 1 to a maximum of 12, with a mean number of offspring per female of 8.8 (variance = 5.4). For males, the number of



offspring ranged from 0 to 14, with a mean of 2.4 (variance = 5.6). Therefore, there was higher variance in reproductive success for males than females. The standardized variance in male reproductive success ( $I_m$ ) was 0.97 ( $5.6/(2.4)^2$ ) and for females ( $I_f$ ) it was 0.30 ( $8.8/(5.4)^2$ ). Male reproductive success was positively related to the number of mates ( $R^2 = 0.72$ ,  $p < 0.001$ ), but there was no such relationship for females ( $R^2 = 0.08$ ,  $p = 0.11$ ) (Fig. 2.3).

### *Correlates of male reproductive success*

Linear regressions revealed no relationship between male RRS and relative orange colouration ( $R^2 = 0.01$ ,  $p = 0.51$ ,  $n = 81$ ), relative black colouration ( $R^2 = 0.007$ ,  $p = 0.45$ ,  $n = 81$ ), iridescent colouration ( $R^2 = 0.001$ ,  $p = 0.94$ ,  $n = 81$ ), body length ( $R^2 = 0.012$ ,  $p = 0.32$ ,  $n = 81$ ) or sperm velocity ( $R^2 = 0.03$ ,  $p = 0.23$ ,  $n = 81$ ). However, there was a significant positive relationship between male RRS and relative gonopodium length ( $R^2 = 0.14$ ,  $p = 0.002$ ,  $n = 81$ ) (Fig. 2.4). The relationship between male RRS and gonopodium length remained even when not controlling for body size ( $R^2 = 0.14$ ,  $p = 0.001$ ,  $n = 81$ ).

## **Discussion**

We found that the mean reproductive skew was 0.14, 94% of the broods were multiply sired and the mean number of sires per brood was 2.9. These are similar to results found by Neff *et al.* (2008), who found that the mean reproductive skew was 0.14 and the proportion of broods that were multiply sired was 100% in this same population, although they found a higher mean

number of sires per brood (3.6), possibly due to a higher mean brood size in their study. In contrast, Kelly *et al.* (1999) found a proportion of multiply sired broods in this population of only 35% (they did not examine reproductive skew and the number of sires contributing to broods). The discrepancy between our results and Kelly *et al.* (1999) is most likely due to the fact that we had a much greater power to detect multiple paternity by using more microsatellite loci (see Neff & Pitcher 2002).

The frequency distribution of the number of offspring was significantly different between males and females, as was the distribution of the number of mates. For females, the number of offspring and the number of mates were both normally distributed, an indication that reproductive success was relatively evenly distributed among females. For males, on the other hand, both the number of offspring and the number of mates were highly skewed toward the right, indicating that most males were able to secure very few mating partners and sire few offspring. These findings are similar to those of Becher and Magurran's (2004) lab study of multiple paternity and reproductive skew in guppies that found that there was a significant difference in the distribution of offspring number, but not in the number of mates, between sexes. Because a small number of males appear to have sired the majority of offspring in our study, there appears to be considerable opportunity for sexual selection. The standardized variance in female reproductive success was relatively low (0.30), but for males it was relatively high (0.97). Reproductive success of females guppies in our population is relatively even and largely related to body size, whereas male reproductive success appears to be highly skewed. Compared to two other studies of wild fish populations, the Atlantic salmon (*Salmo salar*) with a standardized variance in male reproductive success of 0.59 (Garant *et al.* 2001) and the Green swordtail

(*Xiphophorus helleri*) with a standardized variance in male reproductive success of 2.50 (Tatarenkov *et al.* 2008), male guppies appear to be intermediate in terms of their variance in reproductive success. The relatively high variance in reproductive success in male guppies begs the question of which male traits are correlated with reproductive success in our wild study population.

We found that male relative area of orange colouration, relative area of black colouration, area of iridescent colouration and body length did not predict male reproductive success. It is possible that an analysis of colouration based on qualities such as chroma or brightness would yield different results; however, previous lab based research on female choice in guppies have primarily focused on the area of coloured spots. These studies have thus far indicated that female choice, particularly for male colour patterns, is an important determinant of male mating success, and many studies have demonstrated a strong preference for area of orange colouration (Kodric-Brown 1985; Houde 1987; Endler & Houde 1995; Brooks & Endler 2001; Pitcher *et al.* 2003). Studies examining the area of black and iridescent spots are more contradictory, with some studies showing a female preference for these colours and others showing an aversion or indifference (Kodric-Brown 1985; Brooks & Caithness 1995a,b; Endler & Houde 1995). Evidence regarding preference for body size is similarly contradictory (Reynolds & Gross 1992; Endler & Houde 1995). These contradictions in the literature may be due to differences in experimental conditions among studies, but also to variation in female preferences among populations (Endler & Houde 1995) and among individual females (Brooks & Endler 2001). In spite of the considerable body of work on female choice in guppies, very few studies have examined to what extent male traits preferred by females translate into reproductive success.

Becher and Magurran (2004) conducted paternity analysis on offspring born under controlled laboratory conditions, with all mating taking place in aquaria, and found that smaller males sired more offspring than larger males, but the relative area of orange, black and iridescent colouration was not significantly related to reproductive success (also see Houde 1988). It is possible that small male body size led to higher reproductive success in the Becher and Magurran (2004) study because they examined a high predation population, where gonopodial thrusting is likely to be more frequent in nature. However, because female choice experiments and male sexual behaviour studies have not been performed on our study population, it is not possible to determine whether our results are due to a lack of female preference for these male traits, or minimal female control over paternity due to the prevalence of sneaky mating.

Multiple mating is the norm in this species, so sperm competition is likely to affect male reproductive success; however, we found that sperm velocity did not predict male reproductive success. Recent studies using artificial insemination, with one focal female and two males contributing sperm, have demonstrated that sperm velocity is a predictor of paternity success in guppies (Boschetto, Evans & Pilastro, personal communication), confirming previous artificial insemination experiments by Evans *et al.* (2003), which found that male paternity success was correlated with the amount of orange colouration, which in turn has been associated with sperm velocity (Locatello *et al.* 2006; Pitcher *et al.* 2007; but see Skinner & Watt 2007). Our results indicate that sperm velocity may not be as important a predictor of paternity success in the wild when other male traits are taken into account, or that sperm competition is not a primary determinant of fertilization success in our study population. It is also possible that the artificial insemination experiments described above do not replicate normal sperm competition

mechanisms that occur in the wild, where more than two males are often contributing sperm to a female's reproductive tract (see Zeh & Zeh 1994).

We found that males with relatively longer gonopodia had higher reproductive success than males with relatively shorter gonopodia, a finding that is most likely due to an association between gonopodium length and success at gonopodial thrusts. Evans *et al.* (2009) found that males with relatively longer gonopodia were more successful at gonopodial thrusts than males with relatively shorter gonopodia, and Reynolds *et al.* (1993) demonstrated a positive correlation between gonopodium length and frequency of thrusts. There is also evidence that the shape of the gonopodium, including the angle of the hook and the length of the apical tip, contributes to insemination success (Evans *et al.* 2009; Cheng 2004). The positive relationship between male RRS and gonopodium length did not change when we used absolute length instead of relative length, suggesting that there is a reproductive advantage of longer gonopodia *per se*, not relative investment in gonopodium growth. It would be useful to conduct a more detailed analysis of gonopodium length and shape in relation to reproductive success in a wild population of guppies.

The relationship between gonopodium length and male reproductive success suggests that gonopodium length is a sexually selected trait. Sexual selection on male genitalia is common and is likely to account for the diversity in male genitalia, even among closely related species (Eberhard 1985). In guppies, the association between gonopodium length and gonopodial thrusts, suggests that the mechanism of sexual selection is likely to be sexual conflict over mating decisions. Evans *et al.* (2009) found a positive relationship between male gonopodium

length/shape and the depth/width of the female oviduct across ten wild guppy populations, suggesting a role for sexually antagonistic selection. Future studies that examine whether certain aspects of female oviduct morphology are able to help thwart thrusting attempts by males and whether there is sufficient additive genetic variance underlying the expression of these traits to permit response to selection, are needed.

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Table 2.1 List of microsatellite loci used in paternity analysis of guppy (*Poecilia reticulata*) offspring, references for the loci, number of alleles, observed heterozygosity, and expected heterozygosity.

Locus	Reference	Alleles	H <sub>o</sub>	H <sub>E</sub>
Pr92	Becher <i>et al.</i> 2002	3	0.525	0.578
Pr39	Becher <i>et al.</i> 2002	5	0.757	0.711
Pre15	Paterson <i>et al.</i> 2005	6	0.769	0.770
Pre9	Paterson <i>et al.</i> 2005	6	0.702	0.678
PP_F4	Nater <i>et al.</i> 2008	7	0.792	0.747
PP_H2	Nater <i>et al.</i> 2008	5	0.780	0.772
PP_5	Nater <i>et al.</i> 2008	5	0.654	0.665
AGAT-11	Olendorf <i>et al.</i> 2004	9	0.746	0.785
9-1	van Oosterhout <i>et al.</i> 2006	4	0.575	0.594

Table 2.2. Summary of traits of male guppies (*Poecilia reticulata*), including mean, standard deviation (SD) and range (minimum to maximum) of body length, total area of orange colouration, black colouration and iridescent colouration, gonopodium length, and sperm velocity. Sperm velocity measures include average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell).

Male trait		Mean	SD	Range
Body length (mm)		17.9	1.01	16.1 – 20.4
Orange area (mm <sup>2</sup> )		5.78	2.9	0 – 13.4
Black area (mm <sup>2</sup> )		7.97	3.2	0.92 – 16.72
Iridescent area (mm <sup>2</sup> )		2.58	1.41	0.45 – 6.1
Gonopodium length (mm)		4.08	0.33	3.39 – 5.24
Sperm velocity (µm/sec)	VAP	47.4	5.3	38.3 – 64.7
	VSL	26.5	4.8	13.1 – 40.8
	VCL	106.1	11.5	69.9 – 131.4



Table 2.3. Pearson correlations among traits in male guppies (*Poecilia reticulata*), including the total area of orange, iridescent and black coloured spots, body length, gonopodium length, and sperm velocity. Sperm velocity measures include average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell).

			Orange area (mm <sup>2</sup> )	Iridescent area (mm <sup>2</sup> )	Black area (mm <sup>2</sup> )	Body length (mm)	Gonopodium (mm)	Sperm velocity (µm/s)		
								VAP	VSL	VCL
Orange area (mm <sup>2</sup> )	R <sup>2</sup>		1	.17	.11	.64	.41	-.047	-.087	-.030
	P			.14	.33	<.001	<.001	.75	.56	.84
	N		81	81	81	81	81	47	47	47
Iridescent area (mm <sup>2</sup> )	R <sup>2</sup>		.17	1	.15	.21	.15	.022	-.004	.081
	P		.14		.19	.057	.19	.88	.98	.59
	N		81	81	81	81	81	47	47	47
Black area (mm <sup>2</sup> )	R <sup>2</sup>		.11	.15	1	.28	.14	-.18	.20	-.039
	P		.33	.19		.013	.20	.24	.17	.79
	N		81	81	81	81	81	47	47	47
Body length (mm)	R <sup>2</sup>		.64	.21	.28	1	.42	.047	.046	.14
	P		<.001	.057	.013		<.001	.76	.76	.35
	N		81	81	81	81	81	47	47	47
Gonopodium (mm)	R <sup>2</sup>		.41	.15	.14	.42	1	.13	.031	-.084
	P		<.001	.19	.20	<.001		.38	.84	.57
	N		81	81	81	81	81	47	47	47
Sperm velocity (µm/s)	VAP	R <sup>2</sup>	-.047	.022	-.18	.047	.13	1	.840	.52
		P	.75	.88	.24	.76	.38		<.001	<.001
		N	47	47	47	47	47	47	47	47
	VSL	R <sup>2</sup>	-.087	-.004	-.20	-.046	.031	.84	1	.20
		P	.56	.98	.17	.76	.84	<.001		.18
		N	47	47	47	47	47	47	47	47
	VCL	R <sup>2</sup>	-.030	.081	-.039	.14	-.084	.52	.20	1
		P	.84	.59	.79	.35	.57	<.001	.18	
		N	47	47	47	47	47	47	47	47

## Figure captions

Fig. 2.1 Total number of offspring of male (black bars) and female (grey bars) guppies (*Poecilia reticulata*) from an isolated, wild population in Trinidad.

Fig. 2.2 Total number of mates of male (black bars) and female (grey bars) guppies (*Poecilia reticulata*) from an isolated, wild population in Trinidad. Male number of mates represents the number of females with which each male successfully produced offspring, based on paternity analysis. Female number of mates represents the number of sires contributing to a single brood.

Fig. 2.3 The relationship between number of mates and number of offspring for (a) male ( $n = 81$ ) and (b) female ( $n = 32$ ) guppies (*Poecilia reticulata*). Symbol size is proportional to the sample size at overlapping data points.

Fig. 2.4 The relationship between male relative reproductive success (proportion of offspring produced by each male) and relative gonopodium length (residuals from the regression of gonopodium length on body length) in male guppies (*Poecilia reticulata*).

Fig. 2.1

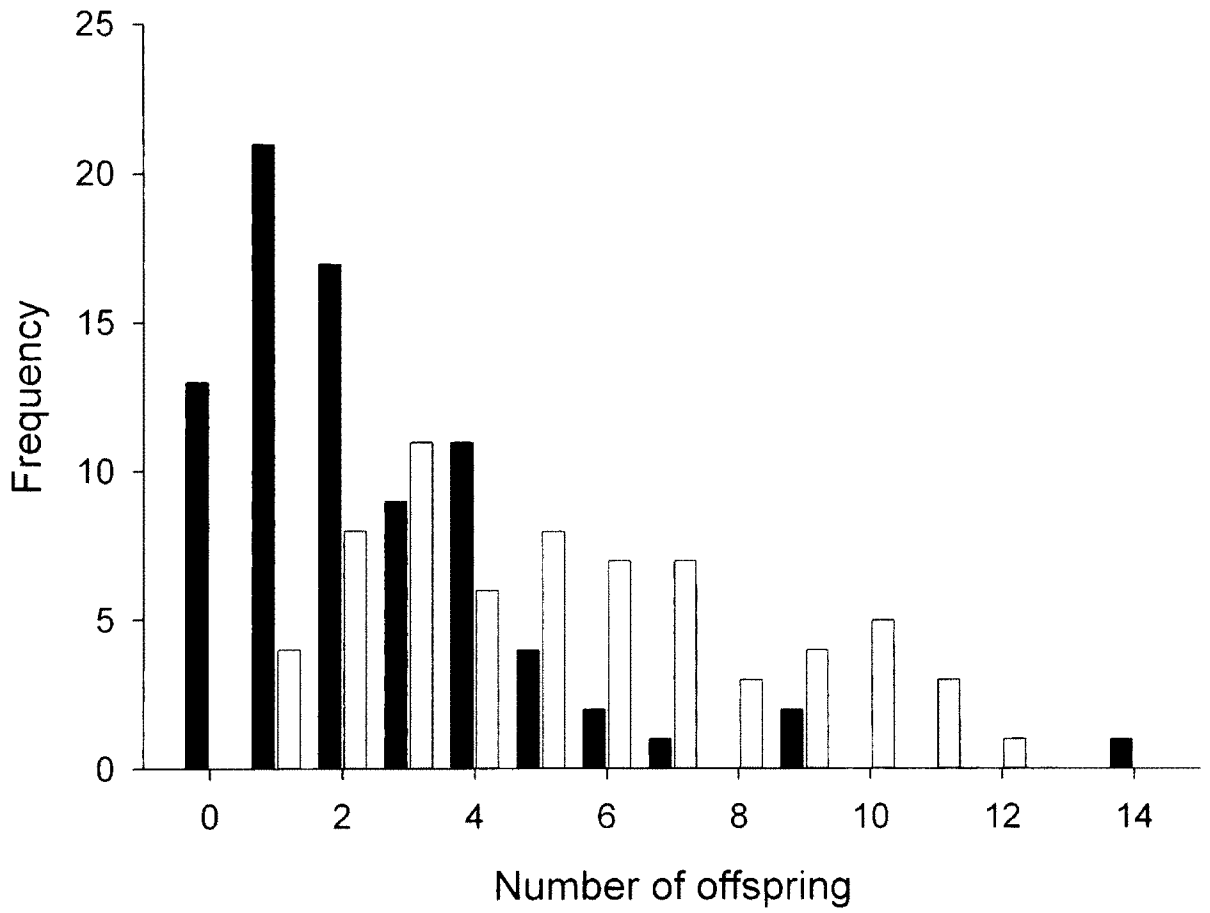


Fig. 2.2

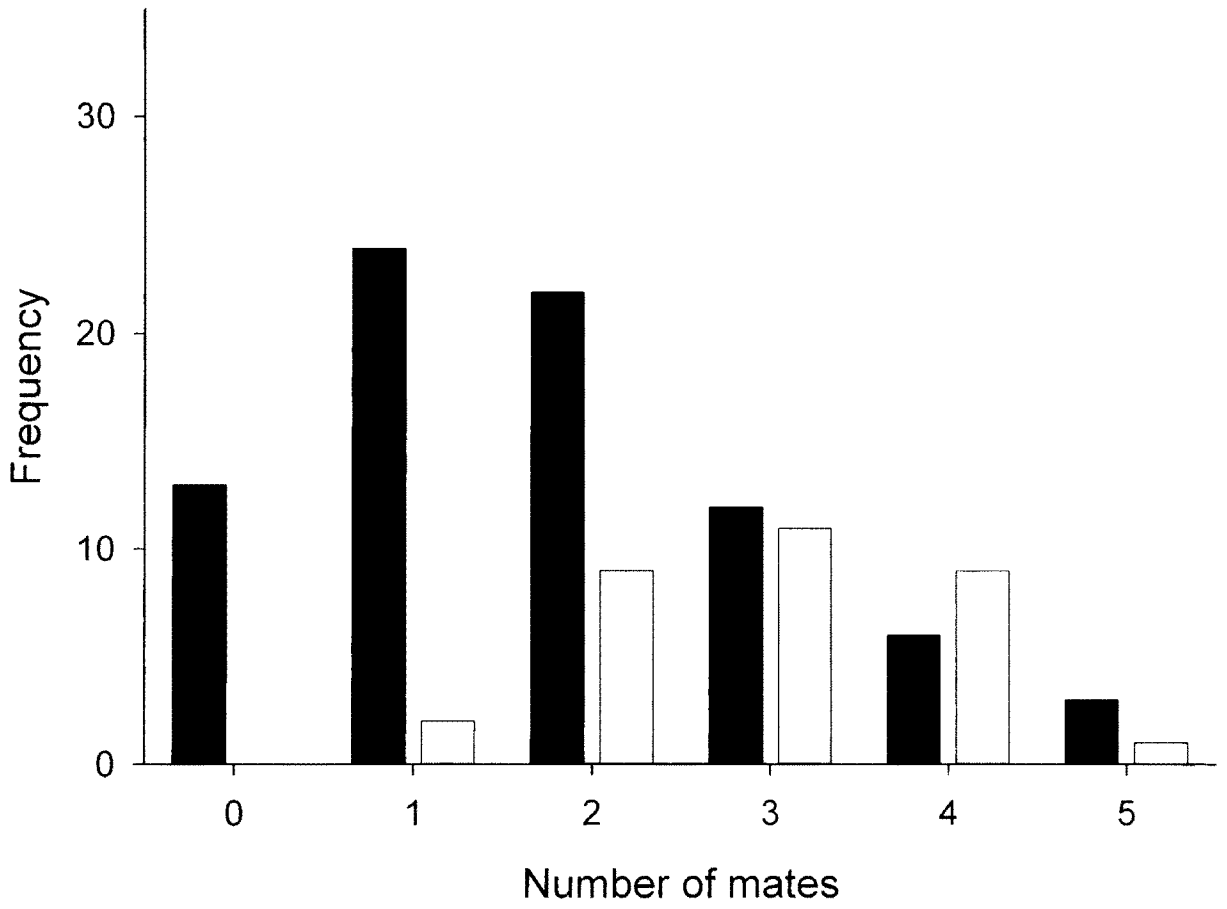
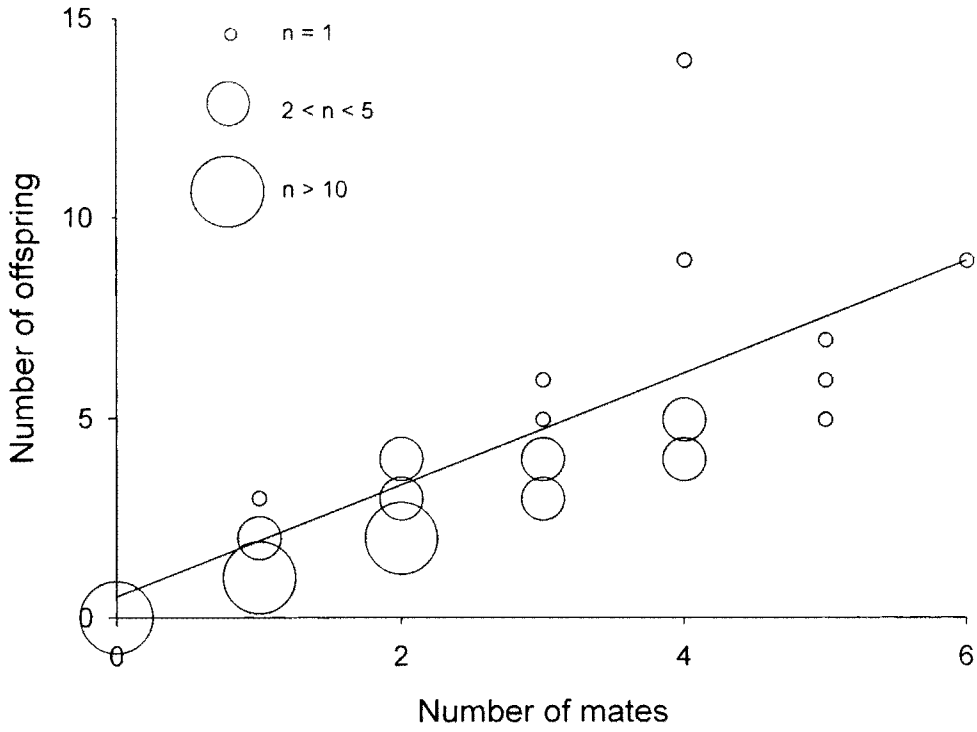


Fig. 2.3

(a) Males



(b) Females

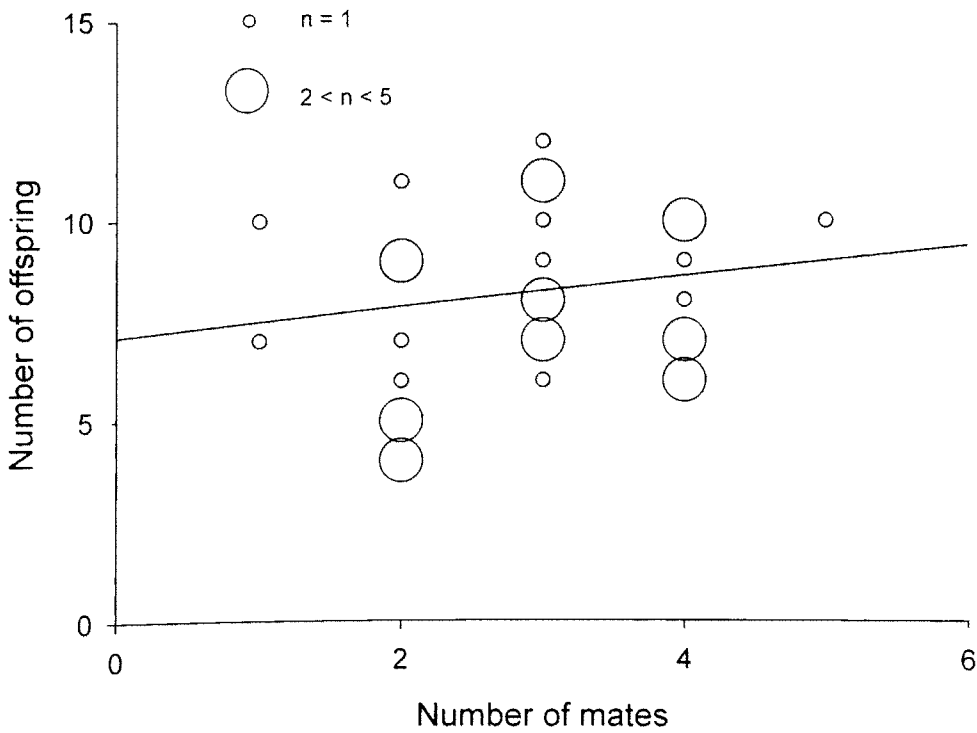
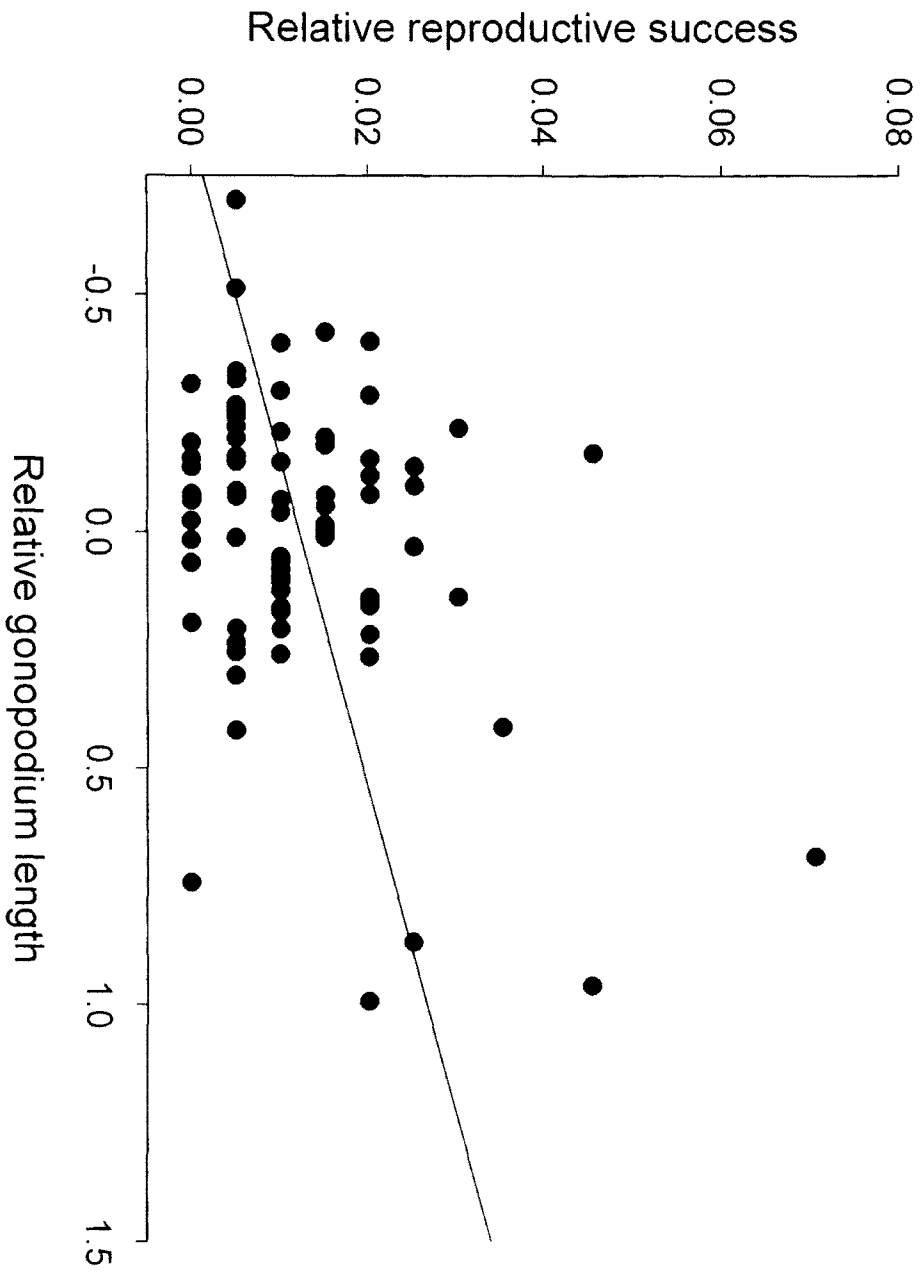


Fig. 2.4



### CHAPTER 3: GEOGRAPHIC VARIATION IN SPERM NUMBER, MORPHOLOGY AND VELOCITY IN THE TRINIDADIAN GUPPY (*POECILIA RETICULATA*)<sup>1</sup>

#### Synopsis

It has long been recognized that mating behaviour can increase the risk of mortality by predation. As a result of predation pressure, males in some species court less and engage more frequently in alternative sneaky mating tactics and females are often less choosy. These predator-mediated changes in mating tactics may result in higher levels of multiple inseminations in females and, thus, in greater sperm competition intensity. We tested the hypothesis that sperm competition intensity and thus, investment in spermatogenesis, positively covaries with predation risk by comparing eleven populations of wild Trinidadian guppies (*Poecilia reticulata*): six populations that experience relatively low levels of predation from a gape limited predator and five populations that experience relatively high levels of predation from a variety of piscivores. We predicted that males in populations facing high predation intensity would have greater sperm numbers, greater sperm velocity and longer sperm heads and midpieces than males from populations facing low predation intensity. As predicted, we found that males in high predation populations had faster sperm and concordantly, longer sperm heads and midpieces. However, contrary to prediction, we found that males in high predation populations possessed lower sperm numbers compared to males in low predation populations. These results suggest that predator mediated changes in sperm competition intensity may have important implications for the opportunity for sexual selection within and across populations.

<sup>1</sup>This chapter is the product of joint research with Dr. Trevor Pitcher and Dr. Indar Ramnarine (University of West Indies)

## INTRODUCTION

The impressive diversity in sperm number, motility and morphology across species is thought to be primarily a result of strong evolutionary pressure from sperm competition, the competition between the ejaculates of two or more males for the fertilization of a single set of ova (Parker, 1970, Birkhead & Moller, 1998; Simmons 2001, Birkhead et al., 2009). The probability of encountering sperm competition (i.e. sperm competition risk; *sensu* Parker et al., 1997) and the average number of ejaculates competing for each fertilization (i.e. sperm competition intensity; *sensu* Parker et al., 1996; Parker et al., 1997), varies broadly among species and environments. Ecological variables can shape mating behaviour, which in turn affects sperm competition. For example, the frequency of extra-pair paternity, and hence sperm competition risk, in birds can be affected by temperature, rainfall (e.g. Bouwman and Komdeur, 2006) and food availability (e.g. Václav et al., 2003). In an insect, the Japanese beetle (*Popillia japonica*), sperm competition intensity covaries with temperature and light conditions, due to changes in male mate guarding behaviour (Switzer et al., 2008).

One ecological variable that has received little attention in studies of sperm competition is predation. It has long been recognized that particular reproductive behaviours can increase the risk of predation (Lima and Dill, 1990; Magnhagen, 1991; Sih, 1994). As a result, predation risk can shape reproductive strategies (Sih, 1994; Magurran & Seghers, 1990), frequency and duration of reproduction (Sih et al., 1990; Rohr & Madison, 2001), and operational sex ratios (Lodé et al., 2004), all of which can affect sperm competition risk and intensity (Birkhead & Moller, 1998; Simmons, 2001). For example, in the agile frog (*Rana dalmatina*), high predation



intensity leads to a reduced probability of multiple mating by females, and thus a lower risk of sperm competition (Lodé et al., 2004). Another species in which reproductive behaviour has been linked to predation intensity is the guppy, *Poecilia reticulata* (Luyton & Liley, 1985; Magurran & Seghers, 1990).

*P. reticulata* is a small, live-bearing fish which can be found in streams throughout the northern mountainous range of Trinidad (Houde, 1997). These streams vary in ecological conditions and are isolated from one another, making this system ideal for studying the effects of various environmental factors on behaviour (Lily & Seghers, 1975; Magurran, 2005). For example, in lowland streams *P. reticulata* face high levels of predation pressure because they co-exist with a number of large piscivores. These include members of the Characidae family, such as *Hoplias malabaricus* and species belonging to the Cichlidae, such as *Crenicichla alta* (Liley & Seghers, 1975). However, waterfalls and rapids act as barriers that preclude upstream migration of these large predators into smaller tributaries. *P. reticulata* in these streams face low levels of predation because they co-exist only with a smaller gape limited predator, *Rivulus hartii* of the family *Cyprinodontidae*, which is incapable of consuming large adult *P. reticulata*, but is a significant predator of juveniles (Liley & Seghers, 1975).

This variation in predation intensity has been correlated with several behavioural and morphological traits, including mating behaviour (reviewed in Houde 1997). Male *P. reticulata* employ two distinct mating strategies, a courtship behaviour called the sigmoid display, and a form of sneaky mating called the gonopodial thrust (Houde, 1997). Males performing the visually conspicuous sigmoid display are presumably at an increased risk of predation (Endler,

1987), and females become unresponsive to male courtship when predation risk is imminent (Godin & Briggs, 1996; Gong, 1997). For these reasons, males in high predation locations employ sneaky mating with a higher frequency than males from low predation populations (Luyton & Liley, 1985; Magurran & Seghers, 1990). This increase in sneaky mating suggests that females in high predation populations receive sperm from a greater number of males, leading to an increase in sperm competition intensity with increasing predation intensity. This hypothesis is supported by patterns of multiple paternity, with high predation populations having greater numbers of sires per brood than low predation populations (Kelly et al., 1999; Neff et al., 2008).

Sperm competition has been likened to a 'fair raffle' in which the male who enters the most tickets (i.e. contributes the most sperm) is most likely to succeed in fertilizing the majority of a female's ova (Parker et al., 1990). The fair raffle principle predicts that males will respond to sperm competition by increasing ejaculate size and overall sperm production, and this has been supported in comparative (e.g. Stockley et al., 1997) and intraspecific studies (e.g. Gage et al., 1995). In many species, there are several sperm traits in addition to number that can influence fertilization efficiency, creating a 'loaded raffle' (Parker et al., 1990; Snook, 2005). These sperm traits are collectively referred to as 'sperm quality' and may include traits such as viability (e.g. García-González, 2005), velocity (e.g. Birkhead et al., 1999; Gage et al., 2004) and morphology (e.g. LaMunyon and Ward, 1998; Oppliger et al., 2003). Sperm competition in *P. reticulata* appears to follow such a 'loaded raffle' mechanism. Artificial insemination experiments have shown that both the number of sperm transferred to the female and sperm velocity are important predictors of paternity (Boschetto, Evans & Pilastro, personal

communication), as is orange colouration, a trait correlated with sperm velocity (Locatello et al., 2006; Pitcher et al., 2007; but see Skinner and Watt, 2007). There also appears to be a positive relationship between sperm velocity and sperm head and midpiece size (Pitcher et al., 2007; Skinner & Watt; 2007). We took advantage of the natural variation in predation intensity in *P. reticulata* to test the hypothesis that sperm quality and quantity are greater in populations facing greater sperm competition intensity. We predicted that populations of *P. reticulata* facing high predation intensity would have greater total sperm numbers, higher sperm velocity and longer midpieces than populations experiencing lower predation intensity. We tested this prediction by measuring sperm number, velocity and morphology in 11 populations of *P. reticulata* in Trinidad with either high or low predation intensity.

## **MATERIALS AND METHODS**

### **FISH COLLECTIONS**

We collected male guppies from 6 river systems in Trinidad's Northern Mountain Range (see Table 1). In 5 of these rivers (Aripo, Tacarigua, Quare, Oropuche and Turure), we sampled both downstream and upstream populations and in one river (Paria), we sampled only an upstream population. For Tacarigua, the Tunapuna stream was the upstream sampling site. As is typical of rivers in the Northern Mountain Range, all upstream locations exhibited low predation intensity and all downstream locations exhibited high predation intensity (Houde, 1997:

Magurran, 2005); except the Oropuche river, which featured high predation intensity in both the upstream and downstream populations (Endler and Houde, 1995). All sampling sites have been extensively surveyed for predators (Magurran and Seghers, 1994; Endler and Houde, 1995) and subsequently monitored by other researchers (e.g. Evans *et al.*, 2003a, Pitcher unpublished data). We collected 25 males from each population; however, for some males, sperm degraded prior to analysis or the video recordings were not of sufficient quality for velocity analysis (see below) so fewer individuals were used.

#### SPERM TRAIT ASSESSMENT

Males were isolated for three days after capture from the wild to allow sperm reserves to replenish (Kuckuck & Greven, 1997). We collected sperm from males from each of the eleven populations, following Matthews *et al.* (1997). Individuals were placed under a dissecting microscope, and sperm was then extracted by swinging the gonopodium forwards and applying pressure to the side of the abdomen with a blunt probe until all spermatozeugmata (i.e. sperm bundles) were released. Initially, a fixed number of sperm (25 bundles) were drawn up in a pipette and added to 250  $\mu$ L Courtland's saline, which contained bovine serum albumin at 1% v/v (hereafter saline solution) (Evans *et al.*, 2003b). The resulting solution was drawn repeatedly into the pipette to break the sperm bundles and activate the sperm for velocity analysis (see below). Remaining sperm bundles were then collected and diluted in saline solution, as outlined above, for sperm number and sperm morphology analyses. Finally, we took a digital photograph of the left side of each male, which was later used to measure total body length (from the tip of

the mouth to the base of the caudal fin, along the central axis) using ImageJ software (available at <http://rsbweb.nih.gov/ij/>).

Sperm numbers at rest (hereafter sperm number) were calculated by counting sperm cells in an “improved Neubauer chamber” haemocytometer under 400 X magnification (see Pitcher et al. 2007). The distribution of sperm cells across the haemocytometer was checked visually for evenness before counts commenced. If the sperm were unevenly distributed across the haemocytometer then the count was discarded and started over. The numbers of sperm in each of five larger squares on the haemocytometer were counted. There are 25 of these large squares on the haemocytometer and each of these large squares has 16 smaller squares within it. Sperm were counted in the four large corner squares and the large centre one (80 smaller grids). The mean number of sperm per large square count (i.e. mean of the 5 counts) was multiplied by 25 (to obtain the mean per 5 x 5 large-square grid) and again by 10 (the depth of the chamber in  $\mu\text{m}$ ). This number was then multiplied by the initial volume of the sample and added to the estimated number of sperm used in motility analysis (we assumed 25 000 sperm per bundle) (Evans et al, 2003b) to estimate the sperm number. Sperm numbers are expressed as the total number of sperm in a male's stripped ejaculate (see Table 2).

Video recordings for sperm velocity analyses were made using a CCD B/W video camera module at 50Hz vertical frequency, mounted on a digital compound microscope (magnification 400 X, Olympus BX60). We used an 8  $\mu\text{l}$  sample of semen in saline solution on a haemocytometer, covered with a cover slip. To maximize the time until the sperm stuck to the glass, the glass slide and cover slip were pre-coated by immersion in 1% bovine serum albumin

followed by a rinse in distilled water (see Billard *et al.*, 1995). Video-recordings were analyzed using the HTM-CEROS sperm tracking package (CEROS version 12, Hamilton Thorne research, Beverly, MA, USA), an objective tool for studying sperm motility in fish (see Kime *et al.*, 2001, Rurangwa *et al.*, 2004), set at the following recording parameters: number of frames = 25; minimum contrast = 15; minimum cell size = 5 pixels. The variables we assessed for each male's sperm were: average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell) at five seconds post-activation (i.e. breaking of the bundle) (see Table 3). Because the variables describing sperm velocity (VAP, VSL and VCL) were highly correlated, we performed a Principle Component Analysis on these variables, which yielded one PC axis (hereafter referred to as sperm velocity) that explained 65.7% of the variation (PC loadings: VAP = 0.97, VSL = 0.87, VCL = 0.62). The sperm velocity estimates used in the final analyses corresponds to the mean velocity of all motile cells analyzed for each male. Sperm that were stuck to one another or the glass slide and those whose movement beneath the cover slip was caused by convection currents were excluded from analyses. Between 16 and 378 sperm were measured for velocity per male (mean +/- s.e. = 89.8 +/- 7.26).

Sperm morphology assessment followed Pitcher *et al.* (2007). We placed 20uL of preserved sperm (sperm suspended in a 2.5% glutaraldehyde) in saline solution and applied it to a glass slide, from which we took digital images at x1000 magnification using a light microscope and oil immersion. From these photographs, we used ImageJ software to measure the length of the head, midpiece and flagellum of 11 to 16 undamaged sperm for each male (mean +/- s.e. =

14.9 +/- 0.76). The head was measured along the midline from the forward apex to the neck. The midpiece was measured in the same manner from the neck to the insertion of the flagellum. The flagellum was measured from the insertion point to the terminal filament. Total length was obtained by combining the lengths of all three components. Analyses were carried out using mean values for each male (see Table 4).

## STATISTICAL ANALYSES

We used Linear Mixed Models to compare sperm related traits (sperm number, sperm velocity, and sperm morphology) between predation regimes among populations (rivers). In these analyses, sperm related traits were entered as response variables, with predation as a fixed factor (2 levels, high- and low-predation), and river as a random effect (6 levels). In these analyses, we tested for the interaction between fixed (predation) and random (river) factors and found it to be non significant, so removed it from the model. Because sperm number was correlated with body length ( $r = 0.31$ ,  $P < 0.001$ ,  $n = 208$ ) (see Pitcher & Evans 2001), we entered body length as a covariate in the mixed linear model when analyzing sperm number. We also examined differences among upstream (low predation) and downstream (high predation) populations from the same river, using independent t-tests. To control for body size, the t-test for sperm number was carried out using the residuals of the Linear Mixed Model. All sperm related variables were log transformed prior to the analyses to normalize the data.

## RESULTS

There was no significant effect of river (Wald = 1.2,  $P = 0.23$ ) on sperm number, but males in low predation populations had significantly more sperm than males in high predation populations ( $F_{1,186} = 4.0$ ,  $P = 0.046$ ; Figure 3.1a); however, pairwise comparisons showed no significant differences between low predation populations compared to high predation populations (Tacarigua:  $t_{38} = -1.9$ ,  $P = 0.062$ ; Quare  $t_{39} = 1.2$ ,  $P = 0.25$ ; Aripo:  $t_{36} = -0.004$ ,  $P = 1.0$ ; Oropuche:  $t_{34} = 0.15$ ,  $P = 0.89$ ; Turure:  $t_{38} = 0.26$ ,  $P = 0.80$ ; Figure 3.1b).

There was a significant effect of predation ( $F_{1,134} = 20.1$ ,  $P < 0.001$ ; Fig. 3.2a), but not river (Wald = 1.4,  $p = 0.15$ ; Fig. 3.2b), on sperm velocity; males in high predation populations possessed significantly faster sperm compared to males in low predation populations. Pairwise comparisons showed that sperm velocity was significantly higher in high predation populations compared to low predation populations for the Aripo ( $t_{26} = 2.1$ ,  $P = 0.046$ ), Tacarigua ( $t_{23} = 5.0$ ,  $P < 0.001$ ) and Turure ( $t_{30} = 2.3$ ,  $P = 0.028$ ) rivers (Fig. 3.2b). There was no significant difference in sperm velocity in high and low predation populations of the Quare ( $t_{18} = 1.4$ ,  $P = 0.19$ ) and Oropuche ( $t_{17} = 0.18$ ,  $P = 0.86$ ) rivers (Fig. 3.2b).

There was a significant effect of predation intensity, but not river on sperm head length (predation:  $F_{1,99.7} = 5.8$ ,  $P = 0.018$ , Fig. 3.3a; river: Wald = 1.4,  $P = 0.15$ , Fig. 3.3b) and sperm midpiece length (predation:  $F_{1,99.7} = 5.8$ ,  $P < 0.001$ , Fig. 3.4a; river: Wald = 1.3,  $P = 0.18$ , Fig. 3.4b); high predation males possessed longer head and midpiece length compared to low predation males. Sperm head length and midpiece length were significantly longer in high predation males than in low predation males of the Aripo (head:  $t_{16} = -2.2$ ,  $P = 0.042$ ; midpiece:



$t_{16} = -3.8$ ,  $P = 0.002$ ) and Quare (head:  $t_{16} = 4.4$ ,  $P < 0.001$ ; midpiece:  $t_{16} = 6.0$ ,  $P < 0.001$ ) rivers, but not the Tacarigua (head:  $t_{18} = -1.4$ ,  $P = 0.19$ ; midpiece:  $t_{10} = 0.85$ ,  $P = 0.42$ ). Oropuche (head:  $t_{14} = 1.7$ ,  $P = 0.11$ ; midpiece:  $t_{14} = 0.36$ ,  $P = 0.73$ ) and Turure (head:  $t_{18} = 0.78$ ,  $P = 0.45$ ; midpiece:  $t_{18} = 1.3$  and  $p = 0.21$ ) rivers (Fig. 3.3b and Fig. 3.4b). Finally, neither predation intensity nor river were related to flagellum length (predation:  $F_{1,97.4} = 1.5$ ,  $P = 0.23$ , Fig. 3.5a; river: Wald = 1.5,  $P = 0.13$ , Fig. 3.5b) or total sperm length (predation:  $F_{1,97.8} = 0.76$ ,  $P = 0.39$ , Fig. 3.6a; river: Wald = 1.5,  $P = 0.13$ , Fig. 3.6b). Despite the overall model not being significant, sperm flagellum length differed significantly between upper and lower populations of the Quare ( $t_{16} = 3.2$ ,  $P < 0.001$ ) and Turure ( $t_{11.6} = -2.6$ ,  $P < 0.024$ ) rivers, but not the Aripo ( $t_{8.4} = 1.7$ ,  $P = 0.13$ ), Tacarigua ( $t_{18} = -1.6$ ,  $P = 0.12$ ) and Oropuche ( $t_{14} = 2.0$ ,  $P = 0.065$ ) rivers (Fig. 3.5b). Total sperm length differed significantly between upper and lower regions of the Quare ( $t_{16} = 6.0$ ,  $P < 0.001$ ) and Oropuche ( $t_{14} = 3.8$ ,  $P = .002$ ) rivers, but not the Aripo ( $t_{8.8} = 0.59$ ,  $P = 0.57$ ), Tacarigua ( $t_{12.3} = 2.1$ ,  $P = 0.06$ ) and Turure ( $t_{11.8} = -2.1$ ,  $P = 0.05$ ) rivers (Fig. 3.6b).

## DISCUSSION

We found support for the hypothesis that sperm quality is higher in populations with more intense sperm competition. Sperm of *P. reticulata* were faster and had longer heads and midpieces in high predation populations, which have more intense sperm competition, than in low predation populations. Contrary to prediction, sperm numbers were greater in low predation than in high predation populations.

Sperm competition is predicted to select for greater sperm number through a 'fair raffle' mechanism, whereby males with greater numbers of sperm are able to enter more 'tickets' into the contest for fertilization and are therefore likely to outcompete males with fewer sperm (Parker et al., 1990). It follows that males facing increasing risk or intensity of sperm competition invest in producing greater numbers of sperm (Stockley et al., 1997; Gage et al., 1995). Contrary to this prediction, we found that sperm number was greater in low predation than in high predation populations, a finding that is in accordance with Evans & Magurran (1999). This finding may suggest that total sperm number at rest may not be a trait selected for by sperm competition, perhaps because total sperm number is not representative of ejaculate size. Pilastro et al. (2002) found no relationship between sperm reserves at rest and ejaculate size in *P. reticulata*. Ejaculate size in this species appears to be primarily determined by copulation duration, which is apparently under female control (Pilastro et al., 2007).

We also found that sperm velocity was greater in high predation than in low predation populations, therefore the lack of evidence for an increase in sperm number with increasing sperm competition intensity may also be because sperm number is not the primary factor determining fertilization success in *P. reticulata*. There is now increasing evidence that sperm number does not fully explain sperm competition success and that instead of a 'fair raffle' mechanism, many species instead exhibit a 'loaded raffle' mechanism, in which sperm traits other than number influence fertilization efficiency (Parker et al., 1990; Snook, 2005). In *P. reticulata*, there is evidence for such a 'loaded raffle' mechanism. Boschetto, Evans & Pilastro, (personal communication) used artificial insemination to transfer controlled amounts of sperm to female *P. reticulata* and found that sperm velocity was a strong predictor of paternity. This

confirms a similar experiment conducted by Evans et al. (2003b), who found that male paternity in *P. reticulata* was related to the amount of orange colouration, a trait which has been linked to sperm velocity (Locatello et al., 2006; Pitcher et al., 2007; but see Skinner & Watt, 2007).

Functionally, the increase in velocity in high predation compared to low predation populations may be due to differences in head and midpiece length, both of which we found to be longer in high predation populations compared to low predation populations. In *P. reticulata*, Pitcher et al. (2007) demonstrated that sperm head length (a measure that included the midpiece) was positively correlated with sperm velocity. Skinner and Watt (2007) found a similar pattern using sperm midpiece area. The midpiece is the component of the sperm containing the mitochondria, which provide energy for the beating of the flagellum (Baccetti & Afzelius, 1976). Thus, a larger midpiece could potentially provide more energy for locomotion, resulting in faster sperm, although this possibility has yet to be thoroughly investigated.

The conclusion that our findings support the sperm competition hypothesis rests on the assumption that sperm competition in *P. reticulata* is more intense in high predation than in low predation populations. There is evidence that males in high predation populations attempt sneaky mating with higher frequency than males in low predation populations (e.g. Luyten & Lily, 1985; Magurran & Seghers, 1990) and the level of multiple mating, as measured by the mean number of sires per brood, increases with predation intensity (Kelly et al. 1999; Neff et al., 2008). However, other studies have not found evidence that the rate of sneaky mating is higher in high predation populations. Evans et al. (2003c) estimated sneak mating rates by retrieving sperm from the gonoducts of wild female *P. reticulata*. Because female *P. reticulata* only

respond positively to copulation attempts during the receptive phase, Evans et al. (2003c) concluded that unreceptive females with sperm in their gonoducts had been inseminated through sneaky mating. Based on this estimate, Evans and colleagues found no difference between high and low predation populations in the rate of sneaky mating. However, Evans et al. (2003c) determined only the amount of sperm inseminated and not the number of males that had inseminated each female, nor did they have a way of determining the rate of sneaky matings on receptive females. Further research is necessary on the relationship between predation intensity, male behaviour and sperm competition in *P. reticulata*. Our results demonstrate the potential for taking advantage of population level variation in reproductive behaviour to elucidate relationships between sperm competition and environmental factors. Further research on the effects of environmental variables such as predation on sperm traits in *P. reticulata* and other species could provide further information on the evolutionary impacts of sperm competition on the opportunity for sexual selection.

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Table 3.1. List of the 11 guppy (*Poecilia reticulata*) populations from the Northern Range of Trinidad. Data comprise population, collection location (GPS coordinates), and predation intensity.

Population	Location	Predation
Lower Aripo	N10°39.036' W061°13.380'	High
Upper Aripo	N10°41.743' W061°12.406'	Low
Lower Quare	N10°40.418' W061°11.833'	High
Upper Quare	N10°40.553' W061°11.792'	Low
Lower Oropuche	N10°39.570' W061°07.868'	High
Upper Oropuche	N10°43.060' W061°08.800'	High
Lower Turure	N10°39.39' W061°10.059'	High
Upper Turure	N10°40.775' W061°10.002'	Low
Tacarigua	N10°40.736' W061°19.168'	High
Tunapuna	N10°42'38.22" W061°21'20.36"	Low
Paria	N10°44.700' W061°15.704'	Low

Table 3.2. Population identity, samples size, mean and estimates of intraspecific variation (standard deviation (SD) and range) for sperm numbers and body length of male guppies (*Poecilia reticulata*) in Trinidad.

Population	N	Sperm number ( $\times 10^6$ )			Body length (mm)		
		Mean	SD	Range	Mean	SD	Range
Lower Aripo	18	3.38	1.50	.890 – 6.43	16.2	0.94	14.3 17.8
Upper Aripo	21	3.97	1.85	.895 – 8.35	18.4	0.96	16.7 20.6
Lower Quare	20	5.84	3.15	.435 – 11.82	15.9	0.97	14.7 17.6
Upper Quare	22	3.79	1.72	1.25 – 9.43	18.1	1.3	15.6 - 20.7
Lower Oropuche	17	3.44	1.63	.910 – 6.33	17.2	0.96	15.2 19.8
Upper Oropuche	20	3.47	2.02	.970 – 8.06	16.6	0.78	15.1 18.2
Lower Turure	18	2.77	1.05	2.14 – 2.74	16.1	0.90	14.8 18.4
Upper Turure	23	3.07	1.35	1.08 – 5.37	16.2	0.89	14.7 17.8
Tacarigua	21	2.06	.727	.830 – 3.88	16.9	0.84	15.4 18.1
Tunapuna	21	3.57	1.87	.950 – 8.06	17.8	1.2	15.2 20.8
Paria	13	4.19	1.67	1.78 – 8.17	18.9	0.60	17.6 19.7

Table 3.3. Population identity, sample size, mean and estimates of intraspecific variation (standard deviation (SD) and range) for sperm velocity of male guppies (*Poecilia reticulata*) in Trinidad. Sperm velocity measures included average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell).

Population	N	VAP			VSL			VCL		
		Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Lower Aripo	16	54.2	11.7	36.2 - 75.2	30.1	11.6	8.4 - 49.8	125.1	15.3	103.1 - 157.5
Upper Aripo	14	45.7	6.40	31.5 - 51.8	24.2	7.08	8.2 - 31.3	111.8	9.25	89.3 - 121.6
Tacarigua	16	48.2	4.25	41.8 - 56.1	26.3	4.35	20.9 - 35	109.8	13.4	83 - 130.4
Tunapuna	14	39.6	4.25	34.1 - 48.9	18.3	4.79	11.5 - 25.2	109.3	13.1	94.3 - 129.9
Lower Quare	6	60.4	8.53	50.3 - 71.3	36.5	7.40	29.1 - 48.5	123.0	16.3	94.5 - 144.8
Upper Quare	7	53.8	12.33	41.8 - 81.7	28.0	12.9	14.3 - 58.9	136.1	14.7	112.2 - 157.8
Lower Oropuche	11	41.8	6.49	31.4 - 51.7	23.8	6.33	14.6 - 33.8	93.75	11.5	71.1 - 112.8
Upper Oropuch	8	41.1	6.08	32.3 - 48.2	44.7	5.96	10.9 - 24.8	119.9	27.2	98.6 - 180.3
Lower Turure	15	49.7	5.62	42.1 - 59.7	26.3	4.11	18.6 - 32.1	122.6	18.0	99.6 - 158.4
Upper Turure	18	44.7	6.01	33.1 - 57.3	21.4	6.04	7.8 - 33.3	123.6	15.1	112.7 - 166.4
Paria	11	43.3	9.43	37.1 - 69.3	22.7	8.38	16.3 - 45.5	109.6	6.55	99.6 - 121.5

Table 3.4. Population identity, sample size, mean and estimates of intraspecific variation (standard deviation (SD) and range) for sperm morphology (total length, head length, midpiece length, and flagellum length) of male guppies (*Poecilia reticulata*) in Trinidad.

Population	N	Head ( $\mu\text{m}$ )			Midpiece ( $\mu\text{m}$ )			Flagellum ( $\mu\text{m}$ )			Total ( $\mu\text{m}$ )		
		Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Lower Aripo	8	4.3	0.077	4.2-4.4	6.2	0.32	5.6-6.6	44.8	1.4	43.3-46.4	55.2	1.2	53.6-56.7
Upper Aripo	10	4.2	0.082	4.0-4.3	5.7	0.26	5.2-6.1	45.6	0.48	44.8-46	55.4	0.48	54.6-56.4
Lower Quare	10	4.2	0.13	4.1-4.5	6.0	0.49	5.4-6.8	43.3	0.97	41.8-44.5	53.5	1.2	51.8-55.4
Upper Quare	8	3.9	0.15	3.7-4.1	4.8	0.40	4.3-5.4	41.8	0.93	40.3-42.9	50.5	0.80	49.3-51.6
Lower Oropuche	8	4.5	0.16	4.2-4.7	5.8	0.77	4.6-7.0	45.6	1.4	43.2-47.2	55.9	0.88	54.7-57.0
Upper Oropuche	8	4.4	0.15	4.1-4.5	5.7	0.79	4.5-6.7	44.2	1.4	41.9-45.8	54.2	0.86	53.1-55.3
Lower Turure	10	4.4	0.094	4.2-4.5	6.0	0.35	5.5-6.7	44.1	1.7	41.4-45.7	54.5	1.7	51.7-56.4
Upper Turure	10	4.3	0.092	4.2-4.5	5.8	0.41	5.3-6.5	45.6	0.68	44.6-46.7	55.7	0.71	55.1-57.3
Tacarigua	10	4.3	0.11	4.1-4.4	6.2	0.17	5.8-6.4	43.5	0.31	43.0-43.9	54.0	0.31	53.5-54.5
Tunapuna	10	4.4	0.20	4.1-4.7	6.1	0.69	4.9-7.2	44.1	1.0	41.7-45.7	54.5	0.72	53.0-55.5
Paria	10	4.2	0.17	3.9-4.4	6.3	0.60	5.3-7.2	41.4	1.1	40.1-43.3	51.9	1.1	50.5-53.9



Table 3.5. Pearson correlations among body size and sperm traits in male guppies (*Poecilia reticulata*), including velocity, number, total length, head length, midpiece length and flagellum length. Sperm velocity measures included average path velocity (VAP = average velocity on the smoothed cell path), straight line velocity (VSL = average velocity on a straight line between the start and end points of the track) and curvilinear velocity (VCL = average velocity on the actual point-to-point track followed by the cell).

			Velocity ( $\mu\text{m/s}$ )			log Sperm number	log Body length	log Head length	log Midpiece length	log Flagellum length	log Total length
			VAP	VSL	VCL						
Velo-city ( $\mu\text{m/s}$ )	V	R <sup>2</sup>	1	.92	.28	.12	.38	-.052	.17	-.034	.10
	A	P		<.001	.051	40	.008	.72	.24	.81	.49
	P	N	49	49	49	49	48	49	49	49	49
	V	R <sup>2</sup>	.92	1	-.05	-.29	-.36	.018	-.12	.006	-.036
	S	P	<.001		.73	.044	.013	.90	.42	.97	.80
	L	N	49	49	49	49	48	49	49	49	49
	V	R <sup>2</sup>	.28	-.050	1	.17	-.096	.12	.24	-.051	.15
	C	P	.051	.73		.25	.52	.42	.097	.73	.32
	L	N	49	49	49	49	48	49	49	49	49
log Sperm number	R <sup>2</sup>	-.12	-.29	.17	1	.38	.36	.034	-.15	-.19	
	P	40	.044	.25		.009	.010	.82	.31	.19	
	N	49	49	49	49	48	49	49	49	49	
log Body length	R <sup>2</sup>	-.381	.36	-.096	.38	1	.23	.32	-.095	.004	
	P	.008	.013	.52	.009		.12	.025	.52	.98	
	N	48	48	48	48	48	48	48	48	48	
log Head length	R <sup>2</sup>	-.052	.018	-.12	-.36	.23	1	.19	.52	.66	
	P	.72	.90	.42	.010	.12		.18	<.001	<.001	
	N	49	49	49	49	48	49	49	49	49	
log Midpiece length	R <sup>2</sup>	.17	.12	-.24	.034	.32	.19	1	-.20	.17	
	P	.24	.42	.097	.82	.025	.18		.17	.23	
	N	49	49	49	49	48	49	49	49	49	
log Flagellum length	R <sup>2</sup>	-.034	.006	-.051	-.15	-.095	.52	-.20	1	.93	
	P	.81	.97	.73	.31	.52	<.001	.17		<.001	
	N	49	49	49	49	48	49	49	49	49	
log Total length	R <sup>2</sup>	.10	-.036	.15	-.19	.004	.66	.17	.93	1	
	P	49	.80	.32	.19	.98	<.001	.23	<.001		
	N	49	49	49	49	48	49	49	49	49	

## Figure captions

Figure 3.1. (a) Estimated marginal means ( $\pm$ SE) of sperm number across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm number (after controlling for body length) across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.2. (a) Estimated marginal means ( $\pm$ SE) of sperm velocity across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm velocity across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.3. (a) Estimated marginal means ( $\pm$ SE) of sperm head length across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm head length across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.4. (a) Estimated marginal means ( $\pm$ SE) of sperm midpiece length across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates

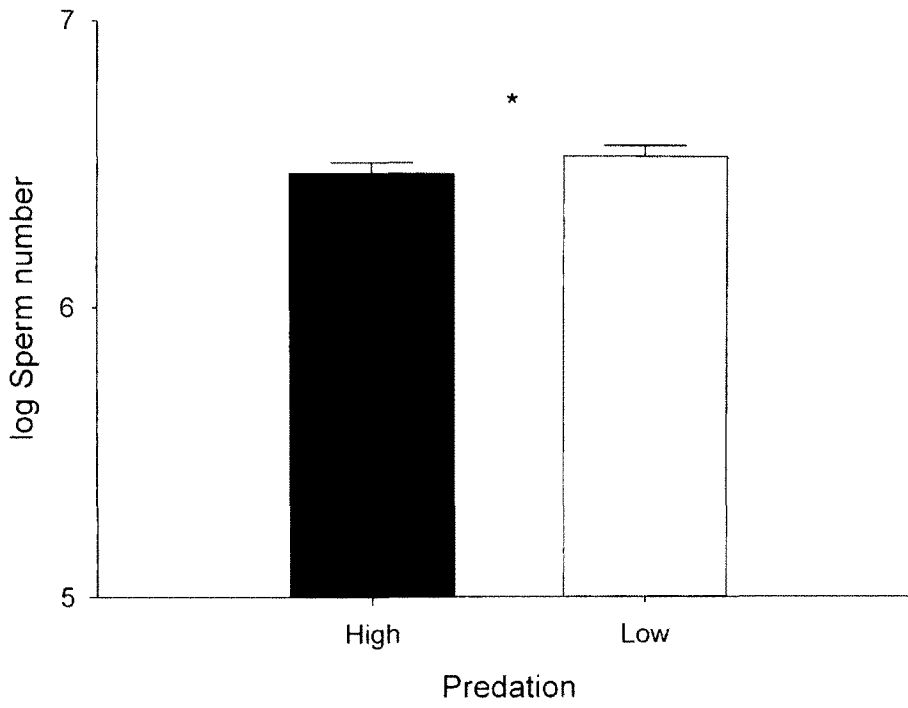
statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm midpiece length across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.5. (a) Estimated marginal means ( $\pm$ SE) of sperm flagellum across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm flagellum across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.6. (a) Estimated marginal means ( $\pm$ SE) of sperm total length across 11 populations of *Poecilia reticulata* in relation to predation intensity. An asterisk indicates statistical significance at  $p = 0.05$ . (b) Mean ( $\pm$ SE) sperm total length across 11 populations of *P. reticulata* paired by river, comparing high predation (black bars) and low predation (grey bars) regions.

Figure 3.1

a



b

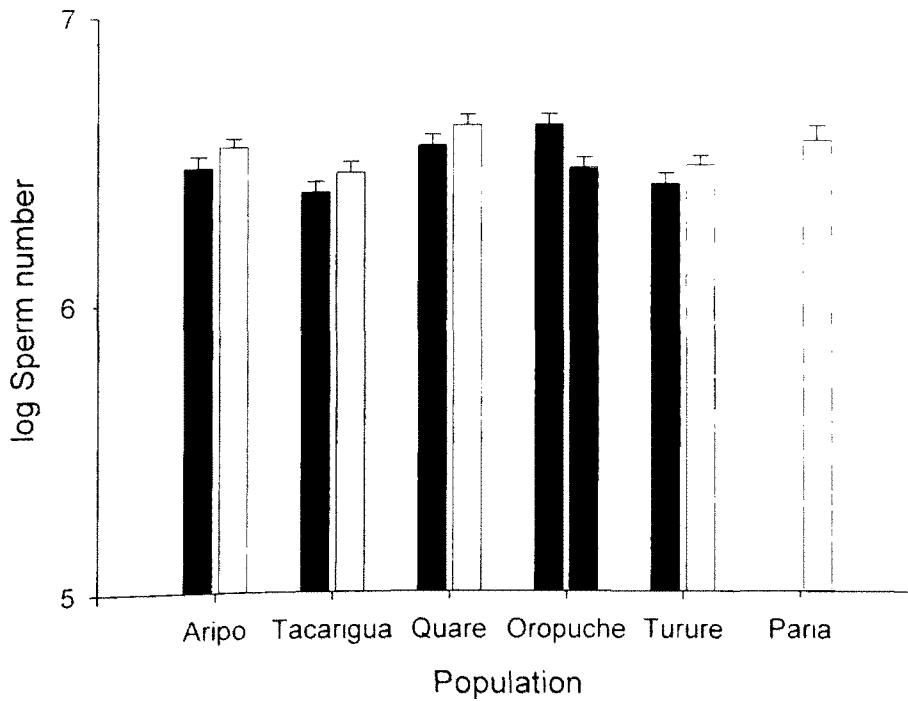
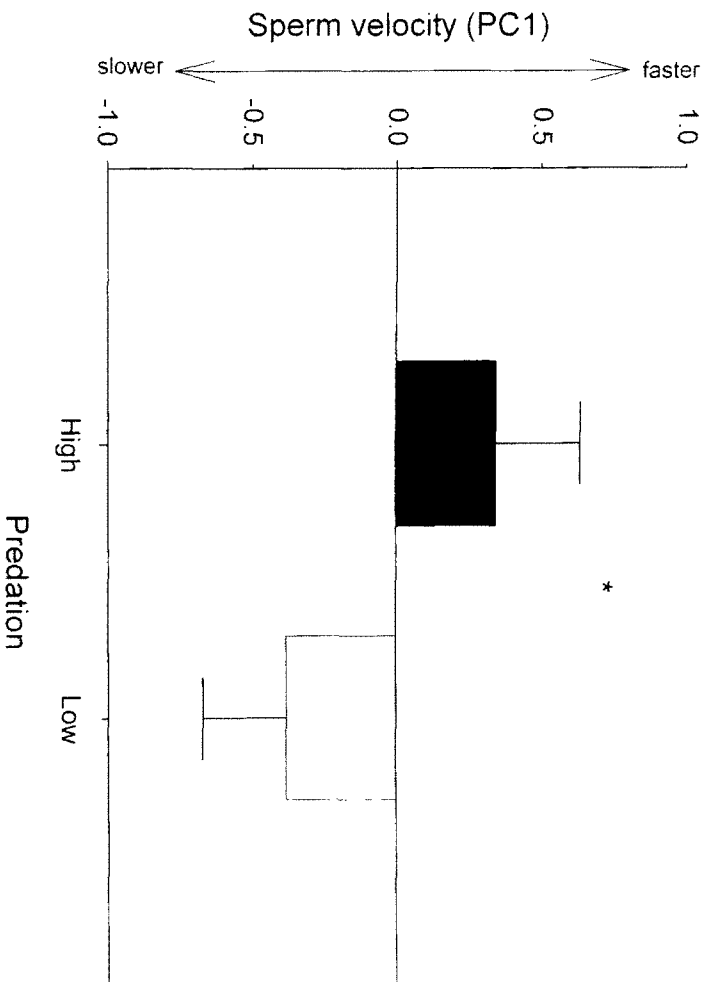


Figure 3.2

a



b

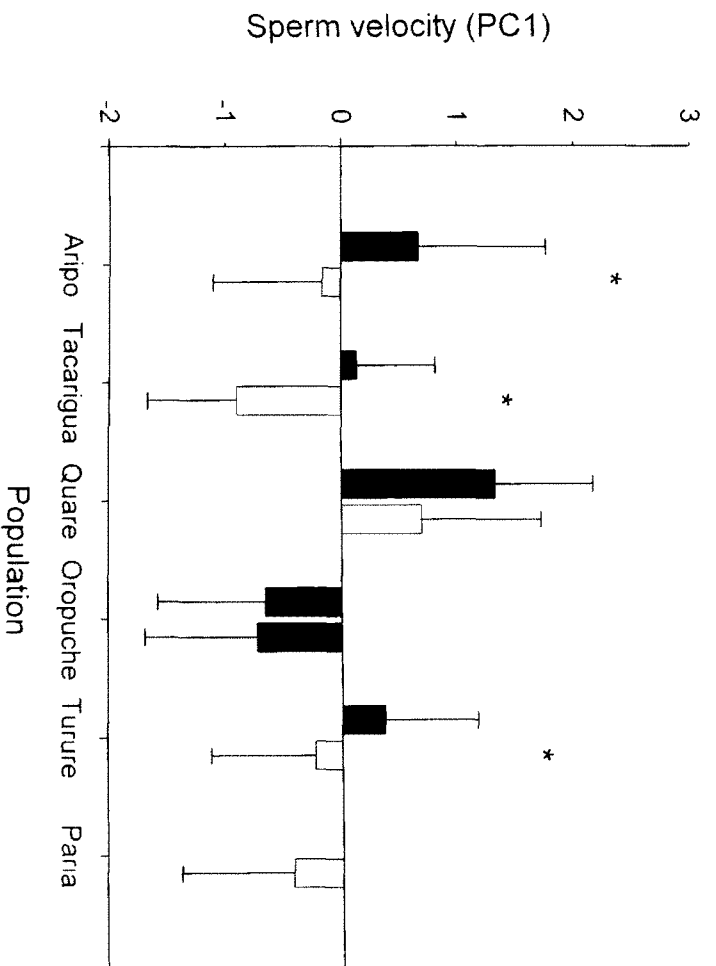
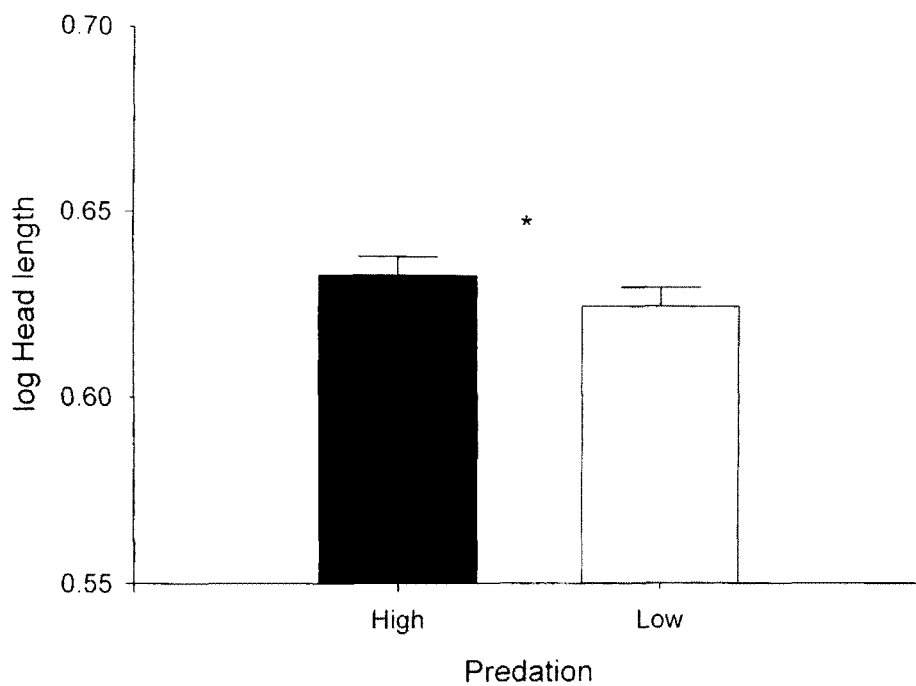


Figure 3.3

a



b

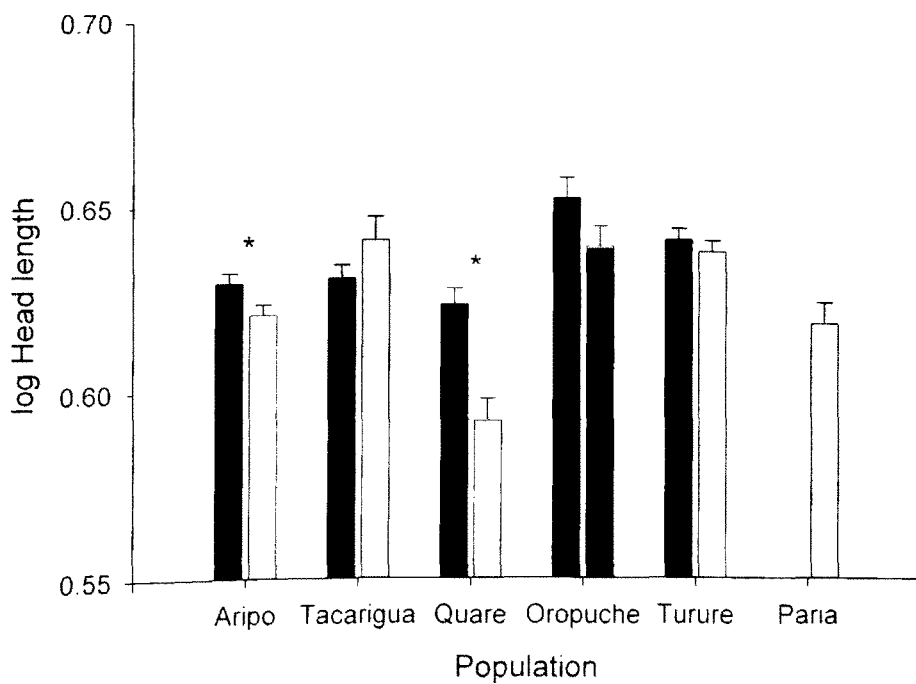
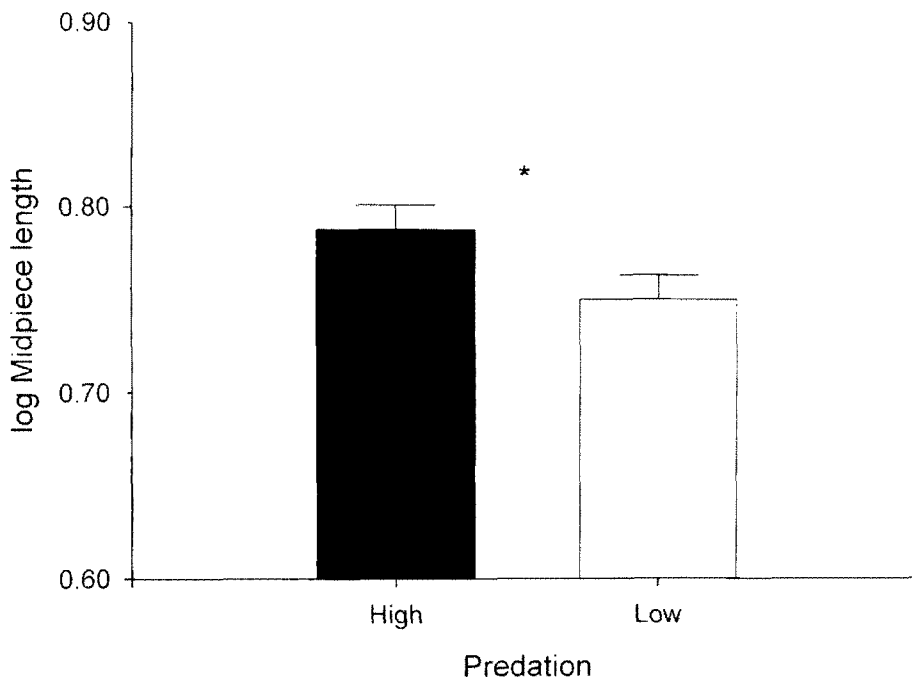


Figure 3.4

a



b

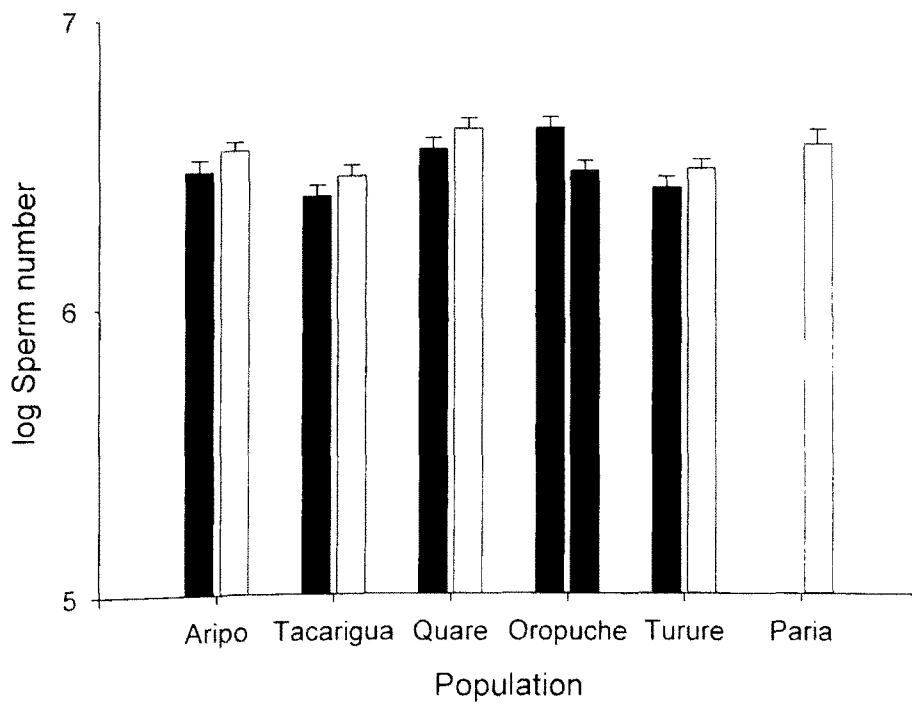
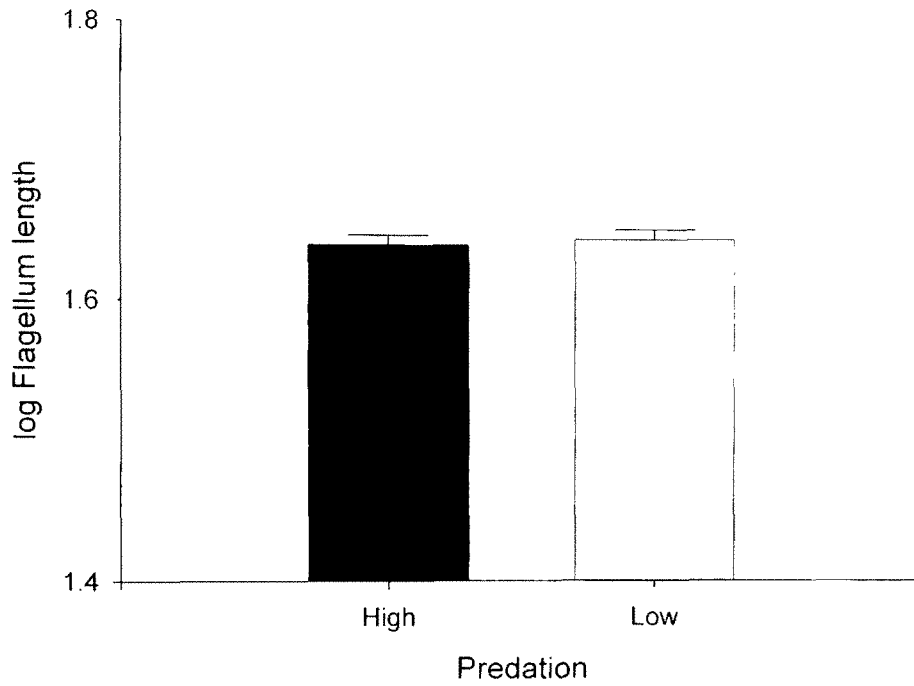


Figure 3.5

a



b

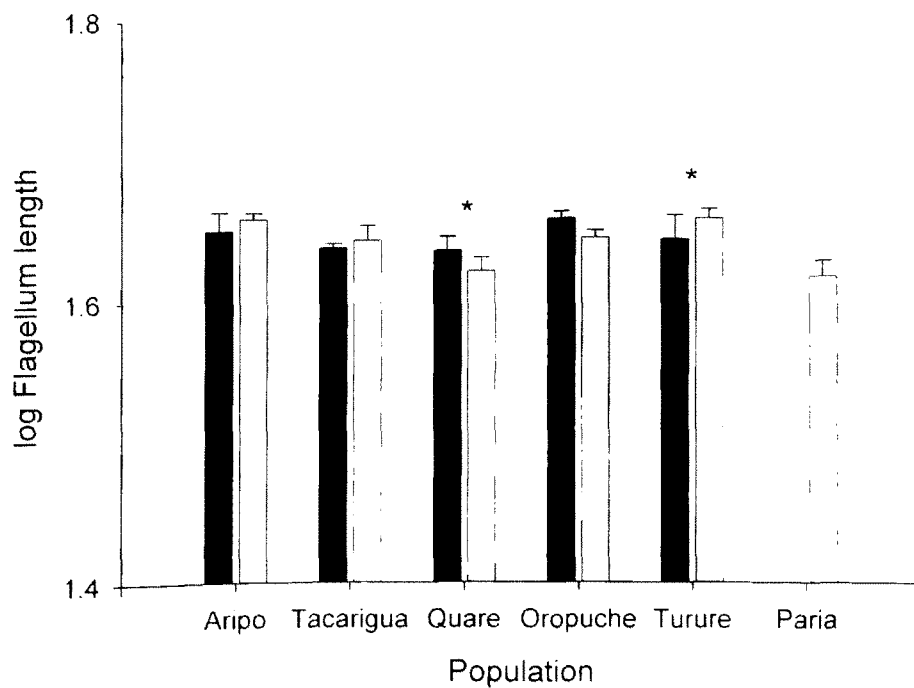
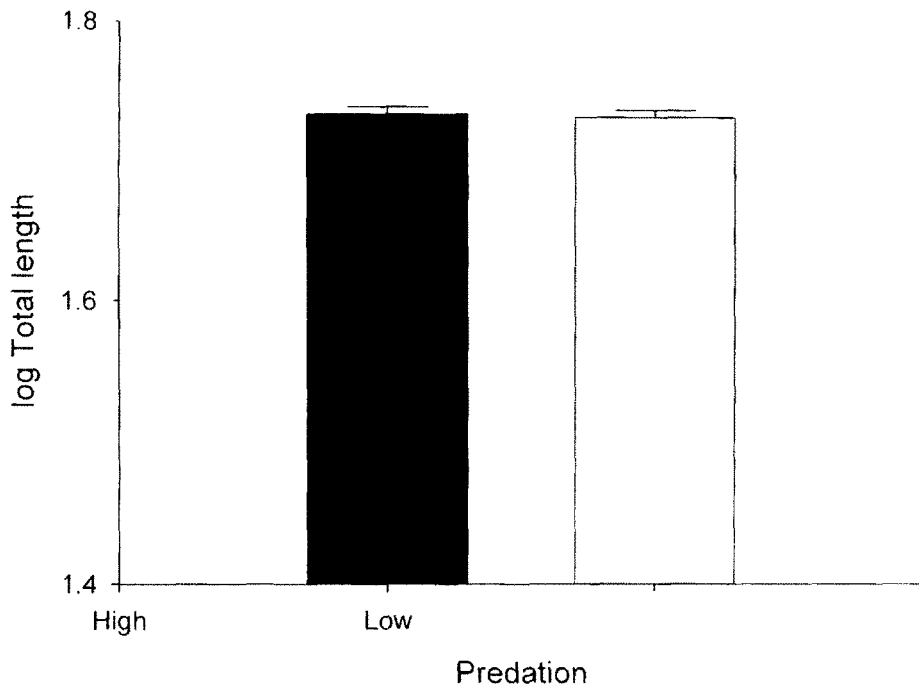


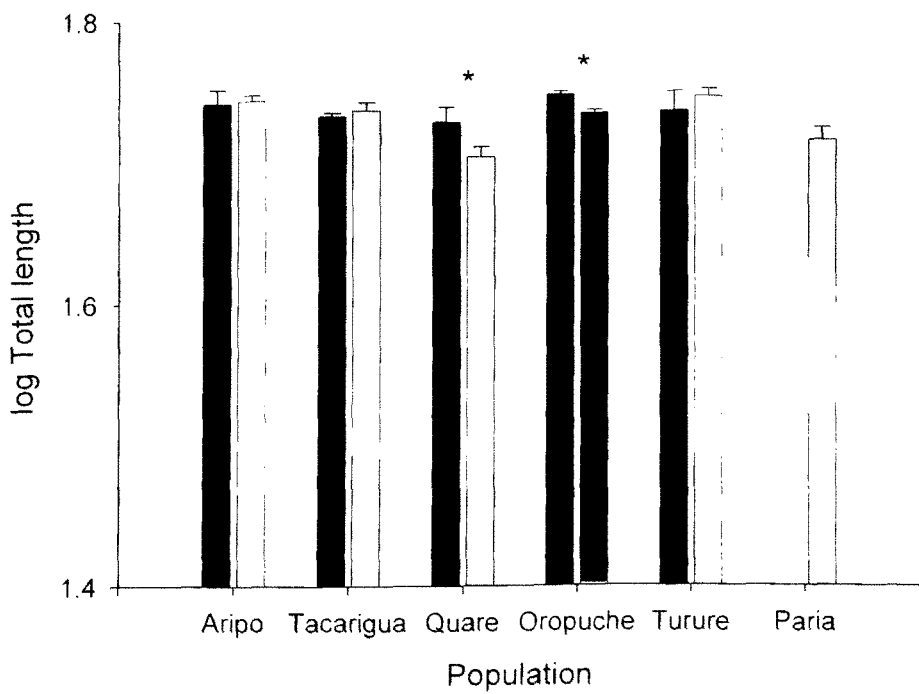


Figure 3.6

a



b



## CHAPTER 4: GENERAL DISCUSSION

### Summary

The overarching theme of my thesis was to investigate sexual selection on male traits in the guppy (*Poecilia reticulata*) and to identify traits subject to sexual selection. My first objective was to determine which traits contribute to male reproductive success in a wild population. In Chapter 2, I found that male reproductive success was strongly skewed, while female reproductive success was more evenly distributed, a pattern indicative of stronger sexual selection on males than on females. Male relative reproductive success was significantly correlated with gonopodium length, but not with the relative area of orange, black or iridescent colouration, body length, or sperm velocity. My second objective was to determine the role of sperm competition in shaping sperm form and function by comparing sperm traits across several populations experiencing different levels of sperm competition. I found that males in high predation populations, which experience more intense sperm competition, had significantly faster sperm with longer midpieces than males in low predation populations, which experience less intense sperm competition.

### Reproductive Skew and Correlates of Male Reproductive Success

Guppies have long been used as a model system for the study of female choice (reviewed in Houde 1997; Magurran 2005), although the consequences of female preferences in terms of male reproductive success have rarely been examined. In Chapter 2, I outline the first investigation of male reproductive success in the wild, and it suggests that female choice is not likely to be as important in this mating system as was previously

thought. Male traits that have been identified as being subject to female choice, such as the area of orange (Houde 1987; Endler & Houde 1995; Brooks & Caithness 1995a), black (Brooks & Caithness 1995b) and iridescent (Kodric-Brown 1985; 1993) colouration and body size (Reynolds & Gross 1992; Magellan *et al.* 2005), were not important predictors of reproductive success. The only male trait that was linked to reproductive success was gonopodium length. It is possible that the relationship between gonopodium length and male reproductive success is the result of female choice. Brooks and Caithness (1995) found some evidence of female preference for gonopodium length in guppies, and Langerhans *et al.* (2005) demonstrated female preference for gonopodium length in two other Poeciliid species, *Gambusia hubbsi* and *Gambusia affinis*.

A more likely mechanism of sexual selection on gonopodium length is gonopodial thrusting. Gonopodium length has been linked to success at gonopodial thrusts in guppies (Evans *et al.* 2009), and males from high predation populations employ gonopodial thrusts at a relatively higher rate and have longer gonopodia than other populations (Kelley *et al.* 2000; Evans *et al.* 2009). Additionally, across the Poeciliid family, species in which males employ gonopodial thrusts as their sole reproductive tactic have longer gonopodia than those that also employ courtship and female choice (Constanz 1989). Attitudes regarding the importance of sneaky mating in guppies have undergone dramatic shifts throughout the history of guppy research. Initially it was assumed that it was the only form of copulation, since female responsiveness to males had not been observed (Breder & Coates 1935). This view was replaced by one of strict female control, once it was demonstrated that gonopodial thrusting only rarely results in sperm transfer (Clark & Aronson 1951; Kadow 1954; Baerends *et al.* 1955), and that

females show preferences for male colour patterns, particularly the area of orange carotenoid pigmentation (Houde 1987; Endler & Houde 1995; Brooks & Caithness 1995a). My study suggests that in spite of strong female preferences, gonopodial thrusting may be the most important factor determining male reproductive success. In a recent examination of gonopodium shape across several populations, Evans et al. (2009) found a correlation between the width of the apical tip of the gonopodium and the width of the female reproductive tract, suggesting a role for sexually antagonistic selection. This suggests that sexual conflict may be an important mechanism of sexual selection in guppies, although this has yet to be tested and further research on female adaptations for avoiding gonopodial thrusts is necessary. Guppies may offer an appropriate system with which to investigate the role of sexual conflict in the diversification of male genital morphology, for which there is limited evidence (Eberhard 2004). Evidence for a role of sexual selection in shaping male genitalia has been demonstrated in a number of insect species (Cordoba-Aguilar 1999; House & Simmons, 2003) and in a comparative analysis in rodents (Ramm 2007).

Sexual selection on male traits results from strong reproductive skew in males (Bateman 1948; Trivers 1972; Shuster 2009), as is evident in the extreme sexual dimorphism of highly polygynous species (Clutton-Brock & Vincent, 1991; Shuster 2009). In most species, greater initial investment by females than males in each offspring leads to greater reproductive potential in males, resulting in intense competition among males and a large degree of reproductive skew (Bateman 1948; Trivers 1972; Shuster 2009). In Chapter 2, I found that this holds true for guppies, as male reproductive success was highly skewed, with most males having sired few or no offspring, while

female reproductive success was relatively evenly distributed and related primarily to body size. This high skew was reflected in the relatively high standardized variance in male reproductive success ( $I_m = 0.97$ ) compared to the relatively low variance in female reproductive success ( $I_f = 0.07$ ). Male guppies appear to have intermediate  $I_m$  values compared to the two other species for which data is available, the Atlantic salmon (*Salmo salar*) with an  $I_m$  of 0.59 (Garant et al. 2001) and the Green swordtail (*Xiphophorus helleri*) with an  $I_m$  of 2.50 (Tatarenkov et al. 2008).

### **Sperm competition**

Predation is known to be an important ecological variable shaping the behaviour of many species, including reproductive behaviour (Lima & Dill, 1990; Magurran & Seghers 1990; Sih et al. 1990; Magnhagen 1991; Sih 1994; Rohr & Madison 2001; Lodé et al. 2004). One variable that can co-vary with predation intensity is the rate of multiple mating by females, and hence the risk or intensity of sperm competition (e.g. Lodé et al. 2004). Multiple mating rates are known to vary with predation intensity in the guppy. Males in high predation populations employ gonopodial thrusts at a higher rate than males in low predation populations (Luyton & Liley 1985; Magurran & Seghers 1990), which suggests that the intensity of sperm competition is greater when predation is higher. This assertion is supported by studies examining multiple paternity rates in the wild (Kelly et al. 1999; Neff et al. 2007). In Chapter 3, I demonstrated that males in high predation populations have faster sperm with longer midpieces, suggesting a role of sperm competition in shaping these traits. Experiments competing sperm from two males using artificial insemination have demonstrated that sperm velocity predicts fertilization success (Evans et al. 2003; Locatello et al. 2006; Pitcher et al. 2007; Boschetto, Evans &

Pilastro, personal communication, but see Skinner & Watt 2007). Furthermore, the midpiece houses the mitochondria of the sperm cell, which provide the energy required for locomotion (Baccetti & Afzelius 1976), so there may be a functional link between sperm velocity and midpiece length in guppies.

Contrary to these results, in Chapter 2 I found that sperm velocity was not a strong predictor of male reproductive success within a population. It is possible that artificial insemination studies that compete sperm from two different males (Evans et al. 2003; Boschetto, Evans & Pilastro, personal communication) do not accurately reflect the conditions under which sperm compete in wild populations due to differences in the number of competing males. There is evidence that patterns of sperm precedence observed in laboratory experiments can break down when females mate with more than two males (Zeh & Zeh 1994), and Neff et al. (2007) found that the mean number of sires per brood was more than 4 in some guppy populations, suggesting that sperm competition between more than 2 males occurs frequently. It is also possible that mating order effects could obscure the effect of sperm quality on fertilization success, as paternity in guppies is usually biased towards the last male to mate in laboratory studies (Evans et al. 2001).

## **Future Directions**

The relative importance of female choice in guppies requires further investigation. It is already known that female preferences vary considerably among populations (Endler & Houde 1995) and among individuals (Brooks & Endler 2001). Females show a preference for novel mating partners (Eakley and Houde 2004) and there is some evidence that they prefer rare males (Farr 1977). In a small population with low genetic

diversity, these effects could increase in importance and obscure any preferences for particular male traits. My research provides a brief snapshot of male reproductive success, but assessing the same variables in other populations and over longer periods of time would provide a more complete picture.

One avenue of research that has not been pursued in guppies is female choice for genetic compatibility. A significant amount of research on genetic compatibility has focused on selection for diversity at the major histocompatibility complex (MHC), a cluster of genes that regulate the immune response. Greater MHC diversity is associated with stronger immunity (e.g. Arkush et al. 2002; Penn et al. 2002), and in many species, it has been demonstrated that females are able to identify and preferentially mate with males with different MHC alleles than their own in order to maximize MHC diversity in their offspring. (e.g. Wedekind et al. 1995; Blomqvist et al. 2002; Freeman-Gallant et al. 2003; Foerster et al. 2003). Mate choice experiments that allow females to choose between males with similar and dissimilar MHC alleles could provide insight into the role that genetic compatibility plays in guppies.

The size and shape of the gonopodium in relation to male reproductive success also requires further investigation. Studies on insects have demonstrated that particular features of male genitalia can have distinct roles (Cordoba-Aguilar 1999; House and Simmons, 2003), and something similar could be at play in the guppy. For example, Cheng (2004) found that removal of the hook resulted in decreased insemination success in guppies, and Evans et al. (2009) found that males with longer gonopodia were more successful at achieving contact with the female gonopore than males with shorter gonopodia.

My results in Chapter 3 suggest that sperm competition in guppies selects for greater sperm velocity and midpiece length. An assumption inherent in Chapter 3 is that the population level differences in sperm traits have a genetic basis. This could be tested by conducting transplant experiments that relocate males from low predation populations to high predation streams and vice versa. Any observed changes in the sperm of transplanted males would be indicative of phenotypic plasticity and not evolutionary change, which would place my conclusions regarding Chapter 3 into question. Another important assumption to test would be the heritability of sperm traits, which have been shown to be high in other species (Birkhead et al. 2009). An additional test for the link between sperm competition and predation intensity would be to compete sperm of males from high and low predation environments using the artificial insemination methods employed by Evans et al. (2003). If the observed differences in sperm traits between high and low predation populations are due to more intense sperm competition, then sperm of males from high predation populations should out-compete the sperm of males from low predation populations.



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