SPERM COMPETITION IN FISH

SPERM COMPETITION IN FISH

By

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ABSTRACT

Sperm competition, the contest between sperm from rival males for fertilizations, is an important evolutionary force shaping sperm characteristics. Theory predicts that males experiencing elevated levels of sperm competition will invest more in sperm number, size and speed. While broad support exists for the idea that elevations in sperm competition lead to increased investment in sperm production, there is mixed support for the role of sperm competition in shaping sperm size and swimming speed. In this thesis, using a combination of within-species and comparative studies, I describe how sperm competition has influenced sperm traits in fishes and critically test a number of predictions from sperm competition theory. In the marine plainfin midshipman fish (*Porichthys notatus*) and the fresh-water shell brooding cichlid *Telmatochromis vittatus,* I show that the males who experience the highest level of sperm competition had faster but not longer sperm. Instead, selection appears to have acted on sperm energetics, increasing energy production to drive sperm movement in males who experience more intense levels of sperm competition. In a comparative study using Tanganyikan cichlids, I show that males in species experiencing high levels of sperm competition (i.e. promiscuous species) had both longer and faster sperm than males of closely related species unlikely to experience sperm competition (i.e. monogamous species). I also uncovered a predicted but previously inadequately tested relationship between sperm size and speed. This relationship holds across, but not within, species and I discuss possible explanations for differences between and within species. Finally, I used directional tests of trait evolution to assess how selection acts to increase sperm swimming speed and provide evidence that the evolution of fast swimming sperm preceded the evolution of long sperm across

cichlid fishes. Together, the results of this thesis show that sperm competition promotes the evolution of faster swimming sperm in fishes and highlights the importance of sperm energetics in determining the competitive success of ejaculates.

ACKNOWLEDGMENTS

The entirety of this thesis was made possible by the guidance, patience, and mentorship of Sigal Balshine and above all I would like to extend my heartfelt thanks for all that she has offered me over the last four years. For an aspiring field biologist my time with Sigal could not have been better. My graduate degree was book ended with two field seasons with Sigal. Within two weeks of starting graduate school I found myself underwater in central Africa wondering if Sigal was regretting her decision to take such an inexperienced young student out to the field. Within two months of handing in my thesis Sigal and I were getting muddy on beaches in British Columbia. I'd like to think that I matured scientifically over the four years separating these field seasons and if I did I owe that maturation in large part to Sigal's mentorship. Sigal's optimism and excitement for science were more than contagious—they made the work enjoyable and helped maintain a level of excitement no matter how tedious the task seemed at the time. One of the greatest gifts she gave me was the freedom to follow new ideas and the confidence to let those ideas grow into different research questions. In every case she acted as a safety net that kept me on target.

I was particularly fortunate while at McMaster to have two homes on campus and I would like to thank Chris Wood for graciously taking me into his lab in Biology. What started as a loose association, where neither Chris nor I had any idea of what the other was doing, soon grew into a collaborative venture. But this dry description does not begin to capture the relationship that developed. Chris generously invited me to join his lab group in Bamfield and before long I was a full fledged member of his lab. And while I am extremely happy with the science that we did together during my time at McMaster I

am equally pleased with the friendship that grew between us. Chris offered his advice and expertise without question and very gently introduced me to the world of physiology.

I am also extremely grateful to Bob Montgomerie who took me into his lab and helped me navigate the world of sperm competition. From the first day that I turned up at Bob's office in Kingston he made me feel at home. He nurtured my ideas and with extreme patience brought me up to speed in the world of statistics. Working with Bob was always enjoyable and I always left our meetings with a smile. In a very humble way Bob offered me amazing opportunities but made me feel like it was I who was doing him the favour—although the reality of the situation was the complete opposite. I will look forward to our continued collaborative projects in the coming years.

Working with so many wonderful people was one of the most rewarding aspects of my graduate studies. Susan Marsh-Rollo buttressed every aspect of the work carried out over the last few years. She was an ever-present rock that was eternally there for me. Susan and I grew together in the lab and I'm excited by the knowledge that our academic relationship is only just beginning. Upon arriving in Hamilton, Julie Desjardins quickly became my closest friend and mentor. Julie taught me more than I could imagine and introduced me to graduate life and, more generally, to the way that science works. I was fortunate to not only share a house with Julie but to spend two field seasons working with her in Zambia. But the best part about my relationship with Julie is that I have found a life-long friend that is even more passionate about behavioural ecology than I am. I also developed a close bond with Carol Bucking, whose friendship and support made my time at McMaster more enjoyable than I thought possible. Carol and I drove across Canada twice, spent two field seasons together and shared a house for two years, and I enjoyed

every aspect of it all. Paul Craig made my time as McMaster particularly enjoyable. From the moment I met Paul I felt like I had found a kindred spirit. And in the years that followed he never stopped making me laugh. Paul's perpetual optimism and desire to have fun made working with him as enjoyable as spending time at the Phoenix with him.

I also want to thank Jim Quinn for lending his supportive ear whenever needed and for being an inspirational role model, Kelly Stiver for being an excellent lab and field mate and teacher, Freddie Chain for constantly offering his friendship and for making my life easier by introducing me to powerful methods of data analysis, Sunita Nadella for being an amazing friend and teaching me humility when cooking Indian food, Niclas Kolm, Iqbal Setiadi and Ben Evans for showing me the ropes in phylogenetic analyses, Ishna Iftikar for her enthusiasm and ability to make everything more enjoyable, Grant McClelland for patiently answering my bizarre questions driven by a total ignorance for all things physiological, the members of the Balshine lab, particularly Julie Marentette, Vicky Mileva and Maz Wong, and the many members of the Wood lab for their support.

Finally, I would like to thank my parents for supporting everything I have done over the past 28 years. Their constant love and reassurance helped me more than I could ever know.

THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format approved by McMaster University and consists of six chapters. **Chapter 1** provides a general overview of the field of sperm competition and describes the goals of this thesis. **Chapters 2-5** are manuscripts that have been either already published (**Chapter 4**), in press (**Chapter 2**), or are soon to be submitted to a journal (**Chapters 3** and **5**). **Chapter 6** summarized the results of this thesis and suggests scope for future research.

CHAPTER 1: **Sperm competition and selection on sperm traits**

Author: John L. Fitzpatrick

CHAPTER 2: **Testes, sperm and sperm competition**

Authors: Robert Montgomerie and John L. Fitzpatrick

Publication: *Reproductive Biology and Phylogeny of Fishes* (in press)

Comments: This book chapter was based on ideas generated by both JLF and RM. JLF contributions included summarizing how sperm competition influences sperm design and speed and RM contribution was to summarize how sperm competition influences testes size and strategic ejaculate investment. The manuscript was thoroughly edited by both authors.

CHAPTER 3: **Sperm competition in a singing fish**

Authors: John L. Fitzpatrick, Carol Bucking, Paul Craig, Chris M. Wood,

Sigal Balshine

Publication: Manuscript will be submitted to Proceedings of the Royal Society London B

Comments: This study was conducted, analyzed and written by JLF under the supervision of CMW and SB. SB, CB and PC assisted in data collection in the field.

CHAPTER 4: **Reproductive tactic-specific variation in sperm swimming speeds in a shell-brooding cichlid**

Authors: John L. Fitzpatrick, Julie K. Desjardins, Nicole Milligan, Robert Montgomerie, Sigal Balshine

Publication: Biology of Reproduction 77: 280-284 (2007)

Comments: This study was conducted, analyzed and written by JLF under the supervision of SB. Data analyses were performed in RM's laboratory. JKD and NM assisted in collection of field data.

CHAPTER 5: **Promiscuity promotes the evolution of faster swimming sperm**

Authors: John L. Fitzpatrick, Robert Montgomerie, Julie K. Desjardins, Kelly A. Stiver, Niclas Kolm, Sigal Balshine

Publication: Manuscript will be submitted to Current Biology

Comments: This study was conducted, analyzed and written by JLF under the supervision of SB. RM provided the tools for measuring sperm swimming speed and wrote a script for the phylogenetic analysis.

NK assisted in phylogenetic analyses. JKD and KAS assisted in collection of field data.

CHAPTER 6: **How sperm competition in fish shapes sperm traits**

Author: John L. Fitzpatrick

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CHAPTER 1

SPERM COMPETITION AND SELECTION ON SPERM TRAITS

1.1 A BRIEF HISTORICAL PERSPECTIVE ON SPERM COMPETITIOIN

Evolution by natural selection acts by promoting the transmission of an individual's genes from one generation to the next (Andersson 1994). However many species have traits that seemingly reduce an individual's ability to survive and reproduce. For example, bright colouration or extensive ornamentation may make an individual more conspicuous to predators and therefore reduce survivorship. Far from being disadvantageous many traits that seemingly reduce fitness actually increase an individual's fitness by making them preferred mates by the opposite sex. Typically, species are characterized by competition between males for access to choosy females and this sexual selection can promote the evolution of traits that increase a male's attractiveness to a female or increase a male's ability to compete in male-male contests (Darwin 1871). However, Darwin's view of sexual selection was steeped in the notion of female sexual monogamy, leading to the prevailing view that male-male competition ended at copulation (Birkhead 2000). This view dominated studies of sexual selection during Darwin's era and guided the field for the next hundred years. A dramatic shift in our understanding of sexual selection took place following the advent of molecular techniques the degree of promiscuity in animals became readily apparent and highlighted the extent of female polyandry that occurs in animals (Birkhead and Møller 1998; Simmons 2001). Today,

female polyandry is recognized as the norm in animal societies rather than the exception (Jennions and Petrie 2000). An important consequence of female polyandry is that sperm from more than one male can overlap in time and space and thus can compete for access to a female's eggs, leading to sperm competition.

Sperm competition was first described in detail by Parker (1970) and is now recognized as a dramatic evolutionary force shaping male behaviour, anatomy and physiology (Birkhead and Møller 1998; Simmons 2001). Parker's revolutionary perspective that intra-sexual competition continues after copulation in the form of sperm competition provided enormous insights and, over time, developed into its own subfield in behavioural ecology. Over the past 40 years the field of sperm competition has grown exponentially, spurred on by a series of theoretical papers by Parker and his colleagues that sought to address how sperm competition influences sperm number, sperm size (which was thought to correspond to sperm speed) and strategic allocation of available sperm stores (reviewed in Parker 1998).

1.2 RESPONSES TO SPERM COMPETITION: THEORY

To promote individual fitness one common response to elevated levels of male-male competition is for males to increase mate guarding, provided females are readily defendable (Parker 1970). By actively guarding a mated female, males can decrease the likelihood of their ejaculates experiencing sperm competition and may be able to ejaculate smaller number of sperm but still ensure fertilizations (Alonzo and Warner 2000). As sperm production can be costly (Dewsbury, 1982; Olsson et al., 1997) and sperm reserves are depleted over successive matings (Nakatsuru & Kramer, 1982;

Preston et al., 2001), it is often more beneficial for males who successfully court females to practice intense mate guarding rather than continuously engage in sperm competition with rival males (Alonzo and Warner 2000).

A rich theoretical framework has been developed which explores how sperm competition influences ejaculate trait evolution (reviewed in Parker 1998). Although specific predictions can depend on the mode of fertilization (internal vs. external) as well as what assumptions are made about how ejaculate traits influence fertilization success, some general predictions are readily apparent. Sperm number is expected to increase with the magnitude of sperm competition, both within and across species (Parker 1990a,b; Parker et al. 1997). This is because larger testes are able to produce greater numbers of sperm (Gage et al. 1995) and males ejaculating more sperm stand a better chance of fertilizing a female's eggs (Martin et al. 1974). On the other hand, responses in sperm morphology to sperm competition vary depending on how sperm size influences fertilization success (Ball and Parker 1996). Theoretical arguments point out that sperm size is likely positively related to sperm swimming speed, as longer flagella will be able to provide greater propulsive force to move the sperm through a medium faster (Katz and Drobins 1990). Therefore males experiencing greater levels of sperm competition should produce longer sperm (Ball and Parker 1996). Conversely, if longer sperm happens to decrease the competitive ability of sperm by, for example, increasing drag, then as the level of sperm competition increases sperm size should decrease (Ball and Parker 1996). Additionally, when sperm compete numerically then theory predicts that sperm size should not be influenced by the strength of sperm competition, as all males should produce sperm of the minimal size that is still functional in order to produce the greatest

number of sperm (Parker 1982; 1993). Finally, theoretical predictions also address how males should allocate their available sperm stores. Males are expected to increase the number of sperm ejaculated when the sperm competition risk (probability of experiencing sperm competition) is high but sometimes reduce ejaculate size when the intensity (number of competing ejaculates) increases above two competing males (Parker 1998). As this final topic is beyond the scope of this thesis I will not dwell on this here but refer interested readers to an excellent review by Wedell et al. (2002).

1.3 RESPONSES TO SPERM COMPETITION: EMPIRICAL EVIDENCE

As argued above, after expending time and energy courting a female, males often protect their reproductive investment by performing mate guarding behaviours (Birkhead 1998). Mate guarding can take several forms, each of which decreases the probability of a male's ejaculate experiencing sperm competition. Males commonly guard females after mating, with males who guard their mates more effectively enjoying higher fertilization rates and lower levels of extra-pair fertilizations (e.g. black-throated blue warblers, *Dendroica caerulescens*, Chuang-Dobbs et al. 2001). Males also tailor the intensity of mate guarding to coincide with the period where they stand the greatest chance of losing paternity to rivals (e.g. marine sculpin, *Hemilepidotus gilberti*, Hayakawa 2007; Seychelles warbler, *Acrocephalus sechellensis*, Komdeur et al. 1999). In some species, males augment postcopulatory mate guarding by placing a mating plug in the female's genital opening after mating to prevent other males from inseminating the female (Dickinson and Rutowski 1989). A recent comparative study in rodents demonstrated that species experiencing higher levels of sperm competition produced larger mating plugs

suggesting that plug size is related to the magnitude of sperm competition (Ramm et al. 2005). While mate guarding can be an effective means of decreasing the strength of sperm competition in some species, it can be ineffective in others, and in these latter species, where sperm competition occurs, selection may act on sperm characteristics in order to increase fertilization success.

Three lines of convergent evidence provide strong support for the idea that the selective force of sperm competition leads to the evolution of larger testes. First, across a wide variety of species where males experience differential levels of sperm competition the relative investment in testes mass (controlling for body mass) positively covaries with the strength of sperm competition (e.g. mammals: Harcourt et al. 1981; Hosken 1997; Rose et al. 1997; Hosken 1998; Ramm et al. 2005; Parker et al. 1997; birds: Møller 1988, 1991; Birkhead and Møller 1992; Møller and Briskie 1995, amphibians: Kusano et al. 1991; Jennions and Passmore 1993; Byrne et al. 2002, insects: Gage 1994; fishes: Stockley et al. 1997; Balshine et al. 2001). Second, intra-specific examinations of testicular investment between individuals in species where males experience different levels of sperm competition also consistently demonstrate that the males who are most likely to experience higher levels of sperm competition invest more in testes (e.g. Peterson and Warner 1998). Third, powerful selection experiments performed with yellow dung fly (*Scathophaga stercaracia*, Hosken and Ward 2001; Hosken et al. 2001) and *Drosophila melanogaster* (Pitnick et al. 2001) where males were subjected to either monogamous (sperm competition absent) or polyandrous (sperm competition present) selection lines revealed that after as little as 10 generations males that were subjected polyandrous treatments had larger testes than males in monogamous treatments.

Contrasting the near unanimity of the response of testes mass to sperm competition, responses in sperm size to sperm competition are far from consistent. Researchers have used the same three approaches that successfully uncovered responses in testes size – inter-specific comparative studies, intra-specific studies, and experimental selection studies– only to find mixed and even contradictory results in response in sperm size, regardless of the approach taken. Studies across species in invertebrates and vertebrates, ranging from mammals to birds to fish have found positive, negative or no relationship when assessing the relationship between sperm length and sperm competition (reviewed by Snook 2005). Similar contradictions abound when looking within a species and comparing males who experience differential intensities of sperm competition. For example, in bluegill sunfish (*Lepomis macrochirus*) some studies reported differences in sperm size between males who experience differential levels of sperm competition (Burness et al. 2004), while others found no differences (Leach and Montgomerie 2000; Stoltz and Neff 2006). Rearing males in selection lines where the selective force of sperm competition was removed did not influence sperm length (Pitnick et al. 2001; Hosken et al. 2001). Yet sperm size can be an important determinant of fertilization success in competitive mating trials. For example, in the cricket *Gryllus bimaculatus* (Gage and Morrow 2003) and the dung beetle *Onthophagus taurus* (Garcia-Gonzalez and Simmons 2007), smaller sperm are competitively superior, while longer sperm outcompete shorter sperm in *D. melanogaster* (Miller and Pitnick 2002). However, other studies found no effect of sperm size in determining fertilization success in competitive mating trials (Simmons et al. 2003; Gage et al. 2004). Further hindering our understanding of how selection acts on sperm size is our relatively inadequate grasp of the relationship between

sperm size and swimming performance (Snook 2005). Despite the mixed results regarding sperm length, researchers remain fixed to the idea that sperm swimming speed should be under intense selective pressure. Strong evidence for this idea comes from competitive mating trials of male Atlantic salmon (*Salmo salar*) paires, where the male with the faster swimming sperm successfully fertilized a greater proportion of available eggs (Gage et al. 2004). Thus, there is little consensus in our understanding of how sperm competition influences sperm size and speed.

1.4 MOTIVATION FOR THIS THESIS

When I began my doctoral research program in 2004, the relationships between sperm size, swimming speed and sperm competition were more unresolved and confusing in fishes than in any other taxon. In the first test of theoretical predictions in fishes, Stockley et al. (1997) documented a negative relationship between sperm size and sperm competition in a broad analysis of both internally and externally fertilizing fishes. This result remained the sole evidence in any taxon for such a negative response in sperm size to sperm competition for the next decade (Snook 2005). In the second comparative study to address this issue, Balshine et al. (2001) found the exact opposite pattern, with species experiencing elevated levels of sperm competition having longer sperm. The strength of Balshine et al.'s work rested in the fact that they studied a group of closely related species, endemic to a single lake, and all of the data were collected by a single research group. Therefore, at a comparative level it was far from clear how sperm size, and presumably swimming speed, respond to differential intensities of sperm competition. Within species analyses were equally ambiguous with no clear patterns emerging.

Equally concerning was the fact that only two species had been studied in detail in an effort to test predictions from sperm competition theory, the Atlantic salmon (Gage et al. 1995; 1998; 2002; 2004; Vladic and Jarvi 2001; Vladic et al. 2002) and the bluegill sunfish (Leach and Montgomerie 2000; Burness et al. 2004). Therefore, the applicability of these findings to fishes in particular, and to the wider field of sperm competition in general, remained unknown. Despite some pioneering efforts, in 2004, studies of sperm competition in fishes remained in their infancy and lagged behind the advances seen in other taxa. Thus, fishes offered excellent opportunities for critically testing theoretical predictions and for making sense of, and expanding on, the existing data that were available.

1.5 AIMS OF THIS THESIS

The overarching aim of this thesis is to increase our understanding of how the selective force of sperm competition shapes male reproductive biology in fishes, with a particular emphasis on sperm morphology and motility. Specifically, in this thesis I focused on:

- quantifying how sperm competition influences testes mass, sperm size and sperm performance both within and between species
- uncovering behavioural and physiological adaptations to sperm competition
- clarifying the relationship between sperm morphology and swimming speed

1.6 FISHES AS A MODEL SYSTEM

Fishes are a speciose group of organisms, consisting of at least 28,000 different species (Nelson 2006) that represent a tremendous diversity in mating systems, life-style and environments (Breder and Rosen 1966; Godin 1999). This variation in mating behaviour

within and across species provides the scope for dramatic differences in the magnitude of sperm competition among species (Stockley et al. 1997; Taborsky 1994). Fishes also exhibit extensive variability in sperm design, as sperm can have either zero, one or two flagella (Jamieson 1991). As a result, fishes offer unparalleled opportunities for studying sperm competition and for assessing how selection shapes gametic traits. Rather than focus on a single species I have taken advantage of the diversity offered by fishes, focusing on two powerful approaches for studying sperm competition: intra-specific analyses in species characterized by male alternative reproductive tactics and inter-specific analyses in species that experience dramatically different levels of sperm competition.

1.6.1 Intra-specific studies: male alternative reproductive tactics

The strength of sperm competition can vary dramatically between males of the same species, owing to the prevalence of male alternative reproductive tactics (MARTs, Taborsky 1994; Mank and Avise 2006). Species with alternative reproductive tactics offer a powerful tool for studying sperm competition for two main reasons. First, males use divergent methods of achieving fertilization and their gametes are exposed to dichotomous levels of sperm competition (Parker 1990b). In species with MARTs, large males, generally called conventional males, build nests and/or defend territories and actively court females while smaller males, generally called sneaker males, attempt to gain some measure of reproductive success without performing costly defense and courtship behaviours. In these species, sneaker males experience sperm competition with every mating as they do not court females themselves, whereas conventional males only

experience sperm competition when a sneaker male is present. Consequently, the magnitude of sperm competition is greater for sneakers than for conventional males. Second, because these males are members of the same species there is no need to account for species-specific effects that can obscure trends in comparative studies and any observed differences between males can more easily be attributed to the selective force of sperm competition. In this thesis I used two species with MARTs, the plainfin midshipman (*Porichthys notatus*) and the shell-brooding cichlid *Telmatochromis vittatus*, to examine tactic-specific responses in sperm characteristics to sperm competition.

Plainfin Midshipman: The plainfin midshipman has been classically described as the "California singing fish" due to the acoustic courtship calls that some males use to serenade females during the breeding season (Cox 1905). However, other descriptions of midshipman have been less complomentary, with adults being described as "grotesque in appearance and...of dirty brown or greenish color" (Arora 1948). Regardless of its appearance, the midshipman represents an ideal model organism, providing many valuable insights into physiology, neurobiology and behavioural ecology (Brantley and Bass 1994; Bass 1992; 1996). Male midshipman adopt one of two reproductive tactics, conventional Type I males and sneaking Type II males (Brantley and Bass 1994). In this thesis, I focused on tactic-specific responses in sperm characteristics to sperm competition while also examining how the magnitude of sperm competition differs between and within male reproductive tactics. Reproductively mature males were collected from various locations around Vancouver Island and on the mainland around Vancouver, British Columbia, Canada (Fig. 1.1). Previous research on reproduction in midshipman was performed mainly at two sites in the United States: Bodega Bay,

California and Hood Canal, Washington State and, in this thesis, I extended this research by studying the reproductive biology of midshipman from a Canadian location.

Telmatochromis vittatus: This species is a small cichlid fish from Lake Tanganyika that breeds in vacant snail shells. Reproductively mature males were collected from Kasakalawe and Wonzye Point in the southern end of Lake Tangayika, Zambia (Fig. 1.2). *T. vittatus* is characterized by four male tactics (pirate, territorial, satellite and sneaker males; Ota and Khoda 2006). Ota and Khoda (2006a,b) described the reproductive behaviour of the four male tactics, which map on to a size hierarchy, with male body sizes among reproductive tactics rarely overlapping. The largest males in the population take the role of pirate males, followed by smaller territorial males, even smaller satellite males, and finally by sneaker males, the smallest reproductive males in the population. The strict size hierarchy of male reproductive tactics, each experiencing a different level of sperm competition, facilitated an excellent opportunity for assessing how sperm competition influences the evolution of sperm traits.

1.6.2 Inter-specific analyses: Comparative studies of cichlid fishes

Fishes exhibit the most dramatic range of sperm competition among vertebrates due to their diversity in both fertilization locations and mating systems (Stockley et al. 1997). Fertilization in fish ranges from massive breeding assemblages where many hundreds of thousands, or even millions, of individuals shed their gametes simultaneously, as in herring (family Clupeidae; Rajasilta et al. 1993), to species where males transfer sperm to females and fertilization takes place internally, as in guppies (family Poeciliidae, Constantz 1984). But regardless of the mode of fertilization male and female mating

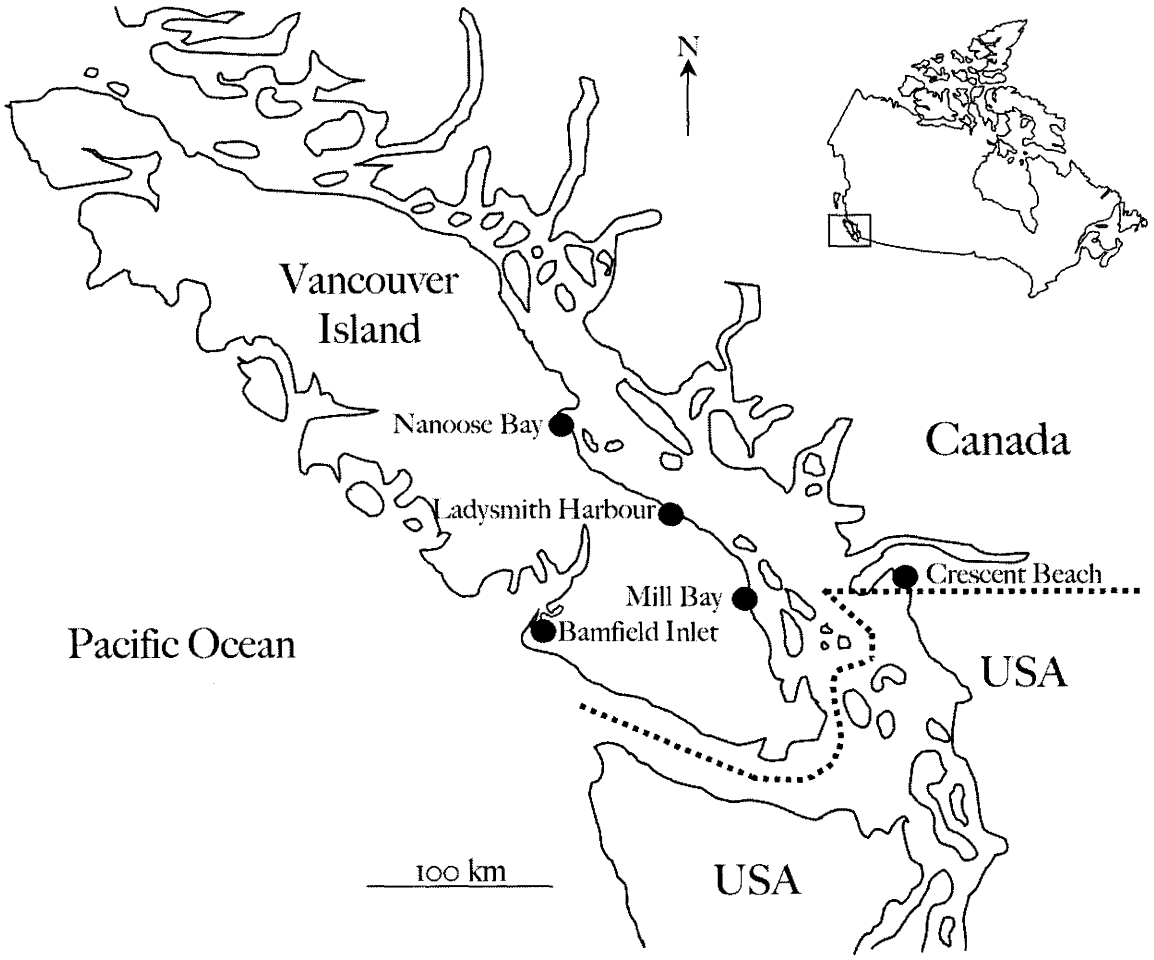

Figure 1.1. Map of Vancouver Island, British Columbia showing (with black circles) the locations of the five field sites used in the studies described in **Chapter 3** of this thesis.

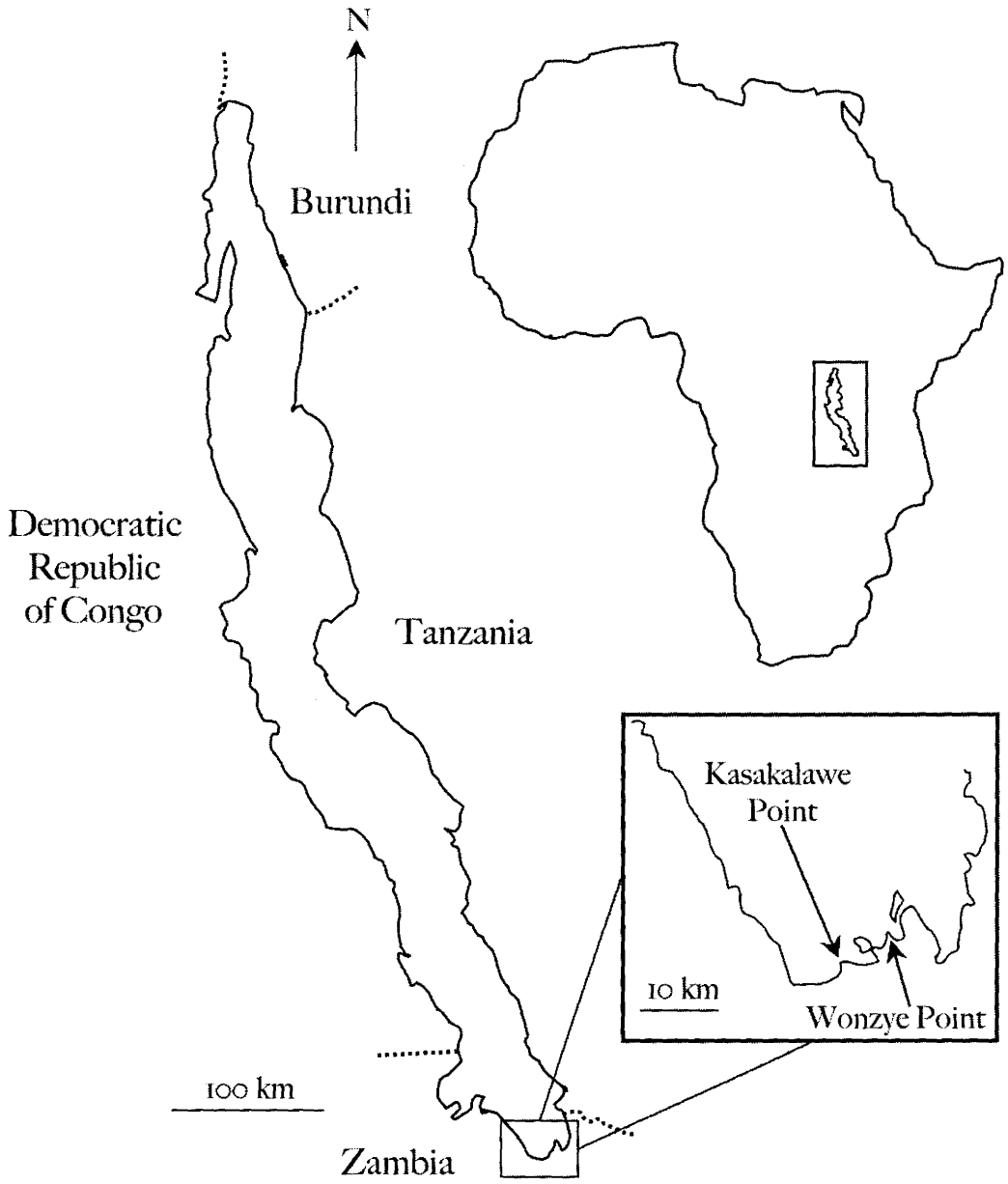

Figure 1.2. Map showing the location of Lake Tanganyika in Africa, the field sites where fish were collected, and the surrounding countries. Modified from Morely and Balshine (2003). Fish were studied and collected from these field sites and described in the study in **Chapters 4** of this thesis.

behaviours modify the strength of sperm competition. While some species practice strict social and genetic monogamy (reviewed by Whiteman and Côté 2004), others are characterized by intense male-male competition during mating and a high degree of female polyandry leading to persistent sperm competition (reviewed by Taborsky 1998; Peterson and Warner 1998). Such diversity facilitates comparative examinations of how the level of sperm competition influences the evolution of sperm traits. Furthermore, recent interest in understanding phylogenetic relationships among families of all fin-rayed fishes (e.g. Mank et al. 2005) as well as phylogenetic exploration of species relationships at the family level (e.g. cichlidae: Cohen et al. 1993; Koblmuller et al. 2004; Day et al. 2007) have allowed researchers to control for evolutionary history when assessing responses in sperm traits to sperm competition in fishes (Stockely et al. 1997; Pyron 2000; Balshine et al. 2001).

Tanganyikan Cichlids: In this thesis I focused my comparative analyses on a group of closely related cichlids from Lake Tanganyika, Africa (Fig. 1.3). For a variety of reasons this group of species is particularly well suited for studying how sperm competition has shaped ejaculates over evolutionary time. First, these closely related species demonstrate an incredible degree of variation in mating system (Kuwamura 1997) and consequently in the level of sperm competition a male is likely to experience (Balshine et al. 2001). Second, because of the explosive speciation observed in African cichlids there is a great deal of information available on the phylogenetic relationships among species (e.g. Staumbauer et al. 2002; Salzburger et al. 2002; Koblmuller et al. 2004; Schelly et al. 2006; Day et al. 2007). This allows for comparative work to be done in a phylogenetically rigorous manner (Felsenstein 1985; Harvey and Pagel 1991; Pagel 1999;

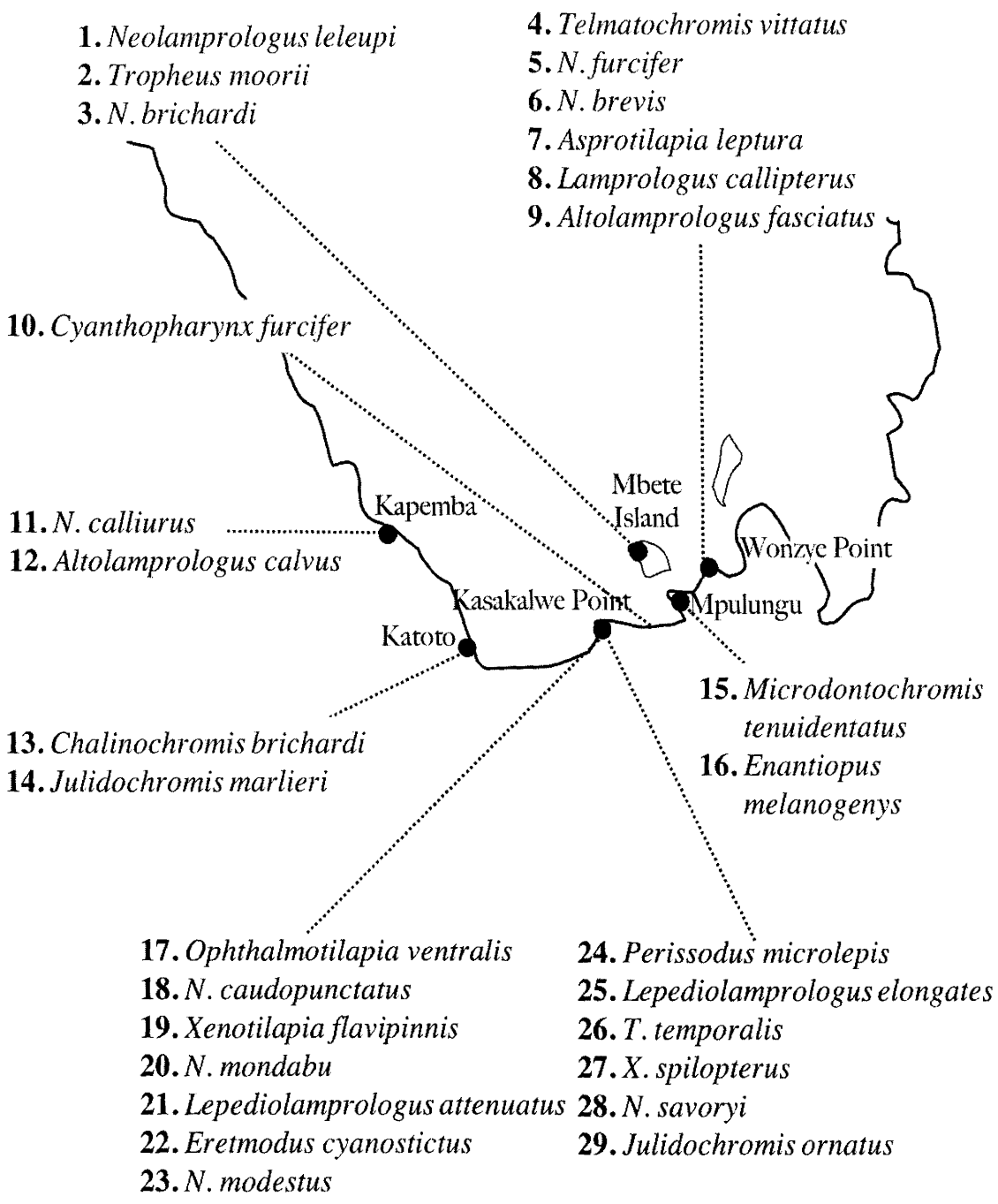

Figure 1.3. Map showing the locations where 29 cichlid species were collected in the southern basin of Lake Tanganyika, Zambia. The coastline of Lake Tanganyika corresponds to the blow-up in Figure 1.2. Fish were collected from these field sites and described in the study in **Chapters 5** of this thesis.

Maddison 2000; Freckleton et al. 2002; Pagel and Meade 2006). Third, Tanganyikan cichlids have been the focus of intensive research detailing their mating systems and reproductive behaviours (e.g. Keeleyside 1991; Kuwamura 1997: Goodwin et al. 1998), allowing for estimates of the magnitude of sperm competition.

1.7 STRUCTURE OF THIS THESIS

In this thesis I employ a variety of techniques, species and a combination of intra- and inter-specific studies to examine responses to sperm competition in fishes. **Chapter 2** reviews the field of sperm competition in fishes and provides a broad overview of the current state of the field that will help situate the subsequent chapters. This is the first such major review of sperm competition in fishes in almost a decade. This chapter attempts to synthesize our current understanding of how sperm competition influences testes size, ejaculate allocation, sperm morphology and sperm swimming performance from studies published primarily over the last 30 years. **Chapter 3** explores how male reproductive behaviour and physiology are influenced by sperm competition in the plainfin midshipman. Although this species has been well studied, and is a classic example of MARTs, surprisingly little was known about tactic-specific sperm characteristics. **Chapter 4** expands on my explorations of how MARTs influence sperm characters by assessing this topic in the shell-brooding cichlid *Telmatochromis vittatus*, which has four MARTs rather than the more usual two or three male morphs. **Chapter 5** addresses predictions from sperm competition theory based on the comparative approach and uses Tangayikan cichlids as a model system. Finally, **Chapter 6** concludes this thesis

by examining the implications of my research, illustrating recent advances in the field, and highlighting likely avenues for future research.

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CHAPTER 2

TESTES, SPERM AND SPERM COMPETITION

2.1 INTRODUCTION

Traditionally, the sexual traits of animals (and plants) have been categorized as primary (gonads, intromittent organs, spermatozoa, ova) or secondary (ornaments, displays), depending upon the form of selection thought to be responsible for their evolution and maintenance (Darwin 1871; Andersson 1994). Thus, primary traits are those influenced mainly by natural selection, optimized to maximize fertilization success, whereas secondary traits are the result of sexual selection, maximizing mating success (i.e., number of mates) even at some cost to survival. Ever since Parker (1970) laid the foundations of sperm competition theory, however, it has become increasingly clear that this distinction is no longer useful, as many 'primary' sexual traits are clearly influenced by both natural and sexual selection (e.g., Harcourt et al. 1981; Eberhard 1985; Briskie and Montgomerie 1997; Montgomerie and Briskie 2007).

In this chapter we look at some of the diversity of gross morphology in testes and spermatozoa in the fishes in the context of sexual selection resulting from sperm competition. Over the past 40 years the study of sperm competition has revolutionized our approach to studying such diversity both within and between species in a wide variety of plants and animals (Smith 1984; Birkhead and Møller 1992, 1998; Simmons 2001; Birkhead et al. 2008), and has often provided insights into the evolution of testis and

sperm morphologies in fish (Constantz 1984; Petersen and Warner 1998; Stockley et al. 1996, 1997; Balshine et al. 2001). However, unlike studies of birds (Birkhead and Møller 1998), mammals (Gomendio et al. 1998) and insects (Simmons 2001), the analysis of sperm competition in fishes has yet to yield a general synthesis. While there have been a few recent comparative studies looking at the influence of sperm competition on the evolution of reproductive traits in fishes (Stockley et al. 1997; Pyron 2000; Balshine et al. 2001; Fitzpatrick et al. **Chapter 5**), the analysis of different taxa sometimes produces contradictory results.

We begin this chapter by attempting to explain why general patterns have been difficult to document in the fishes, and then summarizing some of the relevant information available to date on variation in testis and sperm traits in fishes, especially in the context of phylogenetic patterns and constraints. There is a vast literature on fish reproduction, anatomy, physiology, and behavior that could be brought to bear on this subject but dealing with all of that is well beyond the scope of this chapter. Instead, we have attempted to find and read all of the papers published to the end of 2007 that deal directly with the influence of sperm competition on testis and sperm traits in fishes, but even then have been fairly selective in our coverage. We also draw heavily on our own research, in part because some of the fishes that we have studied are among the best studied species with respect to sperm competition. We hope that this chapter will provide a solid foundation for future work in this area, particularly by pointing the reader to the myriad comparative and empirical studies waiting to be done with this taxon.

The search for patterns in the reproductive traits in fishes is made complex—and interesting—by the large number of extant species worldwide (~28,000; Nelson 2006),

their diverse evolutionary histories, their often long period of growth as adults, an extreme interspecific range of adult sizes (spanning >9 orders of magnitude), the heterogeneous environments in which they live and reproduce, and their varied physiologies and anatomies. For the most part, these factors act as constraints on the evolution of reproductive traits and thus need to be considered both when looking for general patterns and when attempting to explain apparent exceptions. The other chapters in this book all provide a window into this complexity, but the ways that these factors constrain the evolution of reproductive traits is as yet poorly understood.

Any attempt to provide a general synthesis of sperm competition in fishes is made especially complex because they vary interspecifically (i) in modes of fertilization (internal vs. external), (ii) in their external fertilization environment (salt, fresh and brackish waters), (iii) in their life histories (iteroparous and semelparous, sex change during adulthood, clutch sizes from 1 to several million, etc), and (iv) in their body plans. Indeed, the extreme diversity of fish body plans from a thin tube (as in pipefishes, family Syngnathidae) to a flattened sphere (as in ocean sunfish, family Molidae) imposes serious constraints on the evolution of testis size and shape. Moreover, body shape is influenced by phylogeny, foraging mode, and hydrodynamic considerations that have little, if anything, to do with reproduction. Thus the fishes present an unusual challenge when looking for adaptive patterns across the entire taxon, and require careful consideration both of the constraints and the apparent anomalies or exceptions that confound the interpretation of interspecific analyses.

2.2 SPERM COMPETITION IN FISHES

Sperm competition theory. We begin with a brief overview of sperm competition theory and a summary of both the opportunities and challenges for putting the evolution of testes and spermatozoa in fishes into this context. While the potential for postcopulatory (post ejaculatory) processes to influence the outcome of fertilization events in animals had been recognized for some time, it was Geoff Parker (1970) who laid the foundation for sperm competition theory almost 40 years ago. As he defined it, sperm competition occurs when the spermatozoa of two or more males have the opportunity to fuse with an ovum (Parker 1970). In that seminal paper, Parker clearly elucidated both the mechanisms by which spermatozoa might compete for access to ova, and how this perspective led to many novel predictions about the evolution of primary and secondary sexual traits. Then, in an inspired series of papers spanning almost four decades and focusing on both theoretical issues and empirical studies of insects, mammals, and fishes, Parker and his collaborators developed this theory into a major discipline of evolutionary biology. Research on sperm competition has shown exponential growth (Figure 2.1) and has provided profound insights into the evolution of reproductive traits in both animals (Smith 1984; Birkhead and Møller 1992, 1998; Simmons 2001) and plants (e.g., Delph and Havens 1998).

Indeed, sperm competition between proto-male and proto-female gametes is thought to be responsible for the evolution and maintenance of anisogamy, thus giving rise to tiny mobile spermatozoa and large non-motile ova (Parker et al. 1972). Assuming that there was genetic variation in gamete size in an external fertilizer with only one gamete type (isogamy), Parker et al. (1972) modeled the influence of selection for

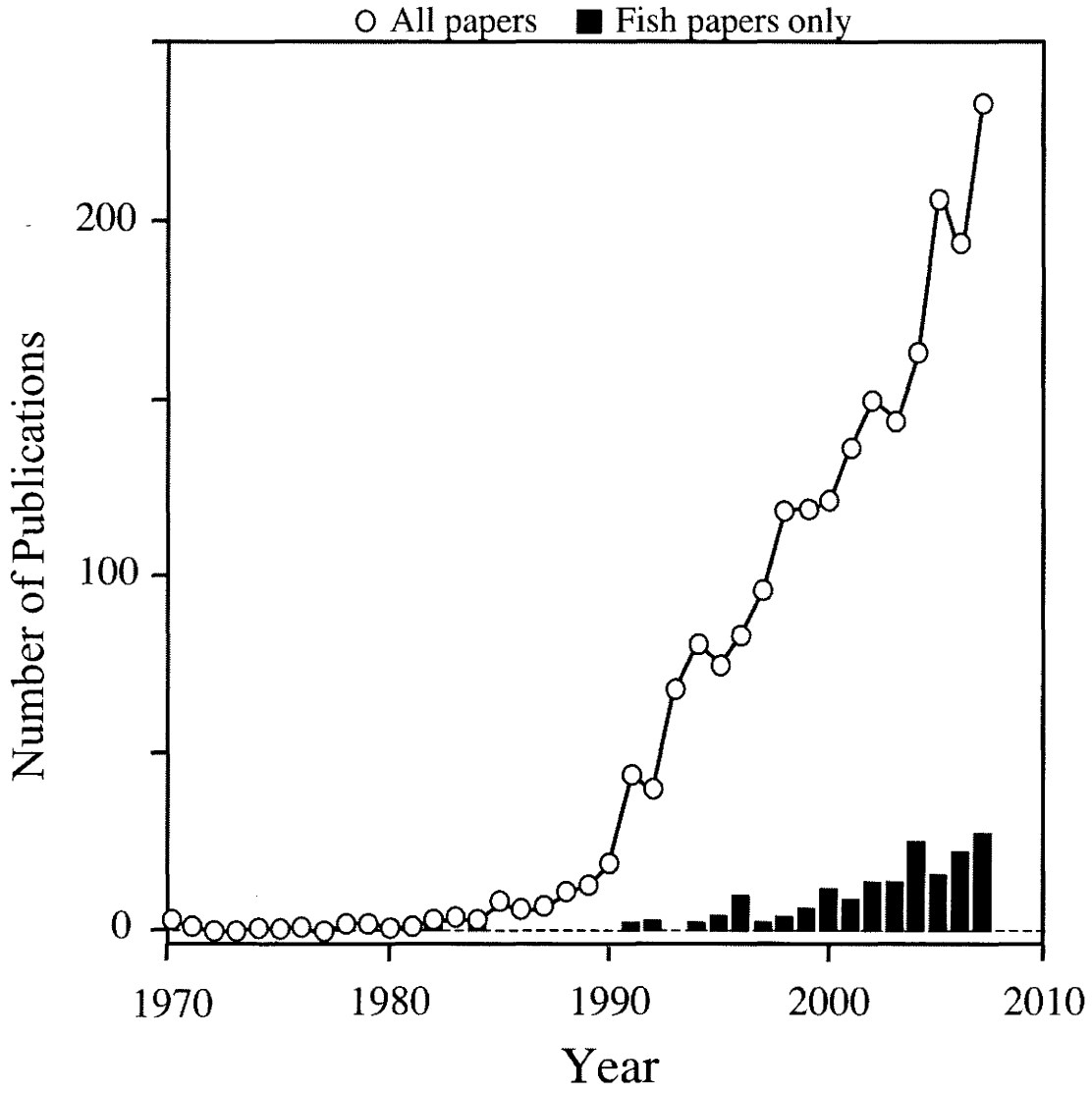

Figure 2.1 Number of publications on sperm competition per year since Parker (1970). These data were compiled by entering the topic "sperm competition" into the Web of Science search engine (at http:// http://apps.isiknowledge.com/) using the Science Citation Index database, then refining this search to find all papers with the topic "fish".

numbers of gametes versus gamete size—both of which were expected to increase the reproductive fitness of their bearers—on gamete morphology. They found that the advantages of disassortative fusion of gametes would rapidly lead to anisogamy (gametes differing in size). After anisogamy—distinctive spermatozoa and ova—becomes established, it is maintained by sperm competition that results in the evolution of ever greater differences between male and female gametes. Thus sperm competition favors the evolution of sperm traits—like smaller size, resulting in greater numbers per unit investment, or increased swimming speed—that increase their ability to encounter and fuse with the limited supply of large ova. Later, Parker (1982) showed how even relatively weak sperm competition between males in internal fertilizers could also maintain anisogamy and lead to further size disparity between male and female gametes. Admittedly, this theory has some detractors and alternative models have been proposed (Dusenbery 2006).

Competition between spermatozoa from the ejaculates of different males has clear fitness consequences for each male, and is thus expected to strongly influence the evolution of male behavior and morphology, as well as both sperm and ejaculate traits. Thus, as the strength of sperm competition increases, four kinds of male traits are expected to evolve. First, sperm competition theory predicts that males will adopt behavioral tactics and morphologies to increase access to, and defense of, females (see **Section 2.3.3**). Second, under sperm competition, the evolution of genitalia that put spermatozoa closer to unfertilized ova, and/or remove the spermatozoa of rival males is expected (Hosken and Stockley 2004). Third, as the risk and intensity of sperm competition increases, males are expected to tailor the size of their ejaculates (see

Section 2.4), and to evolve the necessary morphological (testes and other tissues) and physiological processes to do so. When competing with one other male, a male with larger ejaculates containing more spermatozoa is likely to fertilize more ova, everything else being equal, in a scenario often referred to as a 'fair raffle' (Parker 1990a). Such raffles become unfair when males evolve ejaculate characteristics, like different semen constituents (Poiani 2006), that give them an advantage in the presence of ejaculates of rival males. Finally, the morphology and behavior of spermatozoa themselves are expected to evolve under the influence of sperm competition, favoring increases in swimming speed and/or swimming duration (Ball and Parker 1996). Such changes in sperm behavior require the modification of sperm morphologies and physiologies like sperm size, structure, energy capacity, and energy metabolism, either individually or in concert.

A thorough review of sperm competition theory is beyond the scope of this chapter and we refer the reader to some recent extensive reviews for more detail (Smith 1984; Birkhead and Møller 1992, 1998; Simmons 2001), and especially Petersen and Warner (1998) with particular reference to the fishes. While many of the predictions of sperm competition theory have been well tested and supported, we are only just beginning to understand some of the underlying morphological and physiological mechanisms that influence these traits and their evolution.

Using fish to test the theory. There are several reasons why fish are particularly good for testing some of the predictions of sperm competition theory. Primary among these are the advantages of studying species that fertilize externally (Levitan 1998)—about 95% of fish species are external fertilizers. The advantages of studying external

fertilizers to gain insights about sperm-egg interactions were first recognized by Lazzaro Spallanzani in the late 1700s (Capanna 1999) and capitalized upon by Herman Fol (1879) and Oscar Hertwig (1876), who pioneered the use of sea urchins as model organisms for the study of fertilization. With external fertilizers, sperm movement and sperm-egg interactions can be studied *in vitro* under conditions that closely mimic natural situations, and these behaviors can be observed directly using simple microscopy (Levitan 1998). With respect to sperm competition in particular, the study of external fertilizers facilitates both the examination of the actual mechanisms underlying competitive advantages (e.g., Gage et al. 2004; Burness et al. 2004, 2005) and the experimental manipulation of sperm and ejaculate characteristics that might influence competitive ability (e.g., Hoysak and Liley 2001; Turner and Montgomerie 2002). Moreover, even the existence of sperm competition is more easily quantified in external fertilizers as the presence and behavior of males at spawning events can be readily observed, and the potential strength of sperm competition estimated (Levitan 1998). Similarly the influence of females on sperm behavior can also be readily measured in externally fertilizing fishes (Hayakawa and Munehara 1998; Turner and Montgomerie 2002; Elofsson et al. 2003; Wojtczak et al. 2007), providing some rare insights into cryptic female choice (Rosengrave et al. 2008).

In externally fertilizing species, sperm competition takes place whenever spermatozoa from more than one male are released simultaneously—or are simultaneously active—in the presence of unfertilized ova. Simultaneous release of spermatozoa by multiple males is often easy to observe and has been reported in at least 140 species, belonging to 28 families (Taborsky 1998). Moreover, sperm competition can occur even when spermatozoa are not released simultaneously by different males. For

example, in the rose bitterling (*Rhodeus ocellatus*, family Cyprinidae, Kanoh 1996, 2000) and the three-spined stickleback (*Gasterosteus aculeatus*, family Gasterosteidae, Elofsson et al. 2003), sneaking males practice pre-oviposition sperm release, and as a result fertilize some ova when the female spawns. Without careful behavioral observations, researchers may have previously underestimated the actual level of sperm competition occurring in this species. Similarly, in two species of African cichlid (family Cichlidae), *Lamprologus callipterus* (Taborsky 2001) and *Telmatochromis vittatus* (Ota and Khoda 2006), spawning by sneaker males can be particularly difficult to observe. Following courtship in these species, females enter empty gastropod shells where breeding takes place. Territorial males release spermatozoa into shells containing females while females release eggs from inside the shell. But occasionally, a much smaller sneaker male can be hidden inside the shell with the female, releasing spermatozoa as the female releases eggs. In these species casual observations of territorial males might have led to the assumption that the strength of sperm competition was low.

The study of sperm competition in fish is also facilitated by the widespread occurrence of male alternative reproductive tactics (MARTs; Taborsky 1994; Mank and Avise 2006), wherein some males adopt cuckolder behaviors during spawning, stealing fertilizations from conventional males adopting territorial or dominant tactics. Cuckolders are often both morphologically and physiologically distinct from conventional males and this makes them relatively easy to distinguish in the field (Figure 2.6 B). Most important, cuckolders and conventional males encounter different levels of sperm competition, as described below (**Section 2.3.3**). Thus species with MARTs provide a nice natural experiment in which the effects of sperm competition on sperm and ejaculate traits can be

studied by comparing conventional males to cuckolders (Parker 1990b). Clearly, the existence of alternative reproductive tactics allows the comparison of reproductive traits among individuals within species, obviating the need to control for phylogeny and other potentially confounding variables.

Finally, the tremendous diversity of fishes presents not only a challenge but also a splendid opportunity for the study of sperm competition. Thus the fishes offer an almost unparalleled diversity of spawning environments that might be expected to influence both the nature and the outcome of sperm competition, resulting in a variety of predictions about the evolution of associated reproductive traits. Sometimes this diversity can lead to contradictory conclusions if confounding factors are ignored (as we highlight below), but we believe that these should be treated as exceptions that help us to understand the rules governing selection in the face of sperm competition. Certainly, many of the examples we provide in the following sections suggest that there are some general patterns resulting from sperm competition in fishes, and that the exceptions to those patterns may be particularly instructive.

2.3 TESTES SIZE AND SPERM COMPETITION

In this section we look at the influence of sperm competition on testes, focusing specifically on testes size (specifically, combined testes mass). Testes size variation is, of course, the result of selection on sperm production rate, sperm storage capacity, and other aspects of testis function (Parenti and Grier 2004) but there has been little work to date on the relations between testes size and testis physiology, histology and function. We therefore use testes size as a simple index of all of these testis traits that are expected to

be important in sperm competition.

At the outset, then, we need to ask how well testes size reflects ejaculate size, as a positive relation is assumed in most analyses involving testes size in relation to sperm competition. Unfortunately, this relation has not yet been particularly well studied in animals in general and in fish in particular. While it seems logical that males with larger testes would release more spermatozoa at a spawning event, this is not necessarily true for five reasons. First, testes are not comprised only of spermatogenic tissue (Parenti and Grier 2004), so differences in testes size do not necessarily reflect differences in spermatogenic capacity, particularly when comparing between species (Emerson 1997; Giacomello et al. 2008). In the combtooth blennies (family Blennidae), for example, testes comprise both spermatogenic tissue and an accessory gland that probably contributes to sperm maturation, sperm nutrition and the production of seminal fluid. This accessory gland occupies about 10% of the volume of the testes in *Salaria pavo* but >75% of the testes volume in *Lipophrys dalmatinus* (Giacomello et al. 2008). Nonetheless, some limited evidence from birds (reviewed in Briskie and Montgomerie 2007) and mammals (Møller 1989) shows that there is a positive relation between ejaculate size and testes size among species. Most of the evidence in fishes comes from studies of single species where available milt (sperm and seminal fluid) is stripped by hand from sexually active males, and this is used as an estimate of the number of spermatozoa released during a mating. In the bucktooth parrotfish, *Sparisoma radians* (family Scaridae), for example, combined testes mass accounted for 91% of the variation in the number of spermatozoa stripped from 11 males (Marconato and Shapiro 1996).

Second, the assumption that ejaculate size is highly correlated with testes size

rests on the premise that larger testes produce spermatozoa at a faster rate, and that males with larger testes have the capacity to collect larger sperm volumes for release during a single spawning. There is some interspecific evidence that sperm production rate increases with testes size in flatworms (Schärer et al. 2004), mammals (Møller 1989), and birds (reviewed in Briskie and Montgomerie 2007), but that could result in more frequent, rather than larger, ejaculates if male sperm storage capacity is limited. Conversely, males with smaller testes and concomitantly lower sperm production rates might have relatively large ejaculates if those males release sperm less frequently and have large sperm storage capacity. Neither of these possibilities has been critically examined yet, and both deserve some detailed study both within and between species.

Third, there are some obvious age effects on testes size and function. Thus, testes of the same size in males at different stages of sexual maturation might be expected to have different sperm production rates (e.g., Evans et al. 2002). This could be an issue for both inter- and intraspecific analyses of testes size as an index of ejaculate size because it could add unexplained variation to apparent patterns or obscure them altogether. To the best of our knowledge, there are few data available from studies of fishes on the effect of age on sperm production, controlling for testes size.

Fourth, testes size might well be influenced by sperm size, irrespective of any effect of sperm competition. Larger spermatozoa simply require larger cells for spermatogenesis and so, everything else being equal, the production of larger sperm cells should require a larger volume of spermatogenic tissue. Details of the relations between sperm size and the volume of spermatogenic tissue have yet to be studied, though sperm size can be controlled statistically when modeling the relations between testes size and

sperm competition.

Finally, there is some clear evidence that males adjust ejaculate size itself in response to the magnitude of sperm competition (see **Section 2.4**). While it is still to be expected that maximum ejaculate size will be correlated with combined testes mass, males are expected to adjust actual ejaculate size depending upon the risk and intensity of sperm competition at each mating. Thus testes size might not always correlate with ejaculate size if the risk and intensity of sperm competition is not controlled experimentally or statistically.

Despite all of these caveats, several studies have found some interesting patterns of testes size variation in relation to the strength of sperm competition, and we review these below. In general, the failure to consider these five confounding variables is likely to increase the unexplained variation in any analysis, thus reducing statistical power rather than introducing systematic bias. However, two additional confounding factors—season and body size—can have such a large effect on testes size that they cannot be ignored without seriously compromising the search for patterns, so we deal with these in some detail before proceeding.

2.3.1 Seasonal Variation in Testes Size

Like other vertebrates, most fish species are seasonal breeders with circannual variation in reproductive physiology and anatomy, presumably largely to minimize the costs of maintenance of reproductive tissues and behaviors when these are not needed. One manifestation of this seasonal variation is that an individual male's testes size can vary 100-fold, or more, from the non-breeding season to the height of reproductive activity

(Figure 2.2). Here we summarize some general patterns of seasonal variation in reproduction as a guide to potential difficulties to be encountered in measuring and analyzing testes size in the face of this variation.

Patterns of seasonal variation in fish testes size vary with both the duration of the breeding season (Figure 2.2) and the degree of synchrony among males. Most fish species studied to date have a single annual breeding season—typically a few weeks duration (Figure 2.2 A, B, D) but sometimes much longer (Figure 2.2 C)—during which testes increase dramatically in size compared to their non-breeding state (Figure 2.6 A, B). Tropical species are more likely to have both multiple breeding episodes during the year with concomitant fluctuations in testes size, often related to the lunar cycle (Figure 2.2 A), and protracted breeding episodes with large testes size being maintained over relatively long periods (Figure 2.2 B). Moreover, in some species, the cycles of change in testes size is synchronous among males in the population but in others it is quite asynchronous, though there are few data available on this.

2.3.2 Testes size in relation to body size

As with most other organs and tissues, the mature combined testes mass of male fishes (and other animals) is often determined largely by body size, both within and among species (Figure 2.3). From the perspective of sperm competition, both the absolute size of testes and their size relative to body size (Figure 2.6) are important variables, though from different points of view, as we shall see below. Thus it is important to consider both how body size can influence absolute testes size and then how to measure testes size relative to body size as an estimate of relative investment in gonadal tissue when body sizes vary.

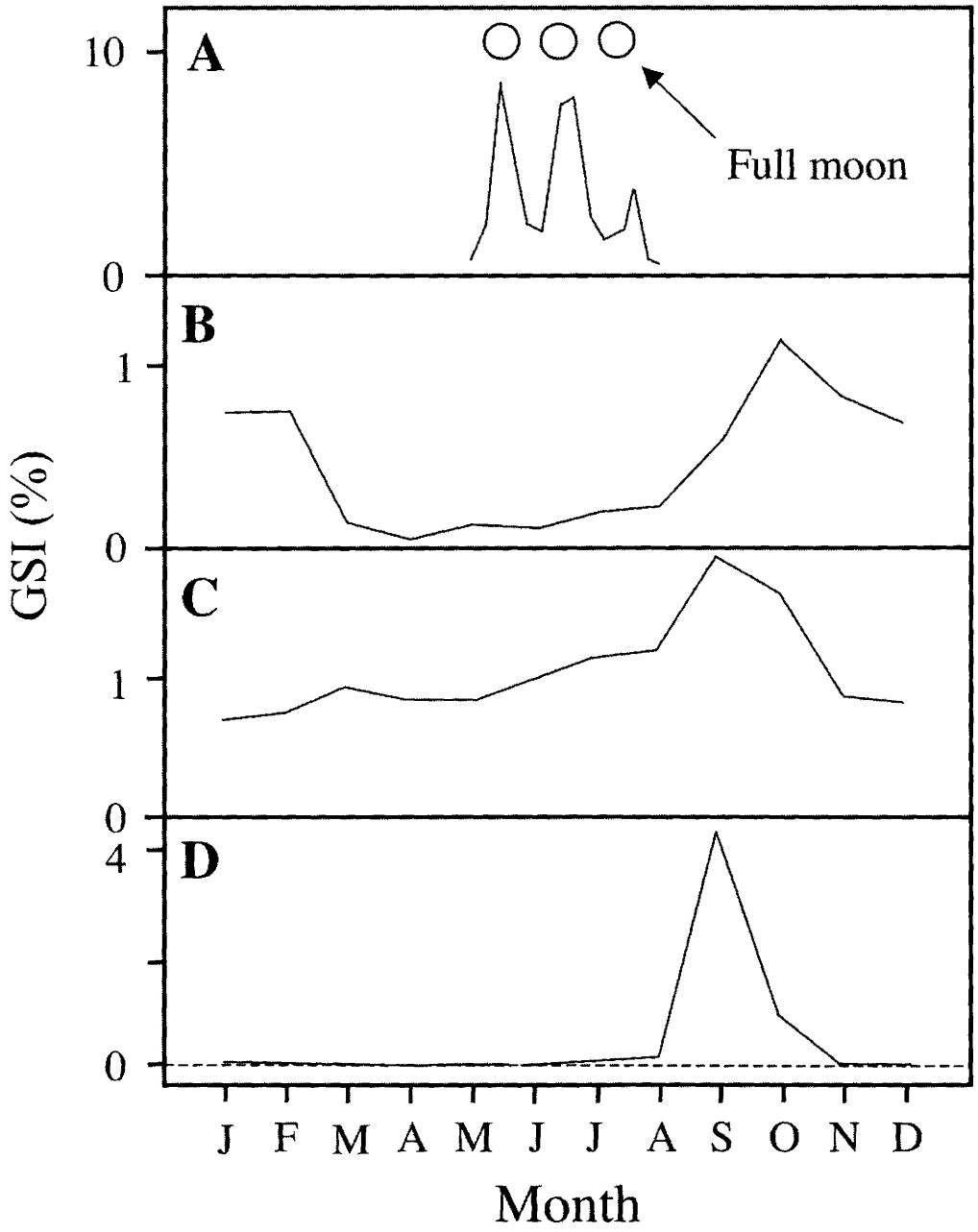

Figure 2.2 Seasonal variation in relative testes size (measured using the gonadosomatic index, GSI). **A.** In the tropical, coral reef-dwelling forktail rabbitfish, *Siganus argenteus* (Siganidae), showing relation to the lunar cycle. Redrawn from data in Rahman et al. 2003, Figure 1. **B.** In the subtropical, oceanic Australian pilchard, *Sardinops sagax*, (Clupeidae). Redrawn from data in Neira et al. 1999, Figure 4. **C.** In the tropical, stream-dwelling rainbowfish, *Cairnsichthys rhombosomoides* (Melanotaeniidae) of northeastern Australia. Redrawn from data in Pusey et al. 2001, Figure 4. **D.** In the subtropical, Mediterranean sharpsnout seabream, *Diplodus puntazzo* (Sparidae), a hermaphroditic species with data here from individuals with testes-predominant ovo-testes. Redrawn from data in Micale et al. 1996, Figure 5.

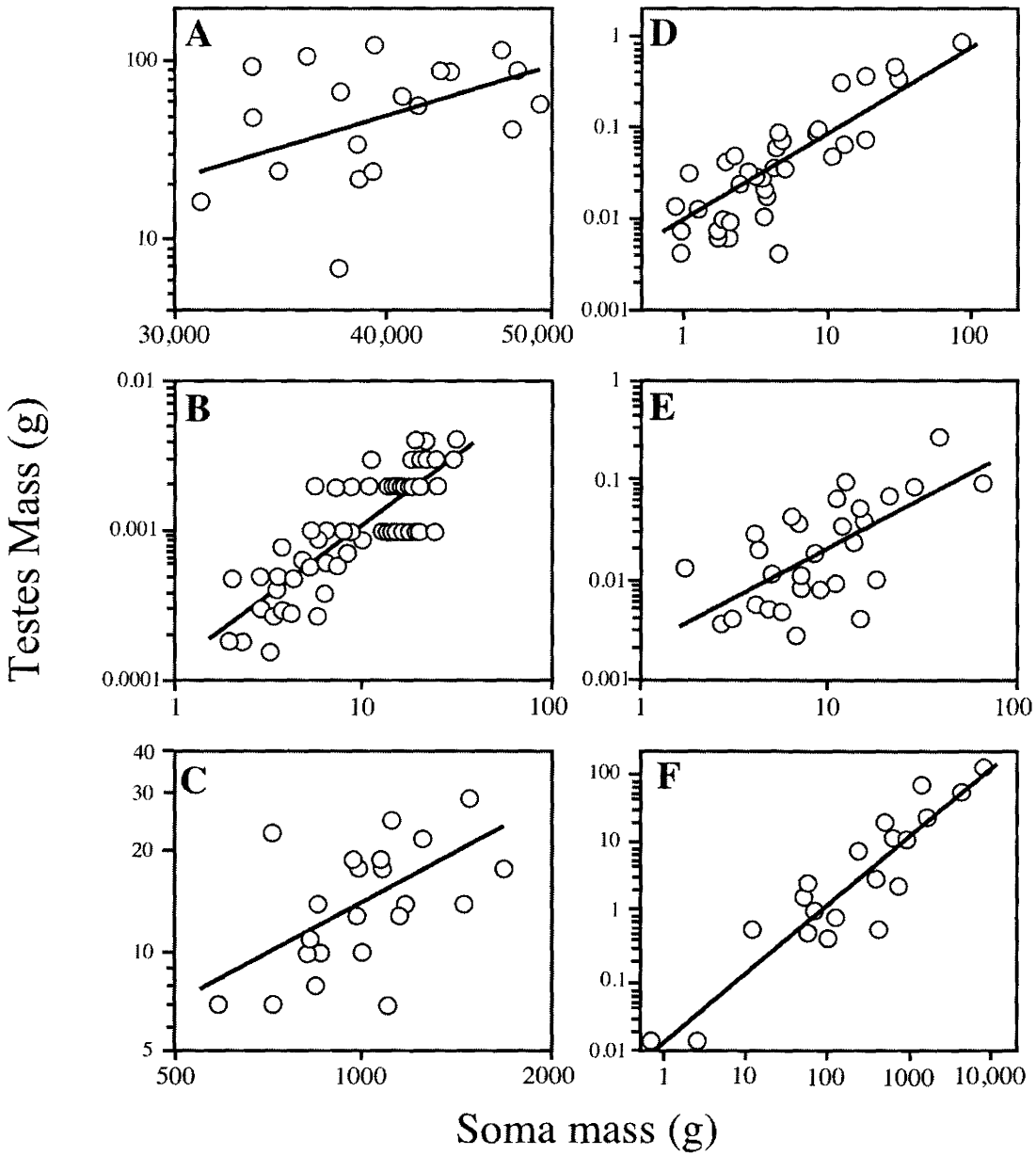

Figure 2.3 Relations between combined testes mass and body (soma) mass. **A.** In 2- to 3-year-old Pacific bluefin tuna (*Thunnus orientalis*, family Scombridae), an ocean-living external fertilizer. Redrawn and reanalyzed from data in Sawada et al. 2007, Table 2. **B.** In the molly, *Poecilia mexicana* (Poeciliidae), a freshwater species with internal fertilization. Redrawn and reanalyzed from data in Schlupp et al. 2006, Figure 1a. **C.** In lake whitefish, *Coregonus clupeaformis* (Salmonidae), a lakedwelling, external fertilizer. Original data, R. Montgomerie. **D.** In 37 minnow species (family Cyprinidae), all of which are freshwater-living, external fertilizers, with a mix of group and pair-spawning species. Redrawn and reanalyzed from data in Pyron 2000, Table 1. **E.** In 29 cichlid species from Lake Tanganyika, all of which all of which are freshwater-living, external fertilizers with a variety of breeding systems. Original data, Fitzpatrick *et al.* **Chapter 5**. **F.** In 20 species of external fertilizers for which intraspecific relations were also known. Redrawn and reanalyzed from data in Stoltz et al. 2005, Figure 1.

Body (soma) mass alone accounts for between 30 and 95% of the intraspecific variation in combined testes mass (different MARTs analyzed separately) in a wide range of fish species, across four orders of magnitude of fish size (Table I in Stoltz et al. 2005; Montgomerie and Fitzpatrick unpublished data). Examples of such intraspecific variation are shown in Figures 2.3 A-C, 2.5, 2.6, and 2.7). Undoubtedly, some of the unexplained variation within species is due to the seasonal and age effects discussed above, but these factors are rarely considered in field studies. Interspecific relations between testes mass and body (soma) mass are also positive (Figure 2.3 D-F), but very little of the unexplained variation among species is likely to be accounted for by age or season since these should have little effect on species means.

Note that we refer to body mass as 'soma mass' in the preceding paragraph, where soma mass is body mass minus testes mass. This distinction is important when analyzing the relation between testes mass and body mass but is rarely made in the literature. Because testes mass is a component of body mass, analysis of the relation between testes mass and body mass results in part-whole correlations that can generate erroneous conclusions (Tomkins and Simmons 2002). Analysis of this relation between testes mass and soma mass is also best done on log-transformed variables (Tomkins and Simmons 2002) to remove the effect of scaling and thus facilitate the comparison of slopes within and between species.

Gonadosomatic Index (GSI). In most studies of fish reproduction, relative testes size is estimated by the gonadosomatic (or gonosomatic) index (GSI; Figure 2.4). GSI is calculated as the mass of the testes divided by a measure of body size (preferably soma mass), usually all multiplied by 100 to express GSI as a percent. As a general index of

relative testes size, GSI is intuitively easy to understand, and is a useful way to describe the differences in relative testes size among species or among males adopting different reproductive tactics, among other things. In this chapter we necessarily refer to GSI frequently as this is often all that is reported in published studies. Where possible, we have used soma mass to reanalyze published values and we report these as GSI' as they differ slightly from the GSI values reported in the original papers.

GSI is not, however, the appropriate measure of relative testes size for statistical analysis, despite its widespread, almost universal, use for this purpose. Most importantly, GSI actually scales with body mass unless the relation between testes mass and body (soma) mass is isometric (i.e., intercept = 0, slope = 1), which it rarely is (Stoltz et al. 2005). As a result, GSI does not actually completely control for body mass (Tomkins and Simmons 2002). In addition, because GSI is a ratio, it has statistical properties that often violate the assumptions of parametric analyses. See Tomkins and Simmons (2002) for a nice overview of the various difficulties encountered when using GSI for statistical analysis. Despite these caveats, the results of most analyses that have used GSI are not qualitatively different from analyses using more appropriate statistical methods, with some important exceptions as described in Tomkins and Simmons (2002). Nonetheless, we encourage researchers to use GSI only for descriptive purposes, and to use appropriate linear models to determine relative testes size, as outlined below.

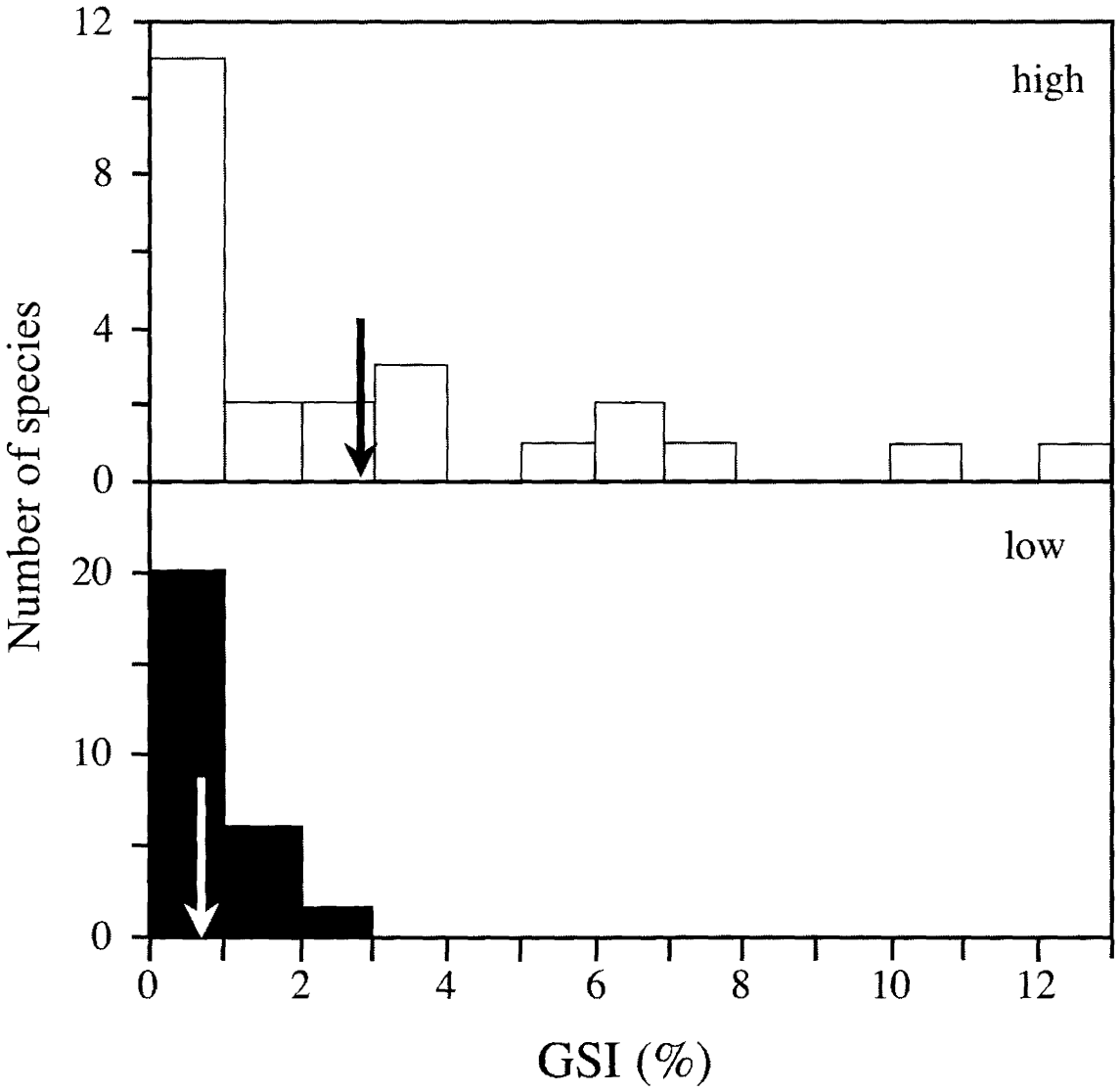

Figure 2.4 GSI of fish species estimated to experience low (lower graph) and high (upper graph) levels of sperm competition. Arrows indicate mean GSI for each category. Redrawn and reanalyzed from data in Petersen and Warner 1998, Figures 11.1 and 11.2, of which categories 0, 1 and 2 in Figure 11.1, and category 1 in Figure 11.2, are shown here as low.

Statistical analysis. Most of the statistical problems encountered when using GSI as a measure of relative testes size are alleviated simply by using general (and generalized) linear models for analysis. Usually, the residuals from the ordinary least squares (OLS, model I) regression of log testes mass on log soma mass are normally distributed and thus satisfy the assumptions of this regression model. Note that residuals from model II regression (e.g., reduced major axis regression) are not appropriate here as these are correlated with soma mass. Thus residuals from the OLS regression (e.g., Figures 2.3, 2.5, 2.7) are a reliable measure of relative testes investment in both intra- and interspecific analyses, with few exceptions, as follows. First, if these residuals are not normally distributed either transformations or a generalized linear model analysis may be required. Second, ANCOVA should be used to compare both slopes and adjusted means when comparing testes size versus body size relations between species or between alternative reproductive tactics (see Tomkins and Simmons 2002). However, this method is only valid statistically when slopes are not significantly different and the ranges of body sizes are overlapping. Finally, to quantify the relative testes size of males adopting alternative reproductive tactics, testes masses for cuckolder tactics could be compared to the regression through conventional male data, as shown in Figures 2.5 and 2.7. Such a comparison assumes that extrapolation of conventional male testes size to smaller animals is reasonable, but this deserves further study.

In the remainder of this chapter, we use GSI mainly as a descriptor because it is readily interpreted, when expressed as [(testes mass/soma mass) x 100], as the percent of body size devoted to gonads. When possible, however, we use (and prefer) ANCOVA for analyses involving relative testes size. Unfortunately most previous analyses of relative

testes investment in fishes have used GSI, and too few of these studies provide enough information (e.g., testes and body masses) to permit the calculation of residuals. A thorough reanalysis of published studies could prove to be rewarding.

2.3.3 Influence of Sperm Competition on Testes

Intraspecific comparisons. Probably the best evidence for the influence of sperm competition on testes size come from studies of fishes with male alternative reproductive tactics (MARTs). In these species, males of different phenotypic (and possibly genotypic; Phillip and Gross 1994; Piché et al. 2008) morphs adopt different MARTs in the breeding season (Taborsky 1994). Typically, conventional males (also called bourgeois, parental, territorial, type I, terminal color phase, or guarding males) are the largest and most aggressive, and defend nests or territories to which they attract females to lay eggs. Cuckolder males (also called parasitic, type II, or initial color phase males), on the other hand, are smaller and either (i) colored and behave like females (satellites), or (ii) cryptic and stealthy about approaching spawning pairs (sneakers). Both cuckolder morphs steal fertilizations when a female spawns with a conventional male, either by pretending to be an additional female visiting to spawn (satellites), or darting toward a spawning female and releasing sperm while the pair is spawning (sneakers). Conventional males often spawn in the absence of cuckolders and thereby achieve the maximum fertilization success in those instances, but cuckolders only release spermatozoa during the spawning of conventional males. Because they always encounter sperm competition while spawning, cuckolders are expected to have evolved traits that would enhance their sperm competitiveness, and this has proven to be the case (Taborsky 1994, 1998; Peterson and

Warner 1998).

This expected conventional-cuckolder male dichotomy in response to sperm competition is based on the notion that sperm production is costly (Nakatsuru and Kramer 1982), and that these costs, as well as the costs of nest-building, courtship, and paternal care, constrain conventional males from matching the adaptations shown by cuckolders. Conversely, cuckolders presumably bear the costs of relatively large testes and high sperm production rates to maximize their own reproductive success. If cuckolder adaptations for sperm competition allowed them to achieve, on average, the same fitness (lifetime reproductive success) as conventional males then these alternative reproductive tactics could be maintained as a genetic polymorphism via frequency dependent selection, as evolutionarily stable strategies (ESSs). The evidence in support of this idea is slim (Gross 1996) and cuckolders may simply be making the best of a bad job, adopting alternative reproductive morphologies and behaviors to maximize their own reproductive success well below that achieved by conventional males. Recent evidence from Atlantic salmon (*Salmo salar*, family Salmonidae) suggests that there is genetic variability in the maturation threshold that influences male reproductive behavior and the adoption of different MARTs (Piché et al. 2008). Thus this study suggests that MARTs are determined by both genetics and environment and that the phenotypic threshold for adopting one or the other tactic may vary among populations. Clearly, gaining an understanding of the proximate mechanisms underlying the maintenance of MARTs will have a profound influence on our understanding of male reproductive characteristics in these species, while also informing and providing boundaries for theoretical predictions.

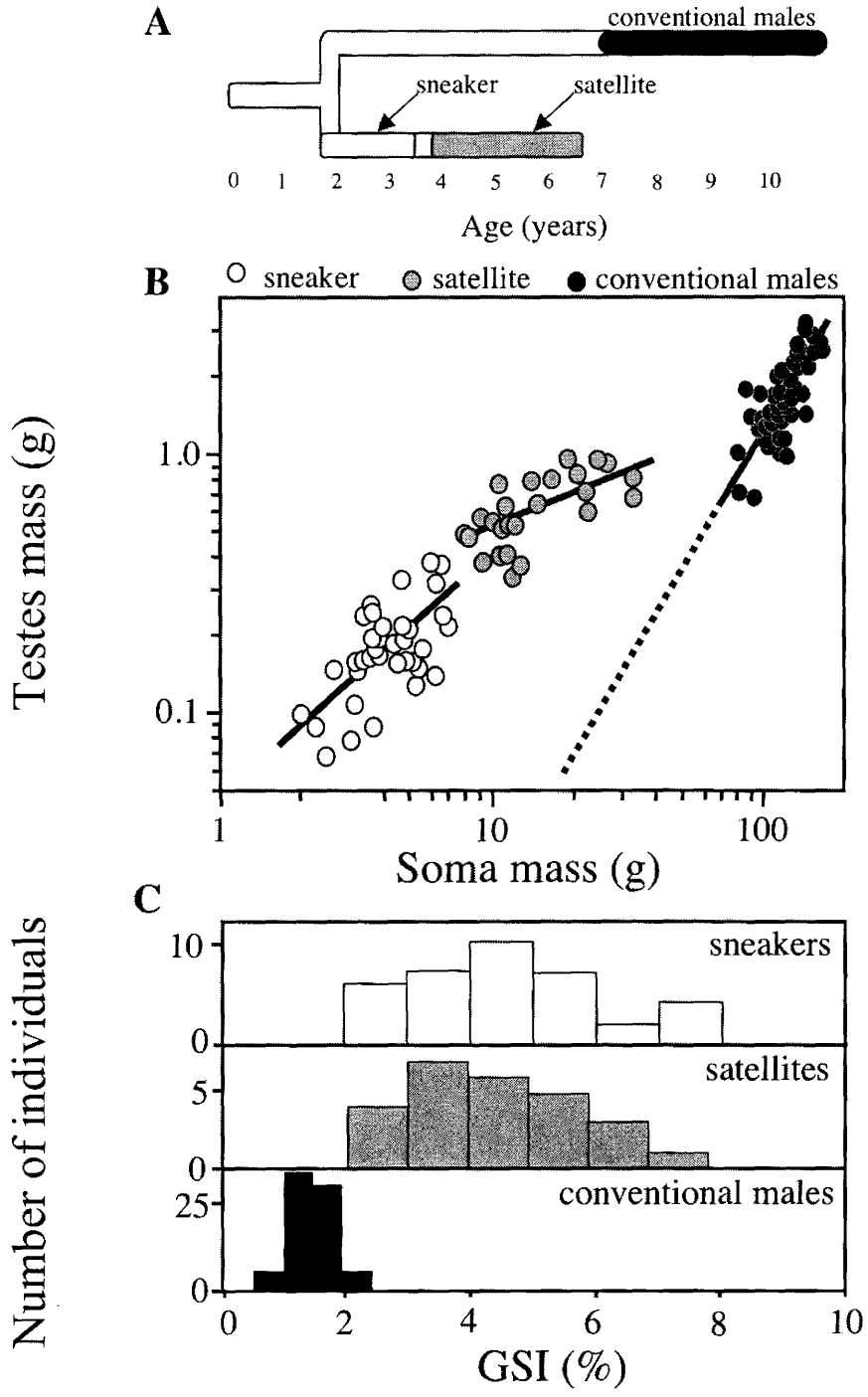

Figure 2.5 Male alternative reproductive tactics in bluegill, *Lepomis macrochirus* (Centrarchidae). **A.** Life histories of the different male morphs. Redrawn from Gross and Charnov 1980, Figure 2, and information in Neff 2004. **B.** Regressions of testes mass on soma mass; dotted line extrapolates regression of conventional male data to illustrate relative size of cuckolder testes (sneakers and satellites). Redrawn from data in Neff et al. 2003, Figure 1. **C.** GSI′ of males adopting each tactic. Original, from data shown in B.

In species with alternative reproductive tactics, then, cuckolder males are predicted to have relatively larger testes than conventional males, as a consequence of selection due to sperm competition (Parker 1990b). Data from many fish species with MARTs provide clear support for this prediction (Table 2.1), and the bluegill (*Lepomis macrochirus*, family Centrarchidae) is probably the best-studied of these (Gross and Charnov 1980).

Bluegills are very commonly found in the freshwater lakes and rivers of eastern North America, breeding in abundance between latitudes 25° and 45°N. At age 2 some males begin to adopt a cuckolder breeding tactic, depending upon their body size, and possibly genetics (Phillip and Gross 1994), whereas smaller males initially become sneakers then switch to adopting a satellite tactic at about age 4 as they get older and larger (Gross 1982) (Figure 2.5). Larger males that do not adopt cuckolder tactics at age 2 continue to grow and eventually adopt conventional male tactics at age 7. In May and June, conventional males usually breed in colonies of up to 300 males nesting together on sandy bottoms in the littoral zone, where males attract females to lay their eggs (Neff

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Table 2.1 Relative sizes of sperm and testes of males engaging in alternative reproductive tactics: conventional (C), sneaker (Sn) and satellite (Sa). In *T. vittatus* males are identified as territorial (T) and pirate (P) and both tactics are expected to face relatively low levels of sperm competition (Fitzpatrick et al. **Chapter 4**).
References: 1. Ota and Khoda 2006, **2.** Fitzpatrick et al. **Chapter 4**, **3.** Katoh et al. 2005, **4.** Gage et al. 1995, **5.** Vladic and Järvi 2001, **6.** Vladić et al. 2002, **7.** Gross 1980, **8.** Leach and Montgomerie 2000, **9.** Neff et al. 2003, **10.** Burness et al. 2004, **11.** Stoltz and Neff 2006, **12.** Rasotto and Mazzoldi 2002, **13.** Locatello et al. 2007, **14.** Marentette et al. submitted, **15.** Svensson and Kvarnemo 2007, **16.** Warner 1982, **17.** Uglem et al. 2000, **18.** Taborsky 1998, **19.** Ross et al. 1983, **20.** Henson and Warner 1997, **21.** Brantley and Bass 1994, **22.** Fitzpatrick et al. **Chapter 3**, **23.** Halpern-Sebold et al. 1986, **24.** Gonçalves et al. 1996, **25.** Santos et al. 1995, **26.** Neat et al. 2003.

Species	Testes Size	Sperm Length	Sperm Swimming speed	Sperm ATP content	Refs.
African cichlid *Telmatochromis vittatus*	Sn>P>T>Sa	Sn=P=T=Sa	Sn>T=Sa>P		1, 2
African cichlid *Telmatochromis temporalis*	Sn>C				3
Atlantic salmon *Salmo salar*	Sn>C	Sn=C		Sn>C Sn=C	4, 5, 6
bluegill *Lepomis macrochirus*	Sn>C	Sn>C Sn=C Sn=Sa=C	Sn>C Sn=C Sn=Sa=C	Sn>C	7,8, 9,10, 11
black goby *Gobius niger*	Sn>C	Sn=C	Sn>C	Sn>C	12, 13
grass goby *Zosterisessor ophiocephalus*		Sn=C	Sn = C	Sn=C	13
round goby *Apollonia melanostoma*	Sn>C	Sn=C	Sn<C		14
sand goby *Pomatoschistus minutus*	Sn>C				15
bluehead wrasse *Thalassoma bifasciatum*	Sn>C				16
corkwing wrasse *Symphodus melops*	Sn>C				17
ocellated wrasse *Symphodus ocellatus*	Sn>Sa>C				18
saddleback wrasse *Thalassoma buperrey*	Sn>C				19, 20
plainfin midshipman *Porichthys notatus*	Sn>C	Sn=C	Sn>C		21, 22
platyfish *Xiphophorus maculates*	Sn>C				23
peacock blenny Salaria pavo	Sn>C				24
Azorean rock-pool blenny *Parablennius sanguinolentus Parvicornis*	Sn>C				25
combtooth blenny *Scartella cristata*	Sn>C				26

2004). After a brief courtship display, females lay their eggs while males release spermatozoa onto the ova that fall into the nest, sometimes while cuckolder males are also releasing spermatozoa on the ova. Conventional males guard and maintain the fertilized eggs, then look after the newly hatched fry for 5-7 d (Neff 2004). Cuckolder males never build nests, always release spermatozoa in the presence of a spawning conventional male, and fertilize about 85% of eggs during spawning events in which they participate (Fu et al. 2001), but overall fertilize only about 25% of the eggs in a colony (Neff 2001). The testes of cuckolder male bluegill are strikingly large for their body size, compared to those of conventional males (Figure 2.5). The GSI' of cuckolder males is 4.7% for sneakers and 4.8% for satellites, compared to 1.5% for conventional males (recalculated from data in Figure 1 of Neff et al. 2003). Comparing cuckolder testes masses to the regression of testes mass on soma mass for conventional males (Figure 2.5), it is clear that average testes investment by satellites and sneakers is much higher than that predicted for conventional males of that body size, assuming that such an extrapolation is reasonable. Note, however, that even though the relative investment in testes by cuckolders is much higher than that of conventional males, the testes of conventional males are still 3 and 9 times the size of the testes of satellite and sneaker males, respectively (Figure 2.5). As a result, conventional males should still have larger ejaculates than cuckolders and by this criterion alone should have a clear advantage under sperm competition. Cuckolders attempt to overcome their disadvantage by positioning themselves closer than conventional males to the ova being released by spawning females, thereby releasing spermatozoa slightly closer to the female than the conventional male at the instant of egg release (Stoltz and Neff 2006), and they also have faster

swimming spermatozoa (**Section 2.5.5**). The result is that cuckolders fertilize a higher proportion of eggs per spawning event, on average, than conventional males (Fu et al. 2001).

Similarly, in Atlantic salmon, parr (cuckolder) males sneak fertilizations when the much larger anadromous (conventional) males are spawning with females that they have attracted to their nests (redds) in fast-flowing streams (Gross 1985). The testes of parr males (GSI′ = 4.5%) are much larger than expected for anadromous males (GSI′ = 2.4%) of that size (Figure 2.7), providing clear support for the predictions of sperm competition theory (Gage et al. 1995). However, as in the bluegill, anadromous male salmon have testes almost sixty times the mass of parr males' testes (293 g vs. 5 g; Vladic and Järvi 2001). Consequently, anadromous males, who often engage in sneak fertilizations themselves, are likely to win the majority of fertilizations in spawnings contested with parr males.

Among species with MARTs, the plainfin midshipman (*Porichthys notatus*, family Batrachoididae) provides an interesting contrast in that cuckolder males, despite their smaller body size, have testes as large as those of conventional males (**Chapter 3**). In this species, Type I (conventional) males usually establish and defend intertidal nest sites under large rocks where they acoustically court females. Type II (cuckolder) males either sneak into nests and release spermatozoa while the pair is spawning, or release spermatozoa just outside the nest rock and fan it into the nests with their pectoral fins (Brantley and Bass 1994). Interestingly, Type I males, especially the smaller ones, also engage in cuckoldry (Lee and Bass 2004). Type I males average 7.6 times the body mass of Type II males, but have much lower GSI (1.2% vs 8.3%; Brantley and Bass 1994).

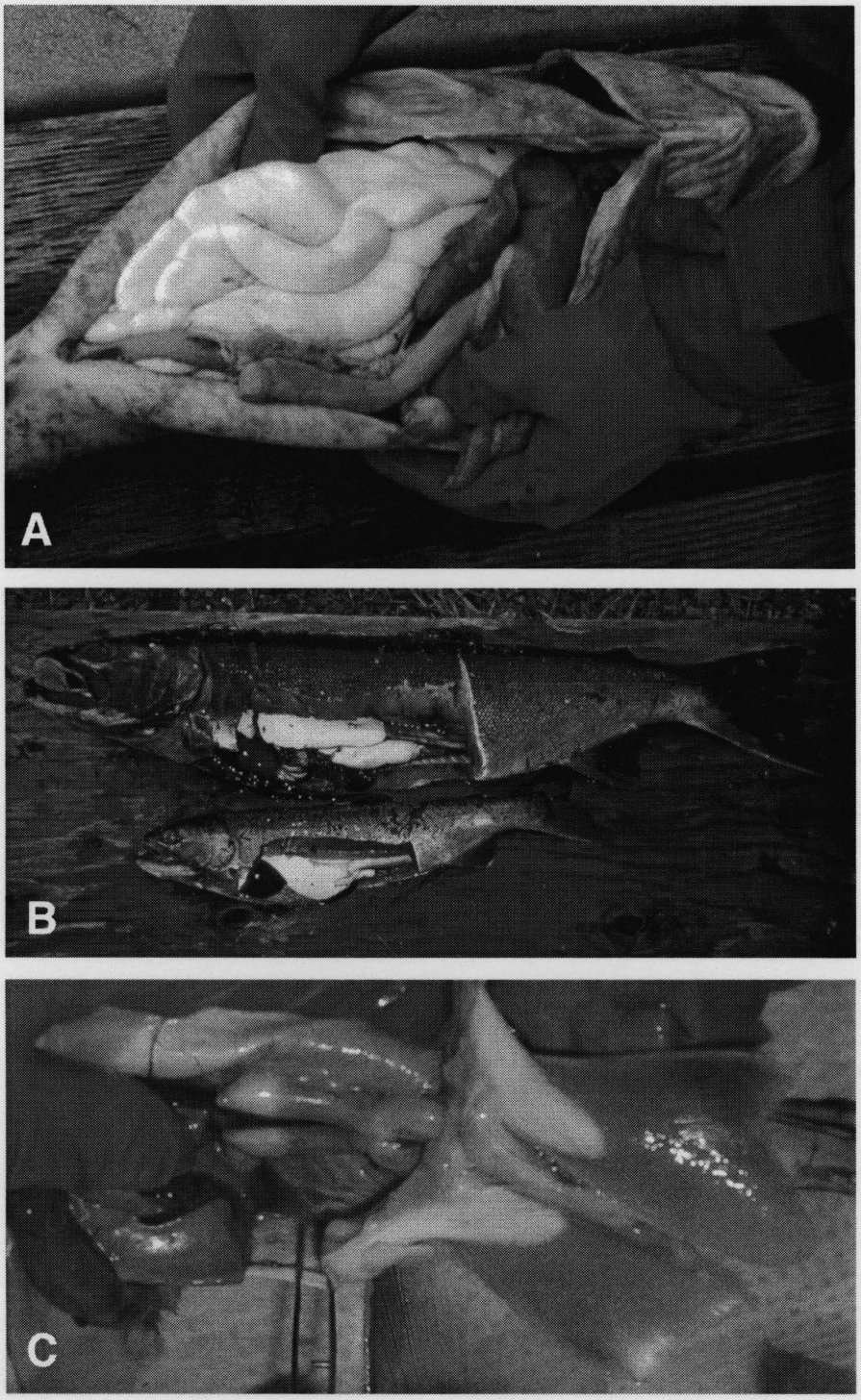

Figure 2.6 Mature testes of sexually active males. **A.** Black cod, *Anoplopoma fimbria* (Anoplopomatidae), showing particularly large investment in gonads. Original photograph by J. L. Fitzpatrick. **B.** Coho salmon, *Oncorhynchus kisutch* (Salmonidae), showing the relatively large testes of a parr male (lower) compared to those of an anadromous male (upper). Original photograph by R. Montgomerie. **C.** Spotted ratfish, *Hydrolagus colliei* (Chimeridae, Chondrichthyes), showing the particularly small and brightly-colored testes of this internally fertilizing species. Original photograph by Nicolas Bury.

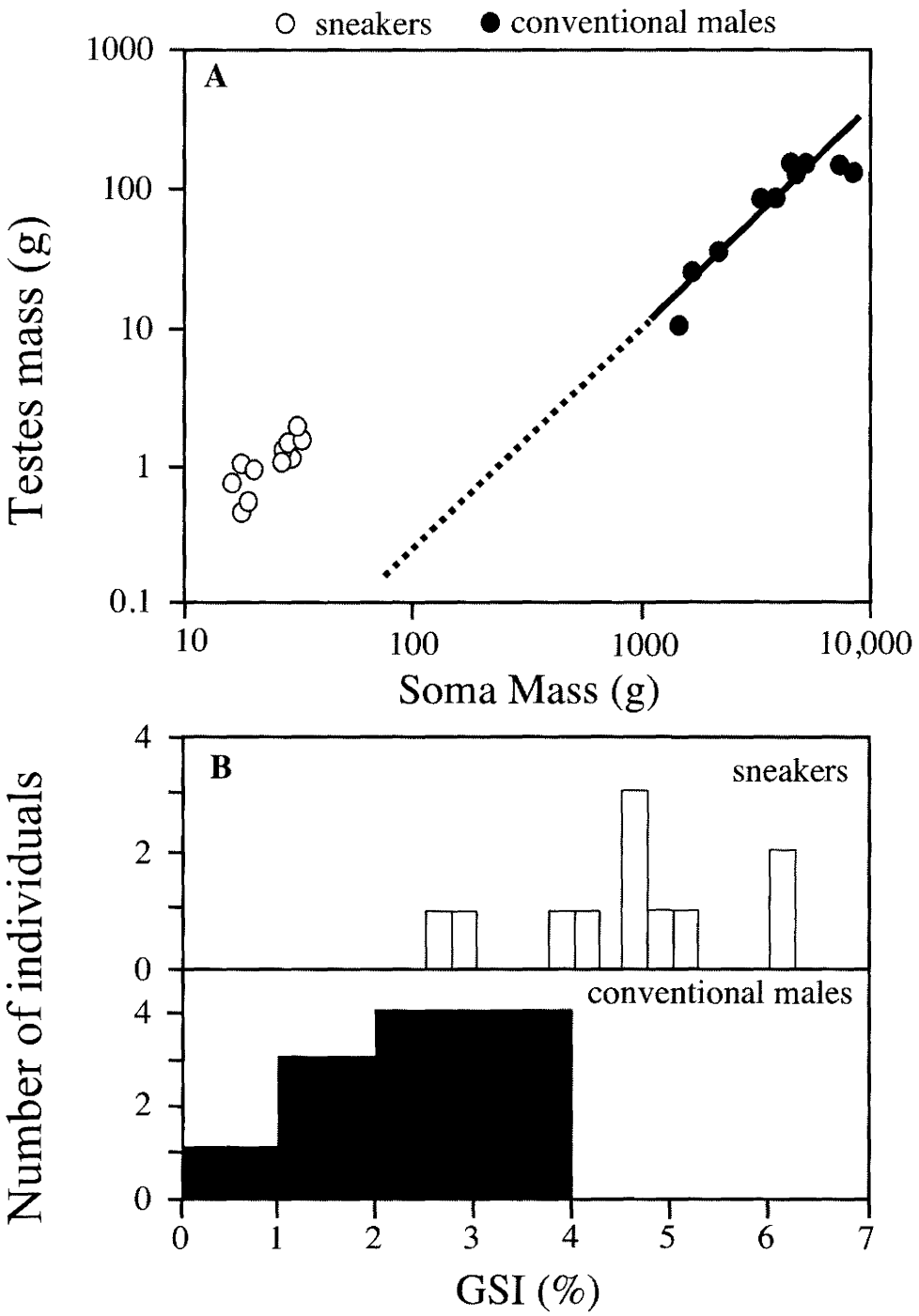

Figure 2.7 Testes size of males adopting alternative reproductive tactics in Atlantic salmon, *Salmo salar* (Salmonidae). **A.** Regressions of testes mass on soma mass for anadromous (conventional) and parr (cuckolder) males. Dotted line extrapolates regression of conventional male data to illustrate relative size of cuckolder testes. Redrawn and reanalyzed from data in Gage et al. 1995, Figure 2. **B.** GSI′ of males adopting each tactic. Original, from data shown in B.

The high investment in testes by Type II males (1.19 ± 0.19 g; mean ± SE) results in the absolute size of their testes being not significantly different from those of Type I males (1.50 ± 0.21 g; **Chapter 3**). As a result, Type II males should be relatively good sperm competitors and are expected to fertilize a relatively high proportion of clutches at which they spawn, although this has yet to be established with genetic paternity data.

Interspecific comparisons. Comparisons among species also show the effects of sperm competition on testes size. For example, species with multi-male spawning bouts have relatively larger testes than those in which a single male spawns with a female at each spawning event (Figure 2.4). On average, the GSI of males that spawn in the presence of other males is 4.6 times that of males that spawn with no other males present where the level of sperm competition appears to be low (Figure 2.4). Most data are currently available on external fertilizers but internal fertilizers are also expected to encounter sperm competition because, at least in some species, females copulate with multiple partners for a single brood and multiple paternity is common (e.g., Evans et al. 2003).

In comparative studies of fish species, Stockley et al. (1997), Petersen and Warner (1998), Balshine et al. (2001), Molloy et al. (2007) and Fitzpatrick et al. (**Chapter 5**) all found that species experiencing greater levels of sperm competition have relatively large testes. Stockley et al. (1997) surveyed the available literature, relying on data from 68 published sources spanning 91 years (from 1902-1993) and controlling for lack of independence due to phylogeny. From that literature they categorized species as belonging to one of six sperm competition ranks, from internal fertilizers with no evidence of polygamy or communal spawning to external fertilizers with no pairing and

high levels of communal spawning. They found that both GSI (n = 24 species) and the number of spermatozoa per stripped ejaculate (n = 17 species) were significantly higher in species classified as having higher levels of sperm competition, compared to their closest relatives having lower levels of sperm competition. Petersen and Warner (1998) restricted their analysis to 32 species from four families of tropical reef fishes, not included in Stockley et al. (1997), and found a similar pattern though they did not control for lack of independence due to phylogeny. The results of these two studies are summarized in Figure 2.4, and show that species experiencing more intense sperm competition have, on average, higher male GSI but also that many species expected to have high levels of sperm competition, actually have rather low GSIs. Balshine et al. (2001) and Fitzpatrick et al. (**Chapter 5**), instead, restricted their analyses to a small clade of cichlids from Lake Tanganyika and focused on residual testes mass rather than GSI. These species show a clearer picture than in previous studies that included a wider variety of taxa: species experiencing sperm competition (polygamous mating systems) have significantly greater residual testes mass than their closest relatives that are unlikely to experience sperm competition (monogamous species). Finally, in a comparison of species with and without sex-change, Molloy et al. (2007) found that relative testes mass was much lower in species where males changed sex than in those with gonochoristic (nonhermaphroditic) males. Species with sex-changing males also have female-biased sex ratios (Molloy et al. 2007), and thus lower levels of sperm competition than gonochoristic species. In contrast to these five studies, Pyron (2000) did not find a significant relation between relative testes size and the magnitude of sperm competition in minnows, controlling for lack of independence due to phylogeny. Pyron (2000)

provides potential explanations for this finding, including the very real possibility that some of these species have MARTs which have so far been undetected. However, further exploration of the constraints and species-specific reproductive biology of these minnow species is warranted if we are to understand this exception to what looks like a general rule.

Based on the general evidence that relative testes investment increases as a result of sperm competition, we can use relative testes size—cautiously—as an index of the strength of sperm competition in fishes. This is a widespread assumption in studies of sperm competition in virtually all animal taxa (see Calhim and Birkhead 2007), based on both the empirical evidence comparing testes investment in species with and without clear evidence for sperm competition, and the logic of testes size as an index of ejaculate size and sperm production rate, presented above. Relative testes size is also thought to be a useful measure of the stength of sperm competition because it does not require direct observation of males competing to fertilize ova, which may be (i) difficult to observe, (ii) even more difficult to quantify with respect to the strength of sperm competition, and (iii) absent at some times and/or some populations even where testes size has been influenced by selection. The assumption, then, is that relative testes size has been shaped by the magnitude of sperm competition, irrespective of male and female behavior that might confound its quantification. In the next two sections, we use this assumption to examine how sperm competition influences the evolution of both ejaculate size and sperm traits, by focusing largely upon relative testes mass as an index of the strength of sperm competition.

2.4 STRATEGIC ALLOCATION OF EJACULATES

As we have argued above, testes size is at least in part the result of selection for maximum ejaculate size necessary for successful fertilization. Sperm competition, however, also influences functional, proximate responses in males with respect to both their behavior and the number of spermatozoa actually released during each spawning event. In a controlled experiment with guppies, for example, males spent more time courting and copulating with females that they had previously seen alone compared to those they had observed copulating with other males (Dosen and Montgomerie 2004). Thus male guppies appeared to be sensitive to the risk of sperm competition, and chose mates where that risk would be minimal. Male fish may also guard females (e.g., Alonzo and Warner 2000) and nests (Svensson and Kvarnemo 2003) more aggressively when the risk of sperm competition increases, suggesting that male fish can often perceive variation in the risks of sperm competition and adjust their behavior according. Theory also predicts that male fish should adjust their ejaculates in response to the intensity of sperm competition, optimizing the release of available sperm stores to maximize their long-term reproductive success (Parker et al. 1996, 1997; Ball and Parker 1996). In this section, we briefly review some of the evidence for such strategic ejaculate allocation in fishes.

Shapiro et al. (1994) were probably the first to look at ejaculate allocation in fishes, in their study of the bluehead wrasse (*Thalassoma bifasciatum*, family Labridae), a small (9 cm) fish that spawns in the water column above tropical coral reefs of the Caribbean and adjacent waters. During 156 pair spawns and 56 group spawns, Shapiro et al. (1994) collected natural ejaculates in large plastic bags by snorkeling to the site of

gamete release. They estimated the number of spermatozoa per ejaculate by filtering the water from the bag and subsampling. When engaged in group spawns involving up to 20 other males, each male released on average six times as many spermatozoa as males spawning alone with a female (Figure 2.8 A). In both cases the number of spermatozoa released increased positively with the number of eggs released by the female (Figure2.8 A). Thus, Shapiro et al. (1994) argued, males appeared to allocate the number of spermatozoa released based on their perception of both the number of ova to be fertilized (possibly assessed by female body size) and their estimate of the intensity of sperm competition. While the conclusions of Shapiro et al. (1994) seemed reasonable, Parker et al. (1996) soon developed a theory of ejaculate allocation under sperm competition that made the opposite, counterintuitive, prediction. According to the 'intensity model' of Parker et al. (1996), for example, a male facing different levels of sperm competition with each spawning event—as was the case the bluehead wrasse studied by Shapiro et al. (1994)—is predicted to release fewer spermatozoa per spawn as the number of sperm competitors increases above two. In this case, Parker et al. (1996) argued that when there are more than two competitors during a spawning event, a male faces diminishing returns on fertilization success as the probability that his spermatozoa will encounter an unfertilized egg is too low to favor the release of more spermatozoa. Thus if a male is faced with strategically allocating sperm stores to several spawning events, he profits most by increasing the number of spermatozoa released when the number of sperm competitors is low. Alternatively, the 'risk model' of Parker et al. (1997) predicts that when the probability of competition between just two ejaculates at a spawning event is low, males should invest more spermatozoa as the risk of sperm competition increases.

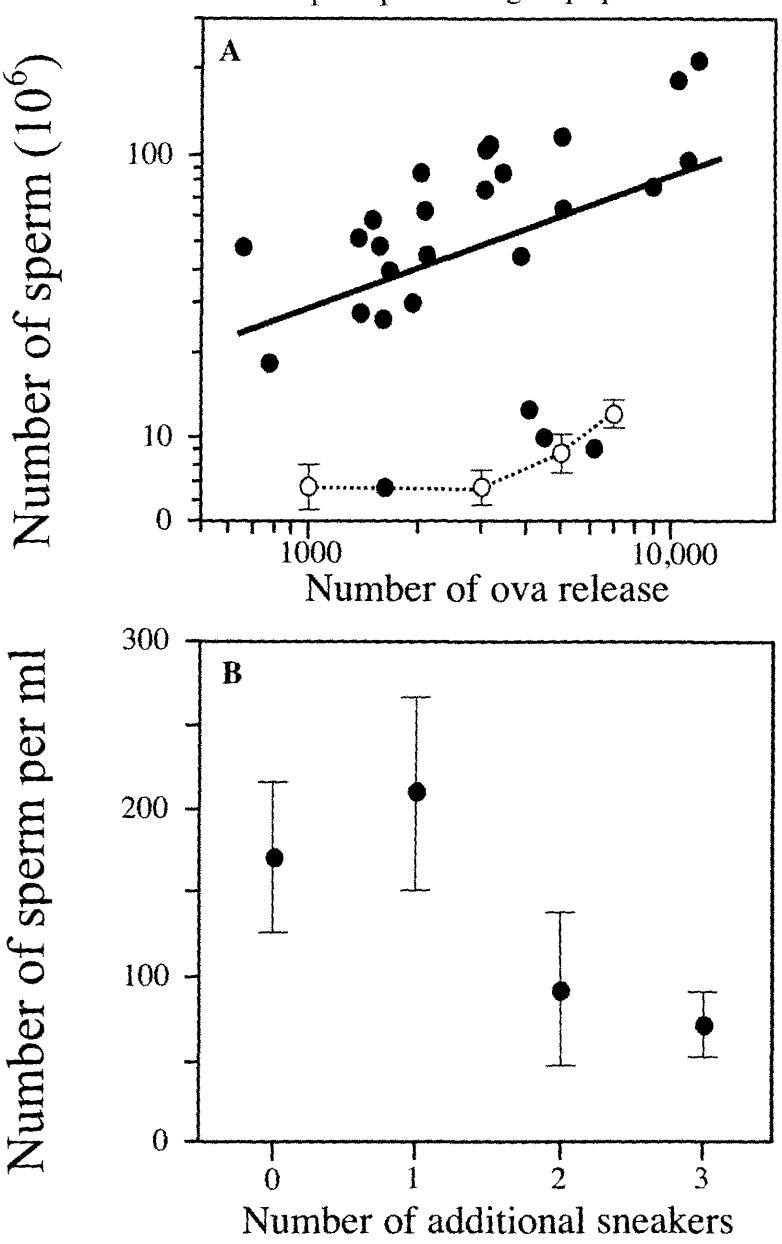

Figure 2.8 Patterns of ejaculate investment in relation to sperm competition. **A.** Relation between numbers of spermatozoa released by each male and number of ova released by the female in the bluehead wrasse, *Thalassoma bifasciatum* (Labridae), at both group-spawns involving up to 20 males and pair spawns involving a single male and female. For group spawns each data point represents a single spawning event, and the regression line shows the trend. For pair spawns, data shown are mean ± 95% CL for each of 4 categories of ova numbers (1-2, 2-4, 4-6, and 6+x103 ova) released by the female, and means are joined by a dotted line. Reanalyzed and redrawn from Shapiro et al. 1994, Figures 2 and 6. **B.** Index of the amount of sperm released (concentration of spermatozoa in controlled volume of water±95%CL) by each black goby, *Gobius niger* (Gobiidae), sneaker male during spawning in the presence of 0-3 other sneakers. Reanalyzed and redrawn from Pilastro et al. 2002, Figure 2.

Pilastro et al. (2002) explicitly tested the intensity model of Parker et al. (1996) in controlled experiments with both grass (*Zosterisessor ophiocephalus*) and black gobies (*Gobius niger*), both in the family Gobiidae. In these experiments, small aquaria were partitioned so that samples of spermatozoa released by a focal male could be collected without contamination, and the concentration of released spermatozoa determined. Focal males were cuckolders (sneakers) induced to spawn while observing a female in a separate compartment, and 0, 1, 2, or 4 other sneakers, who were potential sperm competitors, in other compartments. In both species, the amount of spermatozoa released by focal sneakers was highest when only one potential sperm competitor was present, and declined as the number of such sneakers increased (Figure 2.8 B), just as predicted by the intensity model. Interestingly, when they repeated these experiments, focusing instead on territorial males, they found no differential sperm allocation in response to the number of sneakers present though the territorial males did increase the amount of effort expended in guarding their nests (Scaggiante et al. 2005). To explain this, Scaggiante et al. (2005) suggested (i) that territorial males may have a fixed tactic since they might not always be able to detect the presence of sneakers, or (ii) that territorial male ejaculates are optimized to provide enough spermatozoa for the rather long period of fertilization in these species (which can last several hours) and thus cannot be finely tuned to variation in perceived levels of sperm competition.

Studies of internally fertilizing fishes have also found that males adjust ejaculates in response to the risk of sperm competition. In an experimental study of eastern mosquitofish (*Gambusia holbrooki*, family Poeciliidae), for example, Evans et al. (2003) found that males who copulated with females in the presence of a competitor attempted to

mate more often and released more spermatozoa than males who copulated alone. Similarly, male sailfin mollies (*Poecilia latipinna*, family Poeciliidae) expelled more spermatozoa in a higher risk (1 or 3 other males present) than in a low risk (no other males present) experimental treatment (Aspbury 2007). Aspbury (2007) also tested the intensity model by comparing the number of spermatozoa obtained from males in high (3 competitors), medium (1 competitor), and low (no competitors) intensity. Males in the low intensity treatment actually had more spermatozoa ready to release than males in the medium and high intensity treatments, a result that does not match the predictions of the intensity model (Parker et al. 1996).

Fish have proven to be good subjects for testing predictions of sperm competition with respect to ejaculate allocation in part because so many species experience variation in both the risk and intensity of sperm competition, and these factors can often be controlled experimentally. Moreover, some useful techniques for collecting the spermatozoa released by both internally (Evans et al. 2003; Aspbury 2007) and externally fertilizing species (Shapiro et al. 1994; Marconato et al. 1995; Marconato and Shapiro 1996; Fuller 1998; Pilastro et al. 2002; Zbinden et al. 2003, 2004; Fitzpatrick and Liley 2008) allow accurate quantification of the number of spermatozoa that a male releases in different situations. Most important, the results of studies done so far provide clear support for the risk model but are equivocal with respect to the intensity model. There is clearly scope for more empirical study of this subject and further development of the theory (e.g., Williams et al. 2005).

2.5 SPERM DESIGN AND SPERM COMPETITION

Studies of sperm morphology in fishes, both within and between families, have revealed considerable variation in sperm design, including variation in head size and shape, midpiece size, and the number and length of flagella (Mattei 1988, 1991; Jamieson 1991). While it is likely that sexual selection in the form of sperm competition has influenced the evolution of many aspects of sperm design, we confine our discussion in this chapter to an examination of the selective forces that influence the number and length of flagella. Though some recent studies have looked at the influence of sperm competition on head and midpiece traits in other taxa (e.g., Johnson and Briskie 1999), only sperm size, and particularly flagellum length, has so far been studied in this context in fishes, possibly because it is an obvious morphological feature that is relatively easy to measure.

The primary role of the flagellum, of course, is to propel the spermatozoa toward the ova to effect fertilization. The majority of fish species have spermatozoa with a single flagellum (i.e., monoflagellate), and most of the data currently available with respect to sperm competition are from species with this type of spermatozoon. However, some fish species have spermatozoa that is aflagellate (no flagellum), biflagellate (two flagella), or even a mixture of sperm designs. Our main focus is on variation among monoflagellate sperm designs, but we include sections on the other sperm designs as well and briefly assess how they might also be influenced by sperm competition.

2.5.1 Sperm Length

Gaining an appreciation of the diversity in sperm size across species and placing this diversity in an evolutionary context was not an easy task for early sperm biologists.

When spermatozoa from more than one male compete to fertilize ova, males with more competitive spermatozoa will fertilize a greater proportion of female eggs. Therefore, when sperm competition occurs, selection may act in one of two ways depending on how sperm size influences sperm competitiveness. If sperm number is an important determinant of male fertilization success, then theory predicts that males experiencing elevated levels of sperm competition will increase sperm production while producing smaller spermatozoa, trading off sperm size for sperm number (Parker 1982). Such tradeoffs when producing an ejaculate are expected because sperm production can be costly (Nakatsuru and Kramer 1982; Dewsbury 1982), and effective investment of metabolic resources should be under intense selective pressure as these ejaculate traits directly impact fitness. However, if the competitive ability of spermatozoa is influenced by their flagellum length (either positively or negatively), then theory predicts that sperm size will be shaped by the strength of sperm competition (Ball and Parker 1996).

Sperm length can influence the competitiveness of spermatozoa if greater sperm length allows a spermatozoon to swim faster (Gomendio and Roldan 1991; Fitzpatrick *et al.* **Chapter 5**). Selection for longer and faster spermatozoa may be particularly intense in fishes owing to the fertilization dynamics associated with interactions between spermatozoa and eggs. Spermatozoa of the Neopterygii, and therefore most fishes, lack an acrosome, with very rare exceptions (e.g. Jamieson 1991) and gain entry into the unfertilized ovum by swimming down the micropyle, a narrow canal the leads to the interior of the egg (Jamieson 1991). Because the first spermatozoon that swim down the micropyle successfully fertilizes the ovum (Yanagimachi *et al.* 1992), sperm swimming speed is likely to be the primary determinant of male fertilization success. Under these

conditions, selection should act on increasing sperm swimming speed, which in turn is predicted to increase with sperm length (Ball and Parker 1996). Indeed, in experimental mating trials with Atlantic salmon, when spermatozoa from two males competed, the spermatozoa that swam faster fertilized a greater proportion of the ova (Gage et al. 2004). Thus species experiencing greater levels of sperm competition are expected to have longer sperm flagella, as discussed below.

Fishes show dramatic variation in sperm size across species and surprising variation within both internal and external fertilizers. The size of monoflagellate spermatozoa, for example, varies six-fold in the 139 species of external fertilizers studied so far, ranging from 13 μm in the mullet, *Mugil cephalus* (family Mugilidae; Stockley et al. 1997), to 70 μm in the cichlid, *Pelvicachromis taeniatus* (family Cichlidae; Thünken et al. 2007). Similarly, in 11 species of internal fertilizers, spermatozoon size ranges from 39 μm in the viviparous eelpout, *Zoarces viviparous* (family Zoarcidae), to 143 μm in spotted ratfish (*Hydrolagus colliei*, family Chimaeridae; Stockley et al. 1997), representing more than three-fold variation in sperm size. The actual interspecific ranges of sperm size are undoubtedly larger as we currently have data on fewer than 1% of the 28,000 extant fish species.

Variation in sperm size is also evident even within closely related species. For example, among 35 species of African cichlids (family Cichlidae) endemic to Lake Tanganyika, the longest spermatozoon, from *Telmatochormis vittatus* (33.3 μm), was more than twice as long as the shortest, from *Asprotilapia leptura* (14.6 μm) (Balshine et al. 2001; Fitzpatrick *et al.* **Chapter 5**), despite relatively recent speciation (9-12 million years, Cohen et al. 1993). Again, this represents only about 20% of the species in this

taxon, so even more interspecific variation is to be expected from the full sample of species.

Even intraspecifically, sperm size varies among male fishes, despite little variation within individuals, consistent with patterns reported in mammals, insects, and birds (e.g., Ward and Hauschtech-Jungen 1993; Birkhead et al. 2005; Gomendio et al. 2007). For example, the gross sperm morphology of Atlantic salmon has been examined in three studies, each describing among-male variation (Vladic and Järvi 2002; Gage et al. 1998, 2002). Of these, Gage et al. (2002) reported among-male variation in mean sperm size from 32.3-39.5 μm, representing a 1.22-fold difference between the extremes (Figure 2.9 A). In other species, mean sperm length has been recorded to vary 1.25-1.5 fold among individuals: 24.5-37.5 μm (n = 38 males) in the African cichlid, *Telmatochromis vittatus* (Fitzpatrick *et al.* **Chapter 4**), 60.0-75.7 μm (n = 15 males) in the cichlid *Pelvicachromis taeniatus* (Figure 2.9 B; Thünken et al. 2007), 40.1-48.1 μm (n = 22 males) in conventional male bluegill (Casselman and Montgomerie 2004), and 35.8-49.5 μm (n = 37 males) in the guppy (*Poecelia reticulata*, family Poeciliidae, Pitcher et al. 2007). While it is likely that such among-male variation in sperm size is common in fishes, most published studies present only means and standard errors, making it difficult to assess the extent of variation in sperm size among males, especially if distributions are non-normal. Even in the species in which intraspecific variation in sperm length has been studied in detail, there is usually no relation between a male's sperm length and his own size or alternative reproductive tactic (Figure 2. 9 A)—see Table 1.1 for more information and the single exception.

Detailed behavioral data combined with genetic information on paternity are the

best indices of the strength of sperm competition, but such data are available for only a handful of species. Instead, early studies of the relation between sperm size and sperm competition relied on descriptions of reproductive behavior to estimate the level of sperm competition faced by males. The first such analysis in fishes was performed across a broad range of species, including internal and external fertilizers (Stockley et al. 1997). To assess the wide variety of mating behaviors and associated differences in the threat of sperm competition experienced by males, Stockley et al. (1997) classified the strength of sperm competition across species on a six-point scale as described in **Section 2.3.3**. Contrary to predictions from theory (Ball and Parker 1996), there was a negative relation between sperm size and the level of sperm competition, with species apparently experiencing the lowest levels of sperm competition having the largest spermatozoa (Stockley et al. 1997). While studies on the association between sperm size and indices of the strength of sperm competition have sometimes yielded ambiguous results (reviewed in Snook 2005), Stockley et al. (1997) on fish and, more recently, Immler and Birkhead (2007) on birds, are the only studies to date that have found a significant negative association between sperm size and the level of sperm competition in any taxon.

The perplexing result reported by Stockley et al. (1997) has been cited as an oddity in comparative studies of sperm competition for the last decade, but a closer analysis of these results yields some anomalies. For example, the use of ecological data to categorize the strength of sperm competition is at best approximate, and subsequent research has suggested that some of the classifications used by Stockley et al. (1997) might not accurately reflect the level of sperm competition within some species (see Balshine et al. 2001). Furthermore, two of the species included in the Stockley et al.

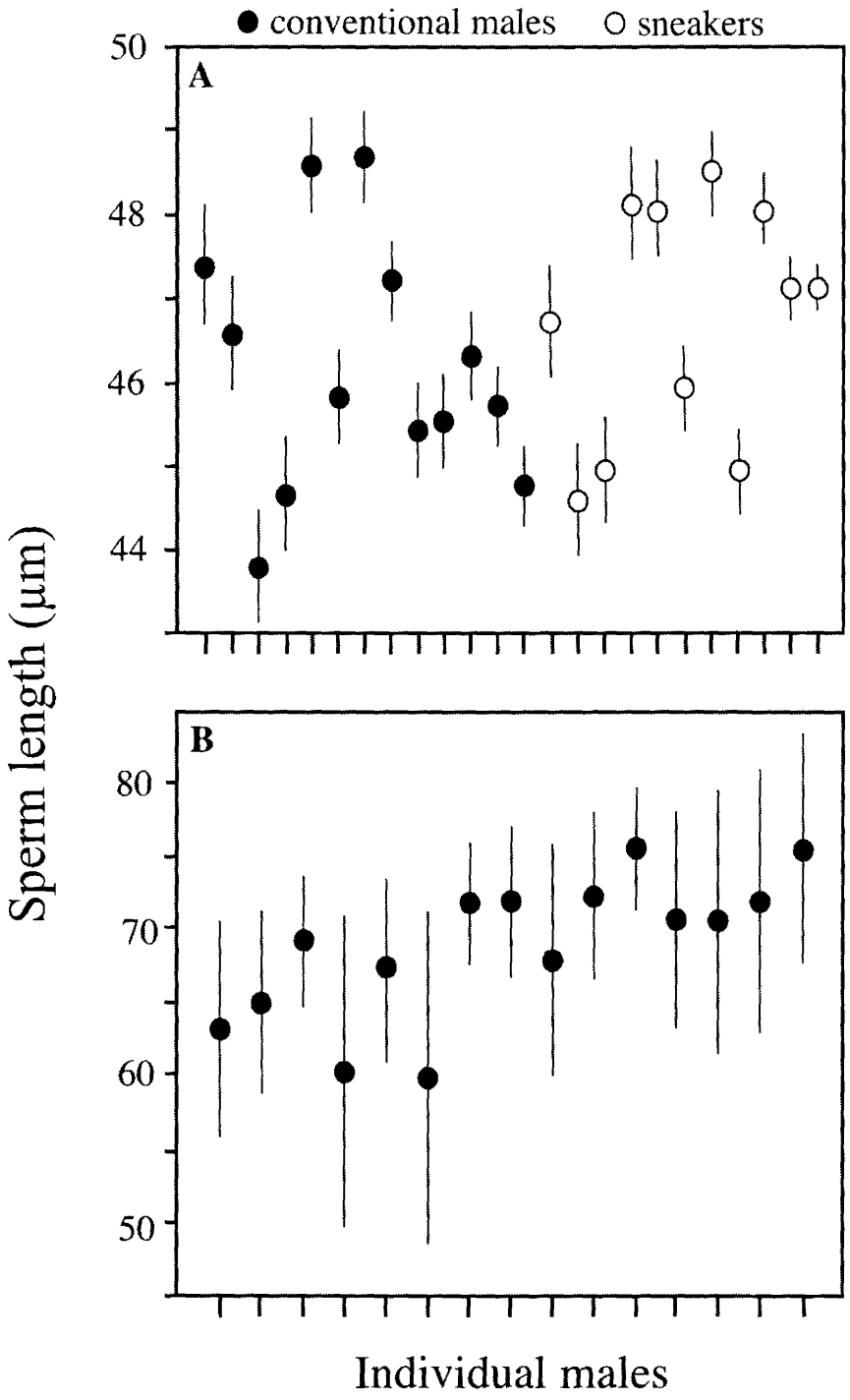

Figure 2.9 Intraspecific variation (mean±95%CL) in sperm length. **A.** In conventional (anadromous) and cuckolder (parr) male Atlantic salmon (n = 30 spermatozoa per male). Redrawn and reanalyzed from data in Gage et al. 1998. **B.** In the cichlid, *Pelvicachromis taeniatus* (n = 6-18 spermatozoa per male). Redrawn and reanalyzed from data in Thünken et al. 2007, assuming 12 spermatozoa measured per male for calculation of CL.

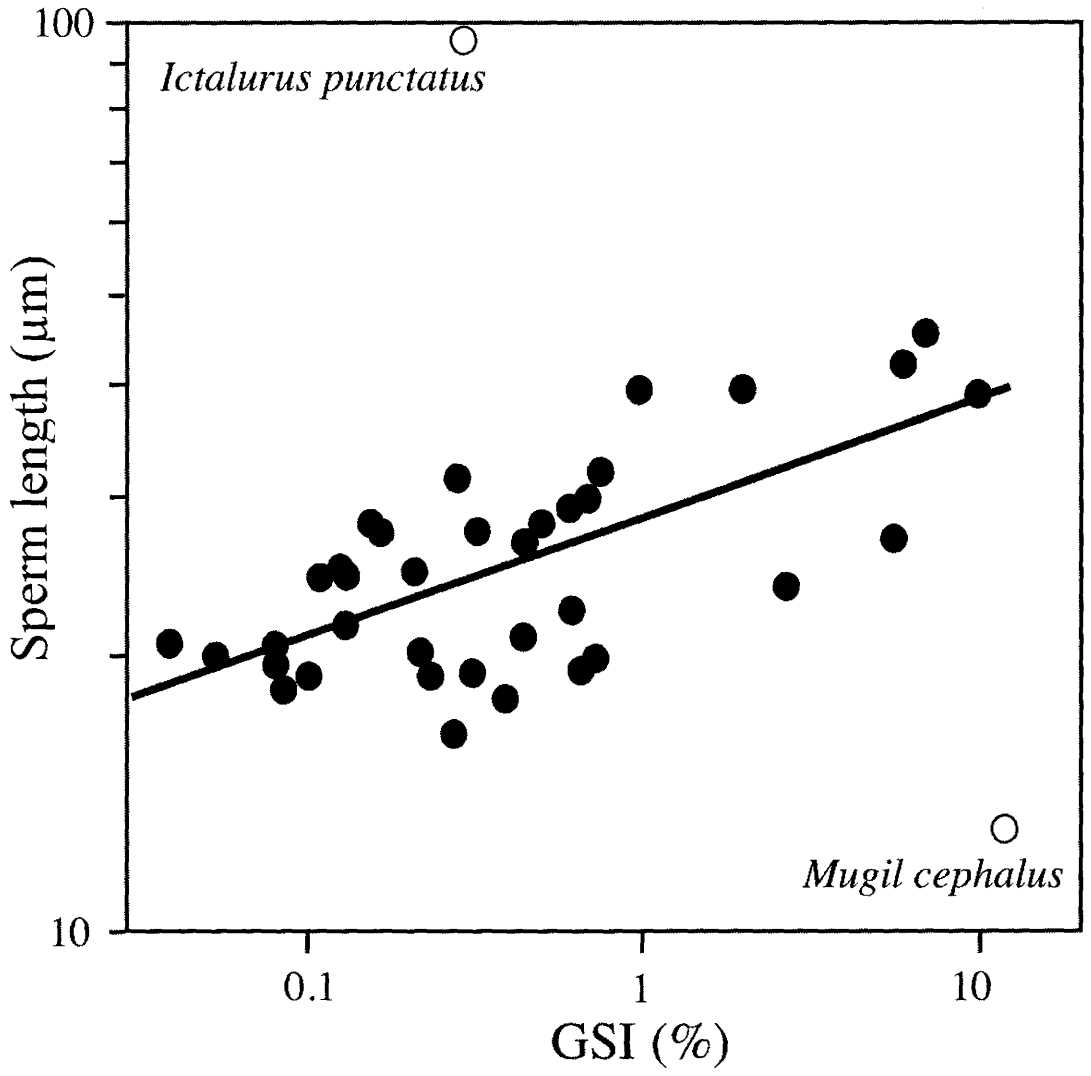

Figure 2.10 Interspecific relation between sperm length and GSI (as a measure of the strength of sperm competition). Two outliers not included in this analysis (see text) are shown as open circles. Original, from data in Stockley et al. 1997, Balshine et al.2001, and Montgomerie and Fitzpatrick (unpublished).

(1997) analysis may have confounded their results. First, the channel catfish, *Ictalurus punctatus* (family Ictaluridae), is a species with biflagellate spermatozoa (Poirier and Nicholson 1982). Given our lack of understanding of the forces that lead to the evolution of biflagellate spermatozoa (see **Section 2.5.2**), this species represents an anomaly that cannot be reasonably compared with species having monoflagellate spermatozoa. Second, the mullet, *Mugil cephalus*, is an extreme outlier in both testes and sperm size, with the smallest spermatozoa (13 μm) reported in any fish species to date, and the largest relative testes investment reported for mature male fish, at up to 20% of male body mass (Render et al. 1995). Excluding these two species from the dataset, and adding information on some additional species studied since Stockley et al. (1997), sperm size shows a positive association with relative testes size across fish species (Figure 2.10; Montgomerie and Fitzpatrick unpublished), thereby supporting predictions from sperm competition theory. Certainly, *Mugil cephalus* is an interesting outlier to this general pattern (Figure 2.10) that should be studied further.

To overcome some of the issues that arise when comparing fishes from a wide variety of phylogenetic backgrounds, spawning environments (e.g., water chemistry and temperature) and ecologies, Balshine et al. (2001) and Fitzpatrick et al. (**Chapter 5**) collected data from the field to examine the relationship between sperm size and the strength of sperm competition in a group of closely related African cichlids from Lake Tanganyika. These cichlids are particularly well suited to study how sperm competition influences male reproductive characteristics as they exhibit tremendous diversity in mating systems, and consequently the intensities of sperm competition experienced by males. Additionally, there is extensive information available on phylogenetic associations

between species based on molecular sequence data, so that analyses can control for lack of independence due to phylogeny (e.g., Balshine et al. 2001; Fitzpatrick et al. **Chapter 5**). In these closely related cichlids, sperm length was positively correlated with relative testes mass (Balshine et al. 2001; Fitzpatrick et al. **Chapter 5**), controlling for phylogeny (Figure 2.11). Balshine et al. (2001) extended their analysis by incorporating mating system information and by using pairwise comparisons showed that spermatozoa from polygamous species were longer than those of closely related monogamous species (Figure 2.11).

Given the difference in the strength of sperm competition facing conventional and cuckolder males, a difference in sperm morphology between males adopting alternative reproductive tactics might also be expected. General sperm competition theory (Ball and Parker 1996) suggests that selection should act to increase sperm length in sneaker males because they face elevated levels of sperm competition when compared to conventional males. Although in one study of bluegill, spermatozoa from sneakers were slightly longer than spermatozoa from conventional males (Burness et al. 2004), this has not been supported by other studies of this same species (Leach and Montgomerie 2000; Stoltz and Neff 2006), nor has this pattern been described in other fish species with MARTs studied to date (Table 2.1). If sperm size is largely determined genetically, with little influence from male phenotype (condition, etc), and MARTs are simply phenotypic responses to slow growth rates, then no difference in the size of spermatozoa from cuckolder and conventional males would be expected. This is an interesting problem that deserves further study.

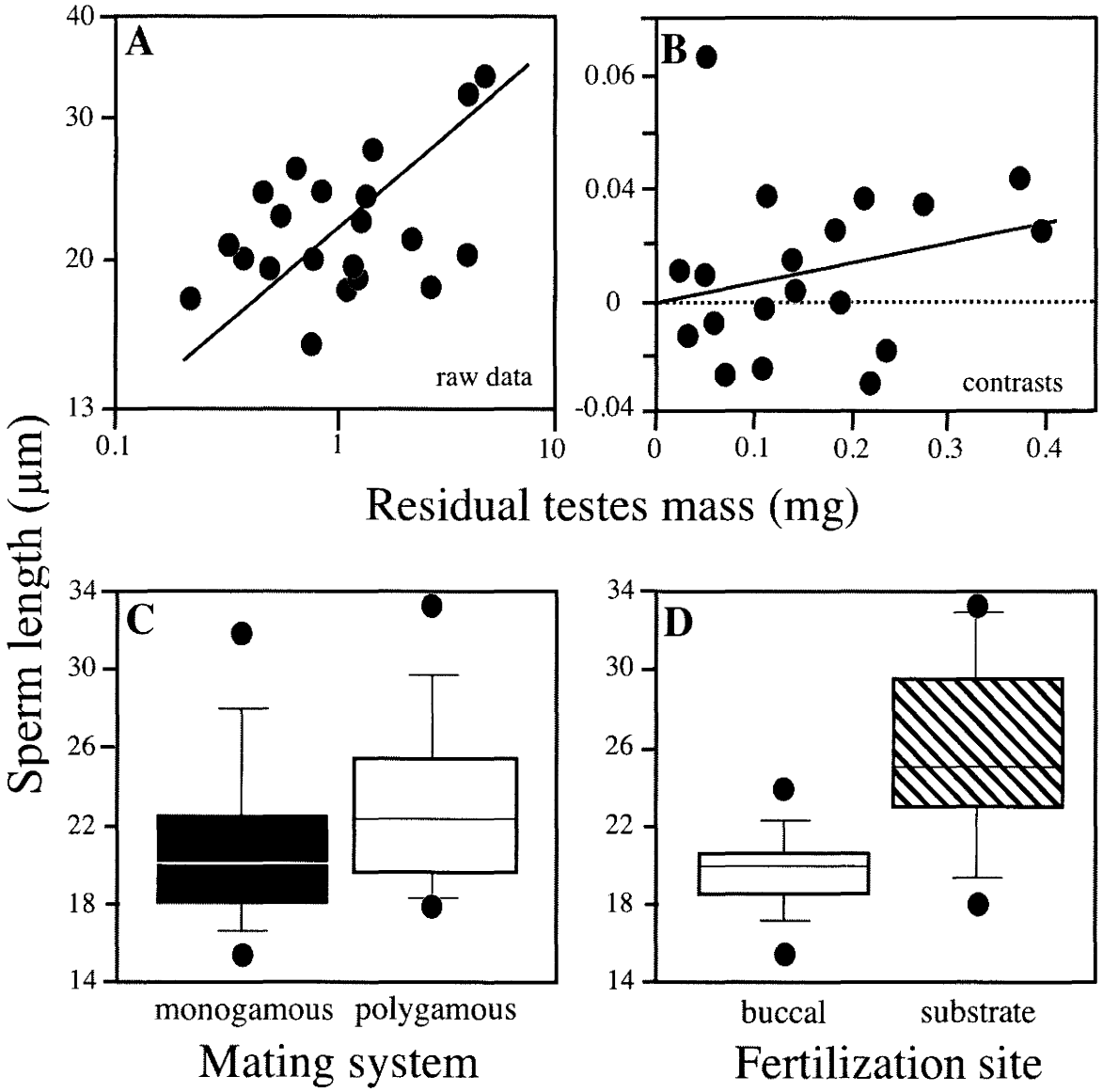

Figure 2.11 Patterns of variation in sperm length among cichlid species from Lake Tanganyika. **A.** Regression of sperm length on relative testes size (residuals of log testes mass on log body mass). **B.** Regression of independent contrasts of sperm length on relative testes size, controlling for phylogeny. **C.** Sperm length varies with mating system (Tukey box plots). **D.** Sperm length varies with site of fertilization (Tukey box plots). Redrawn from Balshine et al. 2001, Figures1 and 2.

2.5.2 Number of Flagella

We now turn our attention to assessing how sperm competition influences the evolution of both fewer and more flagella than the typical number (one flagellum) in fishes. We examine the evolution of both aflagellate and biflagellate spermatozoa in this context, but stop short of looking at inter- and intraspecific variation, as there is currently no information available relative to sperm competition. Instead, we examine evidence that sperm competition may have influenced the evolution of these two sperm morphologies.

Aflagellate spermatozoa. We have just seen that the selective force of sperm competition in species with monoflagellate spermatozoa can lead to the evolution of longer flagella. If we turn this argument around, then, in species where sperm competition is absent, selection may act to reduce sperm length, in extreme cases favoring the evolution of aflagellate spermatozoa, lacking a flagellum altogether. This prediction is based on the assumption that spermatozoa are costly to produce and that males producing aflagellate spermatozoa in the absence of sperm competition will gain a metabolic advantage that translates into higher fitness via increased survival. Ejaculates composed entirely of aflagellate spermatozoa are rare in fishes, described so far only in the families Mormyridae and Gymnarchidae (superfamily Mormyroidea; Mattei 1988; Morrow 2004). The Gymnarchidae, which are basal to the Mormyridae (Van der Bank and Kramer 1996), produce motile aflagellate spermatozoa that move in an amoeboid-like fashion, while the more derived Mormyridae (Figure 2.12) have immotile aflagellate spermatozoa (Mattei 1988; 1991).

Because aflagellate spermatozoa are so rare in extant fishes, it is difficult to assess

the evolutionary forces that may have led to the formation of spermatozoa lacking tails. However, aflagellate spermatozoa are found in at least 36 plant and animal taxa, from algae to invertebrates to fishes (Morrow 2004). Across this wide range of taxa, Morrow (2004) asked whether aflagellate spermatozoa were associated with the reduction of selective forces due to sperm competition, comparing taxa having aflagellate spermatozoa to their nearest relatives that had spermatozoa with flagella with respect to their expected level of sperm competition. Although there were limitations in this analysis due to small sample sizes, Morrow (2004) found some evidence that the evolution of aflagellate spermatozoa was associated with groups in which sperm competition was absent.

Physiological and behavioral data from some species producing aflagellate spermatozoa (in the superfamily Mormyroidea) suggest that it is very unlikely that males encounter any sperm competition (Morrow 2004). For example, in the elephant-snout fish (*Mormyrus kannume* (family Mormyridae), testes mass comprises only about 0.5% of male body mass (Iles 1960), suggesting that sperm competition is rare or absent in this species. Similarly, behavioral studies of spawning behavior in both the elephant-snout fish and the elephant fish (*Pollimyrus isidori,* family Mormyridae) report that males aggressively defend nests, and a single male and female are in close physical proximity during spawning, forming a close association between anal fins during gamete release. Females do mate with more than one male during a breeding season but appear to do so in a sequentially monogamous manner (Iles 1960; Morrow 2004).

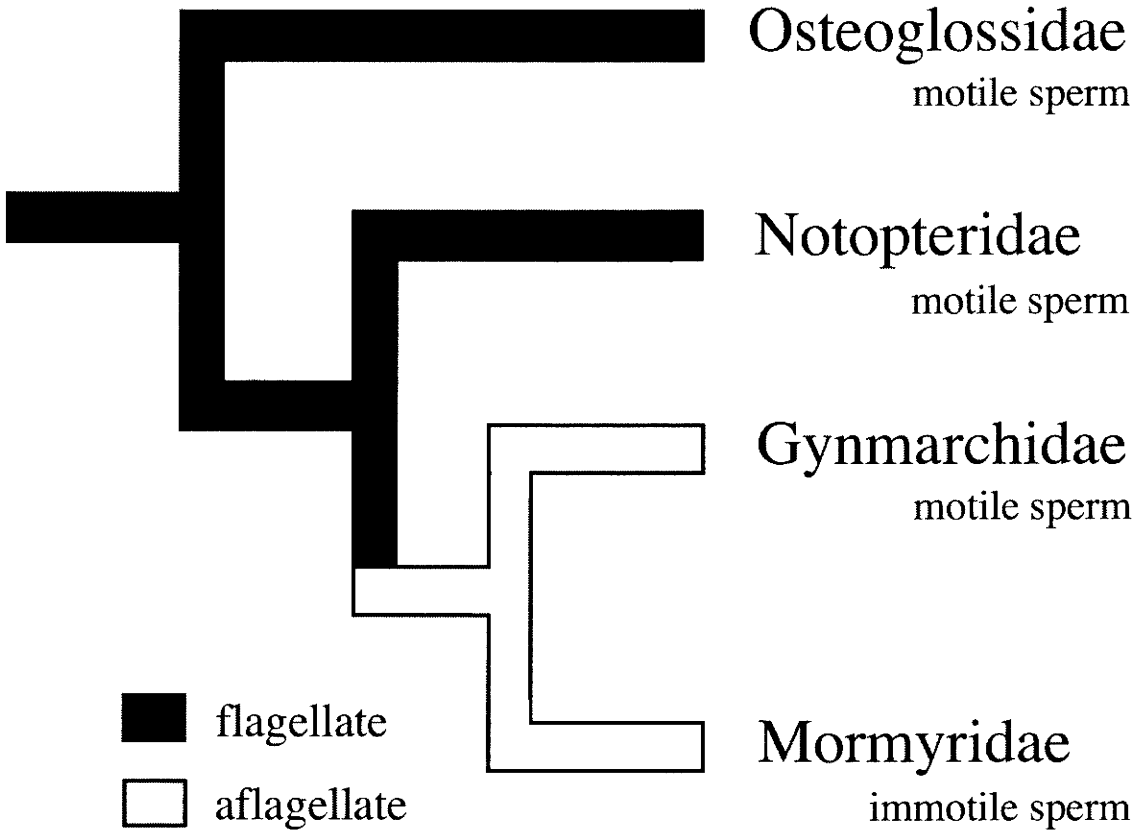

Figure 2.12 Phylogenetic relationship between the superfamily Mormyroidea (families Mormyridae and Gymnarchidae), which produce aflagellate spermatozoa, and their nearest sister families. Modified from Lavoué and Sullivan 2004, Figure 3.

Although a broad phylogenetic analysis of the evolution of aflagellate spermatozoa is not possible in fishes, we can potentially assess the role that sperm competition played in the evolution of aflagellation by contrasting species in the superfamily Mormyroidea (producing aflagellate spermatozoa), to those in their nearest sister family, the Notopteridae, which produce flagellated spermatozoa (Van Der Bank and Kramer 1996; Morrow 2004). However, the breeding behaviors described for the Notopteridae (Morrow 2004) are similar to those described in the Mormyroidea, suggesting that males in this taxon do not experience intense sperm competition either. Therefore, in this comparison, there is no evidence that the evolutionary transition to aflagellate spermatozoa was a response to relaxed selective pressure associated with sperm competition (Morrow 2004). However, assessing the phylogenetic context of sperm characteristics suggests that directional selection may be taking place where, following the removal of the selective pressures associated with sperm competition, an ancestral state of flagellated spermatozoa, in the Notopteridae, gave way to motile, aflagellate spermatozoa, in the Gymnarchidae, which in turn led to the production of immotile, aflagellate spermatozoa in the Mormyridae (Figure 2.12). Further tests of this hypothesis using directional tests of evolution or through path analysis may shed light on this transition.

Biflagellate spermatozoa. Biflagellate spermatozoa have been documented in at least 31 fish species from 16 families belonging to seven orders. These families are distantly related and there is no obvious phylogenetic pattern underlying the evolution of biflagellate spermatozoa. Even within families, some species have biflagellate spermatozoa where others are monoflagellate, suggesting that this trait has evolved

independently many times. For example, catfish of different species in the family Pimelodidae have monoflagellate (Quagio-Grassiotto and Carvalho 2000) and biflagellate spermatozoa (Maggese et al. 1984). The independent evolution of biflagellarity is suggested eight times in the Siluriformes (Burns et al. 2008). Within a single species, ejaculates can also be composed of both mono- and biflagellate spermatozoa, as in several species of cardinal fish (family Apogonidae, Lahnsteiner 2003; Fishelson et al. 2006). Furthermore, production of biflagellate spermatozoa is not specific to the mode of fertilization within a species. While the majority of species with biflagellate spermatozoa are external fertilizers, Yao et al. (1995) documented biflagellate spermatozoa in the internally-fertilizing ocean pout, *Macrozoarces americanus* (family Zoarcidae).

Most studies examining biflagellate spermatozoa have focused on descriptions of sperm ultrastructure in species for which there is no information available on sperm behavior or fertilization dynamics. As a consequence, we can only speculate about the factors that might have favored the evolution of this sperm type under sperm competition. First, it is possible that spermatozoa with two flagella would swim faster than spermatozoa with only one, given the necessary energy input to each flagellum. Evidence from multiflagellar sperm trains in rodents (Moore et al. 2002) supports this idea. Second, biflagellate spermatozoa may be better able to exclude other spermatozoa from the narrow micropylar canal during fertilization, by physically obstructing rival spermatozoa. Finally, the presence of two flagella might increase the maneuverability of spermatozoa, enhancing their ability to find the ova and micropyle more efficiently. Empirical evidence in support of the idea that biflagellate spermatozoa represent an adaptation that enhances sperm competitiveness is, however, quite limited. For example, biflagellate spermatozoa

have been reported in the plainfin midshipman (Stanley 1965; **Chapter 3**), a species characterized by male alternative reproductive tactics and intense levels of sperm competition (Brantley and Bass 1994; see **Section 2.3.3, Chapter 3**). On the other hand, biflagellate spermatozoa are common in the cardinal fishes, a taxon in which a male wraps his anal fin around the female's abdomen, covering her genital papilla during fertilization (Lahnsteiner 2003), presumably reducing the strength of sperm competition. Clearly some detailed studies of species with biflagellate spermatozoa are needed to assess both sperm behavior and the level of sperm competition experienced by males of these species in the wild.

There are likely many more fish species with biflagellate spermatozoa than have currently been recorded since the spermatozoa of so few fish species have been closely examined. Moreover, because the two flagella are so close to one another, identifying biflagellate spermatozoa using a light microscope can be challenging. For example, when examining spermatozoon morphology in the channel catfish, *Ictalurus punctatus*, using a light microscope, Jaspers et al. (1976) described monoflagellate spermatozoa but reported that about 5% were aberrantly biflagellate. A later study, using electron microscopy, revealed that all spermatozoa in this species were indeed biflagellate, although the two tails were closely positioned (Poirier and Nicholson 1982).

Despite the difficulties associated with identifying biflagellate spermatozoa, this interesting sperm morphology is being described in a growing number of species. Careful examination of the phylogenetic relations between species characterized by biflagellate spermatozoa is warranted and may provide insights into the selective forces that promoted the evolution of multiflagellate spermatozoa.

2.5.3 Sperm Heteromorphisms

Males of some fish species produce two distinct types of spermatozoa in a single ejaculate: fertilizing 'eusperm' and non-fertilizing 'parasperm'. As noted by Healy and Jamieson (1981; see also Jamieson 1987), parasperm may be eupyrene, oligogopyrene or apyrene. In fishes, excess chromatin, the hyperpyrene condition, has been described for cottoids (Hayakawa 2007). Eusperm have a complete haploid genome and fertilize eggs, while parasperm have different morphologies and do not fertilize ova. Paraspermatogenesis, like euspermatogenesis, is a distinct developmental process, such that parasperm represent an adaptation and not simply aberrant or deformed spermatozoa unlike those sometimes found in, for example, mammalian ejaculates (Gould 1980).

Sperm polymorphisms are common in invertebrates and their evolutionary origin has been hotly debated (Silbergleid et al. 1984; Snook 1997; Swallow and Wilkinson 2002). Competing theories include a role for non-fertilizing spermatozoa (i) to facilitate the transport of fertilizing spermatozoa to the egg, (ii) to provide nutrients to the female or developing embryo, or (iii) to enhance sperm competitiveness either by blocking the fertilizing spermatozoa of rival males or by delaying female remating rate (reviewed by Silberglied et al. 1984; Jamieson 1987; Swallow and Wilkinson 2002). Such spermatozoon polymorphisms are not common in vertebrates. In the fishes, for example, the presence of parasperm has been suggested or confirmed only in 13 species of sculpin. Of these species, sperm ultrastructure and the functional role of parasperm has been examined in the most detail in the marine sculpins *Hemilepidotus gilberti* (family Cottidae;) and *Blepsias cirrhosus* (family Hemitripteridae; reviewed by Hayakawa 2007).

In both species, eusperm ultrastructure resembles that of typical teleost spermatozoa, having a head without an acrosome, a short midpiece, and a single flagellum (Hayakawa et al. 2002b; Hayakawa and Munehara 2004). In contrast, the parasperm of *H. gilberti* are oval and lack a flagellum (Hayakawa *et al.* 2002b), while those of *B. cirrhosus* have two flagella that are much smaller than the eusperm flagella within the same ejaculate (Hayakawa and Munehara 2004).

Parasperm are thought to play a role during fertilization in *H. gilberti* by enhancing the competitiveness of a male's eusperm. In this species, males defend territories adjacent to one another and attempt to sneak fertilizations in neighboring males' territories (Hayakawa and Munehara 1996). Females spawn only once during the breeding season, laying their eggs, enclosed in a thick layer of ovarian fluid, on the substrate while males release spermatozoa from a distance (~10 cm) beginning about 10 min after the female begins releasing ova. Spawning lasts approximately 20-50 min. Males direct aggressive behaviors against neighboring males prior to sperm release, but gradually reduce this aggression after they begin releasing sperm. Parasperm are released with eusperm during spawning and comprise >50% of the volume of milt (Hayakawa et al. 2002b). Parasperm appear to restrain the dispersal of eusperm away from the egg mass (Hayakawa et al. 2002a) but also play a role in preventing competing males' spermatozoa from reaching the unfertilized ova (Hayakawa et al. 2002c), as follows. When a male's ejaculate comes into contact with the ovarian fluid surrounding the egg mass, eusperm penetrate this viscous layer and swim toward the unfertilized ova, while the parasperm gradually form clumps upon interacting with the ovarian fluid. The aflagellate parasperm engulf eusperm that arrive later at the ovarian fluid, preventing them from reaching the

unfertilized ova. The timing of clumping by parasperm coincides with a period of decreased mate guarding by the territorial male. Presumably, neighboring males attempting sneak fertilizations after the pair male's spermatozoa have contacted the egg mass will have limited success in fertilizing ova, although genetic evidence is still needed to confirm this.

Blepsias cirrhosus has slightly different fertilization dynamics than *Hemilepidotus gilberti*, which may help to account for the morphological differences between their parasperm. *B. cirrhosus* males release spermatozoa onto ovarian fluid covering the genital duct of the female. The female then retracts this sperm-laden ovarian fluid into her ovary (Munehara 1996), where the spermatozoa swim down the micropylar canals of unfertilized ova. The fertilization process is arrested at this point and does not resume until the eggs are released by the female into seawater (Munehara et al. 1991). Because competition between spermatozoa from rival males for access to the micropyle occurs internally in this species, biflagellate, but immotile, parasperm may be better able to interfere with spermatozoa from competing ejaculates simply by taking up more space. However, this notion has yet to be tested experimentally.

2.5.4 Other Explanations for Diversity in Sperm Morphology

Although some of the interspecific variation in sperm size and morphology can be attributed to the selective force of sperm competition, other factors may also be important. For example, Stockley et al. (1996) showed that spermatozoa from internally fertilizing species are longer than spermatozoa from closely related external fertilizers. These authors argued that there might be more intense selective pressure on sperm size in

internal fertilizers in response to the more viscous medium those spermatozoa would encounter within the female's reproductive tract. This argument is based on the notion that longer spermatozoa are able to produce greater thrust with their flagella (Gomendio and Roldan 1991)—an idea that has been recently supported in a study of externally-fertilizing fishes (Fitzpatrick et al. **Chapter 5**)—and, therefore, that longer spermatozoa would be better able to navigate the female's viscous reproductive tract. Similarly, in a group of closely related, externally-fertilizing African cichlids, Balshine et al. (2001) found that sperm length varied significantly with the site of fertilization—species in which fertilization took place on the substrate had longer spermatozoa than species where fertilization took place in the female's buccal cavity. Because the buccal cavity is an enclosed space, spermatozoa may be more likely to encounter an egg than spermatozoa in substrate-fertilizing species where water currents might move sperm away from unfertilized ova, resulting in relaxed selection on sperm size in buccal fertilizers.

Sperm size is also influenced by characteristics of female reproductive biology. For example, Stockley et al. (1996) found a positive correlation between sperm size and the number of ova in externally fertilizing fishes, and suggested that this relation might be driven by the degree to which an egg mass influences spermatozoa:egg interactions.

Most important, the selective forces acting on sperm size may be varied and our treatment of the subject here is cursory at best, given the scarcity of data on this subject. Recent work in other taxa, particularly insects (Pitnick et al. 1999) and birds (Briskie et al. 1997), has revealed important effects of female reproductive tract morphology in shaping the evolution of sperm size. Applying a similar approach to internally fertilizing fish species would be a useful venture and may broaden our understanding of

male/female coevolution in vertebrates.

2.5.5 Sperm Swimming Speed and Energetics

In the previous section, we alluded to the idea that variation in sperm size influences sperm performance. Indeed, males facing higher levels of sperm competition, both within and across species, are expected to produce larger spermatozoa, which in turn should allow for increased sperm swimming speeds (Ball and Parker 1996). Yet, so far we have not critically addressed the relationship between sperm size and speed. As we shall see, confounding intra- and interspecific results complicate our understanding of the factors that influence sperm performance, as they do for so many of the topics discussed in this chapter.

There are relatively few data available on the relation between sperm swimming speed and sperm size (specifically sperm or flagellar length) in any organism. Gomendio and Roldan (1991) were the first to report a positive association based on data from five species of domesticated mammals. Subsequent studies on other taxa have largely failed to reveal such a relation within species. For example, studies of bluegill (Leach and Montgomerie 2000; Burness et al. 2004; Stoltz and Neff 2006), the cichlid *Telmatochromis vittatus* (Fitzpatrick *et al.* **Chapter 4**), plainfin midshipman (Fitzpatrick et al. **Chapter 3**), Atlantic salmon (Gage et al. 2002), guppies (Pitcher et al. 2007), and both grass and black gobies (Locatello et al. 2007) all found no significant relationship between mean sperm length and mean sperm swimming speed among males within a population (e.g., Figure 2.13 A).

Among 29 species of African cichlids, Fitzpatrick *et al.* (**Chapter 5**) uncovered a

positive relationship between sperm size and swimming speed (Figure 2.13 B), supporting the underlying assumptions of sperm competition theory. Within species relations were examined from a total of 289 males across all 29 species but 27 of 29 species showed no intraspecific association between sperm size and speed. In this study, data were all collected by a single research group, so sperm swimming speed was measured at standardized intervals after activation, during the normal period when ova are fertilized, thereby facilitating comparisons across species. Sperm morphology was also measured in a standardized manner. As a result this study was able to reduce the sources of error inherent in compiling data from different studies.

If there is no intraspecific relation between sperm size and swimming speed, are we unlikely to find any differences in sperm performance in species where males perform sneak fertilizations? On the contrary, in the four species with MARTs examined to date, spermatozoa from sneaker males swam faster than those from conventional males (Table 2.1). Similarly, Rudolfsen et al. (2006) added substantial weight to the idea that male reproductive roles influence sperm swimming speed by conducting an interesting experiment with Arctic charr (*Salvelinus alpinus,* family Salmonidae). In this species, social dominance determines access to females, and therefore reproductive opportunities. As a result, subordinate males, who are unable to gain exclusive access to a female, are more likely to experience sperm competition during matings. Rudolfsen et al. (2006) paired two males with initially similar sperm traits for 7 days, then examined their sperm traits at the end of the experiment. The male who became subordinate had faster swimming spermatozoa than did the dominant male of the pair. Rudolfsen et al. (2006) suggested that this rapid alteration in sperm quality reflected the asymmetry in the level

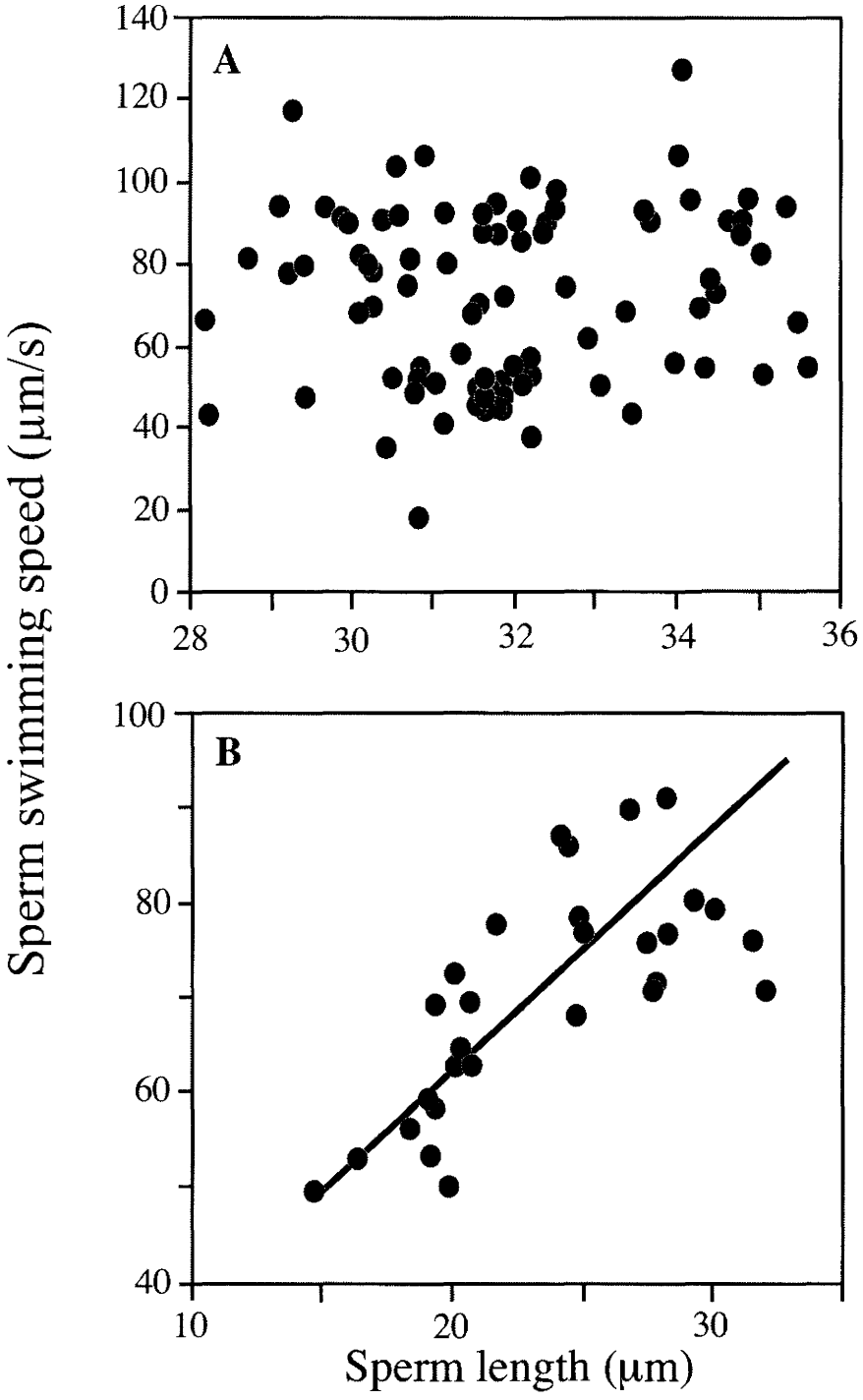

Figure 2.13 Relations between sperm swimming speed and sperm length. **A.** Intraspecific: in individual male Atlantic salmon at 10 s after sperm activation. Redrawn from data in Gage et al. 2002, Figure 2. **B.** Interspecific: in 29 species of cichlid from Lake Tanganyika, each measured at 30 s after sperm activation, from Fitzpatrick *et al.* (**Chapter 5**).

of sperm competition facing males of different social states. Thus males exposed to elevated levels of sperm competition might be expected to have faster swimming spermatozoa, but any increases in sperm speed are unlikely to be the result of alterations in sperm morphology. Table 2.1 shows that this is often, but not always, the case.

Instead, examining sperm energetics appears to be crucial to understanding sperm performance. Recent work with bluegill has demonstrated that adenosine triphosphate (ATP) levels in spermatozoa are positively related to sperm swimming speeds (Burness et al. 2004). In species with MARTs, sneaker males typically have elevated sperm ATP levels relative to conventional males (Table 2.1). Thus sperm energetics may be underlying tactic-specific differences in sperm swimming speed in fishes. However, this idea has only recently been examined in a handful of species. Further exploration of this idea, and incorporation of sperm energetics in theoretical models of sperm competition, will be critical in moving the field forward.

2.6 CHAPTER SUMMARY AND SCOPE FOR FURTHER RESEARCH

This is the third general 'review' of sperm competition in fishes to have appeared in the past 25 years. In the first of these, Constantz (1984) interpreted some of the natural history and anatomy of poeciliid fishes (family Poeciliidae) in the context of sperm competition. At that time, there were few published studies on sperm competition in fish (e.g., Warner and Harlan 1982), so there was little to review (Figure 2.1). The second review (Petersen and Warner 1998) was firmly grounded in sperm competition theory and nicely outlined how that theory might apply to various fish examples. Petersen and Warner (1998) examined, particularly, the alternative reproductive tactics of males, and

concluded that there was ample evidence in species with MARTs that sperm competition influenced their behaviors and morphologies. At that time the first comparative study of potential adaptations to sperm competition in fish had just appeared (Stockley et al 1997), and the promise for such interspecific analyses was evident.

For the present review, we were able to bring together a much larger literature from studies of fishes, though still relatively few fish species have been studied in this context. With information currently available on testes and sperm morphologies, sperm behavior, and/or mating systems for <2% of fish species, there are sure to be interesting discoveries to be made. Most important, the rather haphazard nature of species studied so far with respect to sperm competition makes it difficult to detect and be certain of general patterns, especially because fishes are so diverse. Thus, a major conclusion of this chapter is to show that there are some general patterns within and between species but, for almost all of these, there are exceptions that are, as yet, difficult to explain (e.g., Figure 2.10). We believe, however, that a thorough study of these exceptions will yield some useful insights into the constraints on the evolution of traits under sperm competition.

We conclude by speculating about six subjects that we think will be profitable avenues for further research and could thus form the basis of the next major review of this topic. First, as we have already seen in this chapter, there is much to be gained by looking at sperm competition in a phylogenetic context (Figure 2.11). To do so, future studies should focus attention within clades where species have the same evolutionary background, similar ecologies, morphologies, and physiologies, and are thus more likely to show uncomplicated patterns (Balshine et al. 2001; Fitzpatrick et al. **Chapter 5**). Paying attention to phylogenies, we can also control statistically for lack of independence

among species, thus removing resemblances potentially due to phylogeny (Figure 2.11) and not to the influence of sperm competition *per se*.

Second, we see some real advantage in focusing attention on model systems where we already know much about the behavior, genetics, and physiology of species that can be readily kept, bred and raised in captivity. Work already on guppies (Evans et al. 2003; Evans and Magurran 1999; Pilastro and Bisazza 1999), goldfish (Stacey et al. 2001), sticklebacks (Zbinden et al. 2003, 2004), and cichlids (Balshine et al. 2001; Fitzpatrick et al. **Chapter 3**; Ota and Khoda 2006; Fitzpatrick et al. **Chapter 4**; Katoh et al. 2005) has proven fruitful, as has research on captive-bred but wild-raised salmonids (Gage et al. 1995, 2004; Vladic et al. 2002; Vladic and Järvi 2001; Rudolfsen et al. 2006). Surprisingly, no research has been done on sperm competition in zebrafish (*Danio rerio*, family Cyprinidae) despite its prominence as a model vertebrate system in other contexts (e.g., genetics, development).

Third, we expect that there will be more attention paid to sperm morphology, following the lead taken by research on bird spermatozoa (Birkhead et al. 2005; Immler and Birkhead 2007; Jamieson 2007), and to sperm energetics in fishes (e.g., Vladic et al. 2002; Burness et al. 2004, 2005; Locatello et al. 2007). Fish are, in many ways, excellent research subjects for such study, for the reasons we outlined in **Section 2.2**, especially as recent work suggests that sperm competition acts on sperm energetics within and between species (**Section 2.5.5**). Some focus on the heritability of different morphological traits, the energetic cost of sperm swimming behavior especially in relation to the diverse ways that fish spermatozoa behave, and the sperm and female traits influencing fertilization success will be fruitful to study.

Fourth, fish model systems are particularly well suited to the study of the factors that influence testes morphology, particularly size, and how that relates to sperm production rates and ejaculate size. As we noted above, virtually nothing is known about this subject in any organisms and the diversity of testes sizes, shapes, and colors (Parenti and Grier 2004; Figure 2.6) in fishes is truly astounding and not well understood.

Fifth, the advent of new techniques for the collection of spermatozoa released by males (**Section 2.4**) should encourage studies on the influence of sperm competition on strategic ejaculate investment. There is a rich theory on this subject (e.g., Parker et al. 1996, 1997) and recent tests of theoretical predictions have yielded exciting results, demonstrating sophisticated adjustments in ejaculate expenditure in response to cues of sperm competition in several species of fish (Shapiro et al. 1994; Pilastro et al. 2002; Candolin and Reynolds 2002; Evans et al. 2003; Zbinden et al. 2003; Aspbury 2007; but see Saggiante et al. 2005; Fitzpatrick and Liley 2008).

Finally, we began work on this chapter thinking that we might mine the available fish literature for data relevant to testing aspects of sperm competition theory. While there will be many inconsistencies in the ways that testes, spermatozoa, mating systems, behaviors, physiologies, and phylogenies are measured that will complicate such an analysis, we still think that bringing all of these data together will be rewarding both to assess general patterns and to reveal anomalies and exceptions that deserve more in-depth study.

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CHAPTER 3

SPERM COMPETITION IN A SINGING FISH

3.1 ABSTRACT

In competitive matings, males with superior ejaculates are more likely to fertilize a female's eggs. Therefore, theory predicts that selection will favour increases in sperm number, size and speed when males experience more intense levels of sperm competition. We used the plainfin midshipman fish, *Porichthys notatus*, a species with two well-characterized male alternative reproductive tactics (MARTs) to show the impact of sperm competition on ejaculate characters *between*, as well as *within*, male reproductive tactics. In this species conventional males acoustically court females while sneaker males, who experience more intense levels of sperm competition, do not court females but instead release sperm in the presence of another male. Sneaker males had faster swimming sperm with larger sperm midpieces than conventional nesting males. The largest conventional nesting males attracted more females and had more eggs in their nests but they also had to contend with higher rates of cuckoldry in their nests by both sneaker males and unsuccessful conventional males. Large conventional males appeared to be adapted to this intense level of sperm competition and produced faster swimming sperm than small nesting males. Hence our results extend the view that female choice not only selects for the evolution of MARTS but also amplifies the intensity selection gradient acting on sperm traits within tactics.

3.2 INTRODUCTION

In species with alternative reproductive tactics males within a single population experience different levels of sperm competition (Parker 1970) when mating and hence are excellent models to study the impacts of sperm competition on reproductive behaviour and physiology. Large conventional males build nests and/or defend territories and court females, while smaller males in the population, called sneakers, invest in reproduction rather than courtship (Taborsky 1994; 1998). Sneakers are expected to experience greater levels of sperm competition relative to conventional males, as they only release sperm in the presence of another male. Given this dichotomy in the level of sperm competition experienced during matings, theory predicts that sneakers will invest more in testicular tissue (Parker 1990; Parker and Ball 2005) and produce more competitive (faster) sperm (Ball and Parker 1996). This is because sperm number and sperm swimming speed largely predict fertilization success (Martins et al. 1976; Hoysak and Liley 2001; Gage et al. 2004). Indeed, in a number of species sneakers consistently invest relatively more in testes than conventional males (Peterson and Warner 1998; **Chapter 2**) and sperm from males performing sneak fertilizations typically swim faster than sperm from conventional males who court females (Burness et al. 2004; Rudolfsen et al. 2006; Fitzpatrick et al. **Chapter 4**; Locatello et al. 2007; but see Leach and Montgomerie 2000; Stoltz and Neff 2006a). While empirical studies demonstrate that sperm speed is related to sperm size across species (Gomendio and Roland 1991; Fitzpatrick et al. **Chapter 5**), it remains unclear precisely how selection shapes individual sperm structures within a species.

In addition to producing superior ejaculates, sneakers use a suite of behaviours to

overcome their disadvantaged mating role. Sneakers sometimes dart into a conventional male's territory and release sperm or act as a female mimic, brazenly approaching a conventional male and releasing sperm close to the female (e.g. Stoltz and Neff 2006b). Additionally, sneakers will increase their fitness by targeting their parasitizing efforts on those conventional males that are most likely to have a female visit their nests (Waltz 1982). Sneakers may parasitize based on cues of: 1) female presence, 2) conventional male quality or 3) a combination of 1 & 2. Regardless of the precise process of sneaker choice, the impact of female choice for high quality males, and corresponding responses in sneaker male behaviour, have dramatic implications for the evolution of male alternative reproductive tactics (MARTs) (Henson and Warner 1997; Alonzo and Warner 2000; Luttbeg 2004). An important but overlooked consequence of strong female choice is that the threat of sperm competition will increase with male quality, as high quality males will be preferentially targeted by sneakers. To date all theory and empirical research has assumed that the degree of variance in reproductive tactics would be greatest between, rather than within, a male tactic. But this assumption needs to be re-evaluated in the light of strong female choice for cues of male quality and sneaker males consistently targeting these same high quality conventional males. We predict that the degree of reproductive skew in female mate choice will map onto the variance in sperm traits within the conventional tactic.

To explore the effects of sperm competition on sperm competitive ability, we studied the interaction between male reproductive tactics and female mate choice in the plainfin midshipman (*Porichthys notatus*). Midshipman are an ideal model to study sperm competition, as few species have such well characterized alternative male

reproductive tactics (conventional Type I and sneaking Type II males, Figure 1, Brantley and Bass 1994; Lee and Bass 2004). Although the morphological, physiological and neurobiological differences between the two male tactics have been carefully worked out in great detail (Brantley and Bass 1994; Bass 1992; 1996), surprisingly to date no study has examined how the competition between male morphs may extend beyond the mating stage, in the form of sperm competition. Using the robust framework of sperm competition theory (Parker 1970; 1990; 1998; Ball and Parker 1996), we combined ecological and physiological data to assess tactic-specific responses to competition for mating opportunities. We also investigated what factors (sperm morphology, male body size) influenced sperm performance. Finally, we offer a new perspective for examining sperm competition within a tactic, highlighting that the threat of sperm competition is not equivalent for all conventional males but instead is determined by male quality.

3.3 METHODS

3.3.1 Study Species

The plainfin midshipman is a marine fish that breeds off the west coast of North America from the late spring to early summer (Arora 1948). Midshipman have two male reproductive tactics; conventional Type I males and sneaking Type II males. Type I males build and defend nests under rocks in the intertidal zone (Figure 2) where they acoustically court females by contracting their sonic muscles at high speeds, which vibrates their swim bladders and produces a low frequency 'hum' (Ibara et al. 1983; Brantley et al. 2003). Gravid females are attracted by these male calls (McKibben and

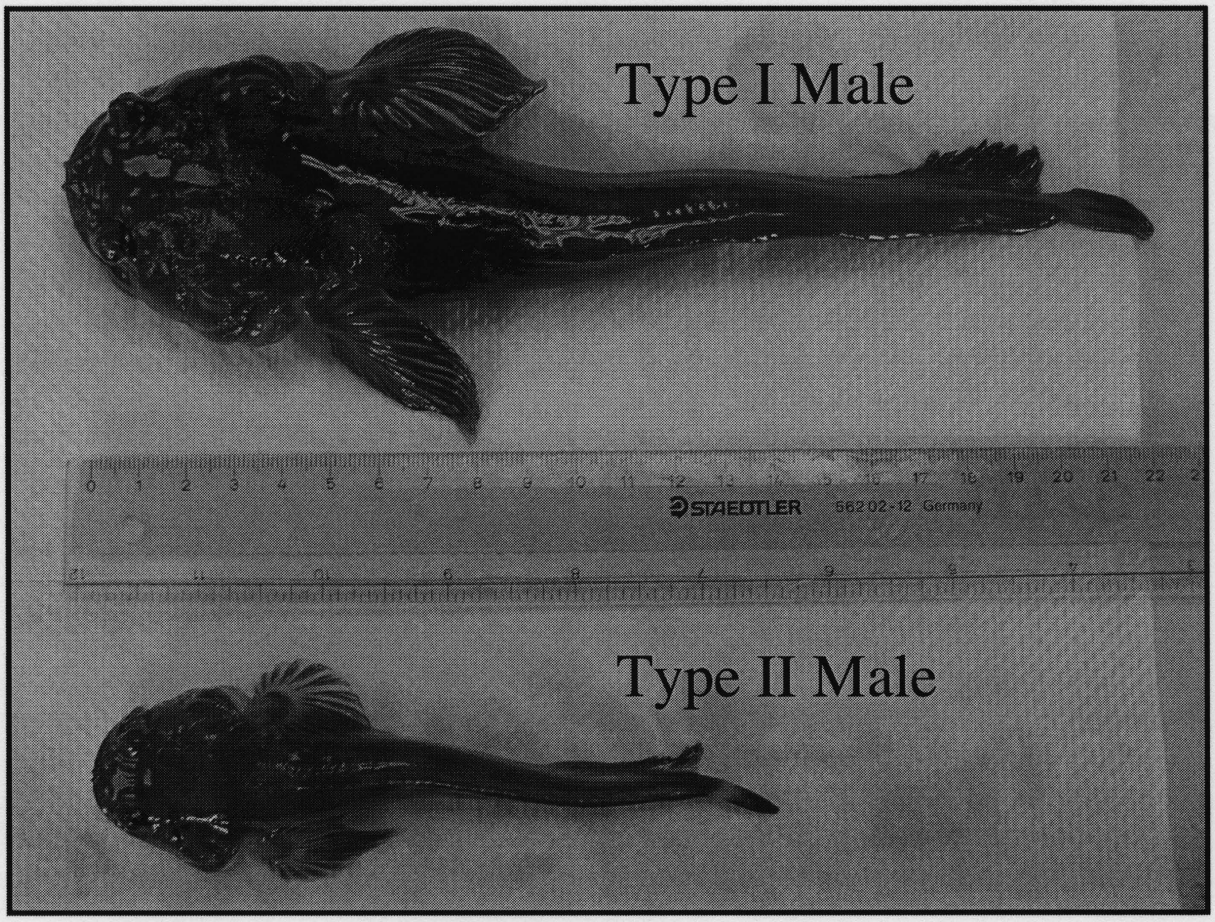

Figure 3.1. Morphological differences between conventional Type I and sneaking Type II male midshipman.

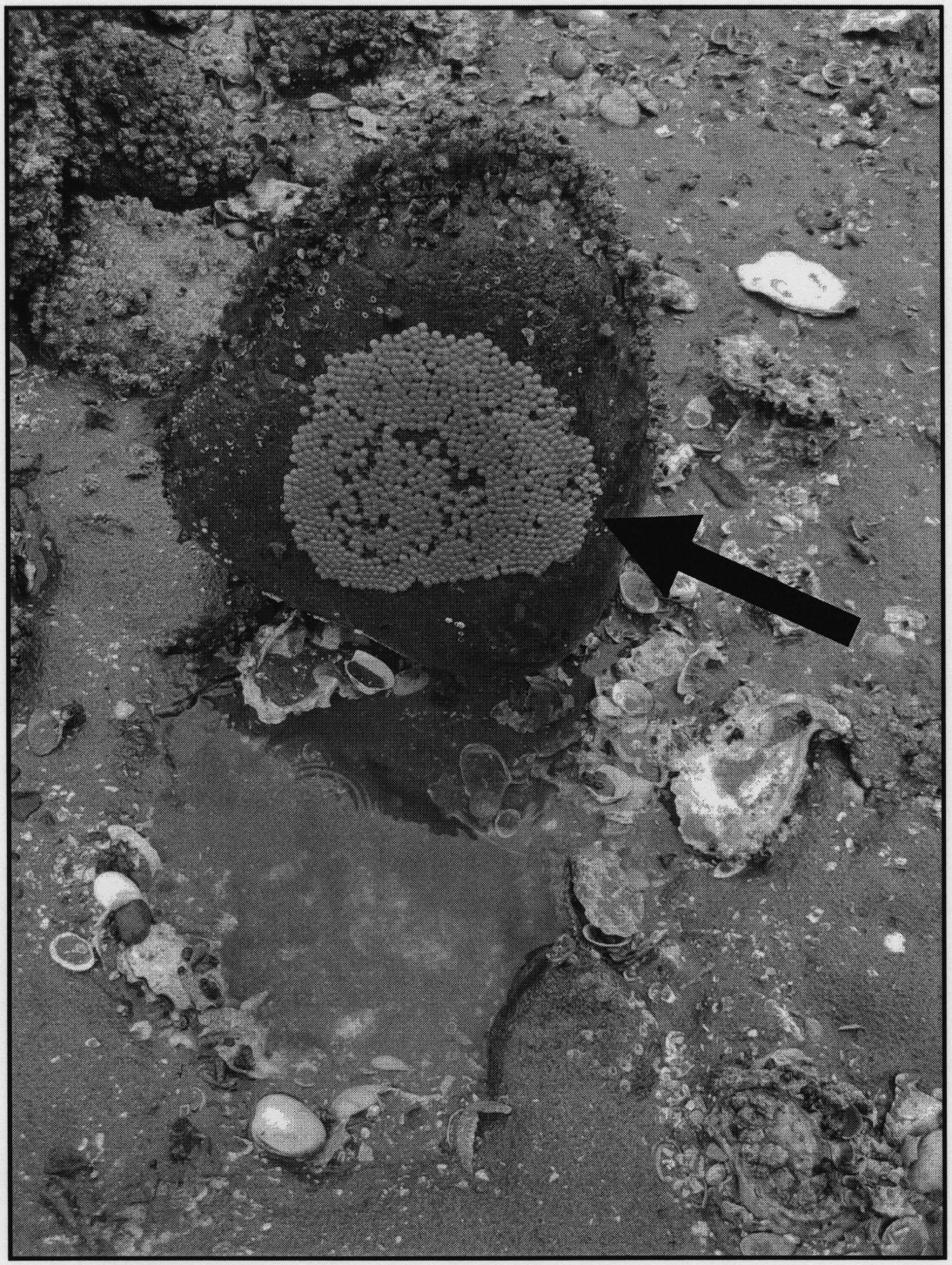

Figure 3.2. Intertidal midshipman nest. The rock has been lifted so that it is perpendicular to the substrate. A black arrow indicates the egg mass that is attached to the underside of the rock. Conventional Type I males remain under the rock in a pool of water during low tides.

Bass 1998). Type II males do not court females but sneak fertilizations by surreptitiously releasing sperm in a Type I male's nests during spawning, sometimes even ejaculating outside the nest and fanning sperm towards the eggs using their pectoral fins (Brantley and Bass 1994).

3.3.2 Field Locations

Sexually mature Type I and II males were collected from nests during low tides in British Columbia, Canada from four sites on Vancouver Island: Bamfield Inlet (48° 81′ N, 125° 14′ W), Nanoose Bay (49° 26′ N, 124° 18′ W), Mills Bay (48° 63′ N, 123° 53′ W) and Ladysmith Harbour (49° 01′ N, 123° 83′ W) and from one site on the mainland: Crescent Beach (49° 04′ N, 122° 88′ W) in June 2006, 2007 and in May and June 2008. For sperm analyses, fish were transported to the laboratory in containers of aerated 10-13°C seawater. In the laboratory, fish were given a lethal dose of anesthetic (MS-222 or Benzocaine), measured for standard length to the nearest mm, and weighed to the nearest 0.1g, and the testes, accessory glands and sonic muscle were removed and weighed to the nearest 0.01g.

3.3.3 Male and Nest Characteristics

At low tides in June 2007 and May and June 2008 we surveyed 123 nests from our study sites. All fish were sexed, measured, marked (with a unique dorsal fin clip) and returned to their nests, with the exception of fish taken to the laboratory for reproductive physiology and sperm characteristics assessment. In each nest, the presence/absence of eggs was noted and a digital photograph taken. Females attach eggs to the upper side of

the rock by an adhesive disc (Arora 1948), which remains intact even after the eggs are gone. We used the photos to calculate the total clutch size and the percentage of live, dead, and no longer present eggs. Orange eyed eggs were classed as living, white eggs and eggs without liquid yolk centers were classed as dead and adhesive disc shells without eggs were classified as missing. Total clutch size was used as a measure of female choice for that nest/male and the percentage of remaining living eggs was used as a measure of the quality of parental care and/or embryo viability.

3.3.4 Testes and Sperm Analyses

We compared testes and sperm characteristics of 19 Type I and 16 Type II males. Testes were removed from each male, placed on a clean, dry weigh boat and split open with a scalpel. A drop of milt (sperm and seminal plasma) was placed in a 2.0-ml microcentrifuge tube and diluted by rapidly adding 0.5 ml seawater to activate sperm. A 60-µl subsample of the sperm/water mixture was placed on a prefocused 1-mm deep welled slide with a cover slip covering half of the depression as quickly as possible. Video recordings of sperm motility began at the moment sperm were diluted with seawater. Sperm motility was captured at 60 frames/s under 200X magnification with a PixeLINK Megapixel PL-A662 digital video camera (PixeLINK, Ottawa, Ontario, Canada) mounted on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, New York, USA). Images were recorded using PixeLINK PL-A600 Series Camera Software (v. 3.1).

Sperm velocity was assessed at 7 time periods; 10, 30, 60, 120, 300, 600, and 900 seconds post-activation. The unusual elongated head morphology of midshipman sperm

prevented the use of computer assisted sperm analysis at the magnification used to capture sperm motility. Consequently, sperm velocity was assessed using the NIH ImageJ software manual tracking function (v. 1.37, available at http://rsb.info.nih.gov/ij/). At each time period 9.06 ± 0.23 (mean ± SE) spermatozoa per male were tracked by following the center of the sperm's head for 74.6 ± 0.77 frames (mean ± SE).

To assess sperm morphology, sperm from each male was placed in a 1.5 ml microcentrifuge tube, diluted with 1 ml seawater, and fixed by adding 0.5 ml buffered formalin. Sperm were examined using a light microscope under 1000X magnification and a digital photograph was captured from clearly visible spermatozoa (9.23 ± 0.27 per male, mean ± SE) using a PixeLINK Megapixel PL-A662 digital camera mounted on the microscope. Midshipman have biflagellate (two-tailed) sperm (Stanley 1965). Because there were no differences in flagella lengths from a single sperm (repeated measures ANOVA, $F_{1,480}$=4.91, p=0.30) the average length of the two flagella was used in all analyses. Sperm head, midpiece, flagellum length and total sperm length (sum of sperm head, midpiece and flagellum) were measured (to the nearest 0.1 μm) from digital images using NIH ImageJ software (v. 1.37) by tracing a freehand line over each sperm component using an Intuos graphic table (Wacom Co. Ltd., Japan).

3.3.5 Statistical Analyses

Statistical analyses were performed with JMP (version 5.0.1, 2002; SAS Institute Inc., Cary, NC). Data were assessed for fit to a normal distribution, transformed when necessary (log transformed or arcsine square root transformed in the case of percentage data, Zar 1999) and whenever possible parametric statistics were used. When data

transformation did not achieve a fit to a normal distribution or when sample sizes were small non-parametric statistics were applied. Mean sperm swimming speed was analyzed using repeated measures ANOVA and differences between type I and II males were assessed at each time period using linear contrast analyses. To avoid observer bias, all sperm samples (speed and morphology) were measured blind to male size or reproductive tactic. As data were sometimes collected over successive years, we included year as a covariate in the analyses. Not all males were dissected; therefore, sample sizes are reduced for some analyses.

3.4 RESULTS

3.4.1 Between-Tactic Differences in Ejaculate Traits

Sperm speed: Swimming speed declined over time in all males (repeated measures ANOVA, $F_{6,122}=31.29$, $p<0.0001$, Figure 3.3a), but the rate of decline differed between conventional (Type I) and sneaker (Type II) males (tactic*time $F_{6,122}=2.21$, $p=0.046$). Post hoc linear contrast analyses revealed that sperm from sneaker males swam faster than sperm from conventional males at two post-activation time periods and nearly did so at two additional time periods (30 seconds $F_{1,122}=3.35$, $p=0.07$; 60 seconds $F_{1,122}=3.86$, $p=0.05$; 120 seconds $F_{1,122}=4.72$, $p=0.03$; 300 seconds $F_{1,122}=2.94$, $p=0.09$). At 10, 600 and 900 seconds post activation no tactic-specific differences were detected in sperm swimming speed ($p \geq 0.34$).

Sperm morphology: Sperm total length did not differ between parental (56.91 ± 1.48 um) and sneaker males (54.93 ± 1.25, $F_{1,26}=0.85$, $p=0.37$). However, individual regions of the sperm differed between tactics (Figure 3.4). Sperm heads from

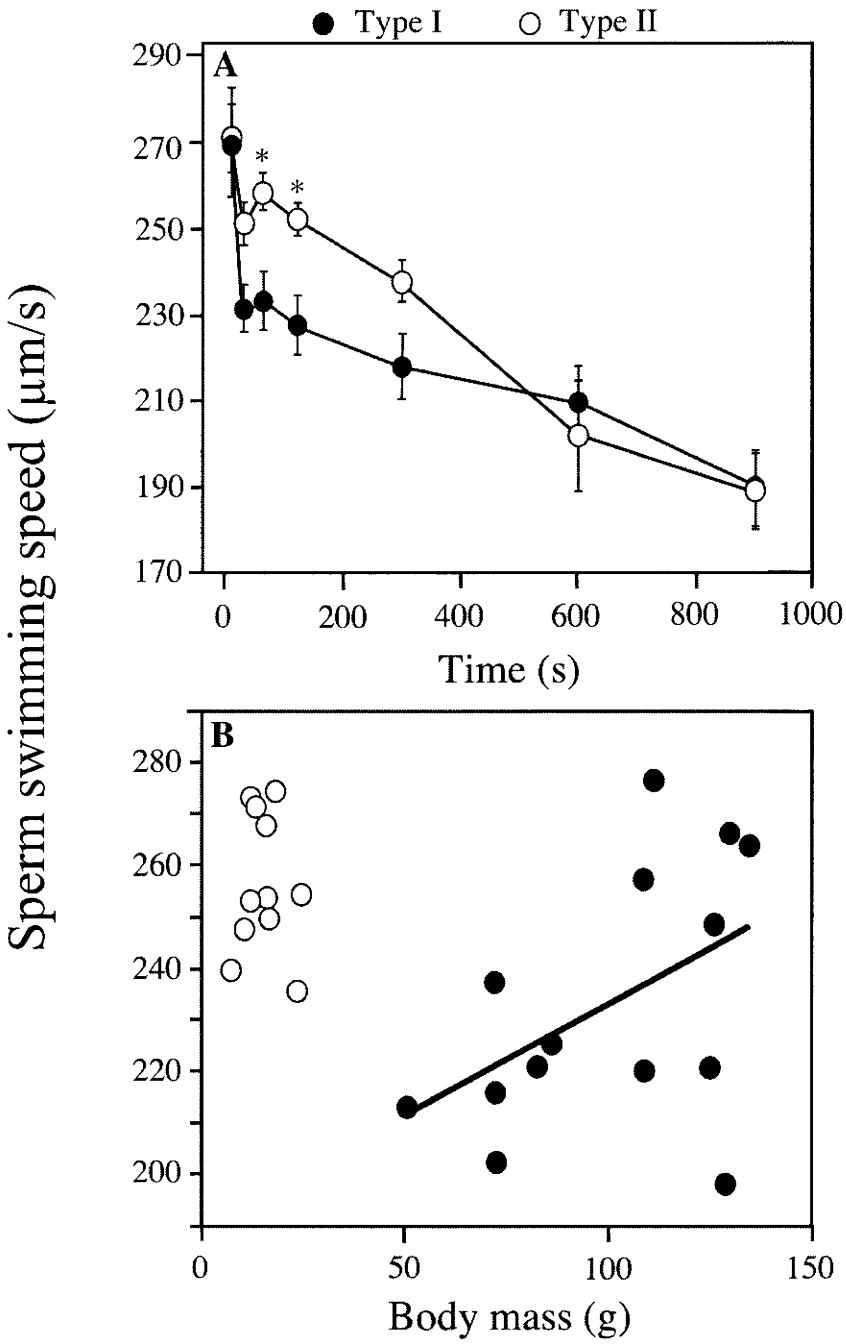

Figure 3.3. (a) Mean ± SE sperm swimming speed at different times post activation for conventional Type I and sneaking Type II males. * indicates significant ($p<0.05$) differences between male tactics at each time period. (b) Relationship between male body size and sperm swimming speed in Type I and II males. Statistical analyses were performed using repeated measures ANOVA but for clarity we have shown data from a single time period (120 seconds post activation) to illustrate the relationship. A best-fit line has been fitted through the Type I male data.

conventional males were 13.5% longer than those of sneaker males ($F_{1,26}$=14.19, p=0.001), while midpieces from sneaker males were 17.3% longer than midpieces from conventional males ($F_{1,26}$=9.28, p=0.006). No differences in average flagella length between conventional and sneaker males were detected ($F_{1,26}$=0.88, p=0.36). Sperm swimming speed was not correlated with any sperm morphology measure (total sperm length, head length, midpiece length or area, or flagella length were all p>0.13).

***Male competitive abilities*:** Sneaker males were almost eight times smaller (in terms of body mass) and had relatively smaller sonic muscles compared to conventional males (Table 3.1). Sneakers had relatively larger testes that did not differ in absolute size from those of conventional males (t=1.09, n=33, p=0.28, Table 3.1). Although conventional males had absolutely larger accessory glands (t=5.24, n=24, p<0.0001), sneaker males invested relatively more in accessory glands (Table 3.1).

Table 3.1. Morphological comparisons of Type I and II males. Data for all variables are presented as mean±SE. The absolute and relative (controlling for body mass) investment in testes, accessory gland and sonic muscle mass are presented. Tactic-specific investment in testes, accessory gland or sonic muscle mass was examined using ANCOVAs with male reproductive tactic, soma mass (body mass minus the variable of interest), year of collection, and the interaction between male tactic and soma mass as covariates. The relative investment in the mass of each organ was calculated as (organ/soma mass)*100, thereby expressing the percentage of the body mass that is made up of testes, accessory glands or sonic muscles.

Characteristic	Type I Males (n=19)	Type II Males (n=16)	n	Tactic		Soma Mass		Year		Interaction	
				F	p	F	p	F	p	F	p
Body mass (g)	121.91 ± 11.03	15.30 ± 1.09	35	416.50	**<0.001**			5.34	**0.01**	6.14	**0.006**
Standard length (cm)	20.44 ± 0.54	10.60 ± 0.30	35	346.90	**<0.001**			4.61	**0.02**	2.57	0.09
Testes mass (g)	1.50 ± 0.21	1.19 ± 0.19	35	7.34	**0.01**	10.41	**0.003**	2.38	0.11	1.48	0.23
Relative testes mass (%)	1.34 ± 0.22	8.20 ± 0.91									
Accessory gland mass (g)	0.63 ± 0.16	0.10 ± 0.02	26	20.06	**<0.001**	62.08	**<0.001**	5.96	**0.02**	1.34	0.26
Relative accessory gland mass (%)	0.40 ± 0.07	0.63 ± 0.08									
Sonic muscle mass (g)	3.37 ± 0.33	0.21 ± 0.02	35	17.99	**<0.001**	52.40	**<0.001**	10.96	**<0.001**	5.31	**0.03**
Relative sonic muscle mass (%)	2.85 ± 0.08	1.36 ± 0.11									

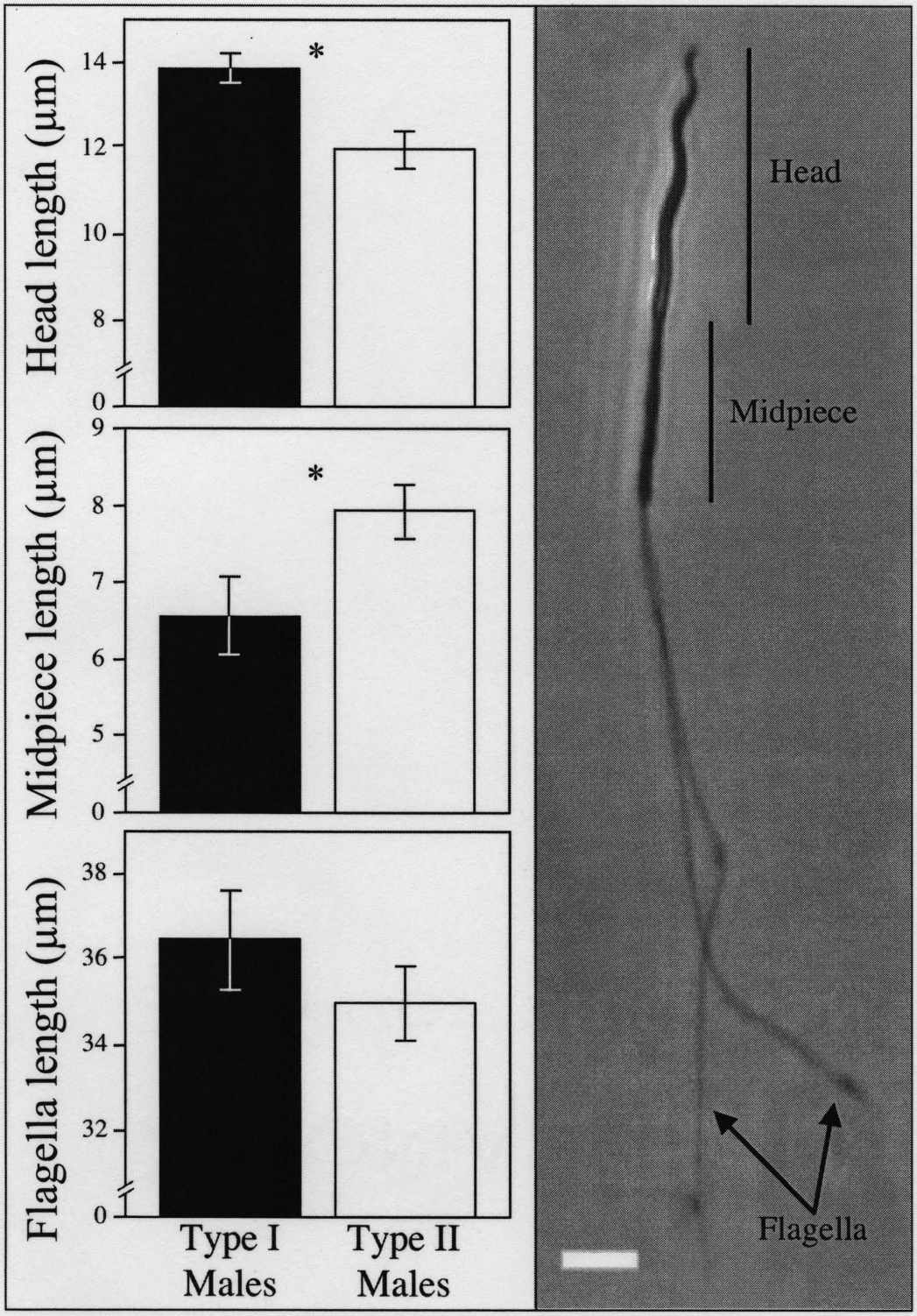

Figure 3.4. Length of sperm head, midpiece and flagella in Type I and II males. * indicates significant ($p<0.05$) differences between male tactics. A representative spermatozoon from a Type I male is presented magnified 1000X. Head, midpiece and flagella are labelled and the while scale bar represents 5 μm.

3.4.2 Within-Tactic Variance in Ejaculate Traits

Across all males, sperm swimming speed was not related to body mass ($F_{1,129}$=1.45, p=0.23). However, because the largest Type I males in the population were subject to greater levels of sperm competition (see below), we examined each tactic independently with the *a priori* assumption that larger males would produce superior ejaculates, and found that larger conventional males indeed had faster swimming sperm (one-tailed test, $F_{1,76}$=3.08, p=0.04, Figure 3.3b). In sneakers sperm swimming speed was not influenced by body size ($F_{1,46}$=0.02, p=0.89). When we classified conventional males as either large (above the average population body mass) or small (below the average population body mass) and compared sperm swimming speed from these males against sneakers we found that small conventional males had sperm that swam significantly slower than sperm from large conventional males and sneakers, which did not differ from one another ($F_{2,128}$=5.14, p=0.007).

3.4.3 Female and sneaker choice for cues of male quality

Early in the breeding season (May), only 26% of Type I males were guarding eggs but female(s) were found on 20% of the nests surveyed. Male body mass predicted whether males had eggs in their nests (logistic regression, χ^2 = 5.72, df = 1, n=82, p = 0.02). In addition, males with two or more females in their nests were larger than males with only one female in their nest (t-test, t=2.73, n=14, p=0.02). Nearly 10% of the nests had two or more conventional males together. One of these males appeared to be the nest holder, positioned centrally under the clutch, while the other males were found in the sneaking position on the outskirts of the nest with their heads positioned outwards and genitalia

oriented towards the eggs (see Lee and Bass 2004 for a more detailed description of conventional Type I males' sneaking behaviour). The conventional nest guarding males found with sneaking conventional males in their nests tended to be larger than average (i.e. > than the average population body mass, paired Wilcoxon Sign Rank Test, H=9.5, n=6, p=0.06), while the conventional males engaged in these apparent sneak fertilizations were significantly smaller than the average population body mass (H=17.0, n=8, p=0.02). Many of these sneaking conventional males were known to have their own nest sites where they had been observed on previous days in their own nests but had not yet received eggs.

Later in the breeding season (June), 84% of conventional males were guarding eggs, but only 12% of these males had female(s) on their nests. Among nesting conventional males where clutch size and organ measurements were recorded, both male body mass and sonic muscle mass of nesting conventional males were positively correlated with the number of eggs present in nests (body mass: r=0.46, n=34, p=0.006; sonic muscle mass: r=0.44, n=33, p=0.03). Larger conventional males, with larger sonic muscles, were more likely to have a female present on their nests (logistic regression; body mass; χ^2=12.37, df=1, n=9, p=0.0004: sonic muscle mass: χ^2= 7.12, df = 1, n = 9, p=0.008). Egg mortality (% of dead eggs) was not related to male body mass (Spearman rho, r_s=-0.11, n=33 p=0.52) or to sonic muscle mass (r_s=0.12, n=33, p=0.50). Sneaker males were more likely to be found on nests of larger, and presumably louder conventional males with larger sonic muscles (χ^2=6.71, df=1, n=41, p=0.01: sonic muscle mass: χ^2 = 6.87, df = 1, n = 41, p=0.009). Sneaker males were found in 7% of nests sampled. Sneakers were associated with the presence of females on nests (Fisher's

exact test: $p<0.001$). Nests with two conventional males, (where at least one male was apparently sneaking fertilizations) were rare in June, representing only 1% of nests sampled.

3.5 DISCUSSION

Female choice has been implicated as a driving force behind the evolution of male alternative reproductive behaviours (Henson and Warner 1997). Following the evolution of MARTs some males will be exposed to an increase in magnitude of sperm competition when mating and a rich theoretical framework has been carefully constructed to model the consequences of sperm competition on male reproductive physiology (Parker 1998). Here we bridge these ideas by describing how female choice influences the strength of sperm competition for different males within a population.

In midshipman, consistent with theoretical predictions (Parker 1990; Ball and Parker 1996), sneaker males invested more in sperm number (this study; Brantley and Bass 1994), and produced faster swimming sperm. The structural sperm differences between tactics detected in this study were not directly related to swimming speed. However the larger sperm midpieces and smaller heads observed in sneakers are consistent with the notion that these sperm have greater adenosine triphosphate (ATP) content (Vladic et al. 2002), the primary source of energy powering sperm movement (Christen et al. 1987). Increased ATP content in sperm from sneakers has been demonstrated in a wide variety of species with MARTs (**Chapter 2**) and may be how midshipman sneakers achieve faster sperm swimming speeds. Further work is now needed to experimentally verify this prediction.

When female preference leads to strongly skewed reproductive success for high quality males, low quality males may be left with few options other than to steal (or sneak) fertilization, yet to date few empirical studies have linked the impact of female mate choice to male reproductive physiology in species with MARTs. We begin to address this oversight in this study. In midshipman, females prefer the largest conventional males in the population (this study and Dimartini 1988). But not only were large males more successful at obtaining females and eggs, they were also more susceptible to cuckolding by Type I (this study and Lee and Bass 2004) and Type II males. Thus the nests of the largest males in the population act as a central hub where both females and cuckolders congregate. Early in the season cuckolding males were smaller than average conventional males, while later in the season the majority of cuckolding males were true sneaker Type II males. Presumably, small conventional males cease to sneak once they received eggs. The coalescence of males and females has dramatic, yet overlooked, implications for our understanding of the level of sperm competition experienced within a tactic. Researchers have traditionally focused on simply contrasting differences in reproductive investment and sperm characteristics *between* conventional and sneaker males. In contrast, our study extends this view by showing differences *within* tactics too. In midshipman, large conventional males appear to have been selected for enhanced reproductive traits, having faster swimming sperm, like the sneaks who parasitize them. Hence, when certain conventional males experience especially elevated sperm competition then tactic-specific differences in reproductive characters and physiology may actually be diminished. For example, Simmons et al. (1999) demonstrated that male reproductive physiology was contingent upon population

demography in two species of dung beetles. In *Onthophagus taurus*, where 60% of the population consisted of sneaker males, no differences in reproductive traits were detected between sneaker and conventional males. In contrast, in *O. binodis*, where sneakers make up only 30% of the population, sneaks had larger testes, produced larger ejaculates and had larger sperm (Simmons et al. 1999). Like Simmons et al. (1999), our study also demonstrates an erosion of tactic-specific differences in sperm swimming speed between sneakers and the largest conventional males in the population within a species. Failure to incorporate within-tactic responses to sperm competition may have stymied previous examinations tactic-specific responses in sperm swimming speed, particularly in cases where studies within a single species have yielded divergent results (e.g. bluegill, *Lepomis macrochirus*: Leach and Montgomerie 2000; Burness et al. 2004; 2005; Stoltz and Neff 2006a).

We present a simple conceptual model describing how female choice may influence sperm quality within a tactic in species with MARTs (Figure 3.5). Sperm competition will be intense for high quality males compared with other males in the population provided two conditions are met. First, females practice mate choice for high quality males, and female mate choice largely determines male reproductive success (Darwin 1871; Andersson 1994). Second, sneakers preferentially target high quality males and/or respond to female behaviours when choosing which conventional male to parasitize (e.g. this study; Kiflawi and Gray 2000; Gonçalves et al. 2002; 2003; Widemo 1998; Brantley and Bass 1994; Oliviera et al. 2002, van den Berghe et al. 1989). Because the prerequisites necessary for creating an asymmetric level of sperm competition between preferred and non-preferred males are commonly observed across a diversity of

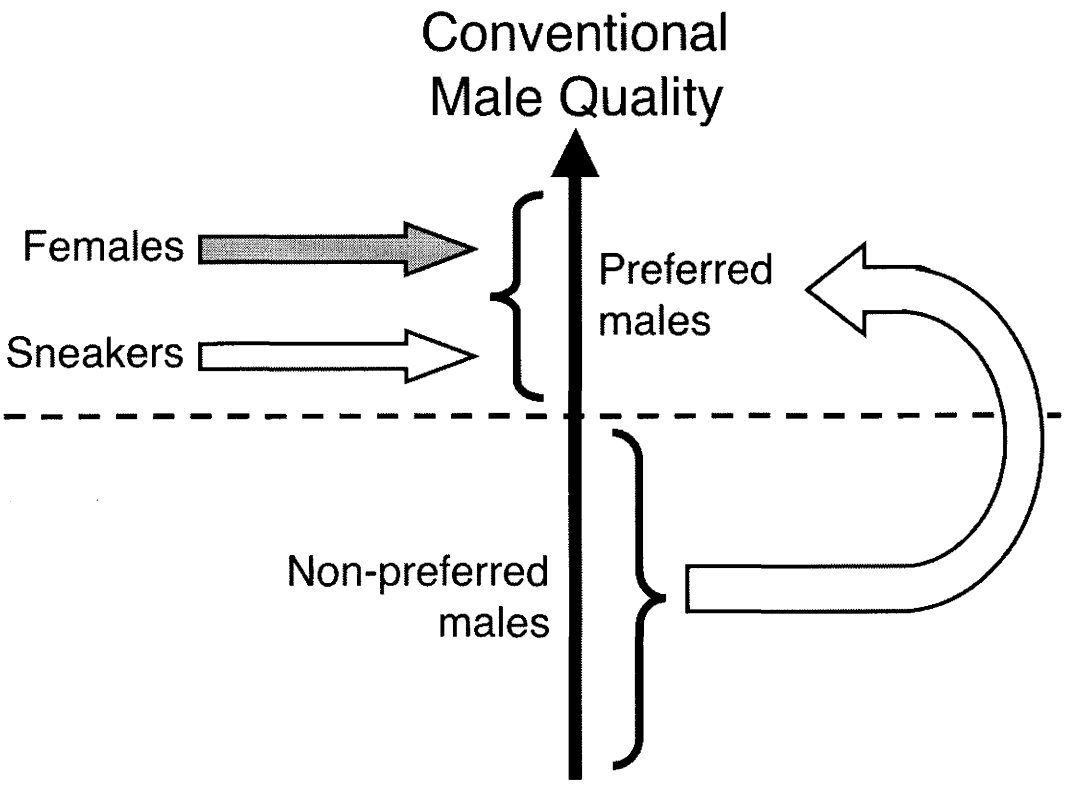

Figure 3.5. Conceptual model illustrating the interaction between preferred and non-preferred conventional males, females and sneaker males in the plainfin midshipman. Both females and sneakers preferentially associate with high quality males. Non-preferred males attempt to sneak fertilizations from preferred males. Therefore preferred males experience an elevated level of sperm competition and must compete with both sneakers and non-preferred males to fertilize a female's eggs. The dashed line represents an arbitrary threshold of male quality above which females prefer males. White arrows indicate males that are competing via sperm competition.

taxa, this conceptual model will be broadly applicable in future studies provided the conditions outlined above are met.

Our results offer a new perspective for understanding how sperm competition shapes male reproductive investment at the population level by highlighting the disparity in the level of sperm competition facing males of different quality. Females are absolutely required for any kind of sperm competition to occur. Therefore, in any species where female choice is predictable and non-preferred males engage in sneak fertilizations, we expect that high quality, preferred males will have superior ejaculates. Female behaviour and physiology in general needs to be more fully explored and integrated when studying male post-copulatory competition and male alternative reproductive tactics. Failure to consider the impact of female choice and within-tactic responses to sperm competition may lead to erroneous conclusions when comparing ejaculate traits between tactics. Incorporating the notion of trade-offs between courtship, parental care and reproduction in species where high quality males perform all of these activities at an elevated level is critically important to furthering our understanding of how selection impacts sperm quality at a population level.

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CHAPTER 4

REPRODUCTIVE-TACTIC-SPECIFIC VARIATION IN SPERM SWIMMING SPEED IN A SHELL-BROODING CICHLID

4.1 ABSTRACT

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

4.2 INTRODUCTION

Sperm competition is recognized as a powerful evolutionary force shaping male reproductive behaviours and physiology (Parker 1970). Males can mate in a favoured role (i.e., gaining primary access to females) or in a disfavoured role (i.e., mating in the presence of another male), and the strength of sperm competition is contingent upon male reproductive roles. Males mating in favoured roles sequester and guard females, experiencing sperm competition only when other males are present during mating. Yet, disfavoured males, who often perform sneak fertilizations, experience sperm competition during every mating, as they always release sperm in the presence of a competing male. Theory predicts that disfavoured males should invest more heavily in ejaculates and allocate greater numbers of sperm during mating (Parker 1990; Parker and Ball 2005), as sperm quality (Gage et al. 2004; Casselman et al. 2006) and number (Martin et al. 1974) influence the probability of successfully fertilizing eggs. As predicted from theory, males performing sneak fertilizations (disfavoured males), in a variety of species, have larger relative testis mass than males in favoured roles (**Chapter 2**). Yet, male reproductive roles are often dependent upon environmental and social conditions, and males can often rapidly adjust ejaculate characteristics, for example ejaculating higher quality sperm in greater numbers when mating in a disfavoured role (Rudolfsen et al. 2006; Cornwallis and Birkhead 2006; Pizzari et al. 2003; Serrano et al. 2006; Wedell et al. 2002). Alternatively, in species with male alternative reproductive tactics (MARTs), some males will consistently mate in either the favoured (conventional males) or the disfavoured (sneaker males) roles, providing a convenient natural system for the study of sperm competition.

In addition to its influence on ejaculate size and allocation, sperm competition is also expected to affect sperm morphology and behaviour, particularly swimming speed (Ball and Parker 1996). Sperm from males mating in the disfavoured role (i.e., sneaker males), and thus experiencing greater levels of sperm competition, are expected to have longer tails (Ball and Parker 1996) that displace a greater volume of water with each undulation, and are thus capable of swimming faster (Gomendio and Roldan 1991). If sperm swimming speed is related to fertilization success, as demonstrated in a number of taxa (fishes, Gage et al. 2004; Casselman et al. 2006; Lahnsteiner et al. 1998, birds Birkhead et al. 1999; Froman et al. 1999; 2001, invertebrates, Levitan 2000, mammals Malo et al. 2005), then longer and faster sperm may increase the fertilization success of sneaker males relative to conventional males. In externally fertilizing fishes, sperm swimming speed may be particularly strongly influenced by sperm competition, as the first sperm cell to reach the micropyle (a narrow opening on the egg surface) of an ovum is most likely to successfully fertilize the egg (Yanagimachi et al. 1992; Snook 2005). Consequently, an appropriate analogy for fertilization dynamics in externally fertilizing fishes is that of a race among sperm to reach the micropyle, with the fastest sperm winning the race.

Despite these predictions, the influence of sperm competition on sperm morphology, swimming speed and longevity remains unclear, with recent studies presenting contradictory results, even within a single species. In support of theoretical predictions, sperm from males performing sneak fertilizations was longer in bluegill (*Lepomis macrochirus,* Burness et al. 2004) and dung beetles (*Onthophagus binodis*, Simmons et al. 1999) and swam faster in bluegill (Burness et al. 2004) and arctic char

(*Salvelinus alpinus*, Rudolfsen et al. 2006; Serrano et al. 2006) than sperm from conventional males. However, contrary to predictions, no differences in sperm morphology or swimming speed were detected between conventional males and sneakers in salmon (*Salmo salar*), roach (*Rutilus rutilus*), or a different study of bluegill (Leach and Montgomerie 2000; Vladic et al. 2002; Burness et al. 2005; Kortet et al. 2004; Stoltz and Neff 2006). Furthermore, because both sperm swimming speed and the duration of sperm motility (i.e., sperm longevity) represent an energetic demand, a trade-off is expected between sperm swimming speed and longevity (Ball and Parker 1996). Therefore, everything else being equal, sperm from sneakers is expected to swim faster, but for shorter time periods, than sperm from conventional males, a prediction that has also received mixed empirical support. Some studies have demonstrated that sneaker sperm are shorter-lived [bluegill, Leach and Montgomerie 2000; Neff et al. 2003; roach, Kortet et al. 2004), where some have failed to detect a difference between male tactics (salmon, Vladic et al. 2002), and still others showed that sneaker males had longer-lived sperm (salmon, Gage et al. 1995; corkwing wrasse, *Symphodus melops*, Uglem et al. 2001; stickleback, *Gasterosteus aculeatus*, de Fraipont et al. 1993). The inconsistency of these findings suggests that further exploration of sperm characteristics in species characterized by alterative reproductive tactics is warranted.

In this study, we examine sperm characteristics in *Telmatochromis vittatus*, a small, shell-brooding cichlid endemic to Lake Tanganyika, with four alternative male reproductive tactics: territorial, pirate, satellite and sneaker (Ota and Khoda 2006a). In most species with alternative reproductive tactics, there are only two male morphs (territorial – parental - conventional males versus sneaker males), so our study species

provides an unusual opportunity to assess differences among four distinct male tactics. Territorial males (50-62 mm standard length) control a nest made of many shells (Figure 4.1a) occupied by several females (density = 1.6 ± 0.8 females/m^2, Ota and Khoda 2006a). These males court females by performing vigorous body-quivers, then lead receptive females to a shell to spawn. Once the female has entered a shell, the male positions his genital papilla directly over the shell opening for 2-3 s, apparently ejaculating sperm into the shell while the female lays her eggs (Ota and Khoda 2006a). Territorial males continue this ejaculatory posture for several hours. Pirate males (61-67 mm, the largest males in the population and >1.3 times the size of territorial males), patrol several shell-nests that they frequently enter, displacing territorial males and fertilizing the ova of any female that the territorial male has attracted. Pirate males are non-territorial, but they exclude other males from the vicinity of the shell while they are spawning. Satellite males (38-56 mm) are submissive to territorial and pirate males and, unlike territorial males, control only a single shell on or near the periphery of territorial males' shell-nests. Satellite males guard their single shell and use it to court and spawn with a female. Satellite males do not attempt to spawn in the territorial male's shell-nest. Sneaker males (22-34 mm) do not court females, but instead have small home ranges around a territorial male's shell-nest (Figure 4.1b). Sneakers parasitize territorial and satellite male spawnings either by entering a shell-nest with the female and releasing sperm, or by ejaculating into a shell-nest when the territorial or satellite male is absent. The reproductive tactics and body sizes of 148 males were studied in detail by Ota and Kohda (2006a; b).

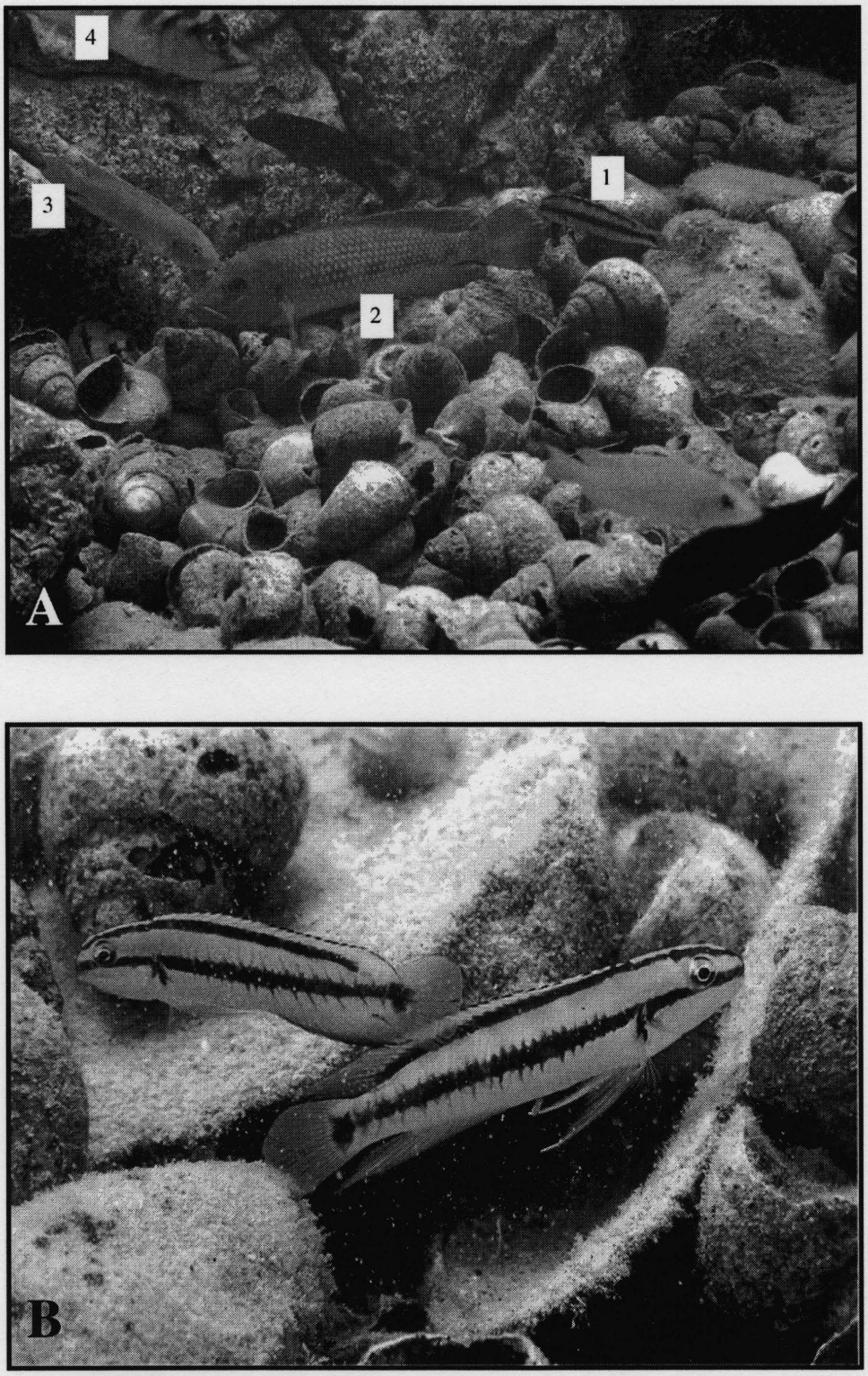

Figure 4.1. (a) Shell nest used by *T. vittatus* for mating and brood care. (1) A territorial male *T. vittatus* controls a single shell nest. (2) Shell nests are built by male *Lamprologus callipterus* (Sato et al. 2004). Shell nests are used for courtship and mating by several cichlid species including *T. vittatus* and (3) *Neolamprologus brevis* and (4) *Altolamprologus fasciatus*. (b) Two *T. vittatus* sneaker males on a shell nest

As both pirate and sneaker males engage in parasitic fertilizations one might expect sperm characteristics to be enhanced in both of these tactics. However, while sneaker males likely perform parasitic spawnings involving simultaneous sperm release with other males, sperm release by pirate males is temporally and spatially separated from that of other males because they exclude all other males from a nest during spawning (Ota and Khoda 2006a). As sneaker males release sperm simultaneously with other males during every spawning, they face higher levels of sperm competition than males adopting the other tactics. Therefore, based on both sperm competition theory (Ball and Parker 1996) and the carefully studied behavioural and physiological differences among males adopting the different reproductive tactics in *T. vittatus* (Ota and Khoda 2006a), we predicted that selection would favor the production of faster-swimming sperm with longer tails in sneaker males (who experience higher levels of sperm competition) compared to all male using other reproductive tactics, particularly compared to pirate males (who likely experience the lowest level of sperm competition).

4.3 METHODS

Between 26 March and 25 April 2005, 38 male *T. vittatus* were collected using SCUBA from depths of 2-7 m at the southern end of Lake Tanganyika, primarily from Kasakalawe and Wnzye Point, near Mpulungu, Zambia (see **Chapter 1**, Figure 2). We located shell-nests and collected males using a 1-mm-mesh fence net (1 m x 5 m) and handnets. In total we collected 5 pirates [standard length = 61.7-67.1 mm], 7 territorial males (50.5-61.1 mm), 15 satellites (38.0-47.6 mm), and 11 sneakers (25.6-37.5 mm). The male reproductive tactics were assigned based on the distributions of body sizes

described by Ota and Khoda (Ota and Khoda 2006a; b). According to Ota and Kohda (2006a), there is a 6-mm (50-56 mm) overlap in the size range of territorial and satellite males, so we classified these tactics with caution. However, only two males fell within this overlap and collapsing territorial and satellite males into a single category, or excluding these two males in this overlap category from our analysis, did not change any of our conclusions.

At the surface, we measured each fish's standard length (SL) to the nearest 0.1 mm and body mass to the nearest 0.001 g. We then anesthetized the fish in benzocaine, quickly sacrificed them via cervical severance, and removed their testes, which we then weighed to the nearest 0.001g. Following Ota and Kohda (2006a), we defined mature males as individuals with testes mass >0.002 g.

4.3.1 Sperm Analysis

After the testes were weighed, one testis from each male was put on a glass slide, and split open with a scalpel, and a drop of milt was collected and placed in a 2.0-ml microcentrifuge tube. Sperm were activated by adding 0.25 ml of lake water (previously boiled and cooled to lake temperature) and gently shaking the tube for 1-2 s (Fitzpatrick et al. 2006). We then placed 60 μl of the sperm/water mixture in a 1-mm deep well on a slide, with a cover slip covering half of the depression so that the sperm/water sample could easily be added to the well. This sample was viewed on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, New York, USA) mounted with a PixeLINK Megapixel PL-A662 digital video camera (PixeLINK, Ottawa, Ontario, Canada) to record sperm motility. Video recordings began as soon as the water was

added to the microcentrifuge tube so that the period since activation could be determined accurately. Videos of active sperm were captured at 60 frames/s at 200X magnification. Images were recorded using PixeLINK PL-A600 Series Camera Software (v3.1).

Sperm velocity was measured for 1 s at 0.5, 1, 2, 3, 4, 5, 6, and 7 min after sperm activation by a single researcher (JLF) blind to male size or reproductive tactic, using a CEROS (v12) video sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA). From some males, sperm continued to swim beyond 7 min post-activation, but because the proportion of a male's sperm that was active past 7 min fell dramatically, we confined our analyses of sperm velocity to the first 7 min post-activation. Due to water movement and variation in video quality, 0.5 min was the earliest time after activation that we were able to reliably measure sperm swimming speed of all males. We analyzed only those spermatozoa whose forward movement was traced for at least 0.33 s (i.e., $\geq$20 frames, see (Lahnsteiner et al. 1998; Burness et al. 2004; Fitzpatrick et al. 2006 for a similar measurement criterion). The median sperm velocity (VAP; median smooth path velocity) and median curvilinear track velocity (VCL) were calculated for all spermatozoa recorded at each time period after activation (mean number of sperm recorded $\pm$ SE, n, range: 76.7 $\pm$ 4.5, n = 261, 5-488). Although VAP and VCL are strongly correlated (see Results), we analyzed and report both of these measures of sperm swimming speed, as they measure slightly different aspects of sperm motion and currently there is no consensus in the literature as to which is more useful. We also measured sperm path straightness (STR), an estimate of the spermatozoon's departure from a straight line while swimming, as STR=VSL/VAP (where VSL is the velocity

along a straight line connecting the start and end of the spermatozoon's path during the 1-s period of each measurement).

Following a period of declining swimming speed, spermatozoa cease forward movement, and begin to vibrate, providing a clear end point to measure sperm longevity. The longevity of sperm movement was measured as the time since activation at which 95% of the sperm no longer exhibited forward movement (Fitzpatrick et al. 2006; Gage et al. 1998; Hoysak and Liley 2001; Gage et al. 2002).

To measure sperm lengths, the remaining testis of each male was split open, and the free flowing milt diluted with lake water, spread over the glass slide, and allowed to air dry. The first ten clear sperm images detected from each male were magnified to 1000x on the computer monitor, photographed and measured using NIH ImageJ software (v. 1.36, available at http://rsb.info.nih.gov/ij/). We traced a freehand line over the tail, measuring tail lengths (to the nearest 0.1 μm) from the centre of the sperm's head to the end of its tail (Balshine et al. 2001). To avoid observer bias, all samples were measured blind to male size or reproductive tactic.

4.3.2 Statistical Analysis

Statistical analyses were performed with JMP (version 6.0.3, SAS Institute Inc., 2006). Data and residuals were tested for normality, and transformed when necessary to improve the fit to normality. Sperm swimming speed measured by smoothed path velocity (VAP), curvilinear track velocity (VCL) and sperm path straightness (STR) were $\log_{10}$ transformed for every analysis and analyzed using repeated measures ANOVAs. We used Tukey HSD tests for post-hoc comparisons. To avoid pseudoreplication and to minimize

the influence of outliers, all statistical analyses involving sperm length were performed using a single median value from each male; we reached the same conclusions using mean sperm length (data not reported here). As we were not able to measure sperm length and longevity from one satellite male, the sample size was reduced to 37 when analyzing those variables.

4.4 RESULTS

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

Smooth path (VAP) and curvilinear track velocity (VCL) of sperm varied significantly among males that adopted different tactics (Figure 4.1, Table 4.1). There was a significant and progressive decline in sperm swimming speed within tactics, with both VAP and VCL at 0.5 min post-activation significantly higher than at all other times post-activation (Tukey tests following separate ANOVAs for each tactic, $p < 0.05$, Figure 4.2, Table 4.1). In general, sperm from pirate males swam slower than sperm from males adopting all other tactics, while sperm from sneaker males was, on average, faster than that of all other tactics at all times post-activation (Figure 4.2). Post hoc tests revealed

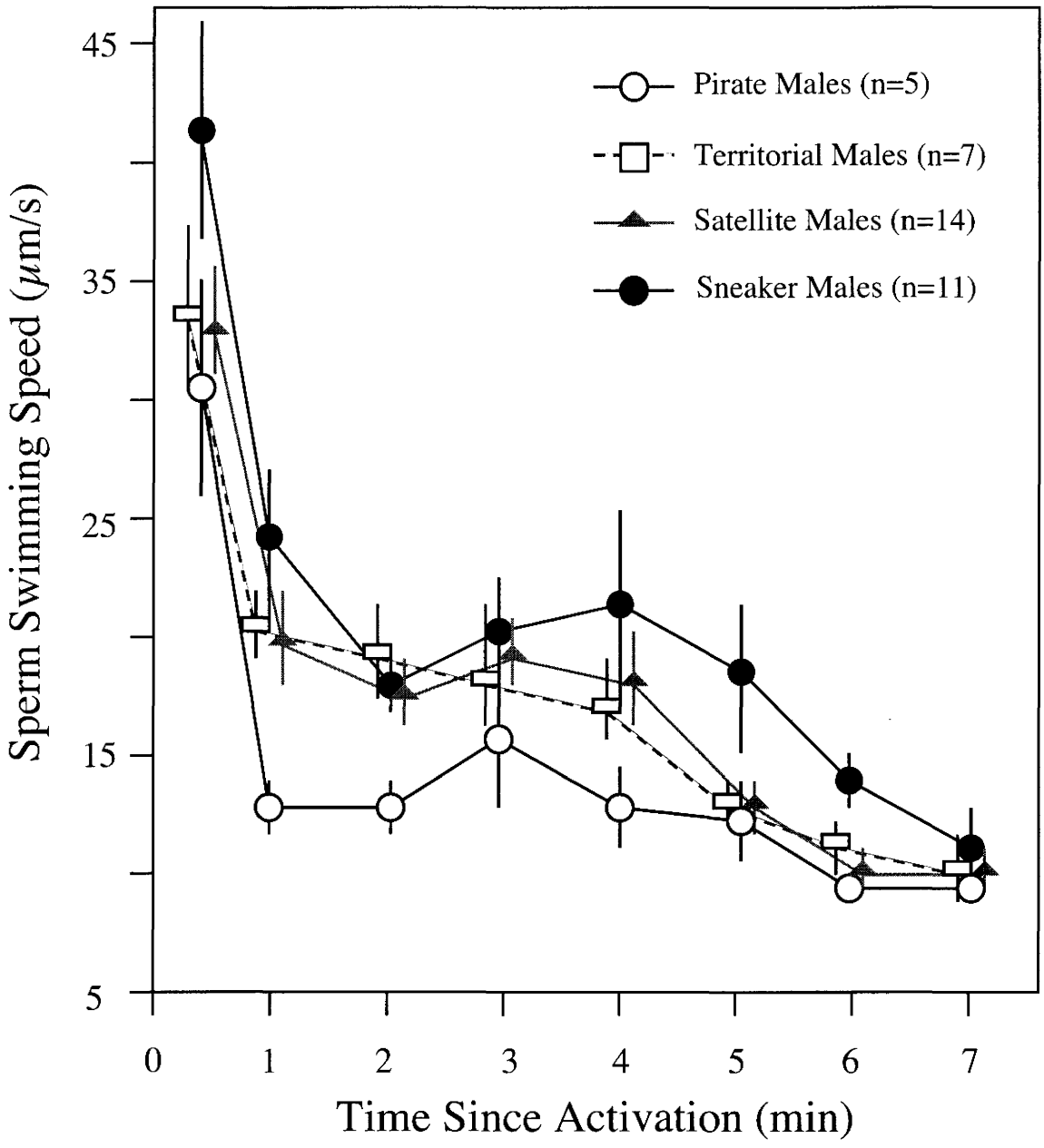

Figure 4.2. Mean (±SE) sperm swimming speed (smooth path velocity, VAP) at different times post-activation for males adopting different mating tactics. For clarity points at each time period are staggered.

that sperm from sneakers swam significantly faster (VAP and VCL) than sperm from pirates, but that none of the other pairwise comparisons between tactics were significant. There was no significant interaction between tactic and time since activation in either model ($p > 0.71$ in both cases), so this term was removed from the final models. Thus, there was no indication that the pattern of decline in VAP or VCL with time since activation differed among the male tactics.

Adding median sperm length to the models shown in Table 4.1 did not change our conclusion that there was significant variation in sperm swimming speed both among male tactics (VAP, $F_{3,31} = 3.5$, $p = 0.03$; VCL, $F_{3,31} = 3.7$, $p = 0.02$) and with time since activation (VAP, $F_{7,226} = 97.0$, $p < 0.0001$; VCL, $F_{7,227} = 75.4$ $p < 0.0001$). The effect of sperm length in both models was not significant (VAP: $F_{1,31} = 1.2$, $p = 0.28$; VCL: $F_{1,31} = 2.4$, $p = 0.13$).

The straightness of the sperm swimming path (STR) also differed significantly among reproductive tactics (indicated by a significant interaction between time since activation and reproductive tactic, repeated measures ANOVA, interaction effect: $F_{21,212} = 2.7$, $p = 0.0002$). At 30 s after activation, STR was not significantly different among tactics ($F_{3.29} = 0.3$, $p = 0.82$), with sperm from males of all tactics swimming in a relatively straight path (mean STR = 0.75-0.82). By 6 min post-activation, however, the sperm of sneakers was still swimming in a relatively straight path (mean STR = 0.67, n = 10 males) whereas that of males adopting the other tactics had a more curvilinear path (mean STR = 0.44-0.55, n = 7-14 males per tactic), and the variation among tactics was significant ($F_{3,31} = 5.1$, $p = 0.006$). At 7 min post-activation, the same pattern of variation in STR according to tactic was apparent and significant.

Table 4.1. Repeated measures ANOVAs examining the effect of male reproductive tactic and time since sperm activation on the median smooth path velocity (VAP) and median curvilinear track velocity (VCL) of sperm recorded at different time periods post-activation.

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

Table 4.2. No significant differences in median sperm length (measured from 10 sperm per male) or sperm longevity were detected among male reproductive tactics in *T. vittatus*. Mean ±SE is shown for each variable

Tactic	*Median Sperm Length (μm)*	*Sperm Longevity (s)*
Pirate (n = 5)	33.7 ± 1.5	406.8 ± 40.7
Territorial (n = 7)	32.3 ± 1.1	456.9 ± 51.9
Satellite (n = 14)	31.6 ± 0.8	393.1 ± 14.1
Sneaker (n = 11)	32.0 ± 0.4	447.3 ± 17.3

There were no significant differences in sperm tail length among tactics ($F_{3,33}$ = 0.81, p = 0.50, Table 4.2). Similarly, although sperm swimming longevity varied across individual males, ranging from 287 to 728 s in duration, it did not differ significantly among reproductive tactics ($F_{3,33}$ = 1.45, p = 0.25, Table 4.2).

There were no significant relationships between VAP or VCL and median sperm tail length (r = –0.01-0.34, p≥0.09, n = 27-36 males) at any time post-activation, pooling across tactics. Sperm tail length was also not significantly related to sperm longevity (r = –0.04, n = 36, p = 0.83) or male body size (standard length, SL: r = 0.23, n = 37, p = 0.18).

4.5 DISCUSSION

Consistent with theoretical predictions, sperm from males encountering elevated levels of sperm competition (sneakers) had the fastest swimming sperm, while males experiencing the lowest levels of sperm competition (pirates) had the slowest (Figure 4.1). The faster swimming sperm of sneakers may be advantageous as fertilization success can be positively correlated with sperm velocity in fishes (Gage et al. 2004; Casselman et al. 2006). Interestingly, our results show that, in *T. vittatus*, sperm from sneakers swam faster initially than sperm from pirate males, but continued to swim at faster speeds than all other tactics throughout the duration of its forward motility. Therefore, the predicted tradeoff between sperm swimming speed and longevity (Ball and Parker 1996) observed in other fishes (Burness et al. 2004), was not detected in *T. vittatus*.

Longer sperm tails are believed to provide greater propelling force than shorter ones (Gomendio and Roldan 1991). However, as in previous studies of fish spermatozoa

(Gage et al. 1995; Leach and Montgomerie 2000; Vladic et al. 2002: Gage et al. 2002), sperm lengths were not correlated with sperm swimming speed, nor were there any differences in sperm length among reproductive tactics in *T. vittatus*. In fishes, differences in sperm lengths between tactics within a species may be small. For example, in bluegill, in one study sneakers had slightly (> 2 μm), but significantly, longer sperm than parental males (Burness et al. 2004). However, our results suggest that sperm from different tactics may differ energetically (i.e., in ATP stores) while not differing in length, and, intriguingly, indicate that selection has acted on sperm energetics in *T. vittatus*, rather than on sperm size. In species with MARTs, ATP levels are usually greater in sperm from sneaker males (**Chapter 2**), suggesting that in order to ensure faster swimming speeds selection has favored increased energy stores in sperm from males performing sneak fertilizations. As ATP levels are correlated with initial sperm swimming speed (Burness et al. 2004), the faster swimming sperm observed in *T. vittatus* sneakers may indeed be due to greater energy stores. While the mechanism underlying the tactic-specific sperm swimming speeds in *T. vittatus* remains unknown, further studies using more refined techniques (e.g. electron microscopy), addressing differences in spermatozoa energy stores and larger sample sizes will improve our understanding of tactic-specific sperm investment. Such studies investigating the tradeoff between sperm energetics and other sperm traits will further reveal how sperm competition has shaped the evolution of sperm phenotypes.

Both life history traits and fertilization dynamics may influence the evolution of sperm characteristics in *T. vittatus*, selecting for faster-swimming sperm as well as sperm that continue to swim faster in sneakers. Prolonged periods of sperm swimming may be

particularly advantageous if sneaker males engage in pre-oviposition ejaculation, as has been reported in other teleosts (Kanoh 1996), where sperm are shed before ova are released. Pre-oviposition ejaculation can be an effective way for sneaker males to fertilize eggs, particularly when territorial males aggressively guard females. For example, in the rose bitterling (*Rhodeus ocellatus*), fertilization occurs within the gill cavity of a freshwater mussel that is defended by a territorial male (Kanoh 2000). Territorial males chase away competing males, leaving their mussel nests vulnerable to sperm release by other males. Sneaker males capitalize on periods of temporarily relaxed mussel-guarding by intruding into the territory and releasing sperm (Kanoh 1996). As the number and timing of female visits and spawning events is unpredictable, sneaker males release sperm whether or not a female is spawning, and are able to successfully fertilize eggs laid for a period of several minutes after the ejaculation (Kanoh 1996; 2000). Similarly, *T. vittatus* sneaker males are likely constrained by the number and timing of spawning opportunities. Indeed, Ota and Kohda (2006a) observed *T. vittatus* sneakers ejaculating into spawning shells while territorial males were absent, suggesting that *T. vittatus* sneakers may also be taking advantage of periods of relaxed shell guarding to gain a measure of reproductive success. In many cichlid species, gametes remain viable for extended periods. For example, in Nile tilapia *Oreochromis niloticus* eggs remain fertilizable for approximately 15 min after release (Myers and Hershberger 1991), while in the Tanganyikan cichlid *Opthalmotilapia ventralis* sperm remains viable for 10-15 min after release from a male (M. Haesler personal communication). Therefore, the prolonged increase in sperm swimming speed observed in sneakers relative to all other tactics may confer a reproductive advantage to sneakers, and should be examined in future studies in

relation to the relative timing of ejaculates in relation to egg-laying among the male tactics.

Asymmetries in the level of sperm competition can select for higher quality ejaculates in males performing sneak fertilizations (Ball and Parker 1996). This study, together with a handful of other studies in fishes (Rudolfsen et al. 2006; Serrano et al. 2006; Burness et al. 2004), demonstrates that males mating in the disfavoured role produce faster swimming sperm than males in the favoured role. These results suggest that differences in sperm swimming speeds among male tactics in *T. vittatus* are an adaptation to sperm competition. We suggest that changes in ATP production or allocation among sperm over an individual's lifetime may underlie differences in sperm swimming speed among reproductive tactics. As it is currently unclear if male reproductive tactics in *T. vittatus* are fixed (genetically determined) or plastic (socially determined), we are unable to assess if differences in sperm swimming speed remain static or change over the course of an individual's lifetime. If tactics are plastic and dependent upon body size relative to conspecifics, as is the case in other fishes (Warner 1988), then sperm swimming speed of *T.vittatus* males would decrease as they grow from sneakers to pirates, largely as a consequence of changes in sperm energetics. In conclusion, our study demonstrates fine-tuned selection on sperm swimming speed due to sperm competition in a species characterized by an unusual number of male reproductive tactics, and sets the stage for further examinations of the factors underlying the phenotypic variation in sperm among male tactics.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

PROMISCUITY PROMOTES THE EVOLUTION OF FASTER SWIMMING SPERM

5.1 ABSTRACT

Theory predicts that increased sperm size (especially in species with external fertilization) and swimming speed make sperm more competitive (Ball and Parker 1996). Yet there is at best mixed support for the role of sperm competition in shaping sperm size, swimming speed and longevity (Snook 2005; **Chapter 2**). Here we examined sperm characteristics from 29 closely related species of cichlid fishes from Lake Tanganyika and show (1) that sperm competition selects for increases in sperm number, size, and both swimming speed and longevity, with no evidence of trade-offs among these traits, (2) that sperm swimming speed is positively correlated with sperm length among species but not within species, and (3) that the evolution of faster sperm preceded the evolution of longer sperm in this clade of fishes. Thus sperm competition can promote the evolution of more competitive spermatozoa by influencing different traits. However, responses to sperm competition differ at the intra- and interspecific level and this appears to explain the lack of consistency among different taxa studied so far.

5.2 RESULTS AND DISCUSSION

Sperm competition theory predicts a positive relationship between sperm size and the strength of sperm competition (Parker 1993; Ball and Parker 1996). This prediction is

based on the assumption that (1) longer sperm size swim faster and (2) faster swimming sperm are more successful during competitive matings (Ball and Parker 1996). While the majority of comparative studies show that species experiencing greater levels of sperm competition have longer sperm (Snook 2005), a well-cited study demonstrated a negative relation between sperm competition and sperm size across fishes (Stockley et al. 1997; but see **Chapter 2**). Furthermore, there is little empirical evidence demonstrating the link between sperm morphology and speed. The reported positive relationship between sperm length and swimming speed is surprisingly based on a single comparative study of five domesticated mammalian species with all sperm data taken from the literature (Gomendio and Roldan 1991). Remarkably, this study remains the only evidence for an interspecific relation between sperm size and speed and is extensively cited in support of this prediction. There have, however, been numerous intraspecific studies using a variety of taxa, aimed at determining if longer sperm swim faster. But, within species, there is almost no evidence that sperm size influences swimming speed (**Chapter 2**). To date, the only intraspecific evidence that longer sperm move faster comes from a study of the nematode *Caenorhabditis elegans*, a species with amoeboid sperm (LaMunyon and Ward 1998). However, the applicability of this result to sperm with flagella (tails), which is the case in most species, is questionable.

In this study, we sought to definitvely examine the relationship between sperm swimming speed and sperm length, and to measure these traits in a methodologically rigorous and consistent fashion, both within and among species in a single family of fishes. To do this we studied wild-caught, reproductively mature, male cichlids from Lake Tanganyika, Africa. Tanganyikan cichlids are ideal species in which to study

adaptations resulting from sperm competition, as males are expected to differ in the level of sperm competition they experience due to the tremendous diversity in their mating systems (Fryer and Iles 1972; Kuwamura 1997; Balshine et al. 2001). Moreover, detailed information on mating behaviours is readily available, facilitating reliable estimates of the strength of sperm competition (see Table 5.1, Supplemental Data Table I). Also, because Tanganyikan cichlid lineages have seeded explosive speciation events in Lake Malawi and Victoria, the evolutionary relationships among species have been extensively studied (Nishida 1991; Staumbauer and Meyer 1993; Cohen et al. 1997; Staumbauer et al. 2002; Salzburger et al. 2002). Furthermore, the natural fertilization environment is easily mimicked in externally fertilizing fish (Levitan 1998), allowing sperm swimming speed and duration to be studied under natural conditions. Finally, all the species that we studied are endemic to a single lake and therefore share a similar fertilization environment. Consequently, we were able to minimize potentially confounding variables, such as family-specific responses to sperm competition (Immler and Birkhead 2007) and differential responses to alternative fertilization environments, which may have unwittingly confused the results of previous studies of sperm traits.

We assessed the impact of sperm competition on various sperm traits using two different indices of the strength of sperm competition, while controlling for lack of independence due to phylogeny (Harvey and Pagel 1991). First we ranked the incidence of sperm competition on a four-point scale, with scores 1-2 indicating that sperm competition was absent or unlikely and scores 3-4 where the probability of ejaculates competing to fertilize eggs was moderate or high (see Table 5.1). Species with high sperm competition rank had relatively heavier testes, longer sperm, faster swimming

Table 5.1. The strength of sperm competition was ranked on a 4-point scale based on a composite score that combined behavioural, ecological and genetic data collected from the literature. See Supplemental Data Table I for a species list and additional information.

Sperm Competition Rank	Species Characteristics		
	Mating System	**Parental Care**	**Fertilization Location**
1 (none)	monogamous	biparental care	buccal
2 (low)	monogamous	biparental care	substrate
3 (moderate)	polygynous	female-only care	substrate
4 (high)	polygynous with male alternative reproductive tactics OR polygyandrous and lekking	female-only care	substrate OR buccal cavity

sperm and longer-lived sperm compared to low-ranking species (Table 5.2). Second, we also used relative testes size (log transformed, controlling for male mass) as an index of the degree of sperm competition. This measure has been widely used in studies of sperm competition (e.g. Parker et al 1997, Gage and Freckleton 2003) and recent work has confirmed its usefulness as an index (Calhim and Birkhead 2007). There was a positive correlation between sperm length and relative testes mass across species (Figure 5.1a). Sperm swimming speed was positively related to relative testes mass during the first 6 min after sperm activation, but none of these correlations was significant (Supplemental Data Table II). Sperm swimming speed was significantly related to sperm longevity at all but the earliest (0.5 min) period after sperm activation (Supplemental Data Table II). Most important, there was no evidence for trade-offs between either sperm size or swimming speed and sperm longevity (Supplemental Data Table II). Instead, species experiencing greater levels of sperm competition had multiple sperm traits enhanced.

Table 5.2. Results of simple and multiple regression analyses of the relationships between testes mass, sperm length, sperm swimming speed, sperm longevity and sperm competition rank while controlling for phylogeny (using GLS). Phylogenetic dependence was assessed using the scaling paramater λ. Superscripts on λ represent significance levels of likelihood ratio tests with λ compared to 0 (first position) and 1 (second position). Significance levels are denoted as ns = not significant, * = $p \leq 0.05$. Sperm competition ranked was assessed on the 4-point scale described in Table I. A *t*-test was used to compare the observed slope against a slope of 0. Effect sizes, *r*, were calculated from *t* values and non-central 95% confidence intervals (CI) are presented. The degrees of freedom, df, used to calculate *r* and CI are presented. CI that do not overlap zero represent significant relationships and are indicated by bold text.

Trait	λ	Predictor	Estimate	*t*	p	*r*	df	CI
testes mass	< 0.001 ^{ns,*}	body mass	0.90	4.92	**<0.001**	0.69	26	**0.44 - 0.82**
		sperm competition rank	0.44	3.37	**<0.01**	0.55	26	**0.23 - 0.73**
sperm length	0.51$^{*,\ ns}$	body mass	-0.04	-1.12	0.27	-0.21	26	–0.52 - 0.17
		sperm competition rank	0.07	2.61	**0.01**	0.46	26	**0.10 - 0.68**
sperm longevity	0.69$^{*,\ ns}$	body mass	-0.02	-0.50	0.62	-0.01	26	–0.37 - 0.35
		sperm competition rank	0.09	2.32	**0.03**	0.41	26	**0.05 - 0.65**
sperm speed	0.89$^{*,\ ns}$	sperm competition rank	0.04	2.10	**0.04**	0.37	27	**0.009 - 0.62**

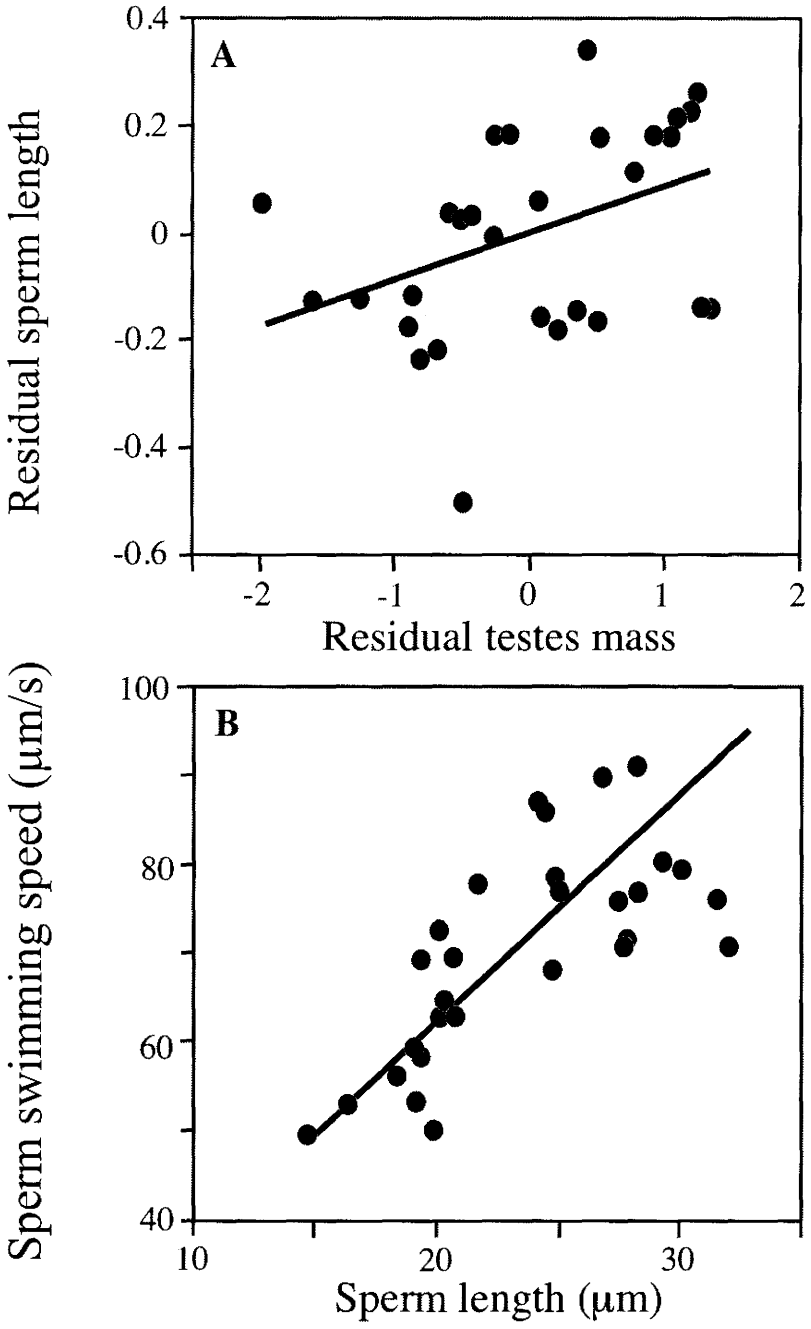

Figure 5.1. (a) Partial residual plot of the relation between sperm size and testes mass, both controlling for the effects of body (soma) mass (all log-transformed). Species that invested relatively greater resources in testes mass had larger sperm (testes mass: estimate = 0.08, t = 18.7, $p < 0.01$, r = 0.96, df = 27, CI = 0.93 - 0.98; soma mass: estimate = –0.10, t = 2.4, p = 0.02, r = -0.43, df = 27, CI = –0.66 - 0.07; $\lambda = 0.71^{*, ns}$). **(b)** Relation between sperm swimming speed at 0.5 min post-activation and sperm length (sperm length: estimate = 0.29, t = 2.53, p = 0.02, r = 0.44, df = 27, CI = 0.08 - 0.66; $\lambda = 0.93^{*, ns}$). See SD Table II for details of regressions between sperm size and speed at all other time periods post-activation. Data in the figures are not controlled for phylogeny.

There was also a significant positive relation between sperm swimming speed and sperm length, among species, controlling for the potential influence of phylogeny (Figure 5.1b, Supplemental Data Table II). Interestingly, only 2 of the 29 species that we studied had a significant positive intraspecific relation between sperm swimming speed and sperm size (Supplemental Data Table III). Thus this general lack of intraspecific patterns is consistent with other studies that have failed to find within-species relationships (**Chapter 2**).

To further explore the co-evolution of ejaculate traits and their evolutionary history within this clade we performed a directional test of trait evolution with these data (Pagel and Meade 2006). These analyses are used to determine which transitions between states are most likely to have occurred first during the evolutionary history of a taxon. To perform these analyses traits are coded into binary states and the most probable ancestral trait must be identified *a priori*. Thus we coded continuous both trait data and sperm competition ranks into 'low' and 'high' categories and determined the probable ancestral states using maximum likelihood methods for phylogeny reconstruction (Pagel 1999), based on molecular traits (see Supplemental Data Table I and Figure 2 for details). This analysis indicates that the ancestral states were (i) low sperm competition, (ii) small sperm size, and (iii) slow sperm swimming speed (likelihood probability estimates= 0.76, 0.78 and 0.78 respectively).

Within these Tanganyikan cichlids, both sperm size and sperm swimming speed increased from the ancestral state of small and slow sperm to the derived state of large and fast sperm, following an initial increase in the strength of sperm competition (Supplemental Data Figure 1). Thus, both larger and faster sperm were evolutionary

responses to increasing levels of sperm competition. We then assessed the evolutionary transition between sperm traits, asking whether sperm size or swimming speed were first to respond to increased sperm competition. These analyses support the conclusion that larger and faster sperm evolved from smaller and slower ancestral sperm via an initial increase in sperm swimming speed, followed by increasing sperm size (Figure 5.2). An alternative pathway suggesting an initial increase in sperm size without an increase in sperm swimming speed is not supported by our analysis.

The most plausible physiological explanation for an initial increase in sperm speed is that selection due to sperm competition acted first on sperm energetics, thereby increasing sperm speed, before acting on sperm size. Sperm motility is powered by ATP, produced by mitochondria in the sperm midpiece (Christen et al. 1987). As ATP production is positively related to sperm swimming speed (Burness et al. 2005), any increase in ATP content per spermatozoon, for example by increasing mitochondrial efficiency or number, will increase sperm swimming speed. Therefore, in the face of intense sperm competition, selection may act by altering sperm energetic output rather than sperm size. Thus we would expect intense selection on sperm midpiece size, as longer midpieces house more ATP (Vladic et al. 2002)—comparative studies have demonstrated a positive relationship between midpiece size and the strength of sperm competition in birds and mammals (Johnson and Briskie 1999; Immler and Birkhead 2007; Anderson et al. 2005; Anderson and Dixon 2002, but see Gage and Freckelton 2003; Immler and Birkhead 2007). Our results indicate that sperm swimming speed preceded evolutionary increases in sperm length and so may also explain why studies on alternative reproductive tactics have generally found that sneaker sperm have greater

ATP content than sperm of conventional males, but there are no evident differences in sperm morphology between male tactics (Locatello et al. 2007; Vladic and Järvi 2001; Burness et al. 2005; **Chapter 4**; reviewed in **Chapter 2**).

Our results provide the first clear evidence that sperm competition selects for faster swimming sperm. In external fertilizing fishes, selection on sperm swimming speed may be particularly intense, as individual sperm must race to a target on the egg surface, a narrow canal known as the micropyle in order to reach the egg's interior. Because the first sperm to successfully navigate this passage fertilizes the egg (Yanagimachi et al. 1992), selection likely places a premium on the production of fast sperm (Gage et al. 2004). Our findings are also likely to be broadly applicable to other taxa. Indeed, a recent study has demonstrated that sperm swimming speed is related to mating system in primates (Nascimento et al. 2008). Given that sperm competition is widespread, the application of techniques described in this study to other taxa should help resolve the importance of sperm competition in shaping sperm traits in general.

5.3 EXPERIMENTAL PROCEDURES

In February-April 2004 and 2005, sexually mature males of 29 species from five tribes (Supplemental Data Table I and III) were collected using SCUBA at depths of 1-15 m near the southern shores of Lake Tanganyika. Males were captured using handnets and a 5 m x 1 m fence net, and taken to the surface for dissection. Fish were exposed to a lethal dose of anesthetic (benzocaine; Ethyl *p*-Aminobenzoate, SIGMA), and their body and testes masses were measured to the nearest 0.001 g. Spawning in these tropical cichlids

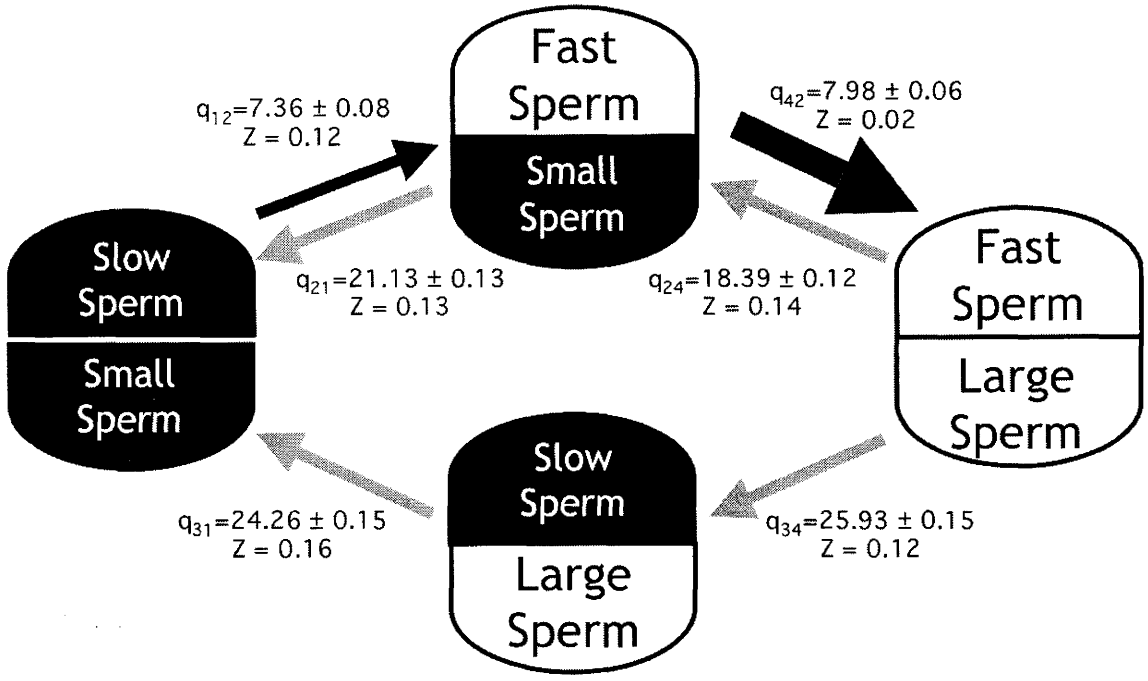

Figure 5.2. Transition diagram illustrating the evolutionary pathways from the ancestral state of slow and small sperm to the derived state of fast and large sperm. Intermediate states are represented in the middle of the diagram. Forward transitions are depicted with black arrows and back transitions with grey arrows. Non-significant transitions were removed from the diagram. The thickest arrow represents the most likely transition. For each transition we report both a q-value (mean ±SE transition parameter), and a Z-value (proportion of iterations assigned to zero); q-values further from zero represent more probable evolutionary transitions and low Z-values indicate more likely evolutionary transition. In general, transitions are considered likely when $Z < 0.10$, meaning that less than 10% of the iterations from the Markov chain are assigned to zero (Pagel and Meade 2006). However, following Kolm et al. (2006; 2007), we also used a more conservative approach to assess trait evolution which accounts for non-significance that may result from low statistical power resulting from small phylogenies (Rolland et al. 1998). In this approach, we considered non-significant ($Z > 0.10$) transitions to be likely evolutionary transitions if the non-significant transition parameter (q-value) was higher than that of the lowest parameter that yielded a significant transition. However, we considered q_{12} to be a likely transition, because comparisons of the q_{12} to q_{13} suggested that q_{12} was the most likely transition from the ancestral to the intermediate state. For this analysis, sperm swimming speed was measured at 0.5 min post-activation.

often occurs year round, so any males with ripe testes and motile sperm were considered reproductively active and used in the analyses presented here.

Sperm size and swimming speed were assessed using the protocols described in Fitzpatrick et al. (2006) and in **Chapter 4**. Sperm lengths were measured by diluting milt (sperm and seminal plasma) in water, spreading this solution over a microscope slide, and allowing it to air dry. Under 400X magnification, we digitally photographed 10 clearly visible spermatozoa from each male and later measured the sperm by tracing a freehand line from the center of the sperm head to the tip of the tail (see Balshine et al. 2001) using NIH ImageJ software (v 1.38, available at http://rsb.info.nih.gov/ij/) and an Intuos graphic tablet (Wacom Co. Ltd., Japan). Sperm swimming speed was assessed by recording a digital video of motile sperm, captured at 60 frames/s under 200X magnification using a PixeLINK Megapixel PL-A662 digital video camera (PixeLINK, Ottawa, Ontario, Canada) mounted on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, New York, USA). Video recordings were started when water was added to a milt sample and the sperm activated, providing an accurate recording of the first instance of sperm movement. Sperm swimming speed was assessed using a CEROS (v.12) computer-assisted sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA) that recorded curvilinear (VCL) and smooth path velocity (VAP). Swimming speeds were recorded for 1 s at 8 time periods (at 30 s and 60 s, then at 1 minute intervals until 7 min post-activation). We focus on sperm swimming speeds at 30 s post-activation data because these are most likely to be relevant to a male's fertilization success (Hoysak and Liley 2001; **Chapter 2**); analyses for speeds at other times are presented in Supplemental Data Table II. We present data only on VCL here but VCL

and VAP are highly correlated measures of sperm swimming speeds (**Chapter 4**) and we obtained qualitatively similar results when analyzing either measure. All sperm lengths and swimming speeds were measured blind to the identity of males. Sperm longevity was measured as the time from activation until 95 % of sperm were immotile (Fitzpatrick et al. 2006).

5.3.1 Phylogeny Construction

We constructed a mtDNA phylogeny for 31 species (29 study species and 2 outgroup species) of Tanganyikan cichlids, belonging to 7 tribes (Boulengerchromis, Bathybatini, Ectodini, Eretmodini, Lamprologini, Perrisodini and Trophini) using NADH dehydrogenase subunit 2 (ND2), cytochrome b (CytB) and non-coding control region (CR) sequences obtained from GenBank. Accession Numbers are listed in Supplemental Data Table IV. These sequences are commonly used for examining phylogenetic relationships among Tanganyikan cichlids (Koblmuller et al. 2004; Day et al. 2007). ND2 sequence data were available for all species, whereas eight CytB and two CR sequences were not (Supplemental Data Table IV). Sequences were aligned using McClade (v 4.06) and a GTR+I+G best-fit model was selected by MrModeltest 2.2 (Nylander 2004). Phylogenetic relationships were determined using Bayesian analyses employing Markov Chain Monte Carlo (MCMC) methods in MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), with a burn-in of 400,000 generations, sampling two million subsequent generations every 100^{th} generation, with *Boulengerochromis microlepis* and *Bathybates fasciatus* set as outgroups. Branch lengths were estimated and incorporated into all subsequent analyses. Support at each node was assessed with Bayesian posterior probabilities. The tree obtained in this study (Supplemental Data Figure 2) is largely

congruent with recently published phylogenetic hypotheses (e.g. Koblmuller et al. 2004; Day et al. 2007).

5.3.2 Phylogenetic multiple regressions

Data were analyzed with regression and multiple regression using a generalized least-squared (GLS) approach in a phylogenetic context (Pagel 1999; Freckleton et al. 2002). This approach controls for any statistical non-independence that can arise from shared ancestry (Felsenstein 1985; Harvey and Pagel 1991). Using likelihood ratio tests we use the scaling parameter λ to assess the degree of phylogenetic dependence exhibited by our data (Pagel 1999; Freckleton et al. 2002). Values of λ can range from 0-1; values not significantly different from 0 indicate that trait evolution occurred independently of the phylogenetic association between species, while values not significantly different from 1 indicate that traits are strongly associated with the phylogeny. GLS analyses are performed using the maximum likelihood value of λ. This scales the analyses to incorporate the degree of phylogenetic correlation present in the data. Prior to analyses all data were log transformed. All analyses were performed using R v 2.6.1 (R Foundation for Statistical Computing 2007).

Because the same variables were used repeatedly in different phylogenetically controlled regressions, we calculated effect size from each test to determine the strength of the relations between the traits of interest (Nakagawa 2004). Effect size, r, was calculated from t values obtained from the GLS model (Nakagawa and Cuthill 2007). The non-central 95% confidence interval (CI) for r was used to assess statistical significance (Nakagawa and Cuthill 2007).

5.3.3 Testing the directionality of evolutionary transitions

We reconstructed probable ancestral states using Mesquite (Midford et al. 2002) with likelihood reconstruction methods (Pagel 1999) in a Markov k-status 1-parameter model with the maximum state restricted to 1 to accommodate our binary data.

We assessed the directionality of sperm trait evolution using BayesDiscrete (www.evolution.rdg.ac.uk; Pagel and Meade 2006). This program examines the evolutionary pathway that gave rise to the observed traits using reversible-jump MCMC (RJ MCMC) methods. Specifically, BayesDiscrete assesses the likelihood that changes in one trait preceded the evolution of another trait. We created binary states from continuous trait data by classifying a trait as "low" (below the mean value for all species) or "high" (above the mean value for all species). For analysis of sperm competition ranks, we collapsed our 4-point scale into "low" (monogamous, rank 1 and 2) and "high" (polygamous, rank 3 and 4) levels. We selected a subsample of trees from the two million generations produced from our Mr.Bayes analysis. After a burn-in of one million generations, we sampled 500 trees at intervals of 20,000 generations. This analysis controls for phylogenetic uncertainty by assessing transitions among a broad subset of trees generated from our Mr.Bayes analysis. We ran the RJ MCMC chain for 5,050,000 iterations, with a burn-in of 50,000 iterations. The chain was sampled every 100^{th} iteration, creating a posterior distribution of 50,000 sample points from which we determined the mean ±SE transition parameter value and the proportion of samples where the transition parameter was assigned to zero. Transition parameters that were frequently assigned to zero were considered unlikely, while those only rarely assigned to zero were

considered to be highly likely evolutionary transitions. Additional details on the logic underlying these analyses can be found in the Supplemental Data (Supplemental Data V).

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5.6 SUPPLEMENTAL DATA

SD Table I. Species assignment to the 4-point scale of sperm competition used in this study. Sperm competition rank was based on a composite score that combined behavioural, ecological and genetic data collected from the literature. All species assigned to rank 1 (none) were monogamous, biparental, buccal (mouth) fertilizers, with the exception of *T. moorii*, which exhibits maternal care[1]. Sperm competition is absent or highly unlikely in these species, as buccal fertilization requires a complicated male-female interaction and protracted mate guarding by both sexes is common in these species[22]. Genetic evidence supports monogamy in *E. cyanostictus*[3] and *T. moorii*[5]. Species assigned to rank 2 (low) were monogamous, biparental, substrate fertilizers. Gametes are shed onto the substrate. Males vigorously defend their territories and guard mates, and there is no evidence from behavioural data that suggests males engage in sneak fertilizations. However, we cannot preclude the possibility that simultaneous sperm release from competing males occurs. There are currently no paternity data available from any of these species. Species assigned to rank 3 (moderate) were polygynous, maternal caring, substrate fertilizers. Males of these species simultaneously guard harems of multiple females, potentially providing an opportunity for sneak fertilizations, with the exception of *J. marlieri*, *N. brichari* and *N. savoryi*[1]. *J. marlieri* is included in this category because, although breeding pairs often appear to be monogamous, females occasionally practice polyandry[8]. The cooperatively breeding cichlids *N. brichari* and *N. savoryi* are included in this category because some authors have suggested that social living may facilitate sneaking by subordinate males[23]. However, there is as yet no evidence of shared paternity in these two species from wild populations. Species assigned to rank 4 (high) were either substrate fertilizers with alternative male reproductive tactics or buccal fertilizers that form leks. In lekking buccal fertilizing species, females take up sperm from one male into their buccal cavity and visit another male within minutes where they receive additional sperm from a different male. Thus sperm competition takes place within the buccal cavity of the female. Each species listed in this group has been individually studied with the exception of *N. brevis*. The existence of male reproductive tactics in *N. brevis* was inferred from a personal communication cited in ref. 16 which stated that small males, presumably sneaker males, invested relatively more in testes than larger males. Subscripts beside each species name correspond to references describing reproductive behaviour in each species.

SD Table I.

Sperm Competition Rank	Species Characteristics	Species
1 (none)	monogamous biparental care buccal fertilization	*Asprotilapia leptura*[1] *Eretmotus cyanostictus*[1,2,3] *Microdontochromis tenuidentatus*[1] *Perrisodus microlepis*[1,4] *Tropheus morri*[1,5] *Xenotilapia flavipinnis*[1,6] *Xenotilapia spilopterus*[1]
2 (low)	monogamous biparental care substrate fertilization	*Chalinochromis brichardi*[1] *Lepidiolamprologus attenuatus*[1] *Lepidiolamprologus elongatus*[1] *Neolamprologus caudopunctatus*[1,7] *Neolamprologus leleupi*[1]
3 (moderate)	polygynous maternal care substrate fertilization	*Altolamprologus calvus*[1] *Altolamprologus fasciatus*[1] *Julidochromis marlieri*[1,8] *Neolamprologus brichardi*[1,9] *Neolamprologus calliurus*[1] *Neolamprologus furcifer*[1] *Neolamprologus modestus*[1] *Neolamprologus mondabu*[1,10] *Neolamprologus savoryi*[1,11]
4 (high)	-polygynous with alternative reproductive tactics -maternal care -substrate fertilization	*Julidochromis ornatus*[1,12,13] *Lamprologus callipterus*[1,14,15] *Neolamprologus brevis*[1,16] *Telmatochromis temporalis*[1,17,18] *Telmatochromis vittatus*[1,18,19]
	-polygynandrous lekking -maternal care -buccal fertilization	*Cyathopharynx furcifer*[1,20] *Enantiopus melanogenys*[1] *Ophthalmotilapia ventralis*[1,21]

SD Table II. Results of simple and multiple regression analyses of the relationships between testes mass, sperm length, sperm swimming speed, sperm longevity and sperm competition rank while controlling for phylogeny (GLS). Phylogenetic dependence was assessed using the scaling paramater λ. Superscripts after the λ value represent significance levels of likelihood ratio tests when λ was compared to 0 (first position) and 1 (second position). Significance levels are denoted as ns = not significant, * = $p < 0.05$. Sperm competition ranked was assessed on the 4-point scale described in Table I. Sperm swimming speed (VCL) was measured at 8 time points following the commencement of sperm motility. A *t*-test was used to compare the observed slope against a slope of 0. Effect sizes, *r*, were calculated from *t* values and noncentral 95% confidence intervals (CI) are presented. The degrees of freedom, df, used to calculate *r* and CI are presented. CI that do not overlap zero represent significant relationships. P-values and CI of all significant relationships are indicated by bold text.

Trait		λ	Predictor	Estimate	*t*	p	*r*	df	CI
sperm speed	0.5	$1.0^{*,ns}$	testes mass	0.01	0.65	0.52	0.13	26	–0.25 - 0.46
time (min)			body mass	0.006	0.20	0.85	0.04	26	–0.33 - 0.39
	1	$1.0^{*,ns}$	testes mass	0.02	0.88	0.39	0.17	26	–0.21 - 0.49
			body mass	-0.02	-0.53	0.60	-0.10	26	–0.44 - 0.27
	2	$1.0^{*,ns}$	testes mass	0.04	1.85	0.08	0.34	26	–0.04 - 0.60
			body mass	-0.07	-1.97	0.06	-0.36	26	–0.62 - 0.02
	3	$1.0^{*,ns}$	testes mass	0.05	1.72	0.10	0.32	26	–0.06 - 0.59
			body mass	-0.08	-2.01	0.06	-0.37	26	–0.62 - 0.008
	4	$1.0^{*,ns}$	testes mass	0.02	0.66	0.52	0.13	26	–0.25 - 0.46
			body mass	-0.07	-1.37	0.18	-0.26	26	–0.55 - 0.12
	5	$<0.001^{ns,*}$	testes mass	0.03	0.56	0.58	0.11	26	–0.27 - 0.44
			body mass	-0.09	-1.29	0.21	-0.25	26	–0.54 - 0.14
	6	$<0.001^{ns,*}$	testes mass	0.06	1.52	0.14	0.29	26	–0.10 - 0.57
			body mass	-0.13	-2.21	**0.04**	-0.40	26	**–0.64 - –0.03**
	7	$<0.001^{ns,*}$	testes mass	-0.04	-0.97	0.34	-0.19	26	–0.50 - 0.20
			body mass	0.002	0.04	0.97	0.008	26	–0.35 - 0.37
sperm speed	0.5	$0.93^{*,ns}$	sperm length	0.29	2.53	**0.02**	0.44	27	**0.08 - 0.66**
time (min)	1	$1.0^{*,ns}$	sperm length	0.23	1.56	0.13	0.29	27	–0.09 - 0.56
	2	$1.0^{*,ns}$	sperm length	0.49	3.88	**0.0006**	0.60	27	**0.30 - 0.76**
	3	$1.0^{*,ns}$	sperm length	0.52	3.24	**0.003**	0.53	27	**0.20 - 0.72**
	4	$1.0^{*,ns}$	sperm length	0.57	2.89	**0.008**	0.49	27	**0.15 - 0.69**
	5	$<0.001^{ns,*}$	sperm length	0.44	2.01	0.06	0.36	27	–0.01 - 0.61
	6	$<0.001^{ns,*}$	sperm length	0.35	1.88	0.07	0.34	27	–0.03 - 0.60
	7	$<0.001^{ns,*}$	sperm length	-0.05	-0.22	0.83	-0.04	27	–0.39 - 0.32
sperm speed	0.5	$0.89^{*,ns}$	sperm comp. rank	0.04	2.10	**0.04**	0.37	27	**0.009 - 0.62**
time (min)	1	$1.0^{*,ns}$	sperm comp. rank	0.06	2.97	**<0.01**	0.50	27	**0.16 - 0.70**
	2	$1.0^{*,ns}$	sperm comp. rank	0.07	3.47	**<0.01**	0.56	27	**0.24 - 0.73**
	3	$1.0^{*,ns}$	sperm comp. rank	0.08	3.47	**<0.01**	0.56	27	**0.24 - 0.73**
	4	$1.0^{*,ns}$	sperm comp. rank	0.07	2.27	**0.03**	0.40	27	**0.04 - 0.64**
	5	$<0.001^{ns,*}$	sperm comp. rank	0.07	1.79	0.08	0.33	27	–0.05 - 0.59
	6	$<0.001^{ns,*}$	sperm comp. rank	0.07	2.18	**0.04**	0.39	27	**0.02 - 0.63**
	7	$<0.001^{ns,*}$	sperm comp. rank	-0.005	-0.10	0.92	-0.02	27	–0.37 - 0.34
sperm speed	0.5	$0.97^{*,ns}$	sperm longevity	0.06	0.71	0.48	0.14	27	–0.24 - 0.46
time (min)	1	$1.0^{*,ns}$	sperm longevity	0.22	2.27	**0.03**	0.40	27	**0.04 - 0.64**
	2	$1.0^{*,ns}$	sperm longevity	0.24	2.48	**0.02**	0.43	27	**0.08 - 0.66**
	3	$1.0^{*,ns}$	sperm longevity	0.38	3.64	**<0.01**	0.57	27	**0.26 - 0.74**
	4	$0.85^{*,ns}$	sperm longevity	0.52	4.17	**<0.001**	0.63	27	**0.34 - 0.78**
	5	$0.10^{ns,*}$	sperm longevity	0.67	3.92	**<0.001**	0.60	27	**0.31 - 0.76**
	6	$<0.001^{ns,*}$	sperm longevity	0.41	2.54	**0.02**	0.44	27	**0.09 - 0.66**
	7	$<0.001^{ns,*}$	sperm longevity	0.36	1.72	0.10	0.31	27	–0.06 - 0.58
sperm length		$0.63^{*,ns}$	sperm longevity	0.07	0.52	0.61	0.10	27	–0.27 - 0.43

SD Table III. Repeated measures ANOVAs examining the effects of time since activation and sperm length on swimming speeds (VCL). Sperm swimming speed decreased over time in every species examined. In 27 of the 29 species examined there was no relationship between sperm swimming speed and sperm tail length. The two exceptions were *L. attenuatus* and *N. brevis* in which positive relationships between sperm length and swimming speed were observed. The sample of males analyzed per species (n), and collection location, are indicated.

			Sperm Swimming Speed (VCL)			
			Effect of Time		**Effect of Sperm Length**	
Species	**n**	**Collection Location**	**F**	**P**	**F**	**p**
Altolamprologus calvus	5	Kapemba	17.87	<0.0001	1.35	0.26
Altolamprologus fasciatus	8	Wonzy Point Kasakalwe Point	33.31	<0.0001	0.34	0.56
Asprotilapia leptura	4	Wonzy Point	25.70	<0.0001	0.0003	0.99
Chalinochromis brichardi	9	Katoto	9.51	<0.0001	0.007	0.93
Cyathopharynx furcifer	8	Kasakalwe Lodge	19.83	<0.0001	0.03	0.87
Enantiopus melanogenys	7	Mpulungu	22.33	<0.0001	2.88	0.10
Eretmotus cyanostictus	7	Kasakalwe Point	11.23	0.0002	0.39	0.54
Julidochoromis ornatus	7	Kasakalwe Point	17.66	<0.0001	1.42	0.26
Julidochromis marlieri	9	Katoto	28.21	<0.0001	0.55	0.46
Lamprologus callipterus	11	Wonzy Point	35.55	<0.0001	1.48	0.23
Lepediolamprologus attenuatus	11	Kasakalwe Point	19.33	<0.0001	5.36	**0.03**
Lepediolamprologus elongatus	2	Kasakalwe Point	70.91	0.003	0.53	0.52
Microdontochromis tenuidentatus	7	Mpulungu	10.44	0.0002	1.37	0.26
Neolamprologus brevis	8	Wonzy Point	56.83	<0.0001	10.97	**0.002**
Neolamprologus brichardi	10		25.86	<0.0001	0.01	0.92
Neolamprologus calliurus	10	Kapemba	38.16	<0.0001	0.11	0.74
Neolamprologus caudopunctatus	9	Kasakalwe Point	16.46	<0.0001	0.23	0.63
Neolamprologus furcifer	4	Wonzy Point	18.39	<0.0001	1.78	0.20
Neolamprologus lelupi	12	Mbete Island	17.12	<0.0001	0.29	0.59
Neolamprologus modestus	9	Kasakalwe Point	23.71	<0.0001	0.55	0.46
Neolamprologus mondabu	5	Kasakalwe Point	45.58	<0.0001	0.83	0.37
Neolamprologus savoryi	5	Kasakalwe Point	33.26	0.002	1.65	0.27
Opthalmotilapia ventralis	13	Kasakalwe Point	44.51	<0.0001	0.0000	1.0
Perrisodus microlepis	5	Kasakalwe Point	4.06	0.01	0.72	0.41
Telmatochromis temporalis	10	Kasakalwe Point	20.64	<0.0001	0.67	0.42
Telmatochromis vittatus	37	Wonzy Point Kasakalwe Point	79.45	<0.0001	1.36	0.24
Tropheus moori		Mbete Island	13.63	0.0002	0.11	0.74
Xenotilapia flavipinnis	7	Kasakalwe Point	22.68	<0.0001	0.07	0.80
Xenotilapia spilopterus	6	Kasakalwe Point	9.93	<0.0001	0.64	0.43

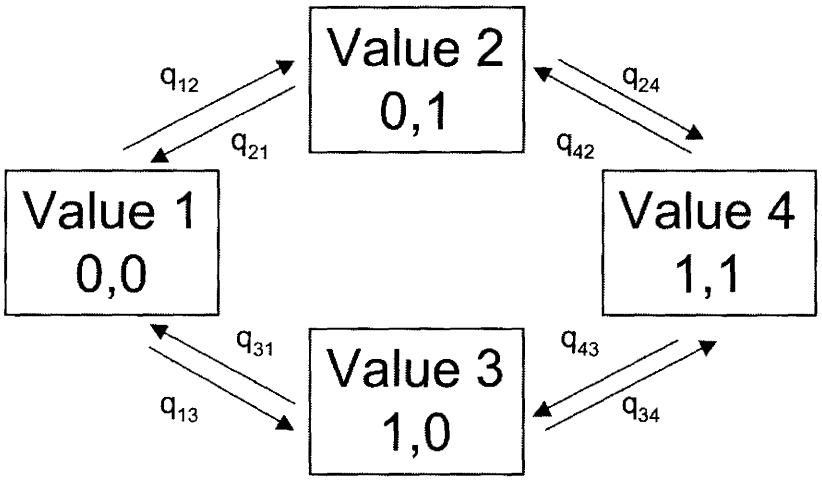
SD Table IV. Genbank Accession Numbers for the sequences included in the phylogenetic analysis. *B. microlepis* and *B. fasciatus* were set as outgroups and were not included in any analyses.

Species	ND2	CytB	Control Region
Boulengerochromis microlepis	AF317229	AF370632	AY929939
Bathybates fasciatus	AY663733	AY663756	
Altolamprologus calvus	EF462256	Z29989	EF462318
Altolamprologus fasciatus	EF462255		EF462317
Asprotilapia leptura	AY337772	Z21758	Z21732
Chalinochromis brichardi	EF462232	Z29991	EF462294
Cyathopharynx furcifer	AY337781	AY337828	AY338981
Enantiopus melanogenys	AY337770	AY337798	AY339022
Eretmotus cyanostictus	AF398220	AF428155	AF400707
Julidochoromis ornatus	EF462229		EF462291
Julidochromis marlieri	EF462227	Z30077	EF462289
Lamprologus callipterus	EF462258	Z29992	EF462320
Lepediolamprologus attenuatus	DQ055037	AB280684	DQ054938
Lepediolamprologus elongatus	EF462268	AB280685	EF462330
Microdontochromis tenuidentatus	AY337784	AY337835	AY339019
Neolamprologus brevis	EF462263		EF462325
Neolamprologus brichardi	DQ055015	AF438804	DQ054917
Neolamprologus calliurus	EF191096		
Neolamprologus caudopunctatus	EF462272		EF462334
Neolamprologus furcifer	EF462249	Z29999	EF462311
Neolamprologus lelupi	EF462251	Z30000	EF462313
Neolamprologus modestus	DQ055012		DQ054914
Neolamprologus mondabu	EF462241		EF462303
Neolamprologus savoryi	EF462247	AF438799	EF462309
Opthalmotilapia ventralis	AY337774	AY337805	AY338993
Perrisodus microlepis	DQ055006	AF428167	DQ054907
Telmatochromis temporalis	EF462234		EF462296
Telmatochromis vittatus	EF462237	Z30003	EF462299
Tropheus moorri	AY930091	Z12035	AY930020
Xenotilapia flavipinnis	AY337794	AY337849	AY339034
Xenotilapia spilopterus	AY337788	AY337841	AY339043

SD V.

Testing the directionality of evolutionary transitions

BayesDiscrete uses discrete binary data to assess all possible transitions between two traits (0 and 1). Traits assigned as binary variables create four possible values (one value per species) that can be described as [trait 1,trait 2] as follows: [0,0], [0,1], [1,0], [1,1]. BayesDiscrete allows forward and reverse transitions between these 4 values. Therefore, there are 8 possible transitions between values: 4 forward transitions and 4 reverse transitions. Each transition parameter q is assigned a unique numerical code describing the direction of change between two states. For example, q_{12} represents a transition from value 1 to value 2 and q_{21} represents the reverse transition from state 2 to state 1. BayesDiscrete assumes that transitions involving simultaneous change in two traits do not occur (i.e. transitions from [0,0] to [1,1] or from [0,1] to [1,0]). The 4 trait values and 8 transition parameters are illustrated as:

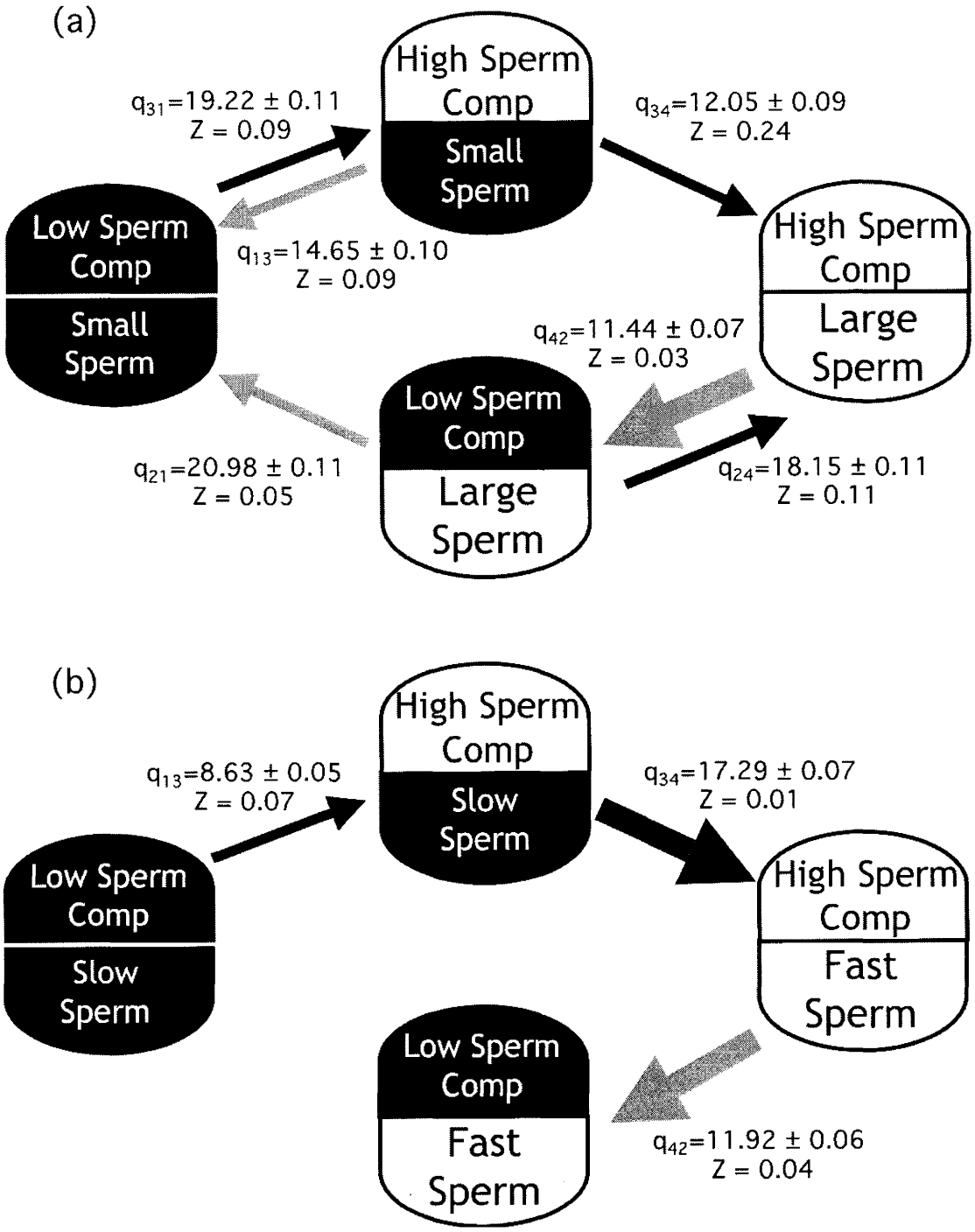


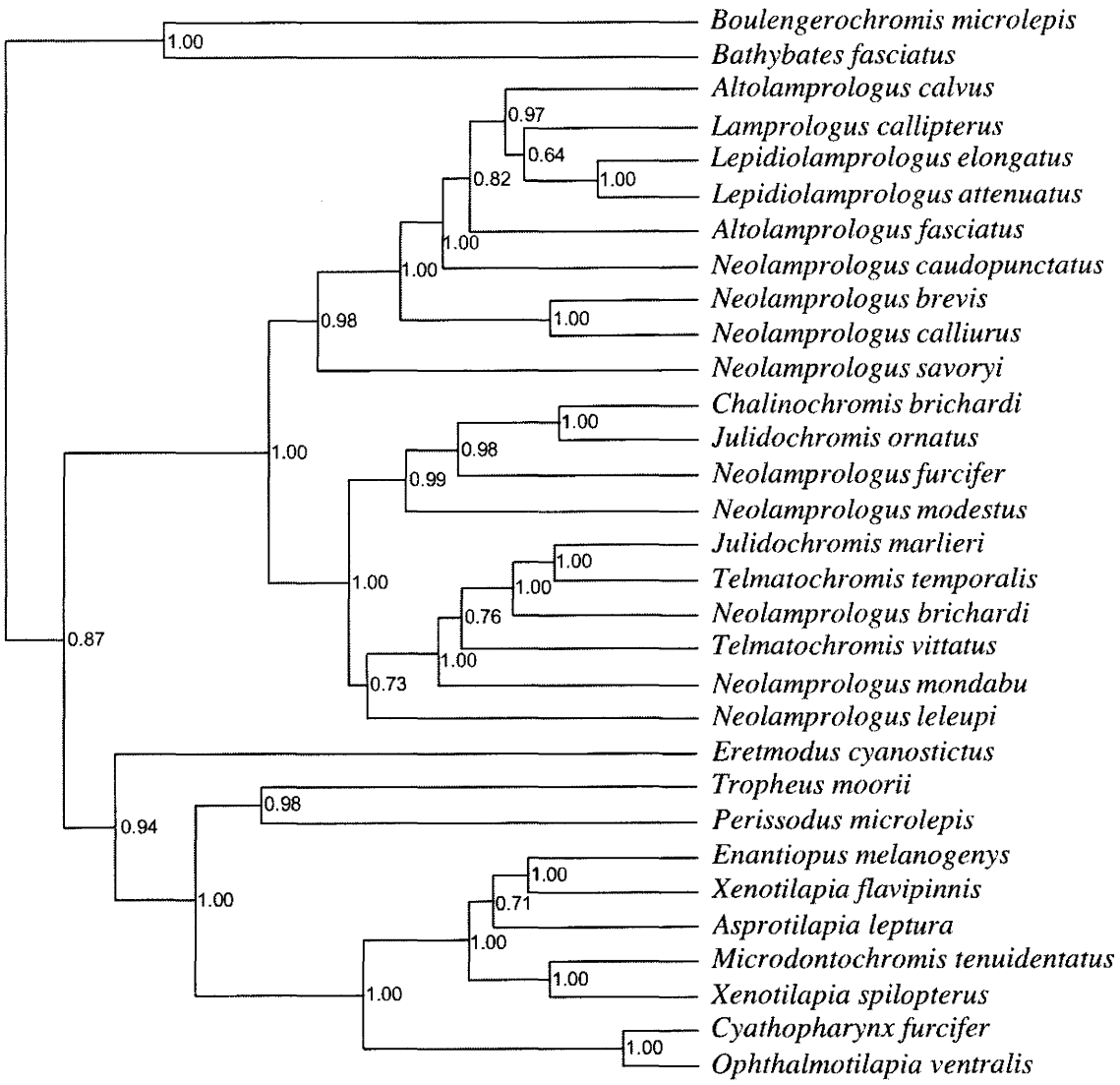
Evolutionary pathways from ancestral to derived values can be determined by assessing the probability of a certain transition parameter having occurred relative to another. For

example, if value 1 was the ancestral trait and value 4 was the derived state than the evolutionary transition from the ancestral to the derived state could have occurred in one of three ways: 1) value 1 leads to value 2 then to value 4, 2) value 1 leads to value 3 then to value 4, 3) value 1 is equally likely to lead to value 2 and 3, which in turn lead to value 4. We can assess these possible evolutionary transitions by examining the transition parameters leading away from value 1. For example, the evolutionary pathway of Value 1 to Value 2 to Value 4, indicates that changes in trait 2 preceded changes in trait 1 because the value 1 traits of [0,0] shifted to value 2 traits of [0,1]. Applying this logic we can assess the other possible evolutionary transitions described in our example above. We assessed the frequency that transition parameters where set equal to zero using a reversible-jump Markov Chain Monte Carlo method which explores the entire parameter space. Transition parameters less likely to have occurred are frequently assigned to zero while those that are likely are assigned to non-zero values. The strength of each transition parameter was also assessed using the average value of each transition parameter.

SD Figure 1: Transition diagrams illustrating the evolutionary pathways from the ancestral to the derived state in (a) sperm size and (b) sperm swimming speed. Sperm swimming speed was measured at 0.5 min post-activation. Intermediate states are represented in the middle of each transition diagram. Forward transitions are depicted with black arrows and back transitions with grey arrows. Non-significant transitions were removed from the diagram. The thickest arrow represents highly likely transition. For each transition we reported a q value that represents the mean ±SE transition parameter as well as a Z value which represents the proportion of iterations that were assigned to zero, or unlikely transitions; q values further away from zero represent more probable evolutionary transitions and low Z values indicate more likely evolutionary transition. Transitions were considered likely when $Z < 0.10$, meaning that less than 10% of the iterations from the Markov chain are assigned to the zero bin (Pagel and Meade 2006), or when the non-significant transition parameter (q value) was higher than that of the lowest parameter that yielded a significant transition.

SD Figure 1.


(a)
High Sperm Comp
Small Sperm
Low Sperm Comp
Small Sperm
High Sperm Comp
Large Sperm
Low Sperm Comp
Large Sperm
q_{31}=19.22 ± 0.11
Z = 0.09
q_{34}=12.05 ± 0.09
Z = 0.24
q_{13}=14.65 ± 0.10
Z = 0.09
q_{42}=11.44 ± 0.07
Z = 0.03
q_{21}=20.98 ± 0.11
Z = 0.05
q_{24}=18.15 ± 0.11
Z = 0.11
(b)
High Sperm Comp
Slow Sperm
Low Sperm Comp
Slow Sperm
High Sperm Comp
Fast Sperm
Low Sperm Comp
Fast Sperm
q_{13}=8.63 ± 0.05
Z = 0.07
q_{34}=17.29 ± 0.07
Z = 0.01
q_{42}=11.92 ± 0.06
Z = 0.04



SD Figure 2. Bayesian consensus tree of 31 species of Tanganyikan cichlids based on ND2, cytB and a mitochondrial control region, with branch lengths proportional to the number of amino acid substitutions. Numbers indicate Bayesian posterior probabilities. *B. microlepis* and *B. fasciatus* were set as outgroups.

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CHAPTER 6

HOW SPERM COMPETITION SHAPES SPERM TRAITS IN FISHES

6.1 THESIS SUMMARY

Throughout this thesis I have critically addressed outstanding issues in the sperm competition literature using a combination of intra- and inter-specific studies in fishes. The results of these studies have shed light on our understanding of how the selective force of sperm competition influences sperm traits in fishes. Moreover, I have provided some of the first empirical validations of predictions regarding sperm size and speed from sperm competition theory. Specifically, my research has provided i) the first phylogenetically controlled empirical demonstration of the positive relationship between sperm size and speed (**Chapter 5**), ii) the second (**Chapter 4**) and fourth (**Chapter 3**) demonstration that sneaker males have faster swimming sperm than conventional males, and iii) a useful paradigm to exploring how female and sneaker mating behaviour influences the selective pressures facing males (**Chapter 3**), while iv) highlighting the importance of incorporating sperm energetics into future studies of ejaculate trait evolution (**Chapter 2**). Furthermore, I have focused on species that were not traditional model systems in sperm competition, thereby expanding our understanding of how sperm compeitoin shapes sperm traits in fish. The results of this thesis, together with several

recent studies (**see Chapter 2**), form the foundation for describing a general synthesis of how selection acts on sperm traits in fishes.

6.2 APPROXIMATING THE STRENGTH OF SPERM COMPETITION

Many researchers use testes mass, controlling for body mass, as an index of sperm competition because, they argue, selection should act on increasing sperm number in the face of stronger competition between ejaculates from rival males (Parker et al. 1997). However, as pointed out in **Chapter 2**, many other factors can influence testes size that has little to do with sperm competition. Therefore, the use of testes size as an index of sperm competition may not always capture the true magnitude of sperm competition (Emerson 1997). The problem with using relative testes size as an index of sperm competition was apparent in **Chapter 5**. In the Tanganyikan cichlids studied in this chapter the behavioural index of sperm competition revealed that, in support of theoretical predictions, males experiencing greater levels of sperm competition had larger testes, and longer and faster sperm. In contrast, while relative testes mass was positively related to sperm length there were no evident relationships between testes mass and sperm performance. This suggests that some of the variance in testes mass may be explained by sperm competition but the use of testes mass alone is not the best proxy of the strength of sperm competition. Consequently, these results suggest that caution should be applied when using relative testes mass as an index of sperm competition and whenever possible additional ecological data should be incorporated into analyses.

6.3 PRODUCTION OF LONGER AND FASTER SPERM

Prior to my thesis research there was a level of uncertainty with respect to how sperm competition has shaped ejaculate traits across and within species. To date, responses in sperm traits have been notoriously variable, differing widely between studies (Snook 2005). In a recent study, Immler and Birkhead (2006) highlighted the importance of taxon-specific responses to sperm competition and cautioned against the use of large phylogenies when examining sperm trait evolution. Indeed, careful assessment of responses in sperm size to increasing levels of sperm competition across fishes reinforced the importance of considering how species-specific effects may influence results, as a single outlier obscured the positive relationship between sperm size and the strength of sperm competition in fishes (**Chapter 2**). Furthermore, as I argued throughout this thesis, species with male alternative reproductive tactics (MARTs) offer a powerful way to test predictions from sperm competition theory at an intra-specific level. However, these studies too have yielded some ambiguous results. Therefore, throughout this thesis I have focused on examining closely related species and males within a species that experience dramatically different levels of sperm competition in order to clarify the influence of sperm competition on the evolution of sperm size and speed in fish.

The results of this thesis, together with recent studies in a wide range of taxa (Birkhead and Møller 1998; Simmons 2001), demonstrate that sperm competition simultaneously promotes the evolution of many ejaculate traits without apparent trade-offs. However, I argue that selection acts to increase sperm competitiveness in a different manner across and within species. In fishes, comparative studies show that species that were more likely to experience sperm competition have larger testes (**Chapters 2, 5**;

Stockley et al. 1997; Balshine et al. 2001), longer sperm (**Chapters 2, 5**; Balshine et al. 2001) and faster swimming sperm (**Chapter 5**). Thus, empirical comparative studies in fishes have validated theoretical predictions (Parker 1998) and provide strong evidence that sperm competition increases the competitive ability of an ejaculate. Similarly, within species examinations demonstrate that a seemingly universal response to sperm competition is an increase in sperm number (**Chapter 2**). Supporting theoretical predictions, in three fish species with MARTs, including one examined in this thesis (plainfin midshipman, *Porichthys notatus*: **Chapter 3),** males performing sneak fertilizations have faster swimming sperm than conventional males (bluegill, *Lepomis macrochirus*: Burness et al. 2004; black goby, *Gobius niger*: Locatello et al. 2007). In the other species with MARTs examined in this thesis, *Telmatochromis vittatus* (**Chapter 4),** pirate males who experience the lowest levels of sperm comeptiton appeared to respond to relaxed levels of sperm competition by reducing sperm swimming speeds rather than sneaker males having faster swimming sperm. However, responses in sperm swimming speed have not been unanimous, as a relationship between sperm size and speed was not found in two species (grass goby, *Zosterisessor ophiocephalus*: Locatello et al. 2007; bluegill: Stoltz and Neff 2006; Leach and Montgomerie 2000; Burness et al. 2005).

Our understanding of how sperm size is influenced by sperm competition within species has progressed slowly. With the exception of bluegill (Burness et al. 2004) and dung beetles (*Onthophagus binodis*, Simmons et al. 1999) where sperm from sneakers is longer, to the best of my knowledge in all other species studied to date (**Chapter 2**), including those examined in this thesis (**Chapter 3, 4**), sperm size remained unaffected

by the strength of sperm competition. Thus post-copulatory competition acts to increase sperm numbers and generally increases sperm speed, but its impact on sperm size varies depending on whether one is making a within or an across species assessment. But to understand why there is a discrepancy in responses in sperm size in intra- and interspecific studies we must first assess the relationship between sperm size and speed.

Do longer sperm swim faster? The answer to this question is not straightforward. Intuitively one might expect that longer sperm swim faster as they are able to provide greater propulsive force to move sperm forward. Theoreticians shared this assumption leading to the prediction that sperm competition will promote the evolution of longer, and therefore faster, sperm (Katz and Drobnis 1990; Ball and Parker 1996). In the only phylogenetically controlled study that addresses this question to date, longer sperm did indeed swim faster than smaller sperm (**Chapter 5**). Yet analyses within species have failed to provide widespread evidence that sperm swimming speed is related to sperm size (**Chapters 3, 4, 5**). In fact, there has yet to be a within-species demonstration of a relationship between sperm size and speed in any species with flagellate sperm (**Chapter 2**). This raises two questions:

1) *Why is there a relationship between sperm size and speed across species but not within species?*

Variance in the degree of sperm competition is greater between than within species, with males in species characterized by monogamy versus polyandry experiencing vastly different levels of sperm competition. For example, in the comparative study presented in **Chapter 5** I contrasted sperm traits from species like

Eretmotus cyanostictus, which are characterized by strict social and genetic monogamy (Morley and Balshine 2002; Taylor et al. 2003) and therefore do not experience sperm competition, against species like *Telmatochromis vittatus*, which are characterized by intense post-copulatory competition with four different males tactics competing for access to females (**Chapter 4**). Therefore, the much greater scope in the degree of sperm competition observed across species may explain why the relationship between sperm size and speed is more apparent when examining many species rather than morphs within a species.

In contrast, when examining sperm size within species with MARTs, where postcopulatory competition is intense, there may be little variation in sperm size between conventional and sneaker males. Indeed, several recent studies demonstrate that the degree of variance in sperm size is reduced in species that experience high levels of sperm competition (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008). Thus if there is little variance in sperm size in species with MARTs then relations between sperm size and speed may be absent or difficult to detect.

Alternatively, our method of assessing sperm size and swimming speed may not be accurate enough to detect subtle within-species relations between these variables. Throughout this thesis, and indeed throughout the literature (e.g. Gage et al. 2002; Leach and Montgomerie 2000; Burness et al. 2004; 2005; Locatello et al. 2007), *average* sperm swimming speed is assessed against *average* sperm size. By using average measures for each male any variability within a male's ejaculate is essentially obscured. In future studies researchers should determine the relationship between sperm size and speed from individual spermatozoon. Several spermatozoa should be measured per male, and several

males from within a species should be assessed. Then the relationship between sperm size and speed should be assessed with data from each individual spermatozoon nested within male identity in a multiple regression. Addressing this overlooked methodological issue will aid in clarifying the relationship between sperm size and speed.

2) *What explains the increased sperm swimming speed observed in sneaker males in species with MARTs?*

Throughout this thesis I have argued that selection acts on sperm energetics in species with MARTs. Males may therefore achieve faster swimming sperm simply by increasing ATP production (Burness et al. 2005), thereby avoiding the metabolically costly production of larger sperm (LaMuyon and Ward 1998). Two lines of evidence support this view. First, the observation that in species with MARTs sneaker males typically have ejaculates with greater ATP content compared to conventional males (Vladic and Järvi 2001; Burness et al. 2004; 2005; Locatello et al. 2007; **Chapter 2**) suggests that increases in energetic content may be sufficient to explain the faster swimming sperm typically observed in sneakers. Both midshipman and *T. vittatus* sneakers had sperm that swam faster than sperm from conventional males but did not differ in flagella length. This too suggests differential energy production rates in sperm from sneakers. Furthermore, in midshipman, sneakers had larger sperm midpieces (**Chapter 3**). As midpiece size is related to energy content (Vladic et al. 2002) this suggest that sperm from midshipman sneakers has greater energy stores. Second, a directional test of trait evolution suggested that selection initially increases sperm swimming speed in the face of more intense sperm competition and acts on sperm size

secondarily (**Chapter 5**). The most plausible way to increase sperm swimming speed without altering flagellum length is to increase energy content in sperm.

6.4 FEMALE CHOICE AND SNEAKING BEHAVIOURS: A NEW PERSPECTIVE

Research in this thesis also suggests that in species with MARTs the strength of sperm competition between male tactics is not as dichotomous as once thought. Typically, sneaker males are expected to experience more intense levels of sperm competition than conventional males. However, this traditional view does not incorporate the effect of female choice on sneaker behaviour. Henson and Warner (1997) recognized that female choice for high quality males would reduce the reproductive success of lower quality males and promote the evolution of alternative reproductive tactics. Other authors argued that males attempting sneak fertilizations would target high quality males, as this will maximize their chance of encountering a female (Waltz 1982). However, the implications of how female choice and sneaker preference for high quality males might influence sperm traits in preferred males had not been addressed. I argue that a combination of female choice for high quality males and responses in sneaker males can promote differential intensities of sperm competition within a reproductive tactic, with high quality males experiencing relatively high level of sperm competition compared to low quality males. In **Chapter 3** I examined this hypothesis and found that large midshipman Type I males, who were preferred by females and preferentially targeted by sneaking males, had sperm that swam as fast as sneaking Type II males. The conceptual framework outlines in **Chapter 3** describing the interactions between conventional

males, sneakers and females applies not only to species with MARTs but to any species where male quality predicts reproductive parasitism.

6.5 FUTURE DIRECTIONS

Throughout this thesis I have advocated for increased focus on sperm energetics and how sperm competition impacts the fuels and engines powering sperm motility. A powerful way to test the importance of sperm energetics in competitive matings would be to create selection lines that faced different degrees of sperm competition, as has been done with insects (Hosken et al. 2001; Pitnick et al. 2001). A critical test of my hypothesis that sperm energetics are at the forefront of responses to sperm competition would be to assess if sperm from males in monogamous lines had lower ATP content than those from polyandrous lines.

In my doctoral research I focused mainly on testes and sperm. Future research should extend this view and examine the non-sperm components of the ejaculate. There is extensive evidence from invertebrates, particularly in *Drosophila*, that accessory gland proteins play a major role in determining paternity in competitive matings (Gillot 2003; Chapman and Davies 2004; Garcia-Gonzalez and Simmons 2005). However, to date few studies have examined how accessory glands are influenced by sperm competition in fish. One notable exception is a recent study demonstrating an association between the degree of polyandry and accessory gland size among 12 goby species (Mazzoldi et al. 2005). Species with MARTs will likely play a major role in understanding whether sperm competition favours the evolution of accessory glands in fishes. For example, in midshipman sneaker males have relatively larger accessory glands than conventional

males (**Chapter 3**) while in other species conventional males invest more in accessory glands (e.g black goby, *Gobius niger*, Rasotto and Mazzoldi 2002). Examining interspecific differences in accessory gland size in species with MARTs while assessing the role of sperm competition in promoting the evolution of accessory glands will provide important insights into the evolution of non-sperm components of an ejaculate.

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