2009

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Recommended Citation

Beausoleil, Jean-Marc, "Mate choice and sperm competition in the redside dace (Clinostomus elongatus)" (2009). *Electronic Theses and Dissertations*. 64.
https://scholar.uwindsor.ca/etd/64

Mate choice and sperm competition in the redside dace (*Clinostomus elongatus*)

By

JEAN-MARC JOSEPH BEAUSOLEIL

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

Windsor, Ontario, Canada

2009

Mate choice and sperm competition in the redside dace (*Clinostomus elongatus*)

by

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: My first data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Stéphanie Doucet. My second data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Daniel Heath. In each case, my collaborators provided valuable feedback, helped with the project design and statistical analysis, and provided editorial input during the writing of each manuscript; however, in both cases the primary contributions have all been by the author. Both Chapter 2 and Chapter 3 have been prepared as manuscripts, and will be submitted to Animal Behaviour for publication.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from my co-authors to include the above materials in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work, completed during my registration as a graduate student at the University of Windsor.

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada

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ABSTRACT

Female choice and sperm competition can be important determinants of male reproductive success. I investigated which ornamental traits females preferred, a potential direct benefit of female choice, and which sperm traits were related to competitive fertilization success, in the redside dace (*Clinostomus elongatus*). I identified male ornaments, female preference for these ornaments, and the relationships between ornaments and sperm traits to determine if females might derive fertility benefits by choosing ornamented males. My findings indicate that females prefer males with more breeding colouration with higher hue values and that those males tended to produce large quantities of high velocity sperm. I also studied sperm competition mechanisms using in-vitro fertilization and found that sperm velocity was the primary determinant of competitive fertilization success. These studies are the first on mate choice and sperm competition in this species, and contribute to our understanding of sexual selection in general.

ACKNOWLEDGMENTS

First and foremost, I need to thank my research advisor, Dr. Trevor Pitcher, for his tireless help and support throughout the entirety of my Master’s. Thank you for being a great mentor and teacher, and always finding the time to help me with my research, both in the lab and in the field, and inviting me to expand my horizons a bit with side-projects. Thank you to my graduate committee members, Dr. Aaron Fisk, Dr. Dennis Higgs and Dr. Chris Wilson, for all their helpful comments and suggestions regarding my research. A big thank you as well to my collaborators, Dr. Stéphanie Doucet and Dr. Daniel Heath, for lending me their advice and expertise, as well as their equipment and personnel, for my two data chapters.

A very special thank you goes to Justin Abbott, my assistant for both field seasons in Ohio, who helped with the myriad of often strenuous tasks that had to be accomplished between dawn and dusk while helping to keep me sane, as well as for all his help taking morphology pictures after we returned. Also for help with my field research, I thank Sara Davidson for coming with us for my second field season. Thank you to Jackie Vandereerden for extracting the first set of DNA from my tissue samples and to Russell Hepburn for subsequent extractions. Thank you to Courtney Beneteau for endless patience and good humour while teaching me how to do genetics properly, as well as to all the other staff and students at GLIER who pointed me in the right direction when I needed it. Thank you to the entire staff at the Brown Family Environmental Center at Kenyon College for making our stay pleasant and providing logistical support. Thank you to Brian Zimmerman for sharing his extensive knowledge of where and how to catch redside dace in Ohio.

Thank you to all of my lab mates and colleagues, past and present, for making my time here educational and enjoyable: Karen Elgee, Michelle Farwell, Sara Davidson, Erin Flannery, Katie Millette, Lex Hand, Margarette Yousef, Caroline Dennis, Justin Abbott, Jackie Vandereerden, Mohsin Khan, Jennifer Smith, Adhan Khan, Emily Carey, Omar Davis and Dane Macri. Thanks for all the comments, feedback, discussion, and lighter moments over the years. Special thanks to Daniel Hanley and again to Stéphanie Doucet, Justin Abbott and Jackie Vandereerden, co-authors with myself on manuscripts published from side-projects with my advisor, Trevor Pitcher. Big thank yous to Nancy Barkley and Usha Jacob for all their help in navigating the bureaucracy of graduate school and graduation.

This work was supported by the University of Windsor and by the Natural Sciences and Engineering Research Council of Canada in the form of Discovery Grants and Research Tools and Instruments grants to T. Pitcher. This work was also supported by the Government of Ontario in the form of an Ontario Graduate Scholarship in Science and Technology awarded to the author.

Heartfelt thanks go to my parents, Marc and Anne-Marie, for twenty four years of patience, encouragement and love. Special thanks also go to my siblings, Brianne, Christopher, Danielle and Matthew, for all their support and love. Thank you as well to all my great friends for their encouragement and love.

Most of all I’d like to thank my girlfriend and my sunshine Jen Low for all of her love, encouragement, support, and patience. Pursuing my Master’s Degree was well worth it just to meet you. I love you and I can’t wait to start the next stage of our life together in London.

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CHAPTER 1: GENERAL INTRODUCTION

Sexual Selection

One of the biggest problems facing Darwin's (1859) theory of natural selection when it was first formulated was the existence of highly elaborated secondary sexual characters in nature. These elaborate traits, which are generally associated with males, include the bright colouration of Trinidadian guppies (*Poecilia reticulata*), the conspicuous call of tungara frogs (*Physalaemus pustulosus*), the excessively large tail feathers of peacocks (*Pavo cristatus*) and the antlers of red deer (*Cervus elaphus*). These types of traits could not be explained by the theory of natural selection because many of them actually appeared to reduce the survival of the bearer; therefore their evolution should be opposed by natural selection. Darwin finally provided an explanation for the existence of such traits with his controversial theory of sexual selection (Darwin 1871), which states that competition between males for access to females and female preference for elaborate traits can also drive evolution by increasing the number of offspring an individual produces. Darwin reasoned that elaborate ornaments such as vivid colouration or exaggerated plumage could be used by females as information to use in choosing mates (intersexual selection), and that weapons such as antlers could be used in competitions between males (intrasexual selection) for access to females for mating. The benefits a male can derive from having elaborate secondary sexual traits are clear; the increase in fitness from increased mating success (i.e. more or higher quality mates) must outweigh the survival costs of the ornament for the elaborate trait to have evolved (Andersson 1994). However, choice also entails costs to the female; therefore, for female choice to be adaptive, the female must also be deriving some benefit from being choosy.

Direct Benefits of Mate Choice

The best documented and least controversial of the models for adaptive female choice are found in resource based mating systems, where males provide energetic resources either to a female or to their offspring. These resources can take many forms, such as food, shelter, parental care (e.g. incubating eggs) or protection from predators (reviewed in Møller & Jennions 2001). If male secondary sexual traits are found to be related to the quantity or quality of resources provided, thereby increasing female fecundity or the survivorship of the brood, then a straightforward answer is provided as to the benefits the female is deriving from mate choice. Evolution of the male trait is then driven by the female preference for the most ornamented males (reviewed in Møller & Jennions 2001).

A commonly overlooked direct benefit that females could derive from mate choice is sperm. Sperm is considered a direct benefit because if a male cannot provide enough sperm or if a male's sperm have low fertilization ability, not all ova are fertilized and female fecundity drops (reviewed in Jennions & Petrie 2000). Sheldon's (1994) phenotype linked fertility hypothesis provides a possible explanation of female benefits of mate choice in species where variation exists in sperm quality among males, resulting in variation in the number of ova that can be successfully fertilized. The phenotype linked fertility hypothesis states that females may be choosing more ornamented males because the elaboration of the ornament correlates with sperm quantity or quality. Several mechanisms have been hypothesized to explain this linkage between secondary sexual and sperm traits. For example, the linkage of ornaments and sperm traits could be explained within the handicap principle, with the linkage arising from the condition dependence of both traits (e.g. Birkhead et al. 1998). Secondary sexual traits and sperm traits could also be linked by the effects of testosterone on each (e.g. Pizzari et al. 2004). Carotenoid pigments that are used in colourful ornaments have also been linked to sperm quality through their antioxidant properties, which shield sperm from oxidative damage (e.g. Peters et al. 2004). Because males with higher sperm quality can fertilize more of the female's ova, the female derives direct fitness benefits through higher reproductive success. Some support for the phenotype linked fertility hypothesis has been found in a variety of taxa (e.g. Roldan et al. 1988; McGary et al. 2002; Masvaer et al. 2004; Peters et al. 2004; Rogers et al. 2008; however see Birkhead et al. 1998; Pizzari et al. 2004; Pilastro et al. 2008).

Indirect Benefits of Mate Choice

In many non-resource based mating systems sperm is not a limiting factor in female fecundity, and yet females are still choosy with regards to potential mates. For example, male Trinidadian guppies display their elaborate colouration in an effort to attract females to mate with them, but provide nothing but sperm to the female at any point (Houde 1997). Extreme examples of female choice in non-resource based mating systems can also be found in lekking species, where males will gather and display their ornaments in fixed courtship territories. Females visiting the lek will generally all choose to mate with the same highly ornamented males (Höglund & Alatalo 1995), but again this male only provides sperm to the females. The term "lek paradox" was coined because it was difficult to understand what had led to the evolution and maintenance of such strong female choice in the absence of any obvious direct benefits (reviewed in Kirkpatick & Ryan 1991). Theory has indicated that in cases such as this, females must be deriving indirect genetic benefits that will increase the survivorship or reproductive success of their offspring in order for mate choice to be adaptive (reviewed in Kokko et al. 2003; Mead & Arnold 2004). I will now examine some of the hypotheses that have been suggested as possible explanations of female choice for elaborate ornamentation where males do not provide direct benefits.

Good Genes Hypothesis

The "good genes" hypothesis is based on the idea that certain alleles (e.g. conferring parasite resistance or metabolic efficiency) will be beneficial regardless of any interactions with the rest of the genome. These alleles are in theory correlated with the elaboration of male secondary sexual traits (indicator traits), and therefore offspring sired by these males will inherit the beneficial alleles and have higher reproductive success or survivorship than offspring of males lacking these alleles (reviewed in Andersson 1994; Neff & Pitcher 2005). The good genes hypothesis was controversial when it was first proposed because theory suggested that strong directional selection on the beneficial alleles and the indicator trait would remove any genetic variation in these traits (reviewed in Charlesworth 1987; Kirkpatrick & Ryan 1991). However, empirical work on non-resource based mating systems has shown that ornaments subjected to mate choice do indeed correlate with survivorship, supporting their use as indicator traits (Jennions et al. 2001), and that benefits of mate choice to offspring survivorship do exist, although these benefits are small (Møller and Alatalo 1999; but see Kirkpatrick & Barton 1997). For example, Sheldon et al. (2003) showed in moorfrogs (*Rana arvalis*) that tadpoles sired by males with brighter blue breeding colouration had a greater chance of escape when experimentally challenged with a predator (also see Petrie 1994; Barber et al. 2001;

Sheldon et al. 2003; Forsman & Hagman 2006; Wedekind et al. 2008a; Wedekind et al. 2008b).

Runaway Selection Hypothesis

The central component of Fisher's (1930) "runaway sexual selection" hypothesis is the possibility of a genetic correlation existing between a male trait and the female preference for that trait. In such a case, the preference could become self-reinforcing and eventually result in highly exaggerated male traits as the males considered most attractive and the females who prefer them produce offspring that continue the cycle. Fisher (1930) proposed that the initial preference would be for some unexaggerated male trait that correlated to some degree with male fitness, accounting for the early spread of the genes; however, as runaway selection leads to more and more elaborate ornaments, the trait would likely have significant negative effects on survivorship. While theoretical work has shown that runaway selection of this sort is possible (e.g. Lande 1981), there is only limited empirical evidence that supports it. For example, Bakker (1993) captured male sticklebacks (*Gasterosteus aculeatus*) with either intense or dull colouration and crossed them with females from the same population, and found that the intensity of red colouration in male offspring correlated with the preference for red of their female siblings (see also Houde 1994; but see Gray & Cade 1999).

Sensory Bias

There is also the possibility that mate choice in some mating systems could be non-adaptive, resulting from a sensory bias in females (reviewed in Ryan 1998). This sensory bias would have evolved in a non-mating context such as foraging, where an attraction to certain stimuli (e.g. shapes, colours, olfactory cues) can increase success; males can then exploit this pre-existing bias by mimicking the stimulus to attract the attention of females. For example, Rodd et al. (2002) tested the responsiveness of both sexes of guppies to coloured inanimate objects (both in mating and non-mating contexts) and found that they were more responsive to orange objects than to those of other colours. Because there is no apparent correlation between the male trait and male quality, females derive no genetic benefits from being choosy, indicating that female choice is non-adaptive. There are several studies that support a role for sensory bias in certain mating systems (e.g. Proctor 1991; Proctor 1992).

Sperm Competition

Sperm competition is a form of post-copulatory competition between males, where sperm from two or more males compete to fertilize a given set of ova (Parker, 1970). In many mating systems, sperm competition is considered a key factor in determining the reproductive success of males (reviewed in Birkhead & Møller 1998; Birkhead et al. 2009), leading to selection on males to increase the competitiveness of their ejaculate or experience decreased reproductive success. The competitive ability of an ejaculate can be augmented by either increasing the number of sperm produced or by increasing the quality of the sperm.

Sperm Competition Mechanisms

Sperm competition was originally believed to follow a “fair raffle” mechanism, where the number of sperm that each male contributes to a competition determines their relative fertilization success. Several studies have found that many species experiencing sperm competition invest more heavily in spermatogenesis, having higher gonadosomatic indexes and levels of sperm production (e.g. Møller 1988; Stockley et al. 1997). For example, Gage and Morrow (2003) provide direct evidence that male crickets (*Gryllus bimaculatus*) that produced more sperm had higher relative fertilization success than competitors with fewer sperm.

A large amount of natural variation also exists in sperm quality, however, which has led to the suggestion that this variation could also help explain differential male reproductive success (reviewed in Snook 2005). Sperm quality can include such traits as sperm velocity, longevity, or the proportion of sperm in movement, and when variation in these traits influences relative fertilization success the resulting process is known as a “loaded raffle,” where each sperm entered into the competition does not have an equal ability to fertilize the available ova (as is assumed in a fair raffle) (Parker 1990; Snook 2005). Sperm quality has been shown to influence relative fertilization success in some internally fertilizing species (Garcia-Gonzalez & Simmons 2005; Garcia-Gonzalez & Simmons 2007; Pizzari et al. 2008). However, due to the possibility of many confounding effects existing within the reproductive tract in internal fertilizers, externally fertilizing species provide the best study systems for determining the relative contributions of sperm number and quality on relative fertilization success. Studies on externally fertilizing fishes have linked relative fertilization success to sperm velocity (Gage et al. 2004; Liljedal et al. 2008), the percentage of sperm that are in motion (Vladić & Järvi 2001; Linhart et al. 2005), the percentage of sperm that were progressive (travelling faster than a threshold speed and in a linear fashion; Rudolfsen et al. 2008) and total sperm length (Vladić et al. 2002).

Overview of the Thesis

The primary objective of my thesis was to evaluate intersexual (female choice) and intrasexual (sperm competition) selection in the redside dace. First, I address female choice in chapter two, where I conducted an experimental behavioural study to determine whether female choice exists in this species, and if so, what might be the underlying benefit of choice that is driving the evolution of the preference. Second, chapter three deals with competition between males in the form of sperm competition, where I conducted in vitro competitive fertilization trials in order to determine which sperm traits (velocity, longevity or density) might be determinants of fertilization success.

The Redside Dace

The redside dace (*Clinostomus elongatus*) is a small, externally fertilizing cyprinid fish exhibiting a promiscuous, non-resource based mating system (Koster 1939). They generally occur in pools and slow-flowing sections of relatively small headwater streams (averaging 5-6 meters wide and 0.3-1 meter in depth) that contain both pool and riffle habitat with gravel-sand bottoms and clear water (McKee & Parker1982; Parker et al. 1988). They are most abundant in areas with abundant riparian vegetation providing overhead cover and prefer the deepest and widest parts of pools (McKee & Parker 1982; Novinger & Coon 2000; Zimmerman, Miner & Pitcher, unpublished data).

Redside dace live up to four years of age, reaching sexual maturity in their second or third year (Parker et al. 1988). Spawning occurs in riffles between mid-May and early June, with both males and females gathering in dense groups downstream of active creek chub (*Semotilus atromaculatus*) nesting sites; individual females will periodically depart from the group and swim upstream, where they are joined by two to six males which crowd around them during spawning (Koster 1939). Any pre-copulatory competition between males appears to be limited to obtaining a position close to the female for spawning (Koster 1939). Redside dace are nest-parasites of creek chub nests and eggs and sperm are released into these, apparently without interference from the creek chub themselves. Once spawning is complete, the group of redside dace moves back downstream to rejoin the main group; spawning groups continue to break off every few minutes, and it is believed that females spawn multiple times per season (Koster 1939). Females have been reported to carry between 409 and 1971 spheroidal eggs when gravid (Koster 1939; McKee & Parker 1982).

Redside dace have a highly conspicuous red ornamental stripe on their side, which is present year-round but becomes much more intense during the spawning season; the colouration of males appears to be more intense than that of females at all times of year, but especially so in spawning season (Koster 1939; Parker et al. 1988). This red colouration is an example of a secondary sexual characteristic which is elaborated in males, and could be the subject of mate choice by females. Sperm competition is likely intense in this species, as several males release sperm in an attempt to fertilize a single clutch of eggs during spawning. Pitcher et al. (2009) conducted a study on structure and function of redside dace sperm and discovered significant between-male variation in sperm total length, head length, flagellum length and sperm head length to width ratio, as well as significant relationships between these morphometric traits and sperm velocity. This variation in sperm quality, along with what is probably intense sperm competition during spawning bouts, indicates that sperm traits may potentially be important determinants of male reproductive success in this species.

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CHAPTER 2: FEMALE CHOICE, SECONDARY SEXUAL CHARACTERS AND SPERM TRAITS IN THE REDSIDE DACE (*CLINOSTOMUS ELONGATUS*)[1]

SYNOPSIS

Female mate choice based on exaggerated male secondary sexual traits of males is widespread in nature. In non-resource based mating systems, where females only receive genes (i.e. sperm) from males, adaptive female choice for secondary sexual traits is commonly attributed to a “good genes” mechanism arising from indirect genetic benefits to the offspring (i.e. offspring inherit beneficial paternal alleles that increase their survival or reproductive success). However, good genes effects only explain a small percentage of the variation in offspring fitness. A more parsimonious explanation of female choice for exaggerated secondary sexual traits of males is potentially provided by direct fertility benefits to the female. The phenotype linked fertility hypothesis suggests that male ornaments may be honest signals of their ability to provide large quantities of high quality sperm, which females can use in mate choice to ensure that high proportions of their eggs are fertilized. The redside dace (*Clinostomus elongatus*) is an externally fertilizing cyprinid that develops intense red integument pigmentation coloration during its spawning season. Redside dace were found to be sexually dimorphic in the relative area (controlling for body size), saturation, brightness and hue of their red spawning colouration; with males having larger values than females for all four traits. Relative female preference for males presented in dichotomous mate choice trials was shown to be

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[1] This chapter is the product of joint research with Dr. Trevor Pitcher and Dr. Stéphanie Doucet.

related to differences in the area and hue of their red spawning colouration. Despite this, neither the relative area nor the hue of red spawning colouration in males were significantly related to any sperm quality metric. Our results provide little support for the phenotype linked fertility hypothesis.

INTRODUCTION

Females from many species discriminate between males when mating, generally preferring males with more elaborate secondary sexual characters (i.e. ornaments, reviewed in Andersson 1994). For adaptive female choice to evolve, there must be some benefit counteracting the costs involved in performing the choice (e.g. the cost of rejecting a potential mate and searching for a new one; see Johnstone et al. 1996; Reynolds & Gross 1990). Female choice of more ornamented males is predicted to result in either direct or indirect benefits, for example, because these ornaments signal the male’s ability to provide material resources that directly benefit females or their offspring (reviewed in Møller & Jennions 2001) or because the ornaments signal a male’s “good genes” genetic quality which will benefit females indirectly via enhanced quality of offspring (reviewed in Neff & Pitcher 2005). In non-resource based mating systems (where males provide nothing but genes (i.e. sperm) to females), female choice for ornamented males is thought to be driven primarily by “good genes” inherited from the father, for increased offspring survival or reproductive success. However, while good genes effects on offspring fitness have been demonstrated in nature (e.g. Barber et al. 2001; Sheldon et al. 2003; Forsman & Hagman 2006; Wedekind et al. 2008a; Wedekind et al. 2008b), they only explain a small percentage of the variation in offspring fitness (reviewed in Møller & Alatalo 1999; Neff & Pitcher 2005).

Sheldon (1994) suggested that a more parsimonious explanation of adaptive female choice for males with more elaborate sexual ornamentation is potentially offered by direct fertility benefits through increased fertilization rates. Despite the fact that the field of evolutionary ecology has long assumed that any male can provide more than enough sperm to fertilize all of a female's eggs, there is mounting evidence that due to low sperm quality (velocity or longevity) or insufficient number of sperm, many eggs remain unfertilized (e.g. Nakatsuru & Kramer 1982; Krokene et al. 1998; Preston et al. 2001; Uller & Olsen 2004; Lifjeld et al. 2007). In response to the high cost of infertility, females should in theory be choosing to mate with males that can fertilize a higher proportion of their eggs. The phenotype linked fertility hypothesis (Sheldon 1994) suggests that females may be choosing males with more elaborate ornamentation because the trait of interest is linked to the male's ability to provide a large number of high quality sperm.

The phenotype linked fertility hypothesis (Sheldon 1994) posits three testable predictions. First, females should prefer males with more elaborate secondary sexual traits. Second, the secondary sexual traits preferred by females positively covary with sperm quality metrics related to fertilization success, such as sperm number (e.g. Marconato & Shapiro 1996; Casselman et al. 2006; Butts et al. 2009) and velocity (e.g. Kime et al. 2001; Casselman et al. 2006; Tuset et al. 2008). Finally, females that mate with males with more elaborate secondary sexual characters should have higher fertility than females that mate with males that possess less ornate secondary sexual characters. Direct or indirect evidence supporting the phenotype linked fertility hypothesis can be found in Cuvier's gazelle (*Gazella cuvieri*, Roldan et al. 1998), greenfinches (*Carduelis* *chloris*, Merila & Sheldon 1999), guppies (*Poecilia reticulata*, Pitcher & Evans 2001; Pilastro et al. 2002; Skinner & Watts 2006; but see Pilastro et al. 2008), chickens (*Gallus gallus*, McGary et al. 2002; but see Pizzari et al. 2004), field crickets (*Gryllus lineaticeps*, Wagner & Harper 2003), Arctic charr (*Salvelinus alpinus*, Masvaer et al. 2004; but see Liljedal et al. 1999), mallards (*Anas platyrhynchos* L., Peters et al. 2004), red deer (*Cervus elaphus*, Malo et al. 2005), and stalk-eyed flies (*Teleopsis dalmanni*, Rogers et al. 2008). However, there are also a number of studies that have found little or no support for the hypothesis, in the zebra finch (*Taeniopygia guttata*, Birkhead & Fletcher 1995; Birkhead et al. 1998), sedge warbler (*Acrocephalus schoebaenus*, Birkhead et al. 1997), and red junglefowl (*Gallus gallus*, Kimball et al. 1997; Parker et al. 2006).

The redside dace (*Clinostomus elongatus*) is a small cyprinid fish native to headwater streams in the basins of the Mississippi river and lakes Michigan, Huron, Erie and Ontario (Parker et al. 1988). Redside dace form spawning aggregations between mid-May and early June near stream riffles, and during the spawning season both sexes develop intense red coloration on their sides which is almost non-existent the rest of the year (Koster 1939; Parker et al. 1988). Females periodically separate from the spawning group and are followed by two to six males (Koster 1939). During spawning, the males will crowd around the female and release their sperm as the female deposits her eggs into a creek chub (*Semotilus atromaculatus*) nest, as redside dace are obligate nest parasites. Pitcher et al. (2009a) have previously demonstrated significant inter-male variance in sperm size and shape metrics (total sperm length, sperm head length, flagellum length and sperm head length to width ratio) and positive relationships were found between these morphometrics and sperm velocity, suggesting that there is potentially significant variation in fertilizing capacity between males. We tested the phenotype linked fertility hypothesis in the redside dace mating system by: (i) establishing what traits are potential targets of female choice (i.e. assessing the extent of sexual dimorphism in a variety of candidate traits), (ii) examining the sexual responsiveness of females to simultaneously presented pairs of males varying in sexually dimorphic traits and (iii) correlating the sexually dimorphic traits preferred by females with the subsequent sperm density and quality of preferred and non-preferred males.

METHODS

After establishing which candidate phenotypic traits were sexually dimorphic, we quantified female sexual responsiveness to pairs of males that varied with respect to these dimorphic traits. Based on the phenotype linked fertility hypothesis, we predicted that the degree to which females are attracted to a male is an increasing function of the difference in phenotype (i.e. secondary sexual characteristic) between the two males; therefore, a female will be relatively more responsive to a male if he is relatively more attractive (i.e. more ornamented). Finally, we inferred from the phenotype linked fertility hypothesis that sexual ornamentation preferred by females would be positively related to sperm quality among males.

Fish Collection

All fish used in this study were wild-caught during the spawning season from Rathburn Run in Wayne County, Ohio, USA (N 40° 48.658’ W 082° 01.400’) between 28 May and 1 June, 2008. Fifty males and twenty two females were caught using standard seining techniques. Individuals were most commonly found in pools between

0.5 and 2 metres in depth with clear water and overhanging vegetation with soft sandy bottoms. Riparian cover was usually relatively extensive and water temperatures were approximately 12° C.

All males and a subset of females (*N*=7, due to time constraints to assess sperm quality in males) were used to test for sexual dimorphism among a suite of candidate phenotypic traits, including standard length, body mass, and metrics related to the red spawning colouration (hue, saturation, brightness, absolute area, and relative area (controlling for body area) (see below)). Forty four males and all twenty two females were used in mate choice trials. Finally, forty five males were used in the comparisons of sexual ornamentation to sperm quality traits because sperm-related data were not available for five of the males.

Behavioural Trials

Mate choice trials were conducted in a Plexiglas tank (see Fig. 2.1) in which the partitions were watertight and all sides of the tank were transparent with the exception of the wall separating the males, which was opaque to prevent interactions between them. The tank was placed on the bed of the streams from which individuals were collected, and was positioned so there would be approximately even levels of light entering both male sections. Trials took place between 1200 and 1600 EST on each day.

Females were chosen haphazardly from those collected and pairs of males were chosen by eye to have similar body size. A removable opaque partition was used to separate the female from the males for a five minute acclimation period. The partition was then removed and twenty minute trials were recorded from overhead using a digital camcorder (Sony Handycam DCR-SR200) with a polarized lens filter (Sony MC Circular PL) to reduce glare from the water surface. After individuals were used in the behavioural trials they were measured for a variety of potential secondary sexual characters and a milt sample was collected from males (see below).

Footage of the mate choice trials was reviewed and the total amount of time the female spent in the neutral and preference zones (see Fig. 2.1) was determined to the nearest second. Preference time with a male was determined as time the female spent in the preference zone (dotted line in Fig. 2.1) on that male’s half of the tank and apparently interacting with that male (approach and visual inspection of males by females was considered to be interaction. Females approached the partition such that they were opposite the male and would follow the male’s movements). Time spent in the neutral zone was counted as not showing preference for either male. Female preference for each male was calculated as the proportion of non-neutral time (total time of the trial minus neutral zone time) that the female spent in each of the males’ preference zones. The favoured male was designated as the male within each pair for which the female spent a greater percentage of time in its preference zone; the disfavoured male was designated as the male within the pair that the female spent a smaller percentage of time within its preference zone. A female’s relative preference for the favoured male was then calculated as her preference for the favoured male minus her preference for the disfavoured male. Three behavioural trials were discarded because either a male escaped from the tank or the female did not inspect both males during the behavioural trial.

At the completion of a trial, all three fish were anaesthetized in a water bath containing 0.15 g l^{-1} MS-222 and standard length was measured for each. Next, each fish was photographed (using a Canon PowerShot A570IS 7.1 megapixel digital camera) to document the body size area and the area of red spawning colouration area (i.e. area of red integument pigmentation). Area of red spawning colouration and total body area on one side was quantified using ImageJ analysis software (available at www.rsb.info.nih.gov/ij/). The total surface area of each fish was measured to calculate the relative area covered by red integument pigmentation (see Pitcher et al. 2003), referred to hereafter as relative red spawning colouration.

The hue, saturation and brightness of the red spawning colouration were also measured for each fish using reflectance spectrometry. The colouration of the red integument pigmentation was measured using an Ocean Optics reflectance spectrometer (USB 4000, detector range 200-1100 nm.) and a xenon pulse lamp (PX-2, illumination range 220-750nm.). Light was delivered to the sample area and reflected light transmitted to the spectrometer via a bifurcated fibre-optic probe (R-400-7-UV-VIS). The probe tip is encased in a matte-black rubber holder of our own design. The system is calibrated using a white standard that reflects >97% of the wavelengths used in our analyses (Labsphere WS-1). Prior to measures being taken, the body surface was wiped with a lint-free paper towel to remove excess water that could cause specular glare by reflecting incident light.

Reflectance was measured at three landmark positions on the red spawning colouration (see Fig. 2.2a); (i) immediately behind the operculum (with the probe positioned so that the entire opening was on the red integument pigmentation and no light could enter through the operculum opening), (ii) midway down the red stripe of integument pigmentation and (iii) at the posterior end of the red stripe of integument pigmentation where there was enough red colouration to fill most of the probe opening.

One reading consisting of 20 consecutive measurements (averaged by the spectrometer operating software, OOIBase 32) was taken at each of the three points along the red spawning colouration. We used the program CLR (see Montgomerie 2008) to calculate three colorimetric variables to approximate the three dimensions of color: hue, saturation, and brightness (Hailman 1977, Montgomerie 2006). We calculated hue as the wavelength at which each reflectance spectrum reached 50% of its maximum value (Montgomerie 2006). We calculated saturation as the difference between maximum and minimum reflectance, divided by brightness (Montgomerie 2006). We calculated brightness as the average percent reflectance across the entire spectrum; lower brightness values are considered indicative of more intense colouration (Montgomerie 2006).

Sperm Trait Assessment

After colour measures were completed, milt was collected by holding each male upside down and applying gentle pressure to the abdomen. The milt was then collected as it emerged using a pipetter and then placed in a 1.6 ml tube. Care was taken to prevent exposure of the milt to activation by water. Tubes containing the milt were stored in a cooler that approximated the temperature of the river water (~12ºC) and transported back to the laboratory for further analysis. Sperm velocity (after activation with 10ul of water at ~12ºC from the river where the fish were collected) was video recorded through a microscope and analysed with sperm-tracking software (see Pitcher et al. 2009b for details). Briefly, video recording was performed using a CCD B/W video camera (at 50Hz vertical frequency) mounted to an external negative phase-contrast microscope (CX41 Olympus) with 10X magnification objective. Recordings were then analyzed using HTM-CEROS sperm tracking package (CEROS version 12, Hamilton Thorne

Research, Beverly, MA, USA), an objective tool for the study of sperm motility in fish (see Kime et al. 2001; Rurangwa et al. 2004). The system was set at the following parameters: number of frames = 60, minimum contrast = 11, minimum cell size = 8 pixels. Three different parameters of sperm velocity are measured by this software: The average path velocity (VAP, the average velocity along a smoothed cell path), the straight line velocity (VSL, the average velocity along a straight line from the starting point to the ending point of the path) and the curvilinear velocity (VCL, the average velocity along the actual path followed by the cell) at six seconds post-activation. These velocity estimates corresponded to the mean velocity of all motile cells analyzed; that is, for each male, the velocity of each individual sperm cell was measured but the estimate used in the final analyses corresponded to a mean over all individual sperm cells. Sperm longevity was also estimated as the time from activation until ~ 95% of the spermatozoa within the field of view were no longer motile, i.e. showing no forward movement (see Table 1). We used Principal Components (PC) Analysis to summarize variation in sperm velocity (VAP, VSL and VCL). One informative PC axis was extracted (hereafter referred to as sperm velocity) that explained 73.9% of the variation in overall sperm velocity.

Estimates of sperm density came from counts taken on an “improved Neubauer chamber” haemocytometer under 400x magnification (see Pitcher et al. 2007; Pitcher et al. 2009b for details). Briefly, the numbers of sperm in 5 of the 25 larger squares were counted (each is further subdivided to simplify counting). The mean number of sperm in 5 squares was then multiplied by 25 (to estimate the number of sperm in all squares), again by 10 (the depth of the chamber in μm) and then by 10 again (the original volume of the sample in μl). The estimated densities were expressed as the number of sperm per millilitre of stripped ejaculate (see Table 2.1).

Statistical Analyses

Because the area of red spawning colouration on males was a function of their body area (Spearman correlation: $r_S = 0.76$, $N = 50$, $P = 0.001$), the residual area of red spawning colouration was used as an index of how much red spawning colouration males possessed. We estimated the residual area of red colouration (i.e. controlling for body size) using the least squares residuals of the regression of the $\log_{10}$ of red stripe area on the $\log_{10}$ of the total area of the body.

Analyses of variance (ANOVAs) revealed that the three landmark positions along the red spawning colouration for males differed significantly from one another in terms of hue (means +/- *SE*) (position one: 598.42 nm +/- 1.02; position two: 594.24 nm +/- 1.30; position three: 589.26 nm +/- 1.58; $F_{2,111} = 12.0$, $P < 0.001$), saturation (position one: 2.01% +/- 0.11; position two: 1.31% +/- 0.07; position three: 1.55% +/- 0.11; $F_{2,111} = 12.55$, $P < 0.001$) and brightness (position one: 19.3% +/- 2.1; position two: 29.2% +/- 2.0; position three: 32.5% +/- 2.3; $F_{2,111} = 12.78$, $P < 0.001$). These significant differences in hue, saturation and brightness along the stripe of red spawning colouration likely exist because the red spawning colouration is generally wider at the anterior landmark position (position one) and narrows toward the middle (position two) and posterior landmark position (position three) resulting in other colours of the fish (e.g. black and yellow) being included in the reflectance spectrometry readings (see Fig. 2.2a for an approximation of the placement and size of the measured positions). In order to ensure that we only measured the reflectance spectrometry readings for the red integument pigmentation, we only used data from landmark position one in our analyses, which does not produce qualitatively different results than if we used the mean of the spot values or any of the other spot landmark locations (data not shown).

We used independent t-tests to examine which of the candidate phenotypic traits were sexually dimorphic. Next, we assayed whether females exhibited a relative preference for any of the significant or nearly significant ($P < 0.08$) sexually dimorphic traits, using Spearman correlations. We then used Spearman correlations to examine the relationship between relative female preference for the favoured male (proportion of time spent near the favoured male minus proportion of time spent near the disfavoured male; larger values indicate that the preferred male is relatively more preferred) and the difference in male traits that were significantly or nearly significantly sexually dimorphic (trait value of favoured male minus trait value of disfavoured male, e.g. difference in hue), which took into account how different the males were in ornamentation. Finally, for traits that were significantly preferred by females, Spearman correlations were used to assess the relationship between the trait that was preferred by females and each of the males' sperm quality metrics (i.e. sperm velocity, longevity, and density).

RESULTS

Sexual Dimorphism

No significant sexual dimorphism was found with respect to standard body length ($t_{55} = -0.44$, $P = 0.66$) or the hue of the red spawning colouration ($t_{55} = 1.88$, $P = 0.06$) (see Table 2). Significant sexual dimorphism was found in the saturation ($t_{55} = 2.52$, $P =$

0.015), brightness ($t_{55} = -4.19$, $P < 0.001$), and the relative area ($t_{55} = 3.42$, $P = 0.001$) of red spawning colouration, with males having larger values than females for saturation and relative area and lower values for brightness (see Table 2).

Behavioural Trials

Relative female preference for the favoured male was not significantly related to differences in male red spawning colouration brightness ($r_S = -0.13$, $N = 19$, $P = 0.61$) or saturation ($r_S = 0.09$, $N = 19$, $P = 0.73$). However, relative female preference for the favoured male was positively related to differences in residual red spawning colouration area ($r_S = 0.53$, $N = 19$, $P = 0.02$, see Fig. 2.3a) and hue ($r_S = 0.57$, $N = 19$, $P = 0.01$, see Fig. 2.3b).

Secondary Dimorphic Traits Preferred by Females and Sperm Quality

Residual red spawning colouration area was not significantly related to sperm density ($r_S = 0.28$, $N = 45$, $P = 0.06$), longevity ($r_S = 0.23$, $N = 45$, $P = 0.13$), or velocity ($r_S = 0.26$, $N = 45$, $P = 0.08$, see Fig. 2.4; with two possible outliers removed, $r_S = 0.43$, $N = 45$, $P = 0.004$). The hue of male red spawning colouration was not significantly related to sperm density ($r_S = -0.05$, $N = 45$, $P = 0.77$), longevity ($r_S = -0.14$, $N = 45$, $P = 0.38$), or velocity ($r_S = -0.15$, $N = 45$, $P = 0.33$).

DISCUSSION

The current study provides little support for the phenotype linked fertility hypothesis in the redside dace mating system. Sexual dimorphism was demonstrated in the saturation, brightness and residual area of the red spawning colouration, as well as a tendency for dimorphism in hue. Females exhibited preference for males with more red spawning colouration and red spawning colouration with a higher hue value. Finally, the dimorphic traits preferred by females were not significantly related to sperm density or any of the sperm quality metrics.

No significant sexual dimorphism in body length was present in redside dace. This finding is consistent with Pyron's (1996) phylogenetic examination of North American minnows, showing that only territory guarding behaviour was a significant predictor of sexual size dimorphism at the species level; this type of behaviour is not known to exist in the redside dace. Sexual dimorphism was found in the saturation, brightness and residual area of red spawning colouration, as well as a near-significant difference in hue, with males having higher values than females (except brightness, for which a lower value is considered more ornamental), indicating that these traits may be used as sexual ornaments.

The first prediction of the phenotype linked fertility hypothesis is that the male ornament of interest must be used by females in the context of mate choice decisions. We found support for this prediction in our experiment; females showed a preference for males with a relatively large area of red spawning colouration and higher hue values in their red spawning colouration. The second prediction of the phenotype linked fertility hypothesis is that the male ornaments preferred by females must be positively correlated with measures of sperm number or quality. We found no significant relationship between sperm quality metrics and any of the traits preferred by females.

The final prediction of the phenotype linked fertility hypothesis is that females that mate with males possessing more attractive ornaments will exhibit higher fertility than those that mate with less attractive males, as a result of sperm successfully fertilizing more eggs. Although there were no significant relationships between traits preferred by females and sperm quality, we did find that there was a trend for male redside dace with larger relative areas of red spawning colouration to have higher sperm velocity and density. Kime et al. (2001) review several studies where sperm velocity was associated with higher fertilization rates, a relationship also found in rainbow trout (*Oncorhynchus mykiss*, Tuset et al. 2008). Positive effects of sperm number on fertilization success have been shown in cod (*Gadus morhua*, Butts et al. 2009) and the bucktooth parrotfish (*Sparisoma radians*, Marconato & Shapiro 1996), and both the velocity and number of sperm were found to have significant effects on the number of eggs fertilized by male walleye (*Sander vitreus*, Casselman et al. 2006). As such, our study provides very weak evidence that female redside dace could in theory achieve higher levels of fertility by choosing males with larger areas of red spawning colouration. However, because we did not directly assay female fertility in relation to sperm metrics as part of this study we cannot definitively determine whether direct fertility benefits are present in the redside dace mating system.

Female choice for more ornate males in the Redside dace mating system could also result in other types of benefits. These other potential benefits of female choice could also include other direct benefits; for example, a female may choose a brightly coloured male because this is indicative of a low parasite load (as carotenoids, that are presumed to make up the red colouration, are not being used for immune response, see

Houde & Torio 1992; Barber et al. 2000), reducing her chances of being infected during spawning. In the absence of any direct benefits, females could also benefit indirectly from choosing a certain male through genetic benefits passed on to her offspring, increasing her fitness through increased offspring reproductive success or survival (see Kokko et al. 2003). These indirect benefits would likely be of an additive or ‘good genes’ type, where certain alleles are always advantageous (Neff & Pitcher 2005). The red colouration in redside dace could act as an indicator trait, advertising the male’s genetic quality (i.e. resistance to parasites, metabolic efficiency, etc.) through its phenotype and thereby attracting mates. Some evidence has been found linking secondary sexual traits to offspring viability in fish (e.g. Barber et al. 2001; Wedekind et al. 2008a; Wedekind et al. 2008b). Alternatively, the observed preference might be a result of runaway sexual selection (Fisher 1930). The runaway sexual selection hypothesis states that the male trait and female preference are correlated genetically through linkage disequilibrium, and that the preference therefore evolves as a response to the evolution of the trait. Finally, it is also possible that the observed relationship between ornamentation and female preference is a result of non-adaptive mate choice resulting from a sensory bias in females (reviewed in Ryan 1998), where a pre-existing bias to prefer certain sensory stimuli (e.g. a colour, shape) that has evolved in a non-mating context is exploited by males, who mimic this in order to stimulate the female’s sensory system and thereby attract mates (e.g. Rodd et al. 2002).

Future research needs to examine whether female redside dace that are mated with more attractive males have a higher proportion of their eggs successfully fertilized. If support is found for the third prediction of the hypothesis, it would provide more substantial evidence that the phenotype linked fertility hypothesis as a whole plays a significant role in female mate choice in the redside dace. However, if no evidence is found of the relationship described in the third prediction, the phenotype linked fertility hypothesis must be discarded and an alternative explanation (see above) of the female preferences shown here sought.

Acknowledgments

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) in the form of Discovery Grants and Research Tools and Instrumentation Grants, as well as grants awarded by the University of Windsor, to T.E.P. and S.M.D. We are grateful to Justin Abbott and Sara Davidson for assistance in the field and lab and Kenyon College and the Brown Family Environmental Research Center for lodging and laboratory space. Fish were collected with a permit (#1041428) issued by the Ohio Department of Natural Resources. We thank A. Fisk, D. Higgs, and C. Wilson for their comments

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Table 2.1. Means and estimates of intraspecific variation (standard deviation (*SD*) and range (minimum to maximum)) for sperm traits of redside dace (*N*=45). Sperm velocity estimates include VAP, which is the average velocity on a smoothed cell path, VSL, which is the average velocity on a straight line between the cell's start and end points, and VCL, which is the actual point to point velocity of the cell.

Trait	Mean	*SD*	Range
VAP (μm/sec)	142.8	23.2	92-188
VSL (μm/sec)	94.8	31.2	12.8-172.9
VCL (μm/sec)	165.4	17.9	129.7-196.2
Longevity (sec)	34.0	4.9	26-50
Sperm density per ml (x10^6)	11.2	4.0	4.4-21.4

Table 2.2. Mean and estimates of candidate dimorphic trait variation (standard deviation (*SD*) and range (minimum value to maximum value)) in male and female redside dace.

Trait	Male Mean +/- *SD* (range)	Female Mean +/- *SD* (range)
N	50	7
Standard Length (cm)	7.6 +/- 0.3 (6.8-8.2)	7.6 +/- 0.3 (7.3-8.2)
Brightness (%)	19.1 +/- 9.4 (6.7-56.9)	35.0 +/- 7.9 (28.0-51.2)
Saturation (%)	1.94 +/- 0.67 (0.8-3.9)	1.16 +/- 0.61 (0.3-2.2)
Hue (nm)	598.1 +/- 6.5 (585-616)	593 +/- 12.6 (574-608)
Relative Red Area (%)	0.20 +/- 0.02 (0.15-0.23)	0.17 +/- 0.01 (0.15-0.18)

Figure captions

Fig. 2.1. Mate choice tank, showing sections containing the two males (left side) and the larger section (comprising the neutral zone as well as preference zones A and B) which contained the female, on the right side.

Fig. 2.2. (a) Male redside dace exhibiting typical sex specific spawning colouration. Circles indicate approximate locations where spectrometer measurements were taken. Black bar at bottom left indicates a 5mm. scale. (b) Mean reflectance spectra of the most anterior spectrometer measurement (see text for details) for male (solid dots, $N = 50$) and female (open dots, $N = 7$) redside dace, with bars indicating standard error.

Fig. 2.3. (a) Relationship between relative red area in males and relative female preference score. (b) Relationship between relative dominant hue in male spawning colouration and relative female preference score.

Fig. 2.4. The relationship between red area and sperm velocity for all males from this population (N=45).

Fig. 2.1

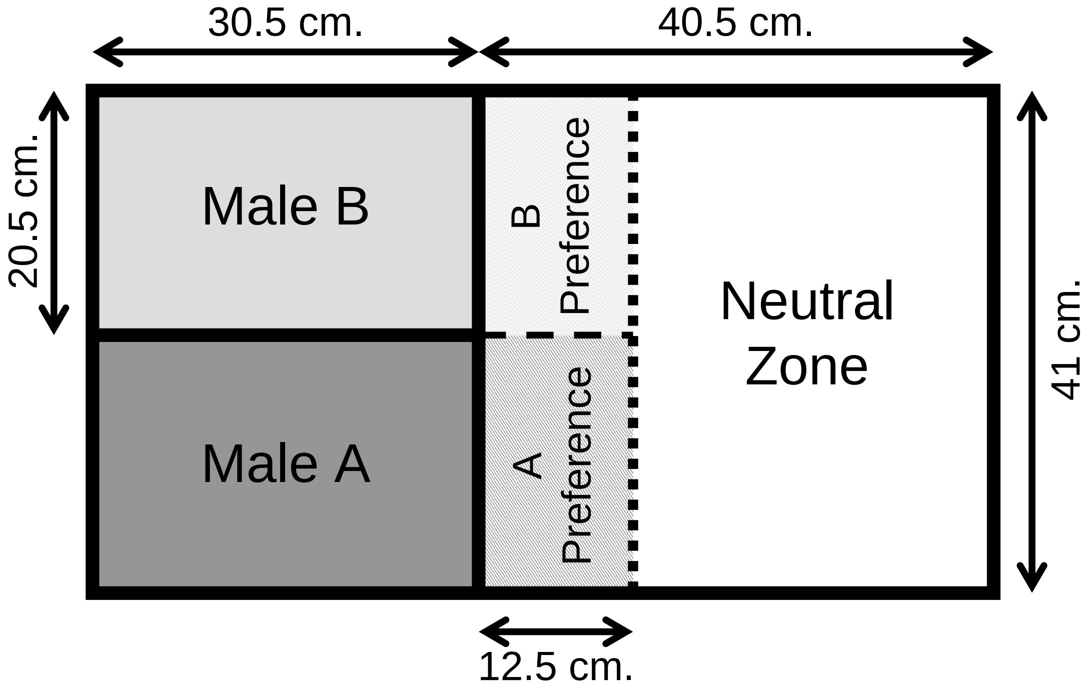

30.5 cm.
40.5 cm.
20.5 cm.
Male B
B Preference
Neutral Zone
41 cm.
Male A
A Preference
12.5 cm.

Fig. 2.2

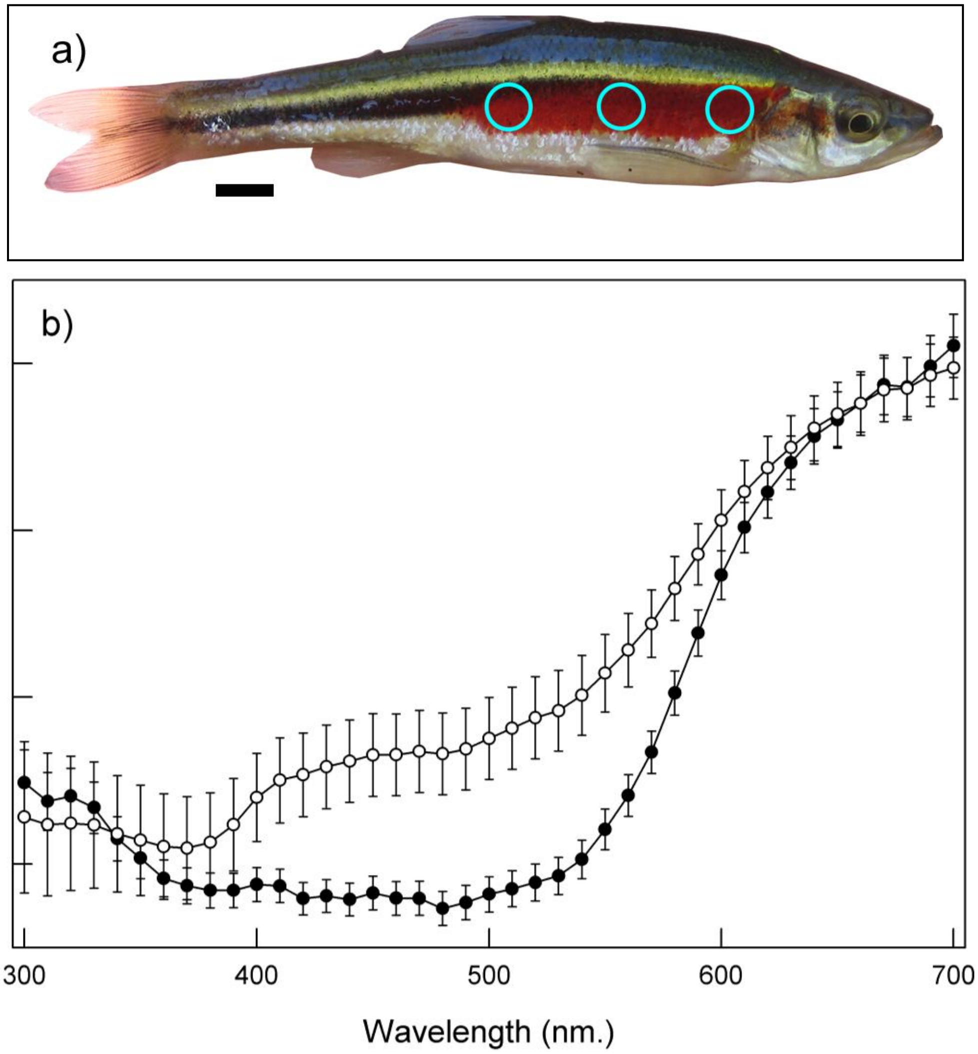

a)
b)
50
40
30
20
Mean Percent Reflectance
300
400
500
600
700
Wavelength (nm.)

Fig. 2.3

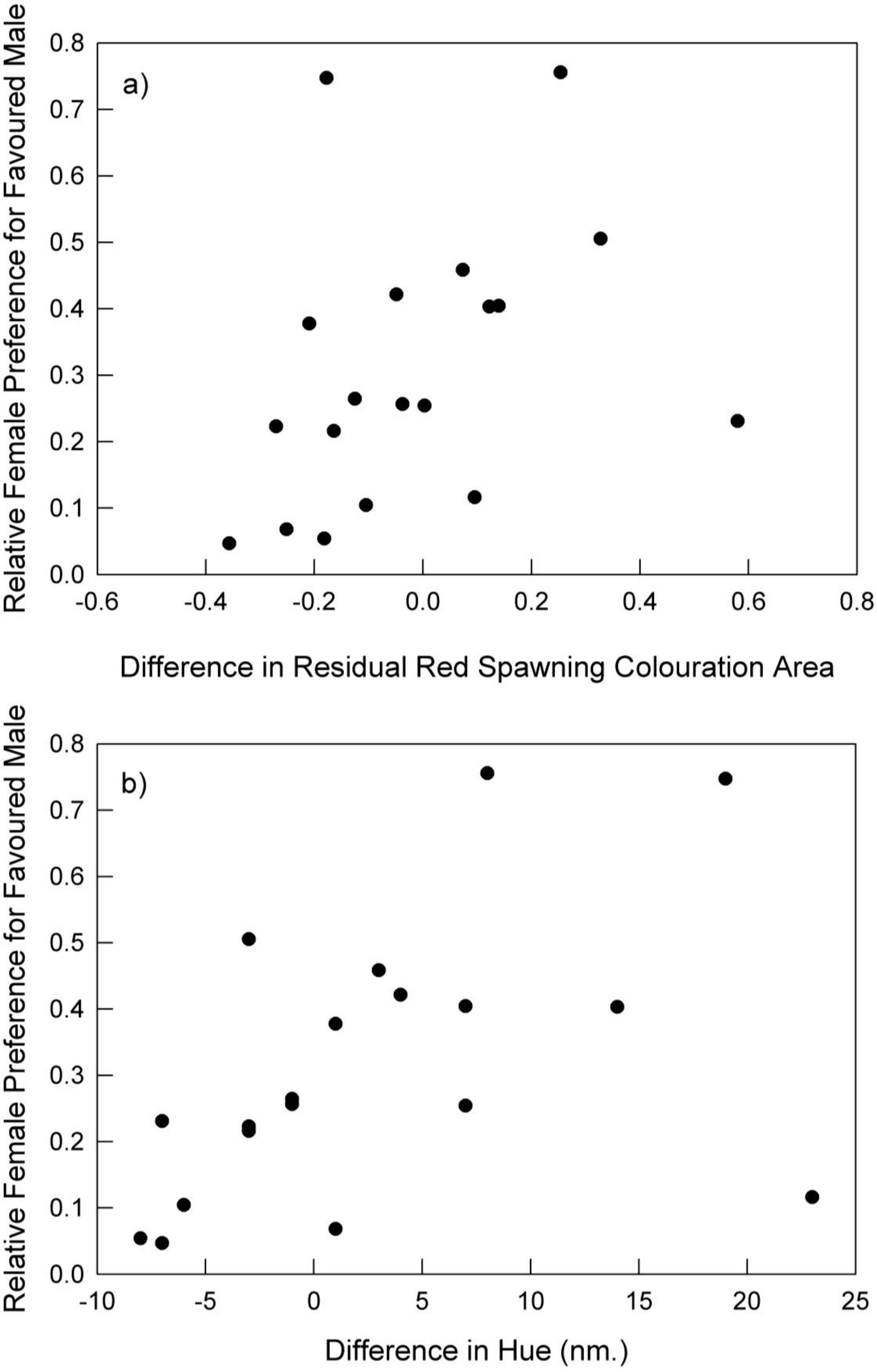
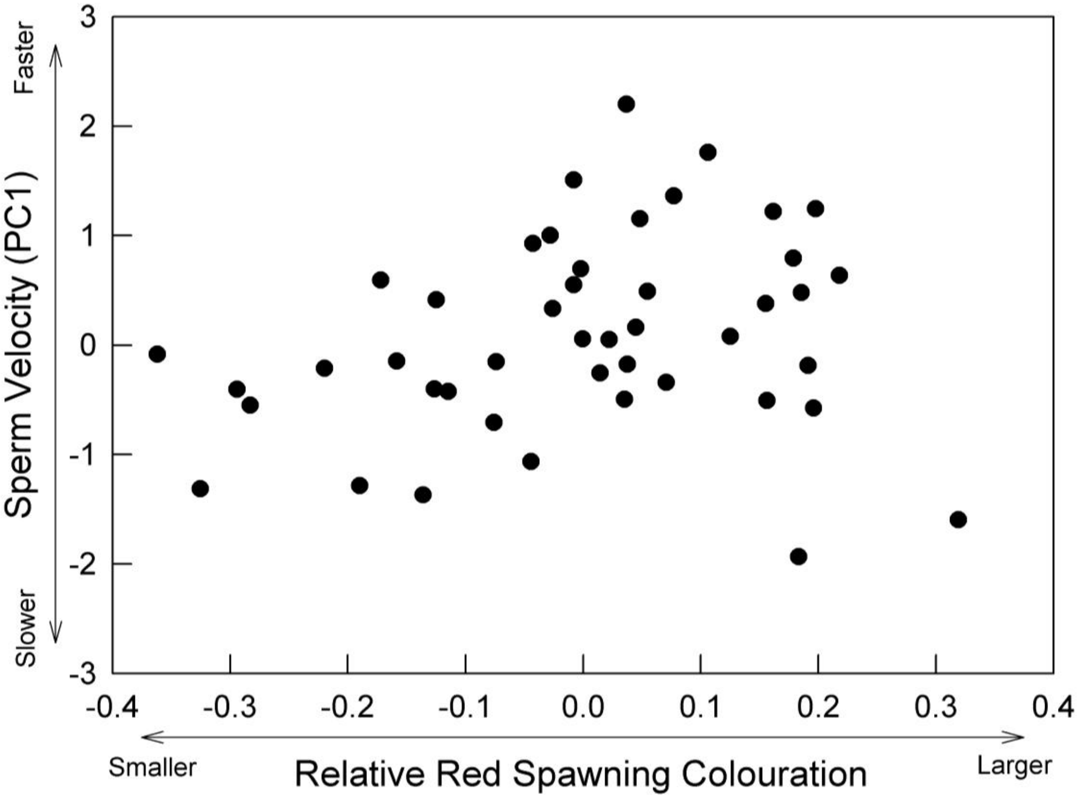

a)
Relative Female Preference for Favoured Male
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
Difference in Residual Red Spawning Colouration Area
b)
Relative Female Preference for Favoured Male
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
-10
-5
0
5
10
15
20
25
Difference in Hue (nm.)

Fig. 2.4


Faster
Slower
Sperm Velocity (PC1)
3
2
1
0
-1
-2
-3
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
Smaller
Relative Red Spawning Colouration
Larger

CHAPTER 3: THE INFLUENCE OF SPERM DENSITY, VELOCITY AND LONGEVITY ON COMPETITIVE FERTILIZATION SUCCESS IN THE REDSDE DACE (*CLINOSTOMUS ELONGATUS*)[2]

SYNOPSIS

In many mating systems, sperm competition is a key factor in determining differential male reproductive success, leading to selection on males to increase their ejaculate competitiveness. Such selection can lead to an increase in the number of sperm produced as well as increases in the quality of the sperm themselves. Two major mechanisms for predicting competitive fertilization success have been proposed; a “fair raffle” and a “loaded raffle.” A fair raffle occurs when sperm number is the only determinant of competitive fertilization success, whereas a loaded raffle occurs when some other sperm trait is important in addition to sperm number. We conducted in-vitro fertilization trials with sperm from pairs of males and eggs from a single female, to examine which sperm traits were linked to competitive fertilization success in the redside dace (*Clinostomus elongatus*). Redside dace are an externally fertilizing cyprinid that spawns in polyandrous groups, exposing males to intense sperm competition. Univariate analyses revealed that competitive fertilization success of redside dace males was significantly associated with the differences in sperm velocity and longevity within pairs of males. There was no significant relationship between competitive fertilization success and the differences in sperm density within pairs. Multivariate analysis revealed that only sperm velocity remained a significant predictor of relative fertilization success, when all other factors were controlled for. Overall, these results suggest that sperm competition in

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[2] This chapter is the result of joint research with Dr. Trevor Pitcher and Dr. Daniel Heath.

redside dace follows a loaded raffle, with sperm velocity being the primary determinant of competitive fertilization success.

INTRODUCTION

Sperm competition, competition between the sperm of two or more males for the fertilization of a given set of ova (Parker 1970), is considered a key factor in determining differential male reproductive success in many mating systems (reviewed in Birkhead & Møller 1998; Birkhead et al. 2009). Sperm competition leads to selection on males to increase their sperm competitiveness in order to increase their reproductive success and ultimately fitness. Such selection has led to increased investment in spermatogenesis in many species, resulting in increased testes size and ultimately sperm production (e.g. Møller 1988; Stockley et al. 1997). These findings were the underpinning for the original sperm competition theoretical framework of a lottery process, a “fair raffle”, in which the male who enters the most tickets (i.e. contributes the most sperm) is most likely to succeed in fertilizing the majority of the female’s eggs (see Parker 1990; Parker 1998). For example, Gage and Morrow (2003) conducted sperm competition trials between male crickets (*Gryllus bimaculatus*) that varied in terms of their sperm number and found that males that produced the most sperm were more successful in terms of achieving higher levels of paternity.

Although many studies have found that sperm competition is determined in large part by the relative numbers of sperm from competing males, substantial variation in sperm quality also exists and many researchers have suggested that this variation may also account for some of the variation in paternity among competing males (reviewed in Snook 2005). Sperm quality, which includes sperm related traits such as velocity, longevity, and viability, can also influence competitive fertilization success, creating a “loaded raffle” (Parker 1990; Snook 2005). A loaded raffle occurs when each sperm entered into the lottery is not equally likely to fertilize the eggs; for example, some sperm may have a higher velocity and as such are more likely to fertilize available ova (e.g. Birkhead et al. 1999). Disentangling the relative importance of sperm number and quality for competitive fertilization success has been hampered by the fact that most studies to date have been on internal fertilizers (e.g. Garcia-Gonzalez & Simmons 2007; Simmons et al. 2003; Pizzari et al. 2008), where it is hard to control for many potentially confounding effects. More recent research on external fertilizing species has allowed investigators to simultaneously assess the relative contribution of sperm number and quality in determining the outcome of sperm competition.

A few studies have examined the effects of different sperm traits on competitive fertilization success in external fertilizing fishes (see Table 3.1). For example, Gage et al. (2004) showed that in Atlantic salmon (*Salmo salar*), the primary determinant of relative fertilization success was sperm velocity; sperm longevity was inversely related to relative fertilization success, and there was no effect of sperm number on relative fertilization success. Similar results were found in Arctic charr (*Salvelinus alpinus*) by Liljedal et al. (2008). Rudolfsen et al. (2008) showed that in Atlantic cod (*Gadus morhua*) the percentage of sperm that were progressive (sperm which travel faster than a minimum threshold speed and in a linear fashion) was positively associated with relative fertilization success, while velocity was negatively associated with relative fertilization success. Finally, in bluegill sunfish (*Lepomis macrochirus*), relative fertilization success is positively related to sperm number, while there is no relationship between sperm velocity and relative fertilization success (Neff et al. 2003; Stoltz & Neff 2006).

The redside dace (*Clinostomus elongatus*) is a small cyprinid fish native to headwater streams in the basins of the Mississippi river and lakes Michigan, Huron, Erie and Ontario (Parker et al. 1988). Redside dace form spawning aggregations between mid-May and early June near stream riffles. Females will separate from this group periodically to spawn and are followed by two or more males (Koster 1939). During spawning between 2 and 6 males will crowd around the female and release their sperm as the female deposits her eggs into a creek chub (*Semotilus atromaculatus*) nest (Koster 1939). Redside dace sperm has already been shown to have significant between-male variation in total sperm length, which was attributable to differences in both head length and flagellum length (Pitcher et al. 2009). Furthermore, all three of these morphological traits correlated with sperm velocity in this species (Pitcher et al. 2009).

In this study we examined which sperm traits are related to competitive fertilization success in the redside dace mating system. We investigated the importance of sperm velocity, density, and longevity with respect to fertilization success by conducting in vitro fertilization competitions using sperm from pairs of males and eggs from a single female. Next, we used microsatellite markers to assign paternity to the offspring, estimate relative fertilization success for each male, and relate relative fertilization success to differences in sperm traits between competing males.

METHODS

Fish and Gamete Collection

Five of the nine triads (each one consisting of two males and a female) used in this study were wild-caught (using standard seining techniques) from Rathburn Run (Wayne county, Ohio, USA, N 40° 48.658’ W 082° 01.400’) between 29 May and 31 May 2008. The other four triads used in this study were wild-caught from the North Fork Licking River (Morrow county, Ohio, USA, N 40° 11.673’ W 082° 42.488’) on June 2, 2008. Individuals were found in pools between 0.5 and 2 meters in depth with clear water, overhanging vegetation and soft sandy bottoms. Riparian cover was usually relatively extensive and water temperatures were around 12° C.

Holding each individual upside down, we collected milt or egg samples after drying the body surface with a lint-free tissue and applying gentle pressure to the abdomen. The milt was collected as it emerged using a pipetter and then placed in 1.6 ml eppendorf tubes. Eggs (and accompanying ovarian fluid) were collected by applying gentle pressure to the females’ abdomen, while holding her over a small Petri dish (55mm. diameter x 13mm. depth). Milt and eggs were kept in a cooler that approximated the temperature of the river water (~12ºC) for transport back to the lab for sperm density and quality analysis and fertilization trials (see below). Fin clips were collected from all adults and preserved in 95% ethanol for paternity assignment genotyping (see below).

Fertilization Protocol

Fertilization trials were conducted in the same small Petri dishes that eggs were initially collected in. Males were paired so that the difference in storage time of their respective milt samples was minimized. Two microliters of milt from each of the two males was applied simultaneously at the same place in the egg mass (from one female) using two micropipettes. River water from the site of collection was then poured to the same level in all dishes (~18ml.) and the mixture was stirred to ensure homogenization of the sample. Petri dishes were then covered and left for ten minutes to allow fertilization to occur; sperm longevity is known to have a mean of 34 seconds in this species (Pitcher et al. 2009). Next, the eggs were transferred to rearing tanks modified from the design of Barber and Arnott (2000) as shown in Fig. 3.1.

At the completion of the fertilization protocol, each batch of eggs was gently poured out into individual submerged egg trays (Fig. 3.1). These trays consisted of short cylinders of transparent plastic (7.5 cm. interior diameter, 2.5cm. tall sides) with bottoms covered with 400 micron mesh to allow water circulation around the eggs. These trays were then placed in a stand designed to hold them in the center of the tank, above an air stone to ensure that highly oxygenated water would circulate around the eggs (see Fig. 3.1); however, water tension prevented the air bubbles from passing directly through the mesh and they escaped to the side, leaving the eggs undisturbed. There were two such setups per rectangular tank, with an extremely fine mesh (~200x150 microns) secured between them to prevent larvae from crossing between sides (see Fig. 3.1). Tanks were filled with river water collected from the Kokosing River, which belongs to the same watershed as Rathburn Run and North Fork Licking River. Half tank water changes were conducted at approximately 8 hour intervals throughout rearing. Forceps were used at every water change to remove any dead or unfertilized eggs (i.e. eggs that were opaque) present. Eyespots were visible to the naked eye approximately 24 hours post-fertilization, and larvae began to hatch approximately 48 hours post-fertilization. A subset of eggs and larvae were collected daily from each family, beginning when eyespots were observed and continuing until no offspring remained in the tank. Offspring were preserved in 95% ethanol for subsequent DNA extraction (see below).

Sperm Trait Assessment

All sperm samples were analyzed less than five hours after collection. A CCD B/W video camera, mounted to an external phase-contrast microscope (CX41 Olympus) with 10X magnification objective and set to 50Hz vertical frequency, was used to record sperm velocity. Recordings were then analyzed using the HTM-CEROS sperm tracking package (CEROS version 12, Hamilton Thorne Research, Beverly, MA, USA), an objective tool for the study of sperm motility in fish (see Kime et al. 2001; Rurangwa et al. 2004; Pitcher et al. 2009a). The system was set at the following parameters: number of frames= 60, minimum contrast= 11, minimum cell size= 8 pixels. Curvilinear velocity (VCL, the average velocity along the actual path followed by the cell) was measured at five seconds post-activation. Five seconds was used as the standard measurement time because while fertilization of eggs is known to decrease after 10 seconds in some fishes, rates do not differ significantly between 0, 5 and 10 seconds in other fishes (e.g. Liley et al. 2002), and redside dace sperm have been shown to only be viable for 34 seconds after activation (see Pitcher et al. 2009a). The velocity estimate as calculated from the mean velocity of each sperm cell analyzed, such that the velocity of each sperm cell was measured individually and the mean used in our analysis. Between 13 and 172 sperm were measured per male (mean +/- *SD* = 50.6 +/- 37.0); these differences arose from the need to quickly find and focus on an area of the slide that was not too dense for the software to analyze. Sperm longevity was estimated as the time until 95% of the cells within the field of view showed no forward movement. Two estimates were made for each male and the mean value used to reduce measurement error (Yezerinac et al. 1992) (see Table 3.2).

Estimates of sperm density were obtained using an “improved Neubauer chamber” haemocytometer under 400x magnification (see Pitcher et al. 2007 for details). Briefly, the numbers of sperm in 5 of the 25 larger squares were counted (each is further subdivided to simplify counting). The mean number of sperm in 5 squares was then multiplied by 25 (to estimate the number of sperm in all squares), again by 10 (the depth of the chamber in µm) and then by 10 again (the original volume of the sample in µm). The estimated densities were expressed as the number of sperm per milliliter of stripped ejaculate.

Paternity Assignment

DNA was extracted from adult fin clips (2 males and 1 female per fertilization trial) and whole larvae or strong-eyed eggs. Fin clips or egg samples were dried of ethanol and placed in 96 well plates with digestion buffer and proteinase K solution before being incubated at 37° C and agitated gently overnight. Extraction was then performed using a Janus Automated Liquid Handling System (Perkin Elmer Life and Analytical Sciences, Dowers Grove, IL USA) following the protocol of Elphinstone et al. (2003). Paternity of offspring was determined using four polymorphic microsatellite markers recently developed for the Redside dace; RSD 42A (dinucleotide repeat motif), RSD-86 (dinucleotide repeat motif), RSD2-91 (tetranucleotide repeat motif) and RSD-142 (tetranucleotide repeat motif) **(**Pitcher et al. 2009b**)**. The loci were amplified using polymerase chain reaction with the following protocol: Denature for 2 minutes at 95° C, followed by thirty five cycles of 30 seconds at 95° C, 30 seconds annealing at 58° C (53°

C for RSD-142), 30 seconds extension at 72° C, then a final extension step of 2 minutes at 72° C. Fluorescently labeled primers were used and the product run by polyacrylamide gel electrophoresis using a Licor 4300 DNA Analyzer system. Allele sizes were called manually using Gene ImagIR (version 4.05) software. Paternity was determined via the exclusion of one possible father; however, not all offspring could be assigned paternity in this manner using these loci (see Table 3.2). Paternity could be assigned to between 10 and 51 offspring per family (mean +/- *SD* = 27.33 +/- 13.47) using available microsatellite loci. Fertilization success was measured for each male by dividing the number of offspring sired by that male by the total number of offspring that could be unambiguously assigned to one of the two males.

Statistical Analyses

To give us a measure of both the direction and magnitude of the difference in each sperm related trait (velocity, longevity and density) as well as fertilization success for the pairs, we subtracted the trait value of the male with the higher ID number from that of the male with the lower ID number (resulting in relative measures of traits, e.g. relative fertilization success, see Fig. 3.2). Because paternity estimates are more accurate when more offspring are examined (e.g. Neff & Pitcher 2002) and there was variance in the number of offspring per pair of males that we could assign paternity to (see Table 3.2), the relationships between the differences in sperm traits and the differences in fertilization success were examined individually using weighted (for offspring number) linear regressions and collectively using a weighted multiple linear regression. Similar results were also found when the non-weighted regressions were used; none of the significant relationships were non-significant using non-weighted regressions (data not shown).

Data were all normally distributed for all of the variables (Kolmogorov-Smirnov tests, $Z < 0.25$, $P > 0.12$) except for sperm density, which was log transformed to normalize its distribution. All statistical tests were performed in SPSS (version 15).

RESULTS

Relative fertilization success was significantly associated with the difference in sperm velocity (R^2=0.62, *F*=13.99, *P*=0.007, see Fig. 3.3) and the difference in sperm longevity (R^2=-0.46, *F*=6.06, *P*=0.043, see Fig. 3.4) among males. Relative fertilization success was not significantly associated with the difference in sperm density (R^2=0.25, *F*=2.38, *P*=0.17) among males. A multiple linear regression was used to assess the relative contributions of each sperm metric to relative fertilization success; there was a significant relationship between relative fertilization success and the difference in velocity of the sperm (ß = 0.63, *P*=0.05), but not differences in sperm longevity (ß = -0.23, *P*=0.42) or density (ß = 0.24, *P*=0.35) (all ß values are standardized; overall model: R^2=0.79, F=6.25, P=0.038).

DISCUSSION

In this experiment we examined which sperm traits are important to relative fertilization success in the redside dace using in-vitro sperm competition trials. We determined that the differences in sperm velocity and sperm longevity were related to relative fertilization success in univariate analyses (positively and negatively, respectively), such that males with faster and shorter lived sperm achieved higher levels of fertilization success than their competitors. We also determined that sperm density had no significant association with relative fertilization success. Multivariate analysis revealed that sperm velocity was the primary determinant of competitive success when other variables were controlled for; all other variables were not significant.

Sperm density can affect sperm competition through a fair raffle mechanism (Parker 1990). In a fair raffle the number of sperm contributed to the competition by each male drives the resulting pattern of paternity, as the sperm from each male are presumed to have the same fertilization ability. In this case, contributing a higher number of sperm to the competition than your opponent results in a higher number of egg contacts per unit time and therefore higher relative fertilization success. By contrast, in a loaded raffle, the relative number of sperm contributed to the competition is important to relative fertilization success, but so is the quality of each male's sperm. These differences in sperm quality can result from different traits in different species, such as sperm velocity (Gage et al. 2004) or the percent of sperm cells that are motile (Linhart et al. 2005). Redside dace do not appear to follow a fair raffle, as sperm density was not related to relative fertilization success in either the univariate or multivariate analyses. However, there was evidence of a loaded raffle, with sperm velocity (and potentially sperm longevity) being the factors of importance to relative fertilization success.

Males with high velocity sperm had high relative fertilization success likely because their sperm travel longer distances per unit time and they make more egg contacts per unit time. Another reason sperm velocity is believed to be an important factor in teleost sperm competition is the existence of a micropyle for fertilization. Unlike mammalian sperm with an acrosome, teleost sperm have no mechanism for penetrating the cell membrane directly and are therefore required to enter the egg by using this canal (micropyle) through the plasma membrane (Iwamatsu et al. 1991). In chum salmon

(*Oncorhynchus keta*) it has been shown that the order of entry of sperm into the micropyle is an important determinant of fertilization success (Kobayashi & Yamamoto 1981). Higher velocity sperm have a higher probability of reaching the micropyle first, which is consistent with our findings. This advantage would be especially pronounced if the sperm were following a chemical gradient released by the eggs (e.g. sea urchin (*Arbacia punctulata*) sperm, reviewed in Kaupp et al. 2008). The difference in sperm velocity between males has been found to be a significant predictor of relative fertilization success in several other fish species (see Table 1); for these species, sperm competition resembles a 'race' between sperm, at least in part. Finally, sperm longevity was negatively related to relative fertilization success. Theory has posited that sperm velocity and longevity may trade-off against one another as they use a common pool of resources (Snook 2005), which would help explain the negative relationship found in the univariate analysis between relative fertilization success and sperm longevity. Contrary to expectation, however, we did not find a significant negative relationship between sperm velocity and longevity ($r_P = 0.075$, $P = 0.77$, $N = 18$) in this study, which would support such an interpretation; Pitcher et al. (2009) also found that there was no significant relationship between these two sperm quality metrics in the redside dace.

While this study examined the results of sperm competition trials where the sperm from both males was applied in a controlled manner to the eggs, this is rarely the case in nature. During spawning, certain males will be able to position themselves closer to the female than others, and some may release sperm more quickly or more in sync with the timing of egg release. These effects could result in the eggs being exposed to the sperm of certain males earlier than others, which could give them a significant advantage, especially if sperm are in a ‘race’ to reach the micropyle first (Gage et al. 2004). Yeates et al. (2007) showed that a delay of two seconds in releasing sperm reduces second-male fertilization success to 30% from an expected 50% in Atlantic salmon. Another factor that needs to be taken into consideration is the effect of ovarian fluid on the measured sperm traits. In Chinook salmon (*Oncorhynchus tshawytscha*), the ovarian fluid of different females has been shown to affect sperm traits (such as velocity or longevity) from individual males differentially (such that a male with a higher sperm velocity than a competitor in one female’s ovarian fluid could have a lower sperm velocity than the same competitor when in another female’s ovarian fluid), providing a potential mechanism for cryptic female choice (Rosengrave et al. 2008; Rosengrave et al. 2009). If redside dace ovarian fluid has similar effects on sperm, female identity may be an important determinant of male fertilization success that we did not explicitly test for in this study. Therefore, future research should aim to more carefully document spawning behaviour in the wild and replicate these effects (e.g. spawning positions) in the lab with proper timing and ovarian fluid gradients to determine how they affect the results found here, as well as conduct replicate competition trials using the same pairs of males and eggs from different females to determine if ovarian fluid differentially affects sperm from individual males.

Acknowledgements

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) in the form of Discovery Grants and Research Tools and Instrumentation Grants, as well as grants awarded by the University of Windsor, to TEP and DH. We are grateful to Justin Abbott and Sara Davidson for assistance in the field, Courtney Beneteau for assistance in the lab, and Kenyon College and the Brown Family Environmental Research Center for laboratory space. Fish were collected with a permit (#1041428) issued by the Ohio Department of Natural Resources. We thank A. Fisk, D. Higgs and C. Wilson for their comments.

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Table 3.1. Summary of existing literature that uses paternity analysis to determine which sperm traits contribute to competitive fertilization success in fishes. (+) indicates that a significant positive relationship was found, (-) that a significant negative relationship was found, and (0) that no significant relationship was found. Blank spaces indicate that the trait was not examined. % progressive is the percentage of sperm with velocity and linearity higher than set thresholds. % motile is the percentage of sperm in movement a short time after activation. Morphology refers to total length of sperm in Atlantic salmon, and to flagellum length of sperm in bluegill.

Common Name	Genus species	Velocity	% Progressive	% Motile	Longevity	Number	Morphology	References*
Atlantic salmon	*Salmo salar*	[a]0, [c]+		[a]+	[c]-	[b,c]0	[b]+, [c]0	[a]1, [b]2, [c]3
Arctic charr	*Salvelinus alpinus*	+		0	0			4
Rainbow trout	*Oncorhynchus mykiss*	0				+		5
Atlantic cod	*Gadus morhua*	[a]-, [b]+	[a]+	[a]0				[a]6, [b]7
Bluegill	*Lepomis macrochirus*	[b]0				[a,b]+	[b]0	[a]8, [b]9
Common carp	*Cyprinus carpio*	0		+		0		10

*1 (Vladić & Järvi 2001), 2 (Vladić et al. 2002), 3 (Gage et al. 2004), 4 (Liljedal et al. 2008), 5 (Gile & Ferguson 1995), 6 (Rudolfsen et al. 2008), 7 (Skjaeraasen et al. 2009), 8 (Neff et al. 2003), 9 (Stoltz & Neff 2006), 10 (Linhart et al. 2005).

Table 3.2. Summary of the mean sperm trait values (velocity, longevity and density) of each male in this study, as well as the number of offspring sired by each male. The percent paternity is calculated as the percentage of the total offspring per trial pair that were sired by the male in question. Relative fertilization success is the difference between the % paternity of male 1 and male 2.

	Male	Velocity (μm/s)	Longevity (s)	Density ($x10^6$)	# Resolved Paternity	# Unresolved Paternity	% Paternity	Relative fertilization success (%)
Pair 1	1	172.6	32.5	9.76	14	14	56	12
	2	166.4	32	11.45	11		44	
Pair 2	3	201	37	7.26	15	2	39.47	-21.1
	4	191.2	32.5	9.37	23		60.53	
Pair 3	5	197.9	33.5	12.96	27	0	84.38	68.8
	6	122.3	37.5	6.25	5		15.63	
Pair 4	7	180.4	41	10.52	30	1	83.33	66.7
	8	150.9	41.5	4.91	6		16.67	
Pair 5	9	129.7	34.5	19.43	1	10	10	-80
	10	155.9	31	20.4	9		90	
Pair 6	11	110.8	37.5	4.85	5	0	29.41	-41.2
	12	82.2	34	7.83	12		70.59	
Pair 7	13	196.2	50.5	14.85	5	3	45.45	-9.1
	14	155.9	31	20.4	6		54.55	
Pair 8	15	182.5	34	14.28	13	2	50	0
	16	154.4	33	6.13	13		50	
Pair 9	17	164.6	37	14.65	11	0	21.57	-56.86
	18	193.4	29	9.54	40		78.43	

Figure Captions

Figure 3.1. A schematic representation of the rearing tanks used to raise eggs and larvae. Plastic disks with mesh bottoms are placed on hollow cylindrical stands over an air stone. There are two such setups per tank, divided by a fine mesh to prevent larvae from mixing.

Figure 3.2. The fertilization success of each male shown as the percentage of the total brood sired by that male (black bars are the males with the lower ID number, grey bars are males with higher ID numbers).

Figure 3.3. The relationship between the difference in sperm velocity among pairs of males and the difference in relative fertilization success among pairs of males. For each trait the difference is calculated by subtracting the trait value of the male with the higher ID number from that of the male with the lower ID number.

Figure 3.4. The relationship between the difference in sperm density (log transformed) and the difference in relative fertilization success among pairs of males. For each trait the difference is calculated by subtracting the trait value of the male with the higher ID number from that of the male with the lower ID number.

Fig. 3.1

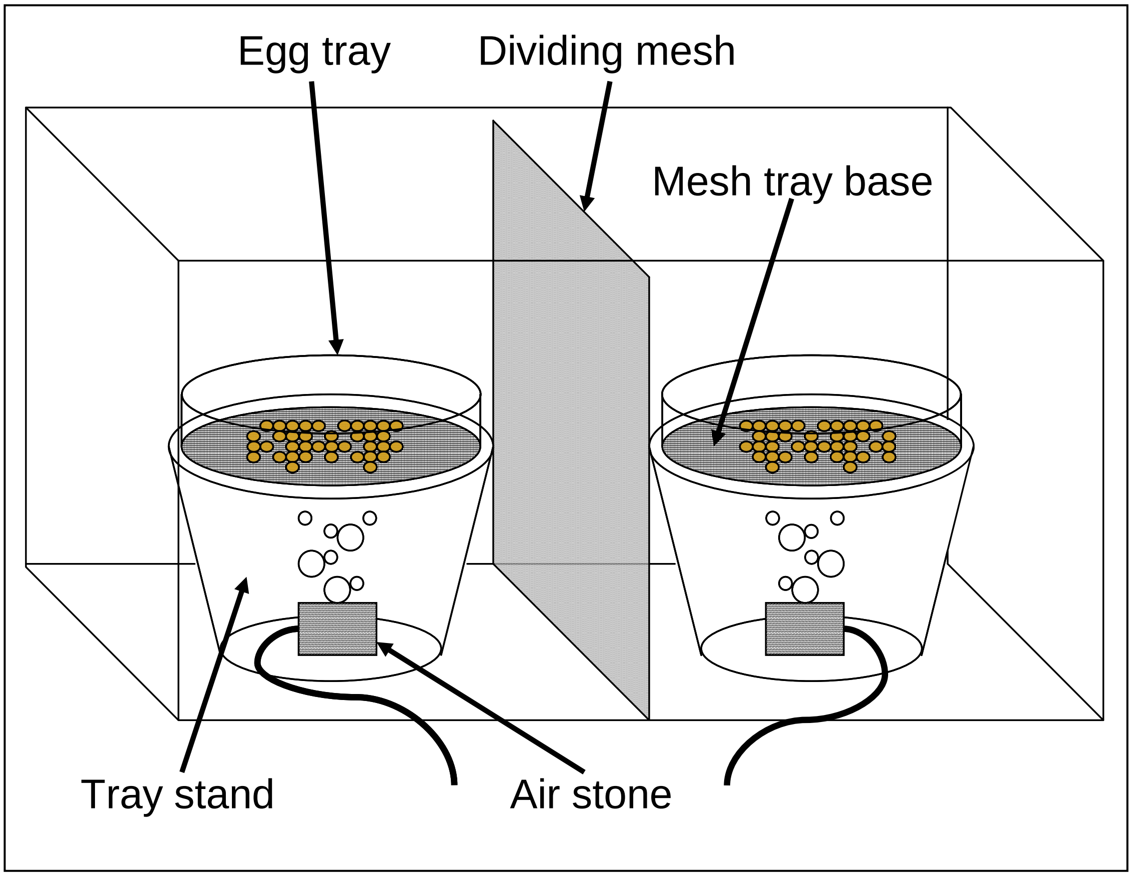


Fig. 3.2

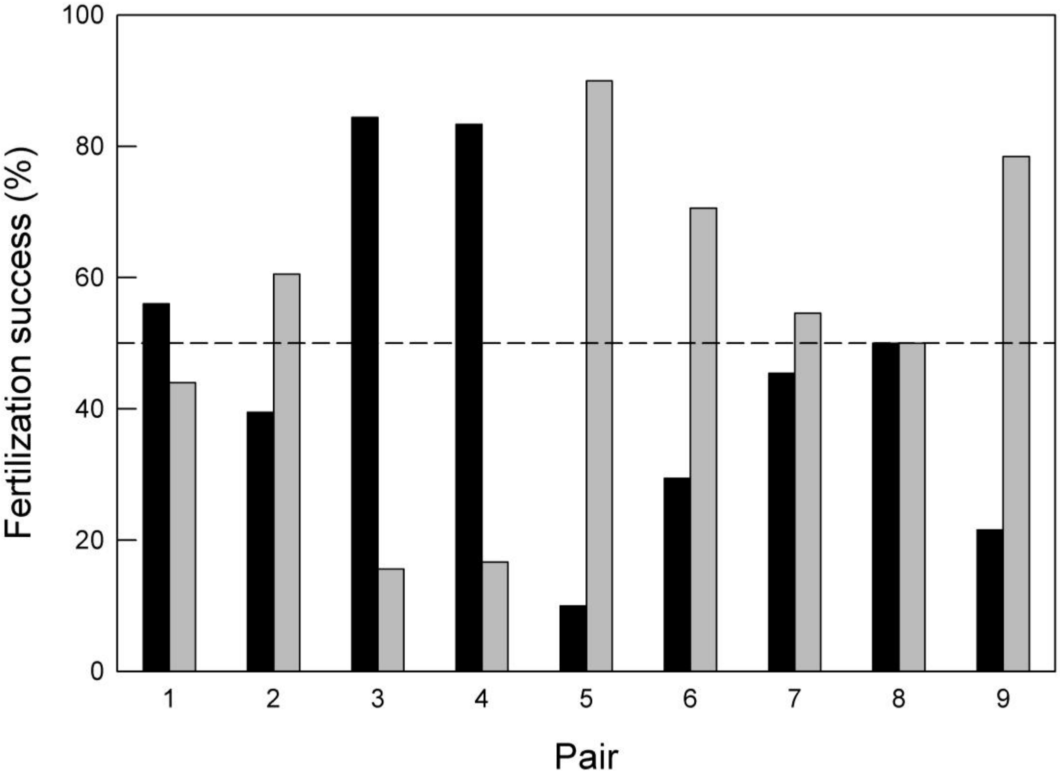


Fig. 3.3

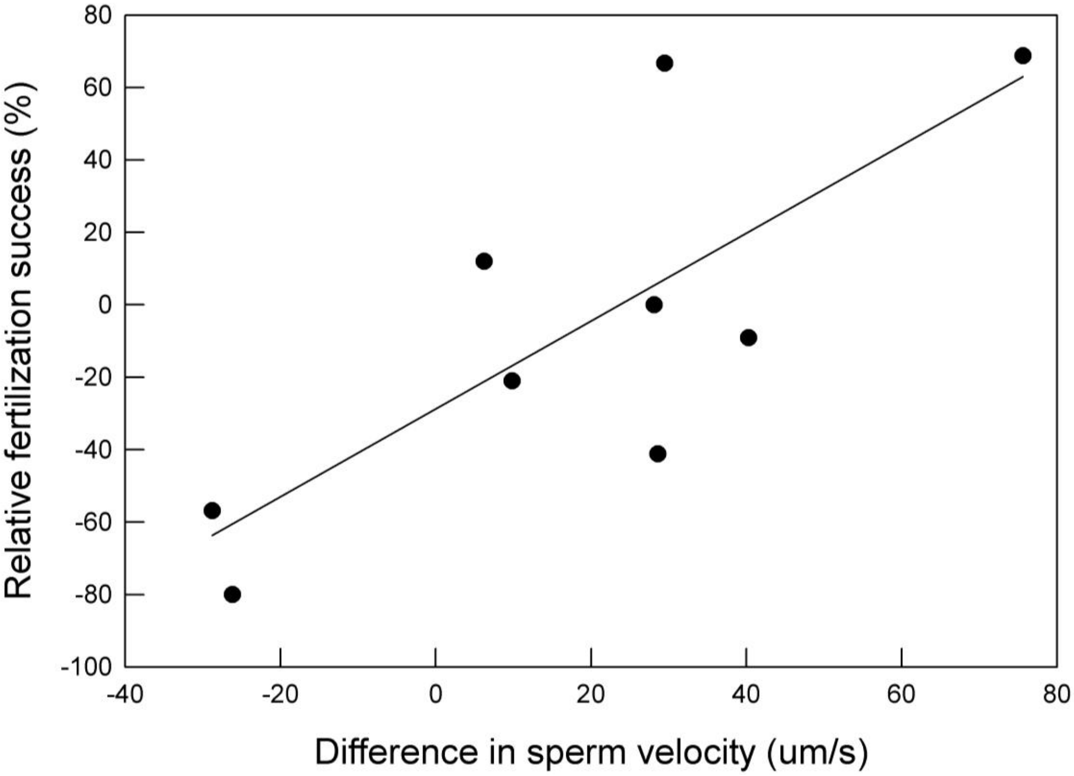
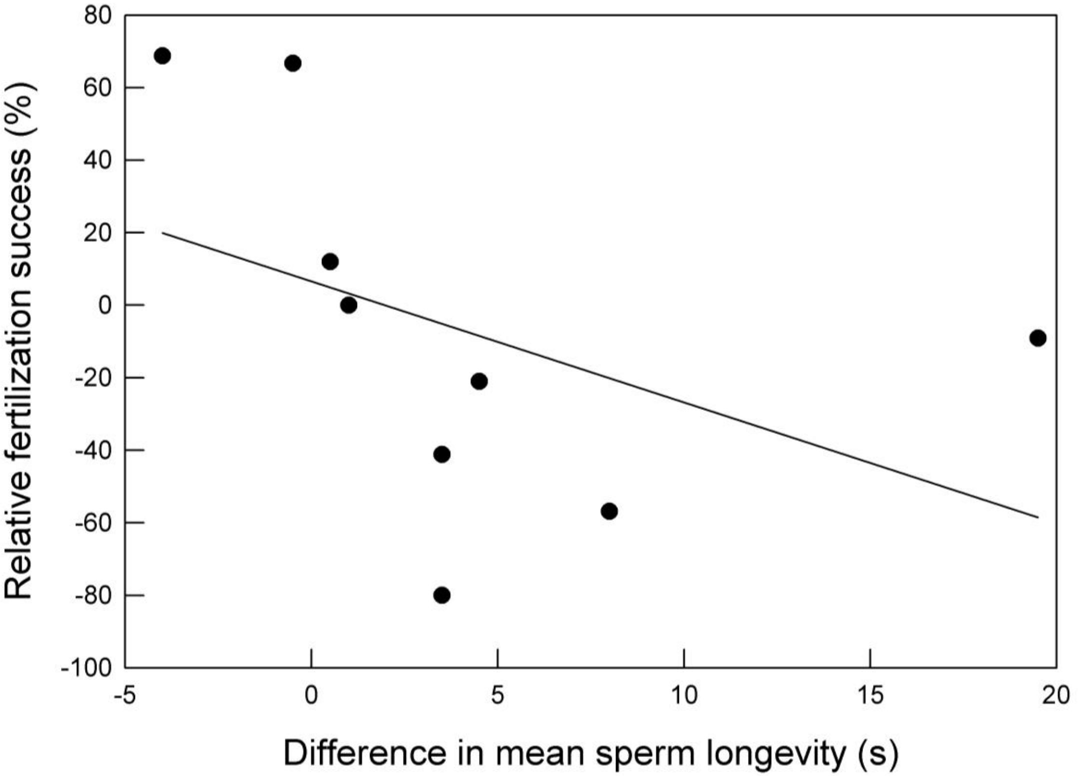

Relative fertilization success (%)
80
60
40
20
0
-20
-40
-60
-80
-100
-40
-20
0
20
40
60
80
Difference in sperm velocity (um/s)

Fig. 3.4


Relative fertilization success (%)
80
60
40
20
0
-20
-40
-60
-80
-100
-5
0
5
10
15
20
Difference in mean sperm longevity (s)

CHAPTER 4: GENERAL DISCUSSION

Summary

In this thesis I present my findings from studies that have addressed female choice and sperm competition in the redside dace mating system. The purpose of this final chapter is to summarize the main findings of my research, address some of the questions that have arisen in previous chapters, and indicate specific directions that future research should take in order to expand on the work done here.

Chapter Two

It is now clear that females prefer males with elaborate secondary sexual characters in many mating systems (reviewed in Andersson 1994). For female choice to be an adaptive trait, however, females must be receiving benefits from being choosy, either directly (reviewed in Møller & Jennions 2001) or indirectly (reviewed in Neff & Pitcher 2005). In a non-resource based mating system like that of the redside dace, female choice for ornamented males is most often thought to be driven by indirect "good genes" benefits to offspring fitness; however, while these effects have been demonstrated in some species (e.g. Forsman & Hagman 2006) their effect size is small (reviewed in Møller & Alatalo 1999). A more parsimonious explanation may be provided by the phenotype linked fertility hypothesis (Sheldon 1994), which suggests that in some mating systems variation may exist between the abilities of males to fertilize all of a female's ova (via differences in sperm number and quality), and that male fertilization ability could be linked to male ornamentation. Females derive direct benefits by avoiding decreased fecundity (i.e. choosing ornamented males that can fertilize all of their eggs).

The phenotype linked fertility hypothesis was tested in redside dace in Chapter Two using behavioural, ornamentation, and sperm data. The residual area, saturation and brightness of the red spawning colouration in redside dace were shown to be dimorphic, and hue nearly so, with males being more ornamented than females in each case. These traits were then tested as candidate ornaments being subjected to female choice using a dichotomous choice behavioural trial. I found that females preferred males with a larger residual area of red colouration and red colouration with higher hue values. These male ornaments were not related to any sperm variable measured, however, although there were similar tendencies for sperm density and velocity to increase with residual area of red colouration.

Without a significant relationship being demonstrated between variation in the ornamental trait and variation in sperm density or quality, I found little support for the phenotype linked fertility hypothesis in the redside dace. The observed preference for more ornamented males could also be explained by an indirect “good genes” type of benefit (reviewed in Neff & Pitcher 2005). One could test for this type of benefit by creating half-sib families using split-clutch in vitro fertilization and then measuring fitness related traits (e.g. growth rate, parasite resistance) of the offspring in each family (see Barber & Arnott 2000). This type of in vitro fertilization experiment would allow one to measure the importance of sire (good genes effects) and dam (maternal effects) effects on offspring performance. Ideally, lifetime reproductive success of the offspring would be measured instead of just survivorship over the early lifespan, as these two variables may not be closely related.

Chapter Three

Sperm competition between males to fertilize a given set of ova is considered a key factor in determining variation in male reproductive success in many mating systems (reviewed in Birkhead et al. 2009). Sperm competition leads to selection on males to increase the competitiveness of their ejaculates in order to reproduce successfully. Two mechanistic models of sperm competition have been formulated, centered around the importance of different ejaculate traits. The "fair raffle" model (reviewed in Parker 1998) suggests that the number of sperm contributed to a competition by each male is the primary determinant of relative fertilization success, while the "loaded raffle" model (reviewed in Snook 2005) suggests that the sperm quality traits (e.g. sperm velocity and longevity) of the sperm that each male contributes can also be important determinants of relative fertilization success. In chapter three I tested for both mechanistic models of sperm competition in the redside dace using paternity and sperm trait data.

I determined that the differences between males in sperm velocity were positively related to competitive fertilization success, and that the differences between males in sperm longevity were negatively related to competitive fertilization success. Sperm density was not found to be related to relative fertilization success. When all three traits were included in a multivariate analysis, sperm velocity was determined to be the primary determinant of success in sperm competition, with longevity and density being non-significant.

These data provides strong evidence that sperm competition in the redside dace follows a loaded raffle model, with sperm velocity and longevity being the sperm quality traits contributing to relative fertilization success. Sperm velocity is believed to be important to sperm success because faster sperm cover more distance per unit time, indicating that more egg contacts are made per unit time. Another reason for the importance of this trait may be the necessity for teleost sperm to enter an egg using the micropyle, a channel through the membrane; higher velocity sperm have a higher probability of entering the micropyle first, an important determinant of fertilization success (Kobayashi & Yamamoto 1981). The advantage of faster sperm becomes even more pronounced if sperm were following some chemical gradient being released by the eggs (e.g. Kaupp et al. 2008). No explanation for the importance of sperm longevity to fertilization success has been put forward, except the possibility that sperm velocity and longevity may trade-off against one another (Snook 2005); however, no significant negative relationship was found between sperm velocity and sperm longevity in this study, or in another study of redside dace sperm (Pitcher et al. 2009). Future work on sperm competition in the redside dace should attempt to replicate the position and timing of gamete release during natural spawning. By competing sperm from the same pair of males with the eggs of different females, future studies could also investigate whether any form of cryptic female choice (via ovarian fluid dynamics, e.g. Rosengrave et al. 2008) may be affecting sperm competition in the redside dace.

Pre-Copulatory, Post-Copulatory and Directional Selection

In my thesis I studied pre-copulatory selection in the form of female mate choice behaviour and post-copulatory selection in the form of sperm competition between males in the redside dace. One of the goals in each case was to determine what traits contribute to reproductive success of individual males; I found that females preferred males with a larger residual area of red colouration, and that males with higher velocity sperm had higher relative fertilization success than their competitors. Taking into account the nearly significant trend for males with a larger residual area of red colouration to have higher velocity sperm, it seems that pre- and post-copulatory selection may both be favouring highly ornamented males.

Directional selection occurs when several different types of sexual selection (i.e. pre- and post-copulatory selection) favour males with the same traits. Under strong directional selection, favoured male traits are under such strong selection pressure that genetic variation for these traits within a population is expected to be rapidly lost. The traits evolve towards their maximum potential values and then become fixed. In my research, however, I found that significant variation exists in both male breeding colouration and sperm traits. Some other selective force must be counteracting directional selection resulting from concordant selection pressure arising from female choice and sperm competition for this variation to be preserved. Female mating preference for males with compatible genes is one mechanism that could be preserving this genetic variation.

Compatible Genes Hypothesis

The genetic compatibility hypothesis was proposed as a form of indirect genetic benefits to female choice in mating systems where each female prefers a different male and traditional indicator traits are absent (Trivers 1972; Zeh & Zeh 1996; Zeh & Zeh 1997). Genetic quality in these systems is based on interactions between parental genotypes rather than single advantageous alleles (as in the good genes hypothesis) (reviewed in Neff & Pitcher 2005). The compatible genes hypothesis suggests that offspring could gain higher survivorship from mechanisms such as heterozygote advantage; however, because there is no congruence in female choice, traditional indicator traits cannot evolve to advertise a male's compatibility (reviewed in Neff & Pitcher 2005). For example, a growing body of evidence suggests that extra-pair copulations in many bird species are used by females in order to have offspring with higher levels of heterozygosity (e.g. Johnsen et al. 2000; Blomqvist et al. 2002; Foerster et al. 2003; Freeman-Gallant et al. 2003). The compatible genes hypothesis could help explain why variation still exists in male residual area of red colouration and sperm velocity in redside dace; since genome compatibility is not likely to correlate with the ornamental trait, some females may choose to mate with a less ornamented male whose genome is more compatible with their own, counteracting the directional selection of mate choice and sperm competition.

Future Research

As outlined above, further work on sperm competition in the redside dace should address two main areas. The first is to carefully document and observe natural spawning behaviour and mimic the positioning and timing of gamete release in vitro, to determine if the results presented here are biologically significant. The second is to study the possibility of cryptic female choice on sperm via ovarian fluid by using the same pair of males to fertilize eggs from several females and examining variation in relative fertilization success.

A powerful experimental design to examine both good genes and compatible genes quality is a full factorial breeding design (Lynch & Walsh 1998). For example, in redside dace one could use replicates of a 2x2 full factorial breeding design in which two males and two females are crossed in all combinations (Lynch & Walsh 1998). Data can then be analyzed using a 2-way ANOVA, which estimates how much of the variation in offspring performance is attributable to sire (good genes), dam (maternal) and interaction (compatible genes) effects (Lynch & Walsh 1998). Assuming some of the variation in genetic quality is due to compatible genes, one could then test for behavioural preferences for loci linked to compatibility.

Another possible study that could be conducted on compatible gene effects would involve mate choice behavioural trials with olfactory cues and the genotyping of loci that are candidates for selection based on genetic compatibility (e.g. Leinders-Zufall et al. 2004; Milinski et al. 2005). Chemical cues appear to be the medium by which information about genetic compatibility is communicated (reviewed in Penn & Potts 1998), and a comparison of female preference in a mate choice trial that included olfaction to my trials which omitted it may reveal different results. One candidate suite of genes that could be screened for genetic compatibility effects would be the major histocompatibiliy complex (MHC); the MHC is a vital component of the immune system, triggering immune responses to infection, and it is believed that heterozygosity for MHC alleles might be beneficial (reviewed in Neff & Pitcher 2005). For example, Landry et al. (2001) studied mate choice in Atlantic salmon in comparison to genetic data and found that females chose their mates in order to increase the heterozygosity of their offspring at MHC loci. The detection of a female preference for compatible genes would help explain why directional selection has not exhausted variation in the residual area of male breeding colouration or sperm velocity in this species. However, MHC markers for redside dace would first have to be developed for such a study to be possible.

In conclusion, redside dace are a good candidate system for studying sexual selection because both intersexual (female choice) and intrasexual (sperm competition) selection have been identified in this species. Female preference for male ornamentation could be driven by a number of potential mechanisms in this species, and mechanisms that preserve genetic variability in the presence of apparent directional selection have yet to be investigated, as does potential cryptic female choice (e.g. ovarian fluid effects).

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