ACCESSORY GLANDS AND SPERM COMPETITION

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By

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A Thesis
Submitted to the School of Graduate Studies
In Partial Fulfilment of the Requirements
for the Degree
Master of Science

McMaster University

MASTER OF SCIENCE (2017)	McMaster University
(Psychology)	Hamilton, Ontario

TITLE: Accessory glands and sperm competition

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SUPERVISOR: Dr. Sigal Balshine

NUMBER OF PAGES: i-xi, 92

ABSTRACT

Sperm competition is a widely-recognized and powerful selective force. Male accessory glands are organs found across animal taxa that can influence sperm performance, and thus may be selected for in competitive contexts. In fishes, these organs are in fact rare, but display great diversity in form and function across species. Although the accessory gland is known to play a role in mate attraction, parental care, fertilization, or post-copulatory competition in a few select species, the role of this organ remains a mystery in most species. Many fishes with accessory glands also exhibit alternative reproductive tactics (ARTs), which add an extra layer of complexity to how species respond to sperm competition. Because males of different ARTs typically experience different levels of sperm competition risk, it's possible they may differentially invest in accessory glands to overcome this competition. In this thesis, I used the plainfin midshipman fish (*Porichthys notatus*), a species with both ARTs and an accessory gland, to experimentally investigate the role of the accessory gland in sperm competition and uncover how this organ may differ between ARTs. Over a two-year period, I studied tactic-specific investment in the accessory gland in fish from the beaches of British Columbia. I also examined the effects of seminal fluid, produced in part by the accessory gland, on sperm performance and morphology. I found that males adopting the 'guarder' male tactic invested more in one region (the lobules) of this organ, while males adopting the smaller 'sneaker' male tactic invested more the other region of the gland (the nodes). Using data collected over five years, I found that guarder males also invested more in their whole accessory glands. Additionally, I report that sperm swam faster in the presence of seminal fluid, and seminal fluid increased sperm head size in both male tactics and increased midpiece size in guarder males. These results suggest that the plainfin midshipman accessory gland may have dual functions, one of which may be to aid sperm competitive ability through enhancements in swimming speed and potentially more successful sperm morphology. Taken together, the results of my thesis improve our knowledge of the role of non-sperm components like seminal fluid and the accessory gland in sperm competition, and demonstrate how species with ARTs can have varying physiological responses to such competition. Only a handful of studies have considered the effects of seminal fluid on sperm performance. By examining sperm competition in a more biologically relevant way and incorporating the effects of a little-studied organ that impacts sperm competition, we should be able to more generally and accurately appreciate the dynamics of post-copulatory competition and fertilization.

ACKNOWLEDGEMENTS

I'd first like to thank my supervisor, Sigal whose support and enthusiasm for research both inspired and motivated me. I couldn't have been graced with a more encouraging and committed mentor. Aneesh Bose, my field season partner in ~~crime~~ mud also deserves my thanks for his instruction, patience, and assistance during our shared work in BC. I'd like to thank Theresa Warriner, Noah Houpt, and Emilie Sadler for their endless positivity and hard work in the field as well. My thanks to Joanna Cunanan, Ephraim David, and Vanessa Ng for patiently and unwearyingly counting and measuring sperm for many, many hours. Many thanks to John Fitzpatrick who taught me the delicate art of the Sperm Tracker and was always happy to provide me with invaluable advice and consistent keenness for sperm. Many thanks as well to Trevor Pitcher who was also willing to share expert sperm-related advice and inspire new research questions. Thank you to both Trevor and Denys DeCatanzaro for serving on my committee. I'd like to thank Mariella Rasotto and Carlotta Mazzoldi for assisting me in many sperm collection and analysis methodologies, as well as providing invaluable insight into the world of accessory glands. Thank you to Natalie Sopkina, Shamaila Fraz, and Kim Mitchell for also sharing your methodological advice and instruction. A big thank you to Francis Juanes and Kieran Cox at the University of Victoria for their part in gaining access to research facilities and their avid collaboration – things wouldn't have gone nearly as well in BC without you. I also wouldn't have been able to accomplish nearly as much in BC without the generous support of John Taylor, Jessica Qualley, and Jeurgen Ehlting who loaned me the use of their scientific instruments and bench space – thank you. To Matt, Kim, Tracy, Christina, Ray, Megan, Michele, and all other UVic Animal Care and Outdoor Aquatic Unit staff that I have might have missed – thank you so much for your all of your hard work, kindness, and interest in the midshipman. Thank you to both Ben Bolker and Jonathan Dushoff for transforming much of my hate for statistics into a love for statistical theory and producing confidence in my statistical abilities. Many, many thanks to my office desk neighbor and confidant Erin McCallum for making me feel welcome as a new grad student, listening to and helping me with my R complaints, and making my grad student life much easier and happier. Thank you to Stephan Gotic (Sauce) for being my coding guru and begrudgingly helping me mass process my sperm video data despite his disinterest in fish sperm. Another thank you to Donna Waxman for generously assisting me with editing electron microscope photos of sperm. A big thank you to all the members of ABEL I haven’t mentioned yet for their helpful feedback on talks, friendly assistance, and enthusiastic participation in the social events and ultimate frisbee teams I organized. Many thanks to Sally Presutti, Wendy Selbie, Nancy Riddell, and Milica Pavlica for helping me navigate the administrative side of graduate school as well as saving me from the copier on many occasions. Thank you to Gary Weatherill and Ken Moyle as well for rescuing me from many technological issues at McMaster. I am forever grateful to my parents Susan and Tom, as well as my “Canadian/Tier II family” Julie, George, Megan, Margaret, Sauce, and Carly (and my cat, Ellie) for their tireless support as I balanced grad school with immigration efforts and health issues – I wouldn’t have survived the last two years without all of your unyielding patience, warm generosity, and hilarious cakes. And last, but certainly not least, I’d like to thank all of the (unwillingly) brave plainfin midshipman that gave their lives to science – without your ultimate sacrifice, none of this would have been possible – thank you.

THESIS ORGANIZATION AND FORMAT

This thesis is organized into three chapters. **Chapter 1** provides the theoretical background and information that motivated this study, as well it introduces the study species and research aims. **Chapter 2** is a manuscript that will soon be submitted for publication. **Chapter 3** summarizes the main results of this thesis, discusses the implications of the findings, and identifies future research areas and directions.

CHAPTER 1: Accessory glands, alternative reproductive tactics, and sperm competition in fishes

Author: Jessica S. Miller

CHAPTER 2: Form and function of the male reproductive accessory gland in the plainfin midshipman, *Porichthys notatus*

Authors: Jessica S. Miller, Aneesh P. H. Bose, Theresa Warriner, John L. Fitzpatrick, Sigal Balshine

Publication: Journal to which the paper will be submitted is still yet to be determined

Comments: This manuscript is based on work conducted by JSM with help in the field and lab by APHB and TW and under the supervision of SB. JLF provided guidance on lab and statistical methods.

CHAPTER 3: The role of accessory glands in plainfin midshipman sperm competition and some future directions

Author: Jessica S. Miller

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CHAPTER **1**

ACCESSORY GLANDS, ALTERNATIVE REPRODUCTIVE TACTICS, AND SPERM COMPETITION IN FISHES

INTRODUCTION

Sperm competition has been recognized as a powerful selective force since the 1970s. In addition to their sperm-producing testes, many male animals have a reproductive accessory gland, and this organ can influence sperm performance during fertilization as well as during post-copulatory competition. However, the degree to which and precisely how these organs influence sperm performance are not well understood, and this is especially true in fishes. In this thesis, I explore the form and function of the male accessory gland and its potential role in sperm competition using the plainfin midshipman fish, *Porichthys notatus*, a marine toadfish. This species is a useful model to study how the accessory gland influences sperm competition because plainfin midshipman not only possess accessory glands, but they also exhibit male alternative reproductive tactics. Because each type of male tactic faces a different level of sperm competition risk, the plainfin midshipman provides a convenient test bed for studying how the accessory gland may influence sperm competitive ability, and how this organ may be differentially invested in by both male tactics according to the level of sperm competitive risk they each face. In this introductory chapter, I detail what is known about this little studied organ, I introduce sperm competition risk theory and describe how the accessory gland can mediate this competition, and then I go on to explain the connections between the accessory gland, sperm competition, and alternative reproductive tactics. I end the chapter by introducing the study species, the plainfin midshipman fish, and finally provide a description of the motivations and aims of my thesis.

THE REPRODUCTIVE ACCESSORY GLAND

The term "accessory gland" names a collection of reproductive organs, glands, and ducts that do not produce gametes and can be found in both males and females across the animal kingdom. These organs appear in many invertebrates such as insects and crustaceans (Adiyodi & Adiyodi, 1988). Amphibians possess appendages of the cloaca, and some lizards, turtles, and snakes have modified kidney tubules that serve similar functions to true accessory glands (Hyman, 1992). Birds do not possess accessory glands. Monotremes and marsupials possess accessory glands called bulbourethral glands like those found in mammals, and true mammals possess a number of complex accessory glands, such as the seminal vesicles and prostate. As will be discussed in more detail below, a number of fishes possess accessory glands as well (Hyman, 1992). In this thesis, I will focus on accessory glands found in male animals only.

Across all these animal groups, the number, morphology, and function of accessory glands differs wildly. Although the names these organs have been given across species can differ (e.g. seminal vesicle, testicular blind pouch, accessory gland, etc.), unfortunately these names do not often serve to accurately describe the origin, morphology, or function of the organ. For example, the name "seminal vesicle" can be given to a mammalian accessory gland and a fish accessory gland, but in fact these two organs are not unified in any way other than by name. Accessory glands in mammals and fishes are derived from completely different areas of the reproductive anatomy and may serve completely different reproductive functions (Rasotto, in prep). For example, the mammalian seminal vesicles are derived from the Wolffian ducts, which are not found in fishes (Miller, 1992; Nagahama, 1983). Because accessory gland morphology is so diverse across animal groups and the functions of these organs are not often not well understood, it is far more accurate to name these organs by their anatomical position (Rasotto, in prep). Following this suggestion or method of nomenclature, two main types of accessory glands arise among fishes: testicular accessory glands, which are outgrowths of the testes, and sperm duct accessory glands, which are appendages of the sperm duct (Fig. 1). Testicular accessory glands can be further described by identifying three sub-categories: mesorchial glands, testicular glands, and dorsal accessory duct glands (Fig. 1). Mesorchial glands are located dorsal to the testes in proximity to the mesorchium (i.e. the tissue that connects the testes to the body cavity wall), testicular glands are located between the spermatogenetic lobules of the testes and the main testicular duct, and dorsal accessory duct glands are located in the testes, but are actually homologous to the ovary and are only found in hermaphroditic species, such as some threadfin bream fishes (Lau & Sadovy, 2001; Fig. 1). Across different fish taxa and even within species, males may possess none, one, or multiple types of these accessory glands, and they can appear as single organs, paired organs, or in even greater numbers (Rasotto, in prep).

This spectacular variation in accessory gland morphology across fishes also gives rise to a diversity of physiological functions, some well described, but most still poorly understood. In some fishes, accessory glands store or maintain sperm within the reproductive tract. For example, sperm duct accessory glands that have high protein content have the ability to immobilize sperm in and near the sperm duct in preparation for release, increasing sperm storage capacity (Chowdhury & Joy, 2007; Mochida et al., 1999). Sperm duct accessory glands are also capable of lytic activity, engaging in autophagocytotic and heterophagocytotic processes, the latter allowing the gland to absorb and eliminate aged sperm cells and thus improving ejaculate quality (Chowdhury & Joy, 2007). Glycogen and lipid production occurs in the sperm duct accessory gland of many fishes as well, these products being important for final differentiation of sperm and as energetic substrates during fertilization. Upon leaving the body in ejaculate, seminal fluid secreted by the sperm duct accessory gland of many fishes acts as an osmotic and ionic shock absorber for sperm entering a new environment and maintains environmental pH immediately surrounding sperm (Chowdhury & Joy, 2007).

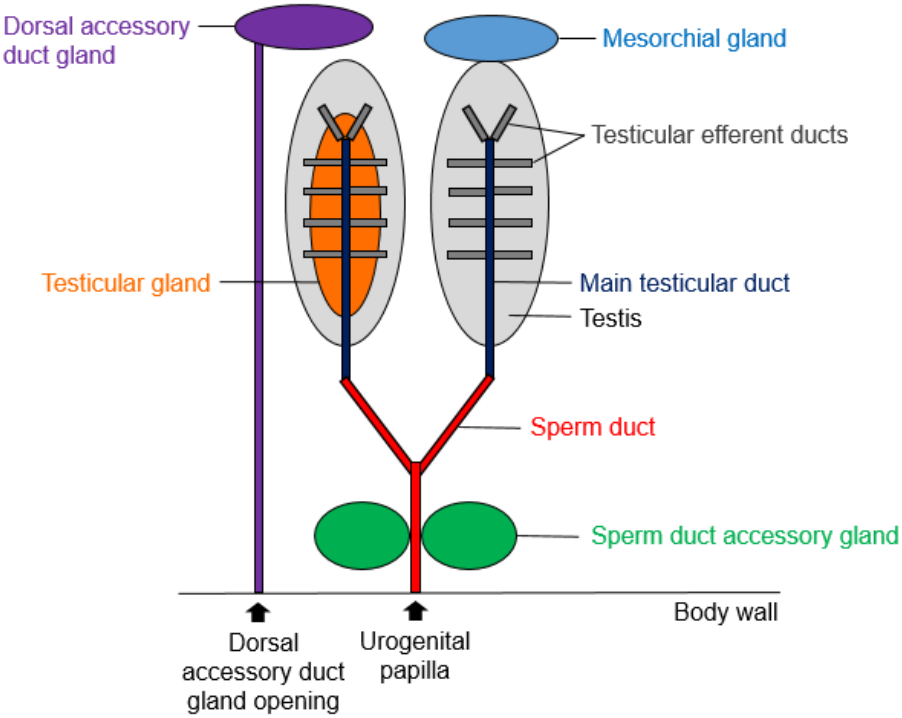

Figure 1. Schematic of the four types of accessory glands in fishes (i.e. testicular gland, mesorchial gland, dorsal accessory duct gland, and sperm duct accessory gland), their positions in the male reproductive anatomy, and other primary reproductive structures.

In some fishes, the accessory gland secretes chemical compounds either in seminal fluid or in separate secretions that serve as pheromones and similar olfactory signals. For example, the mesorchial gland found in some members of the Gobiidae family synthesizes steroid metabolites that act as olfactory signals likely involved in courtship and competition (Arbuckle et al., 2005; Jasra et al., 2007; Locatello, Mazzoldi, & Rasotto, 2002; Serrano et al., 2008). This is also true of the sperm duct accessory gland in the African catfish, *Clarias gariepinus* (Resink et al., 1987a; 1987b; Schnoonen & Lambert, 1987; Schoonen et al., 1987), and evidence supports this function of the accessory gland in the four-eyed sleeper fish, *Bostrichthys sinensis* (Hong et al., 2006), the sperm duct accessory gland in the Lusitanian toadfish, *Halobatrachus didactylus* (Modesto, Freitas, & Canario, 2015), and the dorsal accessory duct gland in two Nemipterid threadfin bream species (Lau & Sadovy, 2001).

One of the most common functions of accessory glands in fishes is the production of seminal fluid, which can play a critical role in fertilization and post-copulatory competition. The testicular gland, present in five families of blenny fishes, is responsible for the maturation of spermatids and the production of sialoglycoproteins called mucins (Lahnsteiner, Richtarski, & Patzner, 1990). Mucins are also produced by the main testicular ducts, testicular efferent ducts, sperm duct, and sperm duct accessory gland of many other fishes (Rasotto, in prep). Mucins are proteins that aggregate in fluid to form gels, and when released in ejaculate increase the viscosity of seminal fluid. This function serves a number of important purposes during fertilization. First, mucin-rich seminal fluid can create an adhesive layer onto which eggs can be deposited, priming the substrate with sperm available for activation and fertilization (Kanoh, 2010; Marconato, Rasotto, & Mazzoldi, 1996; Ota, Marchesan, & Ferrero, 1996; Riehl & Patzner, 1998). Second, mucin-rich seminal fluid disperses more slowly in water, allowing sperm embedded in that seminal fluid to become activated, released from the viscous seminal fluid, and capable of fertilization for longer periods of time (Marconato et al., 1996; Mazzoldi et al., 2000; Rasotto & Mazzoldi, 2002; Scaggiante et al., 1999). This process reduces sperm waste that can occur over long fertilization or asynchronous gamete release events. Mucins may also aid males in avoiding sperm waste in the case of sperm drinking catfishes, in which sperm must survive transport through the female digestive tract (Franceschini-Vincentini et al., 2007; Mazzoldi, Lorenzi, & Rasotto, 2007). Third, during these long fertilization events, mucin-releasing males are also able to multitask. For example, males can guard their nests or females from male competitors while simultaneously fertilizing eggs with the aid of mucins (Marconato et al., 1996; Ota et al., 1996; Scaggiante et al., 1999). Beyond mucins, there are many other seminal fluid proteins produced by accessory glands that have yet to be studied experimentally. These proteins may play a significant role in post-copulatory competition. Seminal fluid itself has shown to enhance self-sperm performance, but reduce competitor sperm performance in at least three fishes (Chinook salmon, *Oncorhynchus tshawytscha:* Lewis & Pitcher, 2016; grass goby, *Zosterisessor ophiocephalus:* Locatello, Poli, & Rasotto, 2013; and Arctic charr, *Salvelinus alpinus:* Rudolfsen, Serrano, & Folstad, 2015). One of these, the grass goby, possesses an accessory gland.

The last known role of accessory glands is egg maintenance or parental care. The sperm duct accessory gland in at least one Gobiidae and two Bleniidae species is known to produce a mucus rich in antimicrobial compounds (grass goby: Giacomello et al., 2008; redlip blenny, *Ophioblennius atlanticus atlanticus*, and peacock blenny, *Salaria pavo*: Giacomello, Marchini, & Rasotto, 2006). Male redlip blennies have been observed releasing secretions from their accessory glands directly onto the surface of developing eggs (Giacomello et al., 2006). These secretions inhibited bacterial growth and increased egg survival in another species, the peacock blenny (Pizzolon et al., 2010). In the peacock blenny, these externally visible accessory glands were not only important for male parental care, but also influenced mate choice - males with larger glands were preferred by females (Pizzolon et al., 2010).

Despite the widely varying functions of accessory glands, these organs are actually relatively rare among teleost fishes. Of the 448 known fish families (Miller & Harley, 2007), only 19 families - approximately 4% of fish families - include species that possess accessory glands (Fig. 2, Table 1). To date, accessory glands have been identified in only 96 out of 33,406 known fish species (Nelson, 2006). Accessory glands appear most commonly in blennies (Blenniidae, Chaenopsidae, Clinidae, Dactyloscopidae, Labrisomidae, and Trypterygiidae), gobies (Gobiidae and Eleotridae), and catfishes (Astroblepidae, Auchenipteridae, Bagridae, Callichthyidae, Clariidae, and Heteropneustidae), and have been described relatively thoroughly in these families. They also appear in a handful of toadfishes (Batrachoididdae), cardinalfishes (Apogonidae), and threadfin bream (Nempteridae) species, as well as in a single wrasse species (*Cheillinus undulatus*) and salamanderfish species (*Lepidogalaxias salamandroides*). Between and even within groups and species, the morphology and function of accessory glands is highly variable. The evolutionary patterns of accessory glands across fishes are a topic of on-going research, but may have been influenced by fertilization mode, mating system, and sperm competition (Mazzoldi et al., 2007; Mazzoldi, Petersen, & Rasotto, 2005; Richtarski & Patzner, 2000). Although this thesis reflects research conducted on a single fish species with an accessory gland, a comparative study of this organ in relation to reproductive behaviours, life history, and environmental factors that might have co-evolved or selected for the evolution of accessory glands across fishes would be a highly worthwhile and interesting future research endeavour.

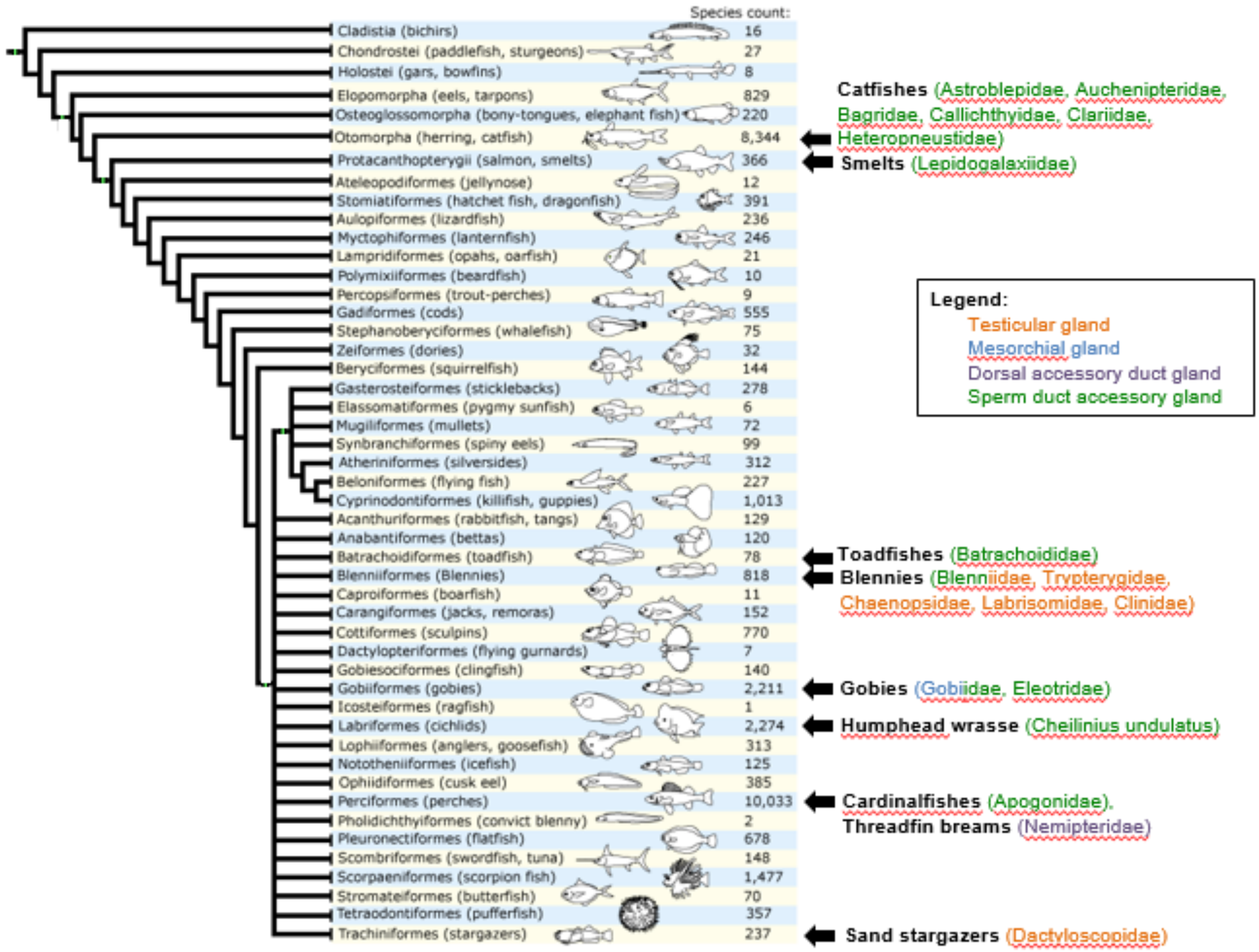

Figure 2. Teleost phylogenetic tree (Berkeley, 2010; Nelson, 2006) with accessory gland presence and types found in indicated fish families.

Table 1. List of known teleost species possessing accessory glands. Accessory gland origin has been indicated as S (sperm duct), T (testes), or H (hermaphroditic sex organ). Accessory gland type has been indicated as S (sperm duct accessory gland), T (testicular gland), M (mesorchial gland), or D (dorsal accessory duct gland).

Family	**Genus**	**Species**	**Common name**	**Accessory gland origin**	**Accessory gland type**	**References**
Batrachoididae	*Halobatrachus*	*didactylus*	Lusitanian toadfish	S	S	Amorim et al., 2006; Modesto & Canário, 2003; Vasconcelos et al., 2012
Batrachoididae	*Opsanus*	*tau*	Oyster toadfish	S	S	Barni, Mazzoldi, & Rasotto, 2001; Gray & Winn, 1961; Hoffman, 1963
Batrachoididae	*Porichthys*	*notatus*	Plainfin midshipman	S	S	Barni et al., 2001; Brantley & Bass, 1994
Lepidogalaxiidae	*Lepidogalaxias*	*salamandroides*	Salamanderfish	S	S	Pusey & Stewart, 1989
Apogonidae	*Apogon*	*crassiceps*	Transparent cardinalfish	S	S	Fishelson, Delarea, & Gon, 2006
Apogonidae	*Apogon*	*evermanni*	Evermann's cardinalfish	S	S	Fishelson, Delarea, & Gon, 2006
Blenniidae	*Aidablennius*	*sphinx*	Sphinx blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Blennius*	*ocellaris*	Butterfly blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Coryphoblennius*	*galerita*	Montagu's blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Lipophrys*	*adriaticus*	Adriatic blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Lipophrys*	*canavae*		S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Lipophrys*	*dalmatinus*		S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Lipophrys*	*nigriceps*	Black-headed blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*gattorugine*	Tompot blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*incognitus*	Mystery blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*rouxi*	Longstriped blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*sanguinolentus*	Rusty blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*sanguinolentus parvicornis*	Rock-pool blenny	T	T	Richtarski & Patzner, 2000
Blenniidae	*Parablennius*	*tentacularis*	Tentacled blenny	S,T	S,T	Richtarski & Patzner, 2000

Blenniidae	*Parablennius*	*zvonimiri*	Zvonimir's blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Paralipophyrs*	*trigloides*		S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Salaria*	*pavo*	Peacock blenny	S,T	S,T	Richtarski & Patzner, 2000
Blenniidae	*Scartella*	*cristata*	Molly Miller	S,T	S,T	Neat, Locatello, & Rasotto, 2003; Richtarski & Patzner, 2000
Chaenopsidae	*Acanthemblemaria*	*spinosa*	Spinyhead blenny	S	S	Rasotto, 1995
Chaenopsidae	*Coralliozetus*	*angelicus*	Angel blenny	S	S	Hastings, 1988; Patzner, 1991
Clinidae	*Clinithrachus*	*argenteus*		T	T	Patzner & Lahnsteiner, 1999
Dactyloscopidae	*Gillellus*	*rubrocinctus*	Saddle stargazer	T	T	Rasotto, 1995
Eleotridae	*Bostrychus*	*sinensis*	Four-eyed sleeper			Hong et al., 2006
Eleotridae	*Gobiomorus*	*dormitor*	Bigmouth sleeper			Hernández-Saavedra et al., 2004
Gobiidae	*Amblygobius*	*rainfordi*	Rainford's goby	S	S	Mazzoldi, Petersen, & Rasotto, 2005
Gobiidae	*Gillichthys*	*mirabilis*	Longjaw mudsucker	S	S	Weisel, 1949
Gobiidae	*Glossogobius*	*giuris*	Tank goby	S	S	Sircar & Har, 1978
Gobiidae	*Glossogobius*	*olivaceus*	Urohaze goby	S,T	S,M	Asahina, Uematsu, & Aida, 1983
Gobiidae	*Gobiodon*	*okinawae*	Yellow clown goby	H		Cole & Hoese, 2001; Nakashima, Kuwamura, & Yogo, 1996
Gobiidae	*Gobiodon*	*quinquestrigatus*	Five-lined coral goby	H		Cole, 1990; Nakashima et al., 1996
Gobiidae	*Gobius*	*niger*	Black goby	S,T	S,M	Mazzoldi et al., 2005
Gobiidae	*Gobius*	*paganellus*	Rock goby	S,T	S,M	Stanley, Chieffi, & Botte, 1965
Gobiidae	*Knipowitschia*	*panizzae*	Adriatic dwarf goby	S	S	Mazzoldi et al., 2005
Gobiidae	*Lythrypnus*	*dalli*	Blue-banded goby	H		Drilling & Grober, 2005
Gobiidae	*Neogobius*	*melanostomus*	Round goby	S,T	S,M	Arbuckle et al., 2005; Marentette et al., 2009
Gobiidae	*Padogobius*	*bonelli*	Padanian goby	S	S	Mazzoldi et al., 2005
Gobiidae	*Paragobiodon*	*echinocephalus*	Redhead goby	H		Cole, 1990; Kuwamura,

						Nakashimn, & Yogo, 1994
Gobiidae	*Pomatoschistus*	*marmoratus*	Mural goby	S	S	Mazzoldi et al., 2005
Gobiidae	*Pomatoschistus*	*minutus*	Sand goby	S	S	Mazzoldi et al., 2005
Gobiidae	*Zosterisessor*	*ophiocephalus*	Grass goby	S	S	Lahnsteiner et al. 1992; Locatello et al., 2013
Labridae	*Cheilinius*	*undulatus*	Humphead wrasse	S	S	Sadovy de Mitcheson, Liu, & Suharti, 2010
Labrisomidae	*Labrisomus*	*bucciferus*	Puffcheek blenny	T	T	Rasotto, 1995
Labrisomidae	*Labrisomus*	*haitiensis*	Longfin blenny	T	T	Rasotto, 1995
Labrisomidae	*Labrisomus*	*nuchipinnis*	Hairy blenny	T	T	Gibran et al., 2004; Rasotto, 1995
Labrisomidae	*Malacoctenus*	*macropus*	Rosy blenny	T	T	Petersen, 1988; Rasotto, 1995
Labrisomidae	*Malacoctenus*	*triangulatus*	Saddled blenny	T	T	Rasotto, 1995
Labrisomidae	*Malacoctenus*	*versicolor*	Barfin blenny	T	T	Rasotto, 1995
Labrisomidae	*Paraclinus*	*nigripinnis*	Blackfin blenny	T	T	Rasotto, 1995
Labrisomidae	*Starksia*	*atlantica*	Smooth-eye blenny	T	T	Fishelson, Baldwin, & Hastings, 2013
Labrisomidae	*Starksia*	*elongata*	Elongate blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*fasciata*	Blackbar blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*fulva*	Yellow blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*galapagensis*	Galapagos blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*greenfieldi*		T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*guadalupae*	Guadalupe blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*guttata*	Spotted blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*hoesei*	Hose blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*lepicoelia*	Blackcheek blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*nanodes*	Dwarf blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*occidentalis*	Occidental blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*rhodophyta*		T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*sangreyae*	Sangrey's blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*sluiteri*	Chessboard blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*smithvanizi*	Brokenbar blenny	T	T	Fishelson et al., 2013

Labrisomidae	*Starksia*	*spinipenis*	Phallic blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*weigti*	Weigt's blenny	T	T	Fishelson et al., 2013
Labrisomidae	*Starksia*	*williamsi*		T	T	Fishelson et al., 2013
Nemipteridae	*Nemipterus*	*japonicus*	Japanese threadfin bream	T	D	Lau & Sadovy, 2001
Nemipteridae	*Nemipterus*	*virgatus*	Golden threadfin bream	T	D	Lau & Sadovy, 2001
Trypterygiidae	*Axoclinus*	*nigricaudus*	Cortez triplefin	T	T	Neat, 2001
Trypterygiidae	*Enneanectes*	*boehlkei*	Roughhead triplefin	T	T	Rasotto, 1995; Thresher, 1984
Trypterygiidae	*Enneanectes*	*carminalis*	Delicate triplefin	T	T	Neat, 2001
Trypterygiidae	*Enneanectes*	*pectoralis*	Redeye triplefin	T	T	Rasotto, 1995; Thresher, 1984
Trypterygiidae	*Tripterygion*	*delaisi*		T	T	de Jonge, de Ruiter, & van den Hurk, 1989
Trypterygiidae	*Tripterygion*	*tripteronotum*	Red-black triplefin	T	T	de Jonge et al., 1989
Astroblepidae	*Astroblepus*	*choate*		S	S	Spadella et al., 2012
Astroblepidae	*Astroblepus*	*sabalo*		S	S	Spadella et al., 2012
Astroblepidae	*Astroblepus*	*trifasciatus*		S	S	Spadella et al., 2012
Auchenipteridae	*Auchenipteris*	*nuchalis*	Moustache cat	S	S	Mazzoldi et al., 2007
Auchenipteridae	*Trachelyopterus*	*galeatus*	Common woodcat	S	S	Meisner et al., 2000
Auchenipteridae	*Trachelyopterus*	*lucenai*		S	S	Meisner et al., 2000
Auchenipteridae	*Trachelyopterus*	*striatulus*	Singing catfish	S	S	Ihering, 1937
Bagridae	*Mystus*	*tengara*	Indian catfish			Rastogi, 1969
Callichthyidae	*Corydoras*	*aeneus*	Bronze cory	S,T	S,T	Kohda et al., 2002; Mazzoldi et al., 2007
Callichthyidae	*Corydoras*	*bondi*	Blackstripe cory	S,T	S,T	Mazzoldi et al., 2007
Callichthyidae	*Corydoras*	*ehrhardti*		S,T	S,T	Mazzoldi et al., 2007
Callichthyidae	*Corydoras*	*potaroensis*		S,T	S,T	Mazzoldi et al., 2007
Callichthyidae	*Hoplosternum*	*littorale*	Fork tailed hoplo	S,T	S,T	Hostache & Mol, 1998; Mazzoldi et al., 2007
Clariidae	*Clarias*	*batrachus*	Walking catfish	S	S	Ridley, 1978; Singh & Joy, 1999
Clariidae	*Clarias*	*gariepinus*	African catfish	S	S	Bruton, 1979; Van den Hurk, Resink, & Peute, 1987
Heteropneustidae	*Heteropneustes*	*fossilis*	Asian stinging catfish	S	S	Nayvar & Bangalore, 1970

SPERM COMPETITION

Geoff Parker brought the existence of sperm competition to light in 1970 when he presented a substantial amount of evidence on the outcomes of multiple matings and adaptations in response to post-copulatory competition in a number of insect taxa (Parker, 1970). Since then, he and his collaborators have developed numerous theoretical models or "sperm competition games" using game theory and the evolutionary stable strategy approach to explain when males should invest resources in ejaculates to overcome sperm competition and when such strategies will be most successful (Parker, 1990a, 1990b, 1993). Parker identified that when there is a high probability that two ejaculates will compete, male expenditure on ejaculate quality is expected to increase, a theory known as sperm competition risk model (Parker, 1982, 1990b). Following this theory, he also coined the terms "fair" and "loaded raffle" in the context of sperm competition risk model (Parker, 1990a). In a fair raffle, each competing male's probability for successful fertilization is proportional to the number of sperm he contributes in an event. According to sperm competition risk model, a male facing a fair raffle would be expected to increase the number of sperm he releases in a fertilization event when faced with a competitor. However, in a loaded raffle, a male's sperm can be of less value relative to his competitor due to variation in sperm quality. Therefore, that male will face a lower probability of successful fertilization even when matched in sperm numbers by a competitor (Parker, 1990a). In both the fair and loaded raffle models, there is pressure for a male to increase his investment in ejaculate quality on both a short scale (e.g. temporarily store and release more sperm in competitively risky contexts) and on a longer, evolutionary scale (e.g. evolve additional sperm storage organs when constantly faced with sperm competition risk).

Parker was a pioneer in describing the many physiological, morphological, and behavioural adaptations and evolutionary responses to sperm competition, but he also birthed a field of research that has led to a substantial amount of evidence of the adaptations he predicted (Parker & Pizzari, 2010; Simmons & Fitzpatrick, 2012; Snook, 2005). One of the earliest described adaptations to sperm competition risk is increased sperm production, which is facilitated by morphological changes. Species that face high levels of sperm competition have larger testes size relative to their body size, or in other words, a higher gonadosomatic index (GSI) (Balshine et al., 2001; Stockley et al., 1997). Furthermore, a higher GSI often correlates with greater sperm numbers (Gomendio & Roldan, 1993; Stockley et al., 1997). Across taxa, males will also temporarily increase their sperm numbers when faced with a sperm competitor (Fitzpatrick et al., 2009; Pilastro, Scaggiante, & Rasotto, 2002; Simmons et al., 2007).

Males not only compete by adjusting their sperm numbers, but also by adjusting the sperm themselves (i.e. sperm morphology). Male can produce sperm that swim faster and/or longer than competitor sperm, and this is often made possible via differences in sperm morphology (Pitnick, Hosken, & Birkhead, 2009). Sperm cells are composed of three regions: the head, midpiece, and tail or flagellum. All three of these regions can be shaped by sperm competition (Simpson et al., 2014; Vladic, 2002). The sperm head contains the genetic material and in many hydrodynamic models represents potential drag, the midpiece contains mitochondria and the energetic capability of the cell, and the tail produces the bulk of forward locomotion and represents propulsive force (Humphries, Evans, & Simmons, 2008).

There is much debate on which sperm morphology produces the fastest and longest swimming sperm. A number of cross-species studies have shown that longer sperm swim faster and experience greater fertilization success (Bennison et al., 2015; 2016; Fitzpatrick, Garcia-

Gonzalez, & Evans, 2010; Gomendio & Roldan, 1991), but there have also been exceptions to this observation (Dziminski et al., 2009; Gage et al., 2002). Some studies show that shorter sperm swim for longer (Gage et al., 2002; Stockley et al., 1997), but there has also been counter evidence to this finding (Vladic, 2002). To overcome these contradictory results on the outcomes of sperm length, researchers have recently opted to investigate the influence of the ratio of sperm head to tail length, since the total length of sperm is often dictated by the length of the tail (Gomendio & Roldan, 1993). After considering this ratio, the relationship between sperm head/tail lengths and successful performance appears to differ between internal and external fertilizers (Simpson et al., 2014), with sperm in internal fertilizers facing morphological constraints imposed by the shape/size of the female reproductive tract (Pitnick et al., 2009). One study has shown that sperm with short heads and long tails swam faster across externally fertilizing species, while sperm with long heads and short tails swam faster across internal fertilizers (Simpson et al., 2014). The influence of the midpiece's shape in sperm energetics has also been debated. The idea that sperm with longer or larger midpieces possess more ATP for locomotion and therefore will swim faster has been supported by a number of studies (Firman & Simmons, 2010; Vladic, 2002). However, in some species, sperm with longer midpiece actually swim more slowly (Malo et al., 2006) and possess less ATP (Bennison et al., 2016). Finally, the morphology of the tail has been of some interest to those aiming to disentangle the relationship between sperm morphology and energetics. The sperm tail end piece at the terminal end of the cell consists of a number of microtubules and influences undulatory movements. In at least Atlantic salmon, *Salmo salar*, longer tail end pieces increase fertilization success (Vladic, 2002).

Recently, there has been growing recognition that seminal fluid and products of the accessory gland can selectively respond to sperm competition. Early work in *Drosophila* *melanogaster* found that several products of the male accessory gland cause females to become less attractive to competitor males, decrease mate receptivity, increase egg-laying rate, shorten female life span, and increase the chances of mated females to store and utilize sperm (Wolfner, 1997). Males with larger accessory glands produced more of these products and experienced greater success than competitors with smaller accessory glands (Wigby et al., 2009). In red junglefowl, *Gallus gallus*, seminal fluid increases sperm velocity, and dominant males will increase seminal fluid volume in their ejaculates to increase their competitive advantage (Cornwallis & O'Connor, 2009). Rainbow trout, *Oncorhynchus mykiss,* seminal fluid contains proteins that prolong and stabilize sperm viability (Lahnsteiner et al., 2004). Several species will strategically allocate seminal fluid as it is energetically costly and can be a limiting factor when mating repeatedly (Linklater et al., 2007; Reinhardt, Naylor, & Siva-Jothy, 2011; Sirot, Wolfner, & Wigby, 2011). Successive competitor males may also adjust investment in seminal fluid based on "last male fertilization advantage", which predicts that the costs of ejaculation will be lower (e.g. reduced sperm mortality) for the second fertilizing male due to the redundancy of seminal fluid in the fertilization environment (Hodgson & Hosken, 2006). This theory assumes that ejaculates buffer sperm, remating occurs while this buffering effect persists, and that there will be a positive outcome of the interaction between competitor sperm and seminal fluid (Hodgson & Hosken, 2006).

However, seminal fluid does not always have a positive influence on sperm competitive capacity. In some species, a competitor's seminal fluid can have a negative effect on sperm performance. This occurrence has been observed in species that exhibit alternative reproductive tactics. For example, in the grass goby, the sperm of sneaker males experienced increased velocity and fertilization rate in the presence of territorial male seminal fluid, while territorial male sperm experienced reduced velocity and fertilization rate in the presence of sneaker male seminal fluid (Locatello et al., 2013). Similarly, Chinook salmon dominant hooknose male sperm swam more slowly in smaller jack male seminal fluid, while jack male sperm swam faster in other rival jack male seminal fluid (Lewis & Pitcher, 2016). The mechanisms responsible for these changes in sperm velocity have yet to be identified, although differences in seminal fluid protein compositions have been observed between Chinook salmon male tactics and are the suspected causes for the observed changes in sperm performance between male types (Gombar et al., in press).

ALTERNATIVE REPRODUCTIVE TACTICS

Sperm competition is often studied in species that have alternative reproductive tactics or ARTs. ARTs are discrete distributions of morphological, physiological, and/or behavioural characteristics that exist within a sex as alternative ways to obtain fertilizations (Taborsky, Oliveira, & Brockmann, 2008). In other words, two or more tactics within a sex can exhibit different traits on a discrete scale and allocate resources in different ways to achieve reproduction. ARTs occur in a number of taxa including insects, crustaceans, molluscs, amphibians, birds, mammals, and other groups, but they may most commonly occur in fishes (Knapp & Neff, 2008; Taborsky et al., 2008; Taborsky, 1998).

Perhaps the most common ART dichotomy across taxa and fishes is the sneak-guard system. Guard males, also called guarder, bourgeois, territorial, parental, or type I males among a number of other terms are typically larger in body size and mass, monopolize resources that are desired by females and/or monopolize females themselves, are behaviourally dominant in mating contexts, and may have a slower growth trajectory than sneaker males (Taborsky, 1994). Sneaker males are the opposite in all described characteristics and will gain access to mating females by sneaking past guarder males and "stealing" fertilizations (Taborsky, 1994).

ARTs provide an interesting context in which to study sperm competition and the evolution of responses to sperm competition risk because sneaker and guarder males by definition face different levels of sperm competition risk. Parker's sneak-guard model predicts that guarder males are entirely capable of mating in the absence of a sneaker male and therefore can mate under zero risk of sperm competition (Parker, 1990b). In contrast, sneaker males can only ever mate once a female has been attracted to a location by a guarder male and must attempt to fertilize in the presence of at least one guarder male competitor during every fertilization event. Assuming guarder males are also often unaware of cuckoldry due to the "sneaky" behaviour of the sneaker male tactic, guarder male perceived risk of sperm competition is even more likely to be less than that of sneaker males. Because of these conditions, the sneak-guard model predicts that sneaker males will invest much more into their ejaculate to increase fertilization success compared to guarder males. Conversely, guarder males should invest more resources into characteristics that enable them to monopolize females and resources, such as large body size and weapons (Parker, 1990b). In simple terms, guarder males invest in their ability to gain access to females (i.e. pre-copulatory competition), while sneaker males invest in the ability to gain access to female gametes (i.e. post-copulatory competition). These tactic-specific responses to sperm competition have been well documented across taxa and are especially common in fishes (Flannery et al., 2013; Neff, Fu, & Gross, 2003; Taborsky, 1998).

Surprisingly, the evolution of accessory glands as a response to sperm competition does not broadly appear to fit Parker's sneak-guard model. Across fishes known to have accessory glands, it is the most often the guarder male tactic that invests more in the size of their accessory glands (Marentette et al., 2009; Mazzoldi & Rasotto, 2002; Neat, Locatello, & Rasotto, 2003; Neat, 2001; Oliveira, Canario, & Grober, 2001; Scaggiante et al., 1999; Utne-Palm et al., 2015). While the positive effects of seminal fluid on sperm competitive ability have been documented across a handful of animal taxa, this has only been reported in a single fish species whose seminal fluid is produced in part by an accessory gland – the grass goby (Locatello et al., 2013). Furthermore, the grass goby accessory gland also plays a role in male parental care by producing antimicrobial compounds that aid in offspring survival during development (Giacomello et al., 2008). Therefore, it is possible that different regions of the accessory gland play different functional roles and investment in the size or mass of the whole organ is misleading as an indicator of investment in a single role. Future research on the role of accessory glands in sperm competition needs to address the potentially complex nature of multi-region accessory glands by identifying the secretions responsible for changes in sperm performance, where these secretions originate from in the accessory gland, and how these secretions and their original secretory tissues may differ between male tactics in species with ARTs. Only by doing so, we can identify the mechanisms responsible for differences in sperm competitive strategies.

THE STUDY SYSTEM: THE PLAINFIN MIDSHIPMAN FISH

To address these questions about accessory glands and sperm competition in species with ARTs, I studied the plainfin midshipman fish, *Porichthys notatus*, a marine toadfish. This fish migrates from the deep ocean to the intertidal zone of the North American west coast to mate in the summer months (Arora, 1948; DeMartini, 1988). Plainfin midshipman males exhibit sneak-guard ARTs, with males adopting either a guarder or sneaker tactic (Brantley & Bass, 1994; Fig. 3). In the intertidal populations that range from British Columbia, Canada to California, USA, 94%

of males found were guarder tactic males. (Cogliati et al., 2014). Guarder males monopolize intertidal nests underneath large rocks, which they excavate, maintain, and defend from other "takeover" guarder males, sneaker males, and predators. Guarder males invest in somatic muscle mass, which consists of muscles that surround and vibrate against the swim bladder when inflated to produce a low frequency "hum" as an acoustic signal to attract fecund females (Brantley & Bass, 1994). Upon finding and choosing an attractive guarder male, females will spend hours laying eggs on the ceiling of the nest cavity (Brantley & Bass, 1994). Plainfin midshipman are external fertilizers, and guarder males will release multiple ejaculates during the fertilization event. The largest, most attractive guarder males may receive clutches of eggs from multiple females (Fitzpatrick et al., 2015). After receiving eggs, guarder males will remain in their nests for up to

Figure 3. A guarder (left) and sneaker male (right) collected during low tide at Ladysmith Inlet, British Columbia.

three continuous months (i.e. the duration of the mating season), consuming energy reserves and caring for their offspring, which reach maturity after approximately 60 days (Bose, McClelland, & Balshine, 2016). In contrast, sneaker males do not invest in female-attracting somatic muscle tissue and do not provide parental care (Brantley & Bass, 1994). Sneaker males are seven times smaller in terms of body mass, but controlling for body mass, possess testes eight times larger than those of guarder males (Brantley & Bass, 1994; Fitzpatrick et al., 2015). Plainfin midshipman sneaker males engage in sneaky fertilization behaviours, releasing ejaculate undetected from inside the nest or from just outside the nest entrance, then fanning their ejaculates into the nest with the use of their caudal fin (Brantley & Bass, 1994).

Intense sperm competition exists between plainfin midshipman male tactics. Sneaker males, at a higher risk of sperm competition, produce faster swimming sperm than most guarder males (Fitzpatrick et al., 2015). However, the largest guarder males in a population experience the highest rates of attempted cuckoldry, and these males produce sperm that swim as fast as the sperm of sneaker males. It has been suggested that the typically superior sperm performance of sneaker males may be caused by tactic-specific differences in sperm morphology. Sneaker males produce sperm with smaller heads, which may reduce drag, and longer midpieces, which may contain greater energy stores than guarder males (Fitzpatrick et al., 2015). Plainfin midshipman sperm have long, helical or corkscrew-like heads and are biflagellate, meaning they possess two tails (Stanley, 1965; Fig. 4). The hydrodynamics of helical, biflagellate sperm in external fertilizers are still poorly understood due to their rarity. For example, biflagellate sperm have only evolved independently six times across fishes (Jamieson, 1991).

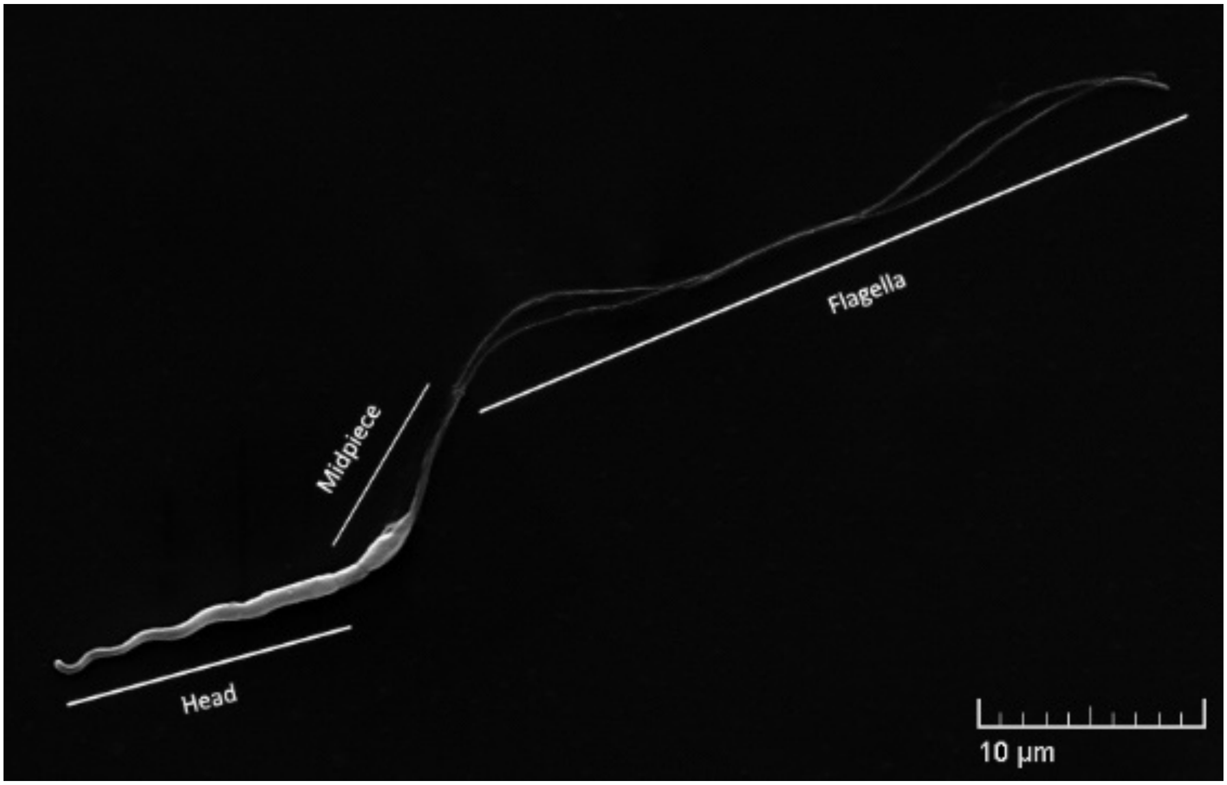

Figure 4. Scanning electron microscope image of a single plainfin midshipman sperm cell taken at 4010x magnification. The helical head, midpiece, and biflagellate regions have all been identified and labelled.

Another mysterious aspect of the plainfin midshipman reproductive biology is the presence of an accessory gland. Plainfin midshipman possess paired sperm duct accessory glands downstream of the testes (Barni et al., 2001; Fig. 5). These glands do not possess glycogen, a source of nutrition for sperm, but do secrete mucins. Barni et al. (2001) suggests that these glands are more developed in guarder males due to increased folding in the epithelium responsible for secreting mucins. In other fishes in which the guarder tactic possesses larger accessory glands than sneaker tactics, the larger accessory gland secretes more mucins that increase sperm longevity, allowing guarder males to both fertilize eggs for long durations and simultaneously defend nests

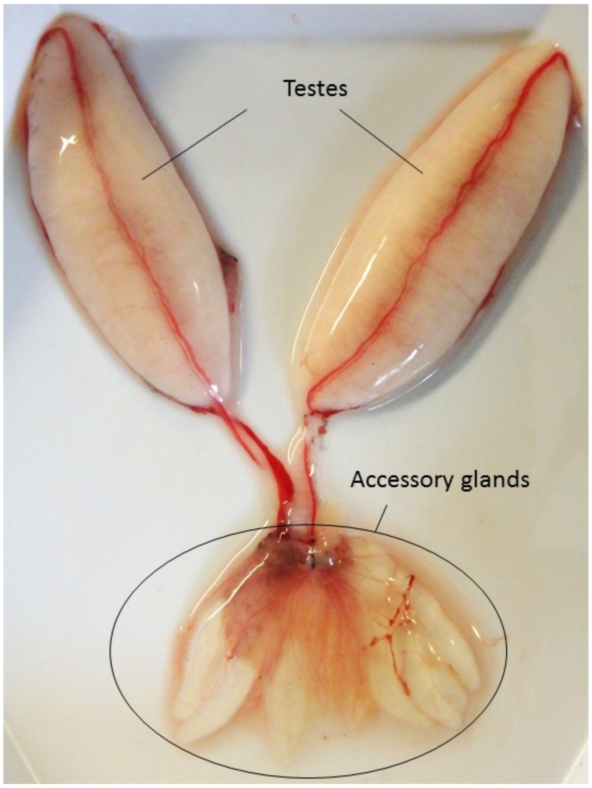

Figure 5. Dissected plainfin midshipman male reproductive anatomy with labelled testes and circled and labelled accessory glands.

(Marconato et al., 1996; Scaggiante et al., 1999). These mucins also benefit guarder males by priming nest surfaces with viable sperm embedded in sticky, mucin-rich "sperm trails" to which eggs adhere (Marconato et al., 1996; Scaggiante et al., 1999). These functions would be of no obvious benefit to sneaker males, which typically spend much less time with females compared to guarder males and fertilize from further away. In contrast to Barni et al.'s claim of larger accessory gland investment in guarder males, a recent study reported that plainfin midshipman sneaker males possess larger accessory glands, relative to their body size (Fitzpatrick et al., 2015). This finding suggests these organs may play an important role in sperm competition. Other functions of the accessory gland such as lytic activity, pheromone production, antimicrobial compound secretion, or secretion of fluids that impact sperm performance have not yet been tested in the plainfin midshipman. It is of course possible that the plainfin midshipman accessory gland has multiple physiological roles.

MOTIVATION AND AIMS OF THESIS

This thesis was motivated by the need to better our understanding of the role of accessory glands in fishes, especially in species with ARTs that experience intense sperm competition. This thesis aims to resolve the questions of 1) which male tactic invests more in the accessory gland and 2) what role the accessory gland may play in sperm competition between ARTs in the plainfin midshipman fish, a species in which males differentially experience selective pressure to invest in ejaculate quality. To answer my first question, tactic-specific reproductive investment was investigated. To address my second question, ejaculate and sperm characteristics were analyzed, and the effect of seminal fluid - produced in part by the accessory glands in this species – on sperm performance and morphology was studied.

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CHAPTER 2

FORM AND FUNCTION OF THE MALE REPRODUCTIVE ACCESSORY GLAND IN THE PLAINFIN MIDSHIPMAN, *PORICHTHYS NOTATUS*

ABSTRACT

Male reproductive accessory glands generally serve to enhance male reproductive success; however, they display great diversity in form and function across taxa. This diversity is perhaps greatest among fishes, with functions ranging from pheromone production for mate attraction to the enhancement of sperm competitive ability in response to post-copulatory competition. Despite this diversity, the role of the accessory gland in most fishes remains a mystery. Here, we explored the form and function of this organ in the plainfin midshipman fish, *Porichthys notatus*, a species with alternative reproductive tactics and a convenient model for investigating sperm competition. We studied tactic-specific investment in the accessory gland and its effects on sperm performance and morphology. We analyzed a five-year data set and showed that males adopting the larger 'guarder' tactic invested more in their accessory glands than did males of the smaller 'sneaker' tactic. We also found that guarder males invested more in one region (the lobules) of the accessory gland, while sneakers invested relatively more in another region (the nodes). We report that sperm swam faster in the presence of seminal fluid, which is in part produced by the accessory gland. Lastly, we show that seminal fluid increased sperm head size in guarder and sneaker males, and increased midpiece size in guarder males only. We propose that the accessory gland in plainfin

midshipman may have dual functions, one of which is enhancing sperm competitive ability. Our results improve our understanding of adaptations to post-copulatory competition and shed light on the largely unexplored role that non-sperm components like accessory gland fluid might play in the fertilization process.

INTRODUCTION

In addition to conventional pre-copulatory competition, males can also face competition in post-copulatory contexts in what is known as sperm competition (Parker, 1970). Geoff Parker's sperm risk model (1982) predicts that when a male faces the risk of his ejaculate competing with another male's ejaculate for a fertilization opportunity, he will increase his competitive investment. Species that exhibit discrete male alternative reproductive tactics (ARTs) are a useful testbed for sperm risk model. ARTs diverge in many important morphological, physiological, and behavioural traits (Taborsky, Oliveira, & Brockmann, 2008; Taborsky, 1998). A common ART dichotomy across taxa is the sneak-guard system (Parker, 1990). Guarder males are typically larger and behaviourally dominant, monopolizing females or resources desired by females, while smaller sneaker males approach a copulating pair using stealth to release ejaculate, steal fertilizations, and escape before detection (Taborsky, 1998). Because of their divergent reproductive strategies, these alternative male types also face different levels of sperm competition (Parker, 1990). Because sneaker males can only gain access to females and their eggs after a guarder male has attracted that female to a location, sneaker males always fertilize in the presence of a sperm competitor and usually do so from a disadvantaged position in relation to the guarder male. In contrast, guarder males are entirely capable of fertilization in the absence of any sperm competitors. Therefore, sneaker males encounter relatively more sperm competition risk, and in consequence, sperm risk model predicts that sneaker males will increase their investment in competitive ejaculate beyond the level observed in guarder males. Sneaker males can increase sperm competitive investment through a number of morphological and physiological changes, such as developing larger testes, producing more sperm per ejaculate, or producing faster swimming and/or longer lived sperm (Parker, 1990). A number of empirical studies across a variety of taxa have found support for these predictions (DelBarco-Trillo & Ferkin, 2006; Gage, Stockley, & Parker, 1995; Simmons et al., 2007).

The complexity of sperm investment strategies employed by male ARTs is further complicated by the existence of the male reproductive accessory gland. This gland is found across a number of animal taxa and has a wide variety of reproductive functions (Adiyodi & Adiyodi, 1988; Chowdhury & Joy, 2007; Hyman, 1992). A growing body of literature suggests that the male accessory gland plays a role in sperm competition. For example, several products of the *Drosophila melanogaster* accessory gland increase female sperm storage and utilization and protect sperm from displacement by competing sperm (Wolfner, 1997), and male *Drosophila* with larger accessory glands experienced greater reproductive success than competitors with smaller accessory glands (Wigby et al., 2009). In external fertilizers like many fish species, seminal fluid, which is produced in part by the accessory glands, can either have a tactic-specific beneficial or negative effect on sperm performance (Locatello, Poli, & Rasotto, 2013). For instance, the seminal fluid of grass goby (*Zosterisessor ophiocephalus*) guarder males has a positive effect on sperm performance for both guarder and sneaker male sperm, while the seminal fluid of sneaker males has a negative effect on guarder male sperm performance (Locatello et al., 2013). Surprisingly, comparative evidence across fishes suggests that the guarder male tactic usually invests more in accessory glands (Marentette et al., 2009; Mazzoldi & Rasotto, 2002; Neat, Locatello, & Rasotto, 2003; Neat, 2001; Oliveira, Canario, & Grober, 2001; Scaggiante et al., 1999; Utne-Palm et al., 2015). Researchers are still trying to understand why an organ that is thought to play a role in sperm competition has greater investment by the male tactic that faces less sperm competition risk, and why tactic-specific seminal fluid can have such different effects on sperm performance. Thus far, the influence of the accessory gland and the differences in its effects on sperm competition has only been investigated in detail in a single species – the grass goby.

The plainfin midshipman fish, *Porichthys notatus,* provides an interesting second model system for further study of the role of the accessory gland in sperm competition. Plainfin midshipman exhibit male ARTs and have a male reproductive accessory gland (Barni et al., 2001; Brantley & Bass, 1994; Fitzpatrick et al., 2015). Guarder males are larger and invest more in sound-producing muscles involved in mate attraction, while sneaker males invest relatively more in their testes (Brantley & Bass, 1994; Fitzpatrick et al., 2015). However, tactic-specific investment in accessory glands is currently debated. Barni et al. (2001) argue that guarder males possess more developed accessory glands based on histological sections of four fish. In contrast and based on a sample of 19 guarder and 16 sneaker males, Fitzpatrick et al. (2015) found that when controlling for body size, sneaker males invested more in accessory gland mass than did guarder males. Also, the accessory glands of plainfin midshipman secrete proteins called sialoglycoproteins or mucins (Barni et al., 2001). Mucins aggregate to form gels, and when released in ejaculates contribute to a thick, viscous consistency in the seminal fluid. Mucins have been shown to increase sperm longevity in the grass goby (Scaggiante et al., 1999). Beyond these findings, little is known about the function of the accessory gland and how this organ differs between alternative reproductive tactics in the plainfin midshipman.

To investigate the form and function of the accessory gland in the plainfin midshipman, we measured tactic-specific reproductive investment across a large sample of fish. We also measured total seminal fluid proteins as well as sperm performance and morphology in the presence and absence of seminal fluid in both male tactics. We predicted that if the accessory gland plays an important role in sperm competition, then sneaker males should invest more in this organ than guarder males and seminal fluid should enhance sperm performance and morphology. However, if instead the accessory glands play a more important role in mate attraction or parental care, then it should be courting, caring guarder males that invest more in this organ and seminal fluid should not necessarily enhance sperm performance or morphology as a response to sperm competition.

METHODS

Specimen collection

Guarder (n = 100) and sneaker (n = 50) males were collected from nests during low tide along the intertidal zone of Ladysmith Inlet, British Columbia, Canada (49°01′N, 123°83′W) between May 17 and July 22, 2016 and May 10 and June 25, 2017. Male tactic was identified in the field by the position of the male in the nest (guarder males are positioned centrally while sneaker males lie in the periphery of the nest), ventral body colour (guarder males are olive grey while sneaker males are yellow), and by body size, which is dimorphic between male types (Brantley & Bass, 1994). Male tactic was further identified after dissection by gonadosomatic index (GSI) and sonic muscle mass, which are also dimorphic traits. Discrete, non-overlapping measurements of body size, GSI, and sonic muscle mass investment between male types are established in early work on this species (Brantley & Bass, 1994). Males appearing intermediate in phenotype (guarder males less than 20 cm and sneaker males greater than 18 cm standard length) were not included in analyses. Males were transported to outdoor holding aquaria at the University of Victoria, British Columbia. Guarder and sneaker males were housed in tactic-specific 400L tanks in 13° C seawater and provided with individual shelters made of bricks. We also had accessory gland and testes mass data from a series of studies performed on an additional 101 guarder and 18 sneaker males that were collected at the same location at Ladysmith Inlet between May and July of 2010, 2011, 2013, 2014, and 2015.

Reproductive investment

To compare testes and accessory gland investment between male tactics, fish were administered a lethal dose of anesthetic MS-222, weighed to the nearest 0.1 g, measured for standard length from the tip of the head to the last vertebra to the nearest mm, and their testes and accessory glands were removed and weighed to the nearest 0.01 g. Differences in body and reproductive organ masses as a result of male tactic were assessed with ANOVAs. To assess whether males of different tactics invested differently in reproductive organs, ANCOVAs were employed using somatic mass (i.e. body mass – organ of interest mass) and male type as covariates. Whole accessory gland investment was analyzed using two separate analyses: 1) all males collected and dissected by a single researcher in 2016 and 2017, and 2) all males collected and dissected in 2010, 2011, 2013, 2014, and 2015 by a number of different McMaster University Aquatic Behavioural Ecology Lab (ABEL) researchers using different equipment and methods. We also determined that plainfin midshipman accessory glands possess two distinct regions: paired, opaque nodes, and numerous, transparent fluid-filled lobules (Fig. 6). To assess tactic-specific differences in investment of the two regions of the accessory gland, ANCOVAs were performed using accessory gland mass and male type as covariates. Data from 2016 and 2017 were used for this analysis because prior to 2016, the two accessory gland regions were not separately measured.

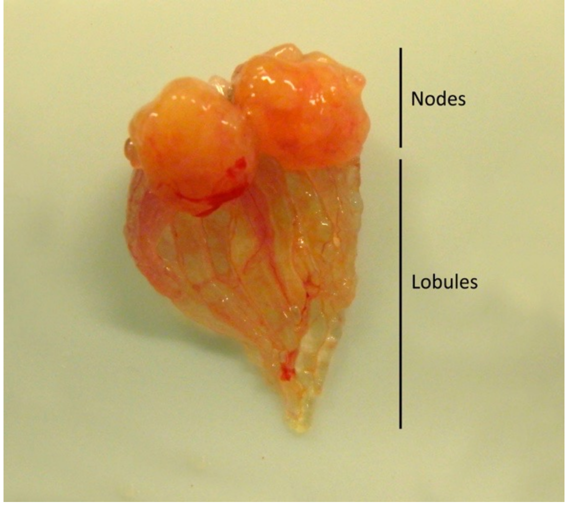

Figure 6. Dissected plainfin midshipman male reproductive accessory gland. The accessory gland nodes and lobules have been identified and labelled.

Sperm and milt collection

To examine sperm in the absence of accessory gland fluid, sperm was collected from the testes and compared to sperm stripped from milt (i.e. ejaculate), which contains seminal and accessory gland fluids. To collect sperm from the testes, fish were administered a lethal dose of anesthetic and either the right or left testis was removed depending on the analysis (e.g. right testis was always used for measuring sperm velocity). The testis was gently sliced open, and the sperm that pooled was collected. To collect sperm from ejaculates, fish were temporarily sedated and placed on their back, the genital area was dabbed dry, and a modified pipette tip was placed over the genital papilla, preventing urine from contaminating the sample. With the papilla held in the pipette tip, gentle pressure was applied to the abdomen along the testes. Pressure was applied until sufficient milt was collected or the fish ceased to release milt. Milt was often collected first, followed by sperm from the testis from the same fish a few days later.

Total seminal fluid proteins

To measure total proteins in seminal fluid, milt collected from 17 guarder and 11 sneaker males was spun in microcapillary tubes in a ZIPCombo Zipocrit portable centrifuge (LW Scientific, Lawrenceville, GA) for 10 min at 7000 rpm to separate seminal fluid. Following separation from sperm, microcapillary tubes were carefully broken to access only the seminal fluid, which was expelled via pipette bulb into cryovials and immediately stored at – 80° C. Total seminal fluid protein concentrations were then determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Sperm density

To investigate tactic-specific differences in ejaculate investment, sperm density in milt was measured in 10 guarder and 13 sneaker males. A known volume of collected milt was diluted and fixed with a 1:2 solution of buffered formalin and filtered seawater. Then 10 uL of fixed milt sample was pipetted into a Neubauer chamber haemocytometer. Video of the four 1 mm^2 squares, each located in the four corners of the haemocytometer grid was captured under 200x magnification by a Lumenera Infinity HD camera mounted on a Leica DME compound light microscope (Leica Microsystems Inc., Buffalo, NY). This procedure was then immediately repeated with a second sample from the remaining preserved milt sample kept at 13° C, providing an analytical repetition. Later, individual sperm cells were counted in each 1 mm^2 square using FlySketch software overlain on video viewing software by an observer unaware of each male's identification/tactic. Sperm counts for each repetition per 1 mm^2 square were averaged and sperm density was calculated for each sample. Tactic-specific differences in sperm density were assessed with ANOVA.

Sperm performance

Sperm velocity was measured until spermatozoa ceased swimming to determine if sperm performance differs in the presence or absence of seminal and accessory gland fluids. To do so, sperm from milt and sperm from testes sampled from 19 guarder and 19 sneaker males were diluted with filtered seawater and immediately incubated at 13° C. Then 10 uL of sample was pipetted into the chamber of a 2X-Cel glass slide (Hamilton Thorne, Beverly, MA) pre-rinsed in 1% bovine serum albumin solution to prevent sperm from sticking to the slide or coverslip. Video recordings of sperm movement were collected at 3 min post-activation and approximately every 15 min following until the observed sperm ceased achieving forward progression. Video was captured at 60 frames/s using the equipment described in the sperm density methods. Later, videos were only analyzed if at least 5 sperm cells were visible and achieving forward progression. Video was analyzed in CEROS sperm tracking software (HTM-CEROS version 12.3, Hamilton Thorne Biosciences) and calculated average sperm path velocity (VAP) was used to represent sperm velocity. Differences in sperm velocity at 3 min post-activation as well as total sperm longevity (i.e. the time at which sperm ceased achieving forward progression) as a result of male tactic and sperm origin were assessed using linear mixed models (LMMs) with individual fish ID as a random factor to control for repeated measures.

Sperm morphology

Differences in sperm morphology as a result of male tactic and presence of seminal fluid were assessed. Sperm from both milt and testes samples from 14 guarder and 14 sneaker males collected in 2016 and 2017 were diluted and fixed using a 1:2 solution of buffered formalin and filtered seawater. At least 10 individual sperm cells per sample were digitally photographed at 1000x magnification on welled slides (MP Biomedicals, Santa Ana, CA) using the microscope equipment previously described. Plainfin midshipman sperm have helical heads and two tails (Fitzpatrick et al., 2015; Stanley, 1965; Fig. 7), a unique morphology that is relatively rare among fishes (Jamieson, 1991). Due to this unique morphology, the surface area of sperm heads was measured instead of the head length and/or width. By measuring surface area, we capture both length and width in one measurement and reduce the number of total morphological parameters analyzed, increasing statistical power in detecting true morphological changes. Sperm head surface area was of particular interest due to the head shape's influence on hydrodynamic movement (Humphries et al., 2008). The number of head turns or gyres was also measured as more helical sperm heads may contribute to greater forward movement and allow sperm to swim in straighter pathways, especially in viscous environments (Pitnick et al., 2009; Vernon & Woolley, 1999). Plainfin midshipman midpieces are tapered in shape, so midpiece surface area was also measured (Fig. 7). Midpiece size can represent available adenosine triphosphate (ATP) or energy production and storage capability of the sperm cell (Cardullo & Baltz, 1991). Flagellum or tail length, reflecting propulsive force capability (Montserrat Gomendio & Roldan, 1991) was also measured for both tails, and an average tail length calculated per sperm cell.

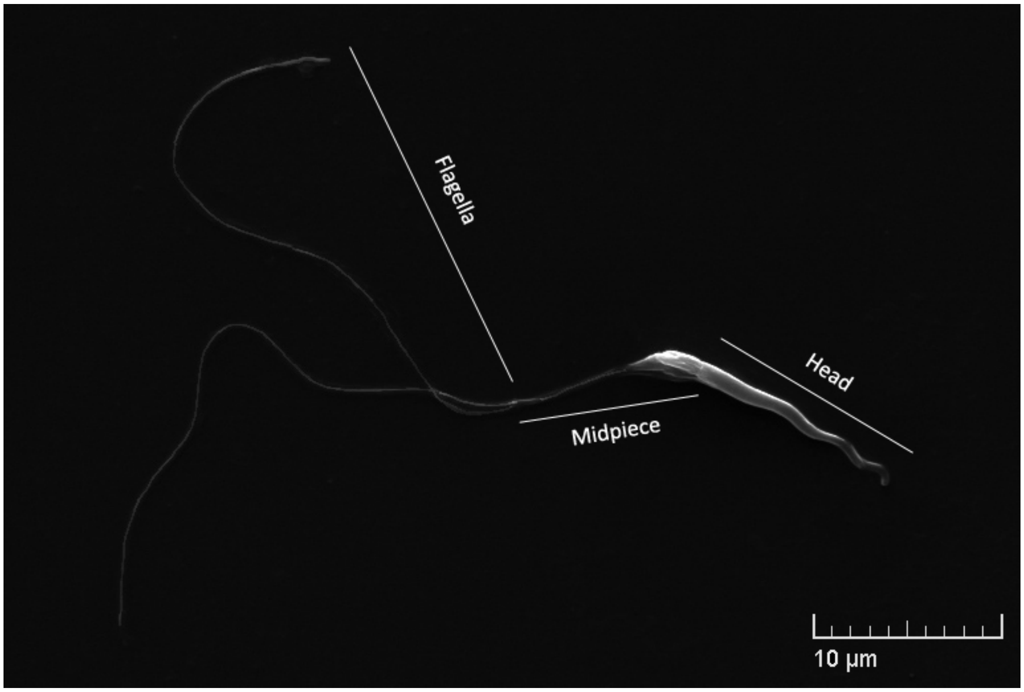

Figure 7. Scanning electron microscope image of a single plainfin midshipman sperm cell taken at 4000x magnification. The helical head, midpiece, and biflagellate regions are labelled.

Finally, the number of sperm head turns per micron of head length and the ratio of sperm head length to tail length were calculated. The number of sperm head turns per length of head was calculated to analyze number of heads turns while taking sperm head length into account. Total sperm length positively correlates with velocity in many species (Montserrat Gomendio & Roldan, 1991); however, the ratio of head length to tail length is often preferred as it is thought to represent the ratio of physical drag to propulsion in biomechanical modeling (Humphries et al., 2008), and provides a better correlate of sperm swimming speed (Simpson et al., 2014).

Photographs were analyzed in NIH ImageJ software (v. 1.50i) using a digital tablet (Wacom Co. Ltd., Japan). Differences in head surface area, number of sperm head turns per micron of head length, midpiece surface area, and tail length as a result of male tactic and sperm origin were assessed using a multivariate linear mixed model (MLMM), followed by univariate linear mixed models (LMM), using individual fish ID as a random factor to control for repeated measures. Differences in the ratio of head length to tail length as a result of male tactic and sperm origin were assessed with a linear mixed model separately due to differences in data scaling against other measured sperm components. Data from 2016 and 2017 were pooled as no differences among any sperm morphological traits as a result of year were detected.

Statistical analysis

All statistical analyses were performed in R version 3.4.1 (R Foundation for Statistical Computing) and significance was assessed at $\alpha = 0.05$. Data was log or power transformed when necessary to achieve normality and homoscedasticity. All measurements were made by observers blind to the tactic ID of the male/sperm sample in question.

Animal ethics

Plainfin midshipman fish are a common intertidal species and their populations are not threatened. All fish were collected in accordance with permits issued by the Canadian Department of Fisheries and Oceans (scientific licenses XR 94 2016 and XR 58 2017). All research procedures were approved by the McMaster University Animal Research Ethics Board (AUP #13-12-52) and the University of Victoria Animal Care Committee (Protocols 2015-009[1] and 2017-003[1]).

RESULTS

Investment in testes and accessory glands

Guarder males ($\bar{x}$ = 169 g) were 6.5 times heavier than sneaker males ($\bar{x}$ = 26.8 g) ($F_{1,149}$ = 1251, $p < 0.001$). Absolute testes mass did not differ between male tactics (ANOVA, $F_{1,149}$ = 0.22, $p = 0.60$); however, sneaker males invested more in testes mass relative to body mass than guarder males (ANCOVA, $F_{1,148}$ = 29.4, $p < 0.001$). In 2016 and 2017, guarder males had larger accessory glands (ANOVA, $F_{1,149}$ = 500, $p < 0.001$), but investment in accessory gland mass relative to body mass did not differ between male tactics (ANCOVA, $F_{1,148}$ = 0.85, $p = 0.30$; Fig. 8a). In contrast, in the 5-year data set collected previous to 2016, guarder males exhibited greater investment in whole accessory gland mass (ANCOVA, $F_{1,100}$ = 41.9, $p < 0.001$). Investment in individual regions of the accessory gland were highly divergent between male tactics. Sneaker males invested more in accessory gland nodes mass (ANCOVA, $F_{1,95}$ = 28.5, $p < 0.001$; Fig. 8b), while guarder males invested more in accessory gland lobules mass (ANCOVA, $F_{1,95}$ = 37.8, $p < 0.001$; Fig. 8c).

Total seminal fluid proteins

We found no difference in protein concentration in seminal fluid collected from guarder and sneaker males (ANOVA, $F_{1,26}$ = 0.1, $p = 0.76$). On average, guarders produced 1666 μg/ml (range: 1021 – 2125 μg/ml) seminal fluid proteins and sneaker males produced 1693 μg/ml (1505 – 2138 μg/ml) seminal fluid proteins.

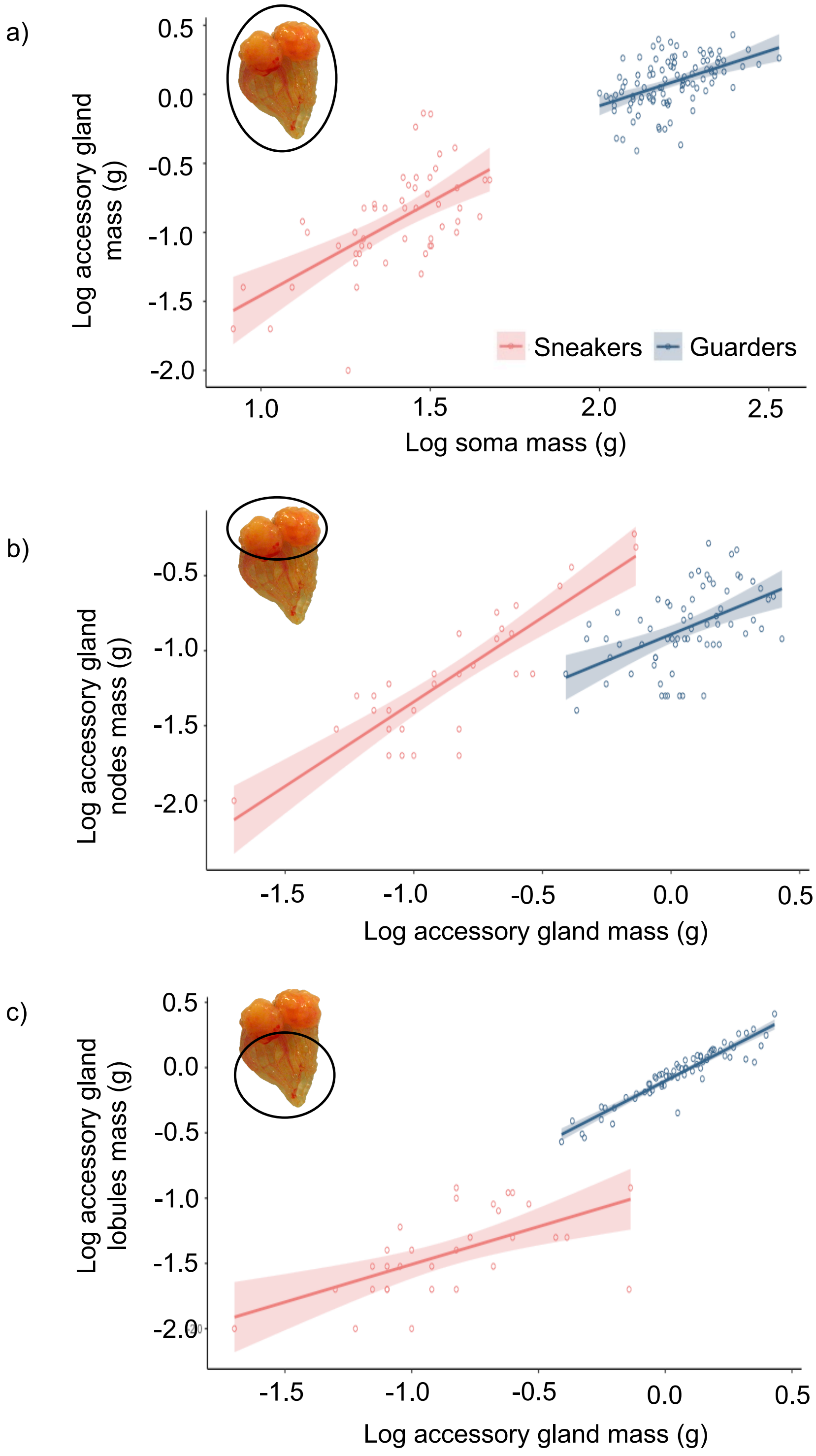

Figure 8. Male tactic-specific investment differences in a) whole accessory gland mass, b) accessory gland nodes mass, and c) accessory gland lobules mass.

Sperm density and sperm performance

Sneaker males produced ejaculates with approximately 3 times more sperm than guarder males (ANOVA, $F_{1,21} = 8.7$, $p = 0.008$; Fig. 9). No tactic-specific differences were observed in the rate of change in sperm velocity over time (Fig. 10). Tactic-specific sperm velocity differences were greatest at 3 min post-activation; however, these differences were not significant (LMM, $X^2 = 0.19$, $p = 0.66$; Fig. 11a). We also found no differences in sperm longevity (LMM, $X^2 = 1.99$, $p = 0.16$; Fig. 11b) as a result of male tactic, but sperm collected from milt were both faster (LMM, $X^2 = 4.03$, $p = 0.045$; Fig. 11a) and swam for shorter durations than sperm collected from testes (LMM, $X^2 = 7.4$, $p = 0.006$; Fig. 11b).

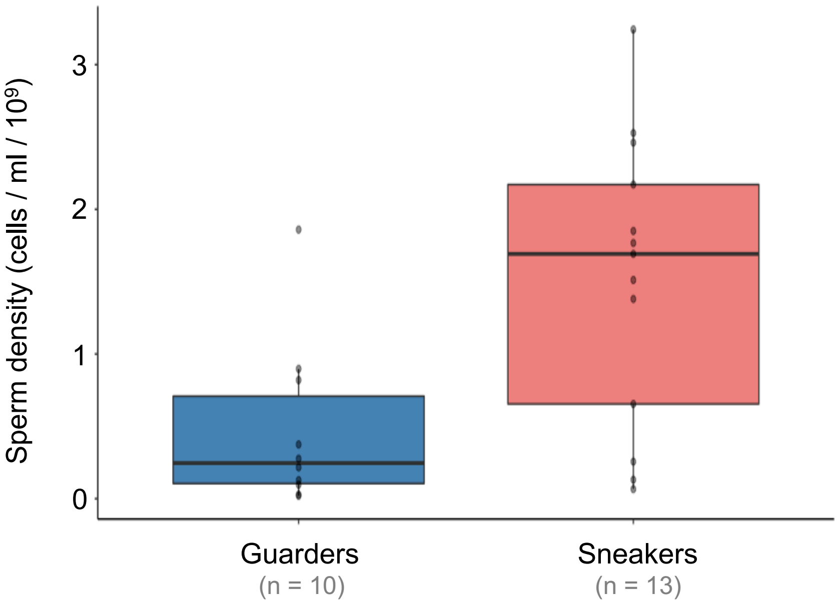

Figure 9. Sperm density in milt of guarder and sneaker males.

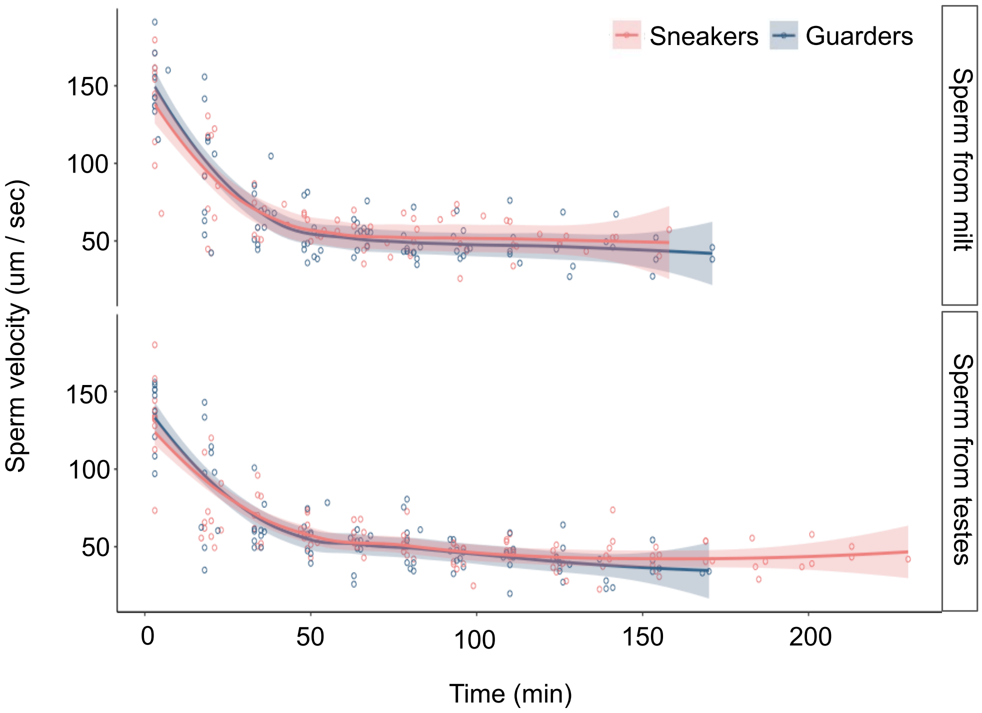

Figure 10. Sperm velocity over the duration of sperm swimming longevity in samples from milt and from the testes in both guarder and sneaker males.

Sperm morphology

We found that sperm morphological features changed with sperm origin (testes or milt) (MLMM, df = 4, $x^2 = 25.5$, $p < 0.001$), but not with male tactic (MLMM, df = 8, $x^2 = 14.2$, p = 0.07), nor with the interaction between male tactic and sperm origin (MLMM, df = 4, $x^2 = 7.32$, p = 0.1). Univariate models revealed that sneaker males had more sperm head turns per µm of sperm head length than guarder males (LMM, $x^2 = 5.15$, p = 0.02; Table 2; Fig. 12). Sperm in milt had larger heads than did sperm collected from testes (LMM, $x^2 = 9.34$, p = 0.002; Table 2). Also, guarder males had larger midpieces (LMM, $x^2 = 4.48$, p = 0.03; Table 2), and guarder male midpieces were larger in milt than from the testes (LMM, $x^2 = 4.53$, p = 0.03; Table 2).

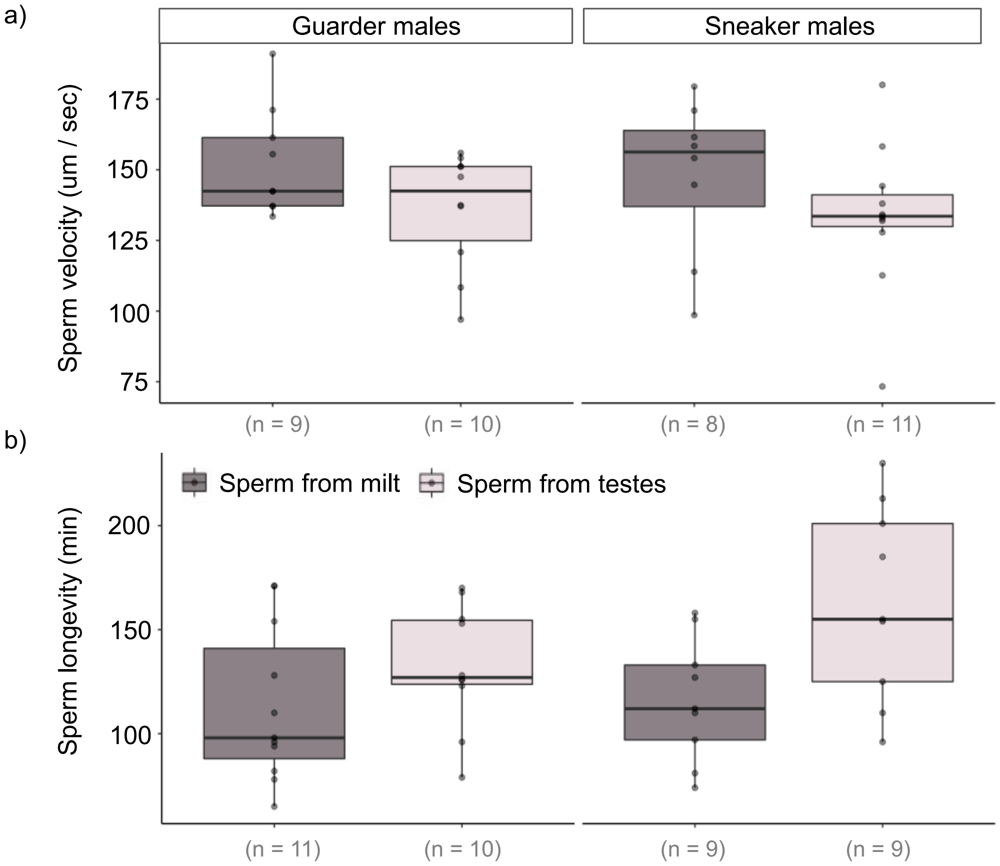

Figure 11. Differences as a result of seminal fluid presence in a) sperm velocity at 3 min post-activation and b) sperm longevity, or total sperm swimming duration.

Table 2. Summary of univariate linear mixed model analyses on individual sperm morphological features.

	Fixed effects					
	Male tactic		Sperm origin		Male tactic : sperm origin interaction	
Response	X^2	p	X^2	p	X^2	p
Head surface area	1.48	0.22	9.34	**0.002**	0.01	0.91
Number of head turns / head length	5.15	**0.02**	0.08	0.77	0.34	0.53
Midpiece surface area	4.48	**0.03**	1.6	0.21	4.53	**0.03**

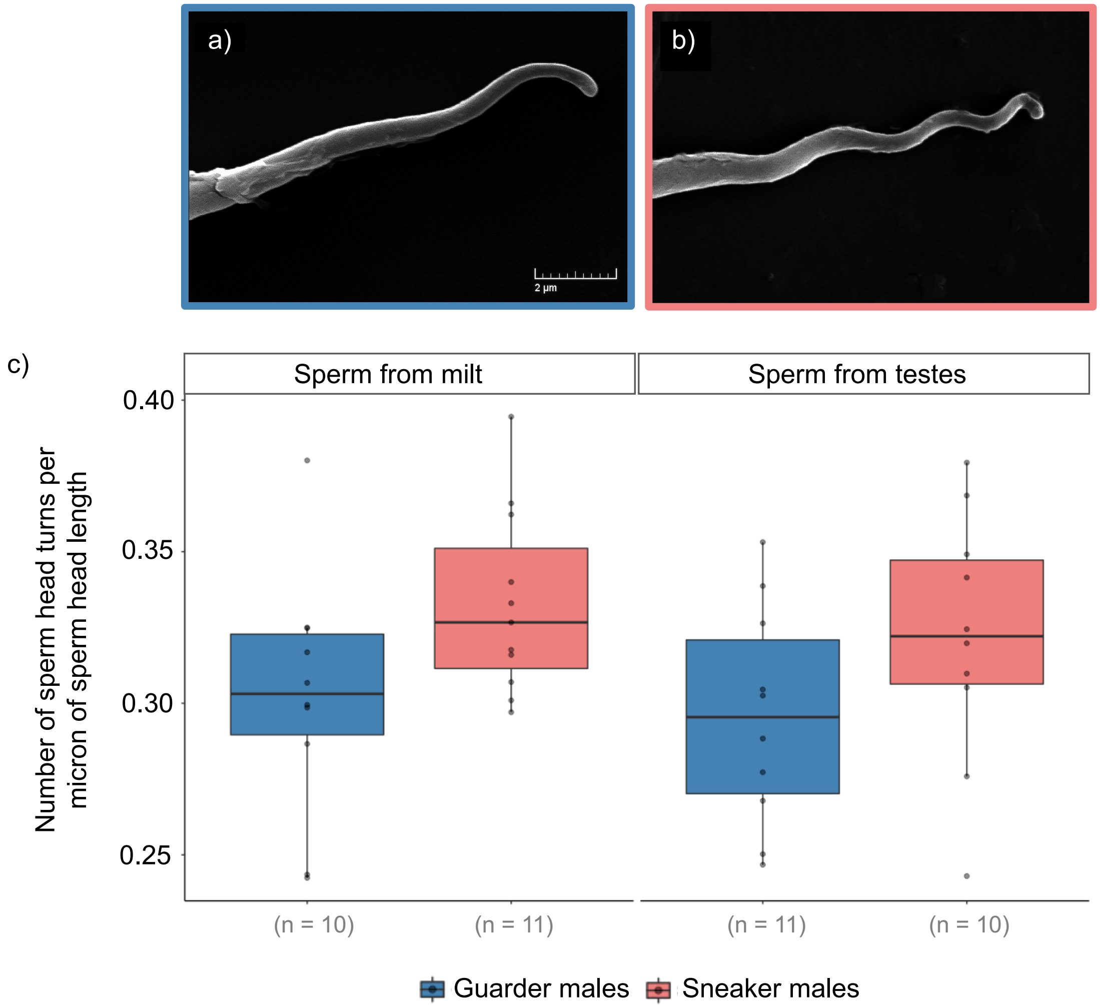

Figure 12. Examples of helical plainfin midshipman sperm heads with a) few turns and b) many turns, taken using scanning electron microscopy at 20,000x magnification, and c) differences in number of sperm head turns per micron of sperm head length between male types in sperm from both milt and testes.

DISCUSSION

The results of this study reveal the possible role of the male reproductive accessory gland in post-copulatory competition between alternative reproductive tactics (ARTs) in the plainfin midshipman. Increasing sperm velocity and modifying sperm morphology to potentially maximize performance, the seminal fluid, secreted in part by the accessory glands, benefits male competitive ability. These findings provide further insight into the effects of seminal fluid on sperm competition, which to date has been investigated in only three other fish species: the grass goby (Locatello et al., 2013), Chinook salmon, *Oncorhynchus tshawytscha* (Lewis & Pitcher, 2016), and the Arctic charr, *Salvelinus alpinus* (Rudolfsen et al., 2015). These findings are also the first report of the effects of seminal fluid on sperm morphology. Not only are these findings significant to a growing literature on sperm competition in the biologically-relevant context of a seminal fluid-added environment, but they also contribute to the literature on divergent morphology and competition between male reproductive tactics.

Tactic-specific reproductive investment

Investment in the whole accessory gland appears to be often greater in guarder males. Assuming that the accessory glands directly play a role in sperm competition, this investment by guarder males is surprising and contrary to Parker's sperm risk model. Because sneaker males experience greater sperm competition risk, their expected investment in reproductive organs benefitting their competitive ability is greater than guarder males. However, across fishes, the guarder male tactic in fact invests more in their accessory glands (Marentette et al., 2009; Mazzoldi & Rasotto, 2002; Neat et al., 2003; Neat, 2001; Oliveira et al., 2001; Scaggiante et al., 1999; Utne-Palm et al., 2015).

This finding may not be as surprising when we consider the idea that the accessory glands could have a dual function, with each region serving a different purpose. We found that guarder males invest more in the lobules of their accessory glands, while sneaker males invest more in accessory gland nodes. In some fishes, the accessory glands plays a role in mate attraction by secreting steroid pheromones (Arbuckle et al., 2005; Hong et al., 2006; Jasra et al., 2007; Locatello, Mazzoldi, & Rasotto, 2002; Resink et al., 1987; Serrano et al., 2008). In other fishes, the accessory glands play a role in parental care by producing antimicrobial compounds that reduce infection-caused offspring mortality (Giacomello et al., 2008; Giacomello, Marchini, & Rasotto, 2006). Perhaps the nodes play a role in sperm competition to the benefit of the sneaker males that invest more heavily in this region, while the lobules play a role in mate attraction and/or parental care, a function that would provide more benefit to the guarder males that invest more heavily in lobules. Future research directed at understanding the role of each accessory gland region and their impacts on fertilization, sperm competition, mate attraction, and parental care is necessary to better understand these reproductive investment differences between male tactics in the plainfin midshipman and other fishes with similar accessory glands. At present, data has already been collected to test the impact of fluid from each region of the accessory gland on sperm performance in the plainfin midshipman, and this data is awaiting analysis. Accessory gland tissue and fluid has also been collected in order to investigate potential roles of this organ in parental care (i.e. does either region secrete antimicrobial compounds?) and/or mate attraction (i.e. does either region secrete pheromones?).

Seminal fluid influences sperm performance

Seminal fluid simultaneously increased sperm velocity and decreased sperm longevity in the plainfin midshipman. In the grass goby, a species with ARTs and accessory glands, guarder males with larger accessory glands produced both faster and longer-lived sperm (Lisa Locatello et al., 2013; Scaggiante et al., 1999). In the plainfin midshipman, perhaps a trade-off between sperm velocity and longevity exists. In addition, grass gobies produce thick ejaculates called sperm trails, which are greatly thickened by significant amounts of gel-forming mucins that prolong sperm longevity (Scaggiante et al., 1999). It is possible that plainfin midshipman males do not produce enough mucins in their seminal fluid to greatly increase sperm longevity like the seminal fluid of grass gobies. This an area that requires further testing.

Seminal fluid changes sperm morphology

Sneaker males produced sperm with more head turns or more tightly helical sperm heads when compared to guarder males. This is a novel finding that contributes to the currently small body of literature on sperm morphological differences between male ARTs in a species. Consistent with sperm risk model, more helical sperm heads may provide an advantage to sneaker males as they theoretically increase forward movement and pathway straightness in viscous environments (Pitnick et al., 2009; Vernon & Woolley, 1999). This morphology may benefit sneaker males who release ejaculates into an environment already occupied by the ejaculates of guarder males, which may be thick with mucins. A more helical sperm head may also aid in egg penetration during fertilization (Thompson, 1966).

This study also is the first investigation into the effects of seminal fluid on sperm morphology. We found that seminal fluid increased head size in both tactics and increased midpiece size in guarder males only. While we do not know how such changes in morphology affect sperm velocity in this species or other species, we can speculate on the possible benefits of these seminal fluid-induced changes.

Seminal fluid increased the surface area of sperm heads in both guarder and sneaker males. In some internal fertilizers, a greater head to tail ratio increased sperm velocity, but how this morphology produces this effect is not understood (Lüpold et al., 2012; Simpson et al., 2014). It is likely that the microenvironment of the female reproductive tract selects for this type of morphology in the context of sperm competition (Lüpold et al., 2012; Simpson et al., 2014). If the plainfin midshipman's sperm morphology mirrors the successful morphology observed among internal fertilizers, then perhaps plainfin midshipman sperm have adapted to a fertilization environment that is similar in some respects to the internal environment of a female's reproductive tract. In what ways might the fertilization environment for plainfin midshipman be similar to the environment for an internal fertilizer? As proposed earlier, the fertilization environment could be highly viscous due to the high mucin content in seminal fluid – akin to the reproductive tract's viscous environment. Also, because this species fertilizes in a small hollow that encounters widely fluctuating intertidal environmental conditions (e.g. periodic flooding, desiccation, changes in pH, dissolved oxygen, temperature, etc.), the fertilization environment is likely highly inhospitable to sperm, much like a female reproductive tract (Arora, 1948; LeMoine et al., 2014). Together, these findings provide a possible explanation for the seminal fluid-driven sperm head enlargement observed in this species. To investigate the validity of these ideas, future studies should attempt to measure the hydrodynamic movements of sperm of different morphologies in naturalistic environments (e.g. *in vivo* in internal fertilizers if possible). Such studies could determine what specific morphologies fare best in their respective fertilization microenvironments and how.

Seminal fluid also increased midpiece size in guarder males. We also found that guarder males produced sperm with larger midpieces than sneaker males regardless of the presence of seminal fluid. While the size of the midpiece has traditionally been thought to translate to the amount of stored energy a sperm cell possessed for movement, and therefore the potential velocity of a sperm cell, this theory has not found consistent empirical support. For example, red deer (*Cervus elaphus*) sperm with shorter midpiece swim faster (Malo et al., 2006), and zebra finch (*Taeniopygia guttata*) sperm with smaller midpieces actually possess more ATP (Bennison et al., 2016). It's difficult to interpret the benefits a larger sperm midpiece might have for plainfin midshipman guarder males without an understanding of the relationship between midpiece surface area and energetic capability (e.g. amounts of ATP, glycogen, etc. produced and stored in the midpiece). Because it's more likely that sneaker males produce sperm with more competitive morphology due to their higher risk of sperm competition, it's possible that sperm with smaller midpieces gain a competitive advantage in this species. Perhaps guarder males can "afford" to have larger midpieces due to their closer proximity to females and less risk of sperm competition. Future tests of the relationship between midpiece size and energetic capability would provide some answer to the question of why only guarder males produce sperm with larger midpieces.

Lastly, the mechanisms potentially responsible for these seminal fluid morphological modifications remain understudied. In humans, the kallikrein-related proteolytic cascade and prostate-specific antigen action in seminal fluid is responsible for the modification and removal of seminal fluid proteins that increase sperm motility (Veveris-Lowe et al., 2007). However, only three studies to date have identified specific seminal fluid proteins in fishes (Gombar et al., in press; Dietrich et al., 2014; Nynca et al., 2014) and none have investigated the functions of those proteins. Given that we know that protein composition differed between male ARTs in at least one species (Gombar et al., in press), and we now report that seminal fluid has different effects on morphology between tactics in this species, despite the fact that guarder and sneaker males do not differ in the amounts of their seminal fluid proteins, it's worth questioning whether plainfin midshipman male tactics have different or differently functioning morphology-modifying seminal fluid proteins. To date, seminal fluid from both plainfin midshipman male tactics, as well as fluid from both regions of the accessory gland has been collected in order to undertake a proteomics analysis. Such an analysis may shed light on sperm performance-influencing proteins produced by the accessory gland.

Conclusion

This study generated as many questions as it answered. Most importantly, isolating the specific effects on sperm of accessory gland fluid from both regions of the organ will help to resolve the concern that the whole seminal fluid has different effects than the portion of the seminal fluid secreted by the accessory glands. Resolving this issue will allow us to better understand the function of the accessory gland regions to then investigate why investment in this specialized organ differs between male tactics. If indeed this organ has dual functions performed by the two regions of the gland (e.g. aiding in parental care or mate attraction), planned future experimentation such as an antimicrobial assay of accessory gland fluid from both regions or behavioural trials on conspecifics in the presence of accessory gland fluid from both regions in the environment could provide interesting insight into these potential functions, in addition to providing a more biologically-relevant context for understanding sperm competition and fertilization dynamics.

This study has obvious implications for aquaculture and fisheries managers, as well as for conservationist biologists. An understanding of the effects of seminal fluid on sperm performance and potentially on offspring development should be incorporated into breeding and rearing practices as this could improve fertilization success and egg survival in commercially important fish stocks. For similar reasons, a thorough understanding of what factors maximize reproductive success in species with accessory glands is of great importance to conservation efforts, where successful breeding of endangered species may rely on this information. Beyond the applications of this research, studying sperm competition in a more biologically relevant context is an interesting and worthwhile goal. The study of sperm performance in the presence of seminal fluid brings us closer to understanding the true dynamics of fertilization and post-copulatory competition.

ACKNOWLEDGEMENTS

We would like to thank Joanna Cunanan, Ephraim David, and Vanessa Ng for their hard work counting and measuring sperm. Thank you to Noah Houpt and Emilie Sadler for their assistance in the field and lab. Thank you to Trevor Pitcher, Mariella Rasotto, Carlotta Mazzoldi, and Kim Mitchell for their instruction and guidance on laboratory sperm analysis techniques. Many thanks to the University of Victoria OAU and Animal Care staff as well as Francis Juanes, Kieran Cox, John Taylor, and Jeurgen Ehlting for making this work possible and lending us equipment. We'd also like to thank Ben Bolker and Jonathan Dushoff for their statistical support, Stjepan Gotic for data processing and coding help, and Donna Waxman for assistance in electron microscopy image editing. This research was supported by grants from the Natural Science and Engineering Research Council of Canada (to SB), the American Museum of Natural History (to JSM), and from the Dept. of Psychology, Neuroscience & Behaviour and the School of Graduate Studies at McMaster University (to JSM).

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CHAPTER 3

THE ROLE OF ACCESSORY GLANDS IN PLAINFIN MIDSHIPMAN SPERM COMPETITION AND SOME FUTURE DIRECTIONS

SUMMARY

This thesis sought to answer the following questions: 1) is there tactic-specific investment in the accessory gland, and 2) does this organ play a role in sperm competition. In Chapter 2, these questions were partly addressed. In this concluding chapter, I summarize the answers to these questions, review the main conclusions of my research, and address the implications of my findings by placing them in a broader context. I end the chapter and thesis by identifying future questions that naturally expand on the research conducted in this thesis.

IS THERE TACTIC-SPECIFIC INVESTMENT IN THE PLAINFIN MIDSHIPMAN ACCESSORY GLAND?

As indicated in the first two chapters of this thesis, prior to my work, two other researchers had investigated tactic-specific investment in the plainfin midshipman accessory gland and reached opposite conclusions (Barni et al., 2001; Fitzpatrick et al., 2015). Barni et al. (2001) argued that guarder males invested more in their accessory glands based on histological differences observed in four males (the number of each male type is not outlined in the paper). In contrast, Fitzpatrick et al. (2015) reported that sneaker males invested more in this organ based on accessory gland mass quantification from 19 guarder and 16 sneaker males. My own research is based on 100 guarder and 50 sneaker males collected in 2016 and 2017 and this sample revealed that while investment in the *whole* accessory gland did not differ between plainfin midshipman male types, there was differential investment in specific regions of the accessory gland. Sneaker males had much larger accessory gland *nodes*, while guarder males had much larger accessory gland *lobules*. My results of differential investment in the two regions of the accessory glands are novel and unique. Although my sample did not show tactic specific differences in the investment of the whole gland, a multi-year sample of 119 males collected between 2010 and 2015 by members of the McMaster Aquatic Behavioural Ecology Lab (ABEL) research group did show that guarder males invested more in their whole accessory gland. This finding is consistent with those of Barni et al. (2001) as well as the majority of reports on tactic-specific accessory gland investment in other fishes (Marentette et al., 2009; Mazzoldi & Rasotto, 2002; F. C. Neat, Locatello, & Rasotto, 2003; Neat, 2001; Oliveira, Canario, & Grober, 2001; Scaggiante et al., 1999; Utne-Palm et al., 2015).

WHAT IS THE FUNCTION OF THE PLAINFIN MIDSHIPMAN ACCESSORY GLAND?

It is tempting to hypothesize that there may be more than one physiological function of the plainfin midshipman accessory gland and that this is the reason that the two different regions of this organ exist. For example, the accessory glands nodes may be responsible for secreting products in the seminal fluid that improve sperm performance, a benefit likely enjoyed by sneaker males that invest more heavily in this region. Seminal fluid, produced in part by the accessory glands, increased sperm velocity, an effect that appears to be driven most by changes in sneaker male sperm velocity, and induced sperm morphological changes that may improve sperm performance. It is currently unknown which region of the accessory gland is responsible for most secretory activity resulting in seminal fluid production. It is also possible that the lobular region of the accessory gland may be responsible for secreting other products that may specifically benefit guarder males that invest more in this region, such as antimicrobial compounds that could aid in parental care or pheromones that operate to attract females. This may explain why guarder males invest so much in these glands.

There has been evidence of dual functions of accessory glands in other fishes. For example, the sperm duct accessory gland of the grass goby plays both a role in sperm competition and parental care (Giacomello et al., 2008; Locatello, Poli, & Rasotto, 2013), although the grass goby accessory gland does not exhibit multiple, distinct regions (Lahnsteiner et al., 1992). Antimicrobial compounds secreted by the grass goby accessory gland protect eggs developing in muddy environments susceptible to infection (Giacomello et al., 2008). Plainfin midshipman also care for offspring in muddy environments where bacterial or fungal infections are responsible for a significant proportion of offspring mortality (personal observation, Fig. 13). The harsh nesting conditions of the intertidal zone where egg and young are exposed to hypoxia, desiccation risk, high ammonia levels, and elevated temperatures may have selected for a physiological mechanism that would reduce offspring mortality. The Lusitanian toadfish, *Halobatrachus didactylus*, a closely related species to the plainfin midshipman has a sperm duct accessory gland with three distinct regions that produces both mucins and steroids, which may act as short-distance pheromones (Modesto et al., 2015). In this species, type I (guarder) males invest more in the size of their accessory glands. More interestingly, accessory gland steroidogenic activity was greater in type I males than in type II (sneaker) males, and type I males exclusively produced at least five steroids not produced by type II male accessory glands (Modesto et al., 2015). This finding supports the idea that regions of the accessory gland may be invested in differently by alternative male types to achieve specific physiological functions. However, more work will be needed to investigate the roles of the two accessory gland regions identified in the plainfin midshipman. Data has been collected to test the impact of fluid from each region of the accessory gland on sperm performance in the plainfin midshipman and this data currently awaits analysis.

Figure 13. Upturned plainfin midshipman nests containing healthy eggs (above) and eggs dead or dying of infection (below).

In order to investigate potential additional functions of the plainfin midshipman accessory gland, two different studies need to be performed. Antimicrobial assays on accessory gland tissue have been previously employed for three goby species (Giacomello et al., 2008; Giacomello, Marchini, & Rasotto, 2006), as well as on the seminal fluid of other taxa (Peng et al., 2016). Comparable assays could be used to study the antimicrobial activity of both regions of the plainfin midshipman accessory gland. Accessory gland tissues and fluids have already been collected with the intention of conducting such an assay. Similarly, steroidogenic activity of both accessory gland regions should be measured via chromatography and/or mass spectrometry (Arbuckle et al., 2005; Asahina et al., 1989; Schoonen et al., 1987). If steroidogenic activity is identified in the plainfin midshipman accessory gland, the role of these steroids in mate attraction and other behavioural responses to pheromones could be further tested in behavioural experiments. For instance, behavioural responses in conspecifics after exposure to ejaculates or accessory gland secretions could be measured to determine if identified steroids appear to act as pheromones. (Locatello, Mazzoldi, & Rasotto, 2002; Resink et al., 1987; Serrano et al., 2008). Similar experiments could be performed using the plainfin midshipman.

DOES SEMINAL FLUID ENHANCE SPERM PERFORMANCE?

As discussed in Chapter 2, plainfin midshipman can now be added to the short list of taxa that produce seminal fluid that aids in sperm performance and potentially sperm competitive ability. While this new addition to the literature increases our understanding of the role of accessory glands in sperm competition across species, we can’t accurately identify which region of the accessory gland produces the beneficial seminal fluid, nor that the accessory gland is wholly responsible for seminal fluid production in this species. While it’s highly unlikely the accessory gland does not contribute in part to the production of seminal fluid in the plainfin midshipman, it must be acknowledged that the secretory activity of this species' reproductive anatomy has not been studied in detail. In order to overcome this deficiency, identifying the physiological machinery behind changes to sperm performance and establishing whether the accessory gland is responsible for this mechanism is still necessary. Identifying the mechanism will confirm 1) whether the accessory glands are involved in sperm competition, 2) if they are, what specific tissues are involved, and 3) how these tissues differ between male types.

Seminal fluid proteins are known to affect sperm performance across taxa, namely by regulating sperm capacitation, swimming velocity, longevity, motility, viability, and by facilitating allospermicidal processes and mating plug formation (Lahnsteiner, Mansour, & Berger, 2004; Minelli, Moroni, & Castellini, 2001; Mochida et al., 1999; Poiani, 2006; Scaggiante et al., 1999). For example, specific seminal fluid proteins in rats are the primary substrate for the process that results in the production of a mating plug, which prevents future sperm competitors from successfully fertilizing (Seitz & Aumuller, 1990). In fishes, glycoproteins inhibit sperm motility in Nile tilapia, *Oreochromis niloticus* (Mochida et al., 1999) and make up one of three groups of proteins likely responsible for positively affecting sperm viability in rainbow trout, *Oncorhynchus mykiss* (Lahnsteiner, 2007). A number of studies have examined the sperm-modifying mechanisms of specific seminal fluid proteins across taxa, but perhaps this has been achieved most comprehensively in insects and mammals (Kordan et al., 1999; Nauc & Manjunath, 2000; Poiani, 2006; Reinert et al., 1997). Unfortunately, we still know very little about the functions of specific seminal fluid proteins in fishes. Seminal fluid proteins have been thoroughly identified and quantified in only three fish species (Chinook salmon, *Oncorhynchus tshawytscha*: Gombar et al., in press; rainbow trout: Nynca et al., 2014; and common carp, *Cyprinus carpio*: Dietrich et al., 2014), yet their roles in fertilization and competition have yet to be studied. We can currently only guess at some of the functions of these proteins based on their roles in other areas of the body where they appear, such as the blood. We also know that seminal fluid protein composition can differ between males of different reproductive tactics in a single species, but the physiological consequences of these differences is very much an area of active research (Chinook salmon: Gombar et al., in press). The plainfin midshipman fish would be an excellent candidate for further studying tactic-specific seminal fluid protein compositions, and how specific proteins influence fertilization success and sperm competitive ability. Seminal fluid as well as fluid from both regions of the plainfin midshipman accessory gland has already been collected in order to undertake such a proteomics analysis.

HOW DOES SEMINAL FLUID INFLUENCE SPERM PERFORMANCE?

Seminal fluid induced morphological changes in plainfin midshipman sperm, but how these changes occur remains unknown. Perhaps a bigger question related to understanding this mechanism is whether specific seminal fluid proteins modify the morphology of sperm or the immediate environment surrounding sperm to produce these benefits. In other words, does seminal fluid enhance performance by containing proteins that modify sperm morphology that result in increased velocity, or does it contain proteins that act as energetic substrates that sperm can incorporate from the seminal fluid-rich environment during fertilization that result in increased velocity?

As mentioned earlier, seminal fluid proteins and their influence on sperm performance have been specifically studied and their mechanisms detailed in a number of mammal and insect species.

For instance, the boar seminal fluid protein Gp-54 (a glycoprotein) and SMIF or sperm motility inhibiting factor (a fragment of a multifunctional glycoprotein complex) bind to receptors at the acrosome and the midpiece to regulate capacitation and tightly bind sperm to the zona pellucida, the membrane that surrounds the mammalian ovum (Kordan et al., 1999). It is possible that some seminal fluid proteins behave similarly in fishes to modify sperm in ways that increase their competitive ability. Also recall that seminal fluid can influence sperm performance by containing nourishment for sperm (Poiani, 2006). For example, Trehalase, a protein produced by the accessory gland of the male mealworm beetle, *Tenebrio molitor,* is incorporated into the spermatophore and is likely an energy source for sperm (Yaginuma et al., 1996). Such findings exemplify how seminal fluid proteins can enhance sperm performance with and without modifying morphology. But what are the physiological processes that occur in seminal fluid and increase sperm velocity in the plainfin midshipman?

Fitzpatrick et al. (2015) found that continuous differences in plainfin midshipman guarder male sperm velocity in relation to body size didn't correlate with any differences in sperm morphology. While Fitzpatrick's study investigated sperm velocity in the absence of seminal fluid, his results suggest that sperm velocity in this species may not be strongly correlated with sperm morphology. Therefore, this is evidence that seminal fluid may provide sperm with energetic substrates or affect the environment in which sperm are fertilizing in some way that results in increased sperm velocity. However, plainfin midshipman have very complex sperm morphology (e.g. helical heads), and it's possible that particular aspects of sperm morphology (e.g. the number of helical sperm head turns) that could make a large difference in swimming velocity might have been overlooked in the previous study. It still may be possible that seminal fluid contains proteins that modify sperm morphology in a way that increases velocity. I argue again that studying seminal fluid protein compositions in the plainfin midshipman and how such proteins influence sperm competitive ability would be paramount to understanding whether seminal fluid enhances sperm performance via changes to sperm morphology or the addition of energetic substrates.

FUTURE RESEARCH QUESTIONS AND DIRECTIONS

Sperm competition researchers are continually trying to better understand the biologically and environmentally-relevant factors that can impact fertilization success and sperm competition. A growing area of research that has resulted from this objective is the study of the impact of female ovarian fluid on sperm competition in externally fertilizing species. Females being capable of influencing mating outcomes (i.e. cryptic female choice) are well documented across internally fertilizing species (Eberhard, 1996; Gasparini & Pilastro, 2011; Pizzari & Birkhead, 2000), but we are only beginning to understand that ovarian fluid released during mating events in external fertilizers also has the ability to affect mating outcomes. In many externally fertilizing fishes, the environment created by female ovarian fluid increases sperm performance (Dietrich et al., 2008; Galvano et al., 2013; Rosengrave et al., 2009; Turner & Montgomerie, 2002). Furthermore, ovarian fluid appears to act as a mechanism for cryptic female choice by aiding sperm produced by attractive males (Butts et al., 2012; Urbach, Folstad, & Rudolfsen, 2005; Yeates et al., 2013). However, there have been mixed results to date about whether ovarian fluid acts as a mechanism for cryptic choice for more attractive male tactics in species with ARTs. One study using ocellated wrasse, *Symphodus ocellates,* revealed that ovarian fluid did not improve the sperm performance of one tactic over another (Alonzo, Stiver, & Marsh-Rollo, 2016), while in another study using Chinook salmon, researchers found that ovarian fluid increased sperm velocity and motility in large, dominant anadromous males, but not in small, sneaking parr males (Makiguchi et al., 2016).

Investigating the role of females in sperm competition between male ARTs is an interesting area of current research that will shed even more light on male and female responses to sperm competition.

This thesis highlights the idea that the evolution of male accessory glands is a strategy to overcome sperm competition. However, sperm competition is only one possible selective force for this evolutionary outcome. The environmental, life history, and social factors favouring the evolution of accessory glands have not yet been investigated across taxa. The question of what factors have led to the evolution of accessory glands is particular interesting in fishes given the extreme diversity in presence/absence and morphology of accessory glands across fishes and even within species. For example, the presence, anatomical position, and morphology of accessory glands vary greatly not only across several species of blennies (Richtarski & Patzner, 2000), but also within a single blenny species (*Salaria pavo*) across three male ARTs (Ruchon, Laugier, & Quignard JP, 1995). Many factors have been suggested as possible explanations for this diversity, including fertilization mode (Mazzoldi et al., 2007), mating system type, sperm competition risk, duration of egg deposition (Mazzoldi et al., 2005), reproductive behaviour, and ecology (Richtarski & Patzner, 2000). An analysis of selective influences on accessory gland variability across 12 goby species revealed that mating system played the greatest role (Mazzoldi et al., 2005). However, a much larger scale phylogenetic analysis across teleost taxa might reveal interesting but different selection patterns for the development of the accessory gland across fishes. While this thesis argues for sperm competition as a selective force behind development and investment in accessory glands, especially in species that exhibit ARTs, it is important to emphasize that there is great variation in sperm competition risk across fishes, as well as variation in accessory gland function. A phylogenetic-based comparative analysis of accessory gland presence and linked selective factors should provide clarity broadly across fishes on the role of these fascinating reproductive organs. Presently, a database containing detailed information about accessory gland presence, anatomical position or type (e.g. testicular accessory gland, sperm duct accessory gland, etc.), and potentially linked selective factors such as mating system and presence of ARTs for almost 250 fishes has been collected with the intention of doing such an analysis.

CONCLUSION

In summary, Chapter 2 of this thesis answered the two outlined primary research questions: 1) is there tactic-specific investment in the accessory gland? – **yes, there is different tactic-specific investment in each region of the accessory gland**, and 2) does this organ play a role in sperm competition? – **seminal fluid produced in part by the accessory gland enhances sperm performance.** The research in this thesis contributes to the growing literature on the role of accessory glands in sperm competition, and helps to explain how these important organs can differ between male alternative reproductive tactics that face different levels of sperm competition risk. Additionally, by investigating sperm performance in a more naturalistic fertilization environment containing not only the activation medium (i.e. seawater) but also the seminal fluid that sperm undoubtedly utilize during fertilization, this research allows us to more accurately study the dynamics of post-copulatory competition and fertilization.

Beyond the academic implications of this work, this research could have applications for the aquaculture and fisheries industries, as well as for conservation efforts. A thorough understanding of the effects of seminal fluid on fertilization outcomes is vital information for fish breeding program managers of aquaculture and conservation operations. Ignoring the effects of seminal fluid on fertilization outcomes could result in unpredictable or unexpectedly low fertilization success in species being raised to produce maximum offspring of the highest quality, especially those species in which ARTs occur. Using the knowledge gained from this research and similar research on commercially important as well as endangered fish species, breeding program methodology can be improved to ensure the greatest probabilities of fertilization success in order to maximize reproductive output.

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