Marine Biology (2019) 166:37
https://doi.org/10.1007/s00227-019-3474-8

ORIGINAL PAPER

Differential investment in male accessory glands: lessons from a marine fish with alternative reproductive tactics

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Received: 4 September 2018 / Accepted: 11 January 2019

Abstract

Male reproductive accessory glands play a number of important roles, including enhancing fertilization success in competitive contexts. Theory predicts that males experiencing greater sperm competition risk (i.e. those adopting the opportunistic tactic) should invest more in accessory glands and ejaculate. However, empirical data show the opposite pattern; males experiencing lower sperm competition risk (i.e. those adopting the conventional guarder tactic) invest more in accessory glands. This pattern has possibly emerged because these organs also function to optimize sperm economy and sometimes also play a role in parental care, which provides more benefits to guarder males. To tease apart these contrasting patterns, we examined tactic-specific investment in and histology of accessory glands, as well as the effect of their fluids on sperm performance in guarder males, using the plainfin midshipman fish (*Porichthys notatus*). We found that midshipman accessory glands consist of two distinct structures: nodes and lobules, differing in organization and secretory characteristics both between structures and male types. Like other fishes with alternative reproductive tactics, guarder males invested more in accessory glands and in lobules specifically compared to opportunistic sneaker males. Fluids from both lobule and nodes increased sperm velocity in guarder males. Moreover, guarder males increased their investment in accessory glands across the breeding season. Our results suggest that accessory glands may have multiple functions and may even play a role in parental care and olfactory signalling. Our study emphasizes the diversity in form and function of accessory glands and highlights the importance of these organs in reproduction.

Introduction

The term "reproductive accessory gland" is used for a collection of reproductive organs, glands, or ducts that do not produce gametes but are closely associated with the testes. Accessory glands are found in a phylogenetically diverse range of taxa from mammals to invertebrates, but are completely absent in birds, amphibians, and reptiles (Adiyodi and Adiyodi 1988; Hyman 1992), and are rare among fishes (Rasotto unpublished ms). In males, accessory glands serve a variety of reproductive functions such as sperm storage and recycling via lytic activity and the production of seminal fluid, which is released with the ejaculate (Hyman 1992; Scaggiante et al. 1999; Chowdhury and Joy 2007). Seminal fluid specifically contributes to fertilization by triggering sperm capacitation, enhancing sperm motility, and acting as an osmotic and ionic shock absorber for sperm entering new environments (Ramm et al. 2005; Poiani 2006; Chowdhury and Joy 2007). Moreover, seminal fluid may influence the outcome of sperm competition, i.e. the competition occurring when ejaculates released by two or more males have the opportunity to fertilize the same group of eggs (Parker 1970). Indeed, seminal fluid can carry constituents like proteins important for the formation of mating plugs, the modulation of oviposition rate and female receptivity, and the modification of own and/or rival sperm performance (Poiani 2006; Chapman 2008; Wigby et al.

Responsible Editor: D. Goulet.

Reviewed by Undisclosed experts.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00227-019-3474-8) contains supplementary material, which is available to authorized users.

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Published online: 13 February 2019

2009; den Boer et al. 2010; Locatello et al. 2013; Lewis and Pitcher 2017; Poli et al. 2018).

Sperm competition is a powerful evolutionary force driving many male morphological, physiological, and behavioural traits (Birkhead and Moller 1998; Pitnick et al. 2009). In particular, theoretical models such as sperm competition risk theory posit that an increase in sperm competition risk should be accompanied by an increase in ejaculate quality (Parker 1998; Parker and Pizzari 2010). To date, experimental studies across species and populations, primarily focused on sperm investment, strongly support this theoretical prediction, showing that higher levels of sperm competition select for increased sperm numbers and/or quality in terms of viability, velocity, longevity, etc. (Parker and Pizzari 2010; Simmons and Fitzpatrick 2012; Smith 2012). In contrast, information on the investment in male accessory glands in relation to sperm competition risk is more ambiguous. For example, in rodents, species with higher levels of sperm competition exhibit larger male accessory structures (Ramm et al. 2005), but the opposite pattern is found across blenny species (Giacomello et al. 2008a). These contrasting results might make sense if, among some species, accessory glands have been shaped by selective pressures other than sperm competition. However, investment in accessory glands and their functions often remain a neglected topic of reproductive biology, especially so in external fertilizers. Much of our current understanding of accessory glands and their role in sperm competition and other selective pressures has been based on internal fertilizers (e.g. rodents, *Drosophila*), which face somewhat different challenges compared to external fertilizers (e.g. blennies), such as the chemical nature of the fertilization environment, the velocity of liquid at the site of fertilization, and the feasibility of sperm competition (Hyman 1992; Poiani 2006).

One of the best ways to test sperm competition theory is to use species with male alternative reproductive tactics (ARTs)—species in which males adopt distinct strategies to achieve reproduction (Taborsky et al. 2008)—because the alternative male types by definition face different levels of sperm competition risk. Male ARTs occur across a wide variety of taxa and perhaps the most common form of ARTs is the sneak-guard system (Parker 1990; Taborsky et al. 2008). Guard or guarder males are typically larger, guard territories and females, and sometimes look after young (Taborsky 1994). In contrast, sneak or sneaker males are small, do not hold territories or compete for females, and instead use stealthy tactics to gain access to mating females to "steal" fertilizations (Taborsky 1994). While guarder males can and often will mate in the absence of a sneaker male competitor, sneaker males only ever mate once a female has been attracted to a location by a guarder male and almost always attempt to fertilize in the presence of at least one competitor, the guarder male (Parker 1990). Therefore, sneaker males face greater sperm competition risk and selection favours their increased ejaculate investment to overcome competition by guarder males (Simmons et al. 2007; Setchell 2008; Olsson et al. 2009). This tactic-specific investment pattern is especially common in fishes, many of which are external fertilizers (Taborsky 1998; Neff et al. 2003; Fitzpatrick et al. 2007; Marentette et al. 2009; Flannery et al. 2013).

Because of the important role accessory glands can play in influencing sperm competitive outcomes, one might expect that in species with ARTs, sneaker males would develop larger accessory glands given their greater risk of sperm competition compared to guarder males. However, in contrast to this prediction, across a wide variety of fish species [a taxonomic group in which ARTs are numerous (Taborsky 2008)], investigation reveals that it is the guarder male tactic that invests more in accessory glands (Scaggiante et al. 1999; Neat 2001; Oliveira et al. 2001; Mazzoldi and Rasotto 2002; Modesto and Canário 2003; Neat et al. 2003; Marentette et al. 2009; Utne-Palm et al. 2015).

The prediction that sneaker males should invest more in accessory glands is predicated on the idea that accessory glands only function to enhance sperm competitive ability. However, accessory glands can have complex reproductive functions that benefit fishes in many different ways. For example, accessory glands often release glycoproteins or mucins that prolong ejaculate longevity in externally fertilizing fishes by slowly dissolving in seawater, thereby delaying the activation of a proportion of sperm, and allowing continuous release of active sperm over time (Scaggiante et al. 1999; Rasotto and Mazzoldi 2002). Such a slow release of sperm reduces sperm waste, and this function is especially important in species where egg laying is prolonged (i.e. many hours). When females have long spawning durations, longer-living ejaculates are an advantageous strategy for guarder males and allows them to defend nests from intruders while fertilization simultaneously occurs (Scaggiante et al. 1999; Rasotto and Mazzoldi 2002). By contrast, sneaker males frequently adopt the strategy of producing short-living ejaculates, richer in sperm that swim faster to overcome the disadvantage they face in spawning further away from the female (Taborsky 1998; Neff et al. 2003; Fitzpatrick et al. 2007; Flannery et al. 2013). In addition to optimizing sperm economy, accessory glands can play a role in parental care. In at least three fish species—the grass goby (*Zosterisessor ophiocephalus*), peacock blenny (*Salaria pavo*), and redlip blenny (*Ophioblennius atlanticus*)—accessory glands are known to produce anti-microbials that caring guarder males release onto developing eggs (Giacomello et al. 2006, 2008b). This fluid prevents or reduces bacterial or fungal growth that can threaten offspring survival (Pizzolon et al. 2010). However, accessory glands may function to store sperm, thus affecting the male capacity to

increase sperm number per ejaculate and/or the number of ejaculates before suffering sperm depletion (Chowdhury and Joy 2007; Rasotto unpublished ms). If accessory glands play a major role in the storage of sperm, sneaker males would be expected to benefit most and invest more in these organs.

To better understand the function of accessory glands in relation to ARTs, we studied the plainfin midshipman fish (*Porichthys notatus*), an externally fertilizing marine fish with accessory glands and well-characterized ARTs (Brantley and Bass 1994). Male tactics are "fixed" in this species, or non-sequential in their development, with guarder males reaching sexual maturity at approximately 2 years of age, while sneaker males reach maturity earlier after 1 year (Brantley et al. 1993). Guarder males excavate and defend nests under intertidal rocks along the Pacific coast of North America (Brantley and Bass 1994). Guarder males also court females by vibrating their swim bladders with highly specialized sonic muscles, producing long duration vocalizations or "hums" (Brantley and Bass 1994; Cogliati et al. 2014; Bose et al. 2015). In contrast, sneaker males are silent, they invest in testes that are eight times larger than those of guarder males relative to their body size, they produce ejaculates with three times the sperm concentrations as guarder male ejaculates, and they produce faster swimming sperm (Miller et al. 2019, in press; Fitzpatrick et al. 2015). The first study of plainfin midshipman male accessory glands by Barni et al. (2001) claimed that these organs were more developed in guarder males. However, a more recent study by Fitzpatrick et al. (2015) reported the opposite pattern: i.e. sneaker males had larger accessory glands relative to their body size than guarder males.

The present study aimed to settle this debate and better understand if accessory gland investment and histological structure differs between the ARTs in the plainfin midshipman, as well as test the possible influence of these organs on sperm competitive ability. Using a 7-year data set, we first investigated which male tactic invested more in accessory glands and if this investment varies across the breeding season. Second, through histological analyses, we compared differences in the structure of the glands between the male adopting different tactics. Third, we collected fluids from guarder male accessory glands and mixed these fluids with sperm to explore whether they impacted sperm performance. We predicted that sneaker males would have larger accessory glands if the primary function of the plainfin midshipman accessory glands is sperm storage, thereby increasing sneaker male ejaculate sperm numbers or ejaculation frequency ability. Instead, if the primary function of the accessory glands is rooted in sperm economy (i.e. limiting sperm release and prolonging ejaculate longevity with mucins) and/or parental care (i.e. producing anti-microbials), then we predict that guarder males would have larger accessory glands. Furthermore, we predicted that accessory gland fluid would increase sperm velocity and thus provide a potential benefit during competitive contexts.

Materials and methods

Specimen collection

We collected guarder ($n = 249$) and sneaker ($n = 80$) plainfin midshipman males from nests during low tides along the intertidal zone of Ladysmith Inlet, British Columbia, Canada (49°01′N, 123°83′W) over a 7-year period. Fish were collected by multiple researchers from the Aquatic Behavioural Ecology Lab (ABEL) at McMaster University (see Supplementary Table 1 for collection and researcher details). In the field, we initially categorized males into tactics based on body size (guarder males are much larger than sneaker males), ventral body colour (guarder males have an olive grey ventral side while sneaker males are more golden yellow), and position in the nest (guarder males are positioned centrally while sneaker males are more typically found in the nest periphery) (Brantley and Bass 1994). Male tactic was later confirmed by dissecting the gonads and the swim bladder and calculating the gonadosomatic index [GSI = (testes mass/body mass − testes mass) × 100] and the investment in sonic muscle mass [(swim bladder mass/body mass − swim bladder mass) × 100]. Sneaker males invest ~ 7 times more in GSI, but ~ 8 times less in sonic muscle mass compared to guarder males (Brantley and Bass 1994). Males were either dissected at the field site or transported to outdoor holding aquaria before dissection. Transported males were housed in tactic-specific gravel-lined 400 L aquaria supplied with 13 °C aerated, filtered seawater fitted to a flow-through system. Each male was provided with a shelter made of bricks.

Reproductive organ investments

To compare investment in the testes and accessory glands between male tactics, we euthanized males with an overdose of MS-222 anaesthetic (> 300 mg/L seawater bath), weighed them to the nearest 0.1 g, measured for standard length (i.e. from the tip of the mouth to the last vertebra) to the nearest mm, and dissected the males. We carefully removed the testes and accessory glands and weighed these separately to the nearest 0.01 g. For all males collected in 2017 ($n = 101$), the accessory glands were further dissected into two distinct structures: paired, opaque "nodes", and numerous, transparent, fluid-filled "lobules" (Fig. 1a). Both of these structures were also weighed separately. The mass of nodes and lobules was measured only in 2017 following histological analysis confirming these two distinct structures. Prior to this, the accessory glands were measured as one single structure.

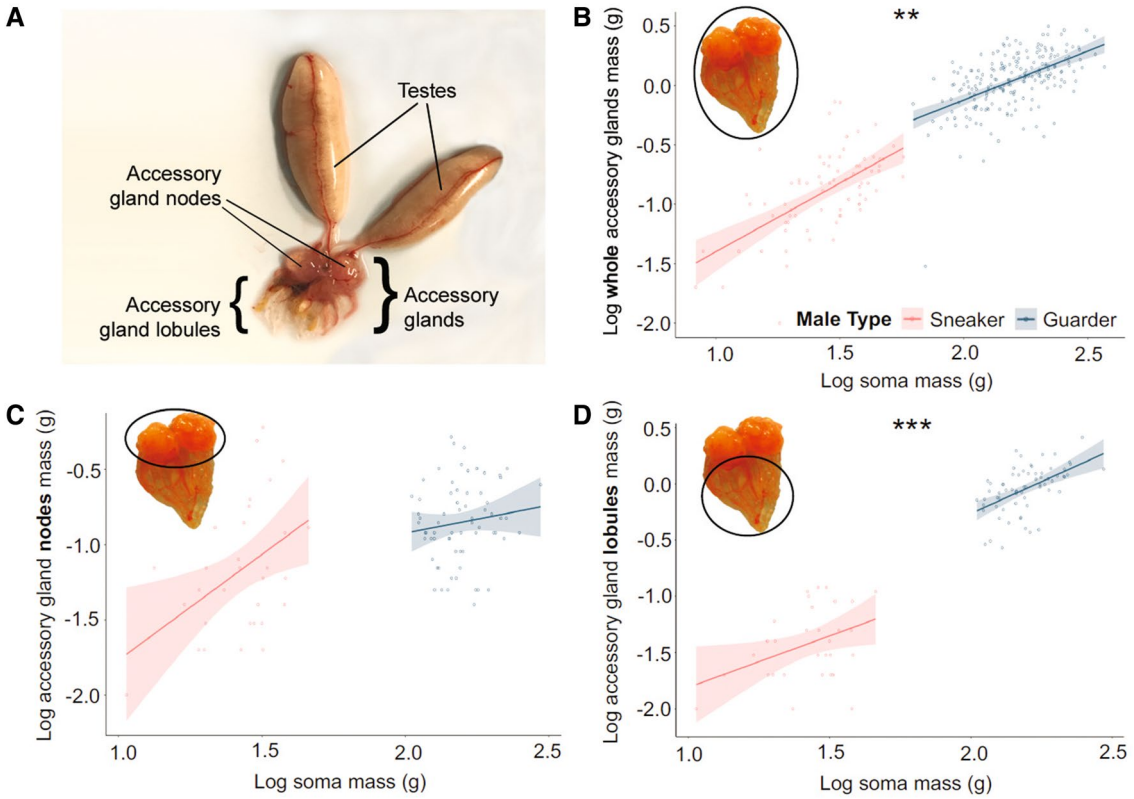

Fig. 1 **a** Photograph of dissected plainfin midshipman gonads with testes and accessory glands with structures denoted, and tactic-specific investment in **b** whole accessory gland mass, **c** accessory gland node mass, and **d** accessory gland lobule mass

Reproductive anatomy histology

To examine differences in the structure and secretory properties of the accessory glands between the male tactics, we euthanized 14 guarder and 6 sneaker males in 2015, removed their reproductive anatomy, fixed the organs in Dietrich's solution, and dehydrated them in ethanol. Then, the accessory glands were separated from the testes, embedded in Paraplast, and sectioned serially at 6–7 µm. Sections from each specimen were stained with haematoxylin and eosin. To detect polysaccharides, sections were stained by the reaction of periodic acid-Schiff (PAS) (Pearse 1950), and for the differentiation of sulphated and non-sulphated mucins by the methods of Alcian Blue at pH 1.0 and pH 2.5 (Pearse 1950).

Measurement of ratio of sperm to non-sperm fluids in ejaculate

To experimentally mix sperm with fluids collected from the accessory glands in a way that mimics natural ejaculate, we needed to determine the biologically relevant proportions of these ejaculate components. Specifically, we needed to measure the ratio of sperm to non-sperm fluids in ejaculate in both male tactics. To do this, we collected ejaculates from 11 guarder and 15 sneaker males in 2017 by temporarily sedating males with a MS-222 bath (250 mg/L seawater), placing them on their backs on a damp towel, and then drying off their genital area. For each male, a tapered gel-loading pipette tip was cut to custom fit 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 ejaculate was collected or the fish ceased to release ejaculate. Due to the viscosity and small volumes of the ejaculate, samples were mixed with bovine serum albumen (BSA) in preparation for separation in microcapillary tubes to ensure complete separation of sperm and non-sperm fluids. A known amount of ejaculate was ejected into an Eppendorf tube along with a known volume of 1.23 mg/mL BSA at approximately a 1:1 volume ratio, then vortexed for 10 min. The ejaculate and BSA mixture was then pulled up into a new microcapillary tube and spun for 30 min at 12,000 rpm in a ZIPCombo Zipocrit portable centrifuge (LW Scientific, Lawrenceville, GA, USA) to separate sperm, non-sperm ejaculate fluids, and BSA.

The lengths of the microcapillary tubes, whole samples, separated sperm, ejaculate fluids, and BSA were

photographed under a dissecting microscope and measured in NIH ImageJ software (v. 1.50i) to calculate the volumes and then percentages of sperm and non-sperm ejaculate fluids in ejaculate. Average percentages of sperm and non-sperm ejaculate fluids for guarder and sneaker males were calculated across individuals of each tactic.

Effects of accessory gland fluids on sperm velocity

To investigate the influence of the accessory glands on guarder male sperm performance, we collected fluids from both structures of the accessory glands (i.e. nodes and lobules) and mixed each of these fluids separately with sperm collected from the testes and tested their effects on sperm velocity. To do this, we euthanized 47 guarder males in 2017 with an overdose of MS-222 (> 300 mg/L seawater bath) and removed their testes. We did not perform these tests on sneaker males due to the characteristics of their accessory gland lobules, which often did not contain enough fluid to conduct the experiment. We then gently sliced open a single testis and collected 0.5 µL of pooled sperm in an Eppendorf tube via pipette. Sperm was always collected from the posterior region of the testis near the main testicular duct to avoid collecting spermatids or undeveloped sperm. Then (based on the results of our study of the ratio of sperm to non-sperm ejaculate fluids in both male tactics—see above section), we added 17 µL of gland fluid collected from either the accessory gland nodes ($n = 15$) or lobules ($n = 15$) of the same male to the sperm sample and mixed the sample gently with the pipettor by pulling the sample up into the pipette tip and releasing it ten times. The selection of either nodes or lobules fluid was randomized for each male. To collect accessory gland nodes fluid, we removed and cleaned the nodes and placed them in a clean Eppendorf tube. Then, we carefully pressed the nodes with forceps to force out fluid, which was collected via pipette. To collect accessory gland lobules fluid, we removed the lobules and inserted a 30G insulin needle into individual lobules to collect the fluid within. We also conducted a third, control treatment, in which no fluid was added to the sperm ($n = 17$).

Following mixing the sperm with or without accessory gland fluid, we pipetted 1 µL of the sample into the chamber of a 2X-Cel glass slide (Hamilton Thorne, Beverly, MA, USA) and immediately activated it with 1.5 µL 13 °C filtered seawater. Video recordings of sperm movement were collected from the time of activation to 15 min post-activation. Video was captured at 60 frames/s by a Lumenera Infinity HD camera mounted on a Leica DME compound light microscope (Leica Microsystems Inc., Buffalo, NY, USA) under 200x magnification. Later, videos were analysed and sperm swimming velocity was measured at the following post-activation time points: 10, 30, 60, 90, 120, 180, 600, and 900 s. Time points during which fewer than three visible sperm cells were moving (with forward progression) were excluded from analysis (Miller et al. 2019, in press). Video was analysed with CEROS sperm tracking software (HTM-CEROS version 12.3, Hamilton Thorne Biosciences) and the average sperm path velocity (VAP) was used to represent sperm velocity.

Statistical analysis

All statistical analyses were conducted in R version 3.4.1 (R Foundation for Statistical Computing) and significance was assessed at $\alpha = 0.05$. When necessary to achieve normality and homoscedasticity, data were log, arcsine, or power transformed based on Box–Cox analyses. Nonsignificant interactions were removed from models whenever possible. Out of the 329 fish, 23 individuals were removed from analyses because they had intermediate phenotypes and sometimes deformities (18 guarder males that were < 18 cm standard length and 5 sneaker males that were > 18 cm standard length). However, the inclusion of these intermediate males did not qualitatively change the patterns observed. All measurements were made by observers blind to the tactic ID of the male/sperm sample in question.

Differences in absolute body mass, absolute testes mass, and absolute accessory gland mass between the two male tactics were assessed using separate general linear mixed effects models (LMMs) [lme4 package 1.1-12 (Bates et al. 2015) and lmerTest package 2.0-32 (Kuznetsova et al. 2017)] with male tactic (guarder or sneaker) as a categorical fixed factor and with year as a random intercept. Differences in absolute accessory gland node mass and accessory gland lobule mass between the two male tactics were assessed with general linear models (LMs) with only male tactic as a categorical fixed factor, since these measurements were taken in a single collection year. To assess whether males of different tactics invested differently in testes and in whole accessory gland masses, LMMs were employed for each organ of interest, where models were fitted with male tactic as a categorical fixed factor, soma mass (i.e. body mass − the mass of the organ of interest) as a continuous covariate, and year as a random intercept. To assess whether males of different tactics invested differently in the two structures of the accessory glands, two separate general linear models (ANCOVAs) were fitted to the accessory gland nodes and lobules data, with male tactic as a categorical fixed factor and soma mass as a continuous covariate.

To assess whether males of different tactics invested differently in accessory gland mass as a function of time across the breeding season, an LMM of the accessory gland mass data was fitted with the following fixed factors: male tactic (categorical), Julian date (continuous), soma mass (continuous), and the interaction between Julian date and male tactic. Year was treated as a random intercept.

To assess differences between male tactics in terms of the percentages of sperm and non-sperm ejaculate fluids, we used separate general linear models with only male tactic as a fixed factor. Differences in sperm velocity as a result of accessory gland fluid type (i.e. nodes fluid, lobules fluid, or no fluid) were assessed using a general linear model with accessory gland fluid type and time point after sperm activation (s) as categorical fixed factors. Post hoc analyses of accessory gland fluid type effects on sperm velocity were completed using Tukey multiple comparisons of means [multcomp package 1.4-8 (Hothorn et al. 2008)].

Results

Reproductive anatomy histology

In both guarder and sneaker males, the testes were elongated, paired organs, suspended from the dorsal wall of the coelomic cavity by a mesorchium. Two main testicular ducts, each running along the testis length, fused in a common sperm duct before reaching the urogenital opening. A pair of accessory structures was connected to the distal portion of the main testicular ducts, posteriorly to the testis and anteriorly to the sperm duct. These structures differed in shape and relative development (Fig. 1a): the anterior being a roundish, spongy structure with a multi-globular irregular surface (hereafter "nodes"), while the more posterior and larger one being composed of several, long, finger-like lobes (hereafter "lobules"). The accessory gland nodes and lobules opened independently into the sperm duct. There were no connections between the nodes and lobules.

Both nodes and lobules were organized in chambers. Neither structure was found to contain sperm cells. Chamber walls consisted, from inside to outside, of a layer of epithelial cells and a layer of connective tissue containing blood capillaries. The chambers of the nodes and lobules differed in size, wall thickness, and secretory characteristics and they varied between male types (guarders and sneakers). In sneaker males, node chambers had an elliptical shape and thick walls (Fig. 2a), with a connective layer that was rich in smooth muscle cells and blood capillaries. Sneaker male node chambers also had an extremely folded epithelial layer with columnar secretory cells, which had empty secretory vesicles. Moreover, the apical parts of these epithelial cells often appeared broken off, with their content extruded in the chamber lumen. A vesiculous, non-homogeneous material was present in the lumen of several chambers (Fig. 2b). In guarder males, node chambers had an elliptical shape, but they were larger in size and had thinner walls than those of sneaker males (Fig. 2c). In addition, guarder male node chambers had connective layers with fewer smooth muscle cells compared to sneakers, and their inner epithelium was only slightly folded and consisted of cuboidal cells. In a few chambers, epithelial cells appeared to have the same apocrine type of secretion observed in sneaker males' nodes. In both guarder and sneaker males, the epithelial cells as well as the material present in the node chamber lumina reacted weakly to staining for the presence of polysaccharides and glycoproteins.

Lobule chambers were large and elongated in guarder males; their walls thinner than that of the nodes. The connective layer of guarder male lobule chambers had very few smooth muscle cells and the epithelial layer, poorly folded, consisted of flat cells (Fig. 2d). By contrast, in sneakers males, lobule chambers were rounded in shape, smaller in size, but had thicker walls than those of guarder males. The connective layer was thick and rich in blood capillaries and the epithelial layer was more folded and composed of columnar cells (Fig. 2e). In both guarder and sneaker males, lobule lumina were often filled with homogeneous material and the apical part of the epithelial cells as well as the material inside the lumina strongly reacted to histochemical staining for sulpho- and syalo-glycoproteins (Fig. 2f).

Reproductive organ investments

Guarder males ($\bar{x} = 169$ g) were approximately 5.5 times heavier than sneaker males ($\bar{x} = 30$ g) (LMM; est. $\pm$ SE $= 2.9 \pm 0.08$; $X^2 = 1140$; $df = 1303$; $p < 0.001$). Absolute testes mass ($\bar{x} = 3.0$ g) did not differ between male tactics (LMM; est. $\pm$ SE $= 0.02 \pm 0.06$; $X^2 = 0.18$; $df = 1.87$; $p = 0.67$); however, relative to their much smaller soma mass, sneaker males invested more in testes mass compared to guarder males (LMM, Table 1). In contrast, guarder males had larger accessory glands in absolute ($\bar{x}_{\text{guarder}} = 1.2$ g, $\bar{x}_{\text{sneaker}} = 0.17$ g) (LMM; est. $\pm$ SE $= 0.9 \pm 0.04$; $X^2 = 640$; $df = 1276$; $p < 0.001$) and relative terms (LMM, Table 1, Fig. 1b) compared to sneaker males. As described above, plainfin midshipman accessory glands had two distinct structures: nodes and lobules (Fig. 1a). Guarder males had larger accessory gland nodes ($\bar{x}_{\text{guarder}} = 0.17$ g, $\bar{x}_{\text{sneaker}} = 0.11$ g) (LM; est. $\pm$ SE $= 0.3 \pm 0.07$; $F = 20.3$; $df = 1.96$; $p < 0.001$) and lobules in absolute terms ($\bar{x}_{\text{guarder}} = 1.0$ g, $\bar{x}_{\text{sneaker}} = 0.05$ g) (LM; est. $\pm$ SE $= 1.4 \pm 0.05$; $F = 648$; $df = 1.96$; $p < 0.001$). Relative to soma mass, guarder males invested more in lobules than did sneakers (ANCOVA, Table 1, Fig. 1d), but there was no difference between tactics in node investment (ANCOVA, Table 1, Fig. 1c).

Guarder and sneaker males had opposite accessory gland mass investment patterns over the duration of the breeding season. Accessory gland mass relative to soma mass increased with Julian date in guarder males (LMM, est. $\pm$ SE $= 5.3 \pm 1.3$, $t = 4.11$, $p < 0.001$), while accessory gland investment decreased with Julian date in sneaker males (LMM, est. $\pm$ SE $= 6.3 \pm 2.5$, $t = 2.49$, $p = 0.01$).

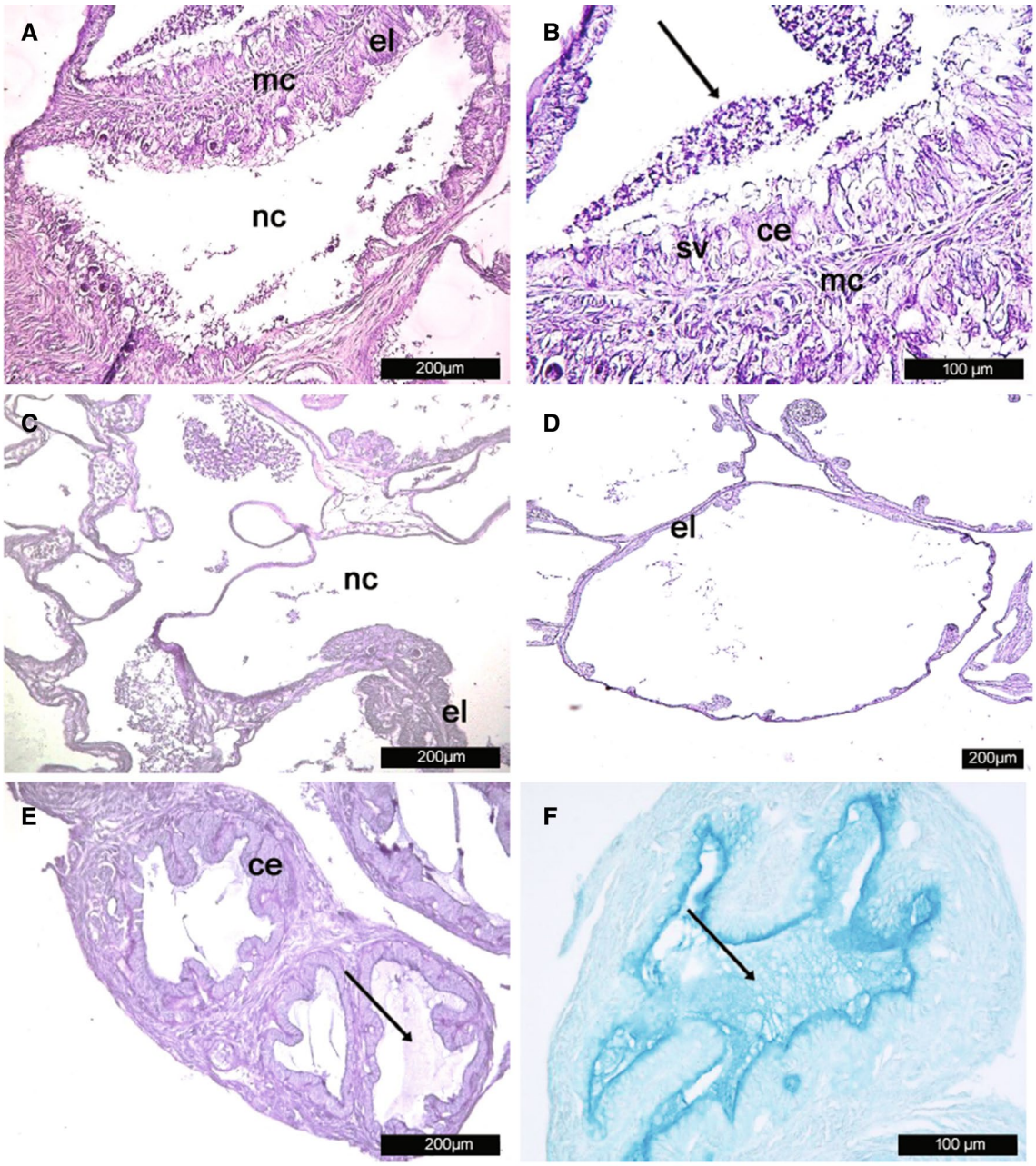

Fig. 2 Photograph of **a** sneaker male accessory gland node chambers, **b** accessory gland node chamber lumen filled with vesiculous, non-homogeneous material (shown with arrow) typical of both male tactics **c** guarder male accessory gland node chambers, **d** guarder male accessory gland lobule chambers, **e** sneaker male accessory gland lobule chambers, and **f** lobule chamber lumen filled with homogenous material that strongly reacted to staining for glycoproteins (shown with arrows). *ce* columnar epithelial cells, *el* epithelial layer, *mc* smooth muscle cells, *nc* node chamber, *sv* secretory vesicle

Ejaculate components and effects of accessory gland fluids on sperm velocity

Sneaker male ejaculate was composed of approximately four times as many sperm ($\bar{x} = 14\%$ of the total ejaculate volume) as guarder male ejaculate ($\bar{x} = 3.1\%$ of the total ejaculate volume) (LM; est. $\pm$ SE $= 0.02 \pm 0.006$; $F = 10.1$; $df = 1.24$; $p = 0.004$). In contrast, guarder male ejaculate was composed of more seminal fluid ($\bar{x} = 97\%$ of the total

Table 1 Results of general linear mixed effects models (LMMs) and general linear models (ANCOVAs) fitted to reproductive organ mass data to investigate tactic-specific investment patterns

Trait	Factor	Estimate ± SE	Test statistic and degrees of freedom	*p* value
Testes mass	Male tactic	0.93 ± 0.09	$X^2 = 115$, $df = 1184$	< 0.001
	Soma mass	1.16 ± 0.09	$X^2 = 141$, $df = 1184$	< 0.001
Accessory gland mass	Male tactic	0.19 ± 0.07	$X^2 = 7.5$, $df = 1276$	0.006
	Soma mass	0.97 ± 0.08	$X^2 = 136$, $df = 1276$	< 0.001
Accessory gland node mass	Male tactic	0.35 ± 0.23	$F = 2.3$, $df = 1.95$	0.10
	Soma mass	0.88 ± 0.29	$F = 9.3$, $df = 1.95$	0.003
Accessory gland lobule mass	Male tactic	0.59 ± 0.16	$F = 525$, $df = 1.95$	< 0.001
	Soma mass	1.02 ± 0.20	$F = 8.6$, $df = 1.95$	0.004

ejaculate volume) than sneaker male ejaculate ($\bar{x} = 85\%$) (LM; est. ± SE = 0.06 ± 0.02; $F = 9.4$; $df = 1.24$; $p = 0.005$).

Sperm velocity (of guarder males) was influenced by both accessory gland fluid (LM; est. ± SE = 17.6 ± 5.5; $F = 16$; $df = 2348$; $p < 0.001$) and the time since sperm activation (LM; est. ± SE = 10.9 ± 9.05; $F = 8.7$; $df = 7348$; $p < 0.001$) (Fig. 3). Specifically, guarder male sperm swam faster when mixed with fluid from their nodes compared to their sperm without accessory gland fluid (est. ± SE = 13.07 ± 5.4, $t = -2.4$, $p = 0.04$), and their sperm swam much faster in the fluid from their lobules compared to their sperm mixed without accessory gland fluid (est. ± SE = 30.6 ± 5.4, $t = -5.6$, $p < 0.001$). Moreover, guarder male sperm mixed with fluid from their lobules also swam faster than sperm mixed with fluid from their nodes (est. ± SE = 17.6 ± 5.5, $t = -3.2$, $p = 0.004$).

Discussion

We initially made two contrasting predictions about which male tactic would invest more in accessory glands based on alternative proposed accessory gland functions. We expected these organs to be more developed in sneaker males if they were mainly involved in sperm storage, but we found no evidence to support this prediction and no sperm was found inside the accessory glands. Our alternative prediction was that if accessory glands function to benefit sperm economy and/or play a role in parental care, then guarder males should invest more in accessory glands. We did indeed find support for this second prediction. As found in the majority of fishes with accessory glands and ARTs, plainfin midshipman guarder males had larger accessory glands in absolute and relative terms compared to sneaker males. Moreover, they invested more in one particular structure of the

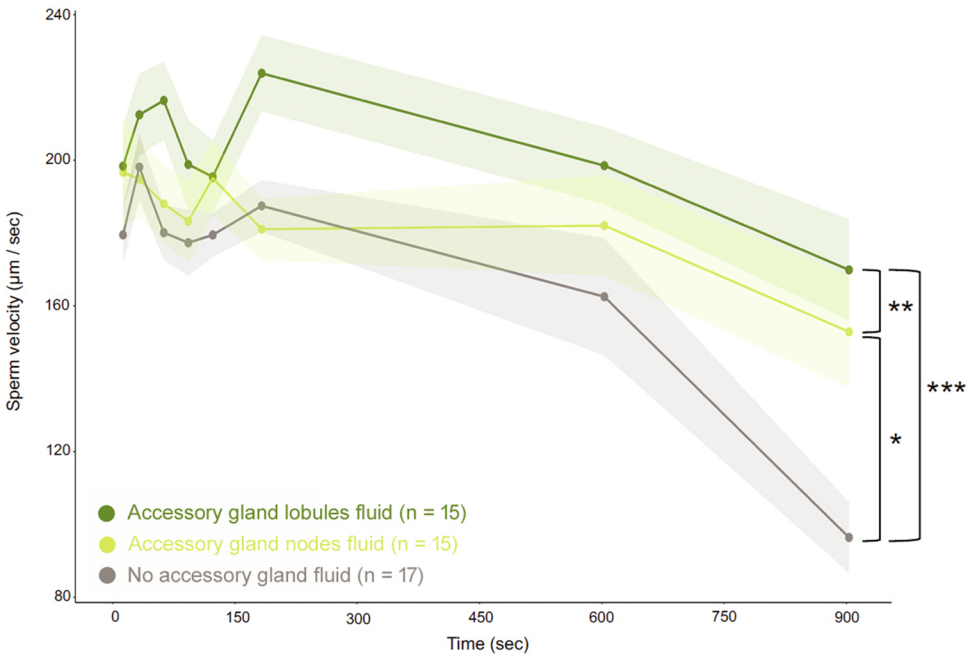

Fig. 3 Velocity of guarder male sperm when mixed with fluid from accessory gland lobules, nodes, or no accessory gland fluid. Significance between treatments (i.e. lines) is denoted with asterisks ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***). The shaded margins outside of the lines denote standard error

accessory glands, the lobules. The lobules contained fluid that increased sperm velocity and did so more than the other structure of the accessory glands, the nodes, which did not differ in relative size between male tactics. Additionally, guarder male accessory gland investment increased over the duration of the breeding season, while sneaker male investment decreased over the season. These results raise a number of important questions about why guarder males, the male tactic that faces less sperm competition risk, would invest more in an organ, let alone a specific structure of this organ, that functions to increase sperm velocity.

First, what is the specific function of the accessory gland lobules and why would guarder males invest more in these structures? We found that this structure reacted strongly to stains for glycoproteins. Glycoproteins or mucins prolong ejaculate longevity in externally fertilizing fishes by releasing trapped sperm slowly (Scaggiante et al. 1999; Rasotto and Mazzoldi 2002). In some fishes with mucin-rich ejaculates like the grass goby (*Zosterisessor ophiocephalus),* ejaculates can release active sperm for over 15 h (Scaggiante et al. 1999). Producing mucin-rich ejaculates to prolong ejaculate longevity is an advantageous strategy for guarder males because they can release fewer sperm in close proximity to a female and do so for the entire duration the female is spawning while simultaneously performing nest guarding duties (Scaggiante et al. 1999; Rasotto and Mazzoldi 2002). By contrast, sneaker males release ejaculates poor in mucins, but rich in fast, albeit short-lived sperm further away from the female compared to those of guarder males (Taborsky 1998; Neff et al. 2003; Fitzpatrick et al. 2007; Flannery et al. 2013). Plainfin midshipman females can lay eggs for up to 20 h, an unusually long spawning duration (Brantley and Bass 1994). Sneaker males, which do produce faster sperm (Miller et al. 2019, in press; Fitzpatrick et al. 2015), had significantly more sperm in their ejaculate compared to guarder males. Parallel to their increased accessory gland lobule investment, guarder males had significantly more seminal fluid compared to sneaker males, and preliminary laboratory experiments confirmed that guarder male ejaculate (including seminal fluid) contained mucins (J. Miller pers obs). Taken together, these results suggest that sneaker males invest in a strategy of short-lived, fast, and numerous sperm, while guarder males produce more mucins in their ejaculates and benefit by optimizing their sperm economy (i.e. reducing sperm waste and prolonging ejaculate longevity).

It is somewhat puzzling, then, that guarder males, the tactic that is usually positioned in closer proximity to a female during spawning compared to sneaker males, would invest in an organ that speeds up sperm. We might expect to see greater investment in an organ that increases sperm velocity in sneaker males because they are usually in the disadvantaged fertilization position (Taborsky 2008; Cogliati et al. 2013, 2014). However, accessory gland fluid that increases sperm velocity might be a by-product of accessory gland fluid constituents, such as proteins, that primarily benefit sperm economy. This by-product idea has been suggested to explain why grass goby sneaker male sperm performed better in guarder male seminal fluid (Locatello et al. 2013). We currently know little about the underlying mechanisms and precisely how constituents in accessory gland fluid like specific proteins affect sperm velocity. However, a great deal of research effort is currently directed at identifying and quantifying specific proteins in ejaculate, especially in species with alternative reproductive tactics (Ciereszko et al. 2017; Gombar et al. 2017). The results of such studies would shed light on precisely how reproductive fluids like seminal fluid influence fertilization outcomes.

Guarder males not only cope with long fertilization periods, but also have extremely long parental care durations. Guarder males can remain in their nest, caring for young for 2–3 months continuously, defending and performing hygienic duties (Cogliati et al. 2013; Bose et al. 2015). It is possible that the accessory glands may also play a role in parental care in the plainfin midshipman, enhancing the parental abilities of guarder males. In support of this proposition, we observed increased guarder male investment in whole accessory glands across the breeding season. The relative size of guarder male accessory glands is largest when they care for offspring and not when they compete with other males or mating with females, suggesting that the products of this organ are used long after of the mating period. Accessory glands are used by care-providing guarder males in at least three fish species that release accessory gland-secreted anti-microbials onto eggs, which prevent or reduce bacterial or fungal growth responsible for offspring mortality (Giacomello et al. 2006, 2008b; Pizzolon et al. 2010). Could midshipman too have accessory gland lobule fluid with anti-microbial or hygienic properties, and do guarder males accelerate lobule fluid production during parental care periods later in the breeding season to increase offspring survival? We cannot yet say, but we are currently conducting experiments to explore if accessory gland lobule fluid, as well as other accessory gland fluids impact fungal and bacterial growth.

The lobules are but one structure of the accessory glands; our histological results suggest that the nodes have a different function than the lobules in this species. Node secretions remained unstained, a sign of high lipid content. Other organs that produce lipid-rich secretions, such as the mammalian sweat gland, produce pheromones. Pheromones are produced by the accessory glands in a number of fishes as well, such as the African catfish (*Clarias gariepinus*), the black goby (*Gobius niger*), the four-eyed sleeper (*Bostrychus sinensis*), and the peacock blenny, (Lambert and Resink 1991; Locatello et al. 2002; Hong et al. 2006; Chowdhury

and Joy 2007; Serrano et al. 2008a, b). The accessory glands of the Lusitanian toadfish (*Halobatrachus didactylus*), a close relative to the plainfin midshipman, have the capacity for pheromone production as well (Modesto et al. 2015). Could plainfin midshipman accessory gland node secretions contain pheromones used to attract females to the nests of guarder males? In the black goby, guarder males attract spawning females by releasing a steroid conjugate pheromone produced in one of their accessory glands, a mesorchial gland, which is highly developed in guarder males (Colombo et al. 1980). In contrast, black goby sneaker male mesorchial glands are reduced, producing low amounts of pheromones, and their ejaculates are pheromonally inconspicuous, thereby avoiding detection by guarder males (Locatello et al. 2002). In the plainfin midshipman, the similar investment of accessory glands nodes in both male tactics suggests that, if these structures produce a female attraction pheromone, then both male types may cooperate in this task. Unlike the black goby, midshipman guarder males might be capable of detecting sneaker male pheromones, but benefit by the sum of their pheromones with those of sneakers to attract more females to their nest and therefore tolerate sneaker male presence. Behavioural and endocrinological experimentation is now required to test for the potential function of olfactory signalling for midshipman accessory glands.

Male reproductive accessory glands have long been known to play a number of important and diverse roles in improving fertilization and post-copulatory competitive outcomes. In this study, we found that plainfin midshipman guarder males invest more in accessory glands than do sneaker males. Thus, the plainfin midshipman can be added to a growing list of species with both ARTs and accessory glands in which the guarder tactic that faces less sperm competitive risk invests more in accessory glands. Our study also showed that there are two distinct structures of plainfin midshipman accessory glands, each with a potentially different function. These organs appear to enhance sperm performance, but may also play a role in parental care and pheromone production. Future studies would be valuable to further our understanding of the potentially multi-faceted role of accessory glands.

Acknowledgements We would like to thank Stz'uminius First Nations for their permission to sample at Ladysmith Inlet, and P. Walker and R. Shepherd for granting us access to our field site. We also thank the University of Victoria OAU and Animal Care staff, H. Hicklin and Dr. H. Kreiberg at the Pacific Biological Station in Nanaimo, BC, as well as Dr. F. Juanes, K. Cox, and Dr. J. S. Taylor for logistical support. We are extremely grateful to Drs. A. P. H. Bose, K. M. Cogliati, J. L. Fitzpatrick, and N. Sopinka for their comprehensive field work, as well as to N. Houpt, E. Sadler, T. Warriner, N. Luymes, E. Balke, H. Kou, H. Howe, A. Hassan, A. Mistakidis, Dr. J. Marentette, and Dr. J. Taves for their assistance in the field and with data collection. We thank C. Breggion for aid with histological analyses and Dr. J. L. Fitzpatrick and Dr. T. Pitcher for their many suggestions and guidance on sperm collection and analysis techniques. We would also like to thank Dr. B. Bolker, Dr. J. Dushoff, and the McMaster "data lunch" crew for their SAGE statistical suggestions, as well as S. Gotic for data processing help. We also thank our three anonymous reviewers for their helpful input and suggestions.

Funding This research was supported by grants from the Natural Science and Engineering Research Council of Canada (Grant no. 10538042), the American Museum of Natural History, and from the Department of Psychology, Neuroscience, and Behaviour and the School of Graduate Studies at McMaster University.

Data availability All reproductive organ, morphological, sperm velocity, and ejaculate component data are available at Pangaea (https://doi.pangaea.de/10.1594/PANGAEA.897160).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

Ethical standards Plainfin midshipman fish are a common intertidal species and their populations are not endangered or threatened. All fish were collected in accordance with permits issued by the Canadian Department of Fisheries and Oceans (scientific licenses XR 50 2010, XR 126 2011, XR 14 2013, XR 121 2014, XR 81 2015, XR 94 2016, and XR 58 2017). All research procedures were approved by the McMaster University Animal Research Ethics Board (AUP's #10-11-70 and #13-12-52) and the University of Victoria Animal Care Committee (Protocols 2015-009[1] and 2017-003[1]).

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