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REGULAR PAPER

Sperm maturation and male tactic-specific differences in ejaculates in the plainfin midshipman fish *Porichthys notatus*

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Using the plainfin midshipman fish *Porichthys notatus*, a species with alternative reproductive tactics (ARTs), we investigated how sperm maturation shapes sperm competitive abilities. We compared sperm performance and morphology before and after final sperm maturation by sampling sperm from the testes and stripped ejaculates of guarders and sneakers. In accordance with sperm competition risk theory, ejaculates from sneaker males had three times as much sperm as ejaculates from guarder males and sneaker males produced faster swimming sperm than guarder males, but this was only the case after final sperm maturation had occurred. Additionally, fully mature sperm found in ejaculates had larger heads and midpieces than sperm found in the testes. These results emphasize the important role played by non-sperm components of an ejaculate in mediating sperm performance and potentially also morphology.

KEYWORDS

alternative reproductive tactics, guarder male, seminal fluid, sneaker male, sperm competition, sperm velocity

1 | INTRODUCTION

Sperm competition occurs whenever ejaculates from multiple males have the opportunity to fertilise the eggs of a single female (Parker, 1970). This phenomenon drives the evolution of many male reproductive behaviours as well as physiological and morphological traits (Birkhead & Møller, 1998; Pitnick *et al*., 2009). For example, when there is a high probability that two ejaculates from two different males will compete, male expenditure on ejaculate quality is expected to increase, a theoretical prediction based on the sperm competition risk model (Ball & Parker, 1997; Parker & Pizzari, 2010) (one of two of the most well-studied models of sperm competition, along with the sperm competition intensity model; see Parker & Ball, 2005 and Simmons *et al*., 2007a). Sperm competition risk theory has been well supported in many species and males repeatedly faced with elevated sperm competitive risk have been shown to increase investment in ejaculate quantity or quality on both short timescales (*e.g*., temporarily release more sperm in competitively risky contexts) and on longer, evolutionary timescales (*e.g*., evolve to produce faster sperm) (Parker & Pizzari, 2010; Simmons & Fitzpatrick, 2012; Smith, 2012).

Species with alternative reproductive tactics represent an attractive model for studying sperm competition theory because the alternative male types by definition face different levels of sperm competition risk (Parker, 1990). Male alternative reproductive tactics (ART) are characterised by discontinuous variation in morphological, physiological or behavioural characteristics that result in two or more distinct means of achieving reproduction (Taborsky *et al*., 2008). While ARTs occur in a wide variety of taxa, perhaps the most common ART dichotomy is the sneak–guard system (Parker, 1990; Taborsky *et al*., 2008). Guarder males (also called bourgeois, territorial, parental, or type I males) are typically larger in body size, court and monopolise females, hold and guard territories, and sometimes look after young (Taborsky, 1994). In contrast, sneaker males (also called parasitic or type II males) are small, do not physically compete for females or resources, and instead use stealth to gain access to mating females to "steal" fertilisations (Taborsky, 1994). 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, 1990). Sneaker males can only ever mate once a female has been attracted to a location by a guarder male and almost always attempt to fertilise in the presence of at least one guarder male competitor (Parker, 1990). Consequently, to increase their chances of fertilisation success, sneaker males typically invest much more into their ejaculate, producing more and faster-moving sperm (*i.e*., investments in post-copulatory

competitiveness; Montgomerie & Fitzpatrick, 2009). In contrast, guarder males typically invest more resources into traits that facilitate the monopolisation of females and resources, such as large body size and weapons (*i.e.*, investments in pre-copulatory competitiveness; Parker, 1990). Evidence of these tactic-specific responses to sperm competition has been well documented across taxa (Olsson *et al*., 2009; Setchell, 2008; Simmons *et al*., 2007b) and is especially common in fishes (Fitzpatrick *et al*., 2007; Flannery *et al*., 2013; Marentette *et al*., 2009; Neff *et al*., 2003; Taborsky, 1998).

While evolutionary adaptations of sperm to greater sperm competition risk (such as more and faster sperm) have been documented across species, especially in those with ARTs (Parker & Pizzari, 2010), sperm are not the only component of the ejaculate capable of influencing competitive outcomes. Sperm develop and mature in the testes, but once they leave the testes, sperm undergo further maturation processes, which prepare them for fertilisation and sometimes for competition with other sperm (Cooper, 2012). Downstream of the testes in other areas of the reproductive anatomy such as the sperm duct, sperm are prepared for the fertilisation environment *via* changes in pH, osmolality, and ion concentrations (Cosson *et al*., 2008; Dzyuba *et al*., 2017; Morisawa & Morisawa, 1988). One critical step in the maturation process is the addition of seminal fluid to ejaculate. Seminal fluid provides buffering capacity, energetic substrates, and proteins that enhance sperm competitive ability and fertilisation success (Cameron *et al*., 2007; Cornwallis & O'Connor, 2009; den Boer *et al*., 2010; Dorus *et al*., 2004; Locatello *et al*., 2013; Poiani, 2006; Wigby *et al*., 2009). These studies have demonstrated how non-sperm components of ejaculate influence fertilisation. However, additional work is still needed to better understand the role of non-sperm components and sperm maturation in sperm competition, especially among species with ARTs and external fertilizers like many fishes.

The plainfin midshipman fish *Porichthys notatus* Girard 1854, an externally fertilising marine fish that exhibits alternative reproductive tactics (Brantley & Bass, 1994; Figure 1a), provides an excellent test bed for understanding sperm competition. *Porichthys notatus* guarder males monopolize nests underneath large rocks in the intertidal zones along the Pacific coast of North America. Guarder males excavate, maintain, and defend nests from predators and other guarder males seeking to usurp nests for their own use (Bose *et al*., 2015; Cogliati *et al*., 2014). Guarder males also court gravid females by vibrating their swim bladders with highly specialized sonic muscles, producing long duration vocalizations or hums (Brantley & Bass, 1994). In contrast, sneaker males, which are much smaller, do not invest in sonic muscles, hum, or provide parental care. Intense sperm competition exists between the two male tactics. Because sneaker males always spawn in the presence of at least one guarder male competitor, they experience more sperm competition risk on average than guarder males that only sometimes spawn in the presence of other males (Brantley & Bass, 1994; Fitzpatrick *et al*., 2016). Sneaker males have traits that have been shaped by sperm competition, such as testes that are eight times larger than those of guarder males relative to their body size (Brantley & Bass, 1994) and their sperm (collected from the testes) also swim faster than the sperm of guarder males (Fitzpatrick *et al*., 2016). Despite this knowledge about plainfin midshipman sperm and ARTs, we still don't know how their sperm performs in the ejaculate after fully maturing. To date, only sperm from the testes have been studied. *P. notatus* not only provides an interesting model system to further study how ARTs respond to sperm competition risk, but also can be used to explore how the sperm of each male tactic mature given the divergent selective pressures that each tactic faces.

To better understand how the inherently different levels of sperm competition risk affect sperm performance and maturation for each male tactic, we conducted a study using *P. notatus*. First, we characterized the ejaculate of both guarder and sneaker males in terms of their tactic-specific investments in sperm density and seminal fluid protein concentration. Then, using both sperm collected from the testes and sperm collected from the ejaculate, we measured and compared sperm performance and morphology between male tactics. We predicted that sneaker males, given their higher risk of sperm competition, should produce ejaculates with more sperm, sperm that swim faster, for longer, and/or that their sperm should have enhanced morphology (*e.g.*, longer tails (Cardullo & Baltz, 1991; Gomendio & Roldan, 1991)) as a strategy to better compete with guarder males. Given that a number of empirical studies have uncovered negative correlations between sperm swimming speed and longevity (Burness *et al*., 2004; Stockley *et al*., 1997; Taborsky *et al*., 2018), we were also prepared for the possibility that only one or some of these sperm characteristics would be enhanced in sneaker males. We also predicted that sperm maturation processes should improve sperm performance. Furthermore, if sperm maturation processes play a role in improving sperm performance in the context of sperm competition risk, then tactic-specific differences in ejaculate sperm performance should be more pronounced following final sperm maturation.

2 | MATERIALS AND METHODS

2.1 | Specimen collection

We collected guarder (n = 57) and sneaker (n = 50) males from nests during low tides along the intertidal zone of Ladysmith Inlet, British Columbia, Canada (49° 01′ N, 123° 83′ W) between May 17 and 22 July 2016 and May 10 and 25 June 2017. In 2016, we collected 21 guarders and 21 sneakers and in 2017, we collected 36 guarders and 29 sneakers. In the field, we initially identified male tactics based on a combination of traits including body size (on average, guarder males are approximately eight times larger in body mass than sneaker males), ventral body colour (guarder males are olive grey while sneaker males are golden yellow), and the position of the male in the nest (guarder males are positioned centrally while sneaker males are found in the nest periphery; Brantley & Bass, 1994). We transported guarder and sneaker males to an outdoor aquatic unit at the University of Victoria, British Columbia and housed them in gravel-lined 400 L aquaria supplied with aerated, filtered seawater at 13°C fitted to a flow-through system. Fish were kept in tactic-specific holding tanks; *i.e.*, guarder males were never housed with sneaker males. Each male was provided with a shelter made of bricks. Following sperm and ejaculate collection, we confirmed male tactics by dissecting the gonads and calculating the gonadosomatic index [GSI = 100(testes mass)(body mass − testes mass)$^{-1}$] and index of investment in sonic muscle mass

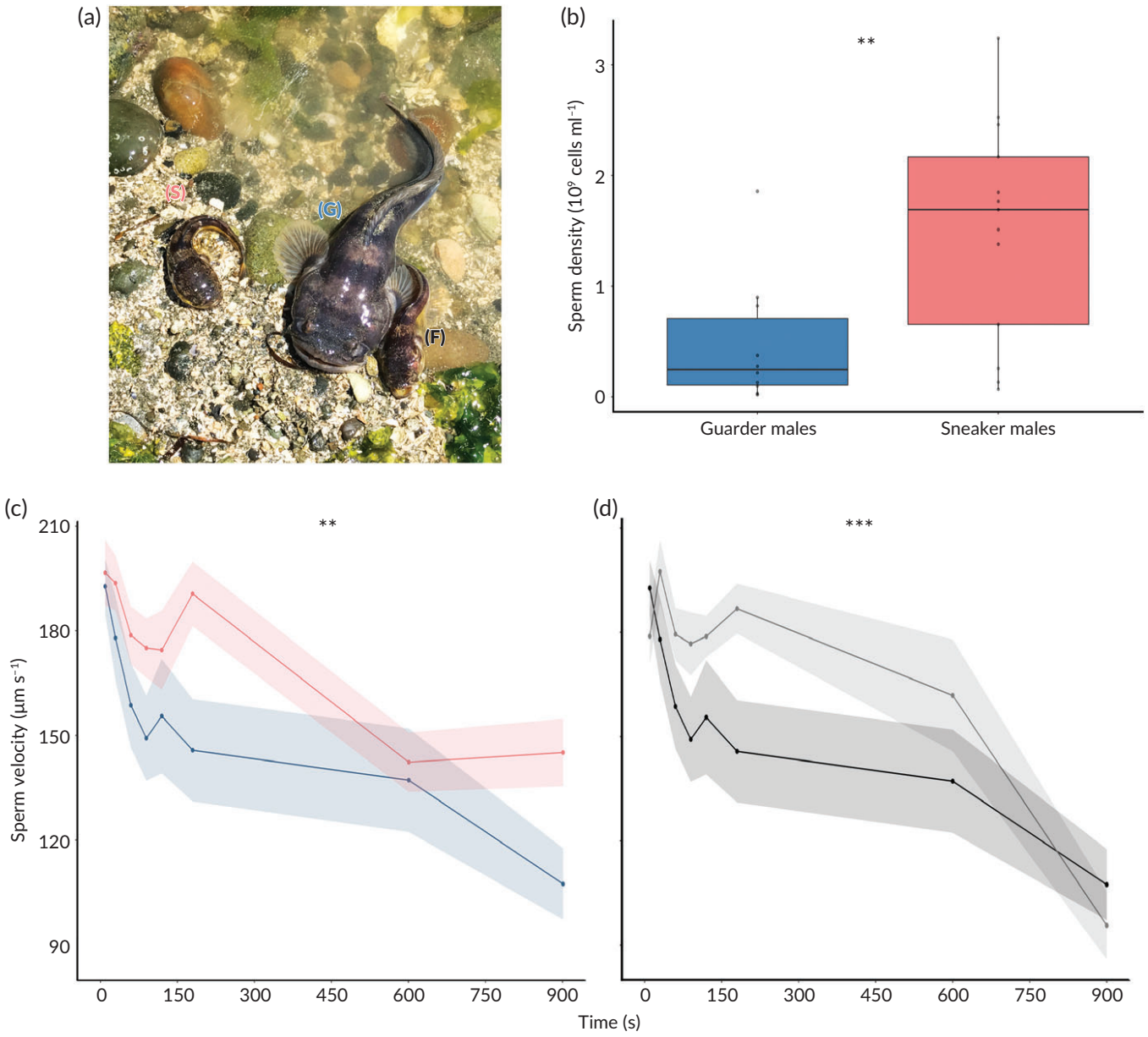

FIGURE 1 (a) Photograph of *Porichthys notatus* sneaker male (S), a guarder male (G) and a female (F) in an uncovered nest. (b) Box plots (——, median; □, 25th and 75th percentiles; |, range) of sperm density in ejaculates of guarder (n = 10) and sneaker males (n = 13). (c) Mean (± SE, shaded areas) velocity of sperm in the ejaculate from guarder and sneaker males against time since activation. (●) Sneaker males (n=14–15), and (●) Guarder males (n=13–15); (d) Mean (± SE, shaded areas) velocity of sperm in the ejaculate and from the testes from guarder males over time since activation (●) Sperm from testes (n=12–17), and (●) Sperm in ejaculate (n=13–15). Significant differences as a result of male tactic or sperm maturation stage: $^{**}P < 0.01$; $^{***}P < 0.001$

[I_S = 100(swim bladder mass)(body mass − swim bladder mass)$^{-1}$]. Sneaker males invest *c.* seven times more in GSI but *c.* eight times less in I_S compared with guarder males (Brantley & Bass, 1994; Fitzpatrick *et al.*, 2016). Such discrete, non-overlapping measurements of body size, GSI and I_S investment between male types have been well established in this species (Brantley & Bass, 1994; Fitzpatrick *et al.*, 2016).

2.2 | Sperm and ejaculate collection

To examine *P. notatus* sperm before and after final maturation processes, we collected sperm from the testes and in ejaculate. In an attempt to reduce the number of fish sacrificed for our study, sperm in the ejaculate and from the testes were collected from some of the same males (this design was later controlled for statistically). Stripped ejaculates (hereafter, referred to as ejaculates) were always collected first from these repeatedly sampled males, followed by sperm collection from the testes typically 3 days later. To collect sperm in ejaculate, fish were temporarily sedated with a MS-222 bath (250 mg L^{-1} seawater), placed on their back on a damp towel and the genital area was dabbed dry. 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. To collect sperm from the testes, fish were euthanized with an overdose of MS-222 (>300 mg L^{-1} seawater bath) and dissected, the testes were

removed and a single testis was gently sliced open. Any pooled sperm from the posterior region of the testis near the main testicular duct was collected to avoid collecting any spermatids or undeveloped sperm. Microscopy was used to verify the absence of undeveloped sperm.

2.3 | Total seminal fluid proteins

To characterise ejaculate and measure total proteins in seminal fluid in both male tactics, ejaculate was collected from 17 guarder and 11 sneaker males in 2017 and was spun in microcapillary tubes in a ZIPCombo Zipocrit portable centrifuge (LW Scientific; www.lwscientific.com) for 10 min at 1085 *g* to separate seminal fluid. Following separation from sperm, microcapillary tubes were carefully broken below the separation point and the remaining seminal fluid was expelled *via* pipette bulb into cryovials, which were immediately stored at −80°C. Total seminal fluid protein concentrations were then determined using a Bradford protein assay (Bio-Rad; www.bio-rad.com).

2.4 | Sperm density

To investigate tactic-specific differences in ejaculate sperm density, ejaculate sperm concentrations were measured from 10 guarder and 13 sneaker males (all collected in 2016). A known volume of the collected ejaculate from each male was diluted and fixed with a known volume of 1:2 solution of buffered formalin and filtered seawater. Then 10 μl of fixed ejaculate sample was pipetted into a Neubauer chamber haemocytometer. Video of the four 1 mm^2 corner squares of the haemocytometer grid was captured under ×200 magnification by a Lumenera Infinity HD camera (www.lumenera.com) mounted on a Leica DME compound light microscope (Leica Microsystems; www.leica-microsystems.com). This procedure was conducted in duplicate for each male. Individual sperm cells were later counted in each sampled 1 mm square using FlySketch software (Flying Meat; www.flyingmeat.com) overlaid on top of the recorded video by an observer blind to the male identity. Sperm counts were averaged between replicates and sperm density was calculated for each sample.

2.5 | Sperm velocity, straightness and linearity

To investigate differences in sperm performance between male types and sperm maturation stages, we measured the velocity, straightness, and linearity of sperm collected from the testes and in ejaculate in both guarder and sneaker males. To do so, we sampled 1 μl of sperm in an ejaculate or sperm from the testes from 31 guarder and 29 sneaker males all collected in 2017. Sperm was pipetted into the chamber of a 2X-Cel glass slide (Hamilton Thorne; www. hamiltonthorne.com,) and immediately activated with 2 μl of 13°C (pH 8.3) filtered seawater sourced from the outdoor aquatic facility at the University of Victoria. Video recordings of sperm movement were taken from the time of activation with seawater to 15 min post-activation. Video was captured at 60 frames s^{-1} using the same equipment described above for quantifying sperm density. 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. Video was analysed with HTM-CEROS 12.3 sperm tracking software (Hamilton Thorne Biosciences) and the average sperm path velocity (VAP) was used to represent sperm velocity (Au *et al*., 2002; Casselman *et al*., 2006). VAP significantly correlated with both straight line velocity (VSL; Pearson correlation coefficient = 0.96) and curvilinear velocity (VCL; Pearson correlation coefficient = 0.87). Sperm path straightness (STR) was calculated in-software by dividing VSL by VAP, then multiplying this value by 100 (HTM-CEROS 12.3). Sperm path linearity (LIN) was also calculated in HTM-CEROS 12.3 software by dividing VSL by VCL, then multiplying this value by 100. Both STR and LIN are expressed as percentages.

2.6 | Sperm longevity

To determine if sperm swimming longevity differed between male tactics or as a result of sperm maturation stage, we measured sperm velocity until sperm ceased swimming. We sampled sperm in the ejaculate and sperm from the testes of 19 guarder and 19 sneaker males collected in 2016. Then 2 μl of sperm was diluted with 750 μl of filtered seawater and immediately incubated at 13°C. Next, 10 μl of diluted sperm sample was pipetted into the chamber of a 2X-Cel glass slide pre-rinsed in 1% bovine serum albumin solution to prevent sperm from sticking to the slide or coverslip. Approximately 2 min video recordings of sperm movement were collected at 3 min post-activation and then every 15 min following until all the observed sperm ceased achieving forward progression. Video was captured and analysed using the same equipment and software as described above for quantifying sperm density and velocity.

2.7 | Sperm morphology

We assessed differences in sperm morphology as a result of male tactic and sperm maturation stage. Sperm in both ejaculate and testes samples were collected from 9 guarder and 14 sneaker males collected in 2016 and another 5 guarder males collected in 2017. Samples were diluted and fixed using a 1:2 solution of buffered formalin and filtered seawater. Between 13 and 24 individual sperm cells per sample (14 cells on average) were digitally photographed at ×1000 magnification on welled slides (MP Biomedicals; www.mpbio.com) using the microscope equipment previously described. *P. notatus* sperm have helical heads and two tails (Fitzpatrick *et al*., 2016; Stanley, 1965; Figure 2a). Due to the unusual sperm head morphology of *P. notatus*, the surface area of sperm heads was measured instead of the traditional head length and width. Sperm head surface area was of particular interest due to the head shape's influence on hydrodynamic movement (Humphries *et al*., 2008). For example, 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). For this reason, the number of head turns or gyres was also measured by counting the number of completed 360° rotations (*i.e*., spirals) in the shape of the head. Midpiece size is thought to represent the available ATP or energy production and storage capability of the sperm cell (Cardullo & Baltz, 1991). Since *P. notatus* midpieces are irregular and tapered in shape (Figure 2a), we measured midpiece surface area. Flagellum or tail length, reflecting propulsive force capability of a sperm cell

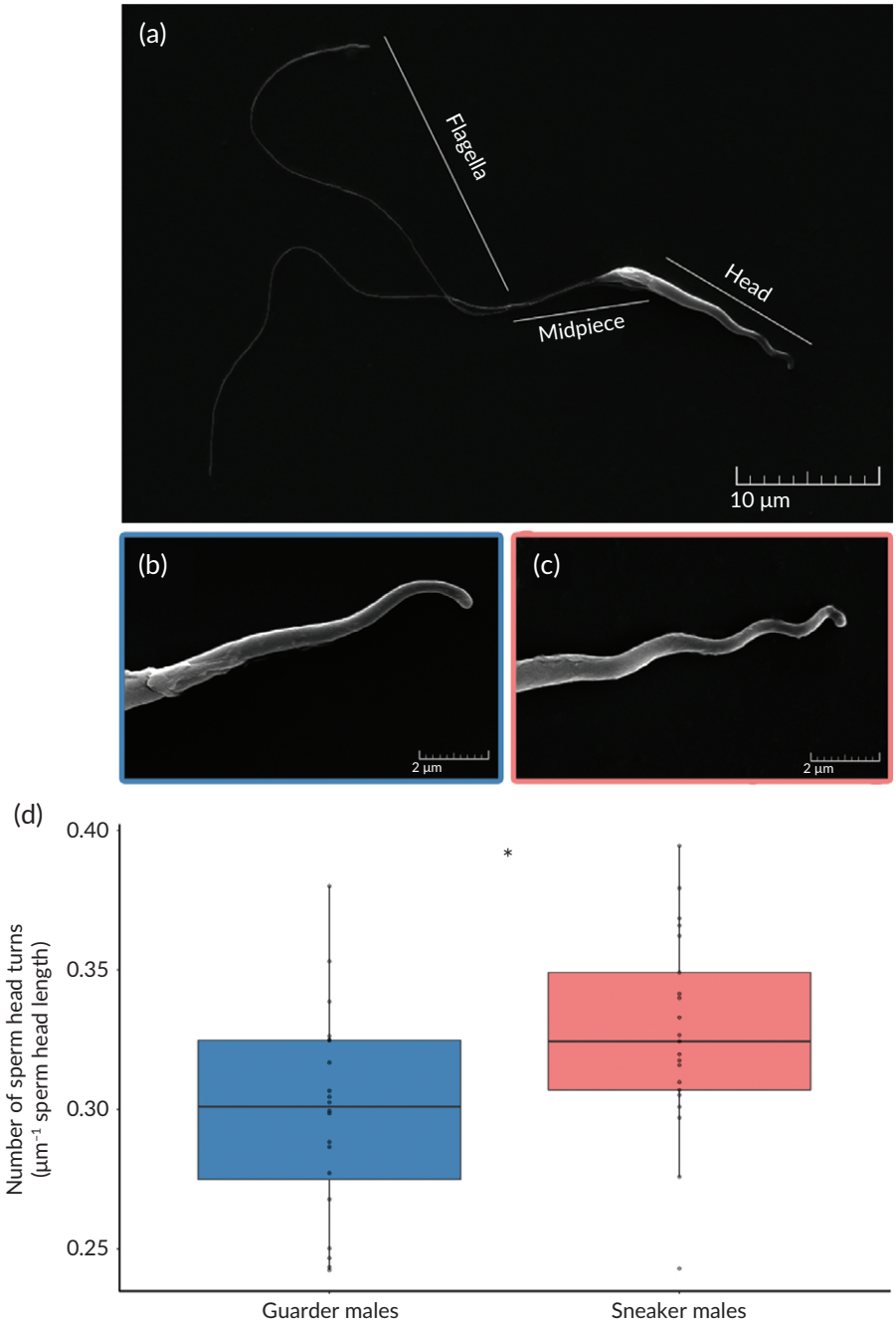

FIGURE 2 Scanning electron microscope (SEM) images of *Porichthys notatus* (a) single sperm cell collected from the testes; (b) helical sperm heads with few turns and (c) with many turns. (d) Box plots (——, median; □, 25th and 75th percentiles; ⟊, range) of differences in number of sperm head turns between guarder (n = 21) and sneaker (n = 21) male sperm from pooled ejaculate and testes samples. Significant differences between guarder and sneaker males: *P < 0.05

(Gomendio & Roldan, 1991) was also measured for both tails and an average tail length calculated per sperm cell.

Finally, the number of sperm head turns per micron of sperm head length and the ratio of sperm head length to tail length were also calculated. The number of sperm head turns per length of head was calculated to analyse number of heads turns while taking overall sperm head length into account. Total sperm length positively correlates with velocity in many species (Gomendio & Roldan, 1991); however, the ratio of head length to tail length is often considered the preferred metric as it is thought to represent the ratio of physical drag to propulsion in biomechanical models (Humphries *et al*., 2008) and thus might provide a better correlate of sperm swimming speed (Simpson *et al*., 2014). Photographs were analysed in ImageJ 1.50i (NIH; www.imagej.nih.gov) using a digital tablet (Wacom Co. Ltd.; www.wacom.com). Sperm morphological measurements and sperm velocity measurements could not be directly compared because these measurements were collected from different samples.

2.8 | Statistical analysis

All statistical analyses were performed in R 3.4.1 (www.r-project.org) and significance was assessed at α = 0.05. All models were tested for

normality and homoscedasticity using Q-Q and residuals *v.* fitted values plots. When necessary to achieve normality and homoscedasticity, data was log or power transformed based on Box-Cox analyses. Nonsignificant interactions were removed from models whenever possible. We removed six individuals from analyses that had intermediate phenotypes [three guarder males that were < 20 cm standard length (L_S) and three sneaker males that were > 18 cm L_S]. However, the inclusion of these intermediate males did not qualitatively change the patterns observed. Guarder males used in this study ranged from 20 to 27.9 cm L_S ($\bar{x}$ = 24 cm) and sneaker males ranged from 9.1 to 16.6 cm L_S ($\bar{x}$ = 13.2 cm). All measurements were made by observers blind to the tactic of the male or sperm sample in question.

We tested whether male tactics differed in ejaculate sperm density as well as total seminal fluid protein concentration by fitting separate general linear models, including male tactic (guarder or sneaker) as a categorical predictor variable. To investigate differences in sperm velocity (VAP), sperm path straightness (STR), and linearity (LIN) as a result of male tactic and sperm maturation stage, we fit separate general linear mixed effects models (LMM) using the lme4 1.1–12 (Bates *et al*., 2015) and lmerTest 2.0–32 (Kuznetsova *et al*., 2017) packages in R for each of these three sperm performance response variables with male tactic and sperm maturation stage as categorical predictor variables and time (s) after sperm activation [log transformed and scaled (mean-centred and divided by SD)] as a continuous predictor variable. To account for repeated sperm sampling (*i.e.*, from both the testes and sperm in the ejaculate) from individual males, a random slope of individual fish identification (ID) across time was fitted to the models. Models were also fit with sum-to-zero-contrasts for male tactic and sperm maturation stage and model results were assessed using type III sums of squares. *Post hoc* analyses were completed using comparisons of least-squares means [emmeans 1.2.3 package in R (Lenth, 2018)] and adjusted for multiple comparisons using the Tukey method.

Differences in total sperm longevity (*i.e.*, the time at which all observed sperm ceased achieving forward progression) as a result of male tactic and sperm maturation stage were assessed using a LMM using the same two R packages used for analysing sperm performance. Similarly, male tactic and sperm maturation stage were fit as categorical predictor variables and to account for repeated sampling of sperm from individual males, fish ID was fitted as a random intercept.

To investigate sperm morphological differences as a result of male tactic and sperm maturation stage, we used a multivariate linear mixed effects model [MLMM; blme 1.0–4 package in R (Chung *et al*., 2013)] to assess sperm head surface area, number of sperm head turns per micron of head length, midpiece surface area and tail length. Data from 2016 and 2017 were pooled as no differences among any sperm morphological traits as a result of year were detected (as tested by individual linear models, all $P > 0.05$). All response variables were scaled (mean-centred and divided by SD) and the model was fitted with male tactic and sperm maturation stage as categorical predictor variables and with individual fish ID as a random intercept.

To interpret the results of the MLMM, univariate LMMs were employed for each response variable indicated with a significant main effect or interaction in the MLMM coefficient plot [dotwhisker 0.3.0 package in R (Salt & Hu, 2017)]. LMMs were created using the same R packages used for analysing sperm velocity and longevity and were fitted with the same predictor variables and random intercept as the MLMM. Post-hoc analyses were completed using comparisons of least-squares means [emmeans 1.2.3 package in R (Lenth, 2018)] and adjusted for multiple comparisons using the Tukey method. Differences in the ratio of sperm head length:tail length as a result of male tactic and sperm maturation stage were not included in the MLMM and were assessed only with a LMM due to differences in data scaling against other measured sperm components.

2.9 | Animal ethics

Porichthys notatus is a common marine fish species and is not endangered or threatened. All fish were collected in accordance with permits issued by the Canadian Department of Fisheries and Oceans (XR 942016 and XR 582017). 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)).

3 | RESULTS

3.1 | Sperm density and total seminal fluid proteins

Sneaker males produced approximately three times more sperm per unit volume of ejaculate than guarder males (ANOVA, mean ± SE = 970 ± 340, $F_{1,21} = 8.2$, $P < 0.01$; Figure 1b). Sneaker male ejaculate contained on average 1.5 billion sperm cells ml^{-1}, while guarder male ejaculate contained only 0.47 billion sperm cells ml^{-1} on average. Seminal fluid collected from guarder and sneaker males contained similar concentrations of proteins (ANOVA, $F_{1,26} = 0.1$, $P > 0.05$). On average, guarders had 1666 μg of protein ml^{-1} of seminal fluid (range = 1021–2125, SD = 219) and sneaker males had 1693 μg of protein ml^{-1} of seminal fluid (range = 1505–2138, SD = 220).

3.2 | Sperm swimming performance

There was a significant three-way interactive effect between male tactic, sperm maturation stage and time on sperm velocity (Table 1). It appeared that sperm in the ejaculate from both male tactics as well as guarder male sperm from the testes slowed at the same rate (*i.e.*, had similar slopes of sperm velocity between 10 s and 15 min), while sneaker male sperm from their testes slowed down faster (Figure 3). However, comparisons among slopes did not reveal significant differences (Table 2).

Because comparisons among slopes did not reveal anything informative, next we examined lower order interactive effects based on the LMM results using type III sum of squares (Table 1). There was also a significant interactive effect between sperm maturation stage and male tactic on sperm velocity (Table 1), which was our main question and was statistically interpretable. Comparisons among intercepts revealed that sneaker male sperm in the ejaculate swam faster than guarder male sperm in the ejaculate (LMM, mean ± SE = 22.1 ± 7.4, $t = 3.0$, $p < 0.05$; Figure 1c). Guarder male sperm from their testes

TABLE 1 Summary of the general linear mixed effects models (LMM) on velocity (VAP), straightness (STR), and linearity (LIN) of sperm collected from both the ejaculate and the testes of guarder and sneaker *Porichthys notatus* males, fitted with male tactic, sperm maturation stage, and time since sperm activation as predictor variables and with individual fish identification (ID) as a random slope

Sperm performance response variable	Predictor variable	Mean ± SE	df	F	*P*
Sperm path velocity (VAP)	Male tactic	4.74 ± 3.32	130	2.03	> 0.05
	Sperm maturation stage	3.37 ± 1.73	1407	3.79	< 0.05
	Time	21.7 ± 2.16	129	100	< 0.001
	Male tactic × sperm maturation stage	6.30 ± 1.73	1407	13.2	< 0.001
	Male tactic × time	0.86 ± 2.16	129	0.16	> 0.05
	Sperm maturation stage × time	2.05 ± 1.74	1418	1.39	> 0.05
	Male tactic × sperm maturation stage × time	3.66 ± 1.74	1418	4.44	< 0.05
Sperm path straightness (STR)	Male tactic	0.97 ± 1.02	130	0.90	> 0.05
	Sperm maturation stage	0.34 ± 0.62	1407	0.30	> 0.05
	Time	4.61 ± 0.65	129	50.5	< 0.001
	Male tactic × sperm maturation stage	1.76 ± 0.62	1407	8.05	< 0.01
	Male tactic × time	0.56 ± 0.65	129	0.75	> 0.05
	Sperm maturation stage × time	1.40 ± 0.62	1418	5.13	< 0.05
	Male tactic × sperm maturation stage × time	0.60 ± 0.62	1418	0.93	> 0.05
Sperm path linearity (LIN)	Male tactic	0.99 ± 1.04	130	0.91	> 0.05
	Sperm maturation stage	0.52 ± 0.58	1407	0.80	> 0.05
	Time	5.71 ± 0.71	129	64.4	< 0.001
	Male tactic × sperm maturation stage	1.89 ± 0.58	1407	10.5	< 0.01
	Male tactic × time	0.52 ± 0.71	129	0.54	> 0.05
	Sperm maturation stage × time	1.01 ± 0.58	1418	3.00	> 0.05
	Male tactic × sperm maturation stage × time	0.91 ± 0.58	1418	2.41	> 0.05

also swam faster than their sperm in the ejaculate (LMM, mean ± SE = 19.4 ± 4.8, t = 4.0, P < 0.001; Figure 1d). We found no difference in velocity of sperm from the testes *v.* ejaculate in sneaker males (LMM, mean ± SE = 5.86 ± 4.98, t = 1.2, P > 0.05).

The effect of sperm maturation stage on sperm path straightness and linearity also varied as a result of male tactic (Table 1). Intercept comparisons revealed that guarder male sperm from their testes swam in a more linear path (LMM, mean ± SE = 4.82 ± 1.62, t = 2.9, P < 0.05) than did their sperm in their ejaculate. However, we did not find that sperm from the testes of guarder males swam significantly straighter than did sperm in their ejaculate (LMM, mean ± SE = 4.21 ± 1.73, t = 2.4, P > 0.05). No differences were observed in sneaker-male sperm path straightness (LMM, mean ± SE = 2.85 ± 1.79, t = 1.6, P > 0.05) or linearity (LMM, mean ± SE = 2.74 ± 1.67, t = 1.6, P > 0.05) when comparing sperm from the testes to those in the ejaculate. We also found no differences in sperm path straightness (LMM, mean ± SE = 5.46 ± 2.38, t = 2.3, P > 0.05) or linearity (LMM,

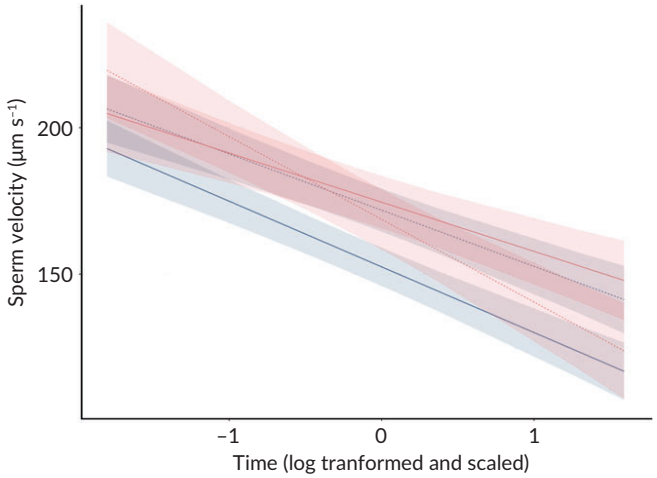

FIGURE 3 Fitted sperm velocity values (± SE) for guarder and sneaker male sperm from the testes and in the ejaculate over log-transformed and scaled (mean-centred and divided by SD) time. (——) Sneaker male ejaculate sperm, (- - - -) Sneaker male testes sperm, (——) Guarder male ejaculate sperm, and (- - - -) Guarder male testes sperm

TABLE 2 Summary of pairwise contrasts among slopes for the categorical predictor variables of male *Porichthys notatus* tactic (guarder or sneaker) and sperm maturation stage (ejaculate or testes) across the continuous predictor variable of time. This *post hoc* test was conducted following a significant three-way interaction between these three predictor variables on the response variable of sperm velocity

Pairwise contrast	Mean ± SE	*t*	*P*
Guarder male ejaculate – Sneaker male ejaculate	5.61 ± 5.37	1.05	> 0.05
Guarder male ejaculate – Guarder male testes	3.22 ± 4.82	0.67	> 0.05
Guarder male ejaculate – Sneaker male testes	5.82 ± 5.73	1.02	> 0.05
Sneaker male ejaculate – Guarder male testes	2.39 ± 5.36	0.45	> 0.05
Sneaker male ejaculate – Sneaker male testes	11.4 ± 5.01	2.28	> 0.05
Guarder male testes – Sneaker male testes	9.04 ± 5.72	1.58	> 0.05

TABLE 3 Summary of the univariate general linear mixed effects model analyses (LMM) on individual sperm morphological features of male *Porichthys notatus*, fitted with male tactic and sperm maturation stage as predictor variables with individual fish identification as a random intercept

Response variable	Predictor variable	Mean ± SE	χ^2	*P*
Number of head turns μm^{-1} head length	Male tactic	0.30 ± 0.01	5.2	< 0.05
	Sperm maturation stage	0.002 ± 0.008	0.08	> 0.05
Head surface area (μm^3)	Male tactic	10.7 ± 9.36	1.3	> 0.05
	Sperm maturation stage	19.6 ± 6.37	9.4	< 0.01
Midpiece surface area (μm^3)	Male tactic	19.0 ± 6.32	4.5	< 0.05
	Sperm maturation stage	15.0 ± 6.21	1.6	> 0.05
	Male tactic × sperm maturation stage	18.7 ± 8.78	4.5	< 0.05

mean ± SE = 5.77 ± 2.38, t = 2.42, P > 0.05) between sneaker male and guarder male sperm in ejaculate.

The sperm of both guarder and sneaker males decreased in velocity over time. The rate of this decrease in sperm velocity over time did not differ between male tactics (ANCOVA, mean ± SE = 0.0002 ± 0.02, t = 0.01, P > 0.05). We also found no differences in sperm longevity as a result of male tactic (LMM, mean ± SE = 16.6 ± 11.8, χ^2 = 1.97, P > 0.05), but sperm in ejaculates swam for shorter durations ($\bar{x}$ = 115 min, SD = 33) than sperm collected from testes ($\bar{x}$ = 147 min SD = 41; LMM, mean ± SE = 31.9 ± 11.8, χ^2 = 7.3, P < 0.01).

3.3 | Sperm morphology

We found that sperm morphological features changed with sperm maturation stage (MLMM, χ^2 = 25.5, df = 4, P < 0.001), but not with male tactic (MLMM, χ^2 = 14.2, df = 8, P > 0.05), nor was there an interaction between male tactic and sperm maturation stage (MLMM, χ^2 = 7.32, df = 4, P > 0.05). While we did not find an effect of male tactic across all sperm morphological features in our multivariate model, we did not eliminate male tactic as a predictor variable; also, we retained the interaction between male tactic and sperm maturation stage in the univariate models due to the suggestive effect of male tactic (*i.e.*, P = 0.07) across all sperm morphological values. Indeed, univariate models revealed that sneaker male sperm had more head turns per μm of sperm head length than guarder male sperm (Table 3 and Figure 2d). Also, while sperm in the ejaculate had larger heads than sperm collected from testes regardless of male tactic (Table 3), the effect of sperm maturation stage on sperm midpiece size depended on male tactic (Table 3). Specifically, guarder male sperm midpieces were larger in their ejaculate compared with their sperm collected from their testes (LMM, mean ± SE = 14.9 ± 6.21, t = 2.4, P < 0.05). Guarder male sperm in ejaculate also had larger midpieces compared with the midpieces of sneaker male sperm in their ejaculate (LMM, mean ± SE = 19.0 ± 6.32, t = 3.0, P < 0.01).

4 | DISCUSSION

This study demonstrates how sperm maturation differs between males adopting alternative reproductive tactics (ART) in *P. notatus* and highlights the competitive adaptations of the alternative tactic, the sneaker male. The inherently different levels of sperm competition risk experienced by guarder and sneaker males makes this species a convenient model to test sperm competition theory. We show that when comparing sperm in ejaculate, sneaker male sperm swam faster than that of guarder males. The sperm in guarder male ejaculate (*i.e.*, after fully maturing) swam more slowly and in a less linear pathway than did their sperm when it was collected from their testes (*i.e.*, before sperm have fully prepared for the external environment). Several sperm morphological traits also differed as a result of male tactic or sperm maturation stage. A number of previous studies have used sperm directly from the testes (*i.e.*, ignoring non-sperm components of ejaculate) to test predictions based on sperm competition theory (Burness *et al.*, 2004; Fitzpatrick *et al.*, 2005, 2007, 2016) and our results suggest that these previous findings should be treated with caution.

Many of our results were anticipated based on sperm competition risk theory, which predicts that sneaker males should invest more in their ejaculate to overcome greater sperm competition risk (Parker, 1990; Parker & Pizzari, 2010). The results of our study imply that sneaker males may have a sperm maturation process that has been shaped by regular and intense sperm competition. In many external fertilizers, sneaker males are forced to fertilise eggs from a disadvantaged position; at a greater distance from a spawning female (Brantley & Bass, 1994; Taborsky, 2008). Under such circumstances, sneaker males may experience selection to produce sperm that swim faster (Taborsky, 2008). Here, we found that sneaker male sperm outperformed guarder male sperm in the ejaculate.

We also observed that guarder male sperm from the testes outperformed their sperm in the ejaculate. Why might this be? One explanation is that sneaker males, more so than guarder males, need to maintain high sperm quality by recycling out poor quality, aged sperm and protecting new sperm. The seminal fluid in the ejaculate completes a number of sperm maintenance processes such as the recycling of sperm and providing protective storage of sperm (Chowdhury & Joy, 2007). *Porichthys notatus* produce and store most of their sperm prior to spawning (Barni *et al.*, 2001; Sisneros *et al.*, 2009); therefore, the capacity to protect and recycle sperm is critical. Because sneaker males are expected to invest more in their ejaculate to overcome greater sperm competition risk, perhaps sneaker males have upregulated non-sperm ejaculate components related to these sperm recycling and storage processes and this results in maintained sperm performance after leaving the testes. In contrast, guarder male ejaculate and their sperm maturation processes have been less shaped

by sperm competition and therefore do not maintain the quality of newer sperm from the testes.

Alternatively, although we diluted our ejaculates in seawater, it remains possible that sticky mucin proteins were more abundant in guarder male ejaculate and these proteins could have been responsible for reducing sperm velocity. In species with mucin-rich ejaculates, sperm are embedded in the ejaculate and are prevented from becoming activated or moving for long periods of time while the ejaculate eventually break-up in seawater (Marconato *et al*., 1996; Rasotto & Mazzoldi, 2002; Scaggiante *et al*., 1999). During this break-up process, ejaculate longevity is prolonged, as only portions of the ejaculate become activated over long periods, sometimes up to 30 h (Scaggiante *et al*., 1999). Therefore, if mucins were responsible for the reduced sperm velocity in guarder males, then we would have expected to see greater longevity of sperm in ejaculates in guarder males compared with sneaker males in our study. However, we did not see this. Instead, sperm from the testes swam for longer periods than sperm in the ejaculate regardless of male tactic. Additionally, if mucins played a significant role in influencing sperm performance in guarder males, then we may have expected to detect differences in protein abundances between the tactics, but we did not. Further study will be required to specifically characterise mucins and detail their effect on sperm performance in this species.

We found that sneaker males had more helical sperm heads than guarder males, a novel finding, and an unusual sperm morphology. The helical shaped sperm heads found in *P. notatus* are extremely rare among bony fishes; most ray-finned fishes have round sperm heads (Jamieson, 1991). Helical sperm heads are more common in chondrichthyan fishes, also appear in most species of passerine birds and rhacophorid tree frogs, and are common in a variety of invertebrates such as insects, crustaceans and molluscs (Fitzpatrick *et al*., 2012; Jamieson, 2005; Pitnick *et al*., 2009). To the best of our knowledge, other than *P. notatus* fish, no other species are known to have both helical sperm heads and ARTs. Theory proposes that having more gyres or turns along the sperm head provides a mechanistic swimming advantage; *e.g*., by enabling the sperm to maintain straighter swimming trajectories through the viscous microenvironment of female ovarian fluid or multiple male ejaculates (Pitnick *et al*., 2009; Vernon & Woolley, 1999). In a meta-analysis of 36 bird species Støstad *et al*. (2018) found that those species with a more pronounced helical sperm form also had faster swimming sperm. Here, in the ejaculate, sneaker male sperm did indeed swim faster than the less helical guarder male sperm, but we have no evidence that sneaker male sperm swam straighter. More work is needed to determine if the more helical heads found on sneaker male sperm result in greater sperm performance regardless of sperm maturation stage. Furthermore, future research is warranted to determine if the morphological differences uncovered by our study represent a greater investment in sperm architecture that can overcome the disadvantaged spawning position of *P. notatus* sneaker males.

Our study also demonstrates how sperm morphology can potentially change as a result of the maturation process; sneaker and guarder male sperm had larger heads once in the ejaculate and guarder male sperm specifically had larger midpieces in their ejaculate. These morphological changes could be the result of the sperm entering a different biotic environment once they leave the testes. Environmental characteristics such as pH and osmotic concentration are known to differ between the testes and in seminal fluid (Chowdhury & Joy, 2007; Rodriquez & Hinton, 2003). Seminal fluid is thought to buffer sperm against the hostile abiotic or biotic environment that they are about to enter (*e.g*., seawater, female reproductive tract, *etc*.) following ejaculation from the male reproductive environment (Poiani, 2006), but these changes in environmental conditions may also cause regions of the sperm cell to expand or shrink (Hadi Alavi *et al*., 2009; Yeung *et al*., 2003). The enlargement of the sperm heads and midpieces observed in this study could be a consequence of the sperm of entering a new environment and seminal fluid entering these cells. On the other hand, components of the seminal fluid can also modify sperm morphology in ways that enhance their performance regardless of environment. In humans, for example, the kallikrein-related proteolytic cascade and prostate-specific antigen action in seminal fluid is responsible for the modification and removal of proteins on the cell surface that increase sperm motility (Dachaux & Dachaux, 2014; Veveris-Lowe *et al*., 2007). These kinds of changes have the potential to be adaptive under the pressure of sperm competition. More experimental work and cross-taxa meta-analyses are needed to better understand the implications of sperm-head size and midpiece size on sperm motility and competitive ability as findings across taxa have revealed sometimes conflicting patterns. For example, midpiece size was thought to positively correlate with ATP content and sperm velocity (Cardullo & Baltz, 1991); however, recent studies across taxa provide conflicting evidence against this relationship (Bennison *et al*., 2016; Firman & Simmons, 2010; Malo *et al*., 2006; Simpson *et al*., 2014; Vladic, 2002). Whether the effects on sperm morphology and performance observed in this study are the result of adaptations to sperm competition risk or are the consequences of simply entering a new osmotic environment remains unclear. More research is now needed to reveal whether these sperm morphological changes result in increased sperm competitive ability and fertilisation success.

In a previous study on *P. notatus*, Fitzpatrick *et al*. (2016) found that sneaker male sperm collected from the testes (*i.e*., before fully maturing) were faster than their competitor guarder males at two time points post-activation. In this current study, however, we did not observe this same pattern. While we did not observe tactic-specific differences in sperm collected from the testes, sneaker male sperm did swim faster than guarder male sperm in the ejaculate. Fitzpatrick *et al*.'s (2016) study used a different sampling protocol (*i.e*., using different activation temperature, seawater, pH, other water quality parameters) and analytical method [*i.e*., selection of all motile cells (this study) *v*. the most motile cells (Fitzpatrick *et al*.) and a different number of cells analysed], making direct comparisons between the two studies difficult. The clear tactic-specific differences in ejaculate sperm swimming speed detected in the current study suggest even more strongly than the Fitzpatrick *et al*. study that there is an adaptive reproductive strategy among *P. notatus* sneaker males. Additionally, these current findings were observed under the most biologically relevant conditions considered so far.

The role of maturation on sperm performance has been examined in only a handful of fish species with ARTs. These other studies show

that seminal fluid specifically can enhance the performance of same-tactic or self-sperm or even reduce the performance of competitor sperm [Chinook salmon *Oncorhynchus tshawytscha* Walbaum, 1792 (Lewis & Pitcher 2017); grass goby *Gobius ophiocephalus* Pallas 1814 (Locatello *et al*. 2013)]. Gombar *et al*. (2017) demonstrated that in *O. tshawytscha*, seminal fluid proteins differ between tactics and these proteins are a possible mechanism to explain the differences in sperm performance. Future studies should continue to investigate the role of seminal fluid, other sperm maturation processes, and even female ovarian fluid in fertilisation dynamics by exploring outcomes with and without sperm competition. In general, our findings expand our understanding of the specialised responses in male reproductive tactics to sperm competition risk and highlight the importance of sperm maturation in sperm competition.

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AUTHORS CONTRIBUTIONS

Jessica S. Miller: funding collection, idea generation, experimental design, data collection and analysis and manuscript preparation. Aneesh P. H. Bose: help with idea generation and experimental design, data collection and manuscript preparation. John L. Fitzpatrick: help with idea generation and experimental design, data analysis and manuscript preparation. Sigal Balshine: funding collection, help with idea generation and experimentation design, help with data collection and analysis and manuscript preparation.

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