

# Nonmating behavioural differences between male tactics in the invasive round goby

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Alternative reproductive tactics (ARTs) exist when individuals of one sex (usually males) adopt different strategies to achieve reproductive success. Although it is well known that the males adopting the different tactics often vary in their morphological, physiological and mating-based behavioural traits, what is not well understood is whether variation also exists in their behaviour outside the mating context. Here, we used the round goby, *Neogobius melanostomus*, a globally prolific invasive species where males exhibit ARTs, to address this knowledge gap. The nonmating behaviour of sneaker and guarder males were compared for differences in boldness, exploration, activity, aggression, sociality and dispersal. We found that sneaker males were more active, explorative and bold, as well as less aggressive compared to guarder males. Additionally, in an exploratory analysis, we found that while many behavioural traits were positively correlated in guarder males, few were correlated in sneaker males, suggesting that sneaker males might be more behaviourally plastic. The results of this study help expand our knowledge of the many ways that alternative reproductive tactics may differ from each other, particularly in nonmating contexts.

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Alternative reproductive tactics (ARTs) are a taxonomically widespread phenomenon, where members of the same sex display two or more divergent strategies to achieve reproductive success (Taborsky, 2001; Taborsky et al., 2008). For example, in taurus scarab beetles, *Onthophagus taurus*, large-horned, resource-holding males guard tunnel entrances that lead to females (Emlen, 1997; Moczek & Emlen, 2000). Smaller short-horned/hornless males instead bypass guarding males by digging adjoining tunnels and moving through these tunnels to 'sneak' access to females (Emlen, 1997; Moczek & Emlen, 2000). While morphological, physiological and mating behavioural differences among males have been well investigated in species with ARTs (Bass, 1992; Emlen, 1997; Gross, 1996; Moczek & Emlen, 2000; Sinervo et al., 2000), outside of the mating context we know little about whether or how the males adopting these tactics differ behaviourally.

Particular behavioural traits may be more common or beneficial for one reproductive tactic over the other. For example, aggression may be necessary for the guarder/territorial tactic to protect against intrusion and reproductive parasitism by sneaker males. In the black goby, *Gobius niger*, and grass goby, *Zosterisessor ophiocephalus*, guarder males provide parental care for offspring and increase their

aggression levels in the presence of sneaker males that exploit this parental care (Scaggiante et al., 2005). However, such aggressive tendencies would likely be disadvantageous for sneaker males trying to avoid detection by guarder males. Moreover, sneaker males are unlikely to win contests because they are often smaller. Also, behavioural traits may be correlated across mating and nonmating contexts, forming what is known as a behavioural syndrome (Sih et al., 2004). Such syndromes have been observed in male water striders (*Gerris gracilicornis*), where males that are more active in a nonmating context also make more mating attempts (Han & Jablonski, 2019). However, if one tactic needs to be opportunistic, then behavioural plasticity may be beneficial for that tactic, potentially resulting in weak or absent behavioural syndromes. This could lead to a situation where behavioural syndromes may not be the same for males across tactics (Wilson & Kelly, 2019).

Alternative reproductive tactics may induce and/or maintain consistent behavioural variation in a population, and as such, influence the likelihood of persistence for that population. Individual behavioural variation within a population is known to have important implications for persistence, especially in unstable environmental conditions (Dingemanse et al., 2004; Dingemanse & Réale, 2005). For example, in great tits, *Parus major*, adult males that are more explorative have higher survival rates after food-plentiful winters but lower survival rates after food-scarce winters, while an opposite trend was observed in females (Dingemanse et al., 2004; Dingemanse & Réale, 2005). This opposing optima in a

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shared trait between the sexes is referred to as intralocus sexual conflict. This concept has been adapted for ARTs and termed intralocus tactical conflict, where behavioural plasticity may be promoted differently among the tactics (Abbott et al., 2019).

The round goby, *Neogobius melanostomus*, is a globally invasive species with two male alternative reproductive tactics: nest-guarding and nest-sneaking males (Bleeker et al., 2017; Bose et al., 2018; Marentette et al., 2009). The round goby's introduction into the Laurentian Great Lakes, U.S.A. is thought to have been facilitated by multiple ballast water expulsions from transoceanic ships arriving from the Black Sea (Jude et al., 1992). Round goby are considered an ecologically disruptive species as they outcompete native benthic fish species for habitat and resources (Bergstrom & Mensinger, 2009; Dubs & Corkum, 1996; Savino et al., 2007), depredate native fish eggs (Chotkowski & Marsden, 1999; Roseman et al., 2006; Steinhart et al., 2004) and accumulate and transfer contaminants up the food chain (Kwon et al., 2006). Currently, round goby are spreading into previously uninvaded habitats in western Europe and North America (Kornis et al., 2012). The success of the round goby invasion on multiple continents make them an ideal study species to understand nonmating behavioural differences between the two tactics and to investigate how ARTs might influence invasion success. Behaviour and the behavioural syndromes associated with each round goby alternative reproductive tactic may in part dictate which individuals are most prone to spreading into new habitats.

There have been a number of studies on round goby behaviour, especially in the context of secondary range expansion (Groen et al., 2012; Myles-Gonzalez et al., 2015; Thorlacius et al., 2015); however, most of these studies have focused on nonreproductive males. To date, only one study – an unpublished thesis – has performed a comprehensive examination of behavioural differences between guarder and sneaker round goby, but in that study the sneaker male sample size was small ( $N = 7$ ) and the reproductive tactics were not fully confirmed by dissection (Nguyen-Dang, 2017). In our study, we completed a robust behavioural scan assessing aggression, sociality, boldness, activity, exploration and dispersal tendencies in a larger sample (~30) of guarder and sneaker male round goby and confirmed reproductive status with dissections. Our objective was to investigate and compare nonmating-related behaviours between sneaker and guarder male round goby. Given their mating strategy, we inferred that sneaker males would likely be more behaviourally plastic and, thus, we also explored behavioural trait correlations in sneaker and guarder males separately.

## METHODS

### *Fish Collection*

Round goby were caught using baited (~25 g of frozen corn) and unbaited galvanized steel silver and vinyl-coated black minnow traps between May and July 2019. Fish were captured at four locations in Hamilton Harbour (LaSalle Marina: 43°18'2"N, 79°50'46"W; Fisherman's Pier: 43°17'48"N, 79°47'47"W; Pier 27: 43°17'4"N, 79°47'30"W; Pier 15: 43°16'12"N, 79°50'2"W) and at Fifty Point Conservation Area (43°13'34"N, 79°37'21"W). Traps were placed approximately 10 m apart and retrieved after 24 h. Any captured round goby were sexed and given a reproductive status via visual inspection of the urogenital papilla (sex and status were later confirmed by dissections; see below). Following the methods outlined in Marentette et al. (2009), males with flat papillae were designated as nonreproductive males while those with a turgid papilla were classified as reproductive. Males with a turgid papilla were further qualitatively differentiated in the field into either guarder males (GM) or sneaker males (SM) based on the following characteristics: males that were black and/or had a wide head were classified as GM and males that had narrow heads,

were generally smaller and had a large papilla relative to their body size were designated as SM. Status was later further confirmed with quantitative internal measures once the fish had been run through behavioural experiments (see below). Identified guarder and sneaker males were placed individually in flow-through containers and transported live within an aerated cooler to McMaster University for behavioural experimentation. Female and nonreproductive male goby were humanely euthanized using a mixture of benzocaine and water whereas any native species captured were promptly returned to the site of capture.

Immediately upon arrival to McMaster, round goby were tagged with subdermal injections of nontoxic acrylic paint (Wolfe & Marsden, 1998), weighed (nearest 0.001 g) and allowed to recover in aerated containers. Fish were then transferred to 75-litre laboratory holding tanks, containing ~1 cm thick layer of gravel and four to six PVC pipes as shelter. Tanks were filtered, aerated and maintained on a 14:10 h light:dark cycle. Water changes were completed every 2 weeks. We separated fish by visually identified status, housing suspected sneaker and guarder males separately from each other. Fish were held for 2–4 days in these tactic-specific holding tanks until behavioural experimentation.

### *Experimental Tank Set-up*

Behavioural tendencies were assessed in one of four identical tri-divided 150-litre (92 × 45 × 38 cm), filtered and aerated experimental tanks. We used six consecutive behavioural assays that were administered in the following order: exit test, exploration, activity, sociality, aggression, startle response and dispersal tendency. We ran the behavioural assays in this set order to standardize the comparison of sneaker and guarder males and to attempt to minimize potential carryover effects (e.g. stress from the startle). In addition, at the study outset, we were uncertain whether we would obtain a large enough sample of each tactic to statistically account for all the permutations that a randomized order would require (Bell, 2013).

The experimental tanks in which the behavioural assays were conducted were divided into sections using both transparent and opaque acrylic barriers (Appendix, Figs A1, A2). Experimental tanks were lined with contact paper on the sides and on the bottom of the tank to minimize glare and interference from neighbouring tanks. Tanks contained no substrate. An opaque curtain was set up between the experimenter and the tanks to minimize disturbance to the fish during trials. Before trials commenced, the filters and airstones were removed from the tanks. Assays were recorded from above each tank using Sony video cameras mounted on tripods. Videos were imported into BORIS video scoring software (Friard & Gamba, 2016), and all videos within a behavioural assay were scored by the same researcher for consistency.

### *Exit Test and Exploration Assay*

Focal round goby in the holding tanks were guided with a hand-net into an opaque tube-shaped black refuge and transferred individually in this refuge to the middle section of one of the four tri-divided experimental tanks. The fish remained submerged in water inside the refuge through the entire transfer process to minimize stress. Once in the experimental tank, the fish was allowed 1 h to habituate and recover from capture and transport. After this habituation period, a door on one end of the refuge was remotely lifted via a pulley system, allowing the focal fish to exit from the refuge (Fig. 1a). Each focal fish was given 15 min to independently exit the refuge, after which the entire refuge was remotely lifted from the closed end via a pulley system, thus forcing any fish still in the refuge to leave. Whether or not the fish willingly left the shelter was recorded, as well as the time taken to leave the

shelter if the fish left of its own volition. The duration of time the fish spent with their heads partially emerged from the shelter was also recorded. Once the refuge was remotely lifted out, the fish was given 30 min to freely explore the middle chamber of the novel experimental tank that had a  $4 \times 4$  grid of  $10 \times 10$  cm squares marked on the bottom (Fig. 1d). The number of unique squares the focal goby visited was recorded as the tendency to explore a novel environment (Jones & Godin, 2010). Furthermore, as an assessment of risk taking, the proportion of time spent in the middle four grid squares (in the open and more exposed tank centre) was also recorded and interpreted as a measure of boldness.

#### Activity Assay

Activity was also measured during the exploration trials. The total amount of time fish spent making any movement was recorded for this 30 min trial.

#### Sociality Assay

Sociality was measured following methods developed and validated in Capelle et al. (2015). Before the trials started, we placed a small tank containing a halved flowerpot that could be used as a refuge in each of the end chambers. In one of these two small tanks (chosen by the flip of a coin), three male conspecifics were also added. The small glass tanks at each end prevented any conspecific odour cues from reaching and influencing the focal fish. Immediately following the exploration/activity trial, two opaque barriers were remotely removed via a pulley system, allowing the focal fish in the middle to see the two end chambers (Fig. 1g). We monitored where the focal fish spent its time within the central chamber over a 30 min trial where it could see both end chambers. The centre focal fish chamber was divided into three zones for this assay: (1) a social zone (area =  $400 \text{ cm}^2$ ), closest to the three stimulus fish, (2) a larger neutral central zone ( $800 \text{ cm}^2$ ) and (3) an asocial zone ( $400 \text{ cm}^2$ ) furthest from the stimulus fish. The total amount of time spent in the social zone was taken as an indication of sociality, where more time spent in the social zone indicated a greater tendency to seek social interactions. We also recorded any aggressive acts (ramming and parallel displays) made by the focal fish during this trial.

#### Aggression Assay

Once the sociality assay was completed, two opaque barriers were remotely lowered, again concealing the two end chambers. One of these barriers (chosen by the flip of a coin) had a  $30.5 \text{ cm}$  square mirror affixed to the barrier's surface (Fig. 1j). The mirror introduced a perfectly size-matched 'competitor conspecific'. Aggressive behaviours towards the mirror image of the 'competitor' were recorded for 30 min. The number of aggressive acts (rams and parallel displays) made by the focal individual was used as a measure of aggressive tendency.

#### Startle Response Assay

Following the aggression trial, two opaque barriers were remotely lowered via a pulley system, one to cover the mirror and the other to balance the disturbance caused by the lowering barrier on the other side of the tank. Movement of each focal fish was then recorded in the central chamber for 15 min, after which a marble was remotely rolled through a PVC tube and dropped into the tank (Fig. 1i). The marble drop was intended to simulate a predator strike. Each fish's response to the marble drop (freeze, dart and freeze, or no response) and their latency to first movement (seconds) post marble drop were recorded. The quicker the fish resumed movement, the bolder the fish was interpreted to be. We

also calculated the time fish spent moving for 15 min after the startle and compared this to the time spent moving before the marble drop as another measure of boldness.

#### Dispersal Assay

Dispersal was assessed in a  $2.5 \text{ m}$  long by  $0.75 \text{ m}$  wide acrylic dispersal chamber (Appendix, Fig. A3). The chamber was divided into three laneways that allowed us to run three separate round goby simultaneously. Each laneway contained light sand substrate approximately  $1.5 \text{ cm}$  deep. Four alternating wall barriers protruded from the sides at  $45 \text{ cm}$ ,  $65 \text{ cm}$ ,  $130 \text{ cm}$  and  $150 \text{ cm}$  from the start zone (Fig. 2a). Two additional canyon-like barriers, each  $21 \text{ cm}$  long, were placed at  $90 \text{ cm}$  and  $170 \text{ cm}$  from the start zone (Fig. 2b). Flow was created in each laneway using  $940 \text{ gallons/h}$  ( $3558.3 \text{ litres/h}$ ) submersible pumps. The start position of each fish was located at the opposite end from the flow so that fish were swimming against the artificial current. Fish were transferred into one of the three laneways, in enclosed and submerged refuges identical to those used in the exit test assay. Fish were given 30 min to habituate in the refuge following the transfer, after which the door was remotely lifted via a pulley (as in the exit test above). Fish were given 15 min to freely exit the shelter before the entire refuge was remotely removed from the dispersal chamber. Then fish were given 1.5 h to disperse and move freely in the laneway. Each trial was recorded from overhead using a GoPro video recorder. A number of measures were recorded, including whether fish reached the end, the time taken to reach the end, total number of laps completed (defined as going from one end section to the other, regardless of flow direction) and the crosses made between zones (from one zone into another). Zones were defined as open sections between wall or canyon barriers, including the start and end sections, resulting in a total of seven zones (Fig. 2a). Canyons were not considered zones.

#### Morphological Differences

After completing the dispersal assay, round goby were immediately euthanized. The following measurements were recorded for each fish: total length, standard length (SL), papilla length, head width, body width, total mass, liver mass, seminal vesicle mass and total gonad mass. Length measures were taken with callipers accurate to the nearest millimetre. Mass was taken in grams using an Ohaus Adventurer Pro digital scale, accurate to three decimal places. Males designated as guarder males (GM) were black or extremely dark, had wide heads and were generally larger ( $> 5.0 \text{ cm}$  SL; Marentette et al., 2009; McCallum et al., 2019). Males classified as sneaker males (SM) were lightly mottled, had narrow heads, were typically smaller ( $< 5.0 \text{ cm}$  SL) and had a long papilla relative to their body size (Marentette et al., 2009; McCallum et al., 2019). A variety of indices were calculated from the collected body measurements. Condition was calculated using Fulton's body condition (total mass (g))/(standard length (cm)<sup>3</sup> × 100%) and hepatosomatic index (HSI) (liver mass (g))/(total mass (g) – liver mass (g) × 100%). Gonadosomatic index (GSI) (total gonad mass (g))/(total mass (g) – total gonad mass (g) × 100%) and seminal vesicle (accessory gland) mass were used to further confirm the alternative reproductive tactic. Sneaker males usually have large GSIs and small accessory glands while guarder males usually have relatively smaller GSIs and larger accessory glands (Marentette et al., 2009; McCallum et al., 2019). Sneaker males were classified as such if they exhibited the usual visual characteristics (narrow head, pale or mottled body colour, small-bodied and a large papilla relative to standard length) and had a GSI of greater than 2% (Marentette et al., 2009; Young et al., 2010). Guarder males were classified as such if they exhibited the usual

visual characteristics (wide head, dark or black coloured body, large seminal vesical mass, larger-bodied) and had a GSI of greater than 1% (Marentette et al., 2009; Young et al., 2010). Note that the >1% GSI is a cutoff used to determine reproductively active round goby males in general, while the >2% GSI is a cutoff for sneaker males specifically. However, this does not imply that all goby with >2% GSI are necessarily considered sneaker males; some are guarder males. Finally, age was calculated by visually counting annuli on otolith samples taken from each specimen.

#### Ethical Note

Animal housing, handling and study protocols were approved by the McMaster Animal Research Ethics Board (Animal Utilization Protocol 17-45-12) and adhered to the guidelines of the Canadian Council on Animal Care (CCAC) and ASAB/ABS (2020) regarding the use of animals in research and teaching. Fish were marked with a nontoxic acrylic paint injected just beneath the dermal layer. This marking method did not cause any apparent long-term distress to the fish. Fish were monitored closely after injection and while in the holding tanks for any signs of injury or stress but none occurred.

#### Statistical Analysis

All statistical analyses were performed using R v.3.6.1 (R Core Team, 2020). Data were assessed visually for normality using quantile–quantile plots and for homoscedasticity using residual-versus-fitted plots. Arcsine square-root or log transformations were performed where required and noted for each model. Morphological and age comparisons were performed using linear models. To better visualize the disparity between sneaker and guarder male round goby, we conducted a principal component analysis (PCA) on the quantitative morphological measures excluding age. Behavioural response variables were modelled as a function of status (guarder or sneaker) using univariate linear mixed effects models (LMMs), generalized linear mixed effects models (GLMMs) or Wilcoxon tests (rank-sum tests for unpaired data and signed-rank tests for paired data) if parametric assumptions could not be met after transformations. (G)GLMMs were performed using the (g)lmer function in the package lmerTest (Kuznetsova et al., 2017). Negative binomial models were performed using the glmer.nb function in the package lme4 (Bates et al., 2015). Random effects included capture site, Julian date, total days in laboratory since capture and experimental tank/lane. The side on which the fish started was included as a random effect for the sociality trial. All videos for a behavioural assay were standardized to the same length. Fish excluded from analyses and the resulting sample sizes for each analysis/comparison are provided in the Appendix, Table A1.

Correlation matrices across behaviours were calculated and analysed for guarder and sneaker males separately using the rcorr function in the Hmisc package (Harrell, 2020). All correlations were analysed using Pearson's  $r^2$  after checking for normality. Each matrix consisted of the following behaviours: number of grid squares explored (exploration assay), activity (activity assay), time spent near conspecifics (sociality assay), number of aggressive acts performed (aggression assay), latency to move after the drop of the marble simulating a predator attack (startle/boldness assay) and time taken to reach the end of the dispersal lane (dispersal assay). Sample sizes for each comparison are provided in the Appendix, Table A2.

Significance for morphological and behavioural tests were assessed at  $\alpha < 0.05$  after adjusting for multiple comparisons using the Benjamini–Hochberg method with a false discovery rate of 0.05 (Benjamini & Hochberg, 1995). Original and adjusted  $P$  values are provided throughout. Correlation analyses were exploratory and

therefore the adjustment of  $P$  values to maintain type I error rate was not considered.

## RESULTS

### Morphological Differences between the Tactics

Guarder and sneaker males had clear morphological differences (Table 1), with the PCA further visualizing the morphological disparity between the tactics (Fig. 3). Sneaker males had larger gonadal investment (GSI) and papilla–body length ratios while guarder males had larger head width–body width ratios, liver investment (HSI), ratios of seminal vesicle (accessory gland) mass to gonad mass, body mass and standard length. Guarder males also had slightly higher body condition values. The average age of guarder males was 3.01 years, while that of sneaker males was 2.11 years (linear model, LM: estimate  $\pm$  SE =  $0.90 \pm 0.29$ ,  $t_{39} = 3.148$ ,  $P = 0.003$ ).

### Behavioural Differences between the Tactics

#### Exit test

Guarder males partially emerged (with just their heads) from the shelter and remained in this position for longer than sneaker males (log LMM: estimate  $\pm$  SE =  $0.76 \pm 0.26$ ,  $t = 2.92$ ,  $P = 0.005$ ,  $P_{\text{adj}} = 0.01$ ; Fig. 1b). However, sneaker males were 92.7% more likely to exit the shelter completely compared to guarder males (binomial GLMM: estimate  $\pm$  SE =  $2.53 \pm 1.08$ ,  $z = 2.34$ ,  $P = 0.02$ ,  $P_{\text{adj}} = 0.03$ ; Fig. 1c). While 32% of the guarder males (8 out of 25) never exited the refuge on their own accord, only 8% of the sneaker males (3 out of 40) never willingly exited. Of the fish that did exit the shelter, there was no observed difference in the latency to exit between guarder and sneaker males (Wilcoxon rank-sum test:  $W = 279$ ,  $N = 54$ ,  $P = 0.52$ ,  $P_{\text{adj}} = 0.61$ ).

#### Exploration and activity

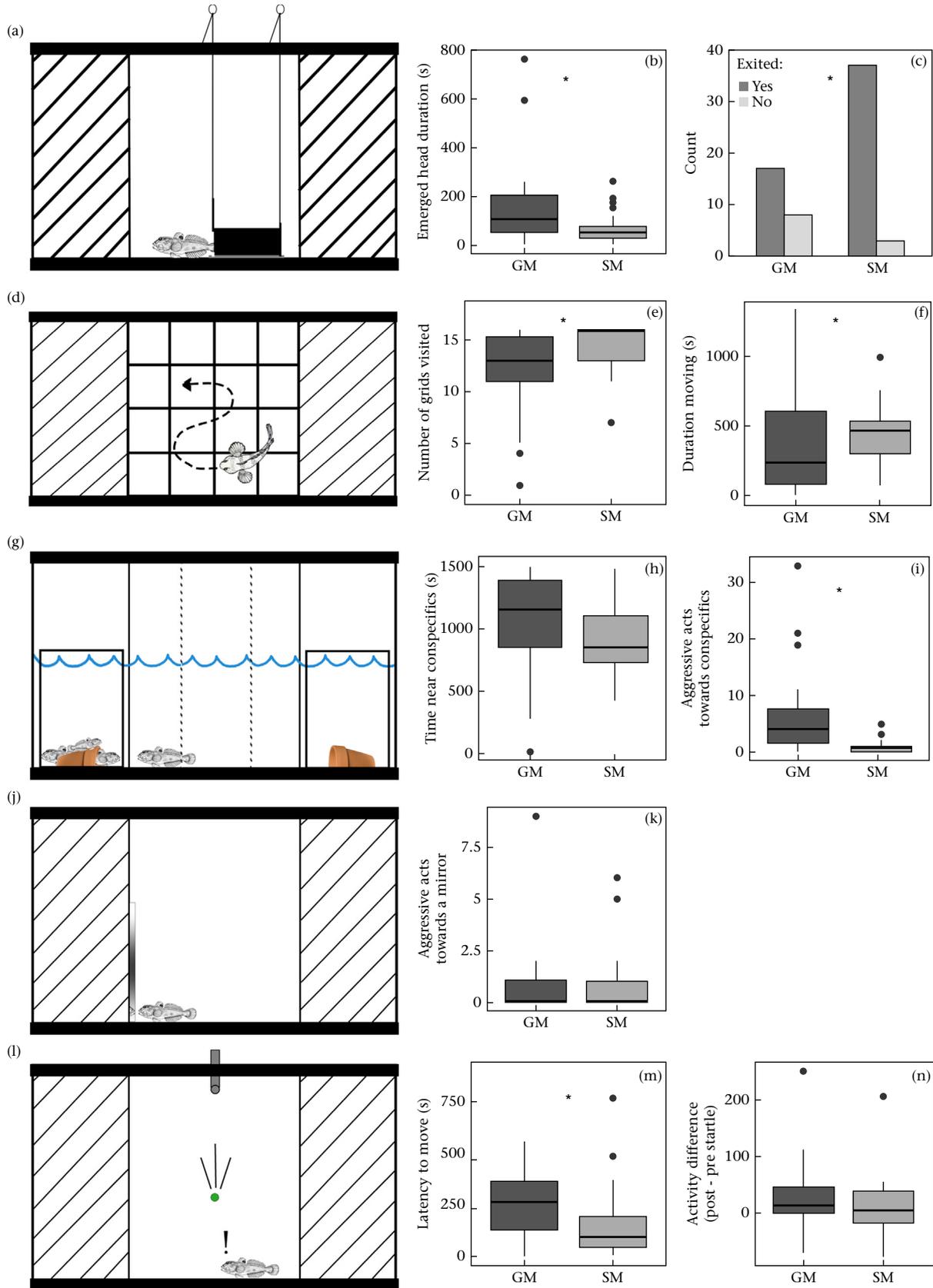
Sneaker males explored more grid squares than guarder males in the first 15 min (arcsine square-root LMM: estimate  $\pm$  SE =  $0.39 \pm 0.10$ ,  $t = 3.77$ ,  $P < 0.001$ ,  $P_{\text{adj}} = 0.002$ ) and over the full assay duration (Wilcoxon rank-sum test:  $W = 189$ ,  $N = 49$ ,  $P = 0.02$ ,  $P_{\text{adj}} = 0.03$ ; Fig. 1e). Sneaker males also spent more time in the centre of the tank (which was more exposed), averaging 78 s in the open centre while guarder males only averaged 26 s in the more exposed central area of the tank (Wilcoxon rank-sum test:  $W = 179$ ,  $N = 49$ ,  $P = 0.02$ ,  $P_{\text{adj}} = 0.03$ ). Sneaker males were more active than guarder males (LMM: estimate  $\pm$  SE =  $178 \pm 80.0$ ,  $t = 2.22$ ,  $P = 0.03$ ,  $P_{\text{adj}} = 0.046$ ; Fig. 1f).

#### Sociality and aggression

Both guarder and sneaker males spent most of the assay near conspecifics and did not differ in how socially motivated they were (LMM: estimate  $\pm$  SE =  $16.78 \pm 89.4$ ,  $t = 0.19$ ,  $P = 0.85$ ,  $P_{\text{adj}} = 0.89$ ; Fig. 1h). Guarder males performed more aggressive acts towards conspecifics during the sociality assay compared to sneaker males (negative binomial GLMM: estimate  $\pm$  SE =  $1.48 \pm 0.44$ ,  $t = 3.35$ ,  $P < 0.001$ ,  $P_{\text{adj}} = 0.002$ ; Fig. 1i). However, the two tactics did not differ in aggression towards the mirror (negative binomial GLMM: estimate  $\pm$  SE =  $-0.02 \pm 0.75$ ,  $z = -0.03$ ,  $P = 0.98$ ,  $P_{\text{adj}} = 0.98$ ; Fig. 1k), with both tactics performing fewer aggressive acts towards a mirror image than towards live conspecifics during the sociality assays (Wilcoxon signed-rank test:  $V = 35$ ,  $N = 30$ ,  $P = 0.02$ ,  $P_{\text{adj}} = 0.03$ ).

#### Startle response

In response to the marble drop (a simulated predator strike), six guarders and two sneakers darted away while 15 guarders and 20 sneakers froze. No fish continued to swim normally. A similar



**Figure 1.** Illustrations of the experimental tank set-ups for the various behavioural assays employed and corresponding results. Each row shows the assay and the associated behavioural results comparing round goby guarder males (GM) and sneaker males (SM). (a) The refuge exit test, with (b) the average duration spent with head partially emerged from the refuge and (c) the number of fish that did and did not exit. (d) The exploration assay with (e) the number of unique grid squares visited and (f) activity. (g) The sociality assay with (h) the time spent near conspecifics (maximum 1500 s) and (i) the average number of aggressive (ramming and parallel displays) acts towards conspecifics. (j) The mirror aggression assay with (k) the number of aggressive acts made towards a mirror. (l) The startle assay with (m) the latency to move after the marble drop and (n) the difference in time spent moving before and after the marble drop. Box plots indicate medians and whiskers extend to the furthest data point within  $1.5 \times$  the interquartile range. Individual points indicate values falling outside this range.  $*P_{adj} < 0.05$ .

number of guarder and sneaker males never resumed moving after the marble drop (binomial GLM: estimate  $\pm$  SE =  $0.69 \pm 0.92$ ,  $z = 0.75$ ,  $P = 0.45$ ,  $P_{\text{adj}} = 0.56$ ). Of the fish that did move after the marble drop (83% of guarders and 91% of sneakers), sneaker males were quicker to resume moving compared to guarder males (LMM: estimate  $\pm$  SE =  $-150.1 \pm 61.6$ ,  $t = -2.44$ ,  $P = 0.02$ ,  $P_{\text{adj}} = 0.03$ ; Fig. 1m). However, the change in time spent moving before versus after the marble drop did not differ between guarder and sneaker males (LMM: estimate  $\pm$  SE =  $-12.2 \pm 22.4$ ,  $t = -0.54$ ,  $P = 0.59$ ,  $P_{\text{adj}} = 0.67$ ; Fig. 1n).

#### Dispersal tendency

About one-third of the fish (11 out of 31 guarder males and 13 out of 34 sneaker males) reached the end of the dispersal chamber (binomial GLMM: estimate  $\pm$  SE =  $0.16 \pm 0.57$ ,  $t = 0.28$ ,  $P = 0.78$ ,  $P_{\text{adj}} = 0.85$ ). Guarder males took on average 1249 s to reach the end while sneaker males took on average 1476 s; this difference was not significant (LMM: estimate  $\pm$  SE =  $-422 \pm 404$ ,  $t = -1.05$ ,  $P = 0.31$ ,  $P_{\text{adj}} = 0.40$ ; Fig. 2c). Sneaker males and guarder males completed similar numbers of laps (negative binomial GLMM: estimate  $\pm$  SE =  $0.36 \pm 0.27$ ,  $t = 1.35$ ,  $P = 0.18$ ,  $P_{\text{adj}} = 0.24$ ; Fig. 2d), but sneaker males crossed back and forth between more zones than did guarder males (square-root LMM: estimate  $\pm$  SE =  $2.34 \pm 0.66$ ,  $t = 3.56$ ,  $P < 0.001$ ,  $P_{\text{adj}} = 0.002$ ; Fig. 2e), suggesting they were more active.

#### Behavioural Correlations across Assays

We uncovered five behavioural correlations in guarder males (Fig. 4a) and only one behavioural correlation in sneaker males (Fig. 4b). In guarder males, there was a negative correlation between activity and the startle response ( $r_{20} = -0.61$ ,  $P = 0.002$ ), and between exploration and the startle response ( $r_{20} = -0.47$ ,  $P = 0.03$ ); fish that were more active and explorative were also quicker to move again after the marble drop. Activity was positively correlated with exploration in guarder males ( $r_{22} = 0.57$ ,  $P = 0.004$ ), and guarders that were more social took longer to reach the end of the dispersal assay ( $r_4 = -0.94$ ,  $P = 0.005$ ). Finally, in guarder males, sociality and aggression were positively correlated

**Table 1**

Average body length, condition, mass and other morphological measures for our sample of round goby guarder males (GM,  $N = 25$ ) and sneaker males (SM,  $N = 40$ )

Measure	GM	SM	$t$	$P$	$P_{\text{adj}}$
Standard length (cm)	7.80	4.60	11.90	<0.001	<0.001
Total mass (g)	13.62	2.35	13.20	<0.001	<0.001
Head width to body width	1.26	1.05	8.64	<0.001	<0.001
Body condition	2.63%	2.29%	3.28	0.002	0.01
Hepatosomatic index	2.13%	0.70%	9.14	<0.001	<0.001
Genital papilla to body length	0.06	0.09	-6.70	<0.001	<0.001
Seminal vesicle to gonad mass	0.37	0.08	11.21	<0.001	<0.001
Gonadosomatic index	2.32%	4.63%	-7.53	<0.001	<0.001

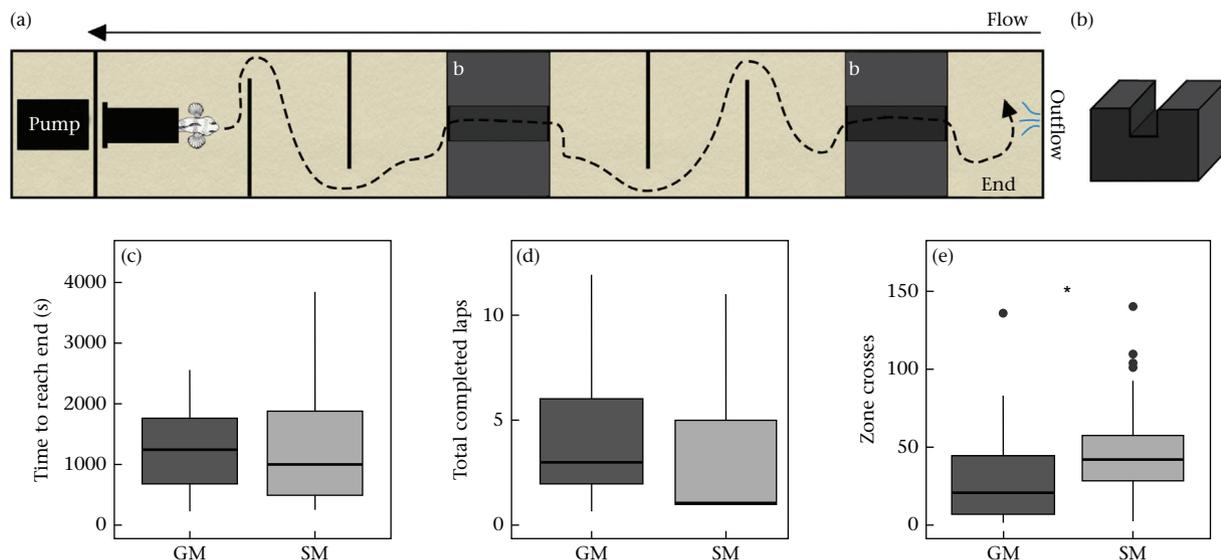
Measures were used in combination to confirm each status. Comparisons were made using linear models and the resulting  $t$  statistics and  $P$  values for each comparison are provided. To account for multiple comparisons, we provide adjusted  $P$  values ( $P_{\text{adj}}$ ) using the Benjamini–Hochberg method with a false discovery rate of 0.05.

( $r_{17} = 0.56$ ,  $P = 0.01$ ). The only significant correlation among behaviours in sneaker males was a negative correlation between exploration and sociality: fish that were more social were less explorative ( $r_{20} = -0.46$ ,  $P = 0.03$ ).

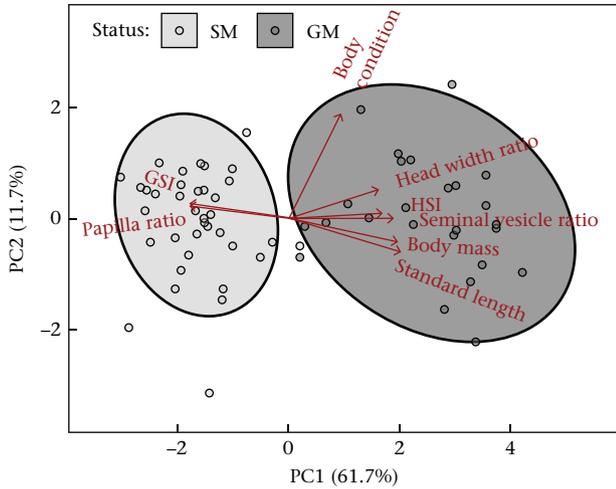
## DISCUSSION

#### Clear Morphological Differences between the Tactics

Sneaker and guarder male round goby differed morphologically. While sneaker males had a larger average gonadosomatic index, guarder males had larger seminal vesicles. Seminal vesicles are thought to produce pheromones that attract females (Jasra et al., 2007) but also appear to play a role in sperm storage (Jasra et al., 2007; Marentette et al., 2009; Miller et al., 2019) and parental care (Pepler et al., 2021). These patterns are consistent with past research conducted on round goby male tactics (Bleeker et al., 2017; Marentette et al., 2009; McCallum et al., 2019). Sneaker males likely invest more into their gonads because they require the presence of a guarder male to reproduce, consequently resulting in sperm competition every time they mate, while guarder males do not always mate in the



**Figure 2.** Illustration of the (a) dispersal assay laneway used to assess dispersal tendency differences between round goby guarder males (GM) and sneaker males (SM). The dispersal tank contained three identical lanes side by side. Vertical lines indicate wall barriers that extended three-fourths of the way across the lane and (b) canyon-type barriers were added to elevate goby closer to the surface to increase perceived risk. Results include (c) the average time taken to reach the end after exiting the refuge (this graph only includes goby that reached the end), (d) the average number of laps (travelling completely from one end to the other) and (e) the average number of crosses made between barriers within the dispersal arena. Box plots indicate medians and whiskers extend to the furthest data point within  $1.5 \times$  the interquartile range. Individual points indicate values falling outside this range. \* $P < 0.05$ .



**Figure 3.** Principal component analysis (PCA) of round goby sneaker male (SM) and guarder male (GM) morphological traits. Together, PC1 and PC2 axes accounted for 73.4% of the total variance. Ellipses indicate the 95% confidence intervals for the SM (in lighter grey) and GM (in darker grey) groups. Red arrows represent the loading and direction of each morphological variable.

presence of a sneaker (Marentette et al., 2009; Parker & Ball, 2005). Guarders were in better body condition than sneakers and also had higher hepatosomatic index scores. This was surprising as we expected guarder males to be in worse condition than sneaker males based on costly parental investment associated with nest guarding and offspring care often depleting energetic reserves in caring parents (Balshine-Earn, 1995). Sneaker males may have lower body condition because searching for nests with spawning females while avoiding the guarding males' aggression is energetically costly.

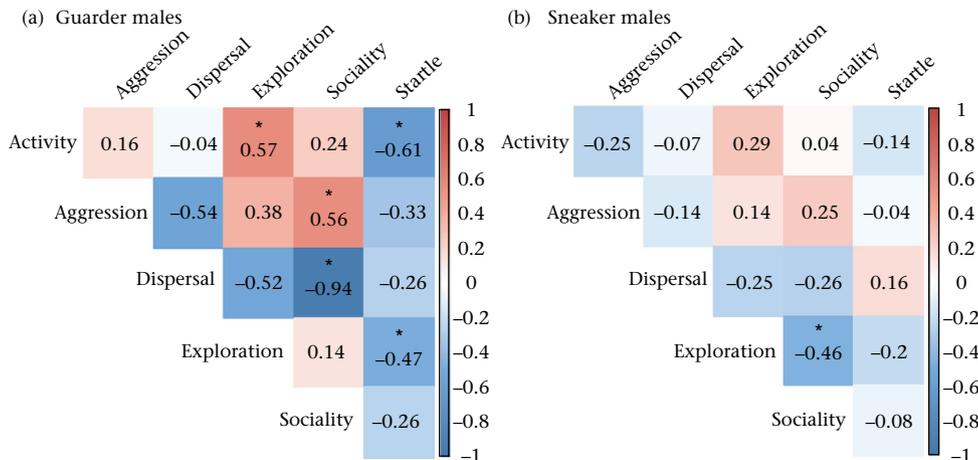
*Behavioural Tendencies Differ between the Tactics*

Guarder and sneaker males differed in terms of exploration, activity, boldness and aggression. Sneaker males were more likely to exit a refuge into a novel tank compared to guarders (92% of sneakers exited versus 68% of guarders). Additionally, sneaker males were more active during the dispersal assay (they made more zone crosses) and explored more of the novel experimental tank than guarder males. Greater exploration in sneaker males may increase the probability of locating a nest containing a spawning female. Conversely,

the territorial nature of guarder males may inhibit explorative behaviour (Dubs & Corkum, 1996; Macinnis & Corkum, 2000). Exploration of a novel habitat generally requires some degree of activity, and while exploration–activity behavioural syndromes have been observed in fish (Cote et al., 2010), we did not find a significant correlation between exploration and activity in sneaker males.

Exploring a novel environment may be risky, often requiring a higher degree of boldness. Structure, such as tank walls, provide fish with more security than do open spaces, such as the centre of a tank (Burns, 2008; Magnhagen et al., 2014). We found that sneaker males spent more time in the central portion of the experimental tank, away from walls, and were also quicker to begin moving after the simulated predator attack. These metrics are common measures of boldness (Aspbury et al., 2010; Krause & Godin, 1994; Thorlacius et al., 2015) and suggest that sneaker males are less averse to taking risks. Boldness may be inherently required for sneaker males as they may be subject to defensive aggressive responses from the much larger guarder males while intruding into their nests, where injury or even mortality may result (Gross & Charnov, 1980; Magnhagen, 1995; Scaggiante et al., 2005).

We found guarders were more aggressive than sneakers, but only during the sociality assay. Guarding males often display aggressive nest defence behaviours (Gross, 1991; Scaggiante et al., 2005). In contests where there is a disparity in size between competitors, the larger individual often has the competitive advantage (Rowland, 1989; Stammer & Corkum, 2005). We used conspecifics of varying sizes as stimuli during the sociality assay. As some of the conspecifics were smaller than focal guarders, the costs for initiating aggression would be low based on an increased likelihood of a successful outcome. In contrast, sneakers, being smaller than most of the fish in the group of conspecifics, may have faced higher costs when engaging aggressively. Significantly fewer aggressive acts were made towards the mirror compared to towards live conspecifics by both sneaker and guarder males. Mirror assays are an attractive method of studying aggression as they present the focal individual with a perfectly size-matched 'competitor'. This is an important factor as a difference in mass of just 3% reliably predicts victory for the larger round goby (Stammer & Corkum, 2005). Presenting such a size-matched 'competitor' likely dissuaded both sneaker and guarder males from aggression as the probability for victory is less certain. There is some ambiguity about whether mirror assays are a valid test of aggression in fishes (Balzarini et al., 2014; Desjardins & Fernald, 2010). Some researchers argue that mirror assays do not always capture the full complexity of dyadic aggressive interactions (Balzarini et al., 2014).



**Figure 4.** Correlation matrix of behavioural tendencies for round goby (a) guarder males and (b) sneaker males. Red indicates positive Pearson's  $r^2$  values while blue indicates negative Pearson's  $r^2$  values for each correlation. Colour saturation indicates the strength of the correlation, and the Pearson's  $r^2$  values are given inside each square. \* $P < 0.05$ .

This may partially explain why we observed differences in aggressive tendencies between the two assays.

We found no differences in either sociality or dispersal between sneaker and guarder males. In the sociality assay, both guarder and sneaker males spent the majority of the assay near conspecifics. Past research has indicated a preference for associating with larger groups of conspecifics in nonreproductive male round goby (Capelle et al., 2015), and round goby often live in dense populations necessitating a high tolerance towards conspecifics (Pennuto et al., 2012; Ray & Corkum, 2001). However, high densities could also drive dispersal behaviours, especially in guarder males, who require a territory to attract females and care for offspring. There is evidence to suggest large round goby males (>70 mm) seasonally migrate into tributaries during the spring season to spawn (Blair et al., 2019). Additionally, larger body size has been correlated with dispersal tendency in round goby (Thorlacius et al., 2015). While we found that the larger guarder males reached the end of the dispersal assay faster than the smaller sneaker males (1249 s versus 1476 s, respectively), this comparison was not significant. However, given that only a few fish reached the end of the dispersal assay ( $N_{\text{sneaker}} = 13$ ,  $N_{\text{guarder}} = 11$ ), the conclusion of no dispersal differences between tactics could be overturned with additional research. Furthermore, previous studies assessing nonreproductive male round goby dispersal have used arenas longer than 4 m (Myles-Gonzalez et al., 2015; Thorlacius et al., 2015), while our dispersal testing arena was only 2.5 m long and may not have been long enough to adequately track dispersal tendencies.

#### Evidence for Differences in Behavioural Correlations

The behaviours of guarder males were highly correlated while those of sneaker males were not. A difference in the correlated behaviours between tactics has been previously found in the water strider *G. gracilicornis* (Han & Jablonski, 2019). Signalling tactic males (that coerce females into reproduction) show a behavioural syndrome, where increased mating attempts are associated with lower cautiousness (Han & Jablonski, 2019). However, nonsignalling tactic males (those that wait for females to expose genitals) show no behavioural syndrome (Han & Jablonski, 2019). Opposite to our results, Wellington tree weta, *Hemideina crassidens*, males that adopt only a sneaking tactic show a behavioural syndrome, while guarding and flexible males (i.e. those that can adopt either a sneaking or guarding tactic) show no such behavioural syndromes (Wilson & Kelly, 2019). It is currently not known whether ARTs in round goby are fixed for life, sequential or flexible. Lastly, our results do not rule out the possibility that guarder males are capable of behavioural plasticity. It is conceivable that the particular assays used in the present study selected for correlated behaviours across contexts in guarder males, but not in sneaker males.

Our results indicate that alternative reproductive tactics introduce considerable behavioural variation. Because round goby males cycle in and out of a reproductive state over a breeding season, behavioural assessments performed only on nonreproductive males (including studies from our laboratory) may miss important variation tied to ARTs. Also, the degree of behavioural consistency between reproductive and nonreproductive states and between guarder and sneaker round goby males has not been fully explored and should be a topic of future work. Our results show that sneakers are typically more active, explorative and bold compared to guarder males. However, it remains unclear whether body size – regardless of reproductive status – dictates these differences. There is conflicting evidence about whether round goby of different sizes show different behaviour. Some studies have found smaller round goby to be more explorative, active and bold (Behrens et al., 2020; Marentette et al., 2012), while other studies found no evidence that differences in exploration, activity, boldness and sociability are associated with

round goby body size (Myles-Gonzalez et al., 2015; Thorlacius et al., 2015). Whether it is body size per se or the reproductive tactic that better predicts behavioural differences requires further investigation. In addition, knowledge of whether one tactic is more prevalent at invasion fronts or is primarily responsible for range expansion could assist in planning habitat protection measures. We encourage researchers to pay careful attention to the ratios of guarder to sneaker males at invasion fronts and compare the ratio to that found in the originating established population. Our findings of differences in behaviours between the tactics could pinpoint the best targeted fish capture techniques to employ based on the behaviour of each tactic (Diaz Pauli et al., 2015). Future studies should seek to concretely link inter- and intrapopulation behavioural differences to potential management strategies of invasive species.

#### Author Contributions

**Caitlyn Synyshyn:** Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project Administration. **Alexandra Green-Pucella:** Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing. **Sigal Balshine:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

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## Appendix

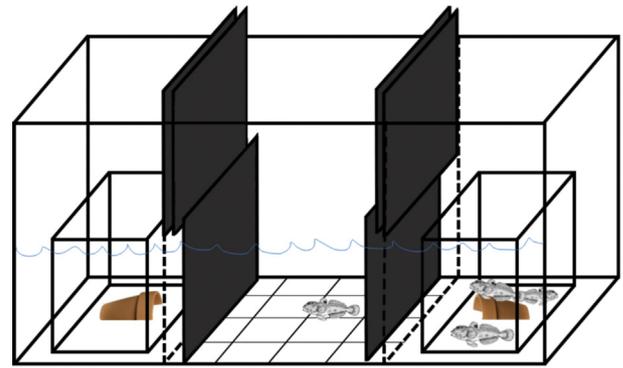
**Table A1**  
Number of sneaker males (SM) and guarder males (GM) used for each behavioural assay and any associated fish exclusions

	Starting sample size	Exclusions	Reason	Final sample size
<b>Morphological measurements</b>	72	5	Unclear reproductive status	40 SM 27 GM
<b>Exit test</b>				
SM	40	0	No fish excluded	40
GM	27	2	Experimental set-up error (excluded from the rest of the assays)	25
<b>Exploration</b>				
SM	40	15	Escaped focal area (excluded from rest of the assays)	25
GM	25	1	Experimental set-up error (excluded from rest of the assays)	24
<b>Sociality</b>				
SM	25	3	Escaped focal area (excluded from the rest of the assays)	22
GM	24	5	Escaped focal area (1) (excluded from the rest of the assays); never moved/switched zones (4)	19
<b>Aggression</b>				
SM	22	4	Never moved/switched zones	18
GM	23	8	Never moved/switched zones	15
<b>Startle</b>				
SM	22	1	Escaped focal area	21
GM	23	0	No fish excluded	23
<b>Dispersal</b>	84	4	Unclear reproductive status	42 SM 38 GM
SM	42	8	Escaped lane (6); conspecific entered lane (2)	34
GM	38	7	Escaped lane (1); conspecific entered lane (4); never moved (1); experimental set-up error (1)	31

If a fish was excluded from an assay due to experimental error (set-up, escape focal area), then it was subsequently excluded from the remainder of the assays due to the sequential nature of the experiment. If a fish was excluded from an assay due to nonparticipation (no movement), it was still included for analysis in subsequent assays. Note that the dispersal assay was run separately from the other assays, and as such, had a different starting sample size.

**Table A2**  
Sample sizes for each behavioural correlation performed on guarder male and sneaker male round goby

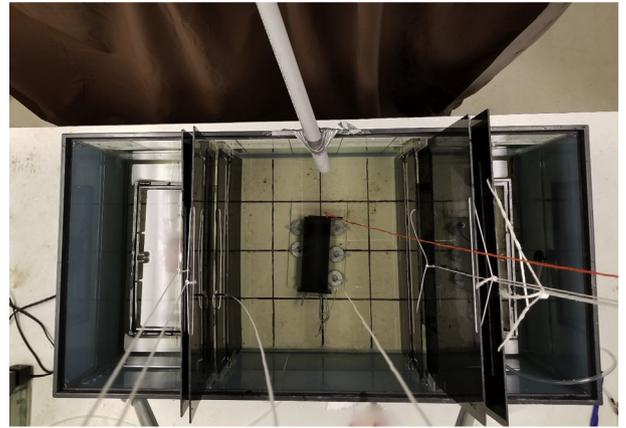
	Activity	Aggression	Dispersal	Exploration	Sociality	Startle
<b>Guarder males</b>						
Activity	–	23	7	24	19	22
Aggression	–	–	7	23	19	22
Dispersal	–	–	–	7	6	7
Exploration	–	–	–	–	19	22
Sociality	–	–	–	–	–	18
Startle	–	–	–	–	–	–
<b>Sneaker males</b>						
Activity	–	23	6	25	22	22
Aggression	–	–	6	23	22	22
Dispersal	–	–	–	6	6	6
Exploration	–	–	–	–	22	22
Sociality	–	–	–	–	–	21
Startle	–	–	–	–	–	–



**Figure A1.** Experimental tank set-up for assessing behavioural tendency differences between guarder and sneaker round goby. The two end sections contained one 2.5-gallon (9.4 litre) tank with a flowerpot half in each. One of the 2.5-gallon (9.4 litre) tanks contained an additional three conspecifics. The separated tanks allowed for visual cues while limiting olfactory cues. Opaque barriers were held on strings and connected to a pulley system behind curtains, allowing for the remote lifting or lowering of barriers with minimal disturbance to the focal individual.



**Figure A3.** Photograph of the dispersal assay set-up just prior to the beginning of the experiment.



**Figure A2.** Photograph of the experimental tank set-up just prior to the behavioural assays.