

REGULAR ARTICLE

Alternative reproductive tactics in goby fishes of the Caspian Sea

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Abstract

Here we report on the reproductive morphology and histology of three Gobiidae species (the Caspian monkey goby, *Neogobius pallasii*; the Caspian goby, *Neogobius caspius*; and the round goby, *Neogobius melanostomus*) from the Iranian coastline of the Caspian Sea. Based on ageing, reproductive histology, and internal and external morphological measures, it appears that all three of these goby species have two types of reproductive males, a large courting, territorial, male type and a small cuckolded parasitic male type, a phenomenon known as alternative reproductive tactics (ART). Although ARTs have been reported previously for one of these species, the round goby, all reports stem from its invasive range; ARTs have never been reported before in any fish species in the Caspian Sea. In all three goby species there was a large, older male type, with a wide head, dark body colouration, and a large investment in accessory glands (AG), an organ important for female attraction and parental care. But there was also a small, light, younger male type, with a narrow head, longer urogenital papilla, and little investment in AGs. The Caspian goby were the largest of the three species, and in this species the smaller, lighter, presumably cuckolded male morph was quite rare (only about 5% of the reproductive male population). In contrast, many of the round goby and monkey goby males were the small, lighter parasitic type, making up nearly half the population of reproductive males (48% and 40%, respectively). Round goby and Caspian goby males had a prominent mesorchial gland, a fibrous sheath of pheromone-releasing connective tissue that attaches the testes to the dorsal body wall, but all the monkey goby specimens examined lacked this structure. Although ARTs are well documented across fish species and appear to be particularly common in gobies, our study provides the first evidence for ARTs in goby fishes from the Caspian Sea.

KEYWORDS

distinct male mating morphs, Gobiidae, guarder, Ponto-Caspian, reproductive biology, sneaker

1 | INTRODUCTION

The Ponto-Caspian region, comprising the Caspian Sea, the Black Sea, and the Sea of Azov, is an area that has served as a global source of aquatic invasive species (Grigorovich et al., 2002; Reid & Orlova, 2002; Snyder et al., 2014). Biota from this Ponto-Caspian region are considered *tolerant* of extreme conditions, a trait that can increase the likelihood of successful invasion. Indeed, some of the most well-known freshwater aquatic invasive species in Europe and North America come from this Ponto-Caspian region and are members of the family Gobiidae (Ricciardi & MacIsaac, 2000; Van Deurs et al., 2021). Given their detrimental effects on biodiversity and productivity of invaded ecosystems such as the Laurentian Great Lakes, the European rivers, and the Baltic Sea (Charlebois et al., 1997, 2001; Corkum et al., 2004; Kornis et al., 2012; Ojaveer et al., 2015; Ricciardi & Rasmussen, 1998), numerous studies have been conducted on invasive gobies. However, many aspects of their reproductive ecology, especially in their native range, remain unknown, and this lack of biological information likely stems from geopolitical research barriers of this important source area. Most notably, the existence of alternative reproductive tactics (ART), common in many fishes (Taborsky et al., 2008), has not been determined before in any fish population of the Ponto-Caspian region, the source for several impressive species invasions (Snyder et al., 2014).

A species is said to have ARTs when two or more distinct reproductive phenotypes coexist, each male type achieving reproduction using a different tactic (Gross, 1985). ARTs occur as a result of disruptive selection and are common in many fishes (Taborsky et al., 2008). The prevalence of indeterminate somatic growth in fishes leads to strong mating competition and, when coupled with external fertilization and paternal care, creates the ideal selective conditions for individuals to achieve fertilization using alternative (inexpensive) approaches (Taborsky, 1998; Taborsky et al., 2008; Taborsky & Brockmann, 2010). Two common types of male ARTs are guarder (or territorial/parental) and sneaker types (Oliveira et al., 2008; Taborsky, 1997). Guarder males often court females, guard nests, and are typically larger, with secondary sexual characteristics, essentially investing in the traits that increase their chance in securing mates through male–male overt, physical competition and female choice. In contrast, sneaker males are often smaller, lack these female-attracting or competitive secondary sexual characteristics, and invest more in reproductive traits that allow them to achieve fertilization through sperm competition, deception (appearing like female), or even sexual coercion (Taborsky & Brockmann, 2010). Traits such as relatively large testes and more competitive ejaculates can facilitate the stealing of paternity from guarder males by sneakers/cuckolding males, but sneaker males also rely on guarder males to attract females (Oliveira et al., 2008; Poli et al., 2021).

The ART adopted by a male can be fixed for life, be ontogenetically determined, or switch back and forth throughout a lifespan. Also, ARTs may be determined by inheriting a particular gene or set of genes from one's father, or alternatively tactics will be expressed based on the ecological, social, or developmental conditions experienced by an individual (Godin, 1995; Gross, 1991, 1996; Meunier et al., 2009; Taborsky, 2008; Takegaki et al., 2012). In many fish

species, the tactics expressed change ontogenetically, with smaller younger individuals expressing a sneaker tactic, and then once they have grown large enough or have gained enough body condition to directly compete for mating, they will adopt a guarder tactic (Alonzo et al., 2000). ARTs are extremely common in some families of fish such as sunfishes and salmonids (Gross, 1984; Oliveira et al., 2008) and have also been reported in several members of the family Gobiidae, such as black goby, *Gobius niger* (Rasotto & Mazzoldi, 2002); common goby, *Pomatoschistus microps* (Magnhagen, 1992); sand goby, *Pomatoschistus minutus* (Svensson, 2004); grass goby, *Zosterisessor ophiocephalus* (Mazzoldi et al., 2000); and invasive round goby, *Neogobius melanostomus* (Bleeker et al., 2017; Marentette et al., 2009; McCallum et al., 2019). Because males adopting the different tactics may differ in their movement propensity, survivability, and reproductive success (Córdoba-Aguilar & Munguía-Steyer, 2015; Schulte-Hostedde et al., 2002; Synshyn et al., 2021), determining whether ARTs exist is important for understanding a species reproductive ecology and a species potential to be invasive.

To date, reports of ARTs in the round goby, one of the most invasive of fish species, have been limited to studies of invasive-range populations in North America (Bose et al., 2018; Marentette et al., 2009; McCallum et al., 2019) and in Europe (Bleeker et al., 2017; Kalchauer et al., 2013). Whereas the round goby is one of the most globally successful invasive species (Kornis et al., 2012), the two other species we studied, also members of the genus *Neogobius*, the Caspian monkey goby *Neogobius pallasii* and the Caspian goby *Neogobius caspius*, have been identified as species with the potential to become invasive, based on their salinity tolerance and high commercial shipping in their native range (Snyder et al., 2014). These three species constitute some of the most abundant goby species on the southern Iranian coast of the Caspian Sea (Zarini et al., 2019). To our knowledge, there is no information on ARTs in round goby in their *native range*, nor is there any study on reproductive tactics and morphs for the Caspian monkey goby or the Caspian goby. In this study, we investigated the abundance of all three species from different sites on the southern coast of the Caspian Sea and studied their external and internal reproductive morphology, as well as the histology of their gonads, traits that are typically used to assign reproductive tactics (Garant et al., 2003; Oliveira et al., 2008). Based on previous reports of ARTs in round goby populations from North America and Europe, we predicted to also find distinct round goby male morph types in its native range. We also investigated the possible existence of ARTs in the two other abundant and potentially invasive goby species from the Caspian Sea, the Caspian goby and the Caspian monkey goby.

2 | MATERIALS AND METHODS

2.1 | Field collection and morphological measurements

Goby specimens were collected monthly from six sites on the coast of the southern Caspian Sea (Noshahr, Mazandaran, Iran; 36° N, 51° E; Figure 1) between April and June 2020. This particular sampling

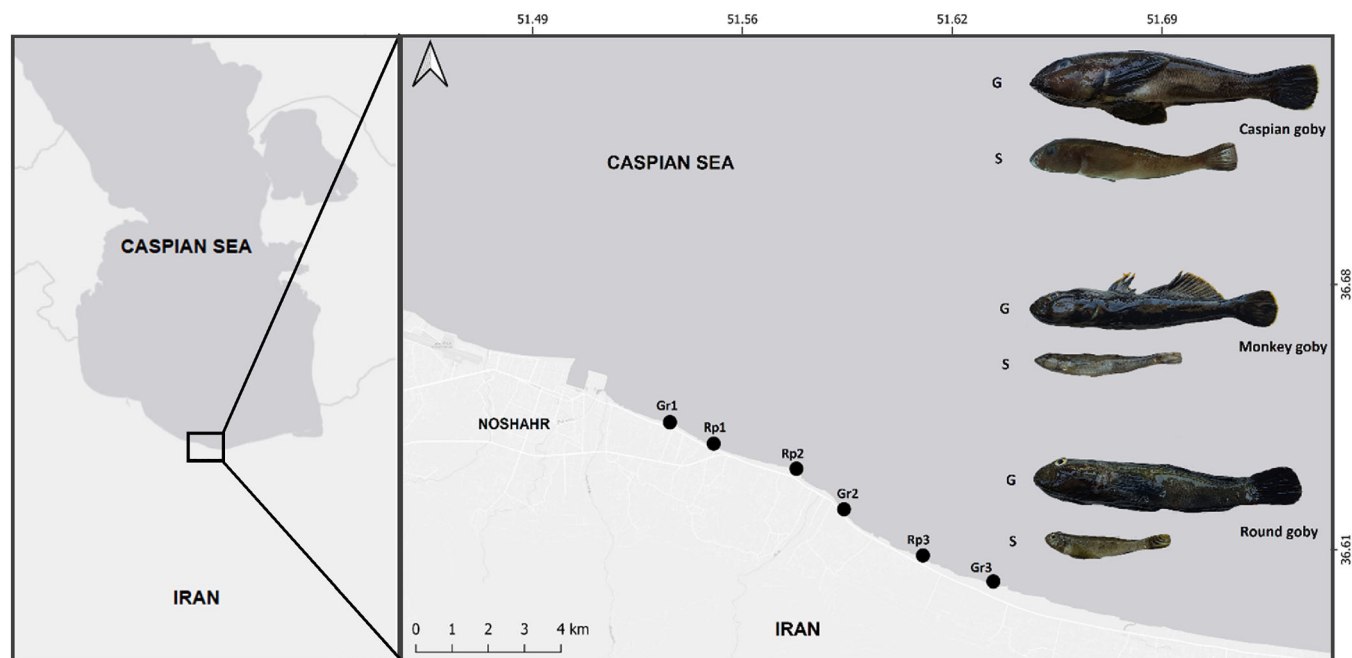


FIGURE 1 Location of the six sampling sites along the Caspian Sea coastline. Gr1 = gravel site 1, Rp1 = riprap site 1, Gr2 = gravel site 2, Rp2 = riprap site 2, Gr3 = gravel site 3, Rp3 = riprap site 3. The latitudes and longitudes of the region are included on the x- and y-axes, respectively. Images were included to illustrate the two male types found in our samples for each of the three species. “S” refers to the small sneaker morph, and “G” refers to the larger guarder morph.

period was selected because it spanned the peak spawning season for all three species in our study area, with Caspian goby breeding between April and May and both round goby and monkey goby having longer reproductive seasons, starting in May and continuing into September with a peak in June (Mahdipour et al., 2020; Miller, 2003). It was essential to examine the fish during the breeding season, as this is the period when males will invest most heavily in gonads and display secondary sexual characters, so it is essentially the only time of the year when we can reliably detect ARTs. Three of the sampled sites had riprap substrate (rocks of >50-cm diameter), and three of the sites had gravel substrate (0.2–5 cm diameter; Figure 1). At each site, specimens were caught using five steel minnow traps (three silver traps and two black minnow traps were used on each sampling trip). Each trap was 42 cm long and 19 cm wide, with two 2-cm-wide entry holes at the top and bottom ends. In addition, we used a single conical trap (made of net, 45 cm high × 80 cm wide, with six 10-cm entry holes) on each sampling site. Traps were always baited with 25 g of frozen corn and deployed in nearshore areas (at c. 1-m depth) at least 10 m apart and were set just after sunrise and retrieved 6 h later.

Only three species were captured in our traps: the round goby (*N. melanostomus*), the Caspian goby (*N. caspius*), and the Caspian monkey goby (*N. pallasii*), with no by-catch. Fish were measured using electric calipers accurate to 0.01 mm for total body length (TL), standard length (SL), head width (HW), body width, and urogenital papilla length. Total body mass and gonad mass were measured on an electronic scale (A&D ET303) accurate to 0.001 g. Based on these measurements a series of indices were calculated: papilla index (papilla length/total length × 100%), testicular somatic index (testes

mass/total body mass × 100%), and accessory gland somatic index (accessory gland mass/total body mass × 100%). Fish were initially sexed externally by visually inspecting the urogenital papillae, which is narrow and pointed in males and square shaped in females (Miller, 1984). We also dissected 205 of the sampled fish and measured the mass of their total gonads (measuring testes and accessory glands [AG] separately). Male goby species often have two sets of paired reproductive organs: two testes (where the sperm are produced) and two accessory organs (where seminal fluid, mucins, and pheromones are produced; Jasra et al., 2007; Miller, 1984; Mazzoldi et al., 2005). Thus, for this dissected fish sample, we calculated the gonado-somatic index (GSI) using the formula: (wet gonad mass [g]/wet total body mass [g]) × 100%. Following Marentette et al. (2009) and Zeyl et al. (2014), males with GSI >1% and females with GSI >8% were considered to be in reproductive status. We also investigated tactic differences in AG investment while controlling for body mass (see later).

The reproductive males of all three species could be further classified into guarder males and sneaker males based on their external morphology and on the differences in the indices of testes and AG investment (Marentette et al., 2009; Mazzoldi et al., 2005; Mazzoldi & Rasotto, 2002; McCallum et al., 2019). Following the methods outlined in Marentette et al. (2009), for all three species, any reproductive males with a GSI >1% that had dark or black body colouration and large investment in AGs were classified as guarders, and reproductive males with a GSI >1% that had a light, mottled body colouration; a long papilla length given their body length; and a relatively small investment in AGs were classified as sneaker males.

2.2 | Ageing

To age the fish both across and within species, two sagittal otoliths from each dissected specimen were extracted, dried, and stored in Eppendorf tubes. We selected otoliths from 20 reproductive males (10 sneakers and 10 guarders) from each of the three species (for a total of 60 fish) for ageing. We selected individuals for ageing that represented the range of body lengths caught for each species (approximately corresponding to 25th, 50th, 75th, and 100th percentiles of the range of total lengths for each species). Ageing was conducted by an experienced otolith reader who remained blind to the sample identity (the size, reproductive status, and even species of the otoliths examined). To establish age, otoliths were immersed in water and then viewed using a Leica GZ6 series stereomicroscope with 80× magnification under transmitted light from a Dolan-Jenner high-intensity fibre-optic illuminator. Otolith images and measurements were taken using a Motic Moticam 1000 camera and software. Yearly annuli were counted, and each specimen was assessed three separate times to ensure that the growth increments were interpreted consistently. Had readings not agreed, that sample would not have been assigned an age, but this never occurred.

2.3 | Histology

To examine the differences in the histological organization of the testes and AGs, as well as the secretory properties of these reproductive organs both among and within species, we selected a few specimens that from an external anatomical examination appeared to be good examples of sneakers and guarders for that species. In total, we examined histology for five sneakers and five guarders of the Caspian goby, four sneakers and five guarders of the monkey goby, and five sneakers and three guarders of the round goby. To examine histology, we removed the testes and AGs from these specimens and fixed them in Dietrich's solution (900 mL of distilled water, 450 mL of 95% ethanol, 150 mL of 40% formaldehyde, and 30 mL of acetic acid), dehydrated them in ethanol, embedded the tissues in Paraplast, and sectioned serially (5–6 µm). Sections were stained using hematoxylin and eosin and, for the differentiation of sulfated and nonsulfated mucins, using Alcian Blue at pH 1.0 and pH 2.5 (Mazzi, 1977; Pearse, 1950).

We originally attributed the guarder or sneaker tactic to specimens based on their external morphology and their investment in testes versus AGs. To test the validity of this attribution, the slides of each specimen's histology were blindly and independently analysed by two experts in fish reproductive histology (Maria Rasotto and Carlotta Mazzoldi). We created a score for the testes, based partly on the occurrence of a mesorchial gland (MG, 1 = present; 0 = absent), a gland containing steroid-secreting cells that attach the testes to the dorsal wall (Colombo & Burighel, 1974) and is thought to be involved in female attraction (Colombo et al., 1980). To characterize the AGs, we scored the various features that have been previously attributed to gland differences between guarder and sneaker males from other goby species (Mazzoldi et al., 2005; Rasotto & Mazzoldi, 2002;

Scaggiante et al., 1999), including (i) the shape of AG chambers, (ii) shape of the epithelium lining AG chambers, (iii) consistency of the mucus in the lumina of AG chambers, and (iv) presence of sperm in the AG chamber lumina. The differences in these features (and the total scores) between the guarders and sneakers are presented in Table 1. Any individual whose histological features were intermediate between sneaker and guarder tactics was categorized as "intermediate," and the degree of mismatch between the macroscopic and microscopic tactic assignment is presented in Table 2.

2.4 | Statistical analysis

All statistical analyses were performed using R (version 3.6.1; R Core Team, 2022). A significance of $\alpha < 0.05$ was used for all tests. Linear models were visually assessed for fit by plotting simulated residuals using quantile–quantile and scale-location plots. Transformations were used to achieve normality when necessary and noted for each model. To account for allometry, the gonadal and morphological indices were not used in statistical analysis, but instead, testes mass, AG mass, HW, and papilla length were log transformed and fit to an analysis of covariance (ANCOVA) model with male morph type (guarder or sneaker) as a factor and total body mass or total length (log transformed) as covariates (Marentette et al., 2009; Tomkins & Simmons, 2002). One-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post hoc tests were used to compare the body lengths of different species. Age, length, and total mass of male morphs were compared using Student's *t*-tests. Effect sizes were included in all analyses.

2.5 | Ethical statement

All fish handling and collection methods were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocols: 18-12-45).

3 | RESULTS

3.1 | Population demographics

A total of 4394 fish were captured (2269 round goby, 1159 Caspian monkey goby, and 966 Caspian goby) across the six sites over the three sampling dates. Caspian goby with an average SL of 93.84 mm were the largest of the three species ($\eta^2 = 0.49$, ANOVA, $F = 377.9$, $p < 0.001$; Tukey's HSD, $p < 0.001$). More males than females were caught for all three species; however, the male bias was much stronger for Caspian goby and monkey goby (96% male and 94% male, respectively), whereas round goby had a more balanced sex ratio (58% male). Most of the male round goby and monkey goby caught were non-reproductive (58% and 58%, respectively), whereas only 29% of the male Caspian goby caught were non-reproductive status with GSIs <1%.

TABLE 1 Scores of anatomical features employed to assign a tactic based on the histological analysis of the gonads and accessory glands for the three goby species studied.

Species	Caspian goby			Monkey goby			Round goby		
	Guarder N = 5	Intermediate N = 4	Sneaker N = 1	Guarder N = 0	Intermediate N = 5	Sneaker N = 4	Guarder N = 3	Intermediate N = 4	Sneaker N = 1
Testis									
Mesorchial gland absent = 0; present = 1	1	0.25	0	NA	0	0	1	0	0
Accessory glands									
Chamber's shape small and not dilated = 0; some small and some enlarged = 1; swollen and enlarged = 2	1.8	1	0	NA	1.2	0.25	2	1	0
Chamber's wall epithelium columnar = 0; some chamber part with columnar epithelium and part with flat epithelium = 1; all chambers lined by flat epithelium = 2	2	1.5	0	NA	1	0.5	2	1.3	1
Mucus in chamber lumina patchy and not filling lumina = 0; only part of the chamber has lumina filled with mucus = 1; mucus completely filling chamber lumina = 2	2	1	0	NA	1	0	2	0.75	0
Sperm in chamber lumina present in more than 10% of chamber lumina = 0; present in less than 10% of chamber lumina = 1; absent in all lumina = 2	1.8	0.75	0	NA	1	0.25	2	0.75	0

Note: The results reflect an average score across the individuals histologically attributed to each tactic.

Abbreviation: NA, not assigned.

3.2 | ARTs, body morphology, and reproductive investment patterns

All three species had males with the sneaker phenotype; these were always small light-colored males, with very large testes but small AGs (Figures 1 and 2). Round goby and monkey goby had fairly similar tactic ratios, with 52% versus 59%, respectively, of the reproductive males being guarders, but nearly all the Caspian goby reproductive males were of the guarder type (95.3%). In all three species, guarder males were longer (Caspian goby: Cohen's $d = 2.05$, $t = 10.7$, $p < 0.001$; monkey goby: Cohen's $d = 2.19$; $t = 13.6$, $p < 0.001$; round goby: Cohen's $d = 3.45$, $t = 24.7$, $p < 0.001$; Figure 2a) and heavier (Caspian goby: Cohen's $d = 1.68$, $t = 11.4$,

$p < 0.001$; monkey goby: Cohen's $d = 1.99$, $t = 12.8$, $p < 0.001$; round goby: Cohen's $d = 2.56$, $t = 18.8$, $p < 0.001$) than sneaker males. Guarder males also had significantly wider heads than sneaker males in monkey goby and round goby (ANCOVAs, monkey goby: $\eta^2 = 0.63$, $F = 54.78$, $p < 0.001$; round goby: $\eta^2 = 0.18$, $F = 27.3$, $p < 0.001$; Figure 2b), but this difference was not statistically clear in Caspian goby (ANCOVA, Caspian goby: $\eta^2 = 0.02$, $F = 0.68$, $p = 0.57$; Figure 2b). In all three goby species, controlling for body size, sneaker males had relatively larger testes compared to guarder males (ANCOVAs, Caspian goby: $\eta^2 = 0.59$, $F = 31.18$, $p < 0.001$; monkey goby: $\eta^2 = 0.77$, $F = 112.84$, $p < 0.001$; round goby: $\eta^2 = 0.23$, $F = 29.92$, $p < 0.001$; Figure 2c) and had a relatively longer urogenital papilla than guarder males (Caspian goby: $\eta^2 = 0.38$, $F = 13.10$,

TABLE 2 The number of individuals identified as guarders, intermediates, and sneakers as indicated by the macroscopic (external and internal features) versus histological (microscopic internal features only) attribution.

Species	Caspian goby			Monkey goby			Round goby		
Tactic attribution									
Histological attribution	Guarder	Intermediate	Sneaker	Guarder	Intermediate	Sneaker	Guarder	Intermediate	Sneaker
	N = 5	N = 4	N = 1	N = 0	N = 5	N = 4	N = 3	N = 4	N = 1
Macroscopic attribution	Guarder	Intermediate	Sneaker	Guarder	Intermediate	Sneaker	Guarder	Intermediate	Sneaker
	N = 5	N = 0	N = 5	N = 5	N = 0	N = 4	N = 3	N = 0	N = 5
Mismatch	N = 0	N = 4	N = 4	N = 5	N = 5	N = 0	N = 0	N = 4	N = 4

Note: We also report the number of individuals for which the tactic attribution showed a mismatch between the macroscopic and histological assignments.

$p = 0.002$; monkey goby: $\eta^2 = 0.35$, $F = 17.49$, $p < 0.001$; round goby: $\eta^2 = 0.12$, $F = 16.46$, $p < 0.001$; Figure 2d). Controlling for body mass, round goby and monkey goby guarder males also invested relatively more in AGs than did the sneaker males of these species (monkey goby: $\eta^2 = 0.34$, $F = 17.15$, $p < 0.001$; round goby: $\eta^2 = 0.10$, $F = 10.72$, $p = 0.001$; Figure 2e), but Caspian goby guarder males did not have relatively larger AGs than did sneakers ($\eta^2 = 0.02$, $F = 0.48$, $p = 0.49$; Figure 2e).

3.2.1 | Ageing

Otolith analysis showed that guarder males in all three species were older than their respective sneaker males (Caspian goby: Cohen's $d = 2.41$, $t = 5.40$, $p < 0.001$; monkey goby: Cohen's $d = 2.68$, $t = 6$, $p < 0.001$; round goby: Cohen's $d = 2.96$, $t = 8.10$, $p < 0.001$; Figure 2f). The otolith analysis revealed that guarder males were aged between 1 and 3 years and were on average about a year older than sneaker males who ranged in age from 0 to 2 years.

3.3 | Histology

All histologically analysed males were sexually mature, with testes in active spermatogenesis, and the examined specimens of all three *Neogobius* species had paired testes and obvious AGs. Testes were suspended, along their length, from the dorsal wall of the coelomic cavity by a mesorchium. In the mesorchial region, testes showed a deep fold (Figure 3) where the main testicular ducts run. The two main testicular ducts fused posteriorly in a common sperm duct entering into the urogenital papilla. Testes were organized in lobules, separated from each other by a thin layer of interstitial tissue. Based on the distribution of spermatogonia along the whole lobule length, in all three species the testes could be defined as an unrestricted lobular type (Grier, 1981, 1993; Grier & Uribe-Aranzábal, 2009; Uribe et al., 2014).

Differences in testis organization were observed both among and within species. In the round goby, the testes of larger individuals, in the mesorchial region, adjacent to the main testicular duct, showed a mass of polyhedral cells, arranged in highly vascularized cords

(Figure 3). Given its position, characteristic shape, and arrangement of endocrine cells, this mass may be considered, according to Arbuckle et al. (2005), to be an MG, composed of putative Leydig cells, as first described by Colombo and Burighel (1974) in the black goby, *G. niger*. In the Caspian goby too, larger individuals showed an MG that was even more extended than the MG of the round goby and was found at the center of the testis, presenting as a cluster of Leydig-like cells with capillaries coursing among them (Figure 3). By contrast, all monkey goby sampled histologically ($n = 9$) lacked an MG (Figure 3), and thus based on the histological features, none of the sampled individuals could be strictly identified as guarders, because their AGs had intermediate histological features representing an in-between (sneakers and guarders) state (see Tables 1 and 2). Therefore, it is possible that the monkey goby lacks an MG, but we cannot exclude the possibility that a true, fully reproductive guarder monkey goby could also have an MG.

In the three species studied, the AGs had a similar general organization. They were multichambered, paired glands (Figure 3) that open into the terminal portion of the sperm duct. Chamber walls consisted of an internal single layer of epithelial cells, a basal lamina, and an external thin layer of connective tissue, containing capillaries. The epithelial cells and the secretions present in the chamber lumina reacted positively to Alcian Blue at pH 2.5, indicating that the epithelial cells secrete nonsulfated sialo glycoprotein. However, differences among males were obvious in chamber shape, epithelium thickness, chamber lumina content (with respect to mucous abundance), and sperm presence/absence. We could differentiate two clearly distinct AG types in each species. In the first type, the chambers were enlarged, swollen with thin walls of flat epithelial cells; lumina were completely filled with secretions; and sperm were not observed. By contrast, in the second type of AGs, chambers were small and round, the chamber walls were thick with columnar epithelial cells, and sperm were abundant in the chamber lumina, whereas the secretions were scanty (Figure 3). Differences in the occurrence of these two accessory types were found at both interspecific and intraspecific levels. In all the three species a few individuals, although identified as sneakers from a macroscopical point of view, showed AGs that were more in line with guarder types, with chambers partially enlarged and partially filled with mucus. In addition, some individuals that had a guarder phenotype showed intermediate

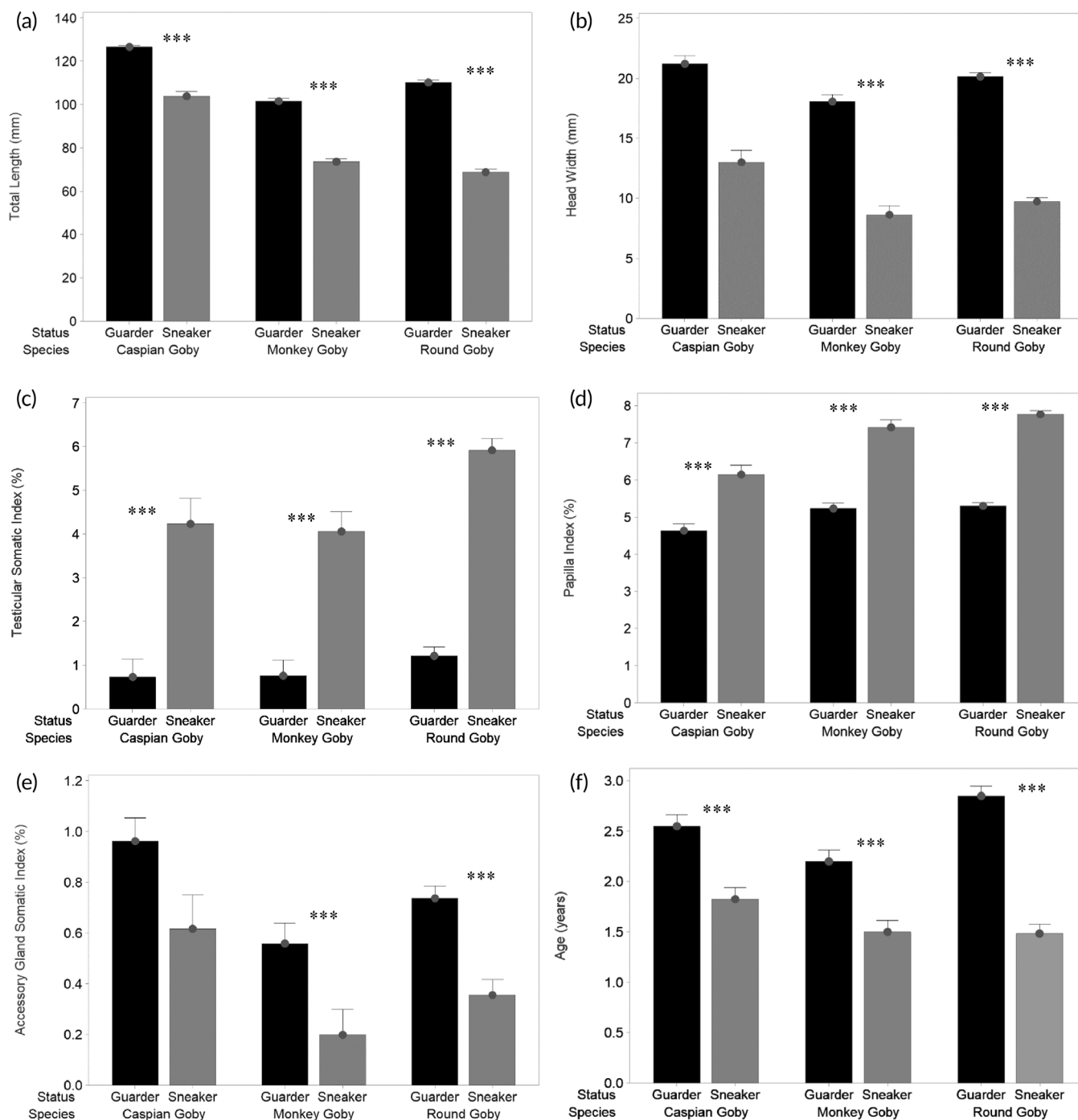


FIGURE 2 Comparison of fish mean (a) total length (in mm), (b) head width (in mm), (c) testicular somatic index, (d) urogenital papilla index, (e) accessory gland index, and (f) age between guarder (black bars) and sneaker males (gray bars) of three goby species, the round goby (*Neogobius melanostomus*), the Caspian goby (*Neogobius caspius*), and the monkey goby (*Neogobius pallasii*), collected from the southern Caspian Sea. Error bars represent one standard error of the mean. Asterisks indicate significant *p*-values.

histological features with AGs that presented as thin or partially empty chambers and/or some sperm visible in the chambers' lumina. These intermediate features suggest that these individuals were likely switching from sneaker to guarder tactics at the time of capture, explaining the mismatch between the histological and macroscopical attribution of the tactic (see Tables 1 and 2).

4 | DISCUSSION

Based on macroscopic features, two distinct reproductive morphs were found in all three goby species from the Caspian Sea, which could be clearly identified as guarder and sneaker males. Guarder males of all three species were older, darker, longer, and heavier and

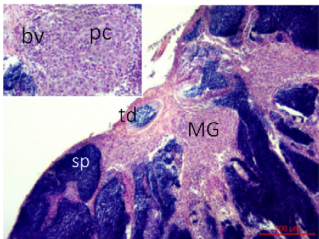
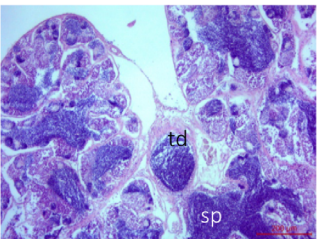
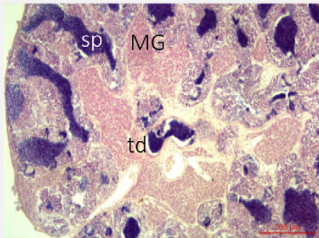
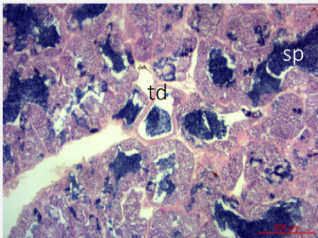
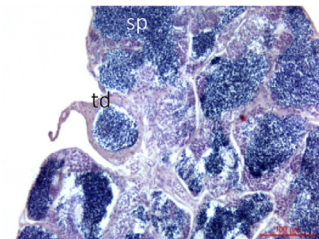
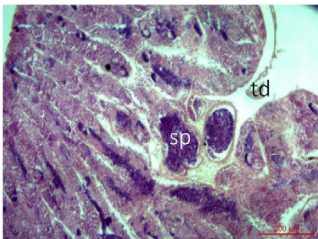
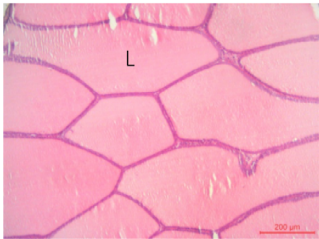

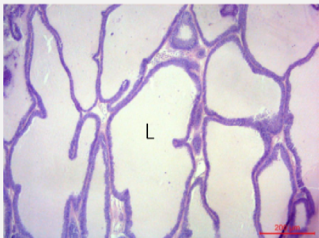
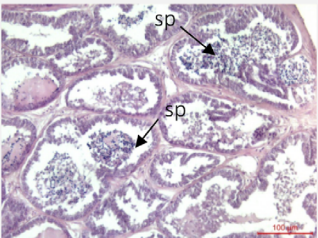
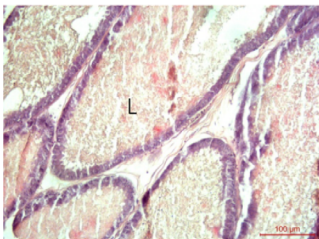
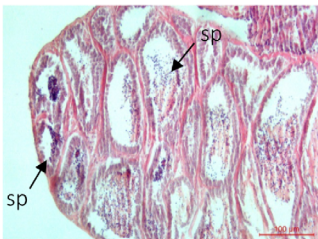
species		GUARDER	SNEAKER
Round goby	TESTIS	(A) 	
Caspian goby			
Monkey goby			
Round goby	ACCESSORY ORGANS		
Caspian goby			
Monkey goby			

FIGURE 3 Histological cross-section of mature testis and accessory organs stained with hematoxylin–eosin from alternative reproductive male phenotypes of three goby species: the round goby, *Neogobius melanostomus*; the Caspian goby, *Neogobius caspius*; and the monkey goby, *Neogobius pallasii*. Sections of testis of guarder males are shown on the left and those of sneaker males on the right. In particular: sperm (sp) and the main testicular duct (td) are indicated in both sneaker and guarder testes, whereas only round and Caspian guarder males present the mesorchial gland (MG). In the round goby, the enlarged MG section (A) of the testis shows the polygonal cells (pc) of the mesorchial glands and the blood vessels (bv). In all the three species, the accessory organs of guarder males show chambers with enlarged lumen (L), whereas the chambers of sneakers' accessory organs contain sperm (sp), indicated by the arrowheads.

had wider heads, whereas sneaker males in all three species had relatively longer papillae, were light colored, and had larger testes (whether or not we controlled for body size). The morphological and colouration differences we found between tactics are in line with observations in other goby species (Mazzoldi et al., 2000; Rasotto & Mazzoldi, 2002; Takegaki et al., 2012) and in other populations of

round goby (Bleeker et al., 2017; Marentette et al., 2009; McCallum et al., 2019). All three species also had multichambered paired AGs that approximately fit into two types: large swollen, thin-walled chambers full of secretions but with no sperm inside as expected for guarder males versus AGs with small thick-walled chambers full of sperm and little secretions, more commonly observed in sneaker

males. However, the histological analysis also revealed that there were a number of males that were intermediate between sneaker and guarder types in terms of their testicular organization.

All the guarder males sampled from all three species were older than the sneaker males, suggesting a possible ontogenetic shift in the expression of male reproductive phenotypes as fish grow (i.e., a transition from sneaker to guarder tactic with fish age). Such ontogenetic shifts are commonly observed in many fish species with ARTs (Taborsky, 1994, 1998), including many gobiid species such as common goby (Magnhagen, 1992), grass goby (Mazzoldi et al., 2000), black goby (Rasotto & Mazzoldi, 2002), and sand goby (Kvarnemo et al., 2010). Working on an invasive round goby population in the Rhine River of the Netherlands, Bleeker et al. (2017) suggested that there is a body size switch point c. 9.35 mm TL, at which the guarder tactic will be more common, but these researchers did not provide an explanation for why this body size should be the switch point, and no ageing was provided for that study. In contrast, another study of an invasive population of round goby from the Laurentian Great Lakes did use ageing analysis and showed a considerable overlap in ages between sneakers ($n = 46$) and guarders ($n = 34$), although otolith shape and weight clearly differed between tactics (Bose et al., 2018). Round goby guarder males from the Great Lakes also had greater initial growth in their first year of life compared to sneaker males, suggesting “birthdate effect” where fish born early in the season may be able to grow more, and this difference in time to grow before winter could serve as a decisive factor in tactic adoption for round goby (McCallum et al., 2019). To settle this debate on whether there is indeed an ontogenetic shift in round goby, the monkey goby, or the Caspian goby, life span studies are now needed to confirm the mechanisms by which tactic adoption occurs.

In the current study we found that about half the reproductive males employed sneaker tactics in both round goby and monkey goby (a tactic ratio of 1:1); however, only a small proportion (4.7%) of reproductive Caspian goby males were sneakers. Why might this be? Previous studies of round goby from invasive populations report a wide range of guarder versus sneaker ratios; for example, anywhere from 51% to 39.3% sneakers have been observed in Hamilton Harbor, part of the Laurentian Great Lakes (Bose et al., 2018; Marentette et al., 2009), 40.6% sneaker males have been reported in the Rhine River in the Netherlands (Bleeker et al., 2017), and only 4% sneaker males have been found in populations of the upper Danube River in Germany (Cerwenka et al., 2021). Marentette et al. (2009) suggested that time since invasion could influence the ratio of guarders to sneakers; the authors of that paper argued that initially in newly invaded habitats there would be less male–male competition for available nesting sites, resulting in a higher frequency of guarder males. However, factors such as differences in sampling habitats, sampling gears, or time in the breeding season could also influence these ratios. For example, minnow traps were used in the current study, and in the Marentette et al. (2009) and Bose et al. (2018) studies, but electrofishing was employed by Cerwenka et al. (2021), whereas Bleeker et al. (2017) used a combination of electrofishing and bottom trawls. Differences in gear will result in differences in size distribution of sampled

fish (Clement et al., 2014), which in turn likely will impact the estimates of tactic ratios. Also, fewer actively defending guarder males would be caught in traps because such males would likely remain in their nests and not explore a trap. It is also possible that minnow traps attract males who are defending a nest or are moving around in search of a nest, which could lead to a higher ratio of guarder males caught compared to other gear types. Another possible explanation for the considerable difference in reported tactic ratio across the three species could be a result of temporal differences in the spawning season. The peak reproductive season for the Caspian goby in the southern part of the Caspian Sea (where we sampled) is April and May (Mahdipour et al., 2020), whereas both round goby and monkey goby have longer reproductive seasons, starting in May, with reproductive peaks in June, but breeding can even continue into September (Miller, 2003). Finally, the population of Caspian goby and monkey goby in our samples exhibited an unusually high male bias (96% and 94%, respectively). This unexpected observation could also be a result of gear selectivity or because of the habitat type sampled, as explained for the tactic ratio biases. Clearly, more work is needed to disentangle the various possible explanations for the tactic and sex ratio differences across species and populations.

In other gobiid species, guarder AGs are commonly swollen and filled with mucin secretion and substances involved in sperm release mechanisms and whole ejaculate performance (Locatello et al., 2013; Marconato et al., 1996; Mazzoldi, 1999; Scaggiante et al., 1999). In AGs of males adopting sneaker-like morphology, spermatozoa are commonly found, suggesting that sneakers use these organs to store sperm rather than to produce mucins to attract females. This difference in the structure of AGs between guarder and sneaker males of same species has been previously reported in black goby (Immler et al., 2004), rock goby (Hajji et al., 2012), and grass goby (Scaggiante et al., 1999). The goby species and male types we studied had a similar distinct AG structure to those previous studies and species, but to confirm the functionality of these different structures, we would need to further examine ejaculates and the secretions from each species and male type.

We found an MG (an aggregation of Leydig-like cells that secrete steroids and pheromones) associated with the testes of Caspian goby and round goby, but the monkey goby apparently lacked this feature. An MG has been found in other goby species such as black goby (Rasotto & Mazzoldi, 2002), *Glossogobius olivaceus* (Asahina et al., 1985), *Gobius pagnellus* (Stanley et al., 1965), and *Rhyacichthys aspro* (Cole & Parenti, 2022). Also, Arbuckle et al. (2005) had previously reported the presence of an MG in round goby from an invasive population; however, in that study the researchers examined only the testes of males with swollen cheeks and black colouration (i.e., guarder male). In other goby species, androgens produced by these MGs are known to be responsible for attracting females to the nest and as a result contributing to the reproductive success of guarder males (Colombo et al., 1980; Marconato, 1980). The absence of an MG or the presence of a very small and underdeveloped MG in sneaker males would mean these males secrete few pheromones, and potentially such a strategy would limit detection by guarder males (Locatello et al., 2002;

Rasotto & Mazzoldi, 2002). We found some mismatches between the macroscopic and histological attribution of the tactics, with some individuals classified as intermediates histologically, as these males had internal AG features that were intermediate between guarder and sneaker phenotypes. In particular, because we did not find any individual monkey goby with extremely well-developed AGs or an MG, all the “guarder-like” males were classified as intermediates. However, it is also possible that the monkey goby lacks an MG.

When examining the testes and AGs of males, we found several intermediate individuals in terms of histological features. A small number of intermediate types have been observed macroscopically in round goby in Europe and North America (Bleeker et al., 2017; personal observations, S. Balshine and S. Zarini). The presence of intermediate phenotypes has also been recorded in other goby species (Mazzoldi & Rasotto, 2002; Scaggiante et al., 1999). In the grass goby, *Z. ophiocephalus*, for example, an intermediate investment in both testes and AGs was found in males with an average total length intermediate between sneaker and territorial tactics. At the end of the breeding season, there can be more opportunities for small males to gain and defend a nest, potentially making it advantageous for sneakers to switch tactic and begin guarding (Scaggiante et al., 1999; Takegaki et al., 2012). It is possible that some of our histologically intermediate males were switching from one tactic to another (Scaggiante et al., 1999). The simultaneous presence of multiple tactics within a species, either genetically or conditionally determined, and the switching between tactics within a breeding season are certainly possible and can be fueled by diverse factors, such as the relative size variation in competitors, which will occur frequently in fish species with indeterminate growth (Engqvist & Taborsky, 2016).

This is the first study to explore the variation in male reproduction resulting from the presence of ARTs in goby fishes from the Ponto-Caspian region, a region responsible for over 70% of the planet's freshwater invasive species (Ricciardi & MacIsaac, 2000). Given that so many invasive species originate from the Ponto-Caspian region, we need to know more about basic species biology and ecological conditions from this invasion super source (Ricciardi & MacIsaac, 2000). Understanding how species have diverged during the invasion process could shed light on basic evolutionary and ecological processes as well as suggest more refined management strategies. Future work is now needed across multiple seasons and geographical locations to map out how sneaker and guarder male morphs vary temporally and spatially. We also still need to document any behavioral differences between the two male reproductive morphs, especially in terms of activity and exploration tendencies, and uncover the extent of paternity loss due to sneakers and hatching success in all three species within their native range.

AUTHOR CONTRIBUTIONS

S. B. and S. Z. were responsible for the development of the study. S.B. provided supervision and direction on the project. S.Z. conducted fish sampling, morphological data collection and statistical tests. F.P. carried out histological analysis and interpretations. All authors contributed to the writing of the manuscript.

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