

Sperm performance limits the reproduction of an invasive fish in novel salinities

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Abstract

Aim: The few fish species able to reproduce across wide osmotic ranges either plastically acclimate sperm performance to, or are locally adapted to, different salinities. The invasive round goby (*Neogobius melanostomus*) is spreading in Eurasia and the Americas, into both fresh and brackish water. We aim to understand if reproduction in different salinities is affected by an ability to acclimate.

Location: Brackish and freshwater systems of northern Europe and the Baltic Sea.

Methods: We cross-exposed round gobies of freshwater and brackish origin to 0 and 16 practical salinity units (PSU), and the fish were given nest boxes in which to spawn. After 4 weeks, we measured their sperm performance in both 0 and 16 PSU; fertilization success of each egg clutch was measured through visual analysis of eggs. Clutches were split and allowed to develop in both 0 and 16 PSU salinity, and reproductive success (zygote development) was measured 20 ± 1 days later. Responses were analysed using generalized mixed models.

Results: After a month, the fish showed no plasticity in sperm performance to their acclimation salinity, regardless of their origin. Sperm velocity was highest in the salinity similar to the males' origin. Significantly lower fertilization success was measured for individuals that reproduced outside their salinity of origin despite recurring spawning events in all treatment groups. Among fertilized eggs, zygote development was similar regardless of salinity treatment of either eggs or parents.

Main Conclusions: Short-term acclimation to new salinities does not affect sperm performance in the round goby. Alternative hypotheses such as local adaptation should be further investigated. Limits to the species' reproductive success, and therefore invasion processes, are likely dependent on environment-phenotype matches. Fish of brackish origin spawned successfully in freshwater, pointing to an increased risk of introducing populations of brackish origin into freshwater.

KEYWORDS

Apollonia melanostoma, condition sensitivity, exotic species, Gobiidae, invasive alien species, phenotypic plasticity, range expansion, reaction norms

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1 | INTRODUCTION

When species shift their ranges to new regions, their success at establishment and population growth is determined by their phenotypic match to the local environment (Valladares et al., 2014). Importantly, since invasion success is dependent on propagule pressure, the ability to reproduce in a particular environment will have large consequences as the population size of the invader commonly increases their effect on their habitat (Dijkstra et al., 2017). As species are increasingly transported across the globe (Sardain et al., 2019), the need to understand what affects their ability to reproduce during establishment in novel environments also increases.

Maintaining body and cellular function in balance with the external environment (homeostasis) is crucial for survival and reproduction (Bateson & Gluckman, 2012). Different ambient conditions inevitably incur different costs of homeostasis, and these apply during all (including gametic) life stages (Willmer et al., 2005). Phenotypic plasticity has increasingly been recognized as important for organisms that experience environmental change, for example global warming (Merilä & Hendry, 2014; Seebacher et al., 2015), and the presence of plastic traits has been shown to facilitate biological invasions (Davidson et al., 2011). For traits that lack plasticity, selection can be stronger (DeWitt et al., 1998). Since many introduced populations are small, they often experience genetic and phenotypic bottlenecks (Golani et al., 2007), which can also increase the potential for selection and local adaptation.

Juvenile and gametic life stages (such as eggs and sperm) are more sensitive than adults (Dahlke et al., 2020), and plasticity or local adaptation to environmental conditions is expected to be more pronounced in these. Freshwater and marine fish have sperm that are adapted to initialize swimming in freshwater and seawater, respectively, but salinity can also affect the velocity of sperm (Browne et al., 2015; Morisawa, 2008; Green, Havenhand, et al., 2020). Since sperm velocity is correlated with fertilization success in externally fertilizing fish (Gage et al., 2004; Gasparini et al., 2010; Stockley et al., 1997), limited sperm velocity due to spawning in the “wrong” salinity can decrease fertilization and subsequent developmental success (Morisawa, 2008; Svensson et al., 2017).

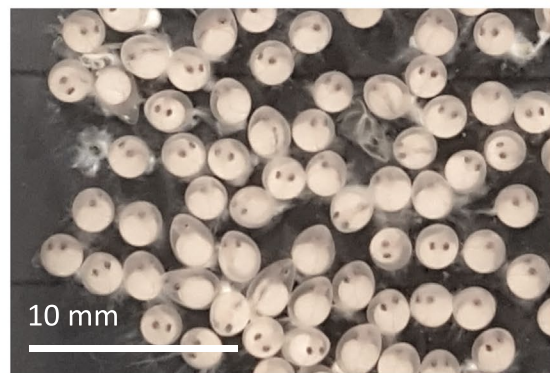
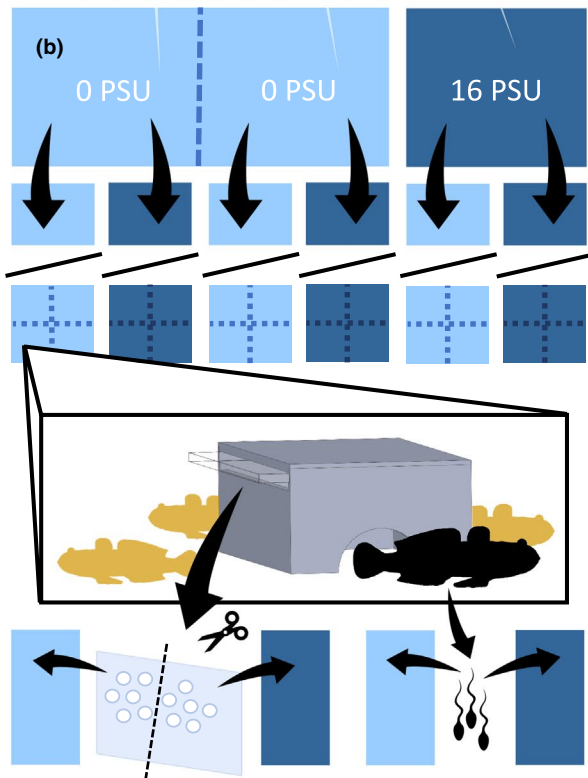
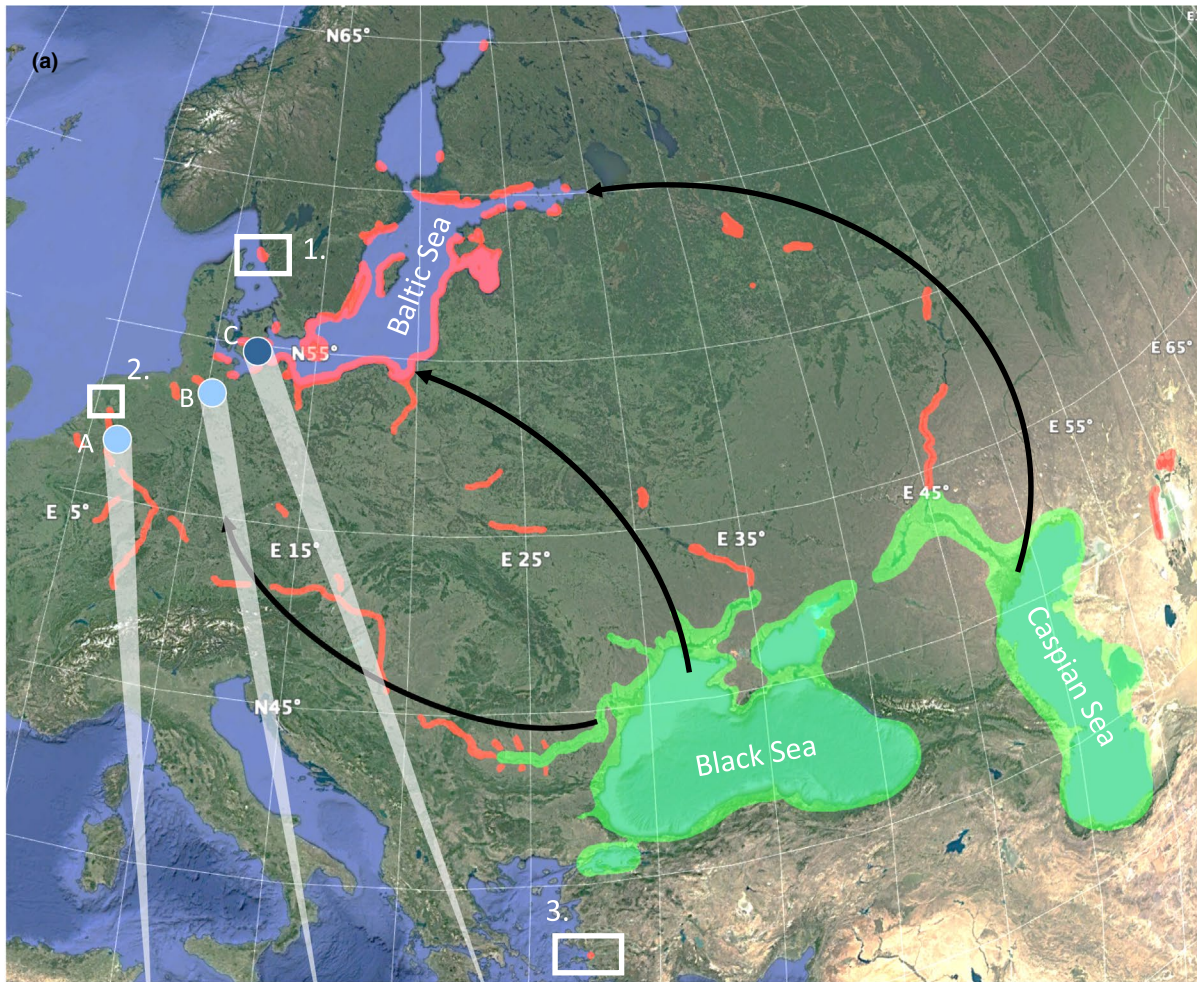
Eggs are more tolerant than sperm, possibly because they are adapted to spend longer time in more varying environmental conditions (Dahlke et al., 2020; Martin & Carter, 2013). Since salinity

treatments can be used to combat fungal pathogens in aquaculture (Martínez-Palacios et al., 2008), research on egg tolerance to salinity has been done in a range of species. Euryhaline species' zygotes commonly develop normally in the natural salinity ranges the species occur in (Lee & Menu, 1981; Santerre & May, 1977), but zygotes of species that spawn in a narrow salinity range can show surprisingly high tolerance to non-native salinities. For example, in freshwater spawning salmonids, zygotes can develop normally in brackish conditions up to 12 practical salinity units (PSU) (Morgan et al., 1992). Eggs, zygotes and juveniles of a marine clown fish (living in 35 PSU), showed normal development between 9 and 42 PSU (Dhaneesh et al., 2012). Adult salinity acclimation can also benefit egg development through plastic responses in euryhaline fishes (Kucera et al., 2002; Lee et al., 1981; May, 1974; Watanabe et al., 1985).

Broad salinity tolerance in eggs and narrow salinity tolerance in sperm predict that sperm and their fertilization ability are the main constraints to reproduction in novel salinities. Studies of plastic responses to salinity in the sperm of externally fertilizing euryhaline fish are few. Among the seven different species tested (see discussion for references), the one species that did not show an acclimation in their sperm to salinity is the sand goby (*Pomatoschistus minutus*, Pallas 1770), native to the North East Atlantic and the brackish Baltic Sea (Svensson et al., 2017). The species belongs to the goby family (Gobiidae), which is the fish family most commonly transported in ballast water, most commonly introduced in non-native areas and most likely to successfully establish following a species introduction (Wonham et al., 2000).

The most widely spread and most impactful of all invasive goby species is the round goby (*Neogobius melanostomus*, Pallas, 1814) (Kornis et al., 2012). This <30 cm benthic fish is native to the brackish Black Sea and associated freshwater tributaries in the Ponto-Caspian region. It now occurs as an invader in many different environments in Europe (Figure 1) and the North American Great Lakes (Azour et al., 2015; Kornis et al., 2012; Puntila et al., 2018; USGS, 2021). During reproduction, the female deposits her eggs in the male's nest, where he fertilizes and subsequently guards them until they hatch (Meunier et al., 2009). Since the round goby has external fertilization, eggs and sperm are exposed to the environment. Despite this, round gobies have successfully established reproducing populations in salinities from 0 to at least 20 PSU (Kornis et al., 2012), pointing to a mechanism by which the species' sperm

FIGURE 1 Study design and European range of the highly invasive non-indigenous round goby *Neogobius melanostomus*. (a) Map of distribution, with sites where fish were collected for the experiment (A: River Rhine, Germany; B: River Elbe, Germany; and C: Guldborgsund, Denmark) and highlighted sites where introduced round goby is known to exist in brackish harbours with access to both freshwater and seawater sites (white boxes 1, 2, 3). Green colour show native occurrences, red colour shows introduced areas. Black arrows show possible routes of introduction into the Baltic Sea and Western Europe. The map is compiled from multiple sources (Hempel, 2017; Kornis et al., 2012; Kvach, 2014; Manné et al., 2013; Mastitsky et al., 2010; Puntila et al., 2018; Teletchea & Beisel, 2018; Vassilev et al., 2012). Also note that the species is introduced and widely spread in the North American Great Lakes (freshwater). (b) Experimental design showing treatment salinities (light blue = 0 PSU, dark blue = 16 PSU) of adults and gametes. Adults were initially placed in communal tubs without nest boxes. They were then separated into smaller spawning groups of 1 male and 2-3 females with access to a nest box. Any spawned eggs were sampled from an acetate sheet in the nest box. The sheet was split in half, and one half-clutch was kept in the adults' spawning salinity, and the other was transferred to the opposite treatment salinity. Sperm were sampled from males and tested in either 0 or 16 PSU salinity. (c) Photograph of a round goby male caught in the river Elbe for the experiment. (d) Zygote development was measured as the proportion of eggs reaching eye spots present zygote stage at day 20, here visible on all normally developing eggs



and eggs function in a range of salinities. Broad tolerance to environmental conditions are common in species originating in the Black Sea region (Casties et al., 2019). A controlled laboratory experiment has shown that with progressive acclimation, adult round gobies can tolerate a broad range of salinities, although with some variation in physiological performance between individuals at the highest salinities (25 and 30 PSU) (Behrens et al., 2017). Brackish round gobies have also been acclimated to freshwater with subsequent successful spawning as a result (Bonisławska et al., 2014).

Studies comparing different sites or regions have also shown population-wide differences in behaviour (Thorlacius et al., 2015) and morphology (Demchenko & Tkachenko, 2017; Kornis et al., 2012), including reproductive traits (Masson et al., 2018). However, it remains unclear whether these documented morphological and behavioural differences among round goby populations are based on pronounced plasticity or local adaptation. Yet, differences in how round goby sperm respond to salinity have been interpreted as signs of rapid local adaptation, based on the observation that sperm velocity increased with the number of generations each population had been present in their local salinity (Green, Havenhand et al., 2020). Notably, no controlled experiment testing potential effects of acclimation or plastic responses has been conducted on sperm traits in round gobies.

The presence of non-native fishes has been shown to be closely associated with environmental conditions (Howeth et al., 2016). Since the round goby is highly invasive in a range of environments, it is a good model organism to better understand in what way the salinity environment affects reproductive traits of invasive fishes and as well as the speciose goby family. In our study, we aimed to test if the round goby can successfully acclimate to, and reproduce in, salinities different from their origin (freshwater or brackish) by measuring the plasticity of traits associated with reproduction. We did this using a classic common-garden design, where we let adult fish of brackish and freshwater origin ("parent origin") acclimate to two salinities, 0 or 16 PSU ("parent treatment salinity"). We subsequently tested their sperm performance in both 0 and 16 PSU ("sperm test salinity"). Furthermore, we investigated the effect of parent origin and parent treatment salinity on fertilization success. Parent treatment salinity thus represents both the salinity to which the adult fish were acclimated and the salinity in which spawning (and fertilization) occurred. Finally, among fertilized clutches, zygote development was investigated, testing for effects of parent origin and parent treatment salinity, together with the salinity in which the zygotes developed ("egg treatment salinity"). The latter was done by splitting each clutch into two and letting them develop in either 0 or 16 PSU.

2 | MATERIALS AND METHODS

2.1 | Fishing and research facility

A total of 412 round gobies (*N. melanostomus*) were captured for the experiment. Fish of brackish origin were captured in the Baltic

Sea during April 2016 (Guldborgsund, Denmark, 54°51'21"N 11°44'59"E, 8–22 PSU salinity range) ($N = 132$) using fyke nets. Fish of freshwater origin were captured in April 2016 in the river Rhine (Bimmen, Germany, 51°51'39"N 6°04'18"E, 0 PSU) ($N = 116$) using fyke nets and in the river Elbe (Hamburg, Germany, 53°32'57"N 9°59'10"E, 0 PSU) ($N = 164$) using baited hook and line (Table S1). Fish were transported in aerated tanks to the laboratory facilities of the Institute of Marine Ecosystem and Fisheries Science, University of Hamburg, Germany. Fish from each respective origin were then put into one of two recirculating water systems, one with freshwater (0 PSU) and one with brackish water (16 PSU). Each system also included purpose-built equipment for rearing of round goby eggs. The 16 PSU salinity corresponds to a median value for the brackish collection site at Guldborgsund (Table S1). All experiments were conducted under the permit nr 59/16 from Amt für Verbraucherschutz, Veterinärwesen und Lebensmittelüberwachung, Hamburg.

2.2 | Salinity change protocol

The fish were first acclimated to aquarium conditions in their salinity of origin (i.e. in 0 PSU for freshwater, and 16 PSU for brackish fish) for a period of 7–11 days in fibreglass holding tubs (140 cm × 140 cm, water depth ~ 50 cm). The turnover volume per hour in each tank was 67%. After this period, salinity was changed from 0 to 16 PSU or from 16 to 0 PSU for approximately a third of the fish from each population, with a ramping protocol of 2 PSU salinity change per day. This was done by first closing off the tanks from the main recirculation system and then replacing ~100 L of water with lower or higher salinity water. When the target salinity was reached, the tubs were reconnected to the main recirculation system of the matching salinity. All groups experienced an initial high mortality during the first weeks in captivity, attributed to injuries sustained during fishing with nets and hooks (see above). Groups that were ramped into new salinities suffered higher mortality rates than control groups (χ^2 test, $\chi^2 = 45.91$, $p < .0001$) (freshwater origin, 0 PSU: 13.0%; freshwater origin, 16 PSU: 44.5%; brackish origin, 0 PSU: 50.7%; brackish origin, 16 PSU: 25%, see Table S1 for population-specific details).

After salinity ramping, fish were transferred to a breeding tank fitted with a nest box (see below) to produce clutches. Small round goby males can engage in sneaking behaviour and often avoid keeping nests of their own (Green, Niemax et al., 2020; Marentette et al., 2009). To avoid sneaking behaviour, males were always paired with females that were of shorter total length. A total of eight breeding groups per origin and treatment were kept at any one time, and when spawning occurred (see below), the group was removed and replaced by new individuals. Since only a low proportion of the breeding groups produced clutches, a second salinity ramping was conducted (as above) to increase the numbers of groups that could be formed. The second ramping was started 15 days after the first, which meant that some fish spent up to 36 days "resting" before being exposed to novel salinities. This resulted in freshwater fish in 0 PSU water (control group, $N = 150$) and 16 PSU water ($N = 130$) and brackish fish in 0 PSU water

($N = 65$) and 16 PSU water (control group, $N = 57$). Since time until spawning varied between breeding groups, the total time individual fish spent in their assigned parent treatment salinity varied between 29 and 36 days for ramped individuals, and 29–63 days for control fish (i.e. fish kept in the salinity of their origin). To estimate effects on adult physiology through growth, total length (TL) in mm was measured as adults were euthanized at the end of the experiment.

2.3 | Egg sampling

For mating, one male and three (occasionally two) females of the same origin and treatment salinity were transferred to a mating chamber constructed by dividing each tub into four sections, using plastic boards. Each mating chamber was fitted with a small artificial nest box (locally constructed, PVC plastic, 210 mm \times 105 mm \times 60 mm, with a semicircular opening of a 25 mm radius) for the male to occupy and use to attract females. This nest box was fitted with a top and a bottom glass pane covered with acetate film attached by rubber bands. When a spawning occurred on the acetate sheet, the sheet with eggs was removed for sampling (see below) and replaced with a new sheet. To increase the total number of clutches, the spawning group was left for another 4–7 days to see if another clutch was produced. Thereafter, the male was euthanized and sampled for sperm parameters (see below). Among the clutches that were sampled, twelve spawning groups produced 1 clutch, and four groups produced two clutches each (two groups of freshwater origin kept in 0 PSU, and two of brackish origin kept in 16 PSU). Nest boxes were inspected every Monday, Wednesday and Friday for eggs. If eggs were spawned on the sides, the eggs were removed and the nest box cleaned with tap-water and a brush before it was put back in the tank. If eggs were laid on one of the glass panes, the pane was removed and the acetate film with eggs was carefully sectioned into two roughly equally sized halves, using a stainless steel scalpel. Each half-clutch was then re-attached to a separate glass pane and marked with a plastic tag with a unique ID number for tracking. After this, both half-clutches were, one after the other, taken to a water bath station to be photographed at a resolution of $2,592 \times 1,944$ pixels at 1:1,025 magnification using a digital camera with fixed focus and exposure time (Caplio R3, Ricoh Imaging Company). The camera was mounted to a static frame that kept the glass pane in the same position in relation to the camera during every photo occasion. After this first photograph, one of the half-clutches was kept in the same salinity it was spawned in, whereas the other was switched to the opposite salinity by drip acclimation (16 PSU change over 2 hr). After acclimation, the half-clutches were kept in a separate flow-through system attached to the main recirculation systems (0 or 16 PSU), where they were exposed to water movement for oxygenation. All clutches were photographed every Monday, Wednesday and Friday to keep track of zygote development until day 20 (± 1 day) or until every zygote in the clutch had stopped development and died. The brief exposure to air when moving the eggs to the tray for photography was not expected

to affect the eggs due to their robustness to air-exposure (Hirsch et al., 2016). Data on zygote development were extracted by visually analysing each egg in the photographs and allocating the egg to one of the following categories: (a) egg present; (b) fertilized (visual separation of yolk and perivitelline space in second photograph taken); (c) abnormal development (opaque or decaying egg); (d) eye spots present on zygote. We focussed our analysis on fertilization and developmental success. The following parameters were therefore calculated for analysis:

Fertilization success = N eggs fertilized in half - clutch/ N eggs in half - clutch

Development success = N eggs with eye spots present at day 20 ± 1 in half - clutch/
 N eggs in half - clutch

2.4 | Sperm sampling

A total of 33 males consisting both of males from spawning groups and males not in spawning groups (total freshwater origin, 0 PSU: $N = 9$; freshwater origin, 16 PSU: $N = 5$, brackish origin, 0 PSU: $N = 9$; brackish origin, 16 PSU: $N = 10$), and that all showed reproductive readiness (erect genital papilla) were sampled for sperm data. After euthanizing a male by a concussive blow to the head, followed by destruction of the brain, the testes were dissected from the fish within 1 min using stainless steel forceps and scissors (curved, sharp point, 4 inch, Sigma-Aldrich Co) and one testis was placed into a 1.5 ml microcentrifuge tube (Eppendorf). To release sperm, the testis was incised five times using scissors and the content diluted with 60 μ l calcium-free Ringer's solution at 10°C (Karila et al., 1993) for a roughly double increase in liquid volume and to prevent sperm activation (confirmed by visual inspection under microscope) (AxioVert. A1, Carl Zeiss AG). The sample was then stirred using a Vortex (Vortex-Genie 2, Scientific Industries) three times for one second in rapid succession. Sperm were activated by transferring 25 μ l of the suspension to a new microcentrifuge tube, filled with 750 μ l filtered water of one of two different salinities (0 or 16 PSU taken from the recirculation systems where the adult fish were housed) and kept at 16°C in a water bath in between vortexing. These two samples were measured immediately after sperm transfer to the filtered water, one after the other and in randomized order.

We focussed our analysis of sperm traits on proportion motile sperm and velocity of the curvilinear path (VCL), allowing comparison with previous studies (e.g. Green, Havenhand, et al., 2020; Locatello et al., 2007, 2013; Marentette et al., 2009). Sperm velocity in round gobies decreases over time: Marentette et al., 2009). Therefore, these sperm traits were recorded as quickly as possible, always within 2 min from activation, in the following way: 45 μ l of the suspension was transferred to a 2% (w/v) albumin coated glass slide fitted with an O-ring. This slide was then covered with a lid (an albumin coated coverslip) to form a suspended drop (Havenhand & Schlegel, 2009; Green & Kvarnemo, 2019). This was repeated for six technical replicates per male and salinity condition. Using a high-speed video camera (PL-D725, PixelLINK) fitted to an inverted

TABLE 1 Spawning success of 100 replicate spawning groups, each consisting of one male and two to three females of the round goby *Neogobius melanostomus*, with parents of freshwater or brackish origin, kept in a parent treatment salinity of 0 or 16 PSU for ~4 weeks. Populations abbreviated as RH = Rhine, EB = Elbe, GS = Guldborgsund

Parent origin:	Fresh	Fresh	Brackish	Brackish	
Parent treatment:	0 PSU	16 PSU	0 PSU	16 PSU	Grand total
Total number of spawning groups	22	23	29	26	100
Groups with no spawnings	15	20	22	16	73
Groups with spawnings	7	3	7	10	27
Groups sampled for egg development ^a	3	2	4	7	16
Number of clutches sampled for zygote development ^b	5	2	4	9	20
Number of clutches sampled (by population)	(1 RH + 4 EB)	(2 EB)	(4 GS)	(9 GS)	20
% of groups that spawned	31.8	13.0	24.1	38.5	Average = 29.6

^aMany clutches were placed on the sides of the nest boxes, which prevented their sampling.

^bFour groups produced two clutches.

microscope (same as above), focus was set at the middle of the suspended drop, and sperm movement was filmed for 15 frames using a 10× magnification objective and standard contrast and illumination (30 frames per seconds, size 2,592 × 2,048 pixels, exposure time 10 ms, gain 0, gamma 0.1). Sperm movement parameters from a total of 309,946 sperm tracks in a total of 793 videos were extracted using the CASA plugin (Wilson-Leedy & Ingermann, 2007) for ImageJ (National Institutes of Health) following a protocol outlined in Green, Havenhand, et al. (2020). Sperm were only classified as swimming if velocity was higher than 25 μm/s, to avoid including non-swimming particles drifting in micro-currents.

2.5 | Data analysis

Data were analysed statistically using mixed and linear modelling through the *lme4* package (Bates et al., 2015) and *lmerTest* package (Kuznetsova et al., 2017) in R version 3.3.3 (R Core Team, 2013). For mixed effects models, terms were first decided and *p*-values were then generated using Satterthwaite approximations, to reduce the risk of Type 1 errors when analysing small sample size datasets of unbalanced data (Luke, 2017), and tested using type III sums of squares. We followed Hendrix et al. (1982) in that non-significant interactions with a *p*-value above .2 were removed sequentially, starting from the highest order, and within order, the interaction with the higher *p*-value was removed first.

The number of spawning groups that spawned per treatment group was analysed using a contingency test. The number of eggs per clutch was analysed using a linear model with parent origin and parent treatment salinity as predictor variables.

The main predictor variables of sperm traits modelled as fixed effects were parent origin (freshwater or brackish), parent treatment salinity (0 or 16 PSU) and sperm test salinity (0 or 16 PSU). These were modelled in a full factorial design with all interactions available. Because sperm from one male were tested multiple

times (i.e. in two different salinities), male ID was included as a random factor to control for the repeated measurements. Population was also included as a random factor to account for differences generated by the separate origin of freshwater fish caught from the rivers Rhine and Elbe. Both random factors were consistently kept in the model. Models were visually explored by inspection of residuals versus fitted values, theoretical and observed quantiles, high influence points and the frequency distribution of residuals, all using the “plot(lm)” function in R. Models were also analysed for multicollinearity by assessing Variance Inflation Factors (Vif), using the “vif(lm)” function from the *car* package in R, and none of concern were found. Sperm motility data were non-normally distributed and did not meet assumptions of sphericity but were not transformed since generalized mixed effects models of non-transformed data are currently regarded as being the best option for proportional data with low sample size (Harrison et al., 2018; Warton & Hui, 2011).

Fertilization success was analysed using a generalized mixed model with parent origin and parent treatment salinity as predictor variables. To control for risk that fertilization occurred after a clutch had been split and exposed to a new salinity, egg treatment salinity was initially included as a fixed factor in the analysis of fertilization success, but as predicted was not found to affect fertilization (*lmer*, egg treatment salinity: $F = 0.079$, $p = .780$). As above, population was included as a random effect to control for differences between fish from the rivers Elbe and Rhine.

Development success was analysed using a generalized linear model. However, since no eggs were fertilized by parents of freshwater origin in 16 PSU, it was not possible to analyse parent origin and parent salinity treatment as independent factors using our original full factorial (two-way) design. Instead, we used “parent treatment group” (ie, parent origin and parent treatment salinity, in a one-way design with three groups) as a factor and included the factor egg treatment salinity to control for half-clutches developing in different salinities.

TABLE 2 (a) Sperm motility (proportion sperm with a velocity above 25 $\mu\text{m/s}$) and (b) sperm velocity (curvilinear path, $\mu\text{m/s}$) in the round goby *Neogobius melanostomus*, analysed with linear mixed effects models in the package “lme4” in R. Results are printed via the tab_model function in the package “sjPlot” and summ function in the package “jtools”. Parent origin, parent treatment salinity and sperm treatment salinity were included as fixed factors. Since sampling was replicated within individuals and two different freshwater populations were used as origin, these factors were also included as random terms. Steps show the order of effect term removal during model simplification. Interaction terms in italics have been removed during model simplification. Variance terms from the random effects and error are from the final model

	Est.	SE	t-value	df	p-value	Sign.	Step
(a) Model: Sperm motility ~ Parent origin \times Parent treatment salinity \times Sperm treatment salinity + (1 Individual) + (1 Population)							
Fixed effects:							
(Intercept)	0.05	0.02	3.47	4.61	.001	**	3
Sperm test salinity (16 PSU)	-0.03	0.02	-1.52	59.00	.128		3
Parent origin (brackish)	-0.04	0.02	-1.78	1.96	.075		3
Parent treatment salinity (16 PSU)	-0.02	0.02	-0.97	59.55	.333		3
Sperm test salinity (16 PSU) \times Parent origin (brackish)	0.11	0.02	4.85	59.20	<.001	***	3
Sperm test salinity (16 PSU) \times Parent treatment salinity (16 PSU)	0.05	0.02	1.99	59.24	.047	*	3
<i>Parent origin (brackish) \times Parent treatment salinity (16 PSU)</i>	0.04	0.02	1.66	58.41	.097		2
<i>Sperm test salinity (16 PSU) \times Parent origin (brackish) \times Parent treatment salinity (16 PSU)</i>	0.04	0.05	0.77	57.34	.443		1
Random effects:							
Individual: 33	Variance	SD					Step
Individual: 33	0.001	0.026					3
Population: 3	0.0000	0.0000					3
Error	0.002	0.044					3
(b) Model: Sperm velocity ~ Parent origin \times Parent treatment salinity \times Sperm treatment salinity + (1 Individual) + (1 Population)							
Fixed effects:							
(Intercept)	53.77	5.05	10.66	4.20	<.001	***	4
Sperm test salinity (16 PSU)	19.42	6.27	3.10	60.31	.002	**	4
Parent origin (brackish)	-5.87	6.71	-0.88	1.82	.381		4
Parent treatment salinity (16 PSU)	-2.62	4.21	-0.62	60.23	.534		4
Sperm test salinity (16 PSU) \times Parent origin (brackish)	42.63	8.37	5.09	60.10	<.001	***	4
<i>Parent origin (brackish) \times Parent treatment salinity (16 PSU)</i>	2.51	8.59	0.29	59.36	.770		3
<i>Sperm test salinity (16 PSU) \times Parent treatment salinity (16 PSU)</i>	-2.73	8.57	-0.32	58.15	.749		2
<i>Sperm test salinity (16 PSU) \times Parent origin (brackish) \times Parent treatment salinity (16 PSU)</i>	6.86	17.45	0.39	57.23	.694		1
Random effects:							
Individual: 33	Variance	SD					Step
Individual: 33	33.11	5.754					4
Population: 3	0.00	0.000					4
Error	356.42	18.879					4

Note: Asterisks mark p-values as * = <.05, ** = <.01, *** = <.001.

Total length of adults at the end of the experiment was analysed using a mixed model (as above) with sex, parent origin and parent treatment salinity as fixed predictor variables and population as a random variable.

Values in the text are presented as mean \pm SE. Effect estimates and SE is presented for models unless otherwise noted.

3 | RESULTS

3.1 | Spawning

Of 100 replicate spawning groups, a total of 27 groups spawned during the experiment. Of these, freshwater fish held in brackish conditions were least likely to spawn, with only 13% of those groups spawning (Table 1); however, the difference in spawning frequency among groups was not significant (χ^2 test, $\chi^2 = 2.095$, $df = 1$, $p = .148$).

Individual clutch size was on average 512 (± 127) eggs for brackish parents in brackish water, 410 (± 205) eggs for brackish parents kept in freshwater conditions, 270 (± 97) eggs for freshwater fish in freshwater conditions and 486 (± 167) eggs for freshwater fish kept in brackish conditions. These clutch sizes did not differ significantly between parents of different origin and parent salinity treatments (*lm*, parent origin: $F_{1,54} = 1.861$, $p = .178$; parent treatment salinity: $F_{1,54} = 1.765$, $p = .190$; origin \times treatment salinity: $F_{1,54} = 0.0026$, $p = .959$).

3.2 | Sperm motility

Males of brackish origin had significantly higher sperm motility when sperm were tested in 16 PSU compared to males of freshwater origin tested in 0 PSU (Table 2a, Figure 2a). Males of freshwater origin showed a trend of slightly higher sperm motility in 0 PSU (Table 2a).

Sperm motility was higher for brackish males' sperm tested in 16 PSU, when they were kept in 16 PSU compared to when they were kept in 0 PSU (Table 2a). This was the only effect of acclimation found on sperm.

No main effects from parent treatment salinity were found in males of either origin.

3.3 | Sperm velocity

Sperm test salinity had a strong effect on sperm velocity (Table 2b). Males of brackish origin had significantly higher sperm velocity when sperm were tested in 16 PSU compared to males of freshwater origin tested in 0 PSU (Table 2b).

We did not find any effect from parent treatment salinity (i.e. male acclimation to 0 or 16 PSU) (Table 2b). This is also visualized by the near horizontal reaction norms (Figure 2b).

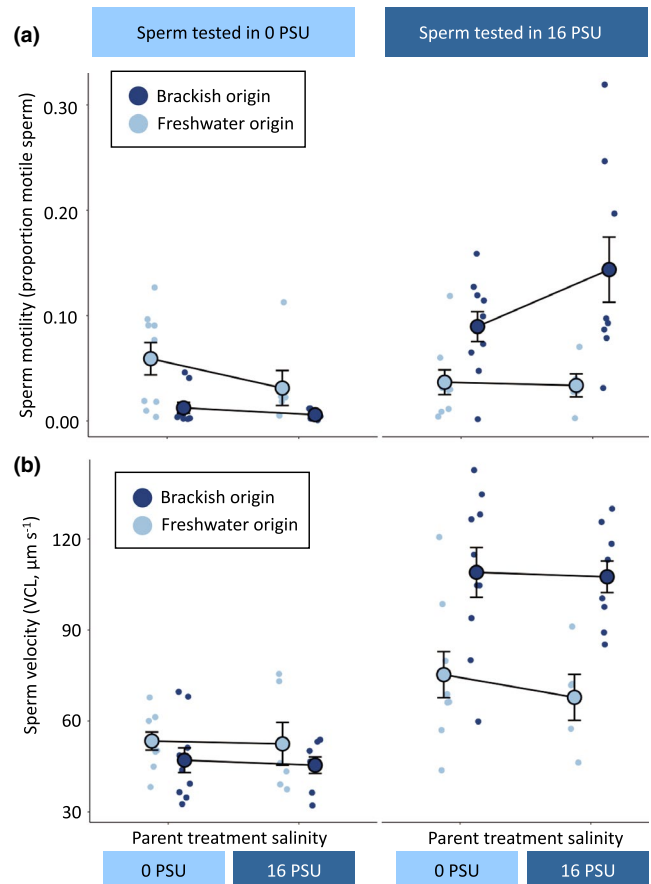


FIGURE 2 (a) Sperm motility measured as the proportion of motile sperm and (b) sperm velocity (VCL, curvilinear path, $\mu\text{m s}^{-1}$), in round goby *Neogobius melanostomus*. The sperm parameters were measured from males of freshwater or brackish origin, kept in a parent treatment salinity of 0 or 16 PSU. The sperm of each male was tested in 0 and 16 PSU salinity. Plot shows mean and standard error. Jittered points show individual averages. Lines between groups of the same origin kept in different salinities show reaction norms. Freshwater origin kept in 0 PSU: $N = 9$, freshwater origin kept in 16 PSU: $N = 5$; brackish origin kept in 0 PSU: $N = 9$; brackish origin kept in 16 PSU: $N = 10$

3.4 | Fertilization success

Fertilization success differed among the four different treatment combinations, and was explained in the model by an interaction effect between parent origin and their treatment salinity (Table 3). Fish of freshwater origin spawning in 16 PSU did not fertilize any eggs and fish of brackish origin also struggled to fertilize clutches in their non-native salinity (0 PSU) (Table 3; Figure 3).

3.5 | Zygote development

There was no statistically significant difference in development success between the three parent treatment groups that had fertilized eggs (*glm*, parent treatment group: $\chi^2 = 0.348$, $p = .840$). Also, we

TABLE 3 Proportion of fertilized eggs in the round goby *Neogobius melanostomus*, analysed with linear mixed effects models in the package “lme4” in R. Results are printed via the `tab_model` function in the package “sjPlot” and `summ` function in the package “jtools”. Parent origin, parent treatment salinity and egg treatment salinity were included as fixed factors. Since two different freshwater populations were used as origin, population was also included as a random term. Steps show the order of effect term removal during model simplification. Interaction terms in italics have been removed during model simplification. Variance terms from the random effects and error are from the final model

	Est.	SE	t-value	df	p-value	Sign.	Step
Model: Proportion of fertilized eggs ~ Egg treatment salinity × Origin of parents × Parent treatment salinity + (1 Population)							
Fixed effects:							
(Intercept)	0.12	0.14	0.84	0.28	.403		4
Parent origin (freshwater)	0.39	0.17	2.23	0.26	.026		4
Parent treatment salinity (16 PSU)	0.66	0.14	4.84	31.00	<.001	***	4
Egg treatment salinity (16 PSU)	0.03	0.10	0.28	31.05	.778		4
Parent treatment salinity (16 PSU) × Parent origin (freshwater)	-1.17	0.23	-4.97	31.59	<.001	***	4
<i>Parent treatment salinity (16 PSU) × Egg treatment salinity (16 PSU)</i>	-0.14	0.21	-0.64	30.06	.521		3
<i>Parent origin (freshwater) × Egg treatment salinity (16 PSU)</i>	0.03	0.24	0.13	29.05	.899		2
<i>Parent origin (freshwater) × Egg treatment salinity (16 PSU) × Parent treatment salinity (16 PSU)</i>	0.02	0.49	0.04	29.00	.97		1
Random effects:							
	Variance	SD					
Population: 3	0.004	0.064					4
Error	0.100	0.316					4

Note: Asterisks mark p-values as * = <.05, ** = <.01, *** = <.001.

found no effect on zygote development success from the salinity in which each half-clutch developed (*glm*, egg treatment salinity; $t = 0.79$, $\chi^2 = 0.578$, $p = .447$, Est. \pm SE: 0.11 ± 0.14) regardless of parent treatment group (*glm*, parent treatment group \times egg treatment salinity; $\chi^2 = 0.420$, $p = .811$) (Figure 4).

3.6 | Adult body size

After the experiment, no difference based on treatment salinity could be found on adult total length (TL) (*lmer*, parent treatment salinity; $t = -0.02$, $p = .980$, Est. \pm SE: -0.15 ± 6.10). Fish of brackish origin were larger than fish of freshwater origin (*lmer*, origin; $t = -7.05$, $p < .001$, Est. \pm SE: 35.81 ± 5.08). Males were on average larger than females within their origin (*lmer*, sex; $t = 3.09$, $p = .002$, Est. \pm SE: 18.24 ± 5.9), males of brackish origin: 143.5 ± 1.9 , males of freshwater origin: 109.0 ± 1.7 , females of brackish origin: 125.3 ± 4.5 , females of freshwater origin: 88.8 ± 1.3 .

4 | DISCUSSION

In this study, we tested if acclimation of adult round gobies to a salinity different from their origin improves sperm motility and velocity

in that new salinity and found no evidence of adaptive plasticity in these traits. After the acclimation to a novel salinity, round gobies still spawned and the egg output did not differ between fish of different origin or in different salinities. However, fish had difficulty fertilizing eggs when spawning in a different salinity from where they originated. This effect was particularly strong for freshwater fish, which were not able to fertilize any eggs when spawning in brackish water. Clutches that were successfully fertilized showed no difference in developmental success regardless of parent origin, parent treatment salinity or egg treatment salinity. Together, these results show that despite adult acclimation, fertilization issues in non-native salinities can limit round goby reproduction and therefore affect invasion processes.

The lack of a strong effect of parental treatment salinity in our experiments shows that round gobies are likely not plastic in the sperm traits measured. Adaptive plasticity in sperm traits has however been documented in other euryhaline fish species following comparable acclimation times to novel salinities as in our study, and responses have occurred as rapidly as within 2 days of acclimation (Sticklebacks, *Gasterosteus aculeatus*) (Taugbøl et al., 2017). In killifish (*Heteroclitus fundulus* and *Fundulus grandis*), sperm responses to salinity were evident after 2 weeks acclimation (Palmer & Able, 1987). In tilapia (*Oreochromis mossambicus* and *Sarotherodon melanotheron heudelotii*), sperm had acclimated when fish were tested after 2 months

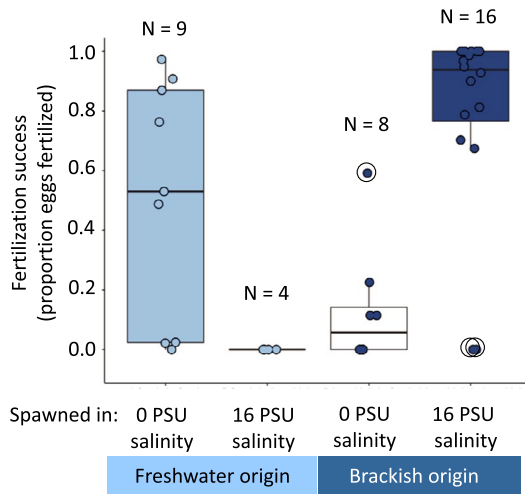


FIGURE 3 Fertilization success of round goby *Neogobius melanostomus* eggs spawned by parents of freshwater (light blue) and brackish (dark blue) origin in a parent treatment salinity of either 0 or 16 PSU. *N* denotes the number of half-clutches in each treatment group. The egg treatment salinity that the half-clutches were reared in after spawning did not affect fertilization, which means that fertilization occurred before the egg treatment started. Therefore, in this figure, half-clutches that were reared in different salinities have been pooled into each parent treatment salinity. Boxes show median, upper and lower quartile, and error bars show max and min with outliers denoted by dots. Jittered points show individual values of each half-clutch. Outliers are circled

(though no tests previous to this time point were performed) (Harvey & Kelley, 1984). The discrepancy between our results from round gobies (i.e. no measured beneficial effect from parent treatment salinity on the sperm traits after ~ 4 weeks) and the abovementioned studies may be due to differences in the evolutionary background and ecology of these species. Life history traits of invasive species have been shown to be highly dependent on taxonomy and phylogenetic background (Alcaraz et al., 2005).

We observed one effect of acclimation on sperm function in fish of brackish origin acclimated to 0 PSU and tested in 16 PSU. These fish had a lower proportion of moving sperm compared to brackish fish continuously kept in 16 PSU and tested in the same condition (Figure 2a, right panel, dark blue dots). We cannot determine an adaptive explanation for this trend, but from a mechanistic perspective sperm motility is directly affected by sperm maturation and energy stores (Schulz & Nóbrega, 2011), which can be costly (Olsson et al., 1997). The decreased sperm motility could be a result of the costs of an increased maintenance metabolism of adults in freshwater (Behrens et al., 2017).

We found that sperm velocity and motility of fish of freshwater origin tested in 0 PSU was lower than for fish of brackish origin tested in 16 PSU. There are two functional explanations for this observation: low sperm velocity is adaptive in a freshwater environment, or the freshwater populations are poorly adapted to the environment where they were caught. A previous study on freshwater round gobies in the American Great Lakes has reported sperm velocities similar to those we report for males of brackish origin

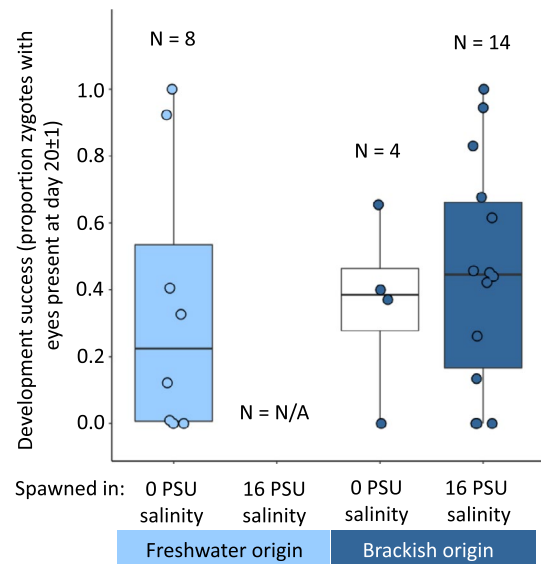


FIGURE 4 Proportion of zygotes reaching the eye spots present stage after 20 (± 1) days of development in the round goby *Neogobius melanostomus*. Parents were of either freshwater (light blue) or brackish (dark blue) origin, and the eggs were spawned in a parent treatment salinity of 0 or 16 PSU. *N* denotes the number of half-clutches in each treatment group. Since the egg treatment salinity that the half-clutches were kept in did not affect the development success (glm, egg treatment salinity: $X^2 = 0.578$, $p = .447$), half-clutches kept in different salinity conditions have been pooled under each parent treatment condition. No eggs were fertilized by fish of freshwater origin that spawned in 16 PSU, so no zygotes could develop in this treatment group (hence marked N/A). The treatment group is kept in the figure to highlight the experimental design. Boxes show median, upper and lower quartile, and error bars show max and min. Jittered points show individual values of each half-clutch

tested in 16 PSU (i.e. above 100 $\mu\text{m/s}$) (Marentette et al., 2009). This contrasts to the idea that low sperm velocity could be adaptive in freshwater environments. The marked difference in sperm velocity between our freshwater populations and those sampled by Marentette et al. could be a reflection of local adaptation (Green, Havenhand, et al., 2020). The Great Lakes populations were introduced from a freshwater source: fish in the Great Lakes were more closely related to fish from the river Dnieper than fish from the Black and Caspian seas (Brown & Stepien, 2009). Round gobies introduced in European rivers have their source population in the lower Danube river (Brown & Stepien, 2008), but also display haplotypes suggesting introductions from the brackish Black Sea itself (Mombaerts et al., 2014). Our sampled freshwater populations could therefore be a hybrid of brackish and river ancestry, and therefore less adapted to freshwater spawning conditions than round gobies in the Great Lakes. Since all these populations are introduced, their ancestral origin likely plays a large role in how well they are adapted to their current local environment. For example, "ecotypes" from ancestral fresh and brackish conditions would allow for different genetic backgrounds and trait selection. This could lead to different mechanisms by which to solve the issue of sperm performance in freshwater.

Great Lakes gobies might have their ancestral origins in freshwater and have sperm velocity sufficient for normal fertilization. The gobies in the European rivers, which seem less adapted to freshwater, could have solved the issue by producing longer-lived sperm. Since trade-offs between sperm longevity and sperm velocity exist in the round goby (Green, Niemax, et al., 2020), investing in longevity could pay off when velocity is compromised by the environment.

Very little is known about how much salinity variation round gobies (of any life stage) experience in their natural environments. Adults can migrate from brackish coastal waters and into freshwater streams (Christoffersen et al., 2019), and under laboratory conditions, they readily tolerate varying salinities (Behrens et al., 2017). Adults can in theory also avoid investing in reproduction during unfavourable conditions (Green, Niemax, et al., 2020). Since nest-holding round goby males are also able to “control” the spawning event via timing of courtship and number of ejaculations, any negative effects of fluctuating environmental conditions may be overcome through behavioural traits instead of plastic physiological responses. Adaptive plasticity in sperm traits could therefore be a more costly option compared to behavioural traits (Murren et al., 2015) and not come under selection during the species’ evolutionary history. A recent study on round gobies showed that salinity affected sperm traits differently depending on the origin of the males (Green, Havenhand, et al., 2020), indicating that adaptation in this trait may occur over generations. In that study, the authors compared fish from two different invasion fronts at ~5 PSU and 10–15 PSU. They found a correlation between increased sperm velocity in local salinity and the number of generations a population had spent in that salinity. When non-plastic traits do not conform to environmental conditions, direct selection on them is expected to be stronger. Our results, which found a lack of plasticity in round goby sperm traits, therefore support the previously described evidence of rapid local adaptation in sperm velocity.

Egg survival in adverse environmental conditions can be highly associated with invasion risk of non-indigenous species (Briski et al., 2011). Eggs of several euryhaline gobies develop well in salinities ranging from 5 to 35 PSU (Fonds & Van Buurt, 1974), and since gobies are associated with coastal regions that vary in their water chemistry, the goby clade has been proposed to be adapted to fluctuating environmental conditions (Thacker, 2009). Furthermore, round goby eggs are extremely tolerant of abiotic stress such as desiccation and temperature (Hirsch et al., 2016). In our study, we found no significant effect of parental acclimation on zygote development. However, in a separate experiment, round goby zygotes in different salinities developed at similar rates, but had different hatching success (J. Niemax, unpublished data). This suggests that zygote survival is an additional barrier to reproduction in different salinities. Our observed differences in reproductive success between fish of freshwater and brackish origins could be explained by differences in energy reserves and cues required to trigger spawning. Fish of freshwater origin kept in 16 PSU were the least likely to spawn, suggesting that despite higher sperm velocity in 16 PSU, these freshwater

fish had limited reproductive ability. Lower energy reserves are commonly seen in freshwater round gobies (Kornis et al., 2012, Table 1; but see Green, Niemax, et al., 2020) and round gobies from brackish origin show increased stress levels in freshwater (Puntilla-Dodd et al., 2021). If more energy is required in adverse salinities (Behrens et al., 2017), initial low energy reserves of freshwater round goby are likely to limit their reproductive output even further.

Despite the robustness of their eggs, male care is key for zygote development in gobies (Patzner et al., 2011). Our experimental design precluded male care during the development of the zygotes. This will have likely impacted the results because males normally remove defected zygotes (Manica, 2002), whereas unfertilized eggs were left in clutches in our experiments. Despite limits to reproduction, normally developing zygotes appeared in most treatment combinations in our experiment, except for freshwater parents that spawned in 16 PSU. This shows the overall tolerance of round goby eggs to different salinity conditions and again highlights the possibility of introduction already during the egg stage (Adrian-Kalchauer et al., 2017; Hirsch et al., 2016; Kotta et al., 2016).

Propagule pressure has been shown to be greater at the invasion front of expanding round goby populations than in established areas (Masson et al., 2018). We show that propagule pressure can be limited by environmentally induced reproductive dysfunction, despite potential for adult acclimation. This is of importance to the biogeographic patterns of round goby invasions. Our results also highlight that the phenotype-environment match can be cryptic and related only to particular life history stages. Population origin arguably plays a substantial role in the invasion process and its ecological consequences. The extensive round goby invasions of Europe and North America, though one species, could be driven by several distinct populations of “ecotypes” that have been previously selected for in a similar environment or subsequently adapted to, the salinity conditions in which they have established. This hypothesis is supported by the differences in sperm velocity found between freshwater fish in our study and that of Marentette et al. (2009) and genetic signatures (Brown & Stepien, 2009). Since no large-scale genomic studies of round goby populations across Europe and North America have yet been conducted, there are important knowledge-gaps in regards to how selection or historical adaptations influence the invasion patterns of this successful fish. The majority of trans-national ports on the latitudinal scale across which round gobies have spread successfully have similar abiotic conditions (low brackish salinity, temperate) (Keller et al., 2011). Monitoring not only species’ presence, but also species’ origins and adaptations, could provide valuable information for management (e.g. Boxes 1, 2, 3 in Figure 1).

In conclusion, we found no evidence that the round goby benefits from plasticity in sperm performance when exposed to novel salinities, and that this has likely limited its reproductive success. This finding is supported by previous work that found rapid adaptive change in sperm traits as the species expanded into novel salinities (Green, Havenhand, et al., 2020). Despite this, round gobies of brackish origin from the Baltic Sea are still able to reproduce in freshwater, albeit with reduced reproductive output. Since adult fish

in the Baltic are known to migrate between brackish and freshwaters, these results highlight the invasion risk into adjacent freshwater tributaries. While fish of freshwater origin seems less likely to spawn successfully in brackish conditions of around 16 PSU, reproduction in lower salinities than this is still in need of study.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are publicly available at Data dryad following this link: <https://doi.org/10.5061/dryad.6djh9w10z>

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BIOSKETCH

Leon Green is interested in understanding the role of environmental adaptations as species move into new habitats, or their habitats change. Utilizing skills from his previous career in underwater documentary film-making, he uses digital video and photo tools to capture data from life stages otherwise difficult to study, such as sperm and eggs in aquatic organisms. He believes in using model systems associated with ecological conservation issues, since this is often where applied and basic research needs overlap. Introduced and invasive organisms provide prime examples as they often threaten the conservation status of other species, while at the same time being under pressure from strong environmental and ecological forces.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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