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Ola Svensson[1,2*], Johanna Gräns[1], Malin C. Celander[1,2], Jonathan Havenhand[2,3], Erica H. Leder[2,4], Kai Lindström[5], Sofie Schöld[1#], Cock van Oosterhout[7], Charlotta Kvarnemo[1,2]

[1]Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE-405 30 Gothenburg, Sweden.

[2]Centre for Marine Evolutionary Biology, University of Gothenburg, Box 461, SE-405 30 Gothenburg, Sweden.

[3]Department of Marine Sciences – Tjärnö, SE-452 96 Strömstad, University of Gothenburg, Sweden.

[4]Department of Biology, University of Turku, FI-20014 Turun yliopisto, Finland.

[6]Environmental and Marine Biology, Åbo Akademi University, Tykistökatu 6, FI-20520 Turku, Finland.

[7]School of Environmental Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK.

[*]Present address: School of Natural Sciences, Technology and Environmental Studies, Södertörn University, SE-141 89 Huddinge, Sweden

[#]Present address: Swedish Meteorological and Hydrological Institute, Folkborgsvägen 17, SE-603 80 Norrköping, Sweden

Author for correspondence: Ola Svensson, email: ola.svensson@sh.se

School of Natural Sciences, Technology and Environmental Studies, Södertörn University, SE-141 89 Huddinge, Sweden

Authors' contribution

OS conceived and coordinated the study, designed and performed experiments, analysed data, performed the statistics, and wrote and edited the article. JG and SS performed experiments and analyses. MCC participated in experimental design and interpretation of the data. OS, JH and CK conceived the sperm analyses that were experimentally designed and performed by JH, EHL, KL and CK. CvO and CK contributed to the analysis, interpretation of data and writing of the article. All authors have revised the article critically for important intellectual content and given final approval for publication.

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Data accessibility

The data will be deposited in the Dryad Digital Repository

ABSTRACT

The distributions of species are not only determined by where they can survive – they must also be able to reproduce. Although immigrant inviability is a well-established concept, the fact that immigrants also need to be able to effectively reproduce in foreign environments has not been fully appreciated in the study of adaptive divergence and speciation. Fertilization and reproduction are sensitive life history stages that could be detrimentally affected for immigrants in non-native habitats. We propose that 'immigrant reproductive dysfunction' is a hitherto overlooked aspect of reproductive isolation caused by natural selection on immigrants. This idea is supported by results from experiments on an externally fertilizing fish (sand goby, *Pomatoschistus minutus*). Growth and condition of adults were not affected by non-native salinity whereas males spawning as immigrants had lower sperm motility and hatching success than residents. We interpret these results as evidence for local adaptation or acclimation of sperm, and possibly also components of paternal care. The resulting loss in fitness, which we call ‘immigrant reproductive dysfunction’, has the potential to reduce gene flow between populations with locally adapted reproduction, and it may play a role in species distributions and speciation.

Key words: Ecological speciation, immigrant inviability, immigrant reproductive dysfunction, local adaptation, multifarious selection, transporter hypothesis

1. INTRODUCTION

A heterogeneous environment can restrict gene flow and increase the opportunity for local adaptation (Berg et al., 2015; Hice et al., 2012; Hohenlohe et al., 2010; Kawecki and Ebert, 2004; Larmuseau et al., 2009a; Limborg et al., 2012; Poulsen et al., 2011). When immigrants to a population perform more poorly compared than the locally adapted residents, natural selection will form a barrier against gene flow that can promote further reproductive isolation. Such isolation caused by natural selection against migrants is termed ‘immigrant inviability’ and encompasses natural selection across the entire life history of individuals (Nosil, 2012; Nosil et al., 2005). An immigrants’ fecundity may also be reduced creating a barrier to gene flow termed ‘immigrant infecundity’ (Lemel et al., 1997; Smith and Benkman, 2007). In addition to acting on individual immigrants, natural selection can also act on gametes before fertilization and on their zygotes immediately after fertilization. In this study, we propose the term 'immigrant reproductive dysfunction' (IRD) to define this potential loss of fitness. IRD is a post-mating barrier to gene flow, which may act as a pre- and/or post-zygotic mechanism promoting reproductive isolation. We advocate for broadening the conceptual role of immigration in reproductive isolation to include immigrant reproductive dysfunction in addition to immigrant inviability and immigrant infecundity.

For organisms with external fertilization, eggs and sperm are exposed to the ambient environment, and hence, fertilization and early development are influenced by external environmental factors such as pH and salinity (Allen and Pechenik, 2010; Byrne et al., 2015; Havenhand et al., 2008; Holliday, 1969; Nissling et al., 2002; Sayer et al., 1993). Local adaptation of reproductive traits results in reduced gene flow as a by-product. With the exception of sexual selection, studies on local adaptation of reproductive traits are scant relative to those on morphological and life history traits. This is surprising because the distributions of species are determined not only by where they can survive, but also by where they can reproduce successfully.

There are several examples from broadcast spawners and flowering plants of co-evolution between male-female gamete recognition proteins causing assortative mating (Clark et al., 2009; Hart et al., 2014; Howard, 1999; Moyle et al., 2014; Palumbi, 2009; Swanson and Vacquier, 2002; Van Doorn et al., 2001). Divergence in gamete recognition proteins can result in gametes of immigrants not being recognized by gametes of residents. However, this does not necessarily preclude the possibility of two immigrants reproducing in the novel environment. IRD caused by e.g. poorly performing sperm (Byrne et al., 2015; Elofsson et al., 2003; Nissling and Westin, 1997), maladapted egg buoyancy (Berg et al., 2015; Nissling and Westin, 1997) or egg coats (Shu et al., 2015), is different from co-evolution between male-female gamete recognition proteins, because immigrants suffering from IRD have difficulties reproducing with both residents and other immigrants.

Although the brackish water environment of the Baltic Sea (Fig. 1) has only existed for ~4500 years (Westman and Sohlenius, 1999), there are several species that exhibit characteristics of local adaptation in this area (Johannesson and André, 2006; Johannesson et al., 2011), including the sand goby (*Pomatoschistus minutus*) (Pallas) (Larmuseau et al., 2010; 2009a; 2009b). The sand goby (Electronic supplement figure S1) is widely distributed and inhabits the shores of the Mediterranean Sea, the North East Atlantic and the Baltic Sea including the very low salinity waters in the Gulf of Bothnia, but it has not been found in fresh waters (Kullander et al., 2012). Several closely related species have, however, entered and speciated in fresh water in the Balkan region (Vanhove et al., 2012). The reproductive behavior of the sand goby includes exclusive paternal care with male nest building (covering a bivalve shell with sand and forming a cavity underneath) (Forsgren, 1999). Before spawning, the male attaches sperm-containing mucus on the nest surface where the females will attach their eggs and the male continues to attach mucus after the female has deposited eggs (Svensson and Kvarnemo, 2005). The mucus of gobiid fish contains compounds that promote sperm performance (Locatello et al., 2002) and protect the eggs from microbial infections (Giacomello et al., 2008). After hatching, the larvae are pelagic for approximately one month (Fonds, 1970). We know from earlier work that small clutches with low reproductive value are often completely consumed by the caring male, as predicted by theory (Manica, 2002), and that this typically happens after approximately two days (e.g. Lissåker and Svensson, 2008). Thus, full clutch cannibalism of larger clutches and occurring after two days would indicate other reasons for cannibalism, such as the eggs being unfertilized, dead or inviable (Manica, 2002).

The aim of the present study was to test the IRD hypothesis, i.e. that gene flow is hampered as a by-product of local adaptation to reproduction at local salinity levels. We conducted a series of laboratory experiments to investigate the sperm motility and reproductive success of sand gobies from a marine population experimentally "immigrating" into a brackish population spawning in brackish salinity, and of sand gobies from brackish populations "immigrating" into a marine population and spawning in marine salinity. We also measured growth and condition of "immigrant" sand gobies to test for effects of immigrant inviability. The IRD hypothesis predicts a loss in reproductive fitness of immigrant individuals spawning in a non-native environment.

2. METHODS

General methods

All fish were collected in sandy and shallow habitats using a hand trawl. Three localities were used: Bökevik, a bay near Sven Lovén Centre for Marine Infrastructure, Kristineberg (Skagerrak, 58°14'55"N 11°26'51"E, salinity 16-35 PSU), Sandviken, a bay near the town of Västervik (Baltic Sea proper, 57°44'15"N 16°42'29"E, salinity 6-7.5 PSU) and the bays Svartvik and Byviken near the town of Härnösand (Gulf of Bothnia, 62°33'08"N 17°52'00"E and 62°35'22"N 17°54'38"E, salinity 3-5.5 PSU). We refer to fish from these localities as marine (Kristineberg) and brackish (Västervik and Härnösand). Fish were stored in tanks of 60 - 240 L before experiments (see below for details of salinity treatments in these tanks). Experiments were conducted in 15-20 L tanks in closed flow-through systems equipped with mechanical, biological, and UV-filters. The fish were fed frozen adult *Artemia* and dry salmon fry food (Nutra HP, Skretting) 3-7 times per week depending on season. In all experiments, apart from the sperm analyses, artificial seawater was used (tap water and CoralMarine reef salt from Grotech, pH 8 regardless of salinity). Because all fish were wild caught, we were not able to distinguish between genetic and non-genetic effects such as parental effects or early-life plasticity.

Growth and condition

Fish (0+ age group) from the three localities were caught during October-November 2011. Salinity for the brackish populations was raised to 20 PSU over the course of a week to avoid *Saprolegnia* infections whereas salinity was kept at 30-32 PSU for the marine population. The following mid-March, 51 individuals were measured for total length (L_T±SD, Kristineberg 57.4±4.7mm, Västervik 53.1±10.3mm and Härnösand 42.2±9.0mm). Thereafter they were divided into two groups, “immigrant” (non-native salinities) and “resident” (native salinities), by slowly decreasing or increasing salinity to 6 PSU or 30-32 PSU over the course of a week. Generally, at the start of the experiment individuals were kept separately but with visual access to each other. A layer of sand and a halved clay-flower pot were provided as shelter. Thereafter, day length and water temperature, were slowly increased from 6.5 h and 4°C, to 18 h and 14°C to mimic natural conditions. By the end of May, approximately two months after the change in salinity started, length and weight (g) were measured again, and the fish were given the opportunity to spawn. Growth was calculated as the increase in total length (mm). During the experimental phase eleven fish had to be euthanized because of poor health. This happened equally often for immigrants as for residents (brackish environment: immigrants from Kristineberg 3/12 vs. residents from Härnösand 1/7 and Västervik residents 0/6; marine environment: immigrants from Härnösand 3/6 and Västervik 1/7 vs. Kristineberg residents 3/13).

Female and male effects on hatching success

At the time of the hatching success experiment (end of May to mid-July), the marine population (Kristineberg) had been subjected to 30-32 PSU for four months initially, and then another 2.5-4 months at 6 PSU (immigrant) or 30-32 PSU (resident). The two brackish fish populations (Västervik and Härnösand) had been subjected to 20 PSU during four months and thereafter 2.5-4 months at 30-32 PSU (immigrant) or 6 PSU (resident). We refer to these fish as being ‘long’ time in treatment. Additional batches of fish were caught from Kristineberg, Västervik and Härnösand in mid May 2012 and again from Kristineberg and

Västervik in mid-June. For these fish the salinities for immigrants were slowly changed over a single week to 6 PSU or 32 PSU. These immigrants had been subjected to the non-native salinity during 1-4 weeks when they spawned. We refer to these fish as being 'short' time in treatment.

One male and one female were introduced to each tank and a halved clay-pot was used as nest site. The salinities were either 6 PSU or 30-32 PSU. The combinations of 'immigrant' and 'resident' males and females, and their sample sizes are shown in Table 1. The fish were inspected daily for nest building (the pot is covered with sand). When a clutch of eggs was found, the female and the clay pot were removed, the eggs were photographed and the clay pot was returned to the male to care for the eggs until hatching. When the eggs were close to hatching, the clay pot was removed and the egg mass was photographed again. The clutch areas were measured using ImageJ 1.48v.

Sperm analyses

To test for possible causes of differences in reproductive success, we compared motility of sperm released by immigrants into non-native salinity with sperm from residents into the same salinity. A set of males, caught in late May 2012 from Kristineberg (marine) and from Härnösand (brackish), were kept in aerated natural seawater at their native salinity at Sven Lovén Centre for Marine Infrastructure, Kristineberg. Within two days of capture, eight males in breeding colour from each site were sacrificed and the sperm from each male were immediately sampled and mixed into 31 PSU filtered natural seawater as well as 6 PSU water (filtered natural 31 PSU seawater diluted with distilled water to this salinity). Sperm motility was recorded at the midpoint of nine 40-µl drops of sperm suspension, placed between microscope slides and coverslips, separated by an O-ring (Havenhand and Schlegel, 2009), at 30 Hz, using a digital video camera (PixelLink 700 series) mounted on an inverted microscope (Leica DM-IL). All recordings were done within 30 s of placing the sample on the slide. Videos were analysed with ImageJ using the CASA plugin (Wilson-Leedy and Ingermann, 2007) to determine the proportion of motile sperm (classified as sperm moving faster than 15 µm/s). Mean values of the nine technical replicates for each male and salinity were used in our statistical analyses.

Statistical analyses

All statistical analyses were performed in SPSS version 23. The number of built nests and spawnings were tested with 2x2 χ^2 tests (Table 1). All other data were analysed with Generalized Linear Models (GzLMs) and Wald statistics. Because sample sizes between groups were uneven and/or the treatment effect was extreme on males breeding as immigrants, covariance matrixes were set on robust estimator (sandwich estimator / Huber-White estimator), to deal with over-dispersion and/or model misspecification (SPSS version 23; Huber, 1967; Luque-Fernandez et al., 2016; White, 1980). A summary of the GzLMs is shown in the electronic supplement tables S1-S5.

Growth and condition were analysed with linear scale response and identity link-function. Growth (mm) was analysed with original length (L_T) as a covariate and condition was analysed as weight (g) with cube transformed final length (L_T) as a covariate. We included treatment ('resident' in native salinity, or 'immigrant' in non-native salinity), population (marine (Kristineberg) and brackish (Västervik and Härnösand)) and sex as factors. The treatment x population and treatment x sex 2-way comparisons were included in the two models.

The hatching success was analysed with a binary logistic GzLM with hybrid method with scale parameter fixed at 1. The final egg area ('hatching') was the dependent variable and original egg area the trial variable in which the event 'hatching' could occur, and the model reports the proportional hatching success. We included male treatment (immigrant vs. resident), female treatment, (immigrant vs. resident) male population, female population and male time in experiment (long vs. short) as factors. The two brackish female populations Härnösand and Västervik were pooled because of the low number of Härnösand immigrant females. Male-treatment x male-population 2-way comparison was included in the model. Given that we wanted to identify the combinations of migrants and residents resulting in IRD, we performed paired analyses with least significant difference (LSD), testing all combinations of immigrants vs. residents within each environment.

Area of lost clutches, time until complete clutch loss (both square-root transformed) and proportional sperm motility (square-root arcsine transformed) were analysed with linear scale response, identity link-function and treatment as factor. The populations were pooled because only 6 clutches were lost in the resident treatment (2 Kristineberg males, 4 Västervik males and 0 Härnösand males) and only treatment was used as factor. Proportions of motile sperm of males from Kristineberg and Härnösand were tested in two separate GzLMs (Kristineberg immigrants vs. Härnösand residents in 6 PSU and Härnösand immigrants vs. Kristineberg residents in 32 PSU) because sperm from each male was tested in both salinities.

We extracted untransformed estimated marginal means as well as 95% Wald CI from the GzLMs. Note, however, that Figure 2 shows raw data and not model estimates. Following Nosil et al. (2005), we also calculated the descriptive value of IRD as:

$$IRD = 1 - (immigrant\ reproductive\ success/resident\ reproductive\ success)$$

using the estimated marginal means in the models. The complete models and their parameter estimates are given in the electronic supplement.

Ethics

Fish were kept in the Zoology building aquarium facility of the Department of Environmental and Biological Sciences, Gothenburg University, except where noted. After the experiment, all fish were euthanized in marine or brackish water containing MS-222. This study complies with Swedish law and was performed under ethical permits 135-2010 and 143-2012.

3. RESULTS

No evidence for immigrant inviability measured as growth and condition

After 2.5 months at 6 and 30-32 PSU salinity, respectively, all 40 fish appeared to be in good health. Immigrants did not show a reduction in growth or condition (Linear GzLMs, growth χ^2=0.005, df=1, p=0.95; condition χ^2<0.001, df=1, p=0.99, Table 2). Growth did not differ between populations but males had grown significantly more than females (population χ^2=3.63, df=1, p=0.16; sex χ^2=3.87, df=1, p=0.049). In contrast, condition did not differ significantly between males and females, but it did differ significantly between populations with Kristineberg > Härnösand > Västervik (population χ^2=11.98, df=2, p=0.002; sex χ^2=0.86, df=1, p=0.35). Two-way comparisons were not significant (Electronic supplement material tables S1-S2).

Significant male IRD – but no female IRD

Male immigrants did not build fewer nests or received fewer spawnings than male residents (2x2 χ^2-tests, df=1, N=111; Nest building χ^2=0.47, p=0.49; spawning χ^2=0.42, p=0.42; Table 1). Female immigrants did not spawn less often than resident females (2x2 χ^2-tests, df=1, N=123; χ^2=0.65, p=0.212). Regardless of immigrant/resident status, males built more nests but did not spawn more frequently in marine water than in brackish water (Nest building χ^2=8.07, p=0.0045; spawning χ^2=0.00, p=0.99). There was no difference in spawning rates between females in marine and brackish water (χ^2=0.24, p=0.63).

Hatching success was not affected by female treatment (immigrant vs. residents), female population, or the time the males had been in the experiment (>2.5 months vs. 1-4 week) (binary logistic GzLM, female immigrant status: χ^2=1.22, df=1, p=0.27; female population χ^2=1.06, df=1, p=0.30; male time in treatment χ^2=0.46, df=1, p=0.50). In contrast, there was a strong treatment effect on males: few immigrant males had any hatching success (χ^2=222, df=1, p<0.001, Figure 2, Tables 1-2). In particular, hatching success depended on whether the immigrant male had a brackish or marine origin: not a single immigrant male of brackish origin successfully hatched eggs, whereas one third of the immigrant males of marine origin succeeded hatching eggs. This was reflected in the significant male treatment x male population 2-way comparison (χ^2=44.61, df=2, p<0.001). The planned comparisons within the model showed that immigrants into non-native salinity from all three populations had significantly lower hatching success than residents (Figure 1, Tables 1-2, Electronic supplement table S3). Using estimated marginal means for hatching success of both males and females (Table 2), for individuals of marine origin immigrating into both brackish water populations the mean IRD=0.58, and for brackish water individuals from both populations immigrating into the marine population mean IRD=0.53.

Patterns of IRD: Timing of clutch failure and clutch size patterns

For complete clutch failures, time until all eggs were lost differed between males spawning as immigrants and residents (time until clutch failure (days): estimated marginal means (and Wald confidence interval), immigrants=3.7 (3.2-4.3); residents=2.0 (1.5-2.5); linear GzLM, N=6+19, χ^2=20.58, df=1, p<0.001). Compared to resident males, immigrant males completely consumed even relatively large clutches, which suggests inviability of eggs in immigrant nests, rather than a paternal-investment trade-off, which can occur when caring for a small number of offspring. (All populations pooled, size of eaten clutches (cm^2): immigrants=8.24 (7.79-9.83); residents=5.24 (3.56-7.26); linear GzLM, χ^2=5.55, df=1, p=0.018). Because eggs of inter-populational spawning immigrant females did not hatch significantly less often than eggs from intra-populational spawning resident females (Table 2, Figure 2), intrinsic genetic incompatibilities between the tested populations are unlikely to be the driving mechanisms behind these clutch failures.

Sperm suffered from IRD

The proportion of motile sperm was significantly lower for immigrants than residents in both marine and brackish water (Table 2, brackish water, N=16, χ^2=32.0, p<0.001; marine water N=16, χ^2=8.3, p=0.004). The sperm-motility based IRD score of the immigrant marine males moving into the brackish water population is IRD=0.90, and immigrant brackish water males moving into the marine water population is IRD=0.74 (Table 2).

4. DISCUSSION

Local adaptation is a prerequisite for ecological speciation, but in order to avoid the subsequent breakdown of such local adaptations, gene flow also needs to be constrained. Our immigrant reproductive dysfunction (IRD) hypothesis provides a mechanism for this, and our data provide empirical support as a 'proof of concept' for IRD.

We found that immigrant males had a significantly lower reproductive success than resident males. In terms of hatching success, immigrant males were either completely unable to reproduce (males of brackish origin in marine environment), or they showed a large reduction (65 %) in their reproductive output (males of marine origin in brackish environment). Clutches spawned by immigrant males were lost later, and were larger, than the few clutches lost by resident spawning males (and reports from previous studies e.g. Lissåker et al., 2003), suggesting different mechanisms e.g. that immigrant males removed unfertilized, dead and/or inviable eggs rather than optimizing their fitness according to clutch size (Manica, 2002). Similar to hatching success, sperm motility of immigrant males was significantly lower than that of residents. Females on the other hand were not significantly affected by treatment (immigrant vs resident) and inter-populational crosses reached hatching stage when the males were residents in native salinity. Therefore, it is unlikely that intrinsic genetic incompatibilities were the mechanism for males failing to reproduce as immigrants. Further, the brackish environment we studied is only 4500 years old (Westman and Sohlenius, 1999), which is expected to be insufficient time for deleterious genetic incompatibilities to accumulate (Stelkens et al., 2010). Moreover, the condition and growth of adults were not significantly affected by salinity treatment. Our data on impaired immigrant male reproductive success therefore support the IRD hypothesis, and extend the observations made on immigrant inviability based on non-reproductive traits (Nosil, 2012; Nosil et al., 2005):

dysfunctional reproduction in the non-native habitat can reduce fitness of immigrants which could have implications for the evolution of reproductive isolation and speciation.

Inviable immigrant spermatozoa can cause IRD

Local adaptation of male reproduction is a likely explanation to why immigrant males were less able to reproduce in the non-native environment. There are several possible mechanisms which could cause the observed clutch failures, such as fertilization failures, impaired embryonic development caused by salinity-induced DNA-damage of spermatozoa, or by direct or indirect salinity-induced effects inflicted by reactive oxygen species on the seminal fluid or accessory gland products (Dowling and Simmons, 2009; O et al., 2006). The fact that sperm were active in all salinities (albeit less so in immigrants), suggests that developmental failure is more likely than fertilization failure. Further, because sand gobies prepare their nests with a thin layer of sperm-containing mucus before spawning (Svensson and Kvarnemo, 2005), the sperm may have been subjected to non-native salinity over an extended period of time.

It is also feasible that local adaptation of paternal care (both behavioural aspects and the mucus that protects the eggs (Giacomello et al., 2008)) selects against immigrants. Even if clutch failure were partly a laboratory artefact caused by the use of artificial seawater diluted with local tap water, etc., clutch failure was nevertheless significantly elevated for males reproducing as immigrants (non-native salinity), regardless of their origin. Osmotic acclimation of sperm during spermatogenesis has been demonstrated in fish (Legendre et al., 2008; Linhart et al., 1999; Morita et al., 2011; Tiersch and Yang, 2012), and sperm have also been shown to respond quickly to selection (Kekalainen et al., 2013). If IRD is caused by acclimation its effects may be limited to the first generation. Nonetheless, all males in our experiments suffered from consistent IRD and loss in reproductive output, irrespective of whether males had been subjected to non-native salinities for weeks or for months. This suggests that this phenomenon is not transient, and that it can play a significant role in creating partial reproductive isolation of organisms of marine and brackish water origins.

IRD compared to mortality-based immigrant inviability

Given that IRD was only observed in males, the reproductive barrier in sand gobies will be incomplete. Consequently, genetic divergence will evolve only in loci responsible for the local adaptation and the nearby linked genomic regions, but would not result in genome-wide divergence (Wu, 2001). In the present study, IRD was approximately 0.5 (overall average), which is equivalent to a 50% reduction in fitness. Comparing the present study with other study systems (Hereford, 2009; Nosil, 2012; Nosil et al., 2005) shows that the magnitude of

IRD we observed in sand gobies is comparable to many cases of mortality-based immigrant inviability. Hence, both processes can contribute to increased reproductive isolation.

IRD caused by environmental factors other than salinity

In the present study, we focused on the effect of salinity on gametes and zygotes, however, IRD may evolve in different species due to different environmental (biotic and abiotic) conditions. Exposure to non-native pH may also cause reproductive failure (Sayer et al., 1993; Schlegel et al., 2015), and gradients in pH between adjacent habitats such as lakes, rivers and streams are well known in habitats with rich biodiversity, including the Amazon River system (Junk, 2007) and lake and stream populations of East African cichlid fish (Kullander and Roberts, 2011; Theis et al., 2014). In amphibians, it has been suggested that local adaptation of egg coats to pH has a role in adaptive divergence (Shu et al., 2015). Other examples are temperature, which affects e.g. sperm performance in the mosquitofish *Gambusia holbrooki* (Adriaenssens et al., 2012), and aquatic hypoxia, which e.g. reduces sperm motility and fertilization success in carp (*Cyprinus carpio*) (Wu, 2009). Terrestrial examples include the yellow dung-fly (*Scathophaga stercoraria*) (temperature effects on eggs, Blanckenhorn et al., 2014), and mammals (hypoxia and/or altitude effects on spermatogenesis and ovarian function, Gonzalo Farias et al., 2008; Parraguez et al., 2014; Vitzthum and Wiley, 2003; Zepeda et al., 2014)). In humans, this is only the case for immigrants, prompting Vitzhum and Wiley (2003) to suggest that the reproduction of humans is locally adapted to high altitudes.

All environmental gradients or shifts described above are likely to be repeated over large geographical scales. Therefore, ecological speciation facilitated by IRD has the potential to occur in parallel via the ‘transporter’ process (cf. ‘transporter hypothesis’, Schluter and Conte, 2009); selected alleles from a locally adapted freshwater population are transported via a marine gene pool into other freshwater populations through hybridization. According to the transporter hypothesis, once a mutation conferring a local adaptation reaches an appreciable frequency in a ‘donor’ population (in our case, a brackish population), it could participate in the transporter process, resulting in parallel evolution and speciation on a large geographic scale (Schluter and Conte, 2009).

Multifarious selection

Besides IRD, other selective pressures can be operating on different traits of immigrants when migrating into a novel environment. For example, selection on both osmoregulation and reproductive ability has been invoked to explain the divergence of Baltic Sea populations from Atlantic populations of Atlantic cod (*Gadus morhua*) (Berg et al., 2015), and European flounder (*Platichthys flesus*) (Momigliano et al., 2017) and this may also be true for other species that have diverged between the two environments (Hemmer-Hansen et al., 2007; Johannesson and André, 2006; Johannesson et al., 2011; Larmuseau et al., 2009a; Limborg et al., 2012; Serrao et al., 1999). IRD may also evolve due to indirect effects of the abiotic conditions affecting the biotic environment. For example, salinity has a significant impact on parasites and pathogens, such as bacteria (Herlemann et al., 2011) and oomycetes (Lehtonen and Kvarnemo, 2015), and the prevalence of parasites and their intermediate hosts changes drastically over salinity gradients (Rogowski and Stockwell, 2006; Zander, 2005). In three-spined sticklebacks (*Gasterosteus aculeatus*), the molecular divergence between marine and freshwater forms of the nesting glue protein *spiggin* has been argued to be driven by selection on functional properties including antimicrobial effects (Seear et al., 2015). In all these systems where we expect IRD to occur, multiple – and very different – traits are expected to be under divergent selection. These traits will be associated by being directly or indirectly linked to an environmental factor, which will aid the build-up of linkage disequilibrium. Such 'multifarious' selection may provide a stronger barrier to gene flow than selection on a single trait (Nosil et al., 2009; Smadja and Butlin, 2011).

Exaptations which release populations from IRD

Lastly, exaptation may enable reproduction in novel environments that would otherwise be impacted by IRD. For example, in several teleost species that are able to reproduce in a wide range of salinities, fertilizations are "protected" in some way: in three-spined sticklebacks, sperm appear to be locally adapted to salinity but it is in fact the ovarian fluid around the eggs that makes males able to breed in both freshwater and marine environments (Elofsson et al., 2003; Elofsson et al., 2006); fertilization in tilapia takes place inside the female mouth (Legendre et al., 2008; Linhart et al., 1999; Morita et al., 2011); whereas poeciliids such as the invasive mosquito fish (*Gambuisa* spp.) and guppies (*Poecilia reticulata*) have internal fertilization within the female reproductive tract (Constantz, 1989). Such exaptations that protect gametes from potentially adverse external environmental conditions – and hence from IRD – may contribute to the success and invasive potential of these species.

5. CONCLUSIONS

Our experimental data show that fertilization and reproduction are sensitive life history stages that can be detrimentally affected in individuals that migrate to non-native habitats with distinct environmental conditions. We call the resulting loss in fitness 'immigrant reproductive dysfunction' (IRD). This fitness loss has the potential to reduce the effective rate of gene flow, resulting in (at least partial) reproductive isolation. We suggest that IRD is a previously neglected aspect of reproductive isolation that may play a role in adaptive divergence, biogeography, biodiversity and speciation and we advocate for broadening the conceptual role of immigration in reproductive isolation to include immigrant reproductive dysfunction.

Data accessibility

The data will be deposited in the Dryad Digital Repository.

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TABLES

Table 1. The nest building, spawning and hatching success of *Pomatoschistus minutus*

	Salinity (PSU)	Males summary (N)				Kristineberg females (N)			Västervik females (N)			Härnösand females (N)		
		N_{tot}	Nest	Spawn	Hatch	N_{tot}	Spawn	Hatch	N_{tot}	Spawn	Hatch	N_{tot}	Spawn	Hatch
Kristineberg males	32	32	22	11	9	28	7	6	**8**	**4**	**3**	**1**	**0**	-
	6	*28*	*14*	*11*	*3*	***14***	***2***	***0***	*9*	*7*	*2*	*2*	*2*	*1*
Västervik	*32*	*15*	*14*	*7*	*0*	*10*	*4*	*0*	***7***	***3***	*0*	-	-	-
males	6	23	12	10	6	**11**	**6**	**3**	18	4	3	-	-	-
Härnösand	*32*	*5*	*4*	*4*	*0*	*4*	*3*	*0*	-	-	-	***3***	***1***	*0*
males	6	8	4	4	4	**1**	**0**	-	1	0	-	6	4	4

N_{tot} is the total number of fish used. Nest is the number of males that built nests. Spawn is the number of the fish that spawned. Hatch is the number of clutches that hatched. If the combination was not implemented it is marked with a dash. If no spawned eggs hatched it is marked as zero (0) whereas if there were no spawnings it is marked with a dash (-). Figures in italics indicate nest building, spawning and hatching of male immigrants into non-native salinity. Figures in bold indicate spawning and hatching of female immigrants into non-native salinity.

Table 2. Measures of immigrant inviability (II) including immigrant reproductive dysfunction (IRD).

	Brackish water: mean (EMM); 95% Wald CI			Marine water: mean (EMM); 95% Wald CI			
Measure	Kristineberg immigrant	Härnösand resident	Västervik resident	Härnösand immigrant	Västervik immigrant	Kristineberg resident	Significant II/IRD
Growth (mm)	1.4; 0.7-2.1	1.7; 0.9-2.4	0.9; 0.2-1.6	1.7; 0.6-2.8	0.4; -0.6-1.3	0.8; 0.3-1.4	no
Condition (g)	1.3; 1.2-1.5	1.2; 1.1-1.3	1.1; 1.0-1.3	1.2; 1.0-1.3	1.1; 1.0-1.2	1.3; 1.2-1.4	no
Female IRD (proportion)	0.42; 0.17-0.74[a]	n/a	0.74; 0.53-0.88[ab]	n/a	0.59; 0.25-0.86[a]	0.60; 0.31-0.84[a]	no
Male IRD (proportion)	0.12; 0.04-0.30	0.56; 0.18-0.88	0.55; 0.30-0.78	0.00; 0.00-0.00	0.00; 0.00-0.00	0.67; 0.39-0.87	**yes**
Sperm motility (proportion)	0.014;0.007-0.025	0.143; 0.089-0.208	n/a	0.021; 0.003-0.055	n/a	0.083; 0.062-0.105	**yes**

We report the GzLM models estimated marginal means (EMM) and 95% Wald confidence interval (CI). Growth is reported in mm and fixed at original length (L_t) 52 mm. Condition is reported in weight (g) with length fixed at 55^{3}mm. Sperm motility proportion is back transformed EMM and CI based on arcsine square root transformed data. See text for statistical calculations.

[a] EMM extracted for females spawning with resident males

[b] Brackish residents i.e. Härnösand and Västervik pooled in the model.

FIGURE LEGENDS

Figure 1. Sample localities and salinity-gradients in the North Atlantic – Baltic Sea region. Salinity data is from Leppäranta and Myrberg (2009).

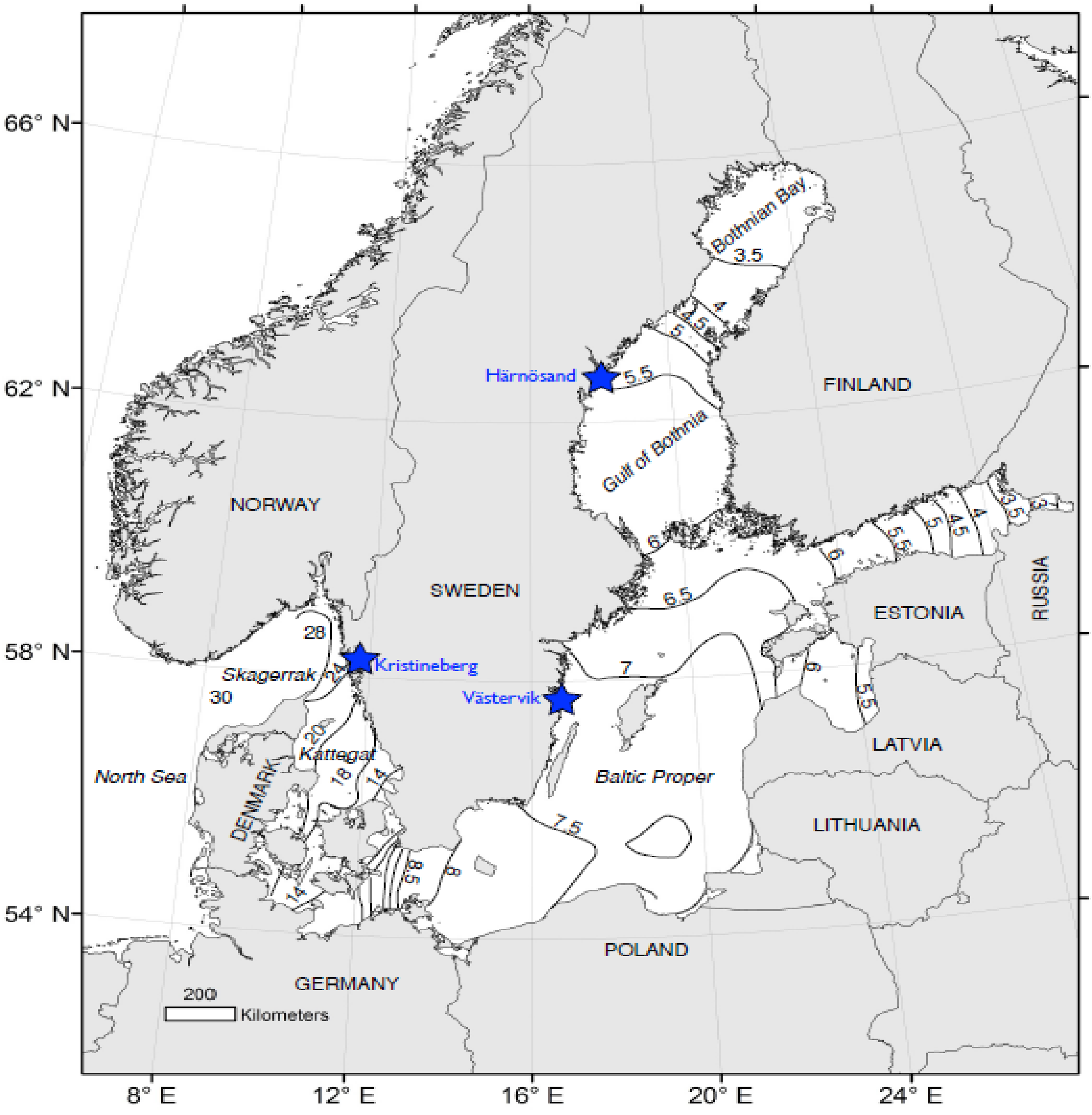

Fig. 2. Proportional hatching success between immigrant and resident sand gobies, *Pomatoschistus minutus*. In the figure we have extracted the data where males were spawning as immigrants in non-native salinity with a resident female native to that salinity and *vice versa*. The boxes show the medians, and the 25th and 75th percentiles and the whiskers the largest and the smallest values of the raw data. Immigrants are shown in red open boxes (immigrant Västervik and Härnösand males into marine water have no boxes because no clutch hatched) and the residents with which they are compared, are shown in filled green boxes. The boxes for clutches where both sexes spawned as residents are identical for males and females (it is the same clutches) and represent the resident populations. N.B. The statistical calculations were performed on the complete dataset that is also containing spawnings in which both sexes were immigrants and not this truncated dataset (see methods and result).