

Reproductive traits in euryhaline gobies
insights into physiology, adaptations and biological invasions

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THESIS ABSTRACT

Organisms are adapted to reproduce in their specific environment by a range of different traits. For many animals, reproduction is tied to specific conditions, that trigger or enable the reproductive events. This thesis deals with traits associated with reproduction, their functions and interactions with the environment. Some traits are plastic, and can change in an individual's lifetime depending on the environment, while other traits are more or less fixed. The variation in trait expression will enable selection when environments change, as some individuals will survive and reproduce to a larger degree. For the gobies, a large family of fishes (ca. 2000 species) found in a wide range of different salinities, reproduction can be affected by the environment. Since their sperm and eggs meet to fertilize externally, surrounded by water, sperm need to be able to swim. The salinity environment can limit this process. However, of all the environmental conditions that can affect reproduction, salinity variation seems to be one that they are highly tolerant towards. This is unusual, since most fishes are adapted to reproduce in a very narrow salinity range. Studying what different environmental adaptations gobies have in their reproductive traits can help us to better understand the eco-evolutionary processes they partake in. For example, the round goby (*Neogobius melanostomus*), is a non-native species in many regions of the world. It is important to understand what limits their reproduction, and if they can adapt to cope with drastically new environmental conditions. Another common species, the sand goby (*Pomatoschistus minutus*), is well studied, but more knowledge on their reproduction will increase the understanding of this budding model organism.

As round gobies are spreading into different salinities, there is likely strong selection for the ability to reproduce in their new environments. This may lead to evolutionary adaptations that greatly strengthens the species' invasiveness. In the Baltic Sea, round gobies are spreading into both lower and higher salinities. By measuring their sperm velocity in a range of salinities representing the Baltic salinity gradient, I found that males from these different invasion fronts showed different responses to salinity. Also, the more generations a population had spent in their local salinity, the higher their sperm velocity when spawning there. This change in sperm velocity is most likely an adaptive, evolutionary response to natural selection.

To understand if a population can also acclimate and increase their reproductive success in a new salinity, I exposed adult round gobies to novel salinities and enticed them to spawn. Despite a lack of acclimation in their sperm traits, they were still able to reproduce. A key to this could be the finding that, in freshwater, low sperm velocity seems to increase fertilization success, while the opposite is true for brackish conditions. Moreover, round goby eggs grew equally well in low and high salinity, although eggs from Baltic Sea parents had better overall development, regardless of what salinity the eggs were spawned or treated in.

Gobies have a pair of special organs called sperm duct glands, that produce chemical substances contributing to the male's ejaculate. In the marine sand goby, I found that these substances increased their sperm velocity, but they did not help sperm survive. However, when testing these substances on sperm in round gobies from freshwater and brackish water, there was no positive effect on sperm. Importantly, gland contents did not improve poor sperm velocity in freshwater. Round gobies have 'sneaker' males, which reproduce by parasitizing nest-holders for fertilizations. I found that in the sampled freshwater river, these males invested little energy in testes growth, compared to the brackish males. This suggests that the elsewhere naturally occurring 'sneaker' tactics are limited by poor sperm performance and could be rare in the freshwater river that was sampled.

Overall, I show that salinity influences round goby reproduction, and that in some cases they can adapt to salinity conditions, while in others it limits their behaviour and physiology. Similarities between the sand goby and round goby exists, but they likely have different uses for their sperm duct glands. Since round gobies from brackish waters can reproduce in both brackish conditions and freshwater, management should try to limit round goby spread from brackish environments into freshwater.

Keywords: Sperm | Reproduction | Life-history Strategies | Local Adaptation | Fish | Invasion Biology | Salinity

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Arter är begränsade till olika typer av livsmiljöer genom anpassningar till dessa. Vissa arter klarar en större bredd av förhållanden, medan andra har snävare krav. Anpassningarna gäller dels interaktioner med andra arter som lever där, så som rovdjur eller bytesdjur, men även den fysiska miljön. Miljöfaktorer som temperatur eller salthalt kan starkt begränsa vilka arter som finns på en plats. Vi hittar ju till exempel inte precis samma arter i Östersjön som på Västkusten, just på grund av Östersjöns låga salthalt. Till stor del beror begränsningen på att djurens känsligaste livsstadier, till exempel yngel, ägg eller spermier, i regel inte klarar lika stora skillnader i salthalt som vuxna individer. I korthet kan vi säga att det är djurens egenskaper i deras känsligaste stadie som begränsar dem till var de kan leva. Men trots dessa begränsningar finns det vissa arter som kan överleva och föröka sig i en mängd av olika miljöer, eller över tid anpassa sig till nya lokala förhållanden. Världens artrikaste grupp av havsfiskar, smörbultarna (med ca 2000 arter), är sådana djur. Dessa små fiskar (normalt 3–10 cm) är vanliga i kustbanden världen över, där miljövariation är vanligt. Längs Sveriges kuster har vi 14 smörbult-arter, flertalet strikt havslevande men några är toleranta mot, eller lokalt anpassade till, lägre salthalter.

Sveriges senaste smörbult är den invasiva svartmunnade smörbulten (*Neogobius melanostomus*). Den liftade med båttrafik från Svarta Havet till Östersjön i början av 1990-talet, och är klassad med den högsta risken i ArtDatabankens klassificering av främmande arter, eftersom den både har hög invasionspotential och stora ekologiska effekter. Den kan sprida sig både i brackvatten (så som i Östersjön) och i sötvatten och verkar klara stora skillnader i salthalt. Skillnader i salthalt finns inte bara mellan sötvatten och havsvatten, utan också inom Östersjön. I detta innanhav går en gradient ifrån norra delen, med låg salthalt, ner till sydvästra, där vattenutbyte med Atlanten sker och salthalten därför är högre. Svartmunnad smörbult finns längs med hela gradienten upp till en salthalt runt 20‰ i de danska sunden. Eftersom spermiesimhastighet verkar vara en viktig begränsande faktor för flera arters reproduktion i Östersjön så finns det anledning att tro att miljön utövar en stark naturlig selektion på spermier. För att undersöka det så provtog vi fisk ifrån flera platser med låg salthalt och hög salthalt i mellersta och södra Östersjön. Sedan tog vi deras spermier och behandlade spermierna i sju olika salthalter, från 1‰ till 30‰, representativa för Östersjöns salthaltsgradient. Vi jämförde hur snabbt spermier simmade i de olika salthalterna för fisk från hög och låg salthalt. Hanarna ifrån hög salthalt hade den högsta spermiesimhastigheten i 15–20 ‰, dvs samma salthalt som de själva fångades i. Fisken fångad i låg salthalt (ca. 5‰) lever och förökar sig idag i ett område som är en tredjedel så salt som i fiskens ursprungsområde i Svarta Havet (ca. 16–18‰). Hos dessa fiskar simmade spermier snabbare ju fler generationer populationen befunnit sig i just den miljön, vilket tyder på att evolution (dvs genetiska förändringar) genom naturligt urval ligger bakom anpassningen till låg salthalt.

Utöver (och ofta föregående) de långsamma evolutionära anpassningarna, så finns det snabbare processer: Andra fiskarter (bland annat spigg) kan anpassa sina spermier till olika salthalter under dagar eller veckor genom acklimatisering. Svartmunnad smörbult har visat sig vara tolerant mot salthaltsförändringar som vuxen och en viss acklimatisering skulle kanske kunna finnas hos deras spermier också. För att ta reda på det så fångade vi fisk från två floder i Tyskland och två brackvatten-lokaler i södra Östersjön. Vi kors-acklimatiserade sedan hälften av fisken till sötvatten (0‰ salthalt) och hälften till brackvatten (16‰ salthalt) och lät dem sedan försöka para sig i sina nya förhållanden. Vi fann att spermie-simhastigheten inte påverkades av vilken salthalt fisken hade acklimatiserats till, och att de kunde para sig och befrukta ägg i en avsevärt högre eller lägre salthalt. Det stämde också för sötvatten, där spermiesimhastigheten var jämförelsevis låg. När vi analyserade samband mellan spermiesimhastighet och hur stor andel ägg som befruktats, såg vi att i sötvatten så gav låg hastighet en ökad befruktningsgrad. I brackvatten däremot så gav högre hastighet en ökad befruktningsgrad. Slutsatsen är att den låga simhastigheten spermier uppvisar i sötvatten kan vara gynnsam i den miljön och att svartmunnade smörbultar, oberoende av ursprung, är flexibelt anpassade för reproduktion i såväl sötvatten som brackvatten.

En annan smörbult som också lever och reproducerar sig i en mängd olika salthalter är sandstubben (*Pomatoschistus minutus*). Denna fisk finns i stora antal längs våra kuster och är viktig föda för flera av våra matfiskar som jagar dem på sandbotten där de lever. Sandstubben är också viktig som modellorganism inom beteendekologi, inte minst när det kommer till att förstå sexuell selektion och parningsbeteenden. Hanar av smörbultar, inklusive sandstubben, har intill testiklarna små slemblåsor vars innehåll bidrar till hanens ejakulat. Hur slemblåsornas innehåll påverkar spermier hos sandstubben har hittills varit okänt, men vi testade detta experimentellt genom att jämföra spermier som blivit behandlade med eller utan slem ifrån blåsorna. Vi kunde se att spermier som blivit behandlade med slem ökade sin simhastighet med 7.3 %. Slutsatsen är att sandstubbens spermier kan få ökad simförmåga, utan en tydlig kostnad i form av sänkt överlevnad hos spermier (vilket annars är en vanlig avvägning hos spermier). Ökad spermiesimhastighet med hjälp av slemblåsor är rapporterad ifrån avlägsna besläktade smörbultarter, och kan vara ursprunglig för gruppen smörbultar vilket kan ha varit till hjälp när smörbultarter har koloniserat och utvecklats i nya miljöer.

Eftersom även svartmunnad smörbult kan föröka sig i många olika salthalter kanske dess slemblåsor kan vara en nyckel till dess framgång. I tidigare studier provtog vi spermier genom dissektion utan påverkan av slemblåsorna. För att undersöka slemblåsornas effekt fångade vi svartmunnade smörbultar från floden Elbe i Tyskland (0‰ salthalt) och en vik med brackvatten i södra Östersjön (~16‰ salthalt). Vi provtog spermier och filmade dem simmande i tre olika salthalter (0‰, 8‰ och 16‰), med eller utan slem från fiskens blåsor. Med hjälp av hanarnas färg, bestämde vi även vilken av artens två reproduktionsstrategier de tillhörde; dels 'Boägare', mörkfärgade hanar som försvarar ett bo, dels 'Smyghanar', ljusfärgade hanar vars strategi går ut på att smygbefrukta ägg i andra hanars bon. Det visade sig att fisk från såväl floden som den bräckta viken hade spermier som simmade dåligt i sötvatten och innehållet från slemblåsorna hjälpte varken simhastigheten eller spermernas överlevnad. Däremot fanns det tydliga fysiologiska skillnader mellan fisk från floden och den bräckta viken. 'Smyghanar' från Östersjön som saknade bohålor hade mycket större relativ testikelstorlek, vilket är vanligt för hanar som vill smygbefrukta ägg. Smyghanar från floden investerade däremot ingen energi alls i testikeltillväxt, men desto mer i kroppsvikt. Slutsatsen är att den låga spermiesimhastighet i flodens sötvatten begränsar möjligheten att smygbefrukta andras ägg, och att unga hanar då hellre investerar i att växa sig stora så att de en dag själva kan försvara ett bo.

Den här avhandlingen ökar vår förståelse för smörbultarnas reproduktionsfysiologi och ekologi. Vi vet nu mer om sandstubbens slemblåsor och deras påverkan på dess spermier. Vi kan också se att olika salthalter verkar ge olika förutsättningar för vilka reproduktionsstrategier svartmunnad smörbult kan uppvisa. Då förhållandena inte är gynnsamma verkar arten kunna anpassa sig till dem. Dessa egenskaper är troligen viktiga för artens förmåga att sprida sig till och inom nya miljöer. Under tiden för detta projekt så har utbredningen av svartmunnad smörbult ökat längs de svenska kusterna och möjligen har de anpassat sig ytterligare till både lägre och högre salthalter. Utanför Göteborgs hamn har vi funnit dem tillsammans med torsk och sjöstjärnor i en ytterst marin miljö med en salthalt runt 29‰. Vi kan i dagsläget inte utesluta att arten kan föröka sig på den svenska västkusten. Beslut kring förvaltning av svartmunnad smörbult bör ta hänsyn till en möjlighet att arten även lokalt anpassar sig till högre salthalter, men att den redan har förmågan att föröka sig i sötvatten, oberoende av ursprung. Åtgärder för att begränsa spridning till sötvattenslokaler behöver därför inkludera populationer av arten som geografiskt sett kan vara avlägsna. Områden som kan bidra till spridning in i sötvattenslokaler är Göteborgs hamn genom Göta älv och Göta kanal till Väneren, samt Stockholms skärgård genom Stockholms slussar vidare till Mälaren.

Nyckelord: Spermier | Reproduktion | Livshistoria | Lokal anpassning | Fisk | Invasionsbiologi | Salthalt

LIST OF PAPERS

This doctoral thesis is based on the following publications and manuscripts, here after referred to as Papers, and referenced by their roman numerals in the text:

- Paper I: Green, L., Havenhand, J. N., Kvarnemo, C. Evidence of rapid adaptive trait change to local salinity in the sperm of an invasive fish. *Evolutionary Applications*. 2020, 13: 533-544. doi:10.1111/eva.12859
- Paper II: Green, L., Niemax, J., Herrmann, J-P., Temming, A., Behrens, J., Havenhand, J., Leder, E., Kvarnemo, C. Invasive round goby shows robustness to novel salinity conditions during reproduction in a common garden experiment. *Manuscript*.
- Paper III: Green, L., Kvarnemo, C. Sperm duct gland content increases sperm velocity in the sand goby. *Biology Open*. 2019, 8 bio037994. doi:10.1242/bio.037994
- Paper IV: Green, L., Niemax, J., Herrmann, J-P., Temming, A., Kvarnemo, C. Alternative reproductive tactics are associated with sperm performance in invasive round goby from two different salinity environments. *Manuscript in revision in Ecology and Evolution*.

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1.0 INTRODUCTION

1.1 REPRODUCING INSIDE THE COMFORT ZONE

Reproduction forms the basis for life as we know it. If there is one thing all organisms are ultimately evolved to do, it is to reproduce. Since this is the only way genes can spread over generations, selection has favored all adaptations that increase an organism's ability to produce surviving offspring. For some organisms, this means elaborate displays showing off one's potential as a partner (Andersson & Simmons, 2006), while for others it means the capacity to travel hundreds of kilometers to the most suitable spawning habitat (Fleming, 1996). The approaches to increase one's reproductive success are almost as many as there are species, but there are some that are common for most. First off, for sexual reproduction (asexual reproduction is beyond the scope of this thesis), the gametes - the cells responsible for fertilization, i.e. eggs and sperm, must function well. This might sound like a simple prerequisite, and for humans and other animals with internal fertilization, it can be. Inside the body, the fertilization environment can be tightly controlled, with the right temperature, pH and other chemical factors that ensure viable sperm (Birkhead *et al.*, 2009). However, the ancestral metazoan mode of reproduction is external, when fertilization occurs outside the animal body in the surrounding water. Many animals (not most, since insects have internal fertilization) still use this classic model of mixing eggs and sperm in water. Even vertebrates, such as amphibians and fishes, are still prevalently external fertilizers. For these animals, it means that they are dependent on a suitable environment for their eggs and sperm to function. As with adult animals, the environmental span that eggs and sperm can function across has limits. As a consequence, animal gametes are adapted to their fertilization environment (Browne *et al.*, 2015). If that environment changes, the animals or their gametes themselves will have to respond in order to maintain the same reproductive success. The first response to a shifting environment is usually through behavior. If the water becomes too warm during the normal reproductive period, for example, one solution can be to start reproducing earlier in the season (Todd *et al.*, 2011). Another approach would be to migrate to a cooler environment (Fogarty *et al.*, 2017). If these behavioral changes are enough to mitigate the negative effects of the environmental change, the response is likely to stop there. But if the change is too large to be overcome by behavioral responses, or if the behavioral repertoire is insufficient to provide a good enough response, the animal's physiology itself will have to change. This physiological change can occur within an individual in which case it is termed phenotypic plasticity (West-Eberhard, 2003). For example, an individual male might respond to different mating opportunities by adjusting the amount or quality of his ejaculate (Scaggiante *et al.*, 2004). Such plastic responses are in themselves adaptations to past ecological and environmental variability, but if the plasticity is not sufficient, the response that remains is evolution by natural selection. If there is any variation among individuals and their ability to reproduce in this new environmental context, the ones that are most successful will have more offspring, and the less successful will have fewer offspring. If the ability to cope with the new environment is heritable, then over generations, the more successful variants have a higher chance to become more common, and spread their abilities to reproduce to the wider population (Darwin, 1859). Since these now successful individuals carry different genetic material, this change in the frequency of individuals with different abilities inevitably also means a change in gene frequencies. Since gene frequency change is the definition of

evolution, we now have an evolutionary response to the environmental change (see Box A for definitions).

1.2 RESPONDING TO RAPID CHANGE

Now imagine a less subtle change. An organism is taken from its natural habitat and transported 900 km to a completely new environment. Can it hope to survive? Well absolutely, if the differences aren't too great. But even if all the environmental parameters the organism cares about was close enough to home conditions, they still have to find food, avoid getting eaten, and find a partner, in order to actually survive more than a generation. Increasing trade and transportation is predicted to move organisms across the globe at an increasing rate (Levine & D'Antonio, 2003; Hulme, 2009), but the vast majority of these are not expected to survive (Sakai *et al.*, 2001). Instead, only a few make it, and establish themselves in the new environment. These populations might persist for some time and then go extinct, but once in a while, they manage to thrive. When a population thrives and can reproduce unchecked, they can reach density levels where they start having severe ecological effects on the community around them (Elton, 1958; Pejchar & Mooney, 2009; Bellard *et al.*, 2016). This is when we start calling them invasive (see Box B for alternative terms).

It has been debated back and forth whether invasiveness depends on plasticity or adaptation to the new local environment. As with most things debated at length, the answer is likely "both" or even worse – "it depends" (Smith, 2009; Davidson *et al.*, 2011). For example, behavioural plasticity seems to aid the invasive lionfish in colonizing new environments (Cure *et al.*, 2014). Increased plasticity in gene transcription in one of two (closely related) invasive gobies has been proposed to explain the round gobies comparatively higher colonization success (Wellband & Heath, 2017). Plasticity has also facilitated historical colonization and subsequent adaptation of algae into low salinities (Johansson *et al.*, 2017). There is also global evidence of plasticity being beneficial to during species invasions in vascular plants (Davidson *et al.*, 2011). Adaption to the new environment has also been shown to be advantageous for species classified as invaders (Lee, 2002; Maron *et al.*, 2004; Novak, 2007). One example are the introduced Chinook salmon in New Zealand that have rapidly evolved distinct breeding periods in different rivers. This limits gene flow between populations and therefore further increases the rate of local adaptation (Quinn *et al.*, 2001). An organism can of course also be plastic in regards to one trait, while it is adapting through selection on another trait at the same time. To make matters even more complex, the two processes can also interact with each other (West-Eberhard, 2005). The plasticity of a trait can allow it to "dodge" natural selection: if all individuals comfortably accommodate environmental changes, no one individual will survive better than anyone else, and no selection will occur (Oostra *et al.*, 2018). On the other hand, if there is a plastic response in a trait to the environment, this response can over time become fixed, and continuously expressed instead of under certain conditions only (Lande, 2009).

Perhaps it can be debated whether understanding the evolutionary and mechanistic processes behind the success of invaders are important or not, since it doesn't say much about the consequences of a species invasion. Funding agencies might argue that resources are better spent looking for a way to control or eradicate invaders, instead of using them as model organisms in some big, uncontrolled experiment on our native ecosystems. They would have

BOX A: THE DEFINITION OF PLASTICITY AND EVOLUTION

Biological change can occur on all organizational levels, from molecules all the way up to the encompassing biosphere, and across time, from within seconds to millions of years. To better describe what biological change we are referring to, we commonly define the timescale and generally what organizational level the change occurs at.

A change that occurs within one genotype (one individual, or several individuals bearing the same genetic makeup) that produces multiple phenotypes (physical or behavioral expressions) is termed **plasticity**. This plastic response happens within one individual's lifetime. When the new phenotype (or parts of it) is carried over and expressed in the individual's offspring, we call this **trans-generational plasticity**.

If the change instead occurs within the genes of individuals across multiple generations, we call this change **evolution**. There is no requirement that this gene-frequency change is translated into new phenotypes, it is simply enough that the genetic code of the studied population has changed. Evolution is therefore constantly occurring, from one generation to the next, as genes are mixed, and some disappear and others mutate. However, these minute and constant changes called genetic drift rarely have any consequences for the population (other than to maintain standing genetic variation), and because of this, they are rarely acknowledged as real evolutionary change. Commonly, it is only when gene frequency differences become large enough for us to detect phenotypic responses from them that we acknowledge them as contributing to evolution.

a point, but there are several examples of conservation projects where an evolutionary perspective on the study of invasive organisms have been key to the projects' successes. One of the most well-known are the mitigation efforts of cane toads (*Rhinella marina*), in Australia. The cane toads (native to the South and Central Americas) were brought in as pest control to the farmlands in the north-eastern territories during the 1930's and onwards. They soon spread, causing ecological havoc by eating native fauna and poisoning enough predators that they by natural selection evolved toxin resistance and avoidance of toads (Phillips & Shine, 2006). Studies of their spread have shown that there is also selection of toads at the invasion front, where toads with longer legs and bolder behavior will reach spawning pools first and therefore increase the rate of spread (Shine *et al.*, 2011). The ingenious idea of transplanting toads with shorter legs and shy behavior from the core-population to the soon-to-be-invaded pools and ponds, have decreased the rate of invasion by outbreeding (Phillips *et al.*, 2016). The traits of bold behavior and long legs, which are selected for during invasion, are now exchanged for shyness and the ability to stay put in a pond with high toad density (Gruber *et al.*, 2017).

The study of evolutionary phenomena as a way to understand the basis for invasions and their success is also crucial (Mooney & Cleland, 2001; Lee, 2002; Prentis *et al.*, 2008; Moran & Alexander, 2014). Species invasions have been important for the understanding of genetic bottlenecks, where an introduction of few individuals depletes genetic variation (but potentially leaves the survivors with the most suitable genetic make-up) (Frankham, 2005). Related to this is the reproduction of the species. When organisms that have adapted to separate conditions again meet, differently adapted traits can be strong barriers to

reproduction, and will keep these locally adapted lineages apart (Svensson *et al.*, 2017; Garlovsky & Snook, 2018). This in turn can help to reduce outbreeding, where the locally adapted traits are “lost” into the crossing populations, due to lack of selection. Other phenomena related to reproduction can also promote biological invasions. We now know that clonal reproduction, though bad for genetic variation, can be effective if the ancestral line is suited to the invaded habitat (Ardehed *et al.*, 2015). Adjacent to these phenomena we find hybridization as a potential stimulus for invasion potential (Schierenbeck & Ellstrand, 2009). Finally, this thesis provides an example of an invasive species that appears to be responding very quickly to natural selection on reproductive traits, showing signs of ongoing local adaptation to the different environments it is invading. In short, though invasive species are excellent to study as “natural” contemporary evolutionary experiments, there are also many benefits to the control efforts from the understanding of the evolutionary processes that invaders are involved in.

1.3 THE ABIOTIC ENVIRONMENT CAN AFFECT ANIMAL PHYSIOLOGY

The interactions between the physical environment and the ecology of the aquatic (and connected terrestrial) ecosystems create incredibly complex systems. A successful reproduction of a fish is the downstream consequence of a myriad interactions between the environment and ecosystem connected to our focal animal. The environment consists in the form of abiotic, physical structures and processes such as rocks, temperature changes and water-currents, but also in part as a chemical world of oxygen content, pH and salinity. The four most commonly studied abiotic factors affecting aquatic animals are oxygen, temperature, pH and salinity. These are often termed ‘master factors’ by their animal ecophysiology proponents, but it is debatable whether or not several others should also be included among these (light, nutrient concentrations and CO₂ are arguably equally important from a plant-perspective). Historically, these factors have made a lot of sense to study. Oxygen is the basis for most life, temperature affects the rate of chemical interactions and therefore determines the speed of biological processes and pH have been changing dramatically in lakes (and recently oceans) through human activity. As greenhouse gas emissions increase, higher global temperatures, decreasing oxygen contents and increasing ocean acidification are all likely to be even more intensely researched in the future. Lastly, salinity divides the world’s aquatic ecosystems into very distinct zones (with some not-so distinct border areas and gradients between them). The end result of a fish successfully reproducing is determined by the cascading effects of chemical and biological interactions as all of the above-mentioned abiotic factors influence the species in an ecosystem.

1.4 SALINITY DIVIDES THE AQUATIC WORLD

In broad strokes, salinity (the amount of salt dissolved into ions in the water, commonly reported in ‰ i.e. g salt / kg water, comparable to ‘practical salinity units’ (PSU) in oceanography and in this thesis’ papers) divides the world’s aquatic ecosystems into two very different environments: the freshwater environment and the marine environment. The main effect of salinity is not necessarily one of what minerals are available for biological processes, but that the amounts of dissolved ions are different in fresh water and salt water. The amounts of dissolved ions first has an effect on the osmotic gradient, as water will travel from a low concentration of a solution (low osmolality) to a high concentration of a solution (high

osmolality). The second effect the amounts of dissolved ions have is on the ion gradient and the electrical currents associated with it. At a cellular level, these gradients have substantial effect on the movement of molecules in and out of the organism. As a consequence, organisms have adapted to the prevailing conditions in each respective environment. The internal osmolality of a fish is around 300 mOsm/L. In contrast, the oceans have a higher osmolality, one of around 1000 mOsm/L. As a consequence, the osmotic forces move water through all cellular membranes (they cannot keep tight, unfortunately), from the inside of the fish (in particular the blood, where a lot of body fluid is held) to the outside environment. As a consequence, a fish living in the ocean has to drink salt water to stay hydrated! Any barrier between the internal environment of the fish, and the outside environment, could potentially leak and cost the fish water. Reasonably enough, they have adapted to this. Fish leak very little through their skin for example. But some barriers also need to be permeable. The gills, where the fish exchanges gases such as CO₂ and oxygen as well as the intestine, where nutrients are taken up and many salts are excreted, cannot function if they don't allow for some permeability. This is of course a dilemma, especially if you wish to move from one salinity to another. As a consequence, fishes that do move between salinities, change their physiology substantially, especially at the interface of water and ion exchange such as the gills (Yang *et al.*, 2009) and intestine (Sundh *et al.*, 2014; Brijs *et al.*, 2017b). These changes typically occur at the cellular level, in the position and type of ion transporters, or their amount, but flood flow rate and gut movement can also help the fish osmoregulate better in novel salinities (Brijs *et al.*, 2017a; Sundell *et al.*, 2018).

When the fish need to spend energy to compensate for the water lost, or the ions gained by being in water with different osmolality to what they are adapted to, this can have consequences on reproduction. All animals have a limited amount of energy (true, they can acquire more, but still a limited amount). This means that when the animal spends a bit of energy doing something, that energy cannot be used for something else. Commonly we refer to this as the animal's energy budget. As the animal requires energy to actively pump ions over their gills and intestine to maintain an internal ion-balance, less can be spent on other processes. The consequence is a continuous trade-off between all kinds of physiological mechanisms and behavioral activities that the animal can engage in. Finding a mate costs energy, subsequently courting the mate takes more (Magnhagen & Magurran, 2008). Investing in eggs can mean that the fish doesn't have energy to grow as fast as competitors (Quinn *et al.*, 2011).

1.5 THE BALTIC SEA AND THE 1000 KM LONG SALINITY GRADIENT

Where land meets water, and especially freshwater run-off meets the sea, there are salinity gradients. One of the of biggest ones in the world is in the Baltic Sea (Jones *et al.*, 2012; Kusakabe *et al.*, 2017). Here, salinity diminishes as you go east/north-east and the community of organisms also changes accordingly (Snoeijs-Leijonmalm *et al.*, 2016). The Baltic Sea is a young sea with the current brackish phase estimated to be about 4,500 years old (Westman & Sohlenius, 1999). New species are increasingly appearing through anthropogenic means (Leppäkoski *et al.*, 2002). Added to this, a few organisms have speciated or locally adapted into the basin (Reusch *et al.*, 1999; Nissling *et al.*, 2002; Tatarenkov *et al.*, 2005; Berg *et al.*, 2015). Most of the Baltic inhabitants however, are simply North Sea vagrants, or freshwater organisms that tolerate the varying salinities (Snoeijs-Leijonmalm *et al.*, 2016).

1.6 SALINITY AFFECTS EGGS AND SPERM

A fish is a fairly complex organism, with a lot of different physiological functions and adaptations that when combined can overcome many of the issues related to osmolality and ion gradients. However, all organisms need to undergo a single cell stage if they are to reproduce sexually. For fish this means that they need to enter the stage of single cell life as a haploid gamete with a single copy of DNA (as opposed to the common diploid cell, with a double DNA copy). This is a very different life stage compared to a multi cellular adult (or even a juvenile or a zygote) life.

Spermatozoa, or sperm cells, are produced in a specialized tissue called testes. In the testes, cellular division of diploid cells results in haploid cells. This occurs in two meiotic cycles eventually forming four haploid spermatozoa from one diploid spermatocyte (White-Cooper *et al.*, 2009). These spermatozoa then go through a phase of development, and in many organisms develop flagella to facilitate movement (Pitnick *et al.*, 2009). In theory, this stage of single cell life is vulnerable. While a multicellular organism has many layers of cells with some redundancy in function, as well as energy reserves to last them many days (if not much longer), a single cell has little of this. Eggs overcome some of these problems through very tight membranes (Alderdice, 1988) and comparatively large energy reserves, but they also bring these to enable the embryo to grow inside the egg (Kamler, 2005). A sperm on the other hand, before it has fertilized the egg, is in essence a small DNA-vector dependent on rapid transport to the safer environment provided by the egg membrane. Sperm are very simple cells and have few organelles (Lessells *et al.*, 2009). A ring of mitochondria provides the energy synthesis for flagellar motion (Cummins, 2009) but apart from this, other organelles are adaptations used for fertilization: an acrosome sheath that carries enzymes aiding in the breakdown of the egg cell membrane to enable the sperm nucleus access to the intracellular space of the egg, and two centrioles provide structures for chromatin arrangement during fertilization (Pitnick *et al.*, 2009).

To increase the difficulty of reaching the safe zone that egg provides, viscosity of water (or any other fluid the sperm swims in) at this microscopic level makes the spermatozoa's world the equivalent of syrup, slowing movement and costing substantial energy to overcome. Fish sperm are adapted to the salinity environment in which fertilization takes place (Browne *et al.*, 2015). This can make sperm sensitive to different environments than the one they are adapted to and though their life expectancy is short, their function during fertilization can be impaired if they are ejaculated into a non-favourable environment (Alavi & Cosson, 2005, 2006; Svensson *et al.*, 2017). The internal osmolality of the sperm cell, just as with the fish, is around 300 mOsm/L, and because of this they too experience the ion gradient imposed by the water medium. In line with the ingenuity that natural selection often results in, sperm have not only adapted to tolerate it, but to use it to kick start their locomotion. The sperm flagellum is in essence a long cell membrane shaped like a tail. Flagellar movement in the spermatozoa is a circular spiralling motion triggered by an action potential originating from the osmotic difference between the intracellular and extracellular environment (Cummins, 2009). This spiralling motion continues to be powered by the mitochondria (cellular power plants) inside the sperm until all the energy is used up, some mechanical damage occurs to the cell, or (extremely improbable) the sperm reaches and fertilizes an egg.

So, sperm movement is powered by the difference in osmolality between the inside of the sperm and the outside environment. Is this true for all fish species? Not necessarily. Some fishes' sperm are activated by substances in the ejaculate or by a specific ion with its own

concentration and gradient, such as K^+ (Alavi & Cosson, 2005, 2006; Browne *et al.*, 2015). What about species that live in an environment that has the same osmolality as the fish itself? This is not very well studied, as few fish are adapted to spawn in brackish conditions, but there are some examples below.

1.7 SPERM CAN COME UNDER SELECTION

Organisms are generally believed to be adapted to their environments, as they would otherwise decline over time (Hendry, 2016). This is further supported by classic studies on local adaptation in isolated populations and/or during periods of fast evolutionary processes as environments change dramatically (Grant & Grant, 2006). Natural selection due to environmental change can be strong, and adaptation to it can be fast, providing there is enough standing genetic variation in a population to allow for a response to selection while maintaining survival (Barrett & Hendry, 2012). As gametes are sensitive and fertilization is important, we can expect strong selection on gametes to adapt to local environmental conditions as well. Local adaptation to abiotic conditions has been found in gametes, for example in the eggs of several aquatic species (Nissling & Westin, 1997; Berg *et al.*, 2015; Momigliano *et al.*, 2017). In Baltic Sea flatfishes, local adaptation in reproductive traits are a fundamental base for population structure (Florin & Höglund, 2008). Also, eggs have been found to be under stronger selection than sperm, since egg buoyancy for some of these species is crucial for the survival of the zygote (Nissling & Westin, 1997). However, a big body of work has been done to examine the role of sperm during post-mating pre-zygotic isolation (Howard *et al.*, 2009; Garlovsky & Snook, 2018), in which they arguably play a role during local adaptation and speciation. This work often focuses on the species barriers themselves, and not on the differences in sperm adaptations due to environmental selection. When it comes to sperm adaptation, the research focus so far has mostly been conducted in the context of sexual selection (Pizzari & Parker, 2009). In these studies, sperm have been shown to adapt to local socio-ecological conditions, such as differences in sperm competition (LaMunyon & Ward, 2002) and female physiology (Pitnick *et al.*, 2003). Sperm and seminal proteins have been shown to be under rapid evolution (Torgerson *et al.*, 2002; Clark & Swanson, 2005; Breckels & Neff, 2014). This further points to sperm being able to readily adapt in response to selection pressures, but it has rarely been studied in the context of environmental adaptation (Breckels & Neff, 2014).

Since sperm are environmentally sensitive, many organisms have evolved ways to shield or boost sperm during fertilization. Most organisms produce an ejaculate that consists of more than just sperm. As sperm motility can decrease with density of sperm cells, diluting sperm numbers can increase their velocity and fertilization success (by avoiding polyspermy), especially for internal fertilizers. The seminal fluid can also provide energy to the sperm, and ATP content is usually high (Poiani, 2006). Even adaptations to parasitize on rivaling males' ejaculate contents have been found (Locatello *et al.*, 2013). Females can also shield sperm from environmental effects in the external environment. Stickleback females produce ovarian fluid that protects the sperm and enables them to fertilize eggs even in environments where sperm motility is severely affected (Elofsson *et al.*, 2003). Some fish embed sperm in mucous substances, possibly to shield them from a challenging environment (and free up the male from timing his ejaculation to the females egg-laying) (Scaggiante *et al.*, 1999). Adaptations that shield sperm from environmental effects can in theory enable organisms to colonize new

regions (Elofsson *et al.*, 2006), but as previously discussed, such plasticity could potentially dampen selection (West-Eberhard, 2003).

Sperm are often studied in the context of sexual selection and mainly sperm competition (Birkhead & Møller, 1998). As males compete for fertilization attempts, the male with the highest number of fertilizations is likely to gain higher fitness than its competitors. Selection on for example sperm velocity (Locatello *et al.*, 2013; Pizzari, 2017), longevity (Dziminski *et al.*, 2009) and complex interactions between them (Fitzpatrick *et al.*, 2012; Bartlett *et al.*, 2017) are therefore common in systems where you have male-male competition. Other reproductive traits such as body size (since it is directly affecting fecundity in many organisms), investment into reproductive tissue, and parental care (Tallamy, 2000; Clutton-Brock, 2007; Lindström & St. Mary, 2008) can also be under strong sexual (as well as natural) selection. The interplay between sexual selection and the environment can also be complex. For example, high energetic costs in a particular environment can limit how much effort individuals can put into reproductive traits and therefore limit how strong sexual selection can be in a population (Locatello *et al.*, 2006; Rasotto *et al.*, 2010).

1.8 ENTER THE ROUND GOBY

So far, we have touched upon a number of phenomena: (1) organisms move around the globe by help from humans, (2) when they do so, they experience new environments, (3) to establish themselves in this new environment the ability to reproduce is key, and (4) there are evolutionary processes at work that can help promote their reproduction in a novel environment. This is true also for the focal species of this thesis, the round goby.

The round goby is a small, inconspicuous, bottom-dwelling fish that has had a huge success as an invader. The species was first described from the Black Sea in 1811 by the Prussian zoologist Peter Simon Pallas by the scientific name of *Neogobius melanostomus* (Pallas, 1811). Despite a brilliant mind (he got his doctors degree at the age of 19!), he erroneously divided the species into several different ones, creating a slew of synonyms and a bit of extra work for later researchers: the species phylogenetic placement (historical relationship to close relatives), as with many other organisms, has been debated and rearranged over time (Stepien & Tumeo, 2006). As a consequence, the name changed during a period to *Apollonia melanostomus* (sometimes *A. melanostoma*) and scientific literature between 2006-2009 often cites the species by these names. At the writing of this thesis, the species resides in *Neogobius* (Genus), *Gobiinae* (Subfamily), *Gobiidae* (Family), *Gobioidei* (Suborder), *Perciformes* (Order). For the layman, Perciformes is the order comprising all “classic fish” that we commonly encounter in our daily lives. Classic food fish such as perch, cod and herring and known aquarium fishes such as cichlids, neon-tetras and zebrafish all belong to Perciformes. The species has recently been divided into two subspecies, one from the Black Sea (and adjacent areas) and the other from the Caspian Sea (both subspecies are invasive) (Brown & Stepien, 2008). It belongs to a clade of Ponto-Caspian gobies, many of which have adapted to both brackish and freshwater environments and as a result, have the potential to survive (and reproduce) in very different conditions. Consequently, many species in this clade are invasive (Stepien & Tumeo, 2006).

The species’ morphology is indicative of its benthic (bottom dwelling) lifestyle: it is relatively small in size (maximum reported ~ 210 mm, though rumors of bigger fish abound), and like all gobies it lacks a swim-bladder, eyes are positioned dorsally, and the pelvic fins are fused into a structure reminiscent of a suction cup. Pigmentation is based on small brown to


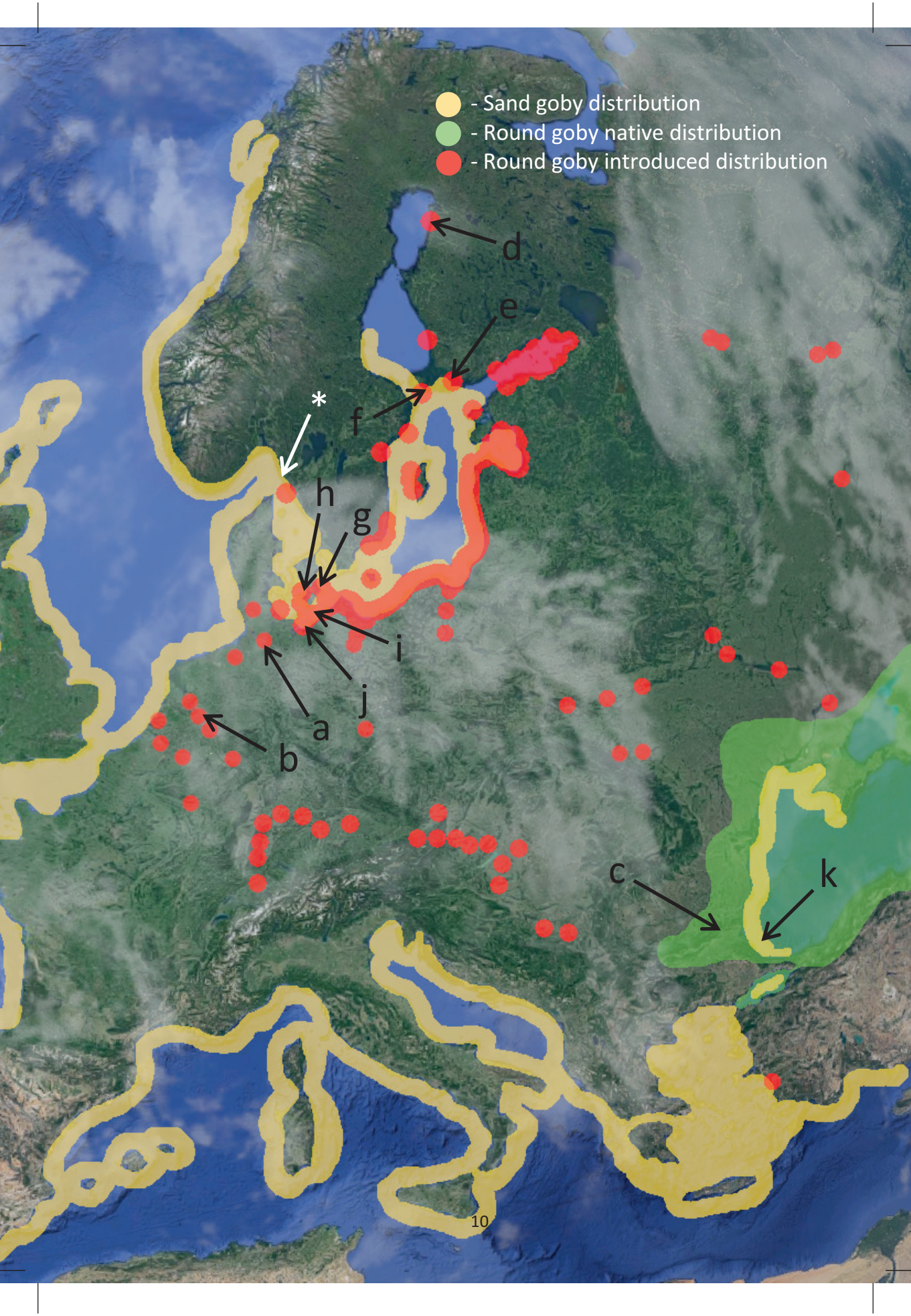


Figure 1. The ranges of the sand goby (*Pomatoschistus minutus*, including a possible cryptic subspecies in the Mediterranean) and the round goby (*Neogobius melanostomus*) in Europe where they occur in habitats suitable for the species. Yellow fields show the sand goby's range, green fields show the round goby's native range, red fields show the round goby's introduced range. The white arrow and star points to where specimens for Paper III was sampled. Letters refer to sampling sites for round gobies corresponding to panels in Figure 12 on page 35 and order is therefore based on salinity of each sampled site: a) River Elbe from Paper II, b) River Rhine from Paper II, c) River Danube, (included in the discussion for comparison; otherwise not part of this thesis), ancestral region, d) Raahe, low salinity invasion front in the Baltic Sea from Paper I, e) Turku, low salinity invasion front in the Baltic Sea from Paper I, f) Mariehamn, low salinity invasion front in the Baltic Sea from Paper I, g) Kindvig, high salinity invasion front in the Baltic Sea from Paper I, h) Karrebæksminde, high salinity invasion front in Baltic Sea from Paper I, i) Guldborgsund, brackish site in Baltic Sea from Paper II, j) Travemünde, brackish site in the Baltic Sea from Paper II, k) Sozopol, brackish site in the Black Sea (included in the discussion for comparison; otherwise not part of this thesis), ancestral region. Ranges and reports are synthesized from Fishbase, accessed February 2020, Kornis et al. 2012, Buřič et al. 2015, Green et al. 2020.

- - Sand goby distribution
- - Round goby native distribution
- - Round goby introduced distribution



BOX B: WHAT IS AN INVADER?

Invasive species have been studied for a long time, and throughout different regions and subfields of biology. As a consequence, the nomenclature for describing an invasive species is not consistent throughout the literature or in contemporary discussions. Exotic species, alien species, introduced species, non-native species are all terms essentially describing the same phenomena.

Non-indigenous species (NIS) is a commonly used term to describe species or populations introduced outside of their historical or present natural range. This term doesn't include any definition of ecosystem consequences as a result of the species introduction.

Another term defining the invasiveness of an organism is **invasive alien species (IAS)**. This term is commonly used for species that have an impact on biodiversity, ecosystem functioning, human health or socioeconomic values where they are introduced. There is no consensus on a definition of the degree of impact, or the direction of impact from an IAS. If an introduced species has a population boom, and large negative economic impacts, say by affecting pine survival in a boreal pine plantation, but increases biodiversity because of increase in dead-wood in the region, is it still deemed as invasive? In reality, this only becomes a problem for organizations in need of categorizing species as non-indigenous and/or invasive. For most scenarios, this is likely very species and context specific. In some regions, species can be introduced but have little impact, and in others, their populations might rapidly expand and the ecosystem around them will be affected.

Throughout this thesis and my papers, I use the term **invasive species** when there are known ecological impacts from the introduction, regardless of their strength. I use the term **introduced species** when ecological impacts are unknown (sometimes due to lack of studies).

grey spots with a few large darker lateral markings, together with a distinct eye-like spot at the posterior part of the anterior dorsal fin. The species often flashes this spot by raising and lowering its fin, for communication and potentially predator avoidance (Kjernsmo *et al.*, 2016). This kind of mark or spot is very common among gobies (evidently also present on the sand goby) (and also other small marine fishes such as juvenile damsel- and angelfishes) but their adaptive significance has been sparsely researched (Kjernsmo *et al.*, 2016). The round goby also has the ability to change color to match the background. Gobies commonly have melanophore structures that allow for some control over how much color is showing on the skin. This ability has not been researched in the species, but in other fishes it is a known adaptive camouflage that decreases predation risk (Nilsson Sköld *et al.*, 2010, 2013). The species' sexes differ in appearance. Morphologically, males are on average larger than females, and their genital papilla are pointed and triangular, as opposed to females that have blunt genital papilla (Kornis *et al.*, 2012). During the reproductive season, large males (with a nest) take on a dark, sometimes inky black, coloration which contrasts well to a bright, almost fluorescent edging on all fins. They also grow proportionally larger cheeks and as a consequence – head morphology changes slightly (Bleeker *et al.*, 2017).

The round goby feeds on a range of benthic invertebrates and smaller fishes (juveniles and larvae) (see below for a summary of ecological effects). The species has been described as a mussel-expert, but experimental trials and stomach sampling from the wild have shown the species to not prefer mussels over other invertebrate prey (Nurkse *et al.*, 2016). In the Black Sea, the species is regularly fished around mussel farms (Apostolos Apostolou, personal communication), and large round gobies caught in the region have been observed to defecate whole (>35 mm total length) mytilid mussel shells (Leon Green, personal observation). Their broad diet probably enables them to easily find food when colonizing new regions and but also facilitate wide ranging effects on the ecosystem they colonize (see below for details on ecological effects).

Growth in the round goby, as with all ectothermic fish, is likely very much dependent on temperature, osmoregulatory costs and food availability. Males are larger than females except during the first year and the sexes therefore have different growth trajectories (excluding sneaker males, more on this later). The species can reach an age of 6 years (reported from the invaded Gdynia in the southern Baltic) (Sokołowska & Fey, 2011). There has not been any study examining if regional temperature or food availability can be a source for differences in size at a given age between populations. Theory does however predict this to be the case. Salinity on the other hand has been proposed to affect individual growth patterns due to its consequences for osmoregulatory costs (Kornis *et al.*, 2012; Behrens *et al.*, 2017). Osmolality in the southern Baltic is close to the internal osmolality of the round goby, and therefore a very “cheap” environment to osmoregulate in. In contrast fresh water has close to 0 osmolality. Round gobies from brackish waters have been shown to have larger size on average compared to fish in fresh water (Kornis *et al.*, 2012). Age at sexual maturity has also been estimated to be one year lower in the fresh water Great Lakes region compared to the native range (MacInnis & Corkum, 2000), though there are no theories as to why (Kornis *et al.*, 2012).

1.9 THE ROUND GOBY NEST

The round goby spawning period starts in the spring, when food, temperature and light cues from the environment trigger physiological changes in both males and females (MacInnis & Corkum, 2000). Like many other demersal (bottom associated) and benthic (bottom dwelling) fishes, the round goby attaches its eggs to a substrate. For gobies, this is typically done in a nest under a rock or similar hard surface, in which the male will guard and care for the eggs for several weeks, if not months (Meunier *et al.*, 2009). Nest sites are competed for by males, and male nest-guarding behaviour has been found to have cascade effects on physiology: growth of puffer cheeks and colour change from a mottled brown morph to the previously mentioned dark morph (Somerville *et al.*, 2019). Males court females both vocally (Rollo *et al.*, 2007) and through visual display (Meunier *et al.*, 2009). As the spawning starts, the male will coat the nest with a substance from his sperm duct glands, specialized organs adjacent to the testes (see Figure 3). In other species, this substance contains mucins (Scaggiante *et al.*, 1999), proteins (Young & Fox, 1937) and ATP (Locatello *et al.*, 2007), and most likely a few sperm (Marconato *et al.*, 1996; Scaggiante *et al.*, 1999). The female follows the male, slowly placing her eggs onto the mucus coated substrate. In some species of goby, the spawning lasts an hour or longer (Marconato *et al.*, 1996; Meunier *et al.*, 2009). As the female continues to place her eggs, the male shifts between ejaculating his sperm over them, and guarding the nest from parasitic spawners. Some of these parasitically spawning males lack a nest because

of their inability to compete due to small size, but in other goby species, nest holding males also sneak (Singer *et al.*, 2006), which could also be the case for round gobies. A male exposed to sneaking will lose some of the fertilizations to other males (and likely spend energy protecting and raising his rivals' young!). The benefits for a male engaging in sneaking are high enough to make these tactics common in the animal kingdom, and especially among demersally spawning fish (Taborsky, 1998). In the round goby, there is evidence for specialized sneaker male morphs, with body weights of around 30 percent of an average nest holding male and a proportional testes investment that can be close to three times that of a nest holding male (Marentette *et al.*, 2009). Round gobies have male care, meaning that the male will stay in close vicinity to the nest, protecting the eggs from predators, fanning them to provide enough oxygen for their growth, and remove dying or sickly eggs (often by eating them) to ensure the survival of the rest of the clutch. A successful male can keep at this process throughout the summer. He will sequentially court many females and keep their eggs (often developing at different stages) safe in his nest. Studies have shown that (sand) goby males with eggs are more attractive to females than those without (Forsgren *et al.*, 1996) and this could be true for round goby as well. The largest amount of eggs from any one nest recorded in the literature is around 10,000 (Charlebois *et al.*, 1997). This is most likely an effect of several females spawning in the nest of a (very successful) male. The species' eggs are oval in shape and about 3.9 mm long with a diameter of 2.2 mm. As the eggs develop over 19 to 145 days (depending on temperature, Jan Niemax, *personal communication*), they will eventually hatch and swim out of the nest and into the watery world outside. Compared to many other gobies, round goby young take a long time to develop and hatch at a very developed stage. They are about 5 mm long, have fully functioning digestive system, pigmented eyes and have completely absorbed their yolk sacs. They also have fully formed fins and a completely flexed urostyle (caudal fin base composed from the skeleton) and comfortably change between resting on the bottom (just like adults do) and swimming in the water column (personal observation). In essence they hatch as juvenile fish rather than as larvae, ready to start feeding from the first or second day.

1.10 THE SAND GOBY – A COMPARABLE EURYHALINE FISH MODEL

Gobiidae is an enormous family, with 1850 species divided into 258 genera (<http://www.fishbase.se>, March 2020), making them the largest teleost family of marine origin presently known. It is estimated that there are more than 2000 species globally, and many undescribed species are still being found and described every year (Thacker, 2015). As an example, since writing my licentiate thesis in October 2017, the fishbase Gobiidae list has grown by 79 species! Gobies occur in marine, brackish and freshwater ecosystems and the greatest species count can by far be found in the tropics (Patzner *et al.*, 2011; Thacker, 2015). That said, they are abundant in temperate climates as well, and Sweden alone has 14 species, making it the most species rich teleost family also in this region (Kullander *et al.*, 2012).

The sand goby (*Pomatoschistus minutus*, Pallas) is one of the most commonly studied gobies in the world (a search on Web of Science yields over 300 publications at the time of writing). Most of the research conducted on the species concerns its reproduction, both behaviour and physiology, as they readily accept captive conditions and are easy to mate in aquaria (Forsgren, 1999; Lehtonen & Lindström, 2007; Svensson *et al.*, 2017). The fish is

BOX D: ANTHROPOGENIC VECTORS OF SPREAD

There have been several studies trying to assess how round gobies are transported. Besides models of the potential introduction when using round gobies as bait-fish (Drake & Mandrak, 2014), the two main theories are that round gobies spread during adult or juvenile stages with big ships in ballast tanks, or alternatively as eggs attached to the hulls of ships. Studies have shown that the occurrence of round gobies is strongly associated with shipping intensity, and the species is typically found in harbours, which support these theories (Kotta *et al.*, 2016).

A study of small recreational crafts as vectors for egg transport proved that round goby eggs are able to remain attached and survive several hours out of water (Hirsch *et al.*, 2016). There is currently no direct evidence for egg transport (Adrian-Kalchhauser *et al.*, 2017a), but in theory, sea-chests (an intake water reservoir in the hull of a ship) could form good environments for nesting.

Ballast tank transport is the other, perhaps more likely vector of transport. There are reports of many different aquatic organisms that have been found in ballast water, and subsequently introduced in the area where the ship has discharged their tanks (or the other way around). In a synthesis from 2002, Marjorie Wonham and co-authors found gobies to be the most common family of fishes reported from ballast tanks. Other small fishes such as blennies (Blenniidae) were also commonly found (Wonham *et al.*, 2000) and these are also known to sometimes become invasive (Gerhardinger *et al.*, 2006). However, adult round gobies are benthic and are not reported to venture up into the water column. Juveniles on the other hand have been found to exhibit some vertical migration (Hensler & Jude, 2007), and perhaps use this as a way to travel in currents meters above the sea or river bed (Jost *et al.*, 2016). This could in theory increase the risk of a ship acquiring them in its ballast water.

There have been experimental studies investigating the effects of rapid salinity exposure on adults and juveniles, but not on larvae. In an effort to simulate a ballast discharge filled with round goby and their subsequent survival as the salinity abruptly changes Karsiotis and co-authors shock-exposed (and step-wise drip-acclimated) freshwater round goby to higher salinities (5-35 ‰), and measured their survival. Only acute and step-wise exposure to salinities above 20 ‰ had strong effects on short-term survival (Karsiotis *et al.*, 2012), which shows that the species likely survives ballast discharge provided the novel salinity is 20 ‰ or below.

extremely abundant along the coastlines of the North Sea and Baltic Sea, where they form benthic aggregations and communities in their shallow sandy or muddy habitat (Rajasilta *et al.*, 1999; Kullander *et al.*, 2012). During the reproductive period (late April to late June), the largest sand goby males develop mating coloration (Figure 1) and establish nests that they court females to spawn in (Forsgren, 1999; Blom *et al.*, 2016), much like the round goby. Sand gobies also have sperm duct glands (Kvarnemo *et al.*, 2010), and males attach mucus trails to the nest substrate prior to spawning (Svensson & Kvarnemo, 2005, 2007), although the presence of sperm in those trails has not been fully investigated. Depending on body size and food availability, a female will lay about 1000-3000 pear-shaped eggs of about 1 mm length



Figure 2a. Illustration of a nest holding (dark morph) round goby (*Neogobius melanostomus* Pallas) male. Note that colouration can vary substantially and image is illustrated to show features of the animal rather than accurate patterns. Notes: spot on first dorsal fin is barely visible in dark morph, but pigmentation is still there (on light male morphs, females and juveniles, the spot is striking and ringed by a white area, very similar to the sand goby below). The fins are pigmented rather than translucent, often grey or dark grey to black. Note also the lighter trim on the second dorsal fin, anal fin and caudal fin. Sometimes also visible on the pectoral fins. This trim varies in presence and is at its most pronounced almost pale blue and iridescent. Mottled patterns of light brown can still be visible in some individuals. Pigmentation often appears as spots or blotches under stereoscope. Ventral colouration often remains lighter than the rest of the body. The fused pelvic fins are often light grey and lightly pigmented. Cheeks are puffy and pronounced in larger males. In this image, gills are visible behind the gill-covers, but this is not always the case. Image to scale. Illustration by Leon Green (thesis author).

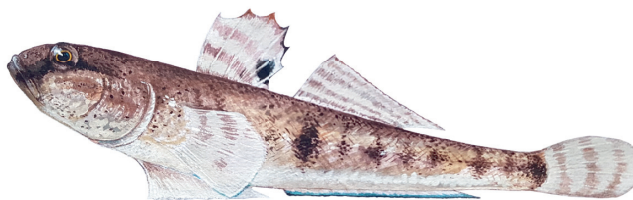


Figure 2b. Illustration of a nest holding sand goby (*Pomatoschistus minutus* L.) male. Colouration accurate. Notes: Pigmentation mainly on main body, with numerous small spots of grey to brown colour. Patterned 'marks' along the lateral sides. Fins mainly translucent with light brown or grey patterning. Anal fin is strikingly coloured with an iridescent turquoise, which is hinted at when the fin is folded as in the drawing. The black spot on the first dorsal fin also has a subtle iridescence. This dark spot is often very small in females. A dark line runs through the eye down to the mouth. Image to scale. Illustration by Leon Green (thesis author).



Figure 2c. Examples of habitats where round gobies were sampled. Top panel shows site h) Karrebæksminde, high salinity invasion front in Baltic Sea from Paper I. Bottom panel shows site a) River Elbe from Paper II and IV. Round goby is strongly associated with shipping traffic and anthropogenically disturbed habitats, but they do occur in natural areas also.

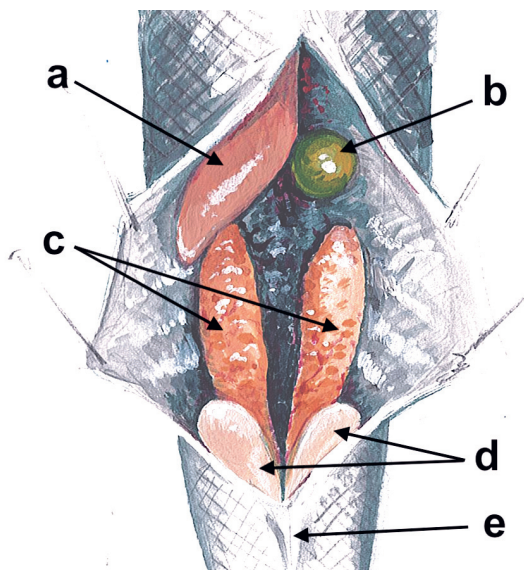


Figure 3. Schematic of a dissection of a goby, with the gut removed and head towards the top of the drawing. Several organs are prominent and indicated by letters: a) Liver, an organ with various functions including fat storage, b) gallbladder, a storage organ for bile, used during digestion, often very visible during dissection of gobies, c) testes, where sperm are produced and stored, d) sperm duct glands (SDGs), also called accessory glands, that likely produces and/or stores mucins, adenosine triphosphate (ATP) and proteins, and e) genital papilla from where sperm and accessory fluids are ejaculated. The testes and SDGs are connected to the genital tract (not shown in drawing) that exits through the genital papilla.

(Breder & Rosen, 1966). Alternative reproductive tactics have been estimated at 10% of the population (Kvarnemo *et al.*, 2010). 50% of all nests contain some parasitically fertilized eggs and nest holding males also parasitize the nearby nests (Jones *et al.*, 2001; Singer *et al.*, 2006). Eggs develop demersally during 5-20 days depending on temperature and larvae hatch at 3 mm total length (Fonds & Van Buurt, 1974; Kvarnemo, 1994). Larvae have a short (days) yolk-dependent stage, and then start feeding in the plankton community. Settlement occurs after 4 weeks of pelagic life and growth into adult size occurs over the season for the fish to mature the next year (Kullander *et al.*, 2012). Sand gobies rarely live more than a year, and often die after the first reproductive season, though a small percentage of the population can live and reproduce for a second year (Healey, 1971).

1.11 ECOLOGICAL CONSEQUENCES OF GOBY INVASIONS

Round gobies interact with many species, not only as competitors, but also as predators and prey. Since the round goby is comparatively aggressive, it often displaces fish species with similar niches or life-styles from their preferred habitats. Sculpins of several different species have been shown to be at risk of displacement from their shelters (Jude *et al.*, 1995; Dubs & Corkum, 1996). In the American Great Lakes several other species are also negatively affected by round goby establishment, such as darters (*Etheostoma nigrum*, *E. caeruleum*) (Jude *et al.*, 1995; Krakowiak & Pennuto, 2008), logperch (*Percina spp.*) (Balshine *et al.*, 2005) and yellow perch (*Perca flavescens*) (Duncan *et al.*, 2011). In the Baltic Sea, the examples include flounder (*P. flesus*) (Karlson *et al.*, 2007), eelpout (*Zoarces viviparous*), eel (*Anguilla anguilla*), perch (*Perca fluviatilis*), roach (*Rutilus rutilus*) and vimba bream (*Vimba vimba*) (Skora & Rzeznik, 2001). Even birds are outcompeted, as have recently been evidenced in the decline of long-tailed duck (*Clangula hyemalis*) (Skabeikis *et al.*, 2019). Most of these interactions are suspected from dietary and habitat overlap, but direct evidence from experiments also exists (MacInnis & Corkum, 2000; French & Jude, 2001).

Round gobies are prey to many other animals. In the Great Lakes, freshwater drum (*Aplodinotus grunniens*), white perch (*Morone americana*), brown trout (*Salmo trutta*) (Campbell *et al.*, 2009), lake trout (*Salvelinus namaycush*) (Dietrich *et al.*, 2006), rock bass (*Ambloplites rupestris*, *A. constellatus*) (Jude *et al.*, 1995), smallmouth bass (*Micropterus dolomieu*), walleye (*Sander vitreus*), white bass (*Morone chrysops*), yellow perch (*Perca flavescens*) (Johnson *et al.*, 2005), sauger (*Sander canadensis*) and pike (*Esox lucius*) all feed to some extent on round goby (Reyjol *et al.*, 2010). The tubenose goby (*Protherhinus semilunaris / marmoratus*), also introduced, has also been found to feed on the round goby (Jude *et al.*, 1995). In the Baltic Sea, there are considerably fewer species and among fishes, only turbot (*Scophthalmus maximus*) (Sapota & Skóra, 2005), cod (*Gadus morhua*) and perch (*P. fluviatilis*) (Almqvist *et al.*, 2010) have been reported to feed on the round goby in this area. Pike, burbot (*Lota lota*), brown trout (*Salmo trutta*) and walleye (*S. lucioperca*) are abundant in the Baltic and probably feed on round goby when encountered, though this has never been reported. Birds feeding on the round goby in this region include great grey heron (*Ardea cinerea*) (Jakubas, 2004) and especially the great cormorant (*Phalacrocorax carbo*) (Oesterwind *et al.*, 2017). The shift to round goby as prey for the cormorant is expected to relax the predation on other fish species from the birds, which are increasing in numbers across the Baltic region (Engström, 2001).

Another important aspect of the trophic interactions that the round goby participates in, is in the role of predator. The species readily consumes a wide range of prey items, such as

zooplankton (during juvenile phases), benthic invertebrates, previously mentioned fish eggs and also small fishes (Brush *et al.*, 2012; Oesterwind *et al.*, 2017). A majority of studies have focused on the effects on mussel communities in the Great Lakes and in the Baltic Sea (Kornis *et al.*, 2012). *Dressenid* and *Mytilid* mussels are habitat engineers with abundant associated fauna living between the mussel shells of a reef or mussel bed and therefore of particular interest when it comes to biodiversity (Norling & Kautsky, 2008). In the Great Lakes, predation on *Dressenid* mussels from round gobies is likely to be negligible, with round gobies consuming fewer mussels than the native whitefish, and at a consumption rate far below the mussel recruitment rate (Bunnell *et al.*, 2005). In the Baltic Sea, where mussels constitute 65.3 – 88.9 % of their diet, mussel populations can be severely depleted by round goby predation (Skabeikis *et al.*, 2019). The full cascading effects from removing mussels from shallow benthic communities have yet to be studied, but the effective filter feeding function of mussel beds are likely severely compromised when mussel abundances drops. This is further complicated with the increase of another invader, the polychaete *Marenzelleria*, which burrows into and destabilizes sediments (Hewitt *et al.*, 2016). The round goby also feeds on small fish. In the Baltic Sea, smelt (*Osmerus eperlanus*), three-spined stickleback (*Gasterosteus aculeatus*) and sand goby (*P. minutus*) have been found in the gut contents of round goby (Skabeikis & Lesutiene, 2015; Ustups *et al.*, 2016). Juvenile flounder are eaten at smaller sizes (<14 mm width) (Schrandt *et al.*, 2016). These species are likely to decline when round goby populations reach high enough densities, which is observed for the sand goby and the black goby in certain Baltic localities (Andreas Svensson, personal communication).

1.12 SOCIETAL CONSEQUENCES OF GOBY INVASIONS

The substantial ecological impacts can potentially lead to socio-economic consequences as well. In a fisheries context, this can happen in several different ways: resource competition with species that are commercially harvested, predation on the fry of commercial species, and direct effects on fishing gear. Resource competition with the commercially harvested flounder (*Platichthys flesus*) is documented in the Baltic Sea (Karlson *et al.*, 2007). Both species have a preference for the mussels *Mytilus spp.* and *Macoma baltica*. The increasing numbers of round gobies in the habitats occupied by both species indicate that the flounder is at risk of being out-competed. Turbot has also been reported to compete with the round goby in the Baltic Sea (Ustups *et al.*, 2016). Predation on the fry of salmonids has long been a fear since it was experimentally tested (Chotkowski & Marsden, 1999). Salmon rivers have now been reported to be colonized by the species in the Baltic (Verliin *et al.*, 2016). Recently, pike (*E. lucius*) was shown to also be under risk of egg predation by the round goby (Miano *et al.*, 2019). Though the commercial importance of pike is debatable, they still contribute to recreational fishing practices. The indirect effects on fishing gear is poorly reported, but video recordings as anecdotal evidence circulates of gill nets, intended for flounder, filled with round gobies to the extent that other fishes cannot physically get caught by the gear.

A study that modelled eradication efforts and their efficacy in rivers found that the most economic and effective model was to remove adult fish, rather than adults and eggs combined (N'Guyen *et al.*, 2018). Provided that >96% of the adult population is removed, eradication was guaranteed. Below the 95% threshold the effect of removal was strongly influenced by natural survival rates and fecundity. The success of removal was also dependent on stopping propagules (larvae or adults) from invading the habitat. This study was done in a river system, where the round goby invasion was anticipated and subsequently controlled and

the eradication effort was modelled on using cages for removal. Other measures that have proven successful in limiting the species spread upstream in rivers are electric nets (Steingraeber & Thiel, 2000). In brackish environments however, where the populations' limits and habitat connectivity are much harder to predict, eradication have been deemed impossible (Kornis *et al.*, 2012).

2.0 RESEARCH SCOPE AND QUESTIONS

Environmental adaptations in traits associated with reproduction are important in the context of ecology and evolution, not least of all when it comes to processes affecting colonization and distributions. Based on the described background, the overarching aim of this thesis was to increase the understanding of the limits imposed on reproduction by the external environment (in the form of salinity), and if these could be overcome by physiology, plasticity or adaptive processes. More specifically, each of the four different studies (hereafter referred to as Paper I-IV) dealt with several questions related to this overarching theme:

Paper I:

Are the round goby's sperm affected by salinity? Are all round goby populations' sperm traits affected in the same way? Can the round goby show adaptive responses in its sperm to different salinities?

Paper II:

Can the round goby plastically acclimate its sperm to novel salinities? If round gobies relocate from freshwater to brackish water (and vice versa), can they still reproduce? Is sperm velocity related to fertilization in the round goby? Is egg development in the round goby affected by what salinity the parents originated from, or what salinity they spawned in?

Paper III:

What are the adaptive functions of the sand goby's sperm duct glands? By what mechanisms does sperm duct gland contents affect sperm in the sand goby?

Paper IV:

Can low sperm velocity for round gobies in freshwater limit the success of sneaker tactics, compared to brackish conditions? Do round goby sperm duct gland contents support sperm function in fresh or brackish salinity conditions? Are different round goby reproductive tactics more reliant on sperm duct glands in order to reproduce?

3.0 METHODS

Similar methods have been used for the papers in this thesis, in particular, sperm treatment assays and computer assisted sperm analysis (CASA) have been key to sperm trait measurements in all four papers. These methods will be further described below. In general, all studies were done on fish caught in different salinity conditions, and then either sampled directly for trait measurements (**Paper I** and **IV**) or exposed to novel salinities (**Paper II**). The sand gobies (**Paper III**) is an exception from this since fish from a single site were studied (See Table 1 for a summary).

3.1 CATCHING FISH

The studies in this thesis have all been done on fish caught in the wild from different salinities and then kept in aquarium conditions for varying amounts of time. In **Paper I**, round gobies were caught using several different methods depending on the environment that I sampled. For all sites but one, fishing with baited hook and line was attempted, but not always successful. The alternative method of catch was fyke nets, which in some localities was very efficient: overnight, an excess of 60 fish were caught in a single funnel of the net in Kindvig, Denmark. Fyke nets were used to catch fish at site Mariehamn, Åland (site L1), Turku, Finland (site L2), Kindvig, Denmark (site H2). Baited hook and line were used for Raahe, Finland (site L3) and Karrebæksminde, Denmark (site H1). For **Paper II**, fish from the river Rhine and Guldborgsund were caught with help of local fishermen, who used fyke-nets and gill nets for their catches. River Elbe and Travemünde were sampled using baited hook and line for both **Paper II** and **Paper IV**. Different fishing methods can likely affect what phenotypes you catch depending on their movement behaviour and propensity for feeding, but this was not included in the scope for any of the studies.

For **Paper III**, the sand gobies were caught using a beach-seine net. Typically, this method is very effective in catching all the fish in the area trawled, but is only useful for sampling beach habitats. Anecdotally, this method was used extensively at one site in the Black Sea, but only juvenile round gobies were caught (as well as other species) and no data from those individuals are presented in this thesis.

3.2 CAPTIVITY CONDITIONS

The conditions in which the fish were kept varied substantially between studies. For **Paper I**, round gobies were kept in temporary plastic holding containers of ca 50 L (for 2-5 fish), for up to two days before sampling. The water in these containers was from the site of catch, which ensured that the fish did not experience any varying salinity from the captive conditions. Temperature in these was controlled simply by keeping them outdoors, shaded and relying on consistency in weather conditions. Air temperature in the shade fluctuated by one or two degrees around 10°C which simplified practices during sampling in Denmark (sites H1 & H2) and Åland (site L1). Fish caught in Turku (site L2) and Raahe (site L3) were kept in a chilled room at 10°C. Oxygen availability was ensured by constant aeration using battery-driven air-pumps (Elite A-790, Hagen, Holm, Germany). A water change of 50% per day was used to keep waste levels down. During this study, fish were never fed after catch.

In **Paper II**, round gobies were kept for up to several months in an aquarium facility with recirculating water of a salinity of either 0 or 16 ‰ (representing the freshwater or

brackish conditions from where they were caught) at a temperature of 16°C. They were kept in large fiberglass holding tubs of up to 30 fish per m² and fed ad-libitum once per day. Tanks were regularly cleaned to remove waste and fish monitored daily for signs of disease or injury. In this study, recently caught fish were first allowed a resting period of 7-11 days before roughly half of them were acclimated to either 0 ‰ (for fish caught in brackish water) or 16 ‰ (for fish caught in brackish water). This acclimation was done by raising or lower the salinity for the fish by 2 ‰ per day. After this, round goby males and females were selected to form a spawning group in the same salinity conditions, light and temperature regimes. They were given a nest box and monitored for signs of spawning.

In **Paper IV**, fish were kept in the same holding facility as in **Paper II**, but only short term (up to two weeks). They were only kept in the salinity representing their catch conditions (0 ‰ and 16 ‰, temp. 16°C), and never acclimated to any other salinities. They were fed *ad libitum* during this time, but were kept in male only groups and without shelter to ensure that no fish changed their reproductive tactic.

In **Paper III**, sand gobies were kept in glass aquaria with flow through sea water (~32 ‰, 10°C) from the bay they were caught in. The males were in kept large groups (10-30 individuals, several experiments were conducted simultaneously) and they were fed *ad libitum* daily to ensure their health.

3.3 SPERM SAMPLING AND SPERM TREATMENT ASSAYS

Sperm was sampled from the males by dissection of the testes for all the studies in the thesis. This is an alternative method to stripping the fish (commonly done by gently rubbing the belly of male fish and “squeezing” the ejaculate from the male’s testes. However, stripping is not an option in the case of sand goby, due to their small size. Therefore, dissection of the testes was used to obtain the sperm samples. Dissection also allowed the option of separating sperm from sperm duct gland contents, and enabled the studies presented in **Papers I and III**. Since I found measurable differences using this method on sand goby (**Paper III** was the first study in the thesis to be conducted), I subsequently moved on to use it on round goby as well.

In order to obtain a sperm sample to test, the testes were dissected out of the fish (as well as any sperm duct glands for the studies where the effect of their contents were studied). The testis was then placed inside a plastic tube and incised for a fixed number of times (commonly five) with a pair of sharp micro-surgery scissors to allow sperm to run into the sample. The sperm was then diluted with a calcium-free Ringer’s solution (Karila *et al.*, 1993) commonly used to replicate iso-osmotic fluids of fish. This sperm-suspension was then pipetted out of the tube with the testes and then used in different assays depending on the study. For **Paper III** and **IV**, two different suspensions were created, one using only a testis, and the other from a testis and a sperm duct gland.

After this, sperm was treated in a water sample with a set salinity to replicate their potential spawning environment. This water sample was made from filtered sea water (**Paper III**) and diluted with filtered tap water from a natural reservoir (**Paper I**). Or mixed from artificial sea salt (Instant Ocean) and non-chemically-treated, filtered tap water of drinking quality (**Paper II and IV**). In **Paper I**, the sperm-suspension was tested in seven different salinities: 1, 5, 10, 15, 20, 25 and 30 ‰. In **Paper II**, the sperm-suspension was tested in 0, 16, and 32 ‰. In **Paper III** the two different suspensions were each tested in 32 ‰ salinity. For **Paper IV**, the two different suspensions were each tested in 0, 8 and 16 ‰ salinity. These are summarised in Table 1.

Table 1. Summary of studies and their overarching methodological comparisons. Fishing methods, days in captivity and feeding regimes are anecdotally presented and their effects are never evaluated in any of the studies. For Paper III, environment, salinity at catch and sperm test salinity is also anecdotally included for descriptive purposes.

Study	Paper I	Paper II	Paper III	Paper IV
Short summary	Fish caught in different salinities, with sperm tested across a salinity range	Fish caught in different salinities and acclimated to new salinities, then allowed to reproduce	Fish caught from a single site, sperm is tested with and without SDG contents	Fish caught in different salinities, sperm from different tactics tested with and without SDG contents
Species	<i>N. melanostomus</i>	<i>N. melanostomus</i>	<i>P. minutus</i>	<i>N. melanostomus</i>
Environment	Low Brackish Brackish	Freshwater Brackish	Marine	Freshwater Brackish
Fishing methods	Hook and line, Fyke nets	Hook and line, Fyke nets	Beach seine	Hook and line
Salinity at catch (‰)	~ 5 ~ 12.5	0 ~ 16	~ 32	0 ~ 16
Acclimation salinity (‰)	NA	0 16	NA	NA
Days in captivity	≤ 2	29-629	≤ 5	≤ 14
Feeding	NA	<i>Ad libitum</i> daily	<i>Ad libitum</i> daily	<i>Ad libitum</i> daily
Allowed to spawn?	No	Yes	No	No
Sperm assays	Sperm only	Sperm only	Sperm only + SDG contents	Sperm only + SDG contents
Sperm test salinities (‰)	1 5 10 15 20 25 30	0 16 32	32	0 8 16

3.4 MEASURING SPERM MOVEMENT USING CASA

When a sperm had been exposed in the water sample for a set time (~ 1 min, this varied slightly and was randomized between each test salinity), a suspension of this water sample with sperm was transferred to a microscope slide to be filmed. To avoid evaporation and temperature flux, the suspension was “encased” with walls from a rubber o-ring, and was given a lid from a glass cover slip. This method was developed from a study on sand goby (Svensson *et al.*, 2017), but was originally invented to film urchin sperm (Havenhand & Schlegel, 2009). After this, the sperm sample was filmed in an inverted microscope with a 10 x magnification objective and standard contrast and illumination, using the same settings (reported in detail in the Papers methodologies). This was commonly done for a number of technical replicates, and when these had been analysed the measurements were often later used to calculate an average for each male and sperm test condition.

The most important tool in this thesis is the CASA system, short for ‘computer-assisted sperm analysis’. It is a tracking software built as a script in the image-handling software ImageJ (Wilson-Leedy & Ingermann, 2007; Purchase & Earle, 2012; Amann & Waberski, 2014). CASA analyses every video frame and compares the position of the particles between the frames. The software can calculate a range of parameters, either for each sperm, or as an average for each film. Parameters such as velocity, total number, and percentage of motile sperm are the most obvious ones, but more subtle variables such as turning angle, number of flagellar beats and the straightness of the sperm path can also be calculated. The CASA sampling method relies on clean and evenly diluted sperm samples to be optimal. By sampling the testes through dissection and cutting into it to access the sperm, immature spermatids are also

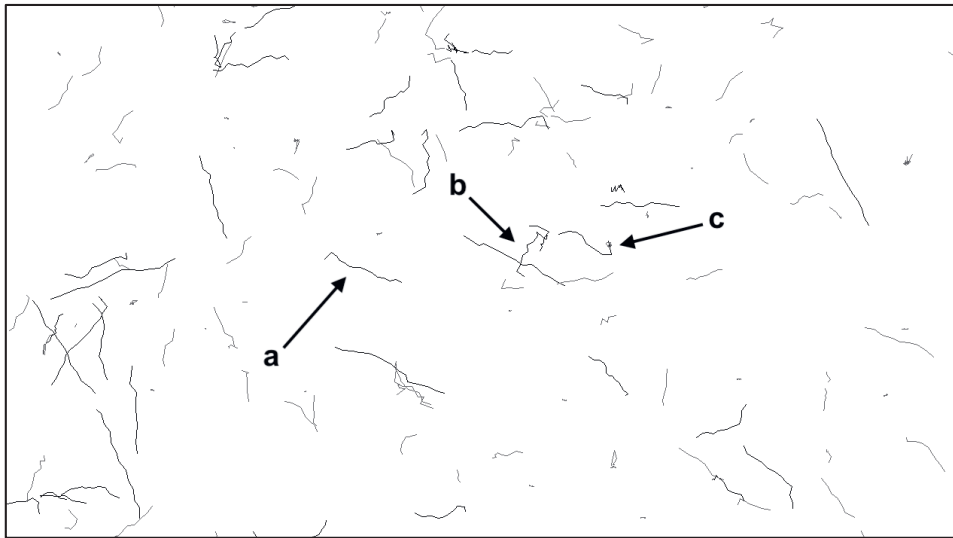


Figure 4. Tracks of sperm from a round goby of brackish origin. Sperm are swimming in 15 ‰ salinity and recorded using CASA in ImageJ. This 0.5 second video revealed 26% of the particles in the sample to be motile (i.e. sperm triggered to swim), with an average sperm velocity of $109.6 \mu\text{m s}^{-1}$. Note a: a free track of one spermatozoon, b: tracks from sperm crossing and c: a spermatozoon likely ending or beginning its track by cork-screwing in the z-plane.

released into the sample. This can be problematic, but can also give valuable data. The percentage of motile sperm in a sample can be seen as a measurement of 'reproductive readiness' since number of mature and moving sperm affects the males' fertilization ability. This needs to be taken into account during the interpretation of the results, most notably during comparison with other studies measuring velocity of stripped sperm.

3.5 MEASURING SPERM VIABILITY USING CELL STAINING

For **Paper III** and **IV**, sperm viability was measured as the proportion of living sperm in the sample. This was done by the use of a cell staining kit that colour live and dead cells with different chemicals depending on the intactness of their cell-membrane. A dead cell quickly loses membrane function, and can then be stained with propidium iodide. A living cell with functioning membrane and intact DNA instead binds cyanine dye (I used the chemical SYBR Green I). These two dyes can be excited using the right wavelength of light, and then photographed for images showing the living cell and dead cells of a select area. For the papers in this thesis, water with sperm was transferred to a new plastic tube, and mixed with the chemicals to stain the sperm. After this, the stained sample was transferred to a microscope slide and photographed for two pictures under the right wavelengths of light to excite the dyes. This was also done for several replicates and commonly averaged for a single value per male and sperm treatment conditions.

3.6 SPAWNING FISH IN NEST BOXES

For **Paper II**, round goby males and females were either kept in their original salinity of catch (freshwater or brackish water) or acclimated to the opposite conditions (16‰ for fish from freshwater, and 0 ‰ for fish from brackish water). From these four treatment groups, I then randomly selected fish to form spawning groups consisting of one male and two to three smaller females. These were placed together in a compartment (roughly 70 x 70 cm bottom area) inside a fiberglass holding tub together with a nest box. These nest boxes were locally constructed out of PVC plastic panes with a length-width-height of 210 mm x 105 mm x 60 mm. The box was also fitted with a half-circle opening of a 25 mm radius to enable fish to swim in and out (see Figure 5). The intention was for the male to defend this box and use it to attract females. This nest box was fitted with a top and a bottom glass pane covered with acetate sheets attached by rubber bands. This enabled the removal of the eggs when they were laid on either pane, as well as cutting the egg clutch in two, since they were laid on the plastic sheet instead of directly on the glass. The fish in the spawning groups were fed *ad libitum* and checked for signs of disease or injury every day. The nest was checked for eggs three times per week.

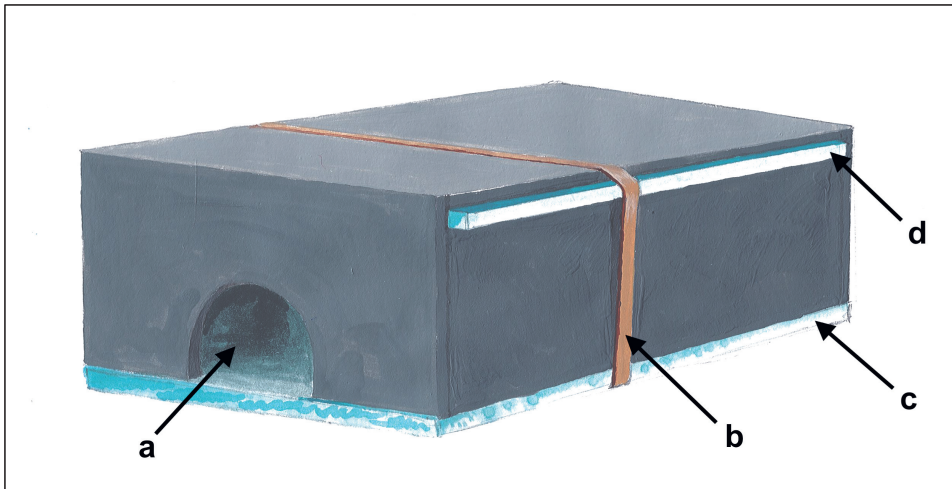


Figure 5. Schematic of the nest box used for spawning fish. Measurements of length-width-height is 210 mm x 105 mm x 60 mm. Note a: 25 mm radius hole for fish to enter and exit through, b: rubber band holding bottom (c) and top (d) glass panes. Acetate film is attached on top of glass pane c and bottom of glass pane d. Glass pane d is inserted through a slit in the nest box wall.

3.7 MEASURING EGG CONDITION

When a group had produced a clutch on the acetate sheets in the nest box, the panes were replaced with new ones, and the acetate sheet with the eggs was removed and cut into two pieces with roughly half the clutch on each piece. One of these pieces of sheet was left on its glass pane, and the other was placed on a new glass pane, and acclimated to the opposite salinity to the salinity that the eggs were first laid in. This was done by dripping water of the opposite salinity for two hours into a bucket with the eggs submerged in their spawning salinity. The aim of this was to measure the effect of parent origin (fresh or brackish), spawning salinity (0 or 16 ‰), and rearing salinity (0 and 16 ‰) on egg development. Unfortunately, few clutches were spawned (and even fewer were spawned on the acetate sheets), which eventually limited the experiment.

After the egg clutches had been divided into two and one of them drip-acclimated to the opposite salinity, both were photographed submerged into about 1 cm of water of their respective salinity under a fixed camera-rig. This camera rig was fixed in relative position to the glass pane in order to photograph the same clutch repeatedly at the same distance and position, for ease of tracking development. The eggs were then inserted into a rack with flow through water from each respective recirculating system, to develop without male intervention. Photography was done three times per week for at least 20 days (but longer when clutches were laid early and continued developing healthily beyond these first 20 days). For each photograph, each pane with a clutch was removed from the rack and transferred to a water bath to be photographed and then transferred back. The seconds spent in air was not

expected to affect their development since eggs develop normally despite hours of air-exposure (Hirsch *et al.*, 2016).

These photographs were later visually analysed and scored into one of several stages for each egg: being present on the pane, developing normally, developing abnormally or infected, and developing black eyes. From these numbers, proportions of normally developing eggs, abnormally developing eggs and eggs reaching the zygote stage where black eyes were visible.

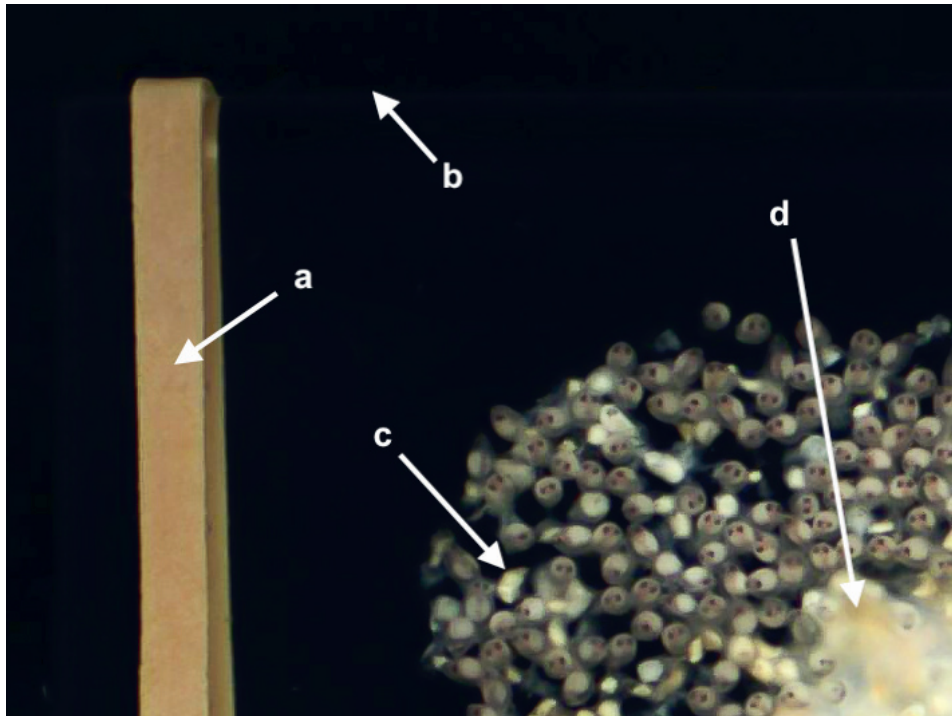


Figure 6. Photograph section of eggs attached to glass pane. Round goby zygotes with visible eyes can be seen in the clutch on the bottom right. These eggs have been laid by brackish fish caught in Guldborgsund, Denmark, spawned in 16 ‰, and reared in 16 ‰. Note a: rubber band keeping acetate sheet on glass pane, b: transparent (and barely visible) edge of glass pane, c: one of several abnormally developing zygotes, d: oomycete infection (likely *Saprolegnia* sp.).

4.0 SUMMARY OF RESULTS

Paper I

When species spread into new environments, they can come under selection and adapt to the local conditions. Several fish species that have colonized the Baltic Sea over historical time have adapted their gametes to the different salinity conditions in the basin. I wanted to investigate if this could also be true for the rapidly invading round goby (*Neogobius melanostomus*), despite its short time (approx. 30 years) in the same region, since this could be crucial for its spread and success within the Baltic Sea. In order to test this, round goby males from several sites at two separate invasion fronts in the Baltic Sea; one into lower salinity (~5 ‰) and one into higher salinity (~15 ‰) regions of the Baltic Sea, were sampled for their sperm. The sperm was then exposed to seven different salinities (1, 5, 10, 15, 20, 25, 30 ‰) where they were filmed for movement traits (sperm velocity and percent motile sperm).

Sperm behaved differently depending on what invasion front the males were caught from. Sperm velocity was the trait that differed the most between the two fronts: males caught in higher salinities showed a peak in sperm velocity in 15-20 ‰, while sperm velocity peaked in 5-15 ‰ for males from the low salinity invasion front (Figure 7).

Sperm motility also differed between the two invasion fronts, with consistently lower motility across all populations sampled from the low invasion front. However, the percentage of motile sperm peaked at 5 ‰ for both fronts.

To assess how sperm velocity changed over generations, I analysed the average sperm velocity in the salinity that best matched the salinity of each separate site that the males were caught in, and the number of generations since the reported year of invasion at each site. From this I calculated the amount of phenotypic change per generation (*Haldanes*). I found that in the low invasion front, there was phenotypic change comparable to what species experience under strong fisheries selection: 0.675 standard deviations per generation. In the high invasion front, there was no change between the sites (but also approximately 1 generation of age difference between them).

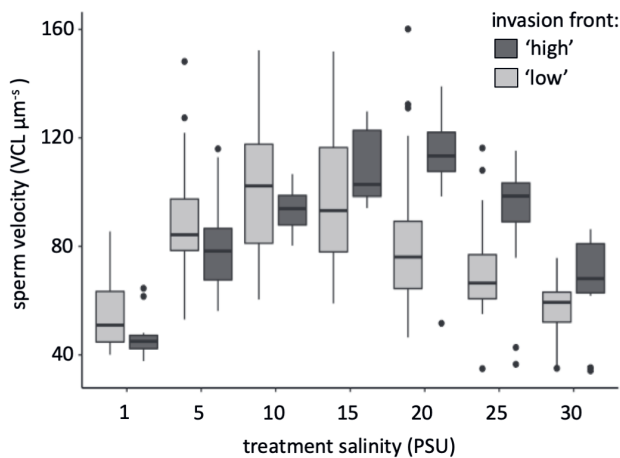


Figure 7. Sperm velocity in round goby males from invasion fronts into high and low salinity have different performance across a range of salinities representing the Baltic Sea salinity gradients. PSU ≈ ‰ salinity. This figure is a greyscale reprint of Figure 2a from Paper I: Green et al. (2020) *Evolutionary Applications*. Reprinted with permission from John Wiley & Sons Ltd.

These results show that in the low and high salinity invasion fronts in the Baltic Sea, the round goby shows different responses to salinity, likely because the fish in the low salinity region are locally adapting to lower salinity conditions. Fish in the high salinity front could experience stabilizing selection on their sperm velocity, since the ancestral region of the Black Sea has a similar salinity (16-18 ‰ compared to the Baltic regions average of 15 ‰). Males in the low invasion front evidently reproduce in salinities around 5 ‰. The velocity sperm showed when tested in this salinity is likely to be sufficient for successful reproduction. A comparable sperm velocity was observed for males from the high salinity invasion when their sperm was tested in 25 ‰. We can therefore expect that high invasion front males will be able to reproduce at least in salinities up to 25 ‰, or at least not be limited due to detrimental sperm velocity. Both fronts showed comparatively low sperm velocity ($< 80 \mu\text{m s}^{-1}$) and motility ($< 10\%$) in sea water ($\sim 30\%$), which might limit them from successfully reproducing in fully marine environments. But since the round goby seems able to adapt in its sperm velocity into lower salinities, it is possible that this can also occur if it is spreading into higher salinities.

Paper II

Though **Paper I** showed strong indications that the round goby is able to locally adapt in its sperm velocity, there can still be an aspect of acclimation to the novel salinity conditions. Acclimation in sperm velocity has been shown in several other euryhaline fishes, which suggests that the round goby might also be able to acclimate its sperm to function better when it is exposed to a new salinity, or between brackish- and freshwater. In order to test the acclimation ability with respect to reproductive traits in the round goby, fish were caught both from two brackish sites, and two freshwater sites. Roughly half of the fish were kept in the salinity they were caught in (0 or 16 ‰), while the other half were acclimated to the opposite salinity. This resulted in four different parent treatment conditions: freshwater fish kept in 0 ‰; freshwater fish acclimated to 16 ‰; brackish fish acclimated to 0 ‰; and brackish fish kept in 16 ‰. After this they were separated into spawning groups of a male and two to three females together with a nest box, and allowed to spawn. After the group produced a clutch, the male was sampled for sperm. Sperm were filmed in test salinities corresponding to the two parent treatments: 0 and 16 ‰, and a sea water salinity (32 ‰). This last test salinity was included to understand if male acclimation to brackish water could also increase sperm velocity in sea water. The clutch itself was split into two, with one half placed in 0 ‰ salinity and the other in 16 ‰ salinity to evaluate if parental origin and acclimation would affect how well the eggs developed in the different conditions. The clutches were photographed roughly every other day for 20 days.

There was no acclimation effect detected on sperm velocity in either males of freshwater or brackish origin in any sperm test salinity. Sperm velocity of males from brackish origin was low in 0 ‰, but increased in 16 ‰ and 32 ‰, and sperm velocity of males from freshwater origin was on average low ($< 75 \mu\text{m s}^{-1}$) independent of sperm treatment salinities (Figure 8).

When analyzing sperm motility, there was an effect of acclimation, in that males of brackish origin acclimated to 0 ‰ showed significantly less sperm motility than males kept in 16 ‰ (Figure 9). The proportion of motile sperm also showed different responses to salinity depending on the male's origin. Males of brackish origin had a higher proportion of motile

sperm in 16 ‰ than males of freshwater origin. Males of freshwater origin had generally few motile sperm across all sperm treatment salinities, similar as for sperm velocity.

Of 101 pairing attempts, 28 % resulted in successfully spawned clutches in the nest boxes. There were no significant differences in what group were the most likely to spawn out of the four parent treatment groups, but freshwater fish acclimated to 16 ‰ salinity had the lowest success of spawning. For these, only 13 % of pairing attempts resulted in clutches,

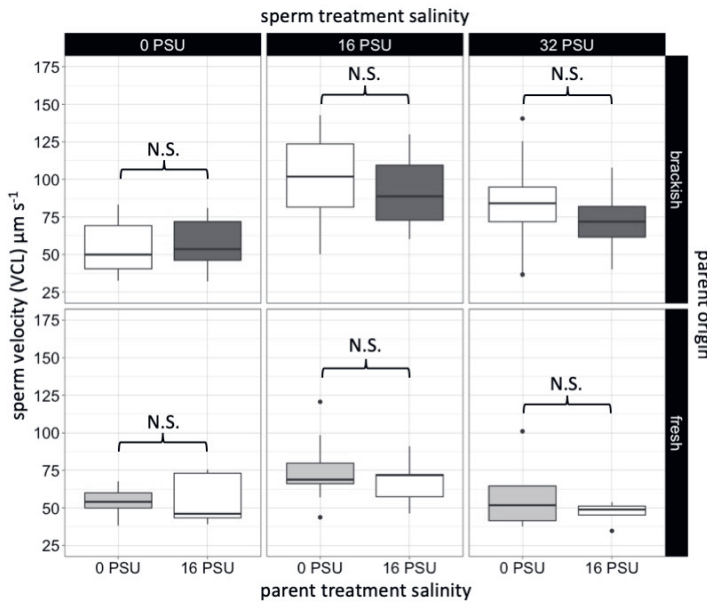


Figure 8. Sperm velocity in the round goby is unaffected by parent treatment salinities, regardless of the origin of the males. N.S. denotes non-significant differences. Salinity units of PSU is equivalent to ‰. This figure is a greyscale reprint of Figure 1b from Paper II.

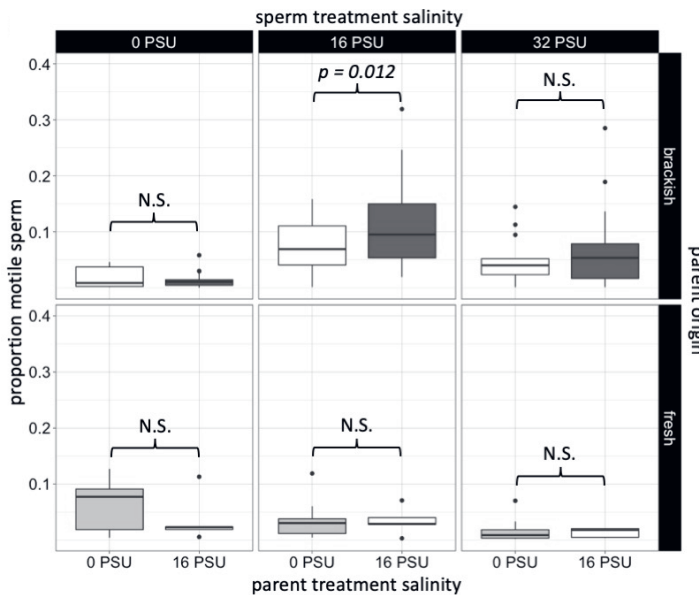


Figure 9. Sperm motility in the round goby is unaffected by parent treatment salinities, regardless of the origin of the males. This was true except for males of brackish origin, which had a lower proportion sperm swimming in 16 PSU (\approx ‰) after the males spent a month in freshwater. N.S. denotes non-significant differences. This figure is a greyscale reprint of Figure 2b from Paper II.

compared to 40% for brackish fish kept in 16 ‰ salinity. Clutch sizes did not differ significantly between any of the parents' salinity treatment groups.

The relationship between sperm velocity and fertilization success was different depending on the environment that the eggs were fertilized in. For clutches spawned in 16 ‰, fertilization success increased with higher sperm velocity and for clutches spawned in 0 ‰, the relationship was the opposite. Similar significant trends were found for the effect of proportion motile sperm on fertilization success.

The development of the eggs was analysed to understand if parental origin, parent treatment salinity (what salinity the eggs were spawned in) or egg treatment salinity was the strongest predictor of successful development. The proportion of fertilized eggs, as expected, had a strong effect as covariate, but I also found that clutches spawned by parents of a brackish origin were more likely to reach to the black eye stage at day 20 than clutches with parents of freshwater origin. There was no effect of egg treatment salinity or parent treatment salinity, but the data was also limited, due to relatively few clutches spawned, which might have influenced the chance of seeing an effect.

In short, I found no positive effect on sperm when brackish males had acclimated to freshwater, or when freshwater males had acclimated to brackish water. However, fish managed to successfully fertilize clutches in both 0 ‰ (fish of both brackish and freshwater origin) and 16 ‰ (fish of brackish origin). The interesting finding that decreasing sperm velocity increases fertilization in 0 ‰ (and that increasing sperm velocity increases fertilization in 16 ‰) shows that low sperm velocity in freshwater can potentially be adaptive. Since males of brackish origin also have low sperm velocity (and motility) in 0 ‰, and had successfully developing clutches there, some round goby populations are evidently very robust to large differences in salinity conditions when it comes to their reproductive traits.

Paper III

Male reproductive success is often tightly linked to sperm performance, and this is especially true for species with sperm competition. Here, I wanted to test the importance of ejaculate components produced in the sperm duct glands (SDG contents) and their effect on sperm. I exposed sperm from nest-holding males of sand goby, *Pomatoschistus minutus*, caught at the Swedish west-coast to two different treatments; either sea water, or sea water mixed with contents from their sperm duct glands (SDG). After this I filmed the sperm for measurements of velocity. I also measured their viability at 10 minutes and 24 hours post-exposure through a cell-staining technique.

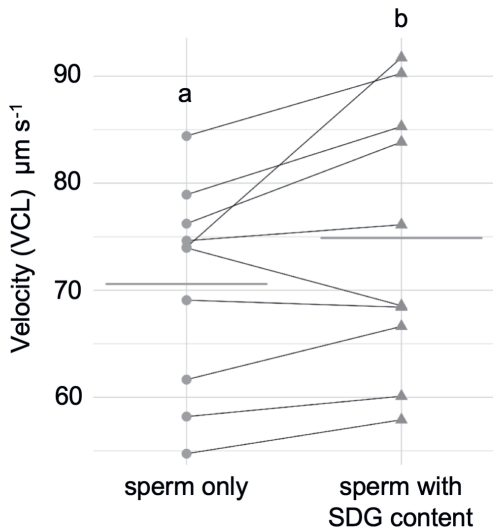


Figure 10. Sperm velocity in the sand goby is positively affected by sperm duct gland contents in sea water. Lines show the individuals average response from three technical replicates in each treatment condition. Letters indicate statistically significant differences. This figure is a greyscale reprint of Figure 1a from Paper III: Green & Kvarnemo (2019) *Biology Open*. Reprinted with permission from The Company of Biologists Ltd.

The treatment with SDG contents significantly increased sperm velocity by an average of $5.12 \mu\text{m s}^{-1}$ (7.3%) compared to when the sperm was treated without. Sperm velocity was also still significantly different between treatments when controlling for the percentage of motile sperm. However, the percentage of motile sperm was unaffected by treatment.

When I compared the proportion of sperm that were alive after 10 min and 24 h, time had a significant effect on the viability of the sperm, but I did not find that treatment with SDG contents in any way affected the viability. However, in both treatments, the sperm had a viability of over 86% after 24 h exposure to seawater.

An increase in sperm velocity by 5% has been shown to affect fertilization success by a similar increase (5%) for other external fertilizers. With increased sperm velocity, there may also be a trade-off between sperm velocity and sperm viability. Yet, since I did not find a decrease in viability from SDG-contents, this mechanism might to some degree enable sand goby sperm to overcome such a trade-off. The extremely long-lived sperm in the sand goby is an interesting phenomenon in itself. It is likely that this is an adaptation to a prolonged spawning session, where a female can take hours to deposit her eggs, and nest-holding males need to guard them from sneakers. If he can release sperm into the water inside his cave-like nest before or early during spawning, he might be able to fend off competing males and still ensure his own fertilization.

Paper IV

In this study, the aim was to investigate if detrimental sperm velocity in freshwater could in some way be offset by SDG contents, or if males possibly had to invest in larger testes in freshwater to overcome environmental limits. If round gobies showed a similar positive effect on sperm velocity as found in the sand goby (**Paper III**), SDG contents may shield sperm from the previously documented negative effects of low salinity in the round goby (**Paper I and II**). Since different reproductive tactics may invest in SDG contents and testes mass differently (Marconato *et al.*, 1996; Kvarnemo *et al.*, 2010), my co-authors and I also compared different colour morphs as a proxy for male reproductive tactics (dark colour morph is indicative of nest-holding, and light colour morph is indicative of sneaker tactics). Reproductively active males were sampled from two separate populations: estuarine males from the brackish southern Baltic Sea, and riverine males living in freshwater in the river Elbe. Sperm from these males were experimentally treated in three different salinities (0, 8, 16 ‰) with or without SDG contents added to the water, measuring the immediate response in sperm velocity and the proportion sperm alive (at < 10 min). In the study observational data on adult physiological parameters such as body mass, testes mass, and energy status were also sampled to compare between fish from different environments.

When first analyzing sperm without SDG contents, similarly to **Paper I and II**, I found that salinity strongly affected sperm velocity, with decreased velocity in 0 ‰ compared to 8 and 16 ‰. I also found relatively low sperm velocity for males of freshwater origin overall while sperm velocity was markedly higher for males of brackish origin. Sperm viability also decreased in 0 ‰ compared to 8 and 16 ‰, independently of whether the fish were from the freshwater river or brackish estuarine environments. When I compared sperm from freshwater and brackish males in 0 ‰, freshwater males had higher sperm viability and velocity than brackish males. In short, the freshwater males likely have sperm that are better adapted to freshwater than brackish males have. That said, freshwater males were overall less well adapted to their local salinity than brackish males are to theirs.

Male body mass was found to determine colour morph. Body mass (rather than length or energy reserves) correlated with dark colour and therefore likely determines nest-holding ability, since dark colour reflects nesting behaviour.

I also found a trade-off in between sperm velocity and viability such that increased sperm velocity resulted in reduced sperm viability. This trade-off was present regardless of what colour morph the males had or what environment they came from.

I then proceeded to investigate how the two colour morphs' sperm were affected by SDG contents in their spawning environment (0 ‰ for freshwater, and 16 ‰ for brackish males). Contrary to my predictions, I found that gland contents did not improve sperm velocity or viability, regardless of male colour morph or what environments the males were expected to reproduced in. Instead SDG contents decreased viability for freshwater males' sperm tested in 0 ‰, while it decreased the velocity of brackish males' sperm tested in 16 ‰.

Freshwater males had higher energy reserves and were bigger than brackish males. This is strong evidence of higher investment in somatic growth. I found no light-coloured males with a gonadosomatic index (a measurement of investment in reproductive tissues) above 2 % among the freshwater males from the river. Light brackish males on the other hand showed an average gonadosomatic index of 4 %, suggesting they were more likely to employ sneaker tactics.

The different negative responses to SDG in sperm velocity and sperm viability for the two separate environments (**Paper IV**, Figure 2) are novel. Despite these interesting findings on SDG contents, there are other strong trends showing life-history differences between round gobies from the two measured environments. Since sperm performance in freshwater was low, and light coloured males in the river invested less in reproductive tissues compared to light males from the estuary, I propose that sneaker tactics are unfavorable in freshwater if the population is not adapted to these conditions, and these tactics are possible selected against. Instead, investment in somatic growth is promoted.

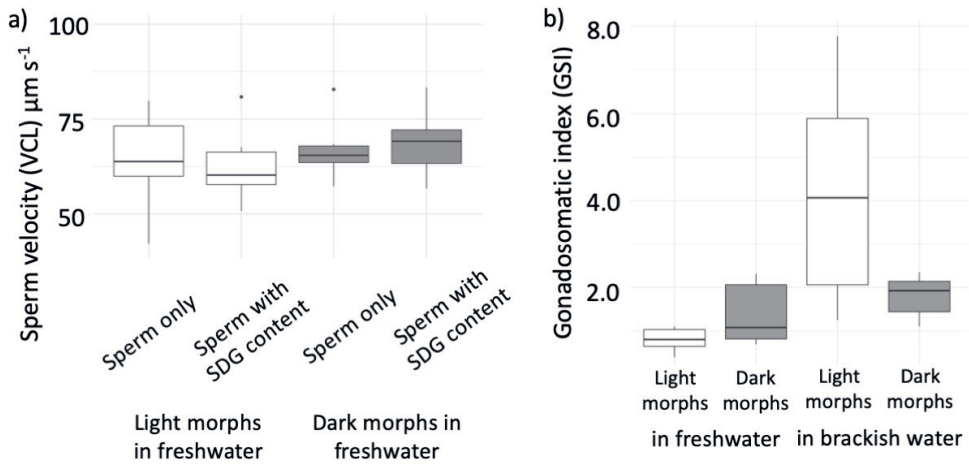


Figure 11. a) Sperm velocity in freshwater round goby is not affected by sperm duct gland contents for males of different morphs. Note also that sperm velocity for the males sampled from the river is comparatively low ($\leq 75 \mu\text{m s}^{-1}$ on average). b) Gonadosomatic indices (GSI) for males of different morphs sampled from both a freshwater river and a brackish estuary. River light morphs do not invest as much energy in reproduction as the other groups. These figures are reworked greyscale versions of a) Figure 3a and b) Figure 4c from Paper IV.

5.0 DISCUSSION

The aim of this discussion is to compare the results in this thesis to some novel data, to provide insights into how they contradict or support each other. On this follows a section of how the findings in this thesis can be viewed in a bigger picture, especially in regards to future colonization processes the round goby, predominantly in Sweden and Scandinavia. Discussions on the specific results from each study are available in the respective papers, but this section also cross-references to results presented in the papers. Therefore, this analysis is best read with those results and discussions at hand.

5.1 ARE ALL ROUND GOBY POPULATIONS' SPERM SENSITIVE TO SALINITY?

Round goby sperm are strongly affected by salinity. Of the different traits I have studied (mainly sperm velocity, motility and viability), sperm velocity seems the most heavily impacted. This is evident from data collected in three separate studies (**Paper I, II and IV**), where sperm velocity was measured in a range of salinities. These Papers all show that the highest sperm velocity ($\sim 110 \mu\text{m/s}$) is found in a narrow range of salinity ($\pm 5 \text{‰}$) for most populations or sampled sites. The sampled populations included in all three studies (see Figure 12) had the highest sperm velocity in brackish conditions (even fish caught in freshwater), most around 15-20 ‰, but those from the northern part of the Baltic Sea had the highest around 10 ‰. The one sample that stands out among these are the fish caught in Travemünde and kept in aquarium conditions long term before in the experiment in **Paper IV** was conducted, more on this below. Apart from the populations sampled during the studies presented in the attached papers on round goby, I have also conducted sampling in the round goby's native Black Sea region with the same methods. Panels c) and k) in Figure 12 show sperm velocity for a freshwater population in the Danube river (0 ‰ salinity), and a brackish population from the southern coast of Bulgaria (16-18 ‰ salinity) measured across a salinity range of 0-30 ‰. These data are unpublished, but support our conclusions that sperm are affected by salinity also for wild native round goby populations. Interestingly, both of these ancestral populations peak at their local salinity, and in contrast to our other "freshwater" populations from **Paper II and IV** (Rivers Elbe and Rhein), the pattern for the Danube fish looks very different in that their sperm velocity peaks in freshwater! This raises the question of the origin of the fish from Elbe and Rhein. The fish colonizing into Elbe and Rhein could have ancestral origins from either freshwater, brackish or a combination of both. A combination of both is supported by the experiment in **Paper II**, which shows that freshwater fish can acclimate to brackish conditions. However, spawning in 16‰ was difficult for these fish, which could be an argument for a freshwater ancestry. Considering the different performance curves of the ancestral and derived freshwater populations, it is unlikely that Elbe and Rhein have solely been colonized by fish with a distinct freshwater ancestry, but this needs further testing with genetic methods.

5.2 IS SPERM VELOCITY A TRAIT THAT RESPONDS TO SELECTION OR ACCLIMATION?

In **Paper I**, I argue that sperm velocity is adaptively changing through selection. I did not test its effect on fertilization success, which is a prerequisite for this assumption. This was instead

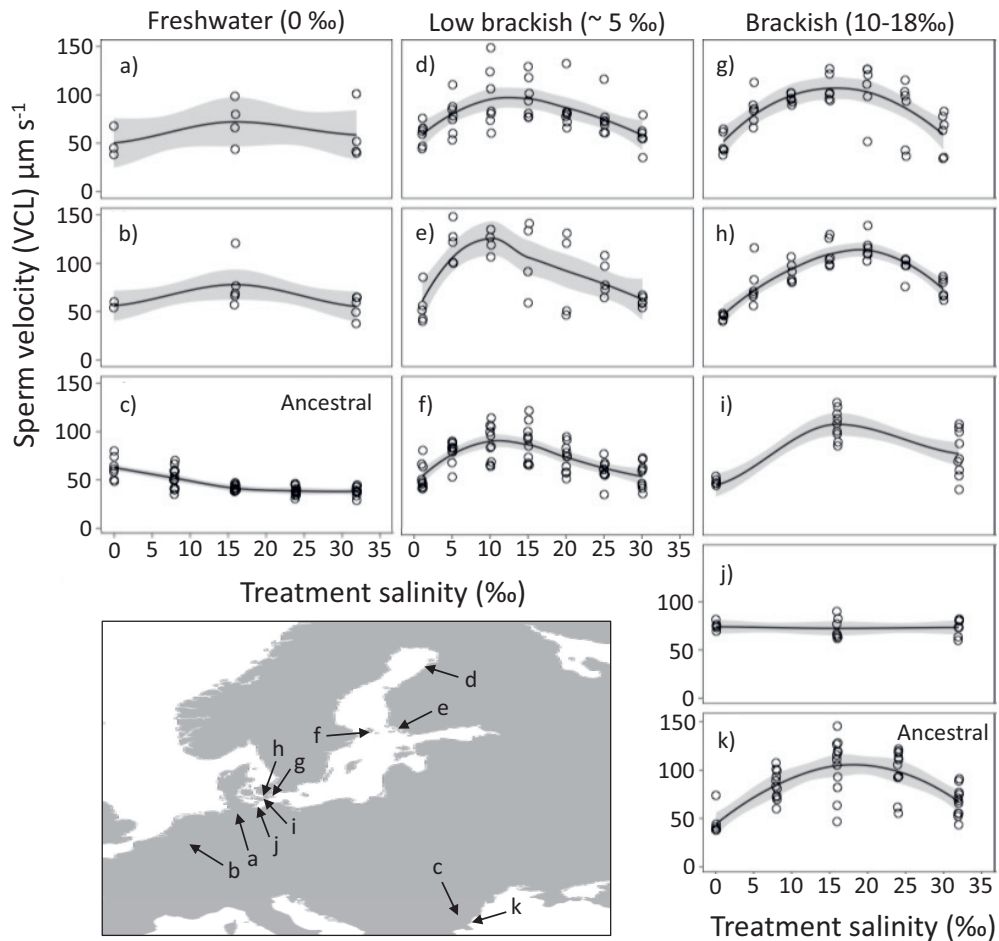


Figure 12. Sperm velocities (VCL, $\mu\text{m s}^{-1}$) of round goby from different environments. a) River Elbe from Paper II, b) River Rhine from Paper II, c) River Danube, (included here for comparison; otherwise not part of this thesis), ancestral region, d) Raahe, low salinity invasion front site (L3) in the Baltic Sea from Paper I, e) Turku, low salinity invasion front site (L2) in the Baltic Sea from Paper I, f) Mariehamn, low salinity invasion front site (L1) in the Baltic Sea from Paper I, g) Kindvig, high salinity invasion front site (H2) in the Baltic Sea from Paper I, h) Karrebæksminde, high salinity invasion front site (H1) in Baltic Sea from Paper I, i) Guldborgsund, brackish site in Baltic Sea from Paper II, j) Travemünde, brackish site in the Baltic Sea from Paper II, k) Sozopol, brackish site in the Black Sea (included for comparison; otherwise not part of this thesis), ancestral region. Lines fitted with 'loess' smoothes in the R package ggplot2; grey areas show 95% confidence intervals. Note that in panels d, e, f, g, and h sperm were tested in a minimum 1 ‰ salinity, while the rest were tested in 0 ‰. Also note that no results from Paper IV are included since sperm from these fish were only tested in 0, 8 and 16 ‰ salinity. Map shows placement of sampling sites. See also page 9-10 for a detailed map of sites in relation to the species distribution.

assumed based on several studies on other fish species that also spawn under sperm competition (Gage *et al.*, 2004; Rudolfson *et al.*, 2008; Gasparini *et al.*, 2010; Beirão *et al.*, 2018; Purchase, 2018), in particular the sand goby, in which low sperm velocity is associated with failed reproductive success (Svensson *et al.*, 2017). The experiment in **Paper IV** allowed the opportunity to test the assumption on round goby. In this study, fish were allowed to spawn, and we could quantify the degree of fertilization. After spawning, sperm velocity was measured and related to fertilization success. Our findings support the idea that increased sperm velocity increases fertilization success (**Paper IV**, Figure 3a), at least in brackish conditions, and thus likely to be under positive selection. However, this was only tested in 16 ‰ salinity, and given that the relationship is the opposite in freshwater (where decreased sperm velocity increases fertilization success) this may not be the case for all brackish conditions where the round goby lives. A larger sample size would provide us with better data,

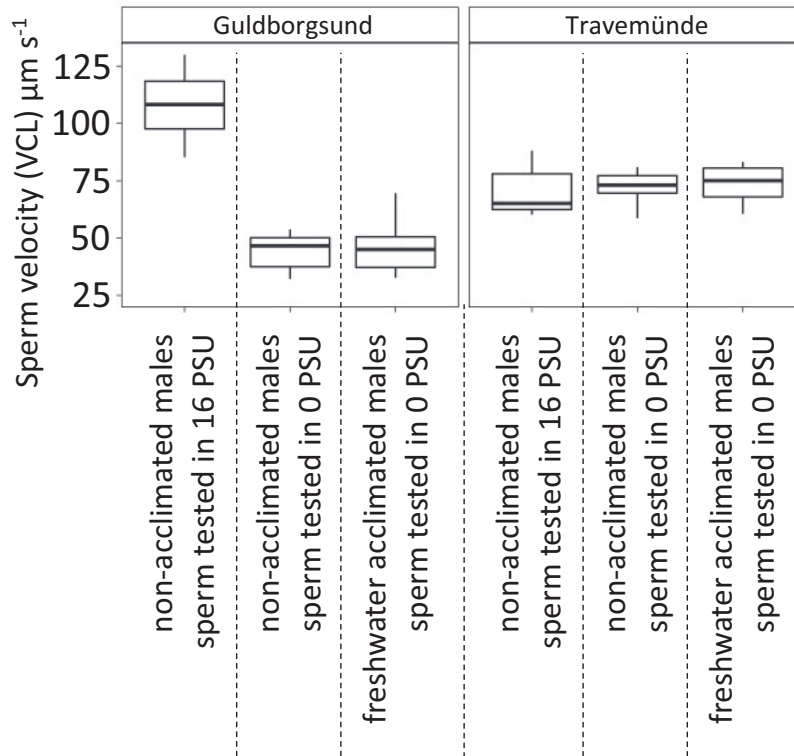


Figure 13. Sperm velocities (VCL, $\mu\text{m s}^{-1}$) of round goby from two different brackish sites in the Baltic Sea, with similar reported salinities ($\sim 16 \pm 2$ ‰), but with different time in captive conditions. Travemünde males were kept in the lab in ‰ and fed *ad libitum* for ca 600 days before acclimation to freshwater, whereas males from Guldborgsund were only kept for 7-11 days before acclimation to freshwater. Data presented is for sperm tested in brackish (origin) conditions of 16 ‰ (first box in each panel, a control of sorts), and freshwater conditions of 0 ‰ for non-acclimated (second box) and freshwater acclimated (third box).

but the mortality associated with acclimation (**Paper IV**, supplementary Table 1), and the limited willingness of the fish to spawn in captive conditions (**Paper IV**, Table 3) makes the required experiments difficult and costly to pursue. The lack of adaptive acclimation found in **Paper IV** (Figure 1b, Figure 8 in this thesis) also supports the relevance of sperm velocity as a trait under strong selection. Plastic responses can limit the strength of selection (and therefore the speed of evolution), since conditions that would normally select for a sample of a population simply induces a shift in expression (Price *et al.*, 2003). Since sperm velocity at a given salinity seems to be a fixed trait (though with some variation in the population), it is more likely to be under selection than if it would have been a plastic response.

In **Paper II**, fish were kept in different salinity conditions to understand their plastic responses to salinity. For the results presented in that study, the two brackish populations (Travemünde and Guldborgsund) were pooled, but included in the analyses as a random effect (similar to **Paper I**). The fish from Travemünde differed from the fish from Guldborgsund in that they had been in captive conditions for considerably longer (ca 600 days as opposed to ca 30 days). In Figure 12 (panel j), the Travemünde shows similar but remarkably low sperm velocities across all tested salinities, likely as a consequence of long time in captivity, and exposure to daily *ad libitum* feeding and lack of seasonal cues. The low sperm velocity may be attributed to immature spermatids, that are all triggered to swim, but fail to gain speed. As discussed in **Paper II**, the potential for different mechanisms triggering onset of sperm swimming and velocity could support this pattern. Since this data make up part of the results on acclimation of sperm velocity presented in **Paper II**, there is a benefit of comparing the data between the two populations sampled for the experiment. As shown by Figure 13 (on the previous side), there is no visible effect from freshwater acclimation in the Guldborgsund population when this data is presented alone. Travemünde fish were kept in the analysis to increase N , and the effect from salinity was significant despite their inclusion. This does however limit our opportunity for interpretation across multiple populations, as the two I have measured are not showing the same response. Extrapolation of our study onto all brackish populations are therefore limited.

There is also sperm velocity data available from the experiments conducted for **Paper IV**. In this experiment, males were sampled from the wild, and their sperm tested across salinities and their physiology measured. Therefore, there was no acclimation of the males to long-term captivity conditions before exposing sperm to different salinities. Yet, males sampled from Travemünde showed a strong response to salinity when measured with the same methods. These data are presented for ease of interpretation below in Figure 14. This comparison shows that the Travemünde population normally has a strong response to salinity (similar to other brackish populations presented in Figure 12). In conclusion, I can show that there is no effect of acclimation to freshwater (or brackish water) in sperm velocity of at least one, but also possibly two populations.

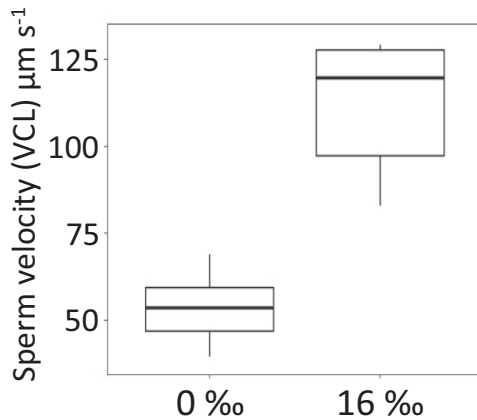


Figure 14. The response in sperm velocity (VCL, $\mu\text{m s}^{-1}$) to salinity of non-acclimated (wild-caught) males from Travemünde used in Paper IV. These data are included for comparison with Figure 13.

In **Paper I**, where we measured sperm velocity in the males' local salinity, and found that this trait increases with generations, selection seems to be the driving force changing sperm velocity for round goby in the Baltic Sea. This analysis was done using synchronous populations (i.e. not the same population at two different time points, but two populations of different age). The use of synchronous populations to calculate "proxy Haldanes" demands some discussion. First, there can be substantial geneflow between the two sites (L1 and L2; e and f in Figure 12) used for the study. These sites are connected by a daily ferry line which could transport larvae and possibly eggs between the sites. Gene-flow between the sites would dampen genetic differences, and any correlated trait differences measured. In isolated populations, the trait change over generations can therefore be higher than our measured H of 0.675. A second issue is that the number of generations for the sites are estimated, and not known. I have used the year that round goby was first reported from the site as the basis for our measurement, but this is a conservative estimate, and the species has likely been present at the sites for longer. These reports from local fishermen and fishery- and environmental agencies at the two sites. Fishing efforts and sampling regimes at the two sites can differ, as can species identification knowledge (round goby is very similar to the Baltic native black goby *G. niger*). If we deem our sources trustworthy, the nature of a species invasion also makes estimations of colonization time problematic. The main issue is that species are unlikely to be reported until population densities are high enough that the fish is observed (either under water or through catch). The estimates of the number of generations at our sites are therefore dependent on population densities, which in themselves can also be an effect of environmental differences (Azour *et al.*, 2015). In my calculations in **Paper I**, I therefore also assume population growth to be similar for L1 and L2. Increased number of generations used in the calculation decreases the H -value. In short, the evolutionary speed reported is to be treated with some caution (as with all models), and should be interpreted as an estimate within a span of higher and lower speeds of change possible for the trait, species and selection conditions.

5.3 WHY THE CONTRASTING RESULTS IN SDG EFFECTS BETWEEN SAND AND ROUND GOBIES?

In **Paper III**, where sperm from sand goby were tested with and without sperm duct gland (SDG) contents, I found a positive effect from sperm duct gland (SDG) contents on sperm velocity. The same methods were also used in **Paper IV**, where I did not find this effect in round goby. Positive effects of goby SDG contents on sperm have been shown in two previous species: grass goby (*Zosterisessor ophiocephalus*), and black goby (*Gobius niger*), both discussed in **Paper III**. In one of these studies (Locatello *et al.*, 2013) conducted on grass goby, the effects were studied from a perspective of male reproductive tactics. However, a control to evaluate the base velocity of sperm without SDG contents was never reported. On the other hand, in black goby, it has been shown that treatment with the nest-holding males' own SDG contents increased their sperm velocity (Poli *et al.*, 2018), similar to our results in **Paper III**. It is possible that the effects, if any, on sperm velocity from SDG contents (or any other non-sperm ejaculate components) are very species specific. For example, in grass goby and the black goby, the SDG of sneaker males functions partly as a sperm storage organ (Scaggiante *et al.*, 1999; Rasotto & Mazzoldi, 2002), and it is still unknown if this is also the case in sand goby or the round goby, and if it occurs in both reproductive tactics. Anecdotally, sperm have been searched for in the SDG contents and inside the organs themselves, in sand goby, but barely any were found (C. Kvarnemo, personal communication). If sand goby indeed store sperm in their SDGs, release of these additional sperm, all mature (since no spermatids would go into the SDG) and possibly fueled by boosting ATP (Locatello *et al.*, 2007), could increase the velocity in a stripped sperm sample compared to one with only sperm from a cut testes. Similarly, if round goby does not store sperm in the SDG, this effect would not show up in the measurements. This could be further researched by continuing the search for sperm in the SDGs, in both round goby and sand goby. For a comparative phylogenetic basis of SDG's in gobies and the placement of sand, black, grass goby, see **Paper III** Supplementary information. Round goby belongs among the Gobiine-like gobies, probably within the *Gobius* lineage together with both grass goby and black goby (Agorreta *et al.*, 2013; Adrian-Kalchhauser *et al.*, 2017b). The lack of similarities is therefore surprising, but again points to the rapid diversification and potential of local adaptation in Gobiidae.

The other trait measured in relation to SDG content in both studies, sperm viability, was in sand goby in **Paper III** tested over time, but on round goby in **Paper IV** only tested once, after a treatment in salinity of about 10 minutes. I did not find any effects at the 10-minute time point in **Paper III**, and neither in **Paper IV**. Viability is expected to drop over time for all species, and since fertilization takes place within seconds (or perhaps minutes) in most externally spawning fishes (Cosson *et al.*, 2008), 10 minutes is a long time to wait until measurement. However, the ecological relevance of testing sperm viability after 10 minutes is threefold: first, the goby nest limits water flow, and sperm released far from the eggs could still be successful in both locating and fertilizing an egg after minutes. The second supporting point for a long-term measurement is the deposition of sperm in SDG-derived mucus trails (for several other goby species), where sperm are released as the mucus dissolves in the water (discussed more in **Paper III**). And third; the females deposit eggs slowly over many minutes, possibly up to an hour (for both the sand and round goby). These phenomena make sperm viability over time a trait that can be relevant for both species to invest in. According to our results however, sperm viability is not positively affected by SDG contents, and the sperm manage to show high viability (in beneficial salinity conditions) by themselves.

In **Paper IV**, where sperm was again tested across several salinities, including freshwater, I found a strong negative effect of the freshwater treatment on sperm regardless of whether the round goby males came from a river environment or an estuary. This also shows that sperm are sensitive to salinity, and would benefit from some mechanism that helps them overcome this problem. I hypothesized that sperm would be aided by SDG contents, but when exposing sperm to freshwater and SDG contents for 10 minutes, there was no clear effect. Since SDG contents do not “boost” sperm in round goby, compared to what was found in sand goby, I expect the negative effect from freshwater to limit the uses of sperm. This led to the conclusion that if a round goby male in freshwater relies on both precise timing and direct proximity to the eggs (i.e. ejaculating directly on top of the eggs) to gain fertilizations, then sneaker tactics, with less control over fertilization timing and proximity (since the nest-holder they parasitize on is guarding the nest), are at a disadvantage. At the moment, we don't know the intricacies of sneaking behavior in the round goby. For sand goby, a range of behaviors expressed by fish of different sizes shows that there is large variation of what constitutes as sneaking. My co-authors and I only included fish that we could catch with a hook and line in the study in **Paper IV**, and therefore, minute sneakers (as those described in Marentette *et al.* 2009) could have been present in the river, but never encountered. Minute sneakers are probably not as limited to imprecise fertilizations, as we can imagine they are small enough to enter a nest without being noticed or chased off. Filming round goby nesting and sneaking behavior can help us understand the variations and limits of male sperm competition in the species.

In **Paper II**, where sperm traits were correlated to fertilization success, I found that low sperm velocity increases fertilization success in freshwater. This data is in part based on fish from river Elbe (see **Paper II**, Table 3), which is the same river fish were sampled from for the study in **Paper IV**. If these results are correct, we can expect that fish in Elbe from **Paper IV**, also express increased fertilization success with decreasing sperm velocity. In this paper we argue that low sperm velocity is a problem when males have little control over the fertilization event (such as when sneaking). However, since the results from Paper II suggests that low sperm velocity is adaptive in freshwater, what effect is this expected to have on sneaking? Since sneakers commonly rely on fast sperm to reach the eggs before the sperm of the nest-holding male, this tactic would still be at a disadvantage, even when slow sperm is advantageous. Importantly, sperm viability in freshwater is also low, which means that while slow sperm might be advantageous, the clock is still ticking, and the longer time they spend searching for an egg, the greater the risk of damage to the sperm. This also supports our conclusions of limits to sneaking success in freshwater. This result can also be highlighted in a comparison of freshwater round goby from the Great Lakes, where sperm velocity was higher than what we measured in freshwater (around $100 \mu\text{m s}^{-1}$ compared to our values of around $70 \mu\text{m s}^{-1}$) (Marentette *et al.*, 2009). Higher velocity in Marentette's study could potentially be attributed to their use of complete ejaculates without spermatids and including SDG contents, or a sign that our measured freshwater population is of a different origin, and relies on low sperm velocity (and perhaps more on sperm viability, due to their trade-off), to enable fertilization.

5.4 WHAT OTHER EFFECTS CAN SALINITY HAVE ON REPRODUCTION BESIDE FROM THOSE ON SPERM?

In **Paper II**, I measured how many spawning groups that initiated spawning after they were either kept in their salinity of catch, or acclimated to freshwater or brackish water (the opposite of their catch salinity for each respective site). This “readiness to spawn” could be considered a crude, but perhaps useable, measurement of condition, since better physiological condition is likely to enable a fish to invest in reproduction. Through these measurement of “readiness to spawn” there are trends in the data that can show us that there are differences in how well fish deal with salinity change. Fish from the brackish environments dealt well with freshwater, as far as measured in their willingness to spawn, and fish from freshwater environments had trouble acclimating to brackish conditions. Mortality was also measured, but not discussed at length in the paper. The highest mortality was found in groups acclimated to a different salinity than their origin (**Paper II**, supplementary table 1), and the most spawnings (although non-significant) was always observed for fish in their salinity of origin. These trends support that acclimating to a different condition is energetically costly. Energetic costs have been shown in the increase of standard metabolic rate, and reduced maximum metabolic rate of round goby with increasing salinities for brackish round goby (Behrens *et al.*, 2017). These costs are mainly attributed to osmoregulation, however, studies

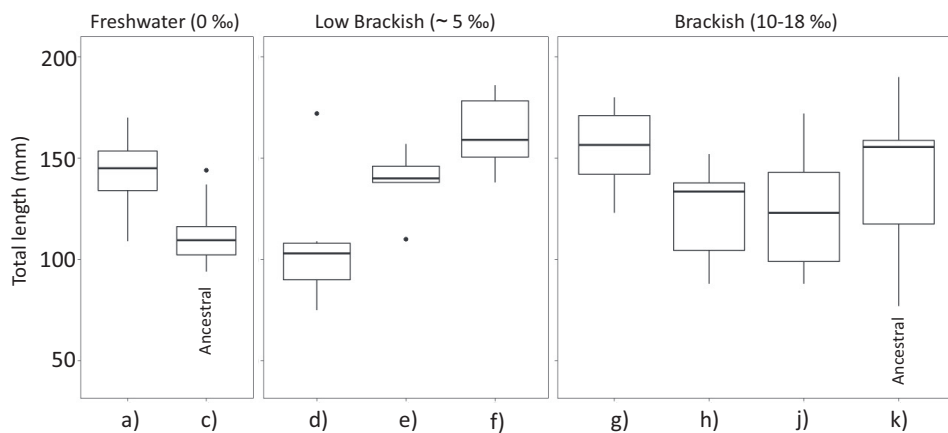


Figure 15. Length as an indicator of size in males used in Paper I and IV, together with males from ancestral populations. Letters correspond to panels in Figure 12. a) River Elbe from Paper IV, c) River Danube, not included in any study, ancestral origin, d) Raahe, low invasion front in Baltic Sea from Paper I, e) Turku, low invasion front in Baltic Sea from Paper I, f) Mariehamn, low invasion front in Baltic Sea from Paper I, g) Kindvig, high invasion front in Baltic Sea from Paper I, h) Karrebæksminde, high invasion front in Baltic Sea from Paper I, j) Travemünde, brackish Baltic Sea from Paper IV, k) Sozopol, brackish Black Sea, not included in any study, ancestral origin. Salinity has no effect (linear model, $F_{2,91} = 0.84$, $P = 0.43$), but populations differ significantly in male total length within each salinity except for brackish (10-18 ‰).

show osmoregulation of brackish and marine fish to make <15% of total energetic costs (Kirschner, 1993). Naturally, these costs would increase in higher salinities for fish adapted to the opposite, but costs have been shown to be very low (< 4 %) in trout acclimating to different salinities (Morgan & Iwama, 1999). Trout (salmonids) are experts at acclimation and equally low costs cannot be expected from other fishes, but these results still point to limited costs for euryhaline fishes compared to stenohaline ones.

Limits on energy budgets can pose potential problems as downstream physiological responses can affect hormone levels and eventually stress responses. There are potentially other long-term costs as well, such as limited energy for immune responses, physiological maintenance, swimming and other energetically demanding behaviors (like courtship). Of course, growth also encompasses energy invested in reproductive organs (and their subsequent gametic output). Energetic status in fish can be indicated by many traits. Size is one, since limited energy allows for less growth. In the sampled populations I can report that size of reproductively active males does not differ between salinity environments (Figure 15). A more detailed description of energy status is the condition factor (Fulton's K) which accounts for an animal's weight to length ratio. When comparing reproductively active males sampled in **Paper IV** and the ancestral region, I find no significant differences in any one group (Figure 16). However, when studying zygote growth in **Paper II**, I found that fish from freshwater origin had significantly worse development, regardless of what salinity they grew in. Are there energy allocation costs to freshwater fish that carry over to reproduction? This is possible. Previous studies have shown round goby from freshwater to be smaller than those caught in estuarine/brackish conditions on average (Kornis *et al.*, 2012). Though I report no such difference in males (rather the opposite, see **Paper IV**), this effect could be wide rather than tied to reproductively active males, but this would need a comparable sampling of females and juveniles from the compared environments. Less energy allocated to into eggs could have effects on zygote growth and development, with potential consequences for adults. In other

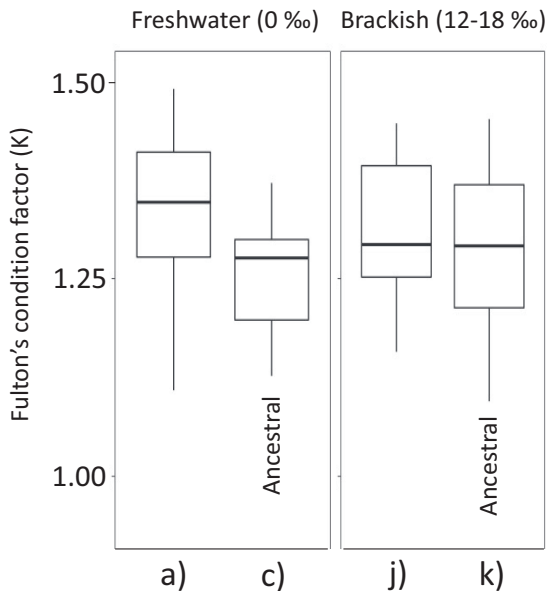


Figure 16. Condition (K) for freshwater and brackish males sampled from the wild. Letters correspond to panels in Figure 12. a) River Elbe from Paper IV, c) River Danube, not included in any study, ancestral origin, j) Travemünde, brackish Baltic Sea from Paper IV, k) Sozopol, brackish Black Sea, not included in any study, ancestral origin. Males from Paper I were not weighed for the study and are therefore not included. Males from Paper II were not weighed before treatments started and are therefore not included. Condition was calculated as $K = 100 \times W / TL^3$ (where W is weight in g and TL is total length in cm).

euryhaline goby species, salinity effects on developmental rate is reported to affect hatching length of larvae (Fonds & Van Buurt, 1974).

If freshwater was a costly environment to colonize, due to osmotic conditions, we could also expect other responses. Round goby population expansions in fresh water have been reported to diminish over time with a 'boom and bust' type growth pattern (Johnson *et al.*, 2005), and this has not been formally reported in brackish areas such as the Baltic Sea (bar the 2018 heat spell, with observed effects on numerous taxa). However, round goby easily invades freshwater, and reports of individuals moving on their own accord into rivers for extended periods of time exists (Christoffersen *et al.*, 2019). Although there seems to be costs, these might be overcome by energy allocation, which I find support for when assessing male condition. In **Paper IV**, I conclude that though the maximum male size is similar for both the river and the estuary, light-coloured males (sneakers) from the river are larger and heavier (better condition) than conspecifics from the estuary. Overall, condition of males is also higher in the river. I interpret this as a consequence of male energy allocation into growth rather than reproductive effort. These results also somewhat support higher costs in freshwater (in **Paper IV**, to male fertilization success, in **Paper II**, to egg development). It has been hypothesized that species from the Black and Caspian seas (sometimes termed the Ponto-Caspian region) are more freshwater tolerant, or more readily adapt into freshwater conditions than species from other brackish regions (Reid & Orlova, 2002; Casties *et al.*, 2016; Paiva *et al.*, 2018). This has been attributed to the geological history of the region, which has at times been brackish, and in times been a freshwater "sea" (the Sarmatian Sea) (Reid & Orlova, 2002). If this contributes to the evolutionary history of round goby, the species could instead be limited in its ability to colonize marine regions.

5.5 WILL SPERM VELOCITY LIMIT ROUND GOBY TO REPRODUCE IN SEAWATER?

There is some support that the species is limited in its ability to fertilize eggs in seawater. Sperm velocity affects how many eggs are fertilized. In **Paper II**, I measured sperm velocity and fertilization success. When looking at males with comparatively low sperm velocity in 16‰ in **Paper II**, there is one male from the Danish straits with an average sperm velocity of $85 \mu\text{m s}^{-1}$ that was able to fertilize a clutch where 90 % of the eggs developed into black eyed zygotes within 20 days. Looking closer at the interval around $85 \mu\text{m s}^{-1}$, roughly 10 % of all males I have tested from the Danish straits in **Paper I** and **II**, in either 30 or 32 ‰, display an average sperm velocity over $90 \mu\text{m s}^{-1}$, and 30 % show a sperm velocity of over $80 \mu\text{m s}^{-1}$ in these conditions. Nevertheless, there is of course variation within this average sperm velocity, with a proportion of sperm swimming with the required velocity for fertilization (a velocity yet to be determined). Provided a given sperm velocity in ≥ 30 ‰ offers the same chance of fertilization as in 16 ‰, we can then expect trait variation for males in the Danish straits to be sufficient to allow a portion of the population to reproduce also in many of the coastal conditions of Norway, eastern Denmark and the Swedish west coast. Combined with the data from Behrens *et al.* 2017 showing a proportion of adults to osmoregulate efficiently even in high salinities, and unpublished data from Niemax *et al.* showing that eggs develop normally (but take longer time) in 30 ‰, it is possible that physiology of adults or gametes are not limiting the expansion of round goby into the North Sea coastlines.

5.6 ARE THERE ECOLOGICAL BARRIERS FOR ROUND GOBY EXPANSIONS?

Another major limit for invasion success is described by the ‘biotic resistance hypothesis’ (Elton, 1958). As ecological complexity increases, niche space becomes limited, and competition for it stronger (Kennedy *et al.*, 2002). In theory, this should limit introduced species as they would become outcompeted by the already locally adapted community. Support for this hypothesis is found on a global scale, as the number of invasive species decreases along the latitudes, with the least number in the tropics. The tropics also harbor the highest species richness. In the marine aquatic environment, this theory is supported in that only two teleosts have ever fully invaded coral reefs (the lion fishes *Pterois volitans* and *P. miles*, both species occupying a niche as piscivorous predators, and also in a globally species poor reef region) (Green *et al.*, 2012; Côté & Bruno, 2015). Interestingly, species richness is generally poor in brackish regions around Europe and brackish regions also suffer the highest number of aquatic species invasions (Paavola *et al.*, 2005). Species richness in the Baltic Sea increases with salinity (Leppäkoski *et al.*, 2002; Snoeijs-Leijonmalm *et al.*, 2016) and niche availability in higher salinities could thus in theory limit round goby distribution. However, its native brackish Black Sea is a complex community of around 180 fish species (at least 9 of which are introduced). In comparison, the North Sea has 201 fish species (in a geographically larger area with more diverse habitats). Species do not only interact with their own infraphylum (or whatever clade level you draw your limit at), but the numbers show us a comparison between regional species richness. One niche in the North Sea is occupied by the closely related black goby, which is very abundant in both simple (sand and gravel bottoms) and complex habitats (eelgrass meadows, rocky shores) along the coastlines. Black goby numbers are decreasing in the Baltic Sea (Andreas Svensson, personal communication), possibly through competition with the larger and more aggressive round goby. If physiological limits from the abiotic salinity environment (or ice-cover and temperature: Leidenberger *et al.*, 2015) are not strong enough, the round goby might be able to annex the niche of the black goby. Ecological consequences from such a species-shift might be minimal, but recent development in the Baltic food webs tells a different story.

6.0 CONCLUSIONS AND FUTURE PERSPECTIVES

The results in this thesis shows that the two studied euryhaline gobies, though similar in ecology and physiology can have different mechanisms that influence their sperm function (**Paper III, IV**). For these externally fertilizing fish, the salinity environment affects their sperm swimming behaviour and survival, and this can influence both evolutionary dynamics (**Paper I**) and life-history trade-offs for some populations (**Paper IV**). Despite these effects, there are adaptations such as low sperm velocity in freshwater (**Paper II**), and mitigation of velocity-viability trade-offs with specific ejaculate substances (**Paper III**), which may be beneficial for reproduction in adverse environments. It is also evident that the round goby, which is highly invasive in many brackish and freshwater regions, have the capacity to reproduce in a wide range of salinities. Population origin matters for where the species can reproduce, but brackish round goby seem robust enough to also spawn in freshwater (**Paper II**). As round goby from freshwater localities did not manage to reproduce successfully in brackish water in our lab experiment (**Paper II**), there is a possibility for differently adapted ‘ecotypes’ or ‘ecomorphs’ from freshwater and brackish origins. Comparisons with freshwater localities in the Black Sea region also supports this (see Figure 12). However, in the highly connected

European water systems (compared to for example the Great Lakes), there is likely a lot of genetic admixture (which is also supported from previous studies: Brown & Stepien, 2008) that can influence what genetic substrate is available, both for the phenotypic expression that limits the species, but also for selection.

There are also several questions left to solve in regards to the studied dynamics. For sand goby, does the SDG contents also affect velocity in the brackish salinities of the Baltic Sea, and is it adaptive in this context? We suggest it could be, but this is still in need of investigation. In the round goby, the limits for its reproduction has not yet been experimentally tested. This is very important for predicting a potential range expansion into the North Sea. During these studies, reports of round goby into salinities close to North Sea coastal ranges have been increasingly documented outside of the city of Gothenburg in Sweden. Though still few in numbers, these observations could represent an increasing ability of this “door-step” population to handle marine conditions, either in their physiology and/or their reproductive capabilities. Since citizen science have been key to many of these observations, further development of these programs and initiatives are likely useful for tracking range expansions. Recent studies looking into the genomic background of the round goby have also highlighted specific genes underpinning osmoregulation (Adrian-Kalchhauser *et al.*, 2020). Looking closer at these across populations, together with these populations’ connectivity, can help us understand where to put in the most effort for range control in order to limit a potential expansion into the North Sea coastlines.

7.0 A FINAL NOTE FOR CONSERVATION AND MANAGEMENT PERSPECTIVES

As mentioned, research on the round goby’s physiology and genome has recently showed it to be tolerant and adaptable when it comes to osmoregulating in different salinity conditions (Behrens *et al.*, 2017; Adrian-Kalchhauser *et al.*, 2020). Compared with the marine sand goby, which has adapted to brackish conditions in the Baltic Sea (Svensson *et al.*, 2017), the round goby mitochondrial genome is potentially pre-adapted for the elevated metabolism demanded by osmoregulation (Adrian-Kalchhauser *et al.*, 2017b). Though we are yet to experimentally test whether the round goby can reproduce in marine conditions, the findings in this thesis show that this is quite likely, at least for some individuals. A further spread along the Swedish west-coast is thus a likely scenario, in which case management stakeholders will have to consider species control in a marine seascape. With our current knowledge, species control of the round goby in marine (as well as in brackish) systems is expected to be costly and unsuccessful (Kornis *et al.*, 2012).

The findings in this thesis also show that round goby of brackish origin can reproduce in freshwater. Therefore, mitigating introduction into freshwater, not only from European rivers, but also from brackish sites, is key to preserving current ecosystems. Control efforts in rivers are possible, but the Great Lakes are telling examples of where lake-systems are heavily impacted by the introduction of round gobies, and management efforts extremely difficult. In a Swedish context, both lake Mälaren combined with adjacent systems, and lake Vänern, are at risk from round goby introductions. Since lake Mälaren has several harbours with trans-European shipping, the risk is potentially greater in this area. Lake Vänern on the other hand lies in direct contact (through the Göta Canal) with the round goby population in the Gothenburg harbour. Efforts to mitigate round goby introductions into these areas should be prioritized. Studies of environmental DNA (e-DNA) provide promising tools for early detection

(preferably already in ballast water) for these scenarios (Adrian-kalchhauser & Burkhardt-holm, 2016; Nevers *et al.*, 2018).

In Sweden we so far have to deal with one single invasive goby, but in mainland Europe, there are another four introduced species; Western tubenose goby (*Proterorhinus semilunaris/marmoratus*), racer goby (*Babka/Neogobius gymnotrachelus*) bighead goby (*Neogobius kessleri*) and Pontian monkey goby (*Neogobius fluviatilis*) (Jakšić *et al.*, 2016). These species are so far only established in river environments. Out of the four, the tubenose and racer gobies are freshwater-only species, but both the bighead and monkey goby also occur in brackish environments in their native Black Sea region. These four species are classified as “knocking on the door” (SV: “Dörrknackare”) in the most recent risk assessment of non-indigenous species in Sweden (Strand *et al.*, 2019). Though the results from this thesis deal in particular with round goby, both the bighead and monkey gobies are closely related to round goby (all reside in the genus *Neogobius*). Therefore, some results can be extrapolated and discussed in the context of bighead and monkey gobies, with some caution. It is likely that both species will be able to survive and reproduce in the Baltic Sea if they are introduced, and it is possible that they can also come under selection and evolve to reproduce better in novel salinities. Though there is a chance that these species will simply be outcompeted by already established round goby, the risk of them spreading to Swedish waters is real, and management plans could benefit from more research on their reproductive ecology.

Increasing global awareness of the human-mediated ecological changes that occurs in the biosphere is likely to trickle down to introduced-species management. This awareness has been growing slowly, but recently at an increasing pace. The treaty of the Ballast Water Convention, adopted by the International Maritime Organization, entered into force in September 2017. The Swedish Species Information Centre undertook a major risk-assessment of introduced species and their ecological effects in 2019 (Strand *et al.*, 2019). School programs to foster citizen science (<http://www.nyaarter.se/>) and mobile applications (<https://www.havochvatten.se/rappen>, <https://artportalen.se/>) designed to simplify species observations and data-management of introduced species are also recent signs of an increasing awareness among stakeholders and society. These signs of awareness and action can be seen in the light of increasing numbers of introduced species (Hulme, 2009), and increasing ecological impacts from these (Doherty *et al.*, 2016). Though the direct societal costs of introduced species are hard to quantify, their impacts on biodiversity are estimated to be second only to direct habitat destruction (Clavero & García-Berthou, 2005). However, once an invasive species is established, management efforts of control are often extremely costly. In a few leading examples, management efforts can be subsidized by the economic uses of the invasive species: in the eastern United States and Caribbean region, lion fish (mainly *Pterois volitans*) are targeted and served in restaurants. On a similar note, a canning industry based on round goby have emerged in Poland. As global fish stocks are decreasing, and introduced species are increasing in number, control efforts based on economic uses of introduced species should be explored in line with our goal of a sustainable future.

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9.0 REFERENCES

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