Ecological, behavioural and molecular aspects enabling invasive round goby translocation via boats

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“It is the unknown that defines our existence. We are constantly searching, not just for answers to our questions, but for new questions. We are explorers.”

– Captain Benjamin Sisko
Star Trek: Deep Space Nine

ABSTRACT

Aquatic invasive species are one of the biggest threats to biodiversity worldwide. Especially in freshwater ecosystems, the introduction of non-native species has led to many detrimental ecological and economic effects. Invasive fish belong to the taxa that exert the most severe impacts on aquatic ecosystems, and globally caused the decline or extinction of native species through predation or competition. Understanding and preventing ways of translocation is crucial to avoid further introductions of invasive fish. The round goby (*Neogobius melanostomus*) is one of the most notorious invasive fish species in European and North American waters. A commonly proposed way of translocation for round gobies is that they lay eggs on boat hulls, which can then be transported within or across water bodies. For example, translocation via recreational boats could help round gobies to spread into the ecologically and economically valuable lakes of Switzerland, which cannot be reached via ballast water transport or active dispersal. However, it is unclear how the bottom-dwelling round goby establishes contact with boats on the water surface. In this study, we investigate potential mechanisms of round goby translocation via recreational boats and derive measures to prevent it by determining which life stages act as propagules, how vector contact is established, and which mechanisms contribute to a successful translocation.

A key behavioural aspect enabling egg deposition on boat hulls is vertical habitat use. Round gobies readily use vertical harbor walls as habitat and use them as beachheads to reach boat hulls, potentially enabling egg deposition there. While practical evidence for round goby eggs on boat hulls is still missing, high breeding frequencies in spawning traps close to the surface confirms that nesting there is a plausible option. Round gobies using vertical habitat are larger and more generalist than those using the bottom substrate during the reproductive season. This differential habitat use by a more competitive subset of the population could help to explain the successful translocation history of round gobies. Another aspect that helps round goby eggs to survive adverse conditions during overland transport is their desiccation tolerance of up to 48 hours. Molecular mechanisms including an enhanced response to oxidative stress, adjustment in protein metabolism and changes in developmental processes contribute to the survival of prolonged desiccation.

The confirmed presence of a round goby larva inside a boat motor shows that there is a second possible mode of translocation via recreational boats. Round goby larvae drift in the water column at night, making their uptake via cooling water intake possible. A compilation of anecdotal reports of fish and other macrofauna inside of boat motor cooling systems from boating forums and the observation of regular presence of boats active after sunset confirms that this mode of translocation can accelerate in-water spread of invasive fish.

Understanding translocation mechanisms of invasive species is crucial for the prevention of their spread. The studies included in this work demonstrate the different ways in which recreational boats can contribute to the introduction of invasive fish into new water bodies and reveal mechanisms that enable vector contact and survival of transport. The results elucidate novel aspects of why certain species are successful invaders and can improve future risk models. The translocation of eggs laid on boat hulls and larvae in cooling water systems can be mitigated by different measures like adjustment of mooring conditions, thorough cleaning of potential nest sites on boats, drying of boats over several days, or emptying and flushing cooling systems before every transport. Applied in management campaigns and communicated to relevant stakeholders, the proposed measures can therefore improve the biosecurity of un-invaded waters.

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Paper II **Bussmann, K.**, Hirsch, P. E., Lehmann, M., Burkhardt-Holm, P. Does differential habitat use contribute to the success of a notorious invader? (manuscript)

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INTRODUCTION

INVASIVE SPECIES IN AQUATIC ECOSYSTEMS

Invasive species are a major threat to biodiversity and species richness worldwide (Gallardo et al. 2016, Mollot et al. 2017, Pyšek et al. 2020). With the help of human transportation, they have colonized terrestrial as well as aquatic realms all over the planet, even the remote and isolated Antarctic Ocean (Sardain et al. 2019, McCarthy et al. 2022). In aquatic ecosystems, invasive species cause a range of ecological and economic problems for the recipient habitats, depending on their ecological niche and lifestyle (Havel et al. 2015, Gallardo et al. 2016, Emery-Butcher et al. 2020). Bivalves, for example, are one of the largest and economically most damaging groups of aquatic invasive species (Sousa et al. 2014). Bivalves act as ecosystem engineers and can transform entire lakes by creating new habitats, acting as a new food resource and filtering the water, thereby changing nutrient content and water clarity, as well as accumulating contaminants that are passed on through the food chain (Emery-Butcher et al. 2020). Their partly excessive growth leads to massive costs for removing them from pipes, boats, and underwater equipment (Sousa et al. 2014, Emery-Butcher et al. 2020).

Even though fish are generally not considered ecosystem engineers, their impacts on recipient ecosystems can be just as altering as the introduction of bivalves (Vitule et al. 2009). Especially the highly diverse and sensitive freshwater ecosystems often suffer from the introduction of novel fish species (Havel et al. 2015, Olden et al. 2021). For example, the introduction of the Nile perch (*Lates niloticus*) into Lake Victoria (East Africa) lead to the extinction of many haplochromine species through predation and competition (Goudswaard et al. 2008). Mosquitofish (*Gambusia affinis, G. holbrooki*) are among the world`s most widespread invasive fish and can diminish zooplankton, fish, and amphibian abundance or richness in wetlands (Haiahem et al. 2017, Tsang and Dudgeon 2021).

The causes of fish invasions are manifold. Many invasive fish species are introduced intentionally for fisheries or biological control agents (Rahel 2004, Pyke 2008, Carpio et al. 2019). Commercially used species often escape aquaculture, ornamental species are released from aquaria (Casal 2006, Vitule et al. 2009, Chan et al. 2019). Another important vector for fish introductions is transport by commercial boats, a major driver of aquatic invasions worldwide (Sardain et al. 2019, Letschert et al. 2020, Olden et al. 2021).

INVASIVE FISH TRANSLOCATION BY BOATS

The two main ways in which commercial ships contribute to the continued global spread of invasive species are hull biofouling and ballast water transport. Ballast water is hereby the better investigated vector when it comes to fish, but even ballast water is poorly characterized regarding the taxonomic potential and extent of fish introductions (Wonham et al. 2000, Bailey 2015, MacIsaac et al. 2015). Many questions about fish translocation via boats therefore remain unanswered.

A successful translocation requires the uptake of a sufficient number of propagules and the survival of propagules during transport (Blackburn et al. 2011, Blackburn et al. 2015). Propagules are hereby individuals of a species that have the potential to found a new population (Lockwood et al. 2005, Cassey et al. 2018) and can refer to any life stage of a fish. To understand fish translocation via boats, it is therefore crucial to know how contact between propagules and their vectors is established, and how they survive transport.

The fish genera which are most commonly translocated by commercial ships (gobies and blennies) are benthic and their tendency to hide in holes and crevices might predispose them for uptake by ballast water (e.g. by hiding in intake systems, (Wonham et al. 2000). Additionally, the specialized lateral line system of Gobiidae might enable them to orientate well and finally survive in ballast water tanks (Parzefall 1986, Wonham et al. 2000).

While ballast water transport as an established primary translocation mechanism of invasive species is already poorly researched for fish, we know even less about potential secondary translocation by small watercraft. Usually, secondary spread happens linearly from a site of first introduction (diffuse or stratified dispersal). This mainly active dispersal however, has its limits: fish cannot overcome certain biogeographic barriers on their own (e.g. large waterfalls or weirs, large patches of unsuitable habitat, land between two water bodies) and without ballast water transport by industrial ships these barriers seem to be the limits of spread for invasive fish. Yet, there are repeated incidences of fish overcoming those biogeographic barriers or exhibit so-called “saltatory dispersal patterns” or “saltation dispersal”, where fish occur in water bodies that are not connected to any established population (Reshetnikov and Ficetola 2011). These movements are often attributed to intentional human activities like angling and bait bucket transport on boats (Drake and Mandrak 2014, Whitfield and Becker 2014, Olden et al. 2021), but rarely to unintentional uptake mechanisms.

For instance, while overland transport via trailered boats is a major distribution pathway for many invasive plants and invertebrates, most of them as part of the biofouling communities forming on boats, transport of fish via trailered recreational boats is less obvious: fish are not sessile and are therefore unlikely to be attached to the exterior of a boat. But there are certain fish that have a reproductive strategy that might allow them to disperse via this unlikely way of transportation: cave-breeders like gobies and blennies, that attach their eggs to hard structures. One species in particular has long been under suspicion to spread with the help of eggs attached to boat hulls: the round goby (*Neogobius melanostomus* Pallas, 1814), a notorious invader in most of Europe and large regions on the North American continent.

THE ROUND GOBY AND ITS SPREAD AS INVASIVE SPECIES

The round goby is native to the Ponto-Caspian region, which is the home range of a number of particularly wide-ranging and disruptive invasive species, successful in a variety of ecosystems. The Ponto-Caspian region extends over the Black, Caspian, and Azov seas and associated drainages. It includes a wide range of salinities, temperatures and habitats, which fluctuated over the past millions of years, and is likely the basis for the especially high environmental adaptability of the inhabiting species (Reid and Orlova 2002, Neilson and Stepien 2009). The Ponto-Caspian invasion was facilitated by the opening of major shipping channels throughout Europe, which opened up new invasion corridors. Meanwhile, the majority of Eastern and Central, and parts of the Western European water systems are colonized by a high number of Ponto-Caspian invaders. Ponto-Caspian invaders also conquered unexpected parts of the world: they colonized large parts of the mostly brackish Baltic Sea and even survived intercontinental ballast water transport to the North American Great Lakes system (Kornis et al. 2012, MacIsaac et al. 2015). The most detrimental Ponto-Caspian species in their invasive ranges include the zebra- and quagga mussels (*Dreissena polymorpha* and *D. bugensis*), the killer shrimp (*Dikerogammarus villosus*) and several species of invasive gobiids, among them the bighead goby (*Ponticola kessleri*), the Western monkey goby (*Proterorhinus semilunaris*) and the most abundant one: the round goby.

The first introduction events leading to the international recognition of the round goby as an invasive fish were in the Baltic Sea at Puck Bay (Gulf of Gdansk) and the St Clair River, which connects Lakes Huron and Erie in North America, both in 1990 (Ricciardi and MacIsaac 2000, Kornis et al. 2012). From there, the round goby colonized most adjacent water systems, and is still increasing its range (Slapansky et al. 2017, Merry et al. 2018, Nogueira Tavares et al. 2020). At most places where they became invasive, other Ponto-Caspian species arrived before them and likely contributed to the rapid establishment and spread of the round goby by providing familiar food resources (“invasional meltdown”, (Ricciardi and MacIsaac 2000, Braga et al. 2018)). But the round goby also has further traits making it a successful invader. Among these traits, the most relevant are a high reproductive output due to high fecundity, batch spawning and parental care (Corkum et al. 1998, Meunier et al. 2009), a high phenotypic plasticity (Meunier et al. 2009, Cerwenka et al. 2014, Hôrková and Kováč 2015, Cerwenka et al. 2017), competitiveness (Balshine et al. 2005, Bergstrom and Mensinger 2009), and a high environmental tolerance (Puntila-Dodd et al. 2020, Christensen et al. 2021).

Round gobies have repeatedly shown saltatory dispersal patterns that are attributed to human transport (Manné et al. 2013, Johansson et al. 2018, Janáč et al. 2019, Bergman et al. 2022). The main secondary translocation mechanisms discussed in the literature are bait bucket release and translocation via recreational boats. Based on a number of early anecdotal reports of round goby eggs attached to boat hulls, several authors have claimed that this way of translocation is likely – however, up to today, no documented evidence for this claim exists (Adrian-Kalchhauser et al. 2017). Still, aspects of round goby biology support the plausibility of egg transport via boats, including the above-mentioned saltatory dispersal patterns. Round gobies readily accept artificial substrate of all kinds as nesting opportunities (Hirsch et al. 2016, N'Guyen et al. 2018). They also exhibit dense populations in harbours (Wiesner 2005, Vélez-Espino et al. 2010), which might increase the probability that a boat is used as nesting opportunity. Taken together, translocation of round goby eggs via boat hulls seems to be possible and warrants attention from a management perspective.

CASE STUDY: THE ROUND GOBY IN SWITZERLAND

The round goby was first recorded in the river Rhine in 2004 in the delta region in the Netherlands (van Beek 2006). The Rhine plays a major role in international shipping and was called a “highway for dispersal of invasive species” (Leuven et al. 2009). In 2012, the round goby was first detected in an industrial harbour in Basel, Switzerland (Rhine km 168, (Kalchhauser et al. 2013). Basel is the most upstream large commercial harbour in the Rhine, commercial ship traffic continues in lower numbers until Rheinfelden, a location 20 km upstream of Basel. Upstream of there, round goby dispersal is limited to either active swimming or passive translocation mechanisms other than ballast water.

The Rhine and its tributaries are highly fragmented by hydropower plants. Fish passes around those hydropower plants are generally seen as a migration barrier to round gobies, which are considered weak swimmers (Wiegleb et al. 2020). Yet, depending on the characteristics of the fish pass, some round goby migration is possible (Egger et al. 2021, Wiegleb et al. 2021). This is corroborated by the slow, but continuous upstream dispersal of round gobies until Laufenburg (46 km upstream of Basel). Switzerland holds large water bodies of concern that are cut off from any route of continuous dispersal by hurdles like the 23 m high Rhine falls (Lake Constance) or an entire lack of connections (e.g. Lake Geneva). In those lakes, the aquatic communities as well as traditional fisheries (Eckmann and Rösch 1998, Gerdeaux 2004, Gerdeaux et al. 2006) might suffer from the introduction of the round goby (Steinhart et al. 2004, Poos et al. 2010, Zorn and Kramer 2021). Preventing the translocation of round gobies into these lakes is therefore the goal of a large Swiss management initiative (goby action plan or "Grundelstrategie" (Dönni 2016)) and was part of the motivation for this study. The investigated questions and results are nonetheless internationally applicable and of relevance for all invaded waterbodies and stakeholder who strive to keep pristine waters uninvaded.

MAIN OBJECTIVES

The main objective of this project was to uncover mechanisms of round goby translocation via recreational boats and to derive measures to prevent it. The following research questions address the main unknowns in the context of these objectives:

1) What are relevant propagules for secondary transport via recreational boats?

2) How is vector contact established?

3) What are the biological mechanisms enabling successful translocation?

ROUND GOBY EGGS AS PROPAGULES

This project started with the hypotheses that round goby eggs can be laid on and transported by boat hulls. Of particular importance for secondary dispersal are recreational boats, which can access smaller rivers and be easily moved between water bodies. A pilot snorkel survey in recreational marinas in the Rhine around Basel did not result in the photographic documentation of round goby eggs on a boat hull. However, two observations provided the basis for the studies presented here. First, we documented a variety of structures on recreational boat hulls, which could be suitable as nesting opportunities for round gobies (Figure 1). Second, we repeatedly observed round gobies using vertical harbour walls, which are close to the moored boats and resemble their hulls. The first observation is important, because round gobies are cave-spawners and it is unlikely that they nest on an open, flat structure like the outside of a boat hull. However, round gobies and other goby species are known to readily use artificial nesting opportunities like tiles, cans, or PVC pipes (Hirsch et al. 2016, N'Guyen et al. 2018). Structures on boat hulls as they are shown in Figure 1 are therefore realistic spawning grounds if round gobies reach boat hulls. The second observation establishes a potential connection between round gobies and their supposed translocation vectors, which is crucial for the plausibility of round goby eggs as propagules, this observation lead to **Paper I**.

Figure 1 Selection of structures on boats that could act as round goby nesting opportunities, photographed between 2018 and 2021 in marinas in and around Basel, Switzerland. Round gobies are cave breeders that readily utilize artificial structures as spawning. Pictures: Karen Bussmann, MGU, University of Basel.

VERTICAL HABITAT ENABLES CONTACT TO BOAT HULLS (PAPER I)

To understand if, and how, round gobies could lay eggs on boat hulls, it is important to find out how the bottom-dwelling round gobies reach boats on the water surface. One clue to this unintuitive connection was the observation that round gobies are not as limited to the ground as they are made out to be. They regularly mount walls up to the water surface. The use of vertical habitats is an important prerequisite for the hypotheses of round gobies on boat hulls, as boat hulls resemble vertical walls more than they resemble the benthic habitat. Additionally, harbour infrastructure is known to act as beachheads for translocation of invasive biofouling organisms (Lacoursière-Roussel et al. 2016, O'Shaughnessy et al. 2020). Yet, the role of harbour walls as habitat and potential beachhead for translocation of invasive fish remains unexplored.

In **Paper I,** we examined differences in habitat use and activity parameters between round gobies on the wall and on the bottom using video recordings in both habitats. Importantly, **Paper I** also documents for the first time direct contact between round gobies and boat hulls. We collected photographic and video evidence of round gobies moving on boat hulls and that they use harbour walls as beachhead to reach them. Additionally, the study revealed that round gobies on vertical habitats score higher in activity parameters like fin beat frequency and movement frequency. This indicates that using vertical walls as habitats comes at the cost of higher energy consumption. According to optimal foraging theory, a behaviour taking up more energy than an alternative behaviour should be compensated by advantages in food abundance, food quality, or other aspects like predator evasion or nesting opportunities. On the other hand, walls might be the energetically inferior habitat and competition drives round gobies to use them. Both possibilities indicate that walls may be used as habitat by different subsets of a population.

ECOLOGY OF VERTICAL HABITAT USE AND CONSEQUENCES FOR TRANSLOCATION (PAPER II)

The potential for differential habitat use by round goby populations living in harbours uncovered in Paper I lead to the questions addressed in **Paper II**. Assuming that round gobies are more likely to ascend boat hulls and reproduce there if they use walls as habitat, we asked: Is wall use limited to a subset of the population? Which characteristics define the subset of the population that utilizes vertical habitats? How does the composition of the population part that utilizes the walls influence the probabilities of successful translocation and invasion dynamics? We used stable isotope analysis and analysis of the population structure to investigate if there are differences in trophic niche and individual diet specialization or in habitat use between the population subsets using harbour walls and bottom. Additionally, we answered the important question if round gobies utilize nesting opportunities on vertical structures, or if spawning is limited to the bottom by quantifying breeding frequencies in spawning traps as a proxy. **Paper II** shows, that the part of the population that uses the walls as habitat during the reproductive season is different from the part of the population using the bottom. Specifically, there are more trophic generalists on the wall and the population is composed of larger and heavier males. Additionally, breeding frequencies are more than 20 times higher on the walls. This confirms that artificial structures off the bottom are readily used for spawning by round gobies, which makes boat hulls an even more relevant vector to consider for their translocation. The results thus point towards a preferential use of vertical habitat by more competitive males, which in turn readily nest in artificial structures close to the water surface. **Paper II** therefore enhances our understanding of the mechanisms enabling round gobies to thrive in harbour habitats, such that they are abundant enough for uptake and translocation. With the potentially translocated clutches being primarily the offspring of more competitive males, this study can also help to explain the high success of introduced round gobies as invaders.

SURVIVAL CAPABILITIES OF ROUND GOBY EGGS DURING OVERLAND TRANSPORT (PAPER III)

After the findings of Paper I and II, there is now ample evidence to suggest that round gobies could realistically nest on boat hulls. Translocation within a water system via boat hulls is therefore plausible and might have occurred on occasion (Manné et al. 2013, Johansson et al. 2018, Janáč et al. 2019, Bergman et al. 2022). Overland transport, however, is another relevant form of translocation for Switzerland, but also anywhere else where an active boating community exists. Overland transport would present a novel challenge to round goby eggs: air exposure and resulting desiccation. In a pioneering publication in the context of round goby translocation by boats, Hirsch et al. (2016) evaluated the risk of overland transport of round goby eggs and their survival after air exposure. They found similar survival rates of desiccated round goby eggs compared to eggs that remained in water for up to 24 h of air exposure. However, it remained unclear what the upper limit of desiccation tolerance for round goby eggs is, and what mechanisms enable them to withstand water withdrawal.

In **Paper III**, we therefore determined the absolute limits of desiccation tolerance of round goby eggs. After finding that some eggs can even survive desiccation up to 48 hours, we additionally investigated the molecular mechanisms behind this remarkable ability to withstand water withdrawal. **Paper III** characterizes the molecular reactions of round goby embryos towards desiccation and finds an increase in protective mechanisms against oxidative stress and DNA damage, adjustments to the cellular protein metabolism, and a short-term attenuation in embryonic development. However, upon rehydration the effects are reversed and at there are no effects of desiccation on the timing of eye spot development or hatching. The ability to withstand water withdrawal will likely prove relevant under future climate change and land use change scenarios: Water levels are projected to decrease and become more fluctuating in the round goby`s current native and invasive range (Grillakis 2019, Konapala et al. 2020). The round goby is, like the majority of fish species, not adapted to extended periods of air exposure, making it an interesting organism to study mechanisms against desiccation damage. The only fish that have been investigated for their molecular reactions and protective mechanisms towards desiccation are the well-adapted species specialized to ephemeral ponds, beach spawning, or semi-terrestrial lifestyle (Tingaud-Sequeira et al. 2013, Thompson et al. 2017, Wagner et al. 2018). In the future, it will be relevant to predict which fish species without these special adaptations are able to adapt to temporally limited desiccation conditions. Especially many freshwater fishes spawn in shallow tributaries or littoral areas that likely will increasingly be influenced by sinking or fluctuating water levels (Lennox et al. 2019). **Paper III** provides a basis for future phylogenetic comparisons of mechanisms promoting survival under air exposure between desiccation-adapted and non-adapted fish.

ROUND GOBY LARVAE AS PROPAGULES

LARVAL DRIFT ENABLES UPTAKE IN BOAT MOTORS (PAPER IV)

While shedding light on the possibility of translocation of round goby eggs on boat hulls, a collaborative project with the local environmental office about boat cleaning practices revealed an additional potential boat part with vector potential (Amt für Umwelt und Energie Kanton Basel Stadt 2019). Thoroughly cleaning the boats also meant disassembling the motors and investigating the contents of the cooling water systems. It turned out that not only can the cooling water volumes be sizeable, but they can also contain sediment and live organisms (Figure 2 C, D). Additionally, the boat mechanics accompanying the project reported that they had seen fish inside the cooling systems before. The grates acting as filters for the water inlets are one to several millimetres wide (depending on the size and brand of the motor), smaller organisms can therefore easily enter the cooling water systems (Figure 2 B). This can either happen actively (the boat stands still and organisms swim inside the cooling systems), or passively (the boat drives and organisms are sucked into the cooling systems).

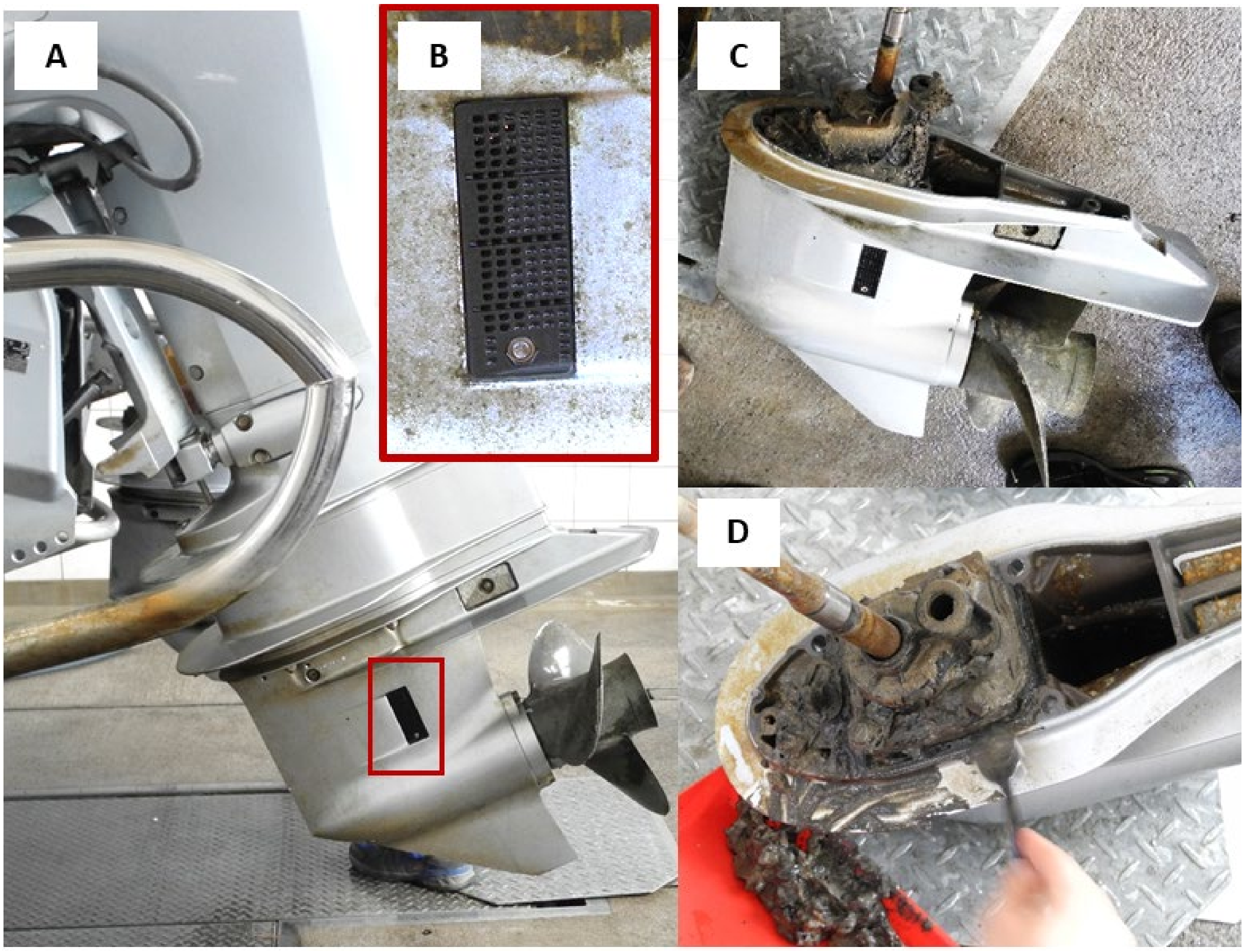


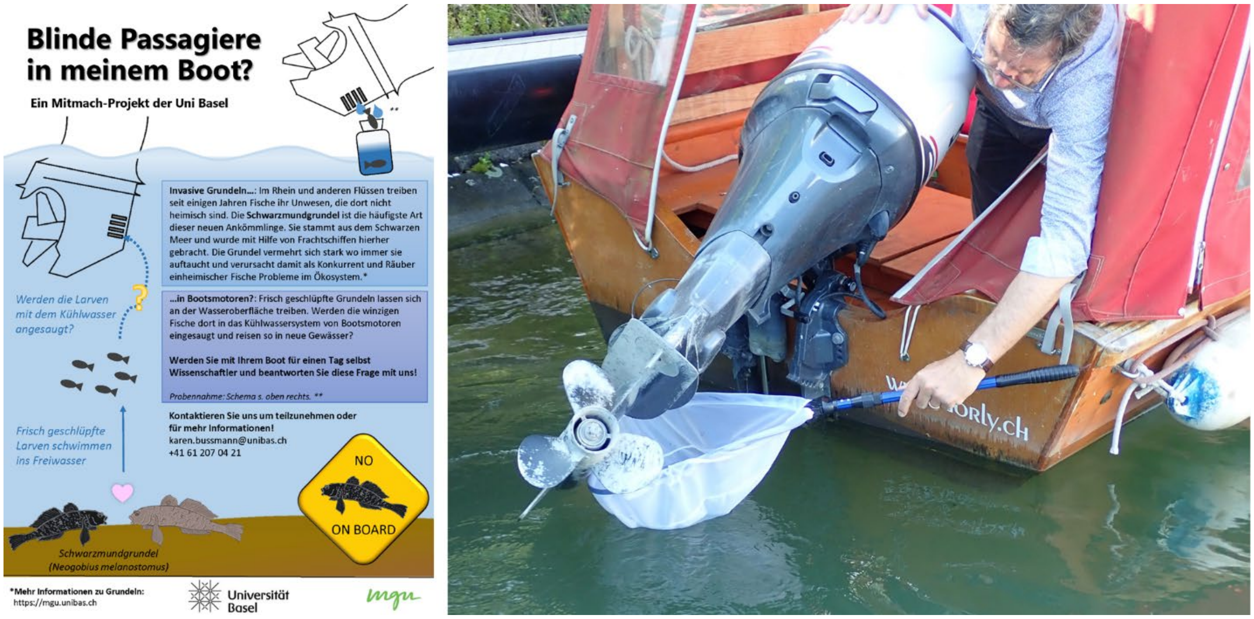
Figure 2 Boat motor with sediments and organisms accumulated in the lower unit. ***A*** *The assembled motor attached to the stern of a boat. The red box indicates the location of the water inlet, which is presented in more detail in* ***B****. The grate in front of the water inlet had a mesh size of 3 mm.* ***C*** *The lower unit of the motor after removal for investigation and cleaning. Sediment can be seen accumulated in the lower unit.* ***D*** *Sampling of the sediment that accumulated in the lower unit.*

The cooling systems and their residual waters have been identified as vectors for the translocation of invasive species before (Darbyson et al. 2009, Kelly et al. 2012, Campbell et al. 2016). However, they did not play a role in the scientific literature about fish translocation. The lack of consideration for small watercraft cooling systems for fish translocation can be attributed to a variety of reasons. First, most studies investigating the potential for small watercraft to translocate invasive species focus on hull fouling (Puth and Post 2005, Murray et al. 2011, Ashton et al. 2014). Second, the majority of studies investigating residual waters only examined boats on land, when most residual water has drained. The volumes left inside the motor then are small and fish that are potentially inside of the cooling systems might not drain with the residual water left, so that they can only be found if the motor is opened. Translocation, however, does not have to happen via overland transport. In-water transport is a major contributor to the spread of aquatic invasive species (Darbyson et al. 2009, Ulman et al. 2019). In this case, boat motors remain submerged, conditions resemble those of the surrounding water, and their vector potential is therefore much higher than during overland transport (Kelly et al. 2012).

A third reason for the underrepresentation of fish translocation via recreational boats in the scientific literature might simply be, that fish do not come to mind when looking for organisms in boat residual waters. How would we expect fish as active swimmers to end up in these residual waters? A phenomenon called larval drift could be the answer to this question. Many fish including the round goby disperse in their larval stages by rising to the water surface during the night and drift with the current (Borcherding et al. 2016, Ramler et al. 2016). In round gobies, this is believed to drive the uptake into ballast water systems of commercial ships and therefore to be a main driver of their long-distance spread (Hensler and Jude 2007, Hayden and Miner 2009). In this work, we aimed to find out whether the same mechanism applies to recreational boats, thereby enabling secondary translocation of round gobies into waterbodies without commercial boating.

EXCURSUS: CITIZEN SCIENCE AS APPROACH TO UNCOVER TRANSLOCATION OF ROUND GOBIES

To investigate the vector potential of recreational boat motors for round goby larvae, we needed the collaboration of boat owners to collect sufficient samples. In a first attempt, we created a citizen science project to motivate boat owners to contribute to this research. Citizen science is an increasingly popular method of conducting research projects including the help of a community of stakeholders and interested people (Fan and Chen 2019, Strasser et al. 2019). The engagement of a non-scientific community thereby furthers mutual understanding and the accessibility of science (Bonney et al. 2015). An established network between our working group at the University of Basel and boating clubs in the High Rhine area seemed a promising starting point for the project. We established and tested a simple method for sampling the motors (Figure 3 right) that should ideally be employed after any boat drive in the evening. Then, we created information material (e.g. Figure 3 left) and contacted the boating clubs in our network and additional clubs in the Upper and High Rhine via email, we personally talked to boat owners in the boat clubs, asked the Basel “Rhine taxis”, boat driving schools, and local shipyards for collaboration. Many boat owners seemed interested in the project during personal conversations about round gobies and their translocation. We deployed sampling material and collection boxes for samples at all collaborators. However, in the end, only seven of them contributed samples, so that we ended up with 48 samples. According to the sampling sheets that the boat owners completed, most of those were collected during the day. While the ratio of contacted to participating people and the deviation from the methods are common problems in citizen science projects (Pocock et al. 2014, Lukas et al. 2017), developing and perfecting the project required more time than we had. We therefore needed a different way to address our research question.



Blinde Passagiere in meinem Boot?

Ein Mitmach-Projekt der Uni Basel

Invasive Grundeln...: Im Rhein und anderen Flüssen treiben seit einigen Jahren Fische ihr Unwesen, die dort nicht heimisch sind. Die **Schwarzmundgrundel** ist die häufigste Art dieser neuen Ankömmlinge. Sie stammt aus dem Schwarzen Meer und wurde mit Hilfe von Frachtschiffen hierher gebracht. Die Grundel vermehrt sich stark wo immer sie auftaucht und verursacht damit als Konkurrent und Räuber einheimischer Fische Probleme im Ökosystem.*

...in Bootsmotoren?: Frisch geschlüpfte Grundeln lassen sich an der Wasseroberfläche treiben. Werden die winzigen Fische dort in das Kühlwassersystem von Bootsmotoren eingesaugt und reisen so in neue Gewässer?

Werden Sie mit Ihrem Boot für einen Tag selbst Wissenschaftler und beantworten Sie diese Frage mit uns!

*Probennahme: Schema s. oben rechts. ***

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***Mehr Informationen zu Grundeln:**
https://mgu.unibas.ch

Figure 3 Left: Flyer for the citizen science project "Stowaways on my boat?" explaining the background, relevance and participation. Right: A participant of the project taking a sample from a boat motor by catching the water in the motor while lifting it, so that as little water as possible is lost.

After the citizen science project was cancelled, we decided to investigate our research question with a different approach, that eventually lead to the first documentation of a round goby larva inside a boat motor and the publication of **Paper IV**. This time, we contacted our network of stakeholders around the Rhine with the question, if they would be willing to lend us a boat and accompany our sampling. Six boat owners agreed and had a boat with a large enough motor to generate considerable volumes of cooling water. Throughout the reproductive season of the round goby in the year 2020 we regularly spent the hours after sunset sampling boat motors while towing a fine-meshed net behind the boat to control for round drifting goby larval abundance close to the water surface. After confirming that round goby larvae can indeed get into the cooling systems of boat motors, we additionally looked for further evidence of fish in cooling systems by conducting a structures internet search. And while the scientific literature did not consider fish translocation via small boat motors, we found evidence in internet boat forums that the uptake of fish, crabs, shrimps or other swimming or drifting animals is not an uncommon incident. Paper IV therefore highlights the potential of drifting larvae to act as propagules for the translocation of invasive fish into uncolonized waters.

PAPER I

Round gobies in the third dimension – Use of vertical walls as habitat enables vector contact in a bottom-dwelling invasive fish

Aquatic Invasions (2020) Volume 15, Issue 4: 683–699

Research Article

Round gobies in the third dimension – use of vertical walls as habitat enables vector contact in a bottom-dwelling invasive fish

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Abstract

Sessile invasive species often efficiently exploit anthropogenic structures, such as harbour walls and pontoons, which can lead to increased vector contact (i.e. contact with boats), and therefore spread rate. The round goby (*Neogobius melanostomus*) is a bottom-dwelling invasive fish species which was never documented on boats or habitats near the water surface. In this study, we wanted to find out if this fish makes use boat hulls and other vertical anthropogenic structures, which could act as invasion beachheads. We inspected boats close to harbour walls in the river Rhine in Basel, Switzerland, to search for gobies on them and documented the position of the boat and the ways the gobies could have reached the hull. We observed round goby presence on three different boats, with up to 28 goby sightings on one boat hull in the course of 45 minutes. Additionally, we recorded gobies on walls between one and five meters above the ground. Based on these observations, we investigated the behaviour of round gobies using vertical walls as habitat and compared the observed behaviours to those exhibited by gobies on the bottom. Gobies used the habitat along a wall in a generally similar fashion to the habitat on the bottom. However, they sat still for less time and moved more on walls than on the bottom, while feeding activity was similar in both habitats. The results raise questions about the drivers for using vertical structures as habitat in the usually bottom-dwelling round gobies and the plasticity of this behaviour. Our study documents round gobies in direct contact with boats for the first time. Potentially, gobies could find hiding places or suitable structures to nest on boats. This study therefore provides support for the theory that boat hulls are potential vectors for the translocation of round gobies. Our observations should lead to an increased awareness about fish and their eggs on boat hulls and stimulate efforts to implement measures like the check-clean-dry routine for commercial as well as private boats.

Key words: *Neogobius melanostomus*, non-indigenous species, translocation, boat, anthropogenic habitat, behaviour

Introduction

Anthropogenic structures can form novel niches in an ecosystem (Connell 2000; Chapman and Bulleri 2003; Bulleri and Chapman 2010). In invasion biology, research focuses on how and by which species these niches are occupied (Tyrrell and Byers 2007; Ruiz et al. 2009; Albano and Obenat 2019). In aquatic environments, anthropogenic structures like walls or pontoons are common in harbor areas, which are the entry point for many invasive species (Connell and Glasby 1999; Airoldi and Bulleri 2011; Foster et al. 2016). Sessile non-indigenous species (NIS) colonize anthropogenic structures easily, where they often outnumber native species (Bulleri and Airoldi 2005; Glasby et al. 2007; Dafforn et al. 2009). Anthropogenic structures may therefore act as critical beachheads, increasing success of establishment and subsequent spread of NIS (Bulleri and Airoldi 2005; Ruiz et al. 2009; Lacoursière-Roussel et al. 2016). Research about the use of anthropogenic structures as habitat mainly focuses on sessile species, while less is known about how mobile species like fish interact with anthropogenic habitats. Studies comparing fish communities between anthropogenic and natural habitats find results ranging from no observable effects, over seasonal differences, up to pronounced effects on species composition and abundance, as well as dependence on type of structure, exposure, or associated epibiota (Rooker et al. 1997; Able et al. 1998; Clynick et al. 2007; Burt et al. 2013; Davis and Smith 2017; Mercader et al. 2018). Little work considered species-specific habitat use and adaptations associated with anthropogenic structures, although the new habitat can cause novel selective pressures. For example, Franssen (2011) showed that anthropogenic habitat alteration can cause persistent population-level differences in body shape of the red shiner (*Cyprinella lutrensis* Baird and Girard, 1853).

Recently, the availability of cheap off-the-shelf underwater cameras has paved a way for direct observations without humans intruding the habitat via e.g. SCUBA diving. Direct observation techniques are among the most effective means for unobtrusively obtaining accurate information about aquatic organisms in their natural surroundings (Sagarin and Pauchard 2010; Thurow et al. 2012; Mallet and Pelletier 2014). Here, we present a case that exemplifies how the application of hand-held and underwater cameras can aid with the detection of conservation-relevant behaviours in an invasive fish species.

The round goby *Neogobius melanostomus* Pallas, 1814 is one of the most notorious invasive fish species throughout Europe and North America (Vilà et al. 2009; Kornis et al. 2012). Round gobies do not possess swim-bladders and are therefore primarily demersal fish. They are especially abundant in harbour areas and readily use artificial materials at the bottom as nesting sites (Corkum et al. 1998; MacInnis and Corkum 2000; Johnson et al. 2005). Together with a single anecdotal observation of round gobies sitting on vertical walls (Hensler and Jude 2007), it seems plausible that they could use pipes, grates and crevices on boat hulls to hide or spawn in spite of their benthic lifestyle (Hoese 1973; Wonham et al. 2000; Johansson et al. 2018). Gobies or their eggs could subsequently be translocated via waterways or even over-land transport (Hirsch et al. 2016). However, the actual observation of how and when this association between propagule and vector occurs is hardly ever made due to a number of limitations, mainly the rareness of the occasion and the impossibility of researchers constantly spending their time in the field to monitor propagule behaviour near vectors. Hence, there is no confirmation of invasive gobies or their eggs on boat hulls published in the peer-reviewed literature until today (Adrian-Kalchhauser et al. 2017).

The main objective of this study was to confirm this postulated, but never documented association: we aimed to find first observational evidence of the presence of round gobies on boat hulls using underwater and hand-held cameras. Additionally, we explored the research question if round gobies show higher activity on vertical habitats than on the bottom by analysing and comparing their movement behaviours on the ground and on concrete walls. Our results document a not yet investigated use of anthropogenic habitat in the round goby, which establishes a connection to potential vectors for translocation.

Materials and methods

Field observations

We visited the industrial harbour Port of Switzerland in Kleinhüningen, Basel, two to three times a week between June and August 2019 (total number of visits: 30) and searched boat hulls for round goby presence in Basel harbour. On each visit, there were between one and three container ships mooring in the harbor that we could investigate. We carried an Olympus Tough TG5 colour video camera (Olympus Corporation, Shinjuku, Tokyo, Japan) to document any round goby presence on boat hulls. We carefully searched for gobies on boats lying at an observable distance to the harbour walkways. To do so, we slowly walked along the side of the boat that was facing the harbour walkway, looking out for gobies moving on the boat hulls. Observations of round gobies on the harbour walls in short distance (20–40 cm) to the boat hulls confirmed the presence of round gobies at the respective mooring sites and our ability to detect them with bare eyes from our position. We took pictures or videos whenever we found round gobies on a boat hull.

We observed a round goby on a boat hull for the first time on 05 July 2019 (Location A, Figure 1). As the boat on which we observed the goby stayed in its position for weeks (private sailing yacht mooring for maintenance), we aimed to repeat the observation and document it on camera. To do so, we carefully searched the boat for a similar event twice a week for *ca.* 15 minutes (total: 24 times) and took photos or videos every time we detected a goby on the boat. Additionally, we installed GoPro Hero 7 black cameras (GoPro, San Mateo CA, USA) at the stern of the boat on five days to film the rudder and stern area for 10–30 minutes (Table 1). We subsequently checked the recorded videos for the presence of round gobies. Differences in recording times arises from the conditions in the harbour. The bottom in the harbour is muddy to sandy, so commercial boat

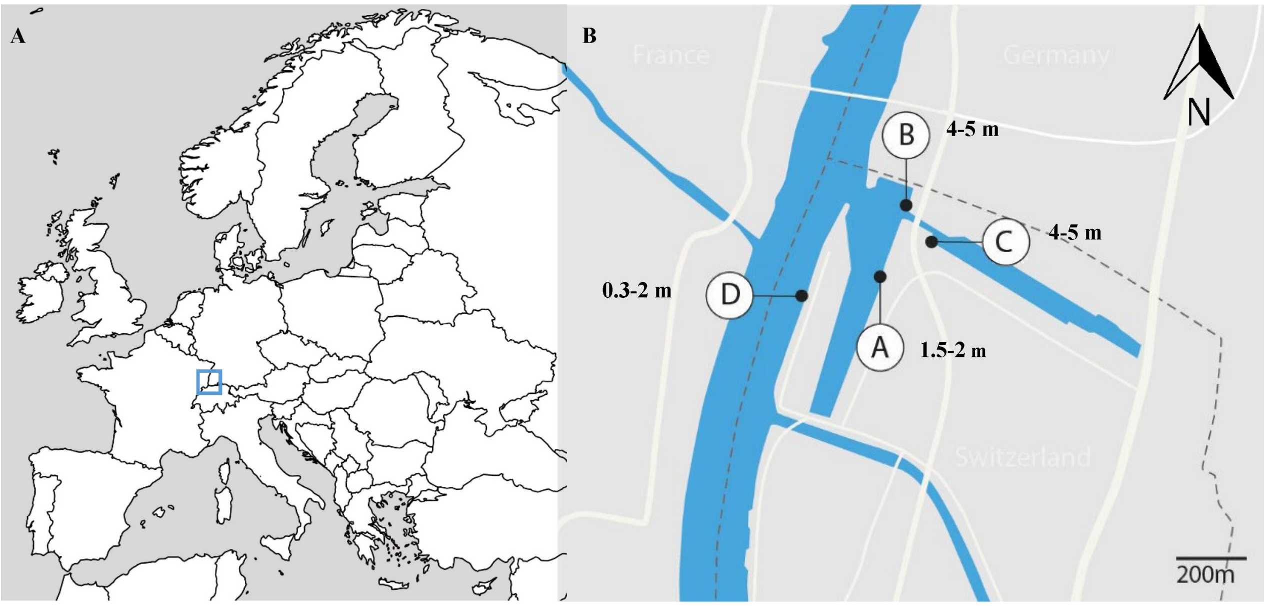

Figure 1. A. Political map of Europe. Blue square indicates the position of Basel, Switzerland; B. Map of the Rhine harbour Port of Switzerland, Basel-Kleinhüningen. Letters indicate the positions of the video recordings of gobies on walls and the bottom below. Numbers next to the letters indicate water depth at the sites. Gobies on boat hulls were present at A, C and D. Videos for behavioural analysis of round gobies on walls and the bottom below were taken at A, B and C.

Table 1. Details of the observations of round gobies on boat hulls. Only observations documented on camera are reported here.

	Date of Record (number of observations)	Location of Record (Figure 1)	Harbour conditions	Boat type	Boat hull
Boat 1 Figure 2	13.08.19 (28)	D	Marina, current (river), no waves, water depth = 0.3–2 m	Commercial ship	Hull entirely covered in biofilm, small patches of macrofouling (algae and mussels). Closest distance to bottom *ca.* 15 cm, hull not moving
Boat 2	10.07.19 (1), 12.07.19 (1), 05.08.19 (5), 09.08.19 (2), 12.08.19 (5), 14.08.19 (2)	A	Industrial harbour, no current, small waves, water depth = 1.5–2 m	Sailboat	Hull almost entirely covered in biofilm, small patches of macrofouling (algae and mussels). Distance rudder-bottom *ca.* 5 cm, hull moving with water motion
Boat 3	17.07.19 (1)	C	Industrial harbour, no current, no waves, water depth = 4–5 m	Container ship	Extensive hull fouling, covered entirely in biofilm and macrofouling, hull in contact with harbour wall, hull not moving

traffic increases turbidity to a point of no visibility at all. Any time a ship entered or exited the harbour, we therefore had to stop recording.

For all three boats on which we found round gobies we recorded characteristics of the boat, location and circumstances under which the observation was made. Specifically, we recorded:

- Boat type
- Hull biofouling presence and composition, based on Floerl et al. (2005)
- Distance of boat to bottom or next harbour wall
- Water depth: estimated based on Port of Switzerland water level particulars

Figure 2. A round goby on the hull of a commercial ship with a layer of biofouling (Boat 1, s. Table 3). The goby was observed to feed and move along the boat hull with as well as against the current.

- Presence of waves and current at time of observation (qualitatively)
- Movement of boat at time of observation

From these observations we developed plausible scenarios, which elucidate how round gobies could have reached the boat hull.

Video recording for behavioural analysis

After observing round gobies sitting on boat hulls we wanted to find out what kind of behaviours they exhibit on these vertical surfaces and if these behaviours are different from the behaviours shown on the bottom. The presence of round gobies on boat hulls was, however, not predictable enough to achieve enough observations of their behaviour. We therefore decided to make use of closely related locations instead, i.e. vertical harbour walls. Gobies on walls were present every time we visited the harbour in our study period between June and August. We collected video material for a behavioural comparison of round gobies on the wall and on the bottom below the wall on four days in July and August 2019 at three different locations in the harbour (Figure 1, Table 2).

The video set-up for the comparative observations of behaviours on walls and the bottom consisted of GoPro Hero 7 black cameras mounted on a concrete block with a 1 m long metal pole protruding in direction of the camera lens, marked with cable straps every 20 cm (Figure S1). We used strings to lower the set-up to the bottom of the harbour, or to the wall close to the water surface. On each site, we placed one camera on the bottom and at the same time one camera on the wall as close as possible to the position of the camera on the bottom. In total, we recorded 16 videos (bottom: 8 videos, wall: 8 videos) suitable for further analysis. We aimed for paired observations, however, on one occasion our set-up for the bottom camera did not reach the ground, so that we could only analyse the

Table 2. Parameters of the videos used for the comparison of behaviours between round gobies observed on either the harbour bottom or vertical harbour walls. Differences in duration of time analysed are due to hindering external conditions (boats in the harbor causing high turbidity). Total N: total number of observations of gobies. Analysed N: Gobies that were in view for > 10 s and therefore part of the statistical analysis. Max N: maximum number of observed gobies in a frame at the same time.

Video Nr.	Habitat	Date	Location	Time Start (hh:mm)	Total time (mm:ss)	Time analysed (mm:ss)	Total N	Analysed N	Max N
1	Bottom	26.07.19	B	12:06	04:38	03:51	15	7	4
2	Wall	26.07.19	B	12:07	04:08	03:32	2	1	1
3	Wall	26.07.19	C	13:24	15:12	11:44	19	14	3
4	Bottom	29.07.19	B	12:09	06:33	05:38	7	3	2
5	Wall	29.07.19	B	11:36	06:58	05:45	20	15	7
6	Bottom	29.07.19	C	11:43	15:28	12:58	50	43	7
7	Wall	29.07.19	C	11:44	13:32	11:14	12	7	3
8	Bottom	05.08.19	A	12:01	18:22	16:22	32	21	4
9	Bottom	05.08.19	A	12:20	17:16	14:41	49	37	5
10	Wall	05.08.19	A	11:59	34:47	34:20	118	85	6
11	Bottom	05.08.19	C	12:01	09:32	08:44	49	40	8
12	Wall	05.08.19	C	11:38	18:09	17:48	6	3	2
13	Bottom	08.08.19	A	12:36	07:23	06:41	2	1	2
14	Wall	08.08.19	A	12:37	06:41	06:00	25	13	4
15	Bottom	08.08.19	B	12:19	09:59	08:50	31	16	4
16	Wall	08.08.19	B	12:22	07:25	07:03	0	0	0

Table 3. Description of the round goby behaviours considered for the analysis of videos taken at the bottom or walls in the harbor. N = number, T = time (s).

Behaviour name	Behaviour type	Description	Variable derived for statistical comparisons
Sitting	State event	Goby sits on one place, with or without fin beats	Percent of time sitting: $\frac{T_{Sitting\ total} \times 100}{T_{in\ view}}$
Fin beat (while sitting)	Point event	Goby moves his pectoral fins: for analysis we only counted fin beats on one side of the body.	Fin beat frequency: $\frac{N_{Fin\ beats}}{T_{Sitting}}$
Hop	Point event	Goby moves forward close to ground, short distance, ≤ 1 pectoral fin stroke	Number of hops per minute: $\frac{N_{Hops} \times 60}{T_{in\ view}}$
Picking food	Point event	Goby picks some food from the bottom/the wall	Number of feeding events per minute: $\frac{N_{Picking\ food} \times 60}{T_{in\ view}}$
Swimming	State event	Goby swims: whole body of goby leaves the bottom, > 1 fin strokes	Percent of time swimming: $\frac{T_{Swimming} \times 100}{T_{in\ view}}$

recording of the wall. On another occasion, we had to relocate the bottom camera amidst the recording, resulting in two videos on the bottom. Table 2 provides details on the videos and the respective circumstances under water. The analysed video sequences were between 4 and 34 minutes long after subtracting camera handling time and time after placement of camera, during which the water was turbid. The difference in times result from increased turbidity due to boat traffic, as described above.

Video analysis

We quantified basic movement and activity parameters that correlate to oxygen consumption and therefore energy expenditure in fish (Trudel and Boisclair 1996; Steinhausen et al. 2005; Tudorache et al. 2008; Table 3). We considered the behaviours of every round goby that stayed within the field of view of the camera for more than 10 seconds. We recorded the duration of the behaviours “sitting” and “swimming” and the number of the behaviours “hop”, “pectoral fin beats while sitting” and “picking food”. We were able to clearly identify the behaviours irrespective of the turbidity of the water with an exception of pectoral fin beats. In videos with a high turbidity we therefore did not count fin beats for gobies of which we could not clearly see the fins. For the quantification of the behaviours we used the program BORIS V.7.9 (Friard and Gamba 2016).

Once an individual left the field of view, it was impossible to decide whether the next individual entering was the same individual or a different one. We therefore counted every round goby entering the field of view as a new observation. The total number of round goby observations was 235 on the bottom and 202 on the wall. Considering only gobies that were in the field of view for more than ten seconds, we ended up with a sample size of 167 for the bottom and 138 for the wall.

We also determined the maximum number (MaxN) of observed gobies in the frame at the same time for every video. MaxN is a widely used, conservative estimate of abundance of a species in video censuses (Whitmarsh et al. 2017). The metal pole that was part of the set-up allowed us to estimate the visibility. With this information we estimated the respective round goby abundance standardized by visibility (MaxN/m).

Statistical analysis

To allow a comparison of the behaviours recorded in the videos on walls and the bottom, we standardized the data gained from the behavioural quantification of a single goby with the time each observation lasted (Table 2). To confirm that there is no difference in the investigated behaviours depending on the duration of the videos, we plotted every behavioural variable against the duration of the video. We did not observe any trends in these plots. Additionally, we took the four videos with a duration of more than twelve minutes and selected the seven first and last individuals that entered the field of view. We conducted t-tests on all considered behavioural variables between gobies observed early and late in the video. There were no significant differences for any of the behavioural variables considered. In total, we recorded 89 minutes of video material on the bottom and 90 minutes on walls.

We inspected each of the derived variables for normal distribution and equal variances visually using histograms and QQ-plots. If the distribution was approximately normal, we used two-tailed student`s t-tests (if variances were equal) or Welch’s t-tests (if variances were not equal) to compare the behaviours that round gobies exhibited on the wall and on the bottom. If the data did not follow a normal distribution, we log-transformed the data before applying *t*-tests. If transforming the data did not result in normal distribution, we used Mann-Whitney *U* tests for the comparisons. We rejected or assumed null hypotheses using a significance level of $\alpha = 0.05$. All statistical analyses were conducted using the stats package in R version 3.5.1 (R Core Team 2019) in RStudio (RStudio Team 2019). Graphs were produced using the package ggplot2 (Wickham 2016).

We are aware that we cannot guarantee complete independence of data for the statistical tests used, as some of the observed gobies could easily have entered the field of view of the camera more than once or could even have been filmed both on the wall and on the bottom. However, this being an exploratory study, we only aimed to quantify observable differences in some basic behaviours that round gobies display on walls as well as the bottom. Therefore, we decided to neglect potential individual pseudo-replication. Considering the high number of observations ($N_{Bottom} = 235$, $N_{Wall} = 202$), and the high goby abundance in the harbour (Bottom: MaxN/m ± SD = 15.67 ± 13.6, Wall: MaxN/m = 9.42 ± 10.0), it is unlikely that behavioural differences between individuals drive major variation in our data. We considered potential site-bias in the data by visually examining all variables separately for each sampling site (Figure S2). We could not fit linear mixed models with sampling site as random factor to quantify the potential site-effect, because with < 5 levels the among-population variance cannot be estimated accurately, and models can be unstable if sample sizes across groups are unbalanced (Harrison et al. 2018). The chosen statistical tests should be able to detect prominent patterns in the data while not taking into account any variability caused by individual or environmental differences.

Results

Round goby observations on boat hulls

Of the *ca*. 40 boats that we inspected, we detected and documented round gobies on three boats mooring in different conditions (Table 1). On boat 1, we documented 28 goby sightings on the hull in the course of 45 minutes. We recorded photos or videos of the hull of boat 2 on eight occasions (Video S3), because it stayed on its position for the entire study period (Table 1). This resulted in six documented instances of goby presence on boat 2 with a total of 16 goby sightings. An initial observation of a goby on the same boat was not documented with a camera. We documented the presence of one goby on the hull of boat 3. All boats had a layer of biofouling on their hulls (Table 1, Figure 2). Round gobies close to the water surface on walls were present every time in varying numbers.

Quantification of behaviours the bottom and on walls

Visual examination of the investigated behaviours for every sampling site separately revealed similar patterns of differences between bottom and wall at every sampling site as in the pooled data, validating the consistency of the measured differences (Figure S2). The only exception to this was mean

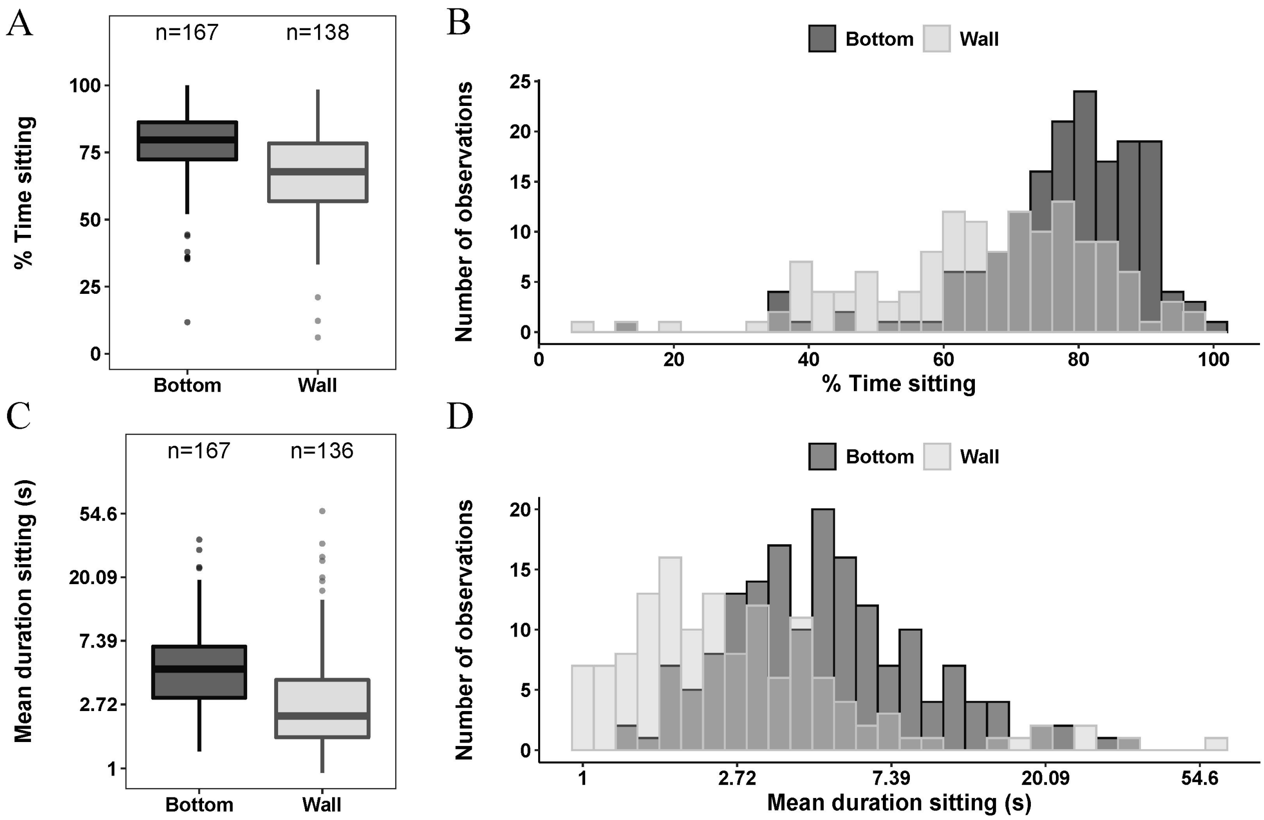

Figure 3. Comparison of time spent sitting still by round gobies on the bottom and on walls. A. Comparison of time spent sitting still (percentage of the total time in view) between gobies observed on the bottom and gobies observed on walls; B. Histograms of the percentage time spent sitting still by gobies on the bottom and on walls; C. Comparison of the mean duration of sitting events between gobies on the bottom and gobies on walls; D. Histograms of the mean duration of sitting events of gobies on the bottom and on walls. Note logarithmic scale in C and D. A, C: Central horizontal lines = median. Boxes = interquartile range. Whiskers = smallest/largest value within 1.5 times interquartile range. Points = outliers. B, D: Overlapping data appears medium grey in histograms.

duration sitting, where no difference in mean duration on walls was visible at sampling site C, whereas the duration was shorter at the other two sites.

Round gobies spent significantly less time sitting still on the wall than on the bottom ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 77.54 \pm 13.3$, $\text{Mean}_{\text{Wall}} \pm \text{SD} = 66.01 \pm 17.4$, *t*-test: $T_{252.18} = 6.39$, $P < 0.0001$. Most of the observed gobies on the bottom spent around 80 percent of the time sitting still, while gobies on the wall spent on average 66 percent of time sitting (Figure 3A, B). The variance in time sitting still was higher for gobies on the wall. Additionally, single sitting events had a significantly lower mean duration on walls than on the bottom ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 5.98 \pm 4.9$, $\text{Mean}_{\text{Wall}} \pm \text{SD} = 4.17 \pm 6.7$, *t*-test: $T_{260.03} = 7.01$, $P < 0.0001$, Figure 3C, D). While sitting still, fin beat frequency was significantly higher on the walls ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 0.01 \pm 0.04$, $\text{Median}_{\text{Bottom}} = 0.00$; $\text{Mean}_{\text{Wall}} \pm \text{SD} = 1.68 \pm 0.57$, $\text{Median}_{\text{Wall}} = 1.78$; Mann-Whitney *U* test: $U = 109.5$, $P < 0.0001$, Figure 4A, B). Additionally, gobies hopped significantly more often on walls compared to on the bottom ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 22.18 \pm 11.9$, $\text{Mean}_{\text{Wall}} \pm \text{SD} = 43.47 \pm 19.3$, *t*-test: $T_{219.01} = -11.31$, $P < 0.0001$, Figure 4C, D).

Round gobies did not feed significantly more often ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 1.81 \pm 4.9$, $\text{Median}_{\text{Bottom}} = 0.00$; $\text{Mean}_{\text{Wall}} \pm \text{SD} = 1.86 \pm 5.3$, $\text{Median}_{\text{Wall}} = 0.00$;

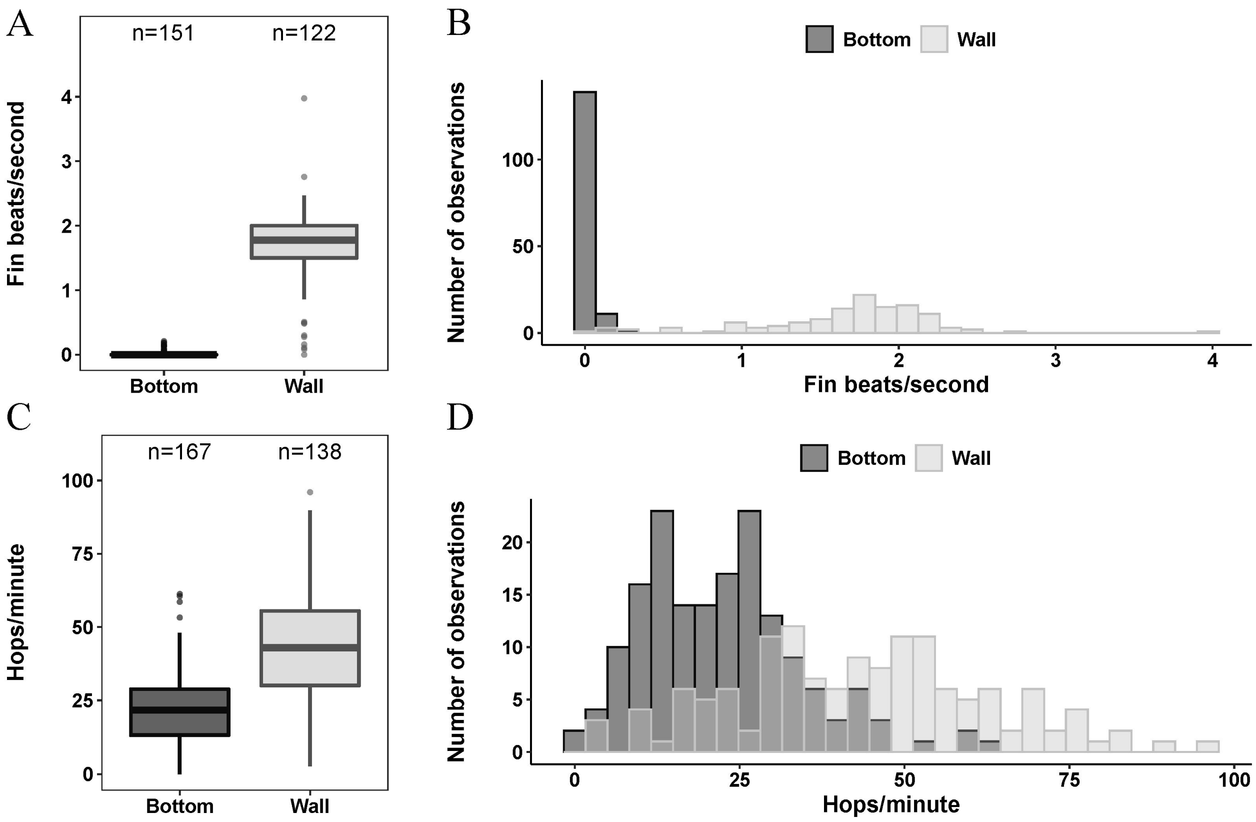

Figure 4. Comparison of activity parameters of round gobies on the bottom and on walls. A. Comparison of fin beat frequency between gobies observed on the bottom and gobies observed on walls; B. Histograms of the fin beat frequency of gobies on the bottom and on walls; C. Comparison of the number of hops per minute between gobies observed on the bottom and gobies observed on walls; D. Histograms of number of hops of gobies on the bottom and on walls. A, C: Central horizontal lines: median. Boxes = interquartile range. Whiskers = smallest/largest value within 1.5 times interquartile range. Points = outliers. B, D: Overlapping data appears medium grey in histograms.

Mann-Whitney *U* test: $U = 11918$, $P = 0.54$), and did not spend significantly more time swimming ($\text{Mean}_{\text{Bottom}} \pm \text{SD} = 1.23 \pm 11.9$, $\text{Median}_{\text{Bottom}} = 0.00$; $\text{Mean}_{\text{Wall}} \pm \text{SD} = 1.27 \pm 2.43$, $\text{Median}_{\text{Wall}} = 0.00$; Mann-Whitney *U* test: $U = 11918$, $P = 0.54$) in any of the habitats.

Further documented behaviours on walls

Apart from the behaviours that we quantified, we also made noteworthy observations of goby behaviours in our videos and in the field. The following list names behaviours that might prove important to understand the use of walls as habitat and for further studies:

Males in spawning colouration: We observed male round gobies with black colouration on few occasions on the bottom as well as on walls. Black colouration is a sign of reproductive activity in nest-guarding male round gobies.

Sitting in holes in walls: We observed round gobies entering holes in harbour walls during fieldwork and in one video. In that video it seems like the hole is used as a nest by a goby, however, due to high turbidity and bad lighting we cannot confirm this without doubt.

Catching insects or feeding above water surface: We observed round gobies sticking their head out of the water or even jumping out of the water on several occasions during fieldwork (partly documented on photo or video). One of these observations included clearly the capture of an insect that was sitting above the water surface (not documented).

Accumulation of round gobies on walls in boat shadow: On very sunny days, we observed round gobies on walls accumulating in the shadows of boats, while we rarely observed any gobies sitting in the open sun.

Sitting upside down: On locations B and C the harbour walls consisted of a concrete wall and an overhanging sheet pile wall 20 cm in front of the concrete wall. In videos that filmed walls sideways, we observed gobies sitting completely upside down on these overhanging sheet pile walls. Gobies also regularly swam up and down between both wall parts.

Holding on to structural elements using the ventral fin: We observed gobies sitting down on the metal pole that was part of the camera set-up, and on structural elements on the walls (e.g. zebra mussels, *Dreissena polymorpha* Pallas, 1771). Thereby, gobies wrapped their fused ventral fin around the respective structure and held on to it for some seconds before moving on.

Bighead gobies (*Ponticola kessleri* Günther, 1861*)* on walls: Although this study only considers round gobies, we also observed bighead gobies on walls. We did not consider bighead gobies in a similar level of detail here, because the number of observations was much lower.

Discussion

Round goby presence on boat hulls

Here, we present the first photographic and video documentation of round gobies on boat hulls. Our study thereby provides support for the hypothesis that gobies can be translocated by boats via mechanisms other than ballast water intake (Hirsch et al. 2016). On a boat, gobies can feed, hide and possibly even lay eggs if they find suitable structures like holes, pipes or other openings. This holds the potential of the unwanted translocation of hidden gobies or attached eggs, like proposed by several authors (Hoese 1973; Moskal'kova 1996; Wonham et al. 2000; Johansson et al. 2018). This mechanism is especially important in areas without commercial shipping, because wherever commercial ships exchange ballast water, this is likely the most important vector for invasive fish (Wonham et al. 2000; Kotta et al. 2016; Johansson et al. 2018). Recreational boats are known as a major vector for a wide range of invasive species, specifically those occurring in biofouling communities (Minchin et al. 2006; Rothlisberger et al. 2010; Murray et al. 2011). However, fish have rarely been associated with this dispersal mechanism. Our data stresses that there are ways in which the invasive round goby can get in contact with recreational boats.

Combining our observations of round goby presence on boats and the characteristics of the boats and their positions, we developed four plausible scenarios how round gobies could have reached the boats:

1. Swimming up a short distance to reach a boat mooring close to the ground. Like this, gobies can reach boats in shallow water. Our observations show that gobies swim upwards short distances also in a moderate current.
2. Using a part of a boat that is close to the bottom to reach the hull (e.g. ascending the rudder). Gobies can reach boats mooring in medium water depths like this. Our observations show that gobies ascend boat parts also when the boat is moving due to wave action.
3. Ascending a wall and moving onto a boat that is in direct contact to that wall. Like this, gobies can reach boats mooring far from the bottom.
4. Ascending walls and swimming to a boat without direct contact to the wall from there. We did not directly observe gobies on a boat in such a position. However, we consider this way to reach a boat plausible, because we observed round gobies swimming small distances side- and upwards to overhanging sections of walls.

We only observed round gobies on boats that were close to the ground or a wall. Walls can therefore indeed be considered as beachheads for vector contact, drastically reducing the distance that gobies have to overcome to reach boat hulls in deeper water. Importantly, this does not exclude the possibility of round gobies reaching boats that are further away from bottom or walls. In the presented work, we were limited to observations from shore. We therefore cannot make statements about boats that were outside of our visible range.

We detected round gobies on only three out of *ca.* 40 investigated boats. However, the high repeatability of the observations on a boat that stayed stationary for more than two months and the high number of round gobies on the boat that was closest to the ground indicate that this behaviour is nothing out of the ordinary if the conditions are right. Limitations for the observations of container ships that can interfere with detailed investigation include for example the limited time they spend in one place, ongoing unloading and loading of goods, or maintenance work on the hull. It is therefore possible that round goby presence is more common than we were able to document. Our data shows that boats that are moored close to the substrate, remain at one place for a while, and show at least patches of biofouling are readily used as habitat by round gobies.

The ubiquity and practicality of nowadays cameras will enable further discoveries and documentation of conservation-relevant behaviours and instances like the herein described ones (Sagarin and Pauchard 2010; Thurow et al. 2012). An increasing interest of volunteers from the general public in observing nature, e.g. in citizen science projects, can also lead to more frequent documentation of infrequent and unpredictable behaviours researchers struggle to detect (Beckmann et al. 2015; McKinley et al. 2017).

Round goby behaviour on walls and the bottom

The use of artificial vertical structures like concrete walls and boat hulls by the round goby is a largely neglected aspect of their behaviour. Apart from a side-note in Hensler and Jude (2007), we are not aware of any literature describing round gobies on vertical walls – Ghedotti et al. (1995) even found that round gobies left any mussels or snails above 20 cm off the bottom untouched in feeding experiments. However, the two mainly bottom-dwelling genera gobies and blennies are worldwide among the most commonly translocated fish families (Wonham et al. 2000). Their high success in establishing populations in their arrival areas has been attributed to their creviculous nature and their resulting ability to make use of harbour habitats (Wonham et al. 2000).

The quantified movement behaviours indicate that using walls as habitat is likely more energy consuming than staying on the bottom for round gobies. Without a swim bladder, moving up several meters and staying there while constantly having to work against sinking down causes gobies to sit still for less time, hop more often, and exhibit an increased fin beat frequency while staying on walls compared to on the bottom. Increased number of movements and increased fin beat frequency are correlated to oxygen consumption and therefore metabolic costs in other fish (Trudel and Boisclair 1996; Steinhausen et al. 2005; Tudorache et al. 2008). Although we did not have the data to calculate bioenergetics models for the determination of the actual metabolic costs in the round goby, it is reasonable to assume a correlation between the measured behaviours and energy expenditure similar to other fish.

If a behaviour takes up more energy than an alternative behaviour, it should be compensated by other advantages like access to more food or more valuable food resources, or higher security from predators, according to optimal foraging theory (McNamara and Houston 1985; Bartumeus and Catalan 2009; Mikheev et al. 2010). We did not observe an increased feeding frequency in gobies on walls compared to gobies on the bottom. This could be due to the short time we got to observe individual gobies before they left the field of view again, or that we recorded them during day time instead of night time, when feeding activities peak in round gobies (Johnson et al. 2008). Another possibility is that there are more energy rich food organisms, or that food organisms are easier accessible on the wall compared to on the bottom. The bottom at all sites was sandy to muddy, while the walls were covered with biofouling, probably harbouring differing invertebrate communities. Zebra mussels, a known major food source for round gobies (Diggins et al. 2002; Lederer et al. 2008; Angradi 2018), were present in both habitats.

Round gobies on walls are seemingly more exposed to predators than round gobies on the bottom due to a lack of refugia and proximity to the water surface, which makes them visible e.g. to fish-eating birds. Although we did see native fish in our videos (e.g. European perch *Perca fluviatilis* Linneaus, 1758, a known predator of round gobies: Liversage et al. 2017), we did not observe any predatory interactions between species in any of the videos. It hence remains unclear whether using walls as habitat actually exposes round gobies to a higher predation risk.

Another reason for using walls as habitat could be high competition at the bottom. If there are not enough resources for the whole population on the bottom of the harbour, individuals could try to migrate out of the area (Chuang and Peterson 2016), or alternatively make use of an unoccupied niche dimension: the harbour walls. For example, food competition is suggested to cause the spread of populations lead by migrating adult round gobies (Gutowsky and Fox 2011; Azour et al. 2015; Brandner et al. 2018). In other regions, juveniles are reportedly more common at the invasion front (Ray and Corkum 2001; Brownscombe and Fox 2012). Similarly, either large, strong gobies could be the ones primarily moving up walls, or rather young and light ones, who might be outcompeted on the bottom. Investigating the distribution of individuals between the two habitats would help to evaluate the ecological and behavioural significance of wall climbing for the respective individuals and populations. Further studies using controlled experimental set-ups should investigate the influence of demographic and environmental variables on the use of walls as habitat. Understanding the use of vertical anthropogenic structures could add important knowledge about behavioural repertoire, population dynamics and invasion progression of the round goby (Mikheev et al. 2010; Wright et al. 2010; Chuang and Peterson 2016).

Conclusions

Round gobies regularly use vertical anthropogenic structures including boat hulls as habitat. Although their energy consumption is likely higher on vertical structures, round gobies display the same behaviours there as on the bottom. Round gobies are therefore likely to use walls and boats as equivalent habitat to the bottom, including behaviours like foraging and possibly nesting. The hypothesis that round gobies or their eggs are translocated on boat hulls gains additional relevance with the herein presented observations. Measures to prevent the spread of round gobies by boats should hence not only consider ballast water, but also the control and cleaning of boat hulls including hard-to-reach areas like pipes and grates.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Camera set-up for the observation of round gobies on harbor walls.
Figure S2. Results of the behavioural comparisons of gobies observed at the bottom and gobies observed on walls separated by sampling site.
Figure S3. Video of a round goby on the rudder of a recreational sailing boat.

This material is available as part of online article from:
http://www.reabic.net/aquaticinvasions/2020/Supplements/AI_2020_Bussmann_Burkhardt-Holm_SupplementaryFigures.pdf
http://www.reabic.net/aquaticinvasions/2020/Supplements/AI_2020_Bussmann_Burkhardt-Holm_Video.mp4

Online supplementary Information

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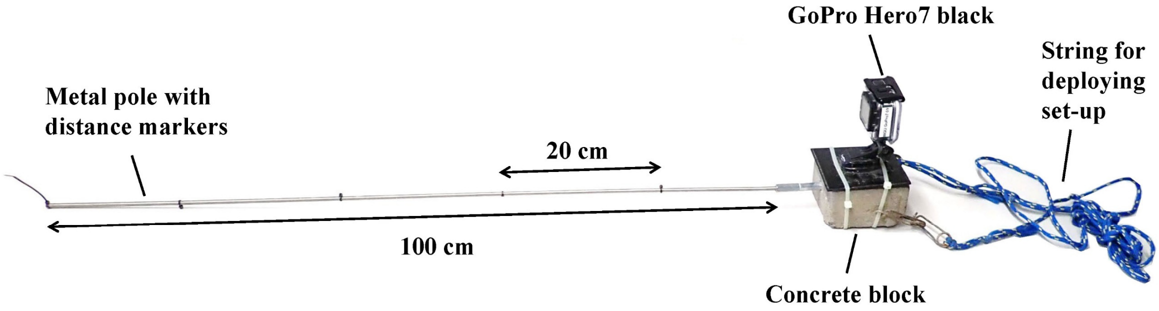

Figure S1. Camera set-up for the observation of round gobies on harbor walls.

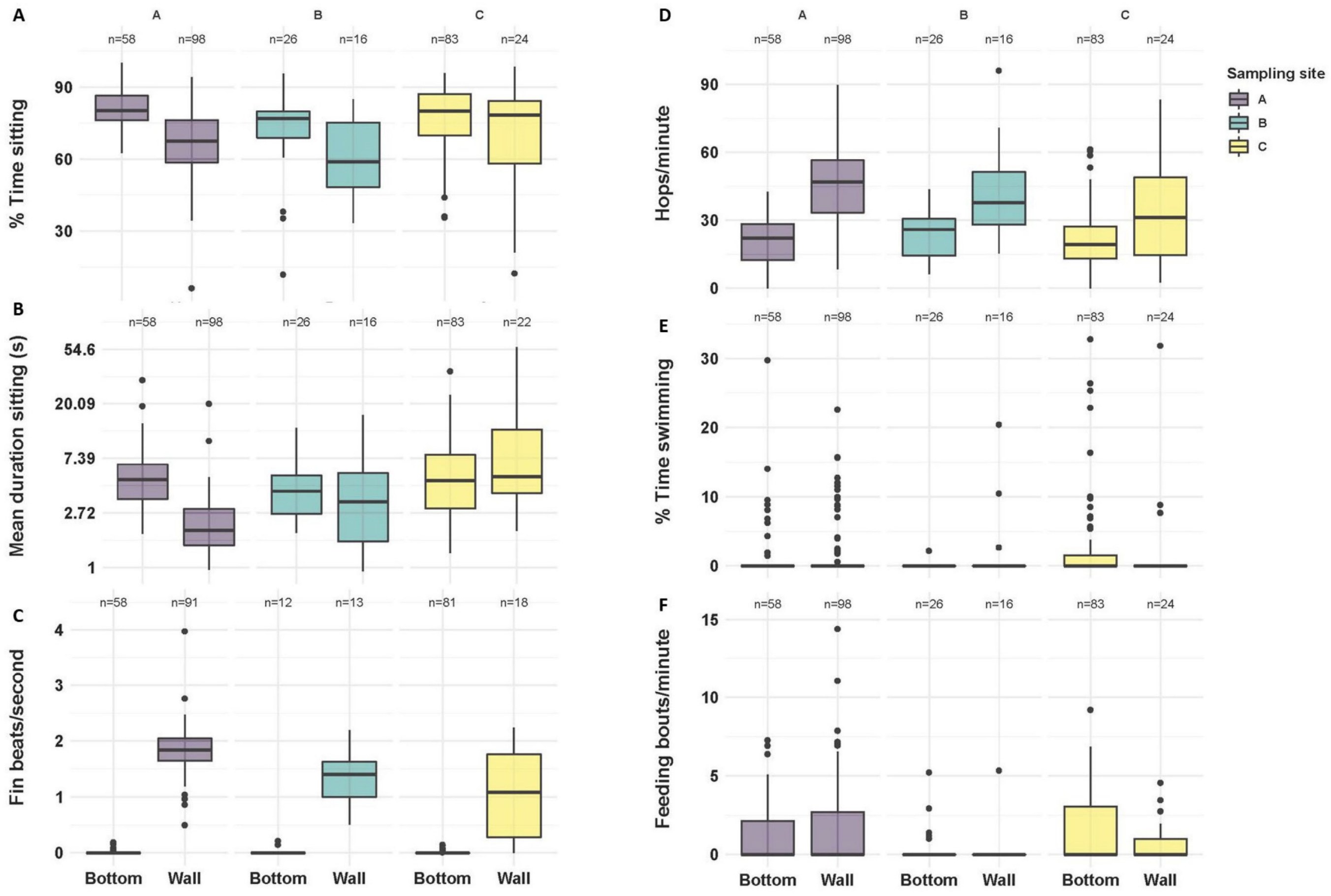

Figure S2. Results of the behavioural comparisons of gobies observed at the bottom and gobies observed on walls separated by sampling site.

S3 Video of a round goby on the rudder of a recreational sailing boat. Video taken on 25. August 2019, Camera: Olympus Tough TG5, frame rate: 25 fps. [Still from the video (duration: 00:00:22, size: 83’186 KB)]

PAPER II

Does differential habitat use contribute to the success of a notorious invader?

DOES DIFFERENTIAL HABITAT USE CONTRIBUTE TO THE SUCCESS OF A NOTORIOUS INVADER?

Karen Bussmann, Philipp Emanuel Hirsch, Moritz Lehmann, Patricia Burkhardt-Holm

ABSTRACT

Anthropogenic structures can form novel niches in ecosystems. Invasion biology in particular deals with species composition and habitat use in these anthropogenic niches, as invasive species are often especially successful in occupying these habitats and use them as beachheads for further translocation. The highly invasive round goby (*Neogobius melanostomus*) readily uses vertical harbour walls as both habitat and bridge to reach boat hulls (potential translocation vectors). As the round goby is inherently a bottom-dwelling fish, this behaviour can be considered as a niche expansion. Here, we investigate vertical habitat use in round gobies compared to their traditional habitat on the bottom. Specifically, we studied whether round gobies caught on wall and bottom differed in trophic niche size and location as well as individual specialization, phenotypic traits, and breeding frequency. Round gobies exhibited habitat partitioning during the breeding season. Trophic niches overlapped but were distinguishable between the habitats. On the walls, there were more trophic generalists than specialists, while on the bottom, there were more trophic specialists. Breeding frequencies were higher on the walls than on the bottom. After the breeding season, a higher similarity in trophic ecology and a different phenotypic composition of the population in both habitats indicated movement from the walls towards the bottom. Taken together, this study reveals the possibility of selective breeding and therefore microgeographic adaptation to either horizontal or vertical habitat use in round gobies. As male gobies using the walls in the breeding season are larger and heavier, we hypothesize that wall climbing behaviour would select for more competitive individuals. Because walls can act as beachheads to reach boat hulls, new founding populations might be dominated by this more competitive subset of the round goby population in harbours. The ability to efficiently exploit anthropogenic habitats and the higher likelihood of competitive individuals to interact with translocation vectors could also be part of the explanation for the high invasion success of round gobies. The use of vertical habitats should also be considered as additional habitat with additional numbers of individuals. Such higher population densities, could lead to higher propagule pressure in harbours.

INTRODUCTION

The ecological niche of a species can be described by multiple dimensions; diet and distribution in space are among the most commonly investigated ones (Ingram et al. 2018). Flexibility in niche use is often considered a typical feature of successful invaders (Wright et al. 2010, Chuang and Peterson 2016). Niche expansion by dispersal and broadened resource utilization is also common under conditions of increased intraspecific competition (Svanbäck and Bolnick 2007, Mateus et al. 2016, Mendes et al. 2019), as it is often the case in invasive species populations. We recently observed that the round goby (*Neogobius* *melanostomus,* Pallas 1814), a benthic fish considered “one of Europe`s 100 worst invasive species” (Vilà et al. 2009), use vertical walls up to the water surface as habitat, foraging ground, and as beachheads to reach potential translocation vectors, i.e. boat hulls (Bussmann and Burkhardt-Holm 2020). Because of their normally bottom-dwelling nature and preference for shallow slopes (Jakubčinová et al. 2018), this behaviour can be considered an expansion of their traditional niche, enabled by the presence of anthropogenic habitat.

Population niche expansion can be driven by all or most individuals each utilizing both the traditional and the novel niche dimension (generalists), or by a partitioning of the population with some individuals specializing on the novel niche dimension and others utilizing their original niche (specialists) (Bolnick et al. 2003). In the first case, the between-individual differences in resource use in a population are low, while the within-individual differences are high. In the latter case, the between-individual differences in resource use in a population are high, while the within-individual differences are low. These options are two ends of a spectrum of possibilities, with the proportion of generalists and specialists within a population being variable. In fact, generalist populations are commonly comprised of specialized individuals utilizing different parts of a niche (Araújo et al. 2011). Which and how many individuals specialize may depend e.g. on the availability of different niches (Bolnick and Ballare 2020), the amount of intra- and interspecific competition, or predation pressure (Araújo et al. 2011). For example, in racer gobies (*Babka gymnotrachelus,* Iljin 1927), spatial niche expansion of the population under conditions of high intraspecific competition is driven by subordinate individuals with lower growth rates, while dominant males occupy shelters in the original habitat (Grabowska et al. 2019).

The round goby`s diet is typically broad (trophic generalists, Borcherding et al. 2013, Brandner et al. 2013), and many exhibit site fidelity during the reproductive season (Ray and Corkum 2001, Lynch and Mensinger 2012, Brandner et al. 2015). The existence of anthropogenic vertical habitat that likely harbours different benthic food organism communities than the bottom, might provide opportunities to specialize in either habitat use, diet, or both. Additionally, vertical habitat use might be correlated to phenotypic traits, as it is likely linked to higher energy expenditure (Bussmann and Burkhardt-Holm 2020). A suspected translocation mode for round gobies is that they lay eggs on boat hulls, which are then moved to un-colonized areas (Adrian-Kalchhauser et al. 2017). Round gobies readily use walls as beachheads to reach boat hulls (Bussmann and Burkhardt-Holm 2020). Based on the resulting assumption that gobies utilizing vertical habitats (or their eggs) are more likely to be translocated, we aimed to understand which part of the population exhibits this behaviour in- and outside of the reproductive season.

In this study, we investigate if the use of vertical walls as habitat is 1) a distinguishable trophic strategy of a part of the population, 2) if the level of individual trophic specialization differs between the habitats, and 3) if habitat choice is correlated to phenotypic traits of the gobies. We expect the results to represent one of three hypothetical scenarios (Figure 1): In scenario A, all individuals in a population exhibit wall climbing (= trophic niches not distinguishable, high within-individual differences and low between-individual differences in trophic resource use, no differences in phenotypic traits between the habitats, Figure 1 A). In this case, wall climbing would indicate generalism in both diet and habitat use. In scenario B, a specific fraction of the population forages on walls in addition to the bottom, while another part of the population stays on the bottom (= overlap of niches with unique regions in wall-utilizing individuals, within-individual variation in resource use larger on the wall than on the bottom, potentially differences in phenotype, Figure 1 B). In this case, wall climbing would be a niche expansion in diet and habitat use of a subset of the population potentially defined by a demographic feature (e.g. sex), a certain phenotype (e.g. size, weight, condition), or behaviour (e.g. foraging strategy). In scenario C, wall climbing is exhibited by a completely separate part of the population than the one using the bottom (= no overlap of niches, low within-individual variation in both habitats, differences in phenotype, Figure 1 C). In this case, wall climbing would be a specialization in habitat use, a sign of development of subpopulations or ecotypes.

We base our hypotheses on scenario B, because we observed some individuals moving between the habitats in the videos used in Bussmann and Burkhardt-Holm (2020). However, the quantification of movement activities of round gobies in Bussmann and Burkhardt-Holm (2020) indicates that individuals utilizing walls use a higher amount of energy than those on the bottom, as they do not possess a swim bladder and exhibit high fin beat frequencies and movement on vertical walls. Using walls for foraging might therefore be a behaviour exhibited by only a subset of the population.

The hypotheses formulated for our study were:

H1: The trophic niche of round gobies caught on the walls is distinguishable in size and position from the niche of gobies caught on the bottom.

H2: The number of individual diet specialists is lower in gobies using the walls as habitat than in those using the bottom.

H3: Round gobies using the walls are distinguishable from those using the bottom by their body size, weight, and condition.

The hypotheses were investigated both during and after the reproductive season, as round gobies show different movement and space use patterns between the seasons that could influence their ecology and their translocation probabilities. Most round gobies exhibit site-fidelity during the reproductive season with males either guarding nests or following a sneaker tactic where they enter a nest while a female spawns and try to “steal” fertilizations from the nest-guarding male (Ray and Corkum 2001, Lynch and Mensinger 2012). Outside of the reproductive season they show higher mobility or even migration patterns (Behrens et al. 2021, Carlson et al. 2021).

To complement the niche-based hypotheses, we compare breeding frequencies of round gobies in vertical and horizontal habitats during the reproductive season to find out if an opportunity for microgeographic divergence based on differential habitat use exists (Richardson et al. 2014, Maciejewski et al. 2020), and if translocation of eggs on boat hulls is a realistic possibility (Adrian-Kalchhauser et al. 2017).

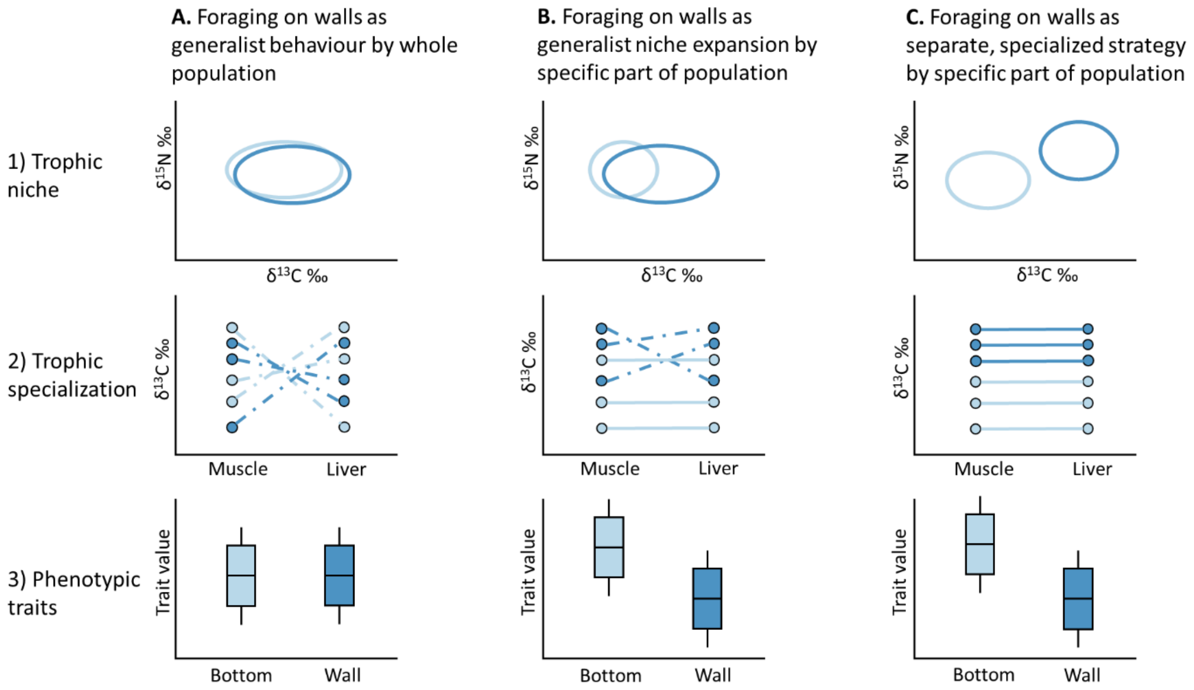

Figure 1 Schematic overview of expected results under different scenarios. ***A.*** *The whole population of round gobies uses both the bottom and the wall habitat flexibly. In the isotope space (1), there would be no difference between size and extension of the trophic niche of specimen caught on the bottom or the wall. All individuals would show a generalist feeding strategy, with high variation between long-term diet (muscle δ*^{13}*C isotopes) and short-term diet (liver δ*^{13}*C isotopes, 2). There would be no difference in body traits between specimens caught on the bottom or the wall (3).* ***B.*** *The whole population uses the habitat on the bottom, but some specific individuals extend their niche to the vertical walls. In the isotope space, the trophic niche of gobies caught on the wall would be larger than the one of gobies caught on the bottom, while there would be a high overlap (1). Individuals utilizing the wall would exhibit larger variation between long-term and short-term diet than individuals only using the bottom (2). The part of the population utilizing the walls would exhibit different traits from the one on the bottom (3).* ***C.*** *The population is separated in two parts: one utilizing exclusively the bottom, the other part exclusively the walls. In the isotope space, the trophic niches would be small and differently located (1). All individuals would show a low variance between long-term diet and short-term diet (2). The part of the population utilizing the walls would exhibit different traits from the one on the bottom (3).*

MATERIAL AND METHODS

TROPHIC ECOLOGY AND POPULATION TRAITS

STUDY SITE AND SAMPLE COLLECTION

The study took place in the commercial harbour Kleinhüningen in Basel, Switzerland (47°35'10"N 7°35'27"E). We sampled the local round goby population twice in the year 2020: 19th August - 04th September (reproductive season); and 28th September - 23rd October (post-reproductive season). We chose a sampling period late in the reproductive season, because we analysed the trophic ecology of round gobies using stable isotope analysis (s. below); a method that integrates dietary information over a period of up to several months (Fry 2006). Sampling in the late breeding season therefore allowed us to analyse the isotopic signatures of approximately the entire breeding season.

We brought out baited minnow traps along a 150 m long stretch of harbour wall (depth 3 - 5 m, Figure S1). We installed minnow traps on the wall at < 1 m depth (reproductive season: 4 traps, post-reproductive season: 8 traps). We submerged minnow traps on the bottom 3 - 8 m off the harbour wall (reproductive season: 2 traps, post-reproductive season: 4 traps). We emptied the traps every second day, euthanized all caught gobies and transported them back to the laboratory on ice.

In the laboratory, we determined the sex of all round gobies by the shape of their urogenital papilla (broad rectangular for females, triangular for males, not identifiable for juveniles, Marentette et al. 2009), measured standard length (SL, ± 1 mm), and recorded wet weight of the blotted dry fish (± 0.01 g). The condition factor Fulton`s K was calculated with the formula K = [100*wet weight, g]/[standard length, cm]^3. We tested for differences in the abundance of males and females using Chi-Square tests. We tested the measured traits for differences between wall and bottom-caught individuals, and between sexes within each season using linear models (trait value as dependent variable, sampling period, habitat and sex as fixed factors including all possible interactions) using the package lme4 version 1.1-27.1 (Bates et al. 2015, R Core Team 2021). For Tukey`s HSD post-hoc comparisons between habitats within season we used the package and emmeans version 1.6.3 (Lenth 2021). We conducted these and all further statistical analyses in R version 4.0.5 (R Core Team 2021).

After taking all measurements, we extracted the whole liver of each goby and removed a ca. 1 x 1 cm piece of skinless and boneless muscle tissue from the caudo-ventral region of the fish. The tissue samples were individually stored on -80 °C until further processing for stable isotope analysis. For stable isotope analysis, we focused on a subset of the round goby population that fell in a standardized range of size and condition. We aimed to eliminate the influence of confounding factors like the well-documented dietary switch of round gobies from arthropods to molluscs around 10-13 cm total length (Brush et al. 2012, Miano et al. 2021), and body condition (Karlson et al. 2018) to focus on habitat-related differences in individual specialization. Therefore, we excluded juvenile individuals (n = 3), individuals with a standard length of ≥ 10 cm (n = 9) and with a condition factor K ≤ 1.7 or K ≥ 2.4 (n = 11). Additionally, we excluded individuals with a visible parasite or skin infection (n = 5) or a notably receded liver (very small and dark red as opposed to large and pink, n = 2). From the remaining 230 round gobies, we chose the individuals closest around the mean of condition factor as well as standard length of the entire population to end up with a sample size of ± 30 individuals per habitat and sampling period.

We collected ten small specimens of the killer shrimp *Dikerogammarus villosus* (Sovinskij 1894) as a representative of amphipods, and of the zebra mussel *Dreissena polymorpha* (Pallas 1771) per sampling season and habitat (27. August and 30. October 2020) to calculate the trophic position of the round gobies and estimate baseline variation of $\delta^{13}C$. *D. villosus* is a known main food organism of round gobies in European waters (Borcherding et al. 2013, Brandner et al. 2013), and incorporates microhabitat differences in $\delta^{13}C$ (Brandner et al. 2015). *D. polymorpha* is a filter feeder and is a baseline representative of the pelagic food web, which we included in the figures as a comparison to the benthic baseline we used for the analyses. We kept the baseline organisms in tap water for > 24 h to empty their guts before processing them (*D. villosus* whole specimen, *D. polymorpha* soft tissue) for stable isotope analysis.

STABLE ISOTOPE ANALYSIS

We prepared tissue samples of round goby muscles and livers as well as *D.* villosus and *D. polymorpha* for stable isotope analysis by drying all samples at 60°C for > 48 h and grinding dried samples to a homogenous powder using mortar and pestle. We weighed 1 mg ± 20 µg of each sample into 5 mm x 9 mm tin cups (*D. villosus* whole specimen). We then ran samples for $\delta^{13}C$, $\delta^{15}N$, C%, and N% on two IRMSs (isotope ratio mass spectrometer). Information on the IRMSs standards used for elemental analysis and instrumental precision are presented in Supplementary document S1.

Because lipids are ^{13}C depleted, a high content of lipids in tissues can influence the $\delta^{13}C$ values of that tissue (DeNiro and Epstein 1978, Focken and Becker 1998). Especially for tissues with high fat content like livers, it is necessary to correct for the lipid content of a sample to enable a comparison with low-fat content tissues like muscle (Post et al. 2007, Logan et al. 2008). We used a subsample of eight round goby livers to establish an equation for lipid correction of the rest of the samples. We ran stable isotope analysis on these eight livers before and after chemical lipid extraction based on a protocol of Bligh and Dyer (1959) as modified by Turschak et al. (2014). In short, a 2:1 chloroform-methanol solution was added to the dried tissue homogenate, the mixture was periodically agitated for 30 minutes and then centrifuged before decanting the supernatant. This procedure was repeated three times. We determined the lipid corrected $\delta^{13}C_L$ using the formula $\delta^{13}C_L = \delta^{13}C_0 + \beta_1 * C{:}N_0 + \beta_0$. In the formula, $\delta^{13}C_0$ and $C{:}N_0$ are the $\delta^{13}C$ and C:N of the sample before lipid extraction, and β_1 and β_1 are the slope and intercept of the linear relationship between $C{:}N_0$ and $\Delta\ \delta^{13}C$ (i.e., $\delta^{13}C_L - \delta^{13}C_0$). Details of the methods and outcomes of lipid correction are presented in Supplementary document S2.

TROPHIC NICHE: SIZE, OVERLAP AND POSITION

To compare the size and position of the trophic niche occupied by round gobies on the bottom and wall during and after the reproductive season, we calculated the so-called standard-ellipse areas (SEA) based on muscle isotopic composition using the package SIBER version 2.6.1 (Jackson et al. 2011). The SEA describes the size of the core niche of a population in a biplot of $\delta^{13}C$ versus $\delta^{15}N$, including 40% of the data points (Jackson et al. 2011). We corrected SEAs for small sample sizes by removing one degree of freedom (SEA_c). For statistical comparisons of niche size and overlap, we fit Bayesian multivariate normal distributions to each group in the dataset (10^5 posterior draws) and then calculated the SEA on the posterior distribution of covariance matrices thereby yielding the Bayesian SEA_B. The SEA_B has been shown to reliably represent the niche size of the real population even when based on small sample sizes (Jackson et al. 2011).

Niche size: To compare the niche sizes between wall and bottom, we calculated the proportion of posterior ellipses in one habitat that were smaller than the posterior ellipses of the other habitat both during and after the reproductive season. This proportion equates to the probability P of the bottom niche being smaller than the wall niche (Jackson et al. 2011). We assumed niche size to be significantly different at a probability of $P > 0.95$.

Niche overlap: We used the mean overlap of the first 1000 posterior ellipses (SEA_B) to calculate the proportion overlap between the niches of gobies caught on wall and bottom during and after the reproductive season. We assumed the overlap of two niches to be significant if it was > 60 % (Wallace 1981, Guzzo et al. 2013, Pettitt-Wade et al. 2015). A high proportion overlap between bottom ellipses and wall ellipses would support scenario A; a smaller proportion overlap of the wall ellipses than proportion overlap of the bottom ellipses would support scenario B; a small or no overlap between the bottom and wall ellipses would support scenario C (Figure 1).

Isotopic ranges: Apart from the SEA_B we also calculated the $\delta^{15}N$ range and of $\delta^{13}C$ range of the gobies caught on wall and bottom habitats. The isotopic ranges hereby represent the isotopic distance between the individuals with the most enriched and depleted $\delta^{15}N$ or $\delta^{13}C$ values in the population, as described in (Layman et al. 2007). We bootstrapped the isotopic ranges (n = 10 000, indicated with a subscript 'b') based on the minimum sample size in the data set (n = 28) to allow comparison among populations (Jackson et al. 2012).

Niche position: To compare the position of the trophic niche between bottom- and wall-caught round gobies, we calculated linear models using the mean $\delta^{13}C$ and $\delta^{15}N$ as dependent variables and habitat and sampling period (including their interaction) as fixed factors. We calculated the same models for the amphipods used as baseline organism and compared differences in round goby isotopes to differences in amphipod isotopes. Nitrogen isotopes of a species are representative of its trophic position, since its value increases stepwise with each consumer by about 3.4 ‰ (Post 2002). As the $\delta^{15}N$ of a consumer is dependent on the $\delta^{15}N$ of the food sources, trophic position of the consumer has to take into account the $\delta^{15}N$ of a system-specific baseline organism. Here, we calculated the trophic position (TP) of round gobies using the equation from Post (2002) for secondary consumers: $TP = \lambda + (\delta^{15}N_{\text{round goby}} - \delta^{15}N_{\text{base}})/\Delta_n$, where λ the trophic position of the baseline organisms (assumed to be 2 for the amphipod *D. villosus*), and Δ_n is the enrichment factor per trophic level (assumed to be 3.4 ‰).

IDENTIFICATION OF INDIVIDUAL TROPHIC SPECIALIZATION

We based our analyses of trophic specialization on temporal variation of carbon isotope composition. Carbon isotopes change little with trophic level of a consumer, but rather reflect their primary carbon source and are therefore suitable to investigate variation in diet choice (Layman et al. 2012). Muscle and liver tissues are frequently used to measure temporal variation in diet, because muscle tissue has a longer turnover time than the metabolically active liver tissue and the two tissues therefore integrate dietary information over different time frames (Boecklen et al. 2011, Bond et al. 2016). We therefore used muscle isotopic composition as proxy for “long-term” and liver isotopic composition as proxy for “short-term” diet. We calculated for each individual the difference between muscle and liver $\delta^{13}C$ ($\Delta\delta^{13}C_{M-L}$) using the formula $\Delta\delta^{13}C_{M-L} = \delta^{13}C_M - \delta^{13}C_L$. We compared $\Delta\delta^{13}C_{M-L}$ between habitats using paired t-tests.

Individual trophic specialization is measured by comparing the contributions of two components of the total niche width (TNW), the within-individual component (WIC) and the between-individual component (BIC, Roughgarden 1972). The WIC describes the variance in resource use within individuals, while the BIC describes the variance in resource use among individuals (Roughgarden 1972). To determine which individual was feeding on a more specialized vs. a more generalized diet, we determined the average Euclidean distance of the absolute values of $\Delta\delta^{13}C_{M-L}$ between all individuals per season. The resulting values represent the variation of diet shifts between individuals (BIC) of a population. $\Delta\delta^{13}C_{M-L}$ was considered the dietary variation of each individual (WIC). We therefore considered an individual a trophic specialist, if $WIC < BIC$, and a trophic generalist, if $WIC \geq BIC$. After identifying which individuals are trophic specialists or generalists, we compared the proportion of specialists between habitats within seasons and between seasons within habitats using a generalized linear model including sex and standard length as covariates (binomial distribution, log link). Additionally, we compared if trophic specialists and generalists were distinguishable by their position in the niche. For this comparison, we calculated linear models with muscle $\delta^{13}C$ or $\delta^{15}N$ as dependent variable and sampling period, habitat and specialization as fixed factors including all possible interactions using the packages lme4 version 1.1-27.1 (Bates et al. 2015), and emmeans version 1.6.3 (Lenth 2021) for Tukey`s HSD post-hoc comparisons.

SAMPLING OF POTENTIAL FOOD ORGANISMS

We collected samples of the macroinvertebrate communities every second week from May to August 2020 on walls and bottom to characterize the habitats. We did not aim for a quantification of food resources in both habitats, but rather for a qualitative description supporting our understanding of the differences in trophic niches and habitat use of round gobies.

Habitat differences and temporal dynamics in the composition of macroinvertebrate communities over the round goby reproductive season can help interpret differences in long and short-term diets. Additionally, differences in composition of food organisms between the habitats help to disentangle round goby trophic niche differences and habitat use. For example, if walls and bottom did not differ in the composition of food organisms, a larger trophic niche of round gobies on the wall would indicate feeding on a higher variety of food resources, but it would be unclear if gobies would use the walls in addition to the bottom, or instead of the bottom. If the habitats differ in food organism composition, a larger niche size on the wall would likely go along with a more distinguishable niche position in the isotope bi-plot. In this case, the larger niche size would also indicate the use of a higher variety of food resources, but could be linked more clearly to differential habitat use.

To sample the walls, we scraped off layers of biofouling at 2-4 spots ad libitum and collected them in a vial filled with 70 % ethanol. To sample the bottom, we used a Van Veen grab sampler and collected the sediment in a bucket. We then sampled the 2 – 3 cm of the sediment into vials filled with 70 % ethanol.

In the lab, we identified the first 100 macroinvertebrates found in a sample under a dissecting microscope. To randomize the choice of organisms, we transferred the entire sample into a container with fresh water, where we gently stirred up the sample and randomly extracted material to examine using a plastic pipette (3 ml). We identified the macroinvertebrates to the lowest practical taxonomical level (Order or Family). We did not quantify sessile macroinvertebrates (e.g. bivalves), as our methods were not suitable to sample organisms firmly attached to the substrate.

USE OF NESTING OPPORTUNITIES

To investigate if gobies use nesting opportunities on vertical surfaces, we built five spawning traps (based on (N'Guyen et al. 2018), Figure S1) that could be deployed along vertical walls as well as on the bottom underneath a wall. For each spawning trap, we attached two batteries of each five PVC pipes (diameter: 4.5 cm, length: 20 cm) to a metal chain. The distance between the two pipe-batteries was 2.5 m. We deployed the traps at five sampling sites in the harbour Kleinhüningen by lowering the traps along the harbour wall until one pipe-battery reached the ground, while the other battery was flush with the wall 2.5 m above the ground (Figure S2). Earlier studies confirmed that round gobies readily accept these traps as nesting opportunities (Hirsch et al. 2016, N'Guyen et al. 2018).

Between 05th May 2020 and 24th August 2020, we removed the traps and searched the PVC pipes for evidence of round goby spawning twice weekly. Evidence of round goby spawning was either the presence of clutches with intact eggs, or traces of clutches (attachment filaments and empty eggshells). After photographing any evidence for spawning, we removed the clutch or the clutch traces and re-deployed the trap with cleaned pipes.

RESULTS

TROPHIC NICHE: SIZE, OVERLAP AND POSITION

We caught a total of 95 round gobies in August and 162 round gobies in October. In August, we caught 46 gobies on the bottom and 49 on walls. In October, we caught 97 gobies on the bottom and 65 on walls. Three gobies caught in August on the bottom were juveniles and we did not use them for further analysis. All other gobies were used for the analysis of phenotypic traits (see below). The standardized subset of all caught gobies, which we used for the analysis of stable isotopes, is presented in Table S1.

Niche size: The trophic niche size of gobies caught on the bottom was significantly larger than the trophic niche size of the gobies caught on the walls during the reproductive season (probability of SEA being larger on bottom = 0.998, Figure 2, Table 1). The trophic niches were similar in size after the reproductive season (probability of SEA being larger on bottom = 0.653 , Figure 2, Table 1).

Niche overlap: The trophic niche of round gobies caught on walls overlapped significantly (> 60 %) with the trophic niche of the round gobies caught on the bottom during the reproductive season (Figure 2, Table 1). On the contrary, the trophic niche of round gobies caught on the bottom did not overlap significantly with the trophic niche of round gobies caught on walls during the reproductive season (Figure 2, Table 1). The trophic niches of round gobies caught on bottom and wall both overlapped significantly with each other after the reproductive season (Figure 2, Table 1).

Isotopic ranges: Round gobies caught on the bottom had a wider $\delta^{13}C$ and $\delta^{15}N$ range during the reproductive season (Figure 2, Figure 3). The $\delta^{13}C$ range of gobies caught on the bottom was wider than the $\delta^{13}C$ range of gobies caught on the wall, while there were no differences in $\delta^{15}N$ range between the habitats after the reproductive season (Figure 3).

Niche position: The mean niche position of round gobies was significantly higher in $\delta^{15}N$ on the walls than on the bottom, but did not differ in $\delta^{13}C$ in both seasons (Table 2, Figure 2). Amphipods did not differ in $\delta^{15}N$ between the habitats, but significantly differed in $\delta^{13}C$ after the reproductive season (Table 2, Figure 2). The direction of change in $\delta^{13}C$ from reproductive to post-reproductive season in round gobies matched the direction of change in amphipods (Figure 2).

The trophic position of round gobies was higher on the wall than on the bottom in both seasons – however, this difference was only significant after the reproductive season (Table 2, Figure 4). In both habitats, the trophic position of round gobies was significantly lower after than during the reproductive season (Table 2, Figure 4), driven by a significant increase in $\delta^{15}N$ in amphipods (Table 2, Figure 2).

It is noteworthy that the results of all isotopic analyses are strongly influenced by outliers of > 2 standard deviations in $\delta^{15}N$ values among the gobies caught on the bottom (two outliers during the reproductive season, one outlier after the reproductive season, Figure 2, Figure 4). There were no technical or biological reasons to exclude these outliers. However, as these outliers are conspicuous, we conducted the same isotopic analyses that are presented here without the outliers. In summary, without the outliers the SEA of the bottom niche was similar in size to the SEA of the wall niche during the reproductive season and smaller after the reproductive season. The wall niche did not significantly overlap with the bottom niche and had therefore a larger unique area in both sampling seasons. The bootstrapped range of $\delta^{15}N$ on the bottom was smaller than the $\delta^{15}N$ range on the walls. All figures and results of the analyses without outliers are presented in Supplementary document S3.

Table 1 Niche characteristics of round gobies caught during and after the reproductive season on either the wall or the bottom. $\delta^{13}C$ and $\delta^{15}N$ (mean ± SD, ‰), SEA_C, Overlap of SEA with the other habitat = mean proportion of the area of SEA_B per habitat *that overlapped with the respective other habitat, Unique area of SEA = mean proportion of the area of SEA_B per habitat that did not overlap with the respective other habitat.*

	Reproductive season		Post-reproductive season	
	Wall	**Bottom**	**Wall**	**Bottom**
$\delta^{13}C$	-26.92 ± 0.34	-27.07 ± 0.55	-26.92 ± 0.32	-26.82 ± 0.39
$\delta^{15}N$	10.06 ± 0.73	9.74 ± 0.67	10.23 ± 0.63	9.91 ± 0.48
SEA_C	0.56	1.18	0.66	0.72
Overlap of SEA with the other habitat	82.05 %	50.20 %	62.39 %	73.04 %
Unique area of SEA	17.95 %	49.80 %	37.61 %	27.96 %

Table 2 Results of linear models for $\delta^{13}C$ and $\delta^{15}N$ of round gobies and amphipods, and round goby trophic position including habitat, reproductive season and their interaction as fixed factors. Pairwise comparisons of contrasts were calculated using Tukey HSD post-hoc tests. Est = estimate, CI = 95% confidence interval, t_n = t ratio$_{\text{degrees of freedom (round goby/amphipods)}}$, p = p-value (printed in bold if $p < 0.05$).

Contrast: Bottom – Wall		**Reproductive Season**				**Post-reproductive season**			
		Est	*CI*	$t_{126/52}$	*p*	*Est*	*CI*	$t_{126/52}$	*p*
$\delta^{13}C$	Round goby	-0.15	-0.35 - 0.05	-1.50	0.136	0.10	-0.09 - 0.30	1.05	0.296
	Amphipods	-0.46	-1.10 – 0.18	-1.44	0.157	1.87	1.02 – 2.72	4.41	**0.0001**
$\delta^{15}N$	Round goby	-0.32	-0.63 - -0.01	2.02	**0.046**	0.32	0.01 - 0.63	2.06	**0.042**
	Amphipods	-0.17	-0.27- -0.63	0.78	0.438	0.12	-0.47 - 0.71	0.41	0.687
Trophic position	Round goby	-0.06	-0.16 - -0.03	-1.39	0.168	-0.12	-0.22 - -0.03	-2.70	**0.008**

Contrast: Rep. season – post-rep. season		**Bottom**				**Wall**			
		Est	*CI*	$t_{126/52}$	*p*	*Est*	*CI*	$t_{126/52}$	*p*
$\delta^{13}C$	Round goby	0.25	0.05 - 0.45	2.49	**0.014**	-0.003	-0.35 – 0.05	-1.50	0.136
	Amphipods	1.53	0.76 - 2.29	3.99	**<0.001**	-0.80	-1.54 - -0.07	2.19	**0.033**
$\delta^{15}N$	Round goby	0.17	-0.14 – 0.48	1.11	0.280	0.17	-0.14 – 0.48	1.11	0.269
	Amphipods	1.02	0.49 – 1.55	3.85	**<0.001**	0.73	0.22 - 1.24	2.86	**0.006**
Trophic position	Round goby	0.15	0.06 – 0.25	3.32	**0.001**	0.10	0.01 – 0.19	2.09	**0.038**

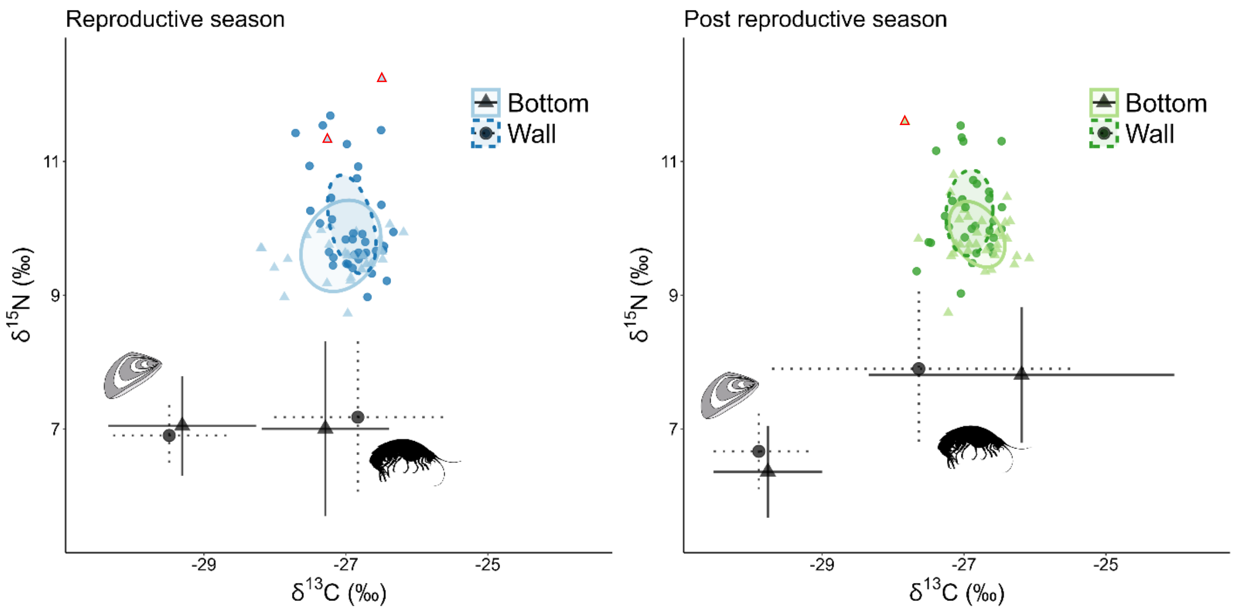

Figure 2 Bi-plot of δ^{13}C and δ^{15}N with standard ellipses (enclosing 40% of the data) of bulk muscle from round gobies either caught during the reproductive season (left) or after the reproductive season (right). Light blue and light green coloured triangles with solid ellipses represent gobies caught on the bottom, dark blue or dark green points with dashed ellipses represent gobies caught on vertical harbor walls close to the surface. Outliers of >2 SD in δ^{15}N in the bottom are marked in red. Mean ± SD of the baseline organism killer shrimps and zebra mussels (indicated by species-symbols) are shown as black triangle (caught on bottom) or black circle (caught on wall).

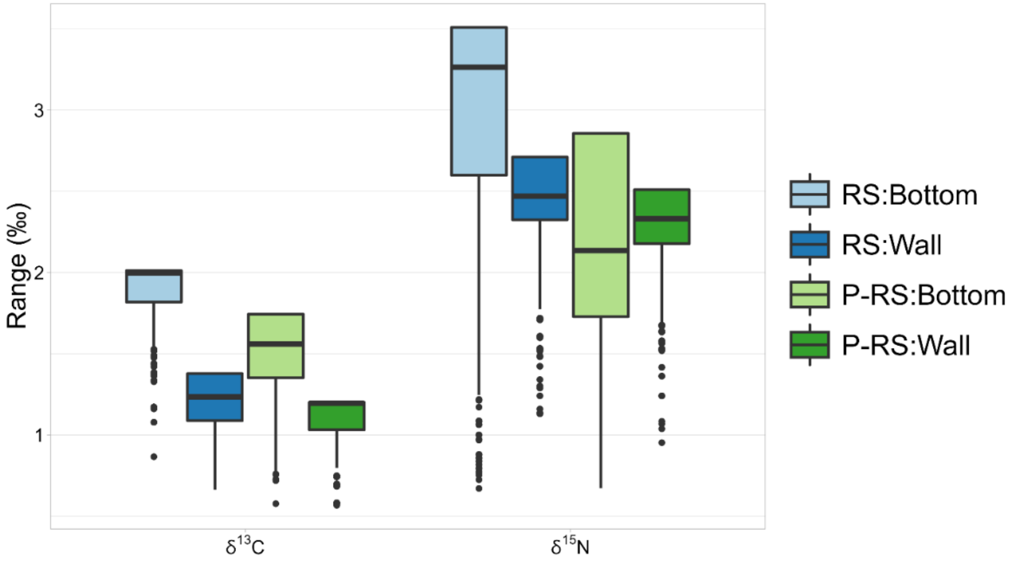

Figure 3 Bootstrapped δ^{15}N and δ^{13}C range (n=10 000, sample size per draw = 28) of round gobies caught on the harbor bottom or the harbor wall during and after the reproductive season based on muscle tissue. RS = Reproductive season, P-RS: post-reproductive season.

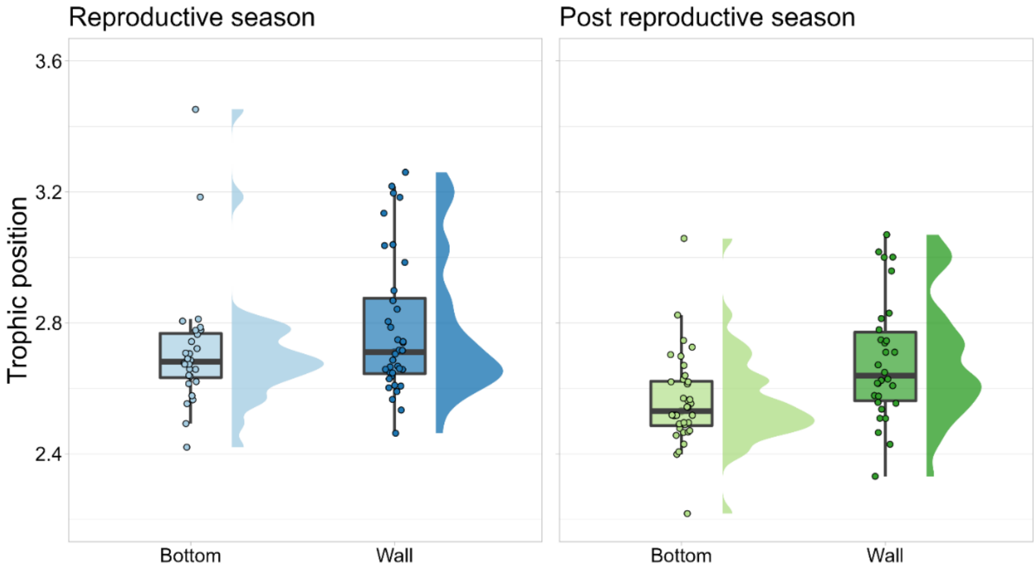

Figure 4 Trophic position of round gobies caught on the harbor bottom or the harbor wall during and after the reproductive season based on muscle tissue. To calculation the trophic position, δ^{15}N values of Dikerogammarus villosus *were used as a baseline.*

INDIVIDUAL SPECIALIZATION

The proportion of trophic specialists in the population was significantly lower on the wall (specialists:generalists = 15:21 (42:58 %), Figure 5 A) than on the bottom (specialists:generalists = 19:9 (68:32 %), Figure 5 B) during the reproductive season (odds ratio = 0.35, CI = 0.12 – 0.96, p = 0.046). The proportion of specialists was similar on the wall (specialists:generalists = 20:10 (67:33 %), Figure 5 C) and on the bottom (specialists:generalists = 20:16 (56:44 %)) after the reproductive season (odds ratio = 0.87, CI = 0.27 – 2.77, p = 0.817). On the walls, the proportion of specialists was significantly higher after the reproductive season than during the reproductive season (odds ratio 4.52, CI = 1.13 – 21.17, p = 0.041). On the bottom, generalists were slightly but significantly larger than on the wall during the reproductive season (odds ratio = 0.48, CI = 0.23 – 0.91, p = 0.034). In none of the other models the covariates sex and standard length were significant.

On the bottom, δ^{13}C was significantly lower in specialists than in generalists during the reproductive season (estimate = -0.43, CI = -0.12 - (-0.75), t_{122} = 2.71, p = 0.008). On the wall, δ^{15}N was significantly higher in generalists than in specialists during the reproductive season (estimate = 0.78, CI = 0.38 - 1.17, t_{122} = 3.89, p = 0.0002). This means, that the unique regions of the trophic niche of the bottom was dominated by trophic specialists, while the unique region of the trophic niche of the bottom was dominated by trophic generalists (Figure 2). In contrast to that, δ^{15}N was marginally higher in specialists than in generalists on walls after the reproductive season (estimate = -0.43, CI = -0.89 - 0.02, t_{122} = -1.89, p = 0.061). On the bottom, there was no significant difference in δ^{15}N between specialists and generalists in either season.

On the wall, there was a significant shift between long-term (muscle) and short-term (liver) δ^{13}C (mean = - 0.40 ‰) during the reproductive season (paired t-test, t_{35} = 6.1, CI = 0.27 - 0.53, p < 0.0001, Figure 5 A), but no shift in δ^{13}C on the bottom (paired t-test, t_{27} = 0.35, CI = -0.14 - 0.19, p = 0.73, Figure 5 B). This shift in isotopic composition between long- and short-term diets was not caused by random variation, but by a parallel shift of a similar amount by many individuals (Figure 5 B lines).

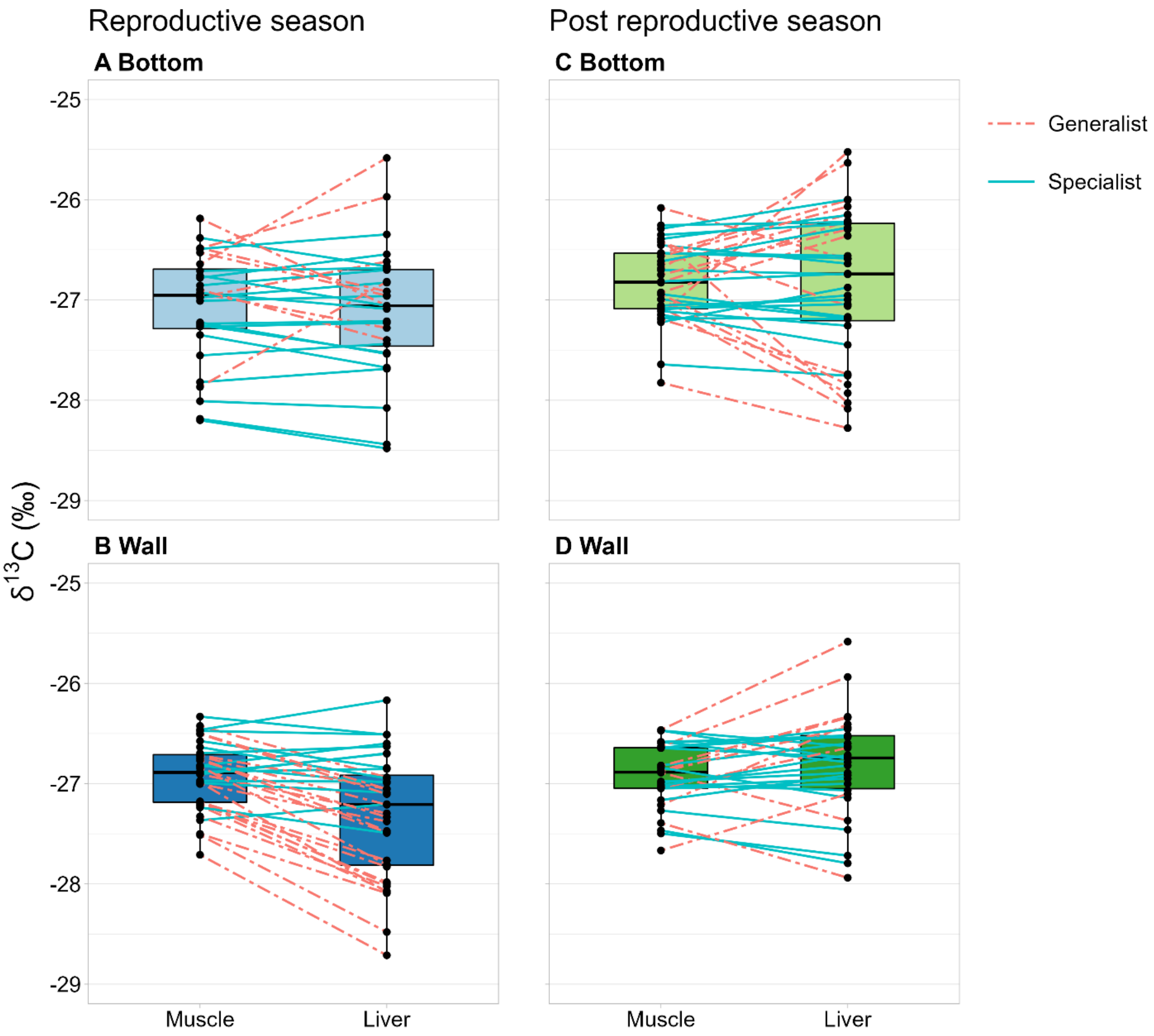

Figure 5 ndividual trophic specialization of round gobies based on the change of $\delta^{13}C$ between muscle (longer turnover time) and liver (shorter turnover time) tissues. A specialist is an individual, for which the difference between muscle and liver $\delta^{13}C$ ($\Delta\delta^{13}C_{M-L}$ = within-individual component, WIC) is smaller than the average Euclidean distance of the absolute values of $\Delta\delta^{13}C_{M-L}$ between all individuals per season (= between-individual component, BIC). A generalist is an individual for which WIC is equal or larger than BIC. ***Reproductive season****:* ***A:*** *Bottom: 9 generalists, 19 specialists.* ***B:*** *Wall: 21 generalists, 15 specialists.* ***Post-reproductive season****:* ***C:*** *Bottom: 16 generalists, 20 specialists.* ***D:*** *Wall: 10 generalists, 20 specialists.*

COMPOSITION OF POTENTIAL FOOD ORGANISM COMMUNITIES

The interpretation of data about niche dynamics and trophic specialization in round gobies was guided by the analyses of the macroinvertebrate communities on bottom and wall as potential food organisms. The composition of macroinvertebrates differed between the habitats throughout the reproductive season of round gobies. On the wall, chironomid larvae and oligochaetes dominated the communities between May and June, while amphipods increased in relative abundance from end of June on and constituted a major part of the community in July and August (Figure 6). On the bottom, we found that oligochaetes and tipulid larvae dominated the communities, with the relative abundance of oligochaetes increasing in late summer (Figure 6).

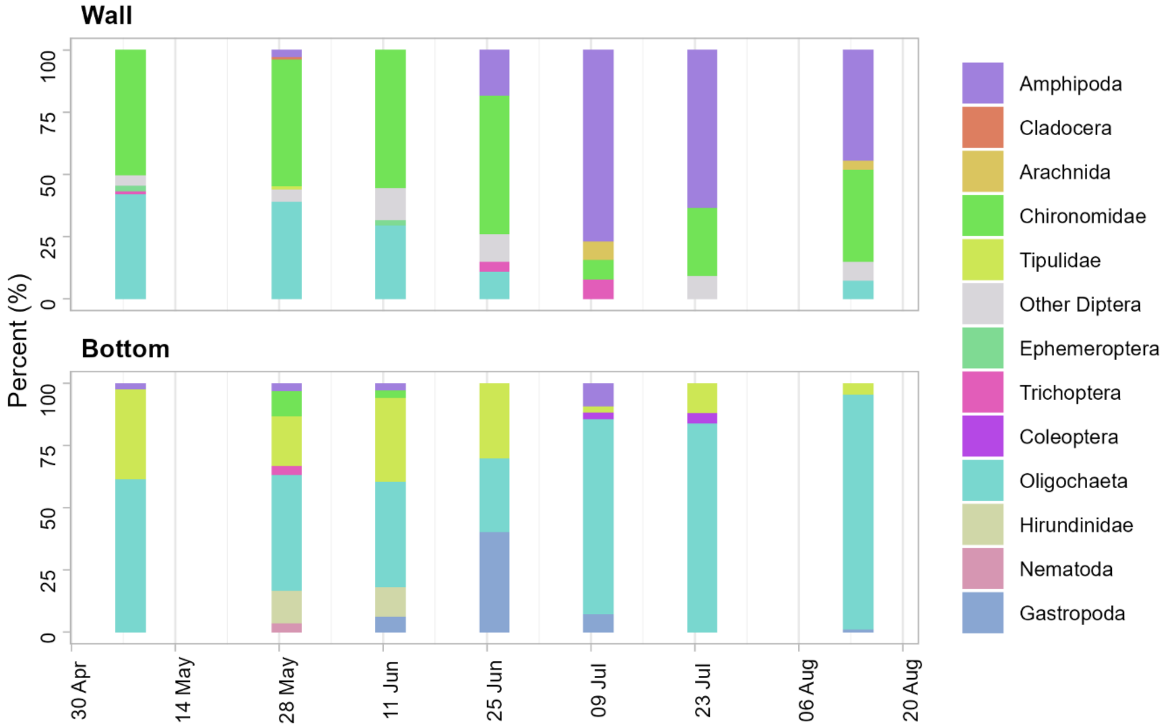

Figure 6 Relative abundance of macroinvertebrates on the harbor walls and the habour bottom. Communities differed between the habitats and changed between early and late summer. Especially on the walls there was a notable increase in amphipod abundance starting in July.

POPULATION CHARACTERISTICS

Females were caught in both habitats in equal numbers in both seasons (reproductive season: $X^2 = 0.08$, df = 1, p = 0.777, post-reproductive season: $X^2 = 0.05$, df = 1, p = 0.829). The number of males caught was slightly, but not significantly higher on the wall during the reproductive season ($X^2 = 1.52$, df = 1, p = 0.217), while it was significantly lower on the wall after the reproductive season ($X^2 = 11.84$, df = 1, p = 0.001). Results for the abundance of sexes are shown in Figure 7 A, B.

Females were similar in size and weight in both habitats, while males were significantly larger and heavier on the wall during the reproductive season (Figure 7 C, Table 2). Contrary to this, both females and males caught on the walls were significantly smaller and lighter than those caught on the bottom after the reproductive season (Figure 7 D, Table 2; differences in standard length for females only marginally significant). Because of the correlation between standard length and log-transformed weight and the resulting similarities of the plots, we only graphically present the results for standard length in Figure 7 C, D.

There were no differences in condition between both habitats, or between female and male gobies during the reproductive season (Figure 7 E, Table 2). The condition factor of males caught on the bottom was higher than the condition factor of males caught on the walls after the reproductive season (Figure 7 F, Table 2). Females did not differ in condition factor in either season.

Table 3 Results of linear models for phenotypic traits of round gobies including habitat, reproductive season, sex, and all interactions as fixed factors. Pairwise comparisons of contrasts were calculated using Tukey HSD post-hoc tests. Weight was modelled using a log-transformation, but results are given on the original scale. Reproductive season: $n_{Female} = 38$, $n_{Male} = 26$, *Post-reproductive season:* $n_{Female} = 38$, $n_{Male} = 28$. *Est = estimate, CI = 95% confidence interval,* t_n *= t ratio*$_{degrees\ of\ freedom}$, *p = p-value.*

Contrast: Bottom – Wall		**Reproductive Season**				**Post-reproductive season**			
		Est	*CI*	t_{246}	*p*	*Est*	*CI*	t_{246}	*P*
Standard length (cm)	Female	0.07	-0.61 – 0.75	0.19	0.848	0.47	-0.05 – 0.99	1.74	0.074
	Male	-0.93	-1-68 – (-0.17)	-2.42	**0.016**	1.17	0.57 – 1.77	3.84	**0.0002**
Condition (Fulton`s K)	Female	0.01	-0.08 – 0.09	0.17	0.868	0.03	-0.04 – 0.09	0.91	0.366
	Male	-0.03	-0.13 – 0.06	-0.69	0.488	0.11	0.03 – 0.18	2.76	**0.006**
Weight (g)	Female	1.03	0.77 – 1.38	0.18	0.856	1.26	1.01 – 1.58	2.04	**0.043**
	Male	0.67	0.48 – 0.93	-2.42	**0.016**	1.65	1.27 – 2.14	3.78	**0.0002**

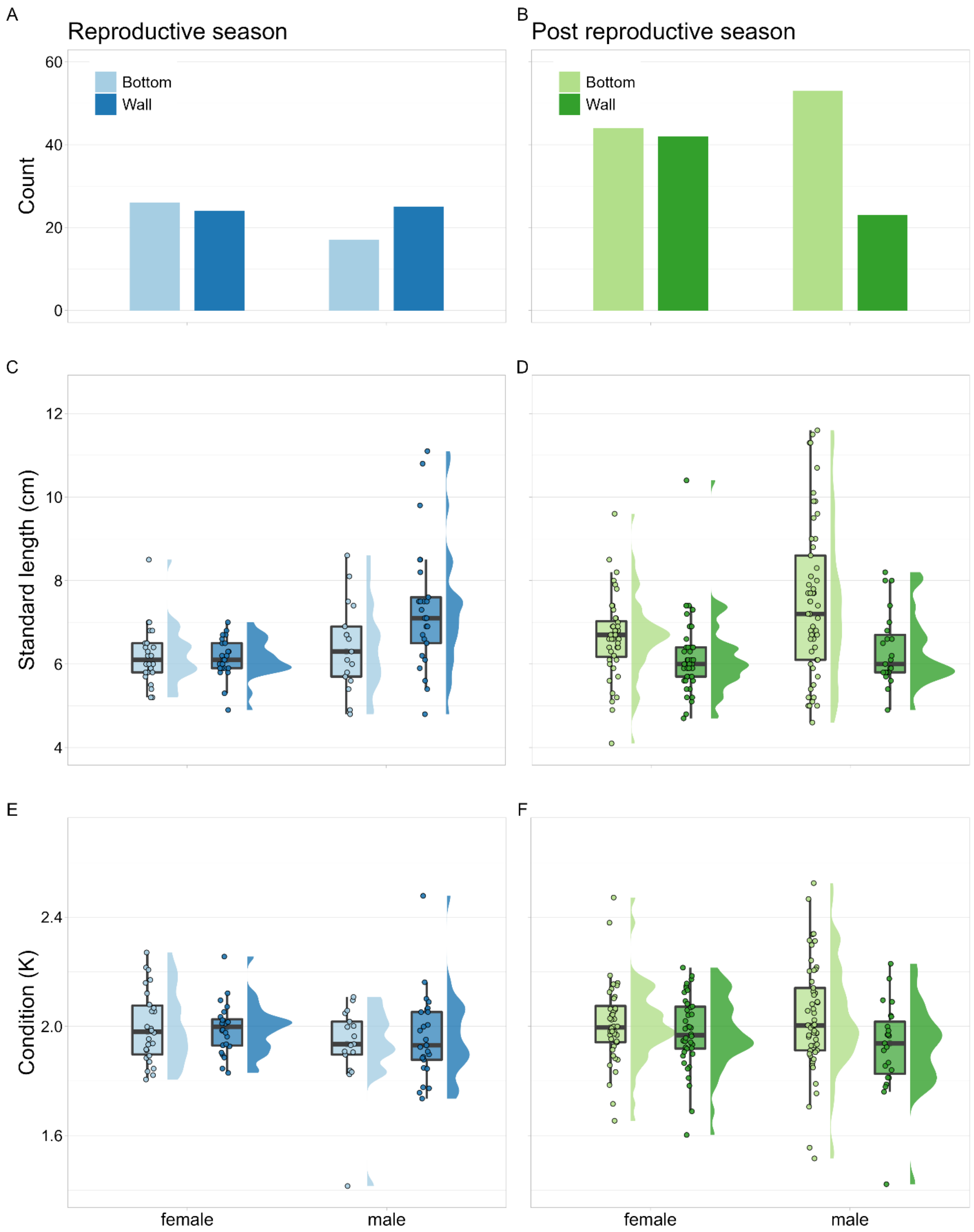

Figure 7 Population characteristics of round gobies caught on the harbour bottom or the harbour wall. ***A, B:*** *Abundance* ***C, D:*** *Standard length, and* ***E, F:*** *Condition factor (Fulton`s K) of female and male gobies of female and male gobies in both habitats in the reproductive season and after the reproductive season. Dots show original data, boxplots show the median (middle line), interquartile range (IQR, box) and values within 1.5 IQR (whiskers). Density plots show the distribution of data.*

USE OF NESTING OPPORTUNITIES

In total, we found signs of spawning (clutches or traces of clutches) in the spawning traps 50 times between 29 May and 14 July 2020. We did not find any signs of clutches in any of the traps before or after these dates. Round gobies spawned 48 times on the traps on the walls, and only twice in the traps on the harbour bottom (Figure 4).

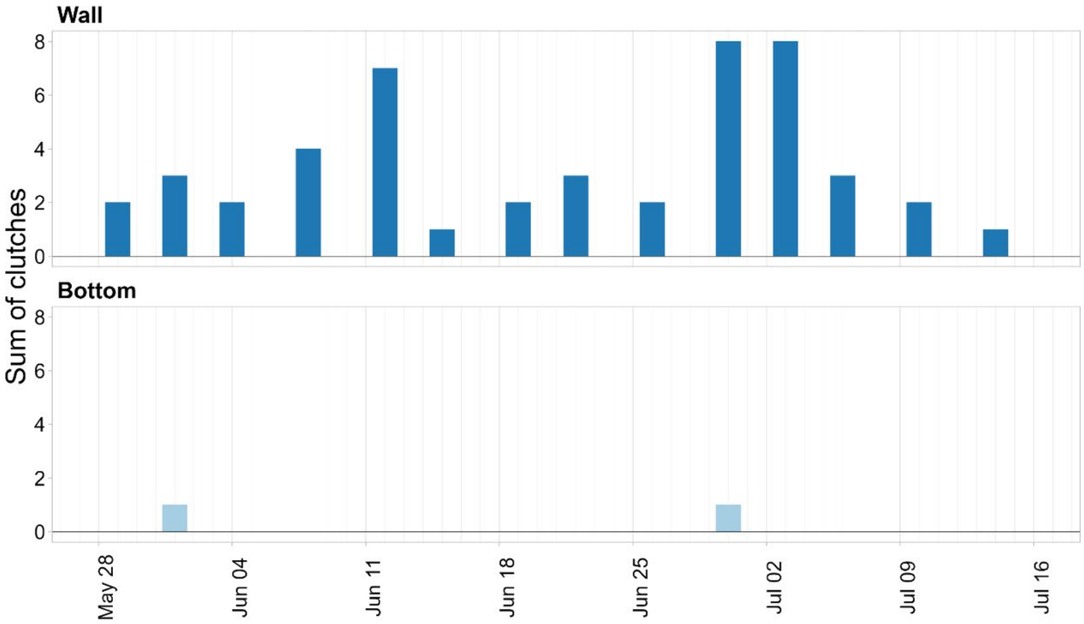

Figure 8 Spawning activities of round gobies in spawning traps deployed on the harbour walls (top panel) or the harbour bottom (lower panel).

DISCUSSION

In this study, we use several indicators for differential habitat use to demonstrate how a subset of an invasive population might utilize a previously undescribed novel anthropogenic niche. Specifically, we compared population trophic niches, individual trophic specialization, phenotypic traits, and breeding frequencies between invasive round gobies using either the bottom or vertical walls as habitat in a harbour ecosystem to find out if vertical walls are a niche extension of the whole population, or a specific subset of the population. Vertical walls as habitat for this bottom-dwelling fish have received little attention from research, despite their function as beachheads to reach potential translocation vectors, i.e. boat hulls. Our results indicate that use of vertical walls is a specialization in habitat use of a population subset defined by higher trophic generalism and larger, heavier males during the breeding season. Additionally, breeding frequencies on walls were higher than on the bottom. After the breeding season, our results show less signs for habitat partitioning due to a higher similarity in trophic ecology, and indicate that larger round gobies that used the walls during breeding season migrate to the bottom towards the winter.

TROPHIC NICHE: SIZE, OVERLAP AND POSITION

The niche size of round gobies on the walls was smaller and significantly overlapped with the niche of round gobies on the bottom. These results would indicate that all individuals forage on the walls and only a subset of the population uses the bottom as a niche extension (reversed scenario B, Figure 1). However, there are isotopic regions that are unique to both habitats and both niche size on the bottom and overlap of the wall niche with the bottom niche decrease substantially if the two outliers in $\delta^{15}N$ on the bottom are removed (Supplementary document S3). Without the outliers, the $\delta^{15}N$ range of the niche of gobies caught on the walls is wider than on the bottom, while the $\delta^{13}C$ range of the gobies caught on the bottom is wider than on the walls. While there was no biological or technical reason to exclude the outliers, it is possible that they were caught in the “wrong” habitat. Some movement between the wall and bottom was observed in Bussmann and Burkhardt-Holm (2020). Individuals temporarily moving from the wall to the bottom might be an explanation for these outliers in the bottom-caught gobies. The composition of available food organisms differs in both habitats, but also includes some of the same macroinvertebrates. Based on the distinguishable, but overlapping food resources, the position and overlap of the trophic niches could indicate consistently differential habitat use, or specialized habitat use by some individuals and flexible habitat use of other individuals.

After the breeding season, the overlap of trophic niches of gobies caught on the walls and the bottom is higher than during the breeding season and levels of individual specialization do not differ between the habitats. Increasing similarity of trophic niches could be caused by a higher mobility of round gobies and migration from the walls to the bottom after the reproductive season. In lake and sea habitats, round gobies migrate to deeper waters (up to > 70 m) in winter (Behrens et al. 2021, Carlson et al. 2021). While we do not know if comparable processes happen in rivers of relatively shallow depths (river Rhine in Basel, Switzerland: ca. 5-8 m depth), our results presented here point towards a seasonally different habitat use, of which we probably only observed the beginning.

INDIVIDUAL SPECIALIZATION

If the bottom niche was an extension of the wall niche (see above), we would expect more generalist individuals on the bottom than on the wall. In fact, however, the opposite is the case and there are more generalist gobies on the walls than on the bottom. Considering the influence of the two outliers on the bottom that might have been caught in the “wrong” habitat, a different conclusion can be inferred from the observed patterns: On the walls as well as on the bottom, there are individuals specializing on using the respective habitat. The specialization in habitat use goes along with different feeding ecologies (more generalist on the walls, more specialist on the bottom). Next to the parts of the population that specialize in habitat use on either wall or bottom, there is probably also a part of the population that is flexible in habitat use and uses both.

The uniform shift in $\delta^{13}C$ isotopic composition between long and short-term diets uniquely occurring in gobies caught on walls indicates that many round gobies consistently use primarily the walls as habitat. A parallel shift in isotopic composition could be explained if these gobies all started feeding on a different food resource during the time that liver tissue integrates over, or if the isotopic composition in the whole food web shifted. The half-life of adult round goby muscle tissue is > 100 days (3-4 months; Brandner et al. 2015). Based on an average turnover rate of livers of 30% of the turnover rate of muscles (Guelinckx et al. 2007, Boecklen et al. 2011), we assumed a half-life of ca. 30 days for round gobies. The shift of relative abundances of food organisms ca. 1-2 months before we sampled the gobies during the reproductive season therefore supports the interpretation that the shift in $\delta^{13}C$ in gobies caught on the walls is related to a change in diet. As we do not observe a similar diet shift in round gobies caught on the bottom, we conclude that round gobies exhibit habitat partitioning during the breeding season. This finding makes sense considering that round gobies are generally territorial during the breeding season with home ranges of ca. 5 ± 1.2 m^2 (Ray and Corkum 2001). Particularly males defend nests and reportedly remain stationary during courtship and nest guarding (Corkum et al. 1998). Although there is less reason for females and non-nest guarding sneaker males to remain in a limited home range, we found no sex differences in the shift in isotopic composition, and other studies support that males and females have similar home ranges during the reproductive season (Marentette et al. 2009). We therefore conclude that specialized habitat use is not limited to nest-guarding males.

The higher number of trophic generalists is a result of the uniform isotopic shift discussed above. If generalist feeding in wall-using individuals was caused by moving between and feeding in both habitats, we would expect the variation between long- and short-term diet of individuals to be random, not uniform (Matthews and Mazumder 2004). The uniform variation between long and short-term diet therefore must results from the uniform variation in diet items within one habitat (wall) and not diet switching between habitats. The timing of the isotopic shift can be related to a change in composition of macroinvertebrates from chironomid-dominated to amphipod-dominated (see above), which supports our conclusion that the observed trophic generalism was caused by a changing relative abundance of food organisms.

Both chironomid larvae and amphipods are common prey organisms for round gobies in European rivers (Borcherding et al. 2013, Brandner et al. 2013). It is therefore likely that a change in relative abundance of the two taxa would cause a dietary shift in round gobies.

We found a correlation between $\delta^{15}N$ and generalist feeding on the walls during the reproductive season. In males, higher values of $\delta^{15}N$ could be correlated to mating strategy: nest-guarding males may feed more opportunistically because of spatial constraints during nesting (McCallum et al. 2018). Additionally, reproductive males may forage less during the breeding season than non-reproductive males, which in turn can lead to higher $\delta^{15}N$ through starvation stress (Bowes et al. 2014, McCallum et al. 2018). If the higher nesting frequencies we found on the walls indicate that more reproductive males are present there, this could explain the correlation between $\delta^{15}N$ and generalist feeding in males. After the reproductive season, a higher $\delta^{15}N$ on the walls (i.e., the isotopic region unique to the walls) correlated with more specialist feeding. This supports the onset of a winter migration dominated by reproductive males: If reproductive males with a more generalist feeding strategy start migrating to the bottom, they leave behind non-reproductive individuals with a more specialist feeding strategy.

POPULATION CHARACTERISTICS AND USE OF NESTING OPPORTUNITIES

One explanation for habitat partitioning during the breeding season could be elevated competition between gobies for energy-rich resources or nesting opportunities. Considering that there was much more breeding activity on the wall than on the bottom and that males caught on the wall were larger during the reproductive season, we conclude that the wall habitat is the preferred one, even though using it might be more energetically costly (Bussmann and Burkhardt-Holm 2020). Advantages in terms of breeding could arise from the quantity or from the quality of nesting opportunities. For example, higher availability of larger nesting sites in one habitat can lead to a preferential occupation of that habitat by larger males in the sand goby (Lehtonen and Lindström 2004). The quantity of nest sites (crevices, holes or pipes etc.) might be higher on the walls than on the bottom (small to medium rocks and other debris from harbour activities), or the nesting opportunities might be generally higher, explaining larger size of males on the wall during the reproductive season. Contrary to this line of argument, the higher breeding frequency in the spawning traps on the wall might indicate that there are less naturally available options for breeding on the walls than on the bottom, so that more gobies use the artificial spawning traps. In this case, larger males might prefer the walls primarily because of other reasons than nesting opportunities (e.g. higher density of zebra mussels, lower turbidity, higher oxygen concentration) and once they encounter nesting opportunities they readily use them. Larger males are more competitive in nest holding potential and they are preferred mating partners for females among ecologically similar goby species (Marconato et al. 1989, Lindström and Pampoulie 2004, Lehtonen et al. 2007). A preferential use of nesting opportunities off the ground by the larger individuals would make boat hulls therefore even more prone to become breeding grounds and eventually translocation vectors for a particularly competitive subset of round gobies.

The interpretation of trophic niche data after the reproductive season as the beginning of round goby migration to deeper habitats (see above) is supported by the population characteristics calculated from the total catch of all individuals. After the reproductive season, both sexes were smaller and lighter on the walls than on the bottom. Additionally, the number of males on the bottom was higher than on the wall and males caught on the walls had a lower condition. The lower condition factor of males on the walls could be explained by a higher energy consumption during the reproductive season due to nesting activities. These results indicate that after the end of breeding activities, formerly reproductive individuals (particularly nest-guarding males) with small home ranges and generalist feeding strategies leave the walls and start inhabiting the bottom, while the smaller individuals with a more specialist feeding strategy remain on the walls. Marentette et al. (2011) also observed a higher mobility of males than of females after the reproductive season. Higher similarity in diets after the reproductive season between alternative reproductive tactics in males could partly explain why the population niche size on the bottom decreases in spite of individuals from the wall migrating to the bottom (McCallum et al. 2018). A decreasing variability of available food resources, as was observed over the course of the summer especially on the bottom, could provide an additional explanation for the smaller niche size as well as for the high level of specialization in general.

RELEVANCE FOR INVASION SUCCESS AND TRANSLOCATION

Taken together, our results indicate the possibility of assortative mating based on habitat use and therefore microgeographic adaptation to either horizontal or vertical habitat use in round gobies. While in theory, gene flow should prevent population differentiation over small spatial scales, non-random dispersal and habitat choice allow for the development of microgeographic divergence within populations (Edelaar et al. 2008, Mortier et al. 2019). This divergence can lead to local adaptations if mating happens selectively in the respectively chosen habitat (Edelaar et al. 2008, Richardson et al. 2014). Indeed our data point towards a non-random habitat use, especially during the breeding season. As male gobies using the walls in the breeding season are larger and heavier, we hypothesize that wall climbing behaviour would select for more competitive individuals. If assortative mating happens between wall and bottom inhabiting gobies, there should be genetic or morphological differences detectable, as has for example been shown in populations of sticklebacks (Bolnick et al. 2009, Maciejewski et al. 2020). Because walls can act as beachheads to reach boat hulls and therefore potential translocation vectors (Bussmann and Burkhardt-Holm 2020), new founding populations might be dominated by this more competitive subset of the round goby population in harbours. Even more, if similar processes happen in the native habitat of round gobies from where they were originally translocated, this might be part of the puzzle of why round gobies are such successful invaders. Adaptive use of anthropogenic habitat like harbour walls might promote invasions, because the translocated part of the population is adapted to similar conditions in their original habitat as in their new habitat after translocation and additionally might be an above average competitive subset of the species (Hufbauer et al. 2012). Apart from adaptive processes, vertical habitat use could allow populations in harbours to be larger than previously estimated in studies only considering the bottom as habitat (e.g. Young et al. 2010, N'Guyen et al. 2018). This higher population size could lead to an increased uptake of propagules, and therefore a higher probability of successful translocation (Cassey et al. 2018).

CONCLUSIONS

With this study, we provide evidence for specialized use of and reproduction in anthropogenic habitats relevant for translocation by a notorious invasive fish. Habitat partitioning and potentially associated assortative mating involving more competitive males could give new founding populations an advantage in uncolonized areas. Additionally, the use of vertical harbour walls as habitat could increase population densities in harbours and therefore increase the likelihood of uptake by ballast water in harbours and consequently propagule pressure. These results could contribute to explaining why round gobies are such successful invaders. Furthermore, this study could increase the accuracy of future models estimating population densities and invasion potential of round gobies in harbours; their typical point of entry into novel ecosystems.

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SUPPLEMENTARY DOCUMENT S1

PREPARATION OF SAMPLES FOR STABLE ISOTOPE ANALYSIS

We ran samples for $\delta^{13}C$, $\delta^{15}N$, C%, and N% at the Department of Environmental Sciences, University of Basel. Due to technical difficulties, we had to run the analyses on two different EA-IRMS (elemental analysis - isotope ratio mass spectrometer): 1. INTEGRA2 instrument (Sercon Ltd., Crewe, UK), 2. Flash 2000 elemental analyzer (EA) coupled to a Thermo Finnigan Delta Plus XP isotope ratio mass spectrometer (IRMS) via a Conflo IV interface (Thermo Fisher Scientific, Bremen, Germany). The second EA was equipped with a thermal conductivity detector (TCD) to measure the abundance of C and N in the sample in the carrier gas stream prior to introduction to the mass spectrometer. Information on standards used for elemental analysis and instrumental precision are presented in Supplemetary document 1.

Either an EDTA (1) or a caffeine laboratory standard (2) was measured every 10th to 12th sample during analytical sequences to check and correct for instrumental drift. These laboratory standards were also used to check and correct for instrument-induced amount effects. Additionally, international reference materials (1. USGS40, USGS64, IAEA-CH-6, IAEA-N-2, 2. Urea, Sucrose, Spirulina, FS-I,) were analysed at the beginning, in the middle, and at the end of each analytical sequence and a calibration using the three standards was applied, taking into account potential instrument drift and sample size linearity effects.

The analytical precision of the instruments based on the difference between the data for the respective standards and certified data for these standards ranged from 0.08-0.17 ‰ (1) or 0.01-0.29 ‰ (2) for N_2, and 0.05-0.11 (1) or 0.01-0.13 ‰ (2) for CO_2. Results were expressed in delta notation (parts per thousand deviation from a standard material): $\delta^{13}C$ or $\delta^{15}N = [(R_{sample} / R_{standard}) - 1] * 1000$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$. The standard reference material for N_2 was atmospheric nitrogen, and V-PDB (Vienna Pee Dee Belemnite) for CO_2.

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SUPPLEMETARY DOCUMENT S2

LIPID EXTRACTION FOR MATHEMATICAL CORRECTIONS OF LIPID CONTENTS

Because lipids are ^{13}C depleted, a high content of lipids in tissues can influence the δ^{13}C values of that tissue (DeNiro and Epstein 1978, Focken and Becker 1998). To compare two tissues with different lipid contents like muscles and livers, it is therefore necessary to correct for the lipid content of a sample (Post, Layman et al. 2007, Logan, Jardine et al. 2008). This can be achieved either by extracting lipids from samples, or by mathematically correcting for the lipid content of each sample (Post, Layman et al. 2007, Logan, Jardine et al. 2008). As lipid extraction is a time-consuming process, which might influence the δ^{15}N of a sample (Pinnegar and Polunin 1999, Sotiropoulos, Tonn et al. 2004), we decided to correct for the lipid content of our samples mathematically. There are formulas published for such a correction (Kiljunen, Grey et al. 2006, Post, Layman et al. 2007). However, it is best practice to individually establish a standard curve between the C:N ratio (proxy for lipid content) of a sample before lipid extraction, and the difference between the δ^{13}C of the sample before and after lipid extraction for the investigated species and tissues (Logan, Jardine et al. 2008).

We used a subsample of eight round gobies for lipid extraction with the goal to establish a mathematical formula for lipid correction. We split each of the muscle and liver samples of the subsample in two, extracted lipids from one half, and ran stable isotope analysis for both halves of the sample.

To extract lipids from round goby muscle and liver samples, we followed the protocol of Bligh and Dyer (1959) as modified by Turschak, Bunnell et al. (2014): A 2:1 chloroform-methanol solution was added to the dried tissue homogenate and mixed. The mixture was then periodically agitated for 30 minutes and centrifuged at 4000 rpm for 10 minutes. Afterwards, the supernatant was decanted. After repeating this process three times, the samples were dried overnight under a fume hood.

According to published literature, round goby muscles have a C:N ratio of < 3.5 and therefore do not need to be subjected to lipid extraction, as the variation caused by lipids is negligible. Our data confirmed this for the herein investigated population (range C:N ratio muscles: 3.32 - 3.37, $\Delta\delta^{13}$C of original and lipid extracted halves = -0.06 ± 0.07) .

Livers had high C:N ratios (10.0 – 16.4). We determined the lipid corrected $\delta^{13}C_L$ using the formula $\delta^{13}C_L = \delta^{13}C_0 - \beta_1 * C{:}N_0 - \beta_0$. In the formula, $\delta^{13}C_0$ and $C{:}N_0$ are the δ^{13}C and C:N of the sample before lipid extraction, and β_1 and β_1 are the slope and intercept of the linear relationship between $C{:}N_0$ and $\Delta\ \delta^{13}C$ (i.e., $\delta^{13}C_L$ - $\delta^{13}C_0$).

The derived mathematical formula for lipid correction for round goby livers was $\delta^{13}C_L = \delta^{13}C_0 + 0.083 * C{:}N_0 + 2.566$. We applied this formula to the measured δ^{13}C of all sampled livers.

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SUPPLEMENTARY DOCUMENT S3

RESULTS EXCLUDING OUTLIERS IN BOTTOM HABITAT

Niche size: The trophic niche of gobies caught on the bottom was of similar size to the trophic niche of the gobies caught on the walls during the reproductive season (probability of SEA being larger on bottom = 0.560, Figure S 1 , Table S 1). The trophic niche on the bottom was smaller than the trophic niche on the walls after the reproductive season (probability of SEA being larger on bottom = 0.054, , Figure S 1 , Table S 1).

Niche overlap: The trophic niches of round gobies caught on the walls and on the bottom did not overlap significantly (> 60 %) during the reproductive season (Figure S 1 , Table S 1). The trophic niche of round gobies caught on the bottom overlapped significantly with the trophic niche of round gobies caught on the wall, while the wall niche did not overlap significantly with the bottom niche after the reproductive season (Figure S 1 , Table S 1).

Isotopic ranges: Round gobies caught on the bottom had a narrower $\delta^{15}N$ range and a wider $\delta^{13}C$ range than round gobies caught on the wall during and after the reproductive season (Figure S 2). The $\delta^{13}C$ range of gobies caught on the bottom was, however, only marginally wider than the $\delta^{13}C$ range of gobies caught on the wall after the reproductive season (Figure S 2).

Niche position: The mean niche position of round gobies was significantly higher in $\delta^{15}N$ on the walls than on the bottom, but did not differ in $\delta^{13}C$ in both seasons (Table S 2, Figure S 3). On the bottom, $\delta^{13}C$ as well as $\delta^{15}N$ was significantly

The trophic position of round gobies was significantly lower on the bottom than on the wall in both seasons (Table S 2, Figure S 3). In both habitats, the trophic position of round gobies was significantly lower after than during the reproductive season (Table S 2, Figure S 3).

Table S 1 Niche characteristics of round gobies caught during and after the reproductive season on either the wall or the bottom after the removal of outliers in $\delta^{15}N$ in the bottom samples. $\delta^{13}C$ and $\delta^{15}N$ (mean ± SD; ‰), SEA_C, Overlap of SEA with the other habitat = proportion of the area of SEA_B per habitat that overlaps with the respective other habitat, Unique area of SEA_B = proportion of the area of SEA_B per habitat that is does not overlap with the respective other habitat. Bold font indicates a significant change after removal of the outliers.

	Reproductive season		**Post reproductive season**	
	Wall (N = 36)	**Bottom (N = 26)**	**Wall (N = 30)**	**Bottom (N = 35)**
$\delta^{13}C$	-26.92 ± 0.34	-27.09 ± 0.56	-26.92 ± 0.32	-26.79 ± 0.35
$\delta^{15}N$	10.06 ± 0.73	9.58 ± 0.33	10.23 ± 0.63	9.86 ± 0.39
SEA_C	0.56	**0.59**	0.66	**0.44**
Overlap of SEA_B with the other habitat	**45.51 %**	55.99 %	**50.89 %**	72.27 %
Unique area of SEA_B	**54.49 %**	44.01 %	**49.11 %**	27.73 %

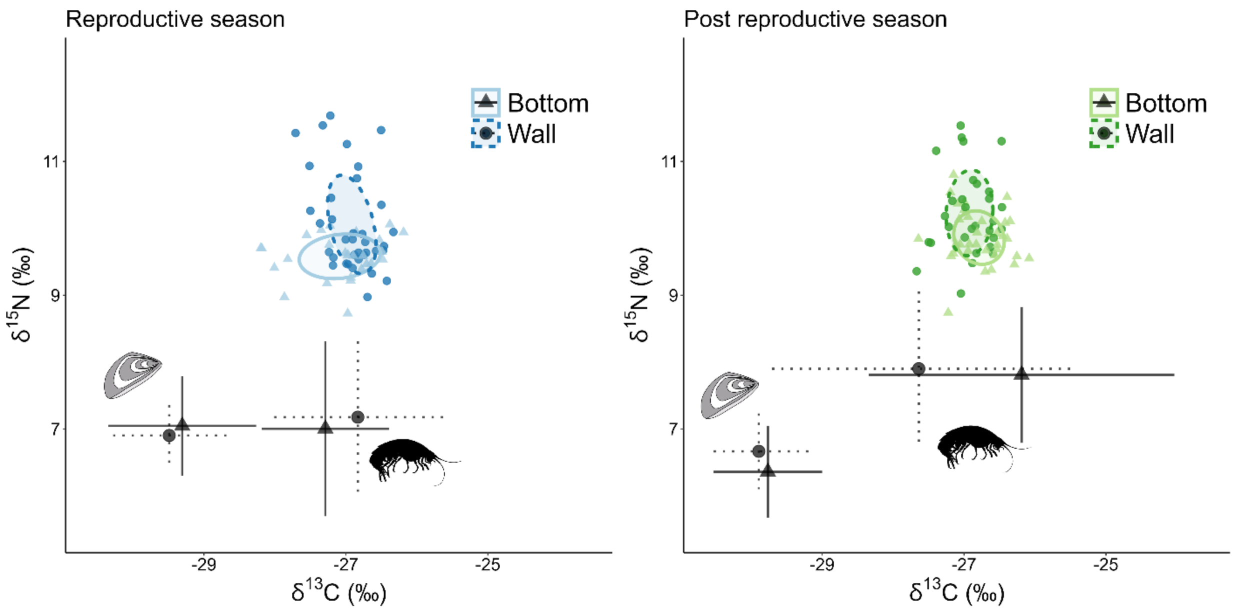


Figure S 1 Bi-plot of $\delta^{13}C$ and $\delta^{15}N$ with Standard Ellipse Areas (SEA$_c$) of bulk muscle from round gobies caught either during the reproductive season (left) or after the reproductive season (right) without outliers. Light blue and light green coloured triangles with solid ellipses represent gobies caught on the bottom, dark blue or dark green points with a dashed ellipse represent gobies caught on vertical harbor walls close to the surface. Mean ± SD of killer shrimps and zebra mussels (indicated by species-symbols) are shown as black triangle (caught on bottom) or black circle (caught on wall).

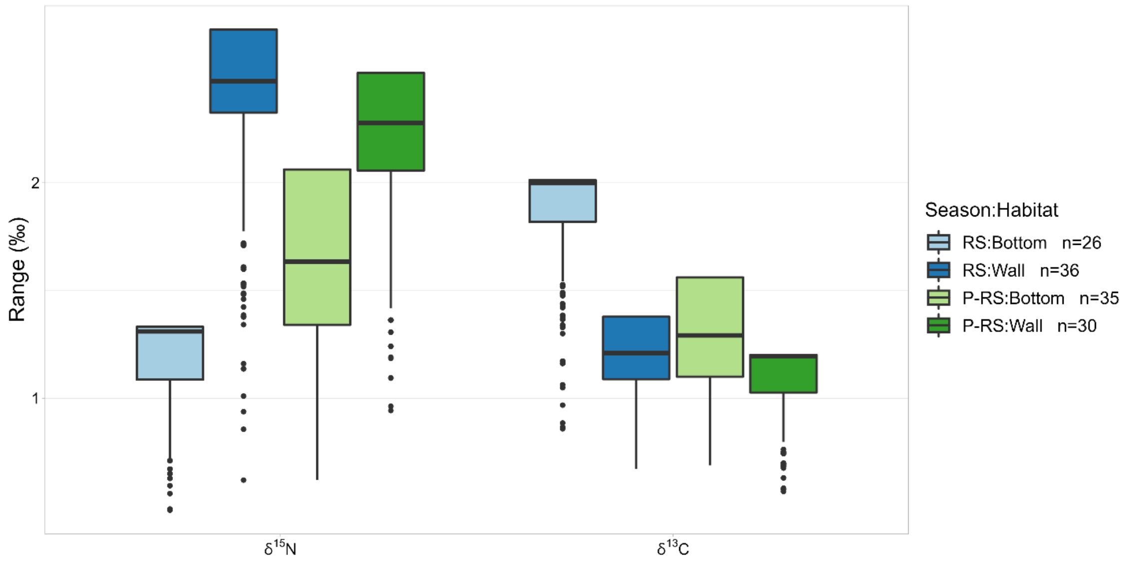


Figure S 2 Bootstrapped Layman metrics (n=10000, sample size per draw = 26). The isotopic ranges were calculated from the distribution of data points in the biplot of carbon and nitrogen stable isotopes without outliers of $\delta^{15}N > 11$ on the bottom. RS = Reproductive season, P-RS = Post reproductive season.

Table S 2 Results of linear models for $\delta^{13}C$ *and* $\delta^{15}N$ *of round gobies and trophic position including habitat, reproductive season and their interaction as fixed factors. Outliers on the bottom with* $\delta^{15}N > 11$ *were removed before running the models. Pairwise comparisons of contrasts were calculated using Tukey HSD post-hoc tests. Est = estimate, CI = 95% confidence interval,* t_n *= t ratio*$_{\text{degrees of freedom}}$*, p = p-value (printed in bold if* $p < 0.05$*).*

Contrast: **Bottom – Wall**	**Reproductive Season**				**Post-reproductive season**			
	Est	*CI*	t_{123}	*p*	*Est*	*CI*	t_{123}	*p*
$\delta^{13}C$	-0.17	-0.37 – 0.32	-1.67	0.100	0.13	-0.06 – 0.33	1.36	0.176
$\delta^{15}N$	-0.48	-0.76 - -0.20	-3.35	**0.001**	-0.37	-0.64 - -0.10	-2.68	**0.008**
Trophic position	-0.11	-0.19 - -0.03	-2.65	**0.009**	-0.14	-0.22 – 0.06	-3.40	**0.001**

Contrast: **Rep. season – post-rep. season**	**Bottom**				**Wall**			
	Est	*CI*	t_{123}	*P*	*Est*	*CI*	t_{123}	*P*
$\delta^{13}C$	-0.30	-0.50 - -0.10	-2.93	**0.004**	0.003	-0.19 – 0.20	0.03	0.974
$\delta^{15}N$	-0.28	-0.57 – 0.00	-1.96	***0.052***	-0.17	-0.45 – 0.10	-1.26	0.208
Trophic position	0.12	0.04 – 0.21	2.91	**0.004**	0.09	0.02 – 0.18	2.38	**0.019**

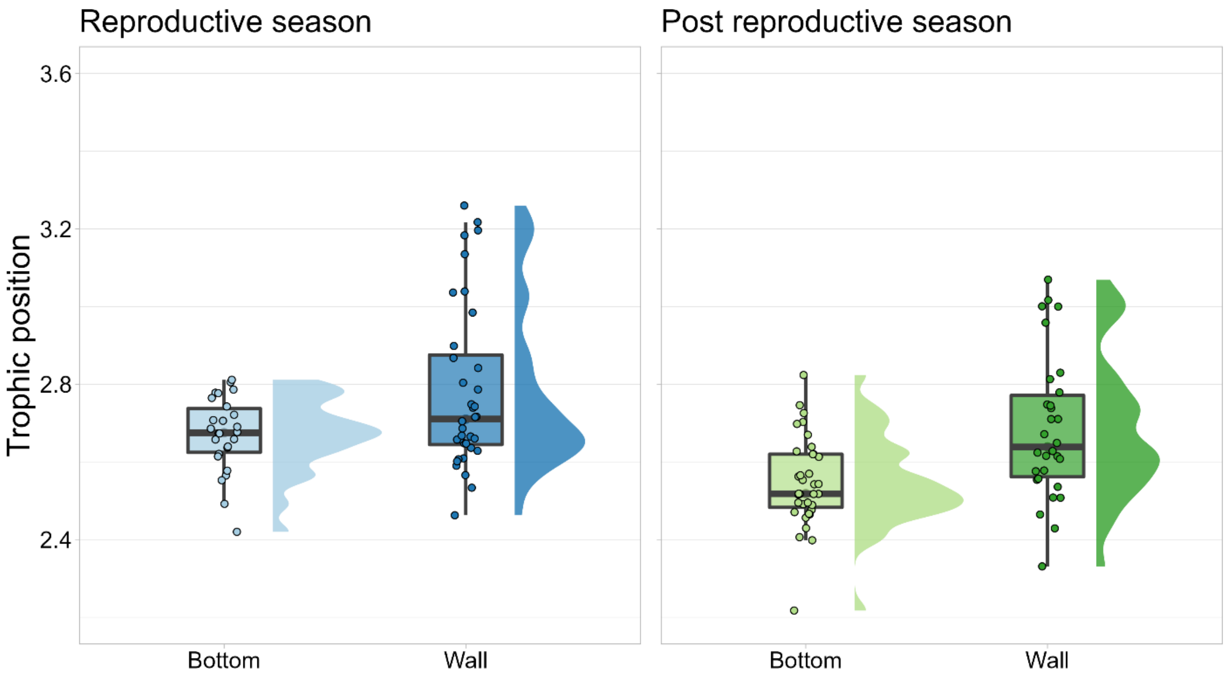
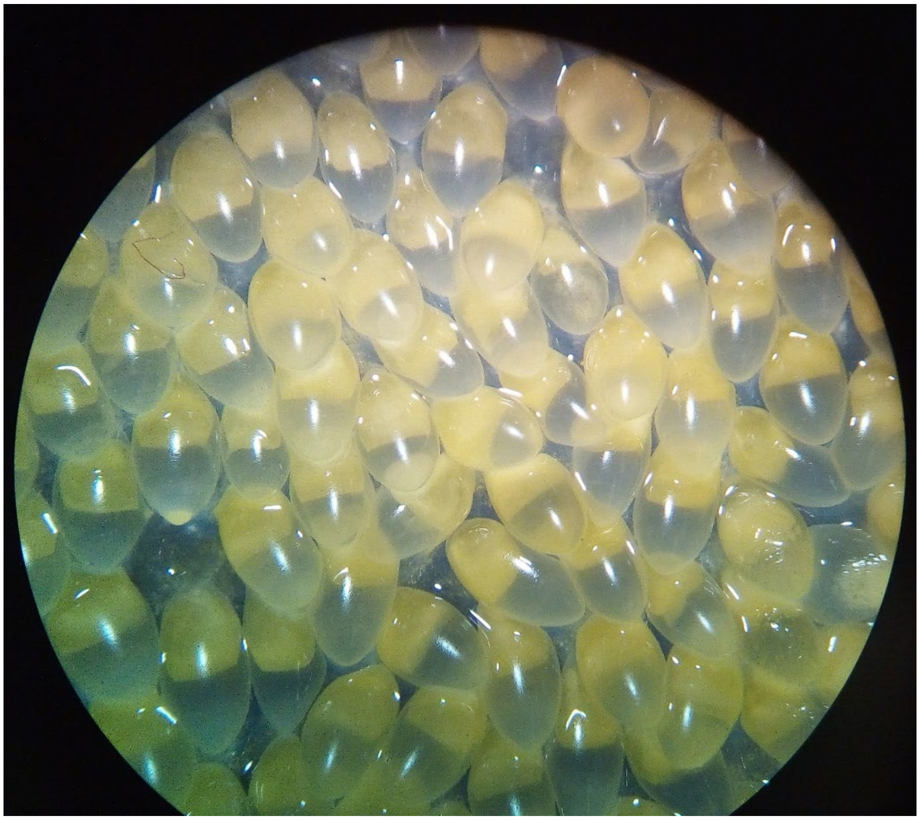


Figure S 3 Trophic position of round gobies caught on the harbor bottom or the harbor wall during and after the reproductive season based on muscle tissue. Outliers with a δ^{15}*N > 11 were removed. To calculate the trophic position, δ15N values of* Dikerogammarus villosus *were used as a baseline.*

PAPER III

Molecular strategies for desiccation tolerance in eggs of the invasive fish *Neogobius melanostomus*



MOLECULAR STRATEGIES FOR DESICCATION TOLERANCE IN EGGS OF THE INVASIVE FISH *NEOGOBIUS MELANOSTOMUS*

Karen Bussmann, Joshua Niklas Ebner, Irene Adrian-Kalchhauser, Patricia Burkhardt-Holm

ABSTRACT

Desiccation is a challenge to aquatic life that will become increasingly relevant in the future under climate change scenarios for freshwater systems. Yet, we know little about the ability of most aquatic organisms to withstand periods of desiccation due to increasingly fluctuating water levels. The eggs of many littoral spawning fish will suffer progressively more from fluctuating water levels that might leave them exposed to air for prolonged times. To predict the effects of climate change on freshwater fish communities it is therefore important to understand which consequences air exposure might have on fish eggs and which potential protective mechanisms help them to overcome the challenge of desiccation. The round goby is an invasive fish species that may face desiccation additionally during their translocation, as overland transport of their eggs on boat hulls is a plausible way of translocation. In this study, we determine how long round goby eggs can survive desiccation and investigate the early transcriptional response (treatment-related changes in RNA expression levels) to air exposure and compare it to the response towards exposure to saltwater as a second osmotic stressor. Round goby eggs survived desiccation up to 48 hours. There were no effects on survival and hatching rates or developmental speed among eggs surviving desiccation. The transcriptional response towards desiccation was strongest after a short exposure time (1.5 h) and changes after continued exposure (3 h). Especially the early desiccation response is distinguishable from the response to saltwater, while the late response includes the differential expression of genes shared between the two stressors. Genes regulating the response to oxidative stress, protein metabolism and embryonic development were among the most important constituents of the desiccation response. After re-submerging the stress-exposed eggs in water, differential expression of genes between treatment and control eggs returned to random baseline levels of differential expression. This study contributes to our understanding of desiccation tolerance in fish and creates a foundation for continued research on responses towards challenges posed by climate change.

INTRODUCTION

Fish spend their entire life in water, and most species do not survive for long outside water. Yet, mechanisms to survive desiccation will become increasingly relevant in a time of climate change with projected scenarios for freshwater systems including lower or more fluctuating water levels (Grillakis 2019, Konapala et al. 2020), which may affect a multitude of fish species in the near future (Lennox et al. 2019) and potentially favour invasive species (Rahel and Olden 2008). Fish eggs are especially vulnerable to fluctuating water levels, as they are not mobile and are often deposited on the ground or on plants that may fall dry when water levels go down (Winfield 2004). On the other hand, fish dispersal patterns suggest that many fish display some degree of tolerance to air exposure: fish repeatedly colonized remote and unconnected water bodies, which requires some sort of overland transport (Hirsch et al. 2018, Martin and Turner 2018) and evolved tolerance mechanisms.

How are inherently water-dependent aquatic organisms such as fish able to deal with water withdrawal? Some species feature special adaptations that allow them to spend some life stage out of the water. Mudskippers, for example, can breathe through their skin, live a semi-terrestrial life, and lay their eggs in air-filled cavities (Chen et al. 2006, Ishimatsu et al. 2007). Some killifish species produce eggs which can enter a dormant stage or diapause and survive months of drought when the ponds they inhabit fall dry (Podrabsky et al. 2010, Polačik et al. 2021). The ability of killifish embryos to adjust embryonic developmental time and hatching evolved at least six times independently of one another (Furness et al. 2015) and is also present in other fish families (Thompson et al. 2017), which indicates a potential for convergent evolution towards desiccation tolerance under similar selective pressures (i.e. repeatedly drying water bodies).

The majority of fish and fish eggs, however, are very sensitive to air exposure. Embryos exposed to desiccation are likely to experience osmotic stress due to water loss and possibly thermal stress. With egg membranes not adapted to avoid water withdrawal, prolonged desiccation is not something we would expect them to withstand. Accordingly, eggs of investigated salmonid species die within few hours of desiccation (Becker et al. 1983, Neitzel and Becker 1985). Yet, other fish eggs of littoral spawners show a medium tolerance towards desiccation, without exhibiting special mechanisms like the development of dormant stages (Marliave 1981, Shimizu et al. 2006, Fisk et al. 2013). Those species often utilize environmental factors like humidity-retaining caves or seaweed covers to increase egg viability under air exposure (Marliave 1981, Shimizu et al. 2006). This medium level of desiccation tolerance over several hours to days may also enable fish to disperse e.g. via birds to unconnected water bodies (Hirsch et al. 2018, Martin and Turner 2018). Little to nothing is known about the molecular processes promoting tolerance to water withdrawal in fish that are not adapted to survive desiccation. The few available studies on fish response to air exposure focus on fish, which have special adaptations to a terrestrial reproductive strategy (Tingaud-Sequeira et al. 2013, Thompson et al. 2017, Wagner et al. 2018).

One fish whose eggs display surprisingly high desiccation tolerance to water withdrawal without a terrestrial life or breeding strategy is the invasive round goby (*Neogobius melanostomus*, Pallas, 1814). The round goby is an invasive species in fresh- and saltwater systems in Europe and North America. Wherever this species appears, it reproduces explosively, thereby competing for resources and habitat with native fish species (Hirsch et al. 2016b). Round gobies readily lay their eggs on artificial structures under water and there are indications that they can use boat hulls as nesting opportunities (Adrian-Kalchhauser et al. 2017, Bussmann and Burkhardt-Holm 2020). The resulting possibility for translocation via overland transport is therefore a plausible way of range extension for the round goby.

The round goby produces eggs that survive at least 24 h out of water (Hirsch et al. 2016a). As long as eggs are re-immersed after air exposure, round goby embryos can hatch healthily at the expected time point. The round goby is therefore an interesting model to study molecular strategies of desiccation tolerance of fish with no adaptation to water stress. Results gained from a species not specialized on withstanding desiccation like the round goby are a relevant complementation to the data generated from desiccation-adapted species like killifish or mudskippers to evaluate whether there is some phylogenetically conserved molecular response to desiccation.

Adverse conditions have previously been shown to trigger phylogenetically conserved transcriptional responses. For example, elevated temperatures trigger the production of heat shock proteins which prevent protein denaturation (“heat shock response”, Richter et al. 2010). Similarly, chemicals trigger the expression of cytochrome P450 enzymes which chemically break down complex molecules and reduce their toxicity (“detox response”, (Goldstone et al. 2006). Both examples illustrate the power of gene expression analyses after exposure to a stressful stimulus to identify organismic responses. The more species are investigated for their molecular response towards desiccation stress, the better we understand if there is a phylogenetically conserved response-mechanism to desiccation. With this knowledge, predictions about the adaptive potential of species towards fluctuating water levels under climate change would be possible, which will allow us to estimate which species will be able to survive in the future. Additionally, this knowledge contributes to the explanation of why round gobies are among the globally most wide-ranging invasive fish (Kornis et al. 2012).

In this study, we investigated 1) the maximum desiccation tolerance of round goby eggs and the influence of desiccation on developmental speed and 2) the transcriptomic response to desiccation stress in comparison with a second osmotic stressor (saltwater). The second experiment aimed to establish whether the detected molecular processes are desiccation-specific or a general response to osmotic stress. We used an RNA sequencing approach to analyse patterns of differential gene expression upon exposure to and recovery from osmotic stress. The overall goal was to identify potential molecular mechanisms promoting desiccation tolerance in fish, which are poorly understood but highly relevant in the face of climate change. Because of the restricted preliminary body of knowledge about desiccation tolerance in non-specialized fish, the set-up of the study is exploratory and aimed to generate an empirical foundation to generate novel hypotheses and design future experiments.

MATERIAL AND METHODS

1. DETERMINATION OF MAXIMUM DESICCATION TOLERANCE

1.1 COLLECTION OF EGGS

For the first experiment, we collected round goby eggs in the industrial Rhine harbour Kleinhueningen, Basel, Switzerland (47°35'10"N 7°35'27"E) between 27. May and 07. August 2018. We used ten spawning traps consisting of ten PVC pipes with each two plastic foil sheets inside (7.5 x 21 cm, initial design described in Hirsch et al. 2016a) to retrieve round goby eggs. The two plastic sheets per PVC tube enabled us to collect every clutch in two parts that were used in either the experimental or the control treatment. We checked the traps every third day and collected all plastic sheets with eggs on them separately in a transport box filled with water from the harbour. We transported the eggs back to the lab, removed dead (i.e. coagulated) eggs and debris from the clutch and took a picture of both parts of every clutch. After taking photos of the clutches, each half was put in a new PVC tube and subjected to treatment conditions. We continued sampling until we did not find new clutches for three consecutive times checking the traps in the field.

1.2 TREATMENT

We exposed one half of every clutch to air for 12 h (n = 12), 24 h (n = 13), 36 h (n = 2), 48 h (n = 11), or 72 h (n = 11), while the other half of the clutch acted as a control and remained submerged in water throughout the entire experiment. We chose these time durations based on a similar experiment presented in Hirsch et al. (2016a), in which no significant effect of desiccation for up to 24 h on hatching was found. Because of the small sample sizes of n = 2-3 in Hirsch et al. (2016a), we included 12 and 24 h as treatments to test the repeatability of their results. However, in the current experiment we aimed to increase desiccation duration until a maximum tolerance could be determined and therefore included the durations 36 h, 48 h, and 72 h. We only exposed two clutches to 36 h of treatment in the beginning of the experiment in order to be able to increase the sample size per treatment for the other four desiccation times. Apart from that, we assigned clutches to desiccation durations randomly while keeping sample sizes approximately equal. For every clutch, we exposed the bigger half to the assigned desiccation treatment. We put each control half in an individual aerated tank filled with filtered tap water (volume: 10 l, water temperature: mean = 17.2, min = 15.7, max = 19.2°C). Tank temperature correlated with Rhine water temperature ($R^2 = 0.83$, $F_{1, 46} = 225.8$, $p < 0.0001$). We exposed the other half of the clutch to air in an incubator (relative humidity 71 – 84 %, temperature 18.0°C). After the respective time of air exposure, we moved the desiccated half to an individual tank similar to the tanks of the control half. For both the rehydrated and the control clutches, we removed dead eggs daily. If all eggs in a clutch died due to the desiccation treatment, we removed that half of the clutch entirely after one day, while we kept observing the control half until seven days after the start of the experiment. If both halves of a clutch survived until seven days after treatment, we kept monitoring the clutches daily until all embryos had hatched. We documented the progression of hatching by removing and counting all hatched larvae daily once hatching started.

1.3 ANALYSIS OF PICTURES

To compare survival rates of eggs exposed to air and eggs submerged in water, we counted the initial numbers of eggs in each half of every clutch from the photos taken at the start and after seven days using the cell counter function in Fiji (Schindelin et al. 2012). If both halves survived (n = 24), we did not take photos after seven days for some clutches (n = 10) to evaluate if photography would lead reduced hatching rates. Additionally, we compared hatching rates for those clutches in which some eggs survived the air exposure treatment.

1.4 STATISTICAL ANALYSIS

We compared the survival rate of eggs after air exposure with the survival rate of control eggs using a linear mixed model including treatment (desiccation – control) and level (12, 24, 36, 48, 72 h) and their interaction as fixed factors and clutch ID as random factor using lme4 version 1.1-27.1 (Bates et al. 2015). We then compared the survival rate of each air exposure time between control and desiccation with Tukey HSD tests using emmeans version 1.7.1-1. We used R version 4.0.5 (R Core Team 2021) for all statistical analyses.

We compared the hatching rate between desiccated and control halves of those clutches that survived air exposure until seven days after air exposure using the same models as used for survival rates.

2. RNA-SEQ EXPERIMENT

2.1 FIELD WORK, SAMPLES, TREATMENTS

For the second experiment, we collected round goby eggs similar to the first experiment between April and August 2019. For the investigation of molecular mechanisms we aimed for more standardized clutches than in the first experiment (eggs by only one female and in an early developmental stage), so we checked the spawning traps daily. Clutches qualified for the experiment if their developmental stage was between 512 cells and completion of epiboly (stage in embryonic development in the late blastula and gastrula period, during which epithelial cells divide and spread around the yolk cell, until it is fully engulfed, Kimmel et al. 1995). Between these time stages, maternal RNAs have been degraded, but organ development has not yet initiated, and embryonic cells are rather homogeneous (Adrian-Kalchhauser et al. 2018). In total, three clutches that met this criterion were processed. Two clutches were in the 1000-cells stage, one was in early epiboly.

We divided each clutch between three treatments by cutting the substrate plastic foil (Figure 1). The three treatments were aerated flow-through freshwater (control treatment), air exposure in an incubator (desiccation treatment), and 10‰ NaCl solution (second osmotic stressor treatment). From each treatment, samples were collected in regular time intervals (Figure 1). After the treatment period, we transferred the eggs subjected to desiccation or saltwater to aerated flow-through freshwater for recovery. During the recovery phase, we collected samples again in regular time intervals (Figure 1). In total, 18 samples were collected from each clutch (15 samples for one clutch that did not have enough eggs to sample at the last time point. Each sample consisted of 10 fish eggs individually removed from the substrate foil with fine forceps, controlled for integrity and developmental stage, and flash frozen together in liquid N2 for storage at -80°C. Samples were later transferred to RNA*later*®-ICE (Frozen Tissue Transition Solution, Ambion) according to manufacturer's protocol to prevent degradation of RNA due to thawing during RNA extraction.

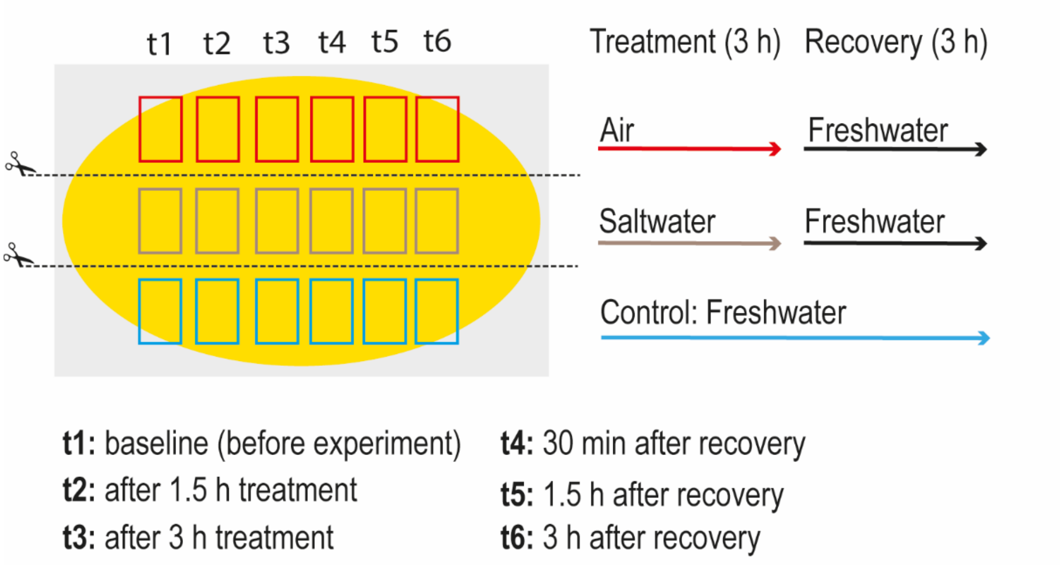


Figure 1 Sampling scheme. Clutches (yellow, attached to a plastic foil in grey) were cut in three parts that were subjected to different treatments: desiccation (air exposure), a control stressor (salt water), and no treatment (continued freshwater incubation). After treatment, all eggs were allowed to recover for 6 hours. Samples for RNA sequencing were taken at the indicated time points.

2.2 RNA EXTRACTION AND SEQUENCING LIBRARY CONSTRUCTION

Total RNA was isolated from 10 embryos per sample using a protocol combining TRIzol reagent extraction with a RNA clean-up using the RNeasy Micro Kit (Qiagen, # 74004) as described in Peterson and Freeman (2009). Embryos were ground in 250 µl TRIzol reagent until they were sufficiently disrupted, then 750 TRIzol was added and the samples were incubated at room temperature for 5 minutes. Afterwards, 0.2 ml chloroform was added, the tube was rocked for 15 seconds to mix, then incubated at room temperature for 2 minutes and centrifuged at 12.000 x g for 15 minutes at 4 °C. The developed top aqueous layer containing the RNA was carefully transferred into a new tube and 0.5 ml isopropanol was added to precipitate the RNA. The samples were incubated for 10 minutes and then centrifuged at 12.000 x g at 4 °C. The supernatant was carefully removed and the pellet containing the RNA was washed with 75% EtOH by gentle inversion. After centrifugation at 7500 x g for 5 minutes at 4 °C the ethanol was removed and the pellet was dried for 10 minutes. Then, the pellet was resuspended by adding 50 RNAse-free water and the samples were incubated at 55°C for 10 minutes while frequently finger-vortexing the tubes. The resulting eluted RNA was then purified using the RNeasy Micro Kit according to manufacturer`s protocol. The recommended DNAse treatment was included.

The quantity and quality of the extracted RNA was assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit RNA BR and HS Assay Kit (Thermo Fisher Scientific, Q10211 and Q32855) and an Advanced Analytical Fragment Analyzer System using a Fragment Analyzer RNA Kit (Agilent, DNF-471) or a FEMTO Pulse system using an Ultra Sensitivity RNA Kit, 275 (Agilent, FP-1202-0275), respectively. Prior to cDNA library generation, probe-based depletion of ribosomal RNA was performed on unquantifiable-100ng ng of total RNA using a RiboCop rRNA Depletion Kit -Human/Mouse/Rat plus Globin (Lexogen #145.96) according to the producer's protocol. Thereafter, the remaining RNA was used as input for a CORALL Total RNA-Seq Library Prep Kit (Lexogen #117.96) in combination with Lexogen workflow A unique dual indexes Set A1 (lexogen UDI12A_0001-0096) following the corresponding user guide (Lexogen document 117UG228V0200). The quantity and quality of the generated NGS libraries were evaluated using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854) and an Advanced Analytical Fragment Analyzer System using a Fragment Analyzer NGS Fragment Kit (Agilent, DNF-473), respectively. Pooled cDNA libraries were paired-end sequenced using a NovaSeq 6000 S1 reagent kit v1.5, 100 cycles (illumina 20028319) on an Illumina NovaSeq 6000 instrument. The quality of the sequencing runs was assessed using illumina Sequencing Analysis Viewer (illumina version 2.4.7) and all base call files were demultiplexed and converted into FASTQ files using illumina bcl2fastq conversion software v2.20. On average, 37 million reads/library were produced. The RNA quality-control assessments, generation of libraries and sequencing runs were performed at the Next Generation Sequencing Platform, University of Bern, Switzerland.

2.3 SEQUENCE DATA PROCESSING AND DIFFERENTIAL GENE EXPRESSION ANALYSIS

Following the merging of technical replicates, the quality of the raw data was assessed using FastQC v.0.11.8 (Babraham Bioinformatics) and MultiQC v.1.7 (Ewels et al. 2016). Following quality assessment, low quality sequences were filtered, UMIs processed, and adapters trimmed using fastp v.0.20.1 (Chen et al. 2018). Three representative samples per treatment group and clutch were subjected to individual *de novo* assembling using Trinity v.2.8.5 (Haas et al. 2013), resulting in a set of 82303 transcripts (mean N50 = 1002). To obtain a set of non-redundant transcripts we applied the following filtering steps: first, we used TransDecoder v.5.3.0 to identify all likely coding regions, and then filtered by selecting the single best open reading frame (ORF) per transcript. Any transcripts with ORFs less than 100 bp in length were removed before performing further analysis. Second, redundancy was further reduced in the remaining transcript set by clustering highly similar sequences with CD-Hit v.4.8.1 (Fu et al. 2012), using a sequence identity threshold of 0.95. . We applied BUSCO (https://academic.oup.com/bioinformatics/article/31/19/3210/211866) to evaluate the assembly completeness by identifying a set of highly conserved Eukaryotic and Actinopterygii orthologs in the assembly. We then used salmon v.1.5.0 (Patro et al. 2017) to quantify the expression value of each transcript across samples in an alignment-free manner. The R package tximport v.3.14 (Soneson et al. 2015) was then used to obtain transcripts counts used for downstream analyses.Differentially expressed genes (DEGs) were identified between treatments and controls using DESeq2 v.1.30.1 (Love et al. 2014). We chose FDR< 0.1 and log-2-fold change = 1 as our thresholds for significant DEGs, as our experiment was of exploratory nature and based on inherently variable field samples, therefore allowing for an increase false positive rate. We conducted differential expression analyses separately for each time point, which is often the recommended approach when dealing with small time-course datasets with only a few time points (Robinson et al. 2009, Love et al. 2014, Ritchie et al. 2015, Varoquaux and Purdom 2020).All sequences were first annotated against the UniProtKB/SwissProt database using stand-alone blastp v.2.11.0 (max_target_seqs = 5, min. e-value = 0.000001). Matches with the lowest E-value were extracted from the results and sequences with no hits were queried against the Trembl database with parameters as above. All sequences were then assigned to Gene Ontology (GO) terms based on UniProtKB/SwissProt mapping and additionally via eggNOG (Huerta-Cepas et al. 2018) v.5.0 using eggNOG-Mapper v.2 (Taxonomic scope: automatic, Orthologs: all orthologs, GO evidence: non-electronic terms, E-value: 0.001, min. hit bit-score: 60). Transcripts of interest that did not result in an annotation were additionally manually annotated with BLASTp against the NCBInr database.

2.4 IDENTIFICATION AND CHARACTERIZATION OF SPECIFIC DESICCATION RESPONSE AND GENERAL OSMOTIC STRESS RESPONSE

We identified which of the DEGs identified in in desiccation and saltwater treatments were unique to that treatment and which DEGs were shared between both treatments at each time point. To test whether any GO terms were overrepresented in DEGs, we sorted transcripts by their adjusted p-values and performed GO category-specific (BP, MF) ranked-based tests for each assigned GO term by applying Kolmogorov-Smirnov tests via package topGO v.2.42.0 (Alexa and Rahnenfuhrer 2019) with the full *denovo* assembled transcriptome as GO background universe (method = “weight01”, significance level α = 0.1).

RESULTS

1. DETERMINATION OF DESICCATION TOLERANCE

In total, we collected 49 clutches of round goby eggs that were used for the determination of desiccation tolerance over the course of summer 2018 (Figure 2).

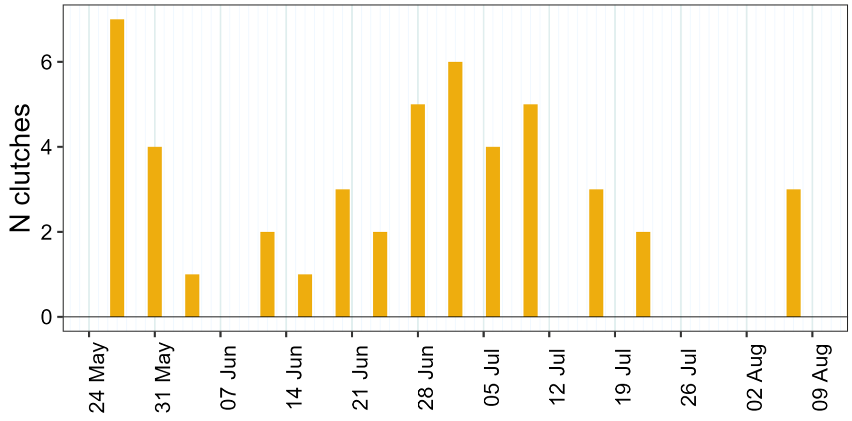


Figure 2 Number of round goby clutches collected daily during the breeding season 2018.

1.1 EFFECTS OF DESICCATION ON SURVIVAL

The longer clutches were exposed, the less eggs survived compared to the unexposed part of the clutch (Figure 3 A). While the survival rate after seven days was not significantly different between desiccated and watered eggs after 12h, it was significantly lower in the desiccated halves of the clutches after 24, 48, and 72 h (Figure 3 A, Table 1). Only in one clutch individual eggs survived air exposure for 48 h, while no eggs survived air exposure for 72 h (Figure 3 A).

If a clutch survived air exposure (survival rate ≠ 0, only available for statistical testing for 12 and 24 h of air exposure), the hatching rate was similar in the control and the air-exposed halves (Figure 3 B, Table 1). Taking the clutches out of the water to take photos after seven days did not influence the hatching rate (linear model, estimate = -3.2, CI = -15.23 - 8.74, df = 15.0, t = -0.51, p= 0.616).

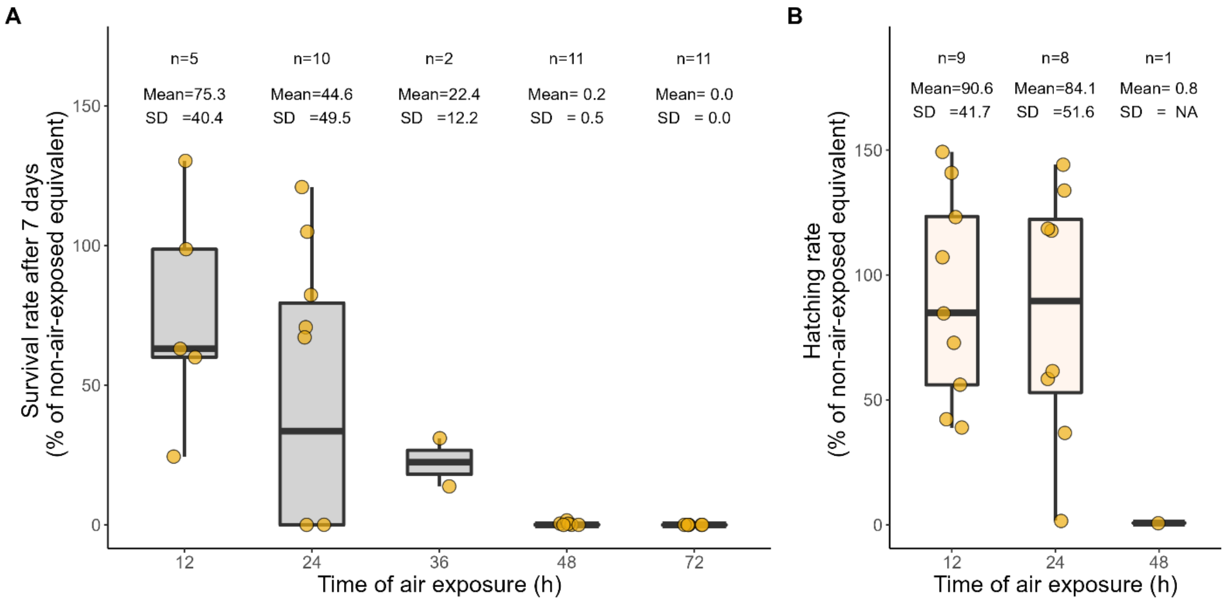


*Figure 3 **A** Survival rate of round goby clutches exposed to air for different amounts of time expressed as percentage of survival rate of a non-air-exposed control part of the clutch. **B** Hatching rate of round goby clutches exposed to air for different amounts of time expressed as percentage of hatching rate of a non-air-exposed control part of the clutch. n = sample size, SD = standard deviation.*

Table 1 Differences in survival rates and hatching rates between clutch halves that remained in water (C) and clutch halves that were exposed to air (D) for 12, 24, (36), 48, and 72 hours. We only had two samples exposed to air for 36 h; the results are therefore to be interpreted carefully. Results for survival rate and hatching rate are of linear mixed models including treatment (C, D) and level (12 -72 h) and their interactions fixed factors and clutch ID as random factor. Pairwise comparisons of each desiccation time to control treatment were calculated using Tukey HSD post-hoc tests. Estimate = mean of differences C – D, CI = 95 % confidence interval, t = t-value, df = degrees of freedom, p = p-value (bold if significant using a significance level of p < 0.5).

Survival rate after 7 days					
	Estimate	*CI*	*t*	*df*	*p*
12 h	22.6	-3.69 – 48.9	1.75	33.2	0.090
24 h	39.1	20.54 – 57.7	4.28	33.2	**0.0001**
(36 h	48.5	6.98 – 90.1	2.38	33.2	**0.0234)**
48 h	62.8	44.67 – 81.0	7.03	33.2	**<0.0001**
72 h	74.9	57.16 – 92.6	8.597	33.2	**<0.0001**
Hatching rate of clutches surviving air exposure					
12 h	-0.3	-16.48 – 15.8	-0.05	16	0.965
24 h	9.6	-8.48 – 27.6	1.12	16	0.278

1.2 EFFECTS OF DESICCATION ON DEVELOPMENTAL TIME

We did not find an effect of desiccation on the onset of eyespot development, the day of start of hatching, the day of completion of hatching, or the number of days until complete hatching (Figure 4) for the samples surviving seven days after air exposure (statistics only for 12 h and 24 h).

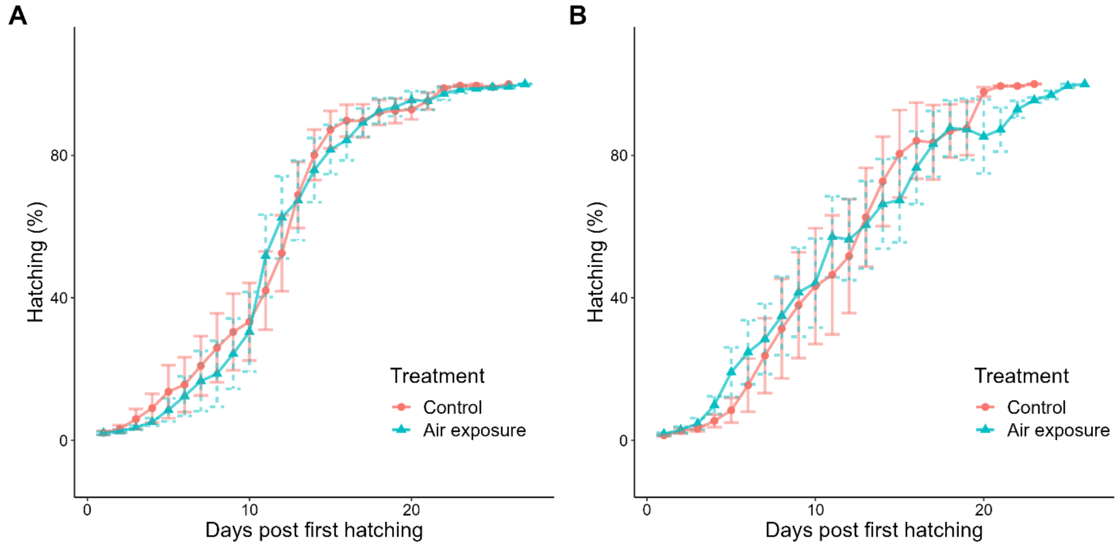


*Figure 4 Effect of air exposure on hatching of round goby embryos. Daily cumulative percentage hatching of round goby clutches exposed to air for **A** 12 hours, or **B** 24 hours. Day 0 represents the first day of hatching. Data show mean ± standard error.*

Table 2 Differences in developmental rates between clutch halves that remained in water (C) and clutch halves that were exposed to air (D) for 12 or 24 hours. Results are of linear mixed models including treatment (C, D), level (12 -72 h) and their interactions, as well as water temperature of the tanks as fixed factors, and clutch ID as random factor. Pairwise comparisons of each desiccation time to control treatment were calculated using Tukey HSD post-hoc tests. Estimate = mean of differences C – D, CI = 95 % confidence interval, t = t-value, df = degrees of freedom, p = p-value (bold if significant using a significance level of p < 0.5).

Onset of eye development					
	Estimate	*CI*	*t*	*df*	*p*
12 h	-0.1	-0.53 – 0.35	-0.428	27	0.677
24 h	0.3	-0.15 – 0.82	1.42	27	0.076
Onset of hatching					
12 h	-41.4	-110.5 – 27.6	-1.30	13	0.217
24 h	-5.3	-89.9 – 79.2	-0.14	13	0.894
Completion of hatching					
12 h	-0.4	-3.77 – 2.89	-0.29	14	0.779
24 h	0.3	-3.49 – 4.06	0.16	14	0.873
Duration of hatching					
12 h	-41.0	-29.8 – 111.8	1.25	13	0.233
24 h	5.8	-80.9 – 92.6	0.15	13	0.887

2. RNA-SEQ EXPERIMENT

2.1 SEQUENCING AND ASSEMBLY

The *de novo* assembly resulted in a set of 82303 quality-controlled contigs with an average length of 660 bp, and an average N50 of 1002 bp. After ORF prediction and removal of redundant sequences, 31237 unique contigs with an average length of 822 bp, an average N50 of 1035 were kept with the following BUSCO completeness results: C:76.8% [S:62.7%, D:14.1%], F:15.3%, M:7.9% for the Eukaryota ortholog set, and C:43.5%[S:39%, D:4.5%], F:6%, M:50.5% for the Actinopterygii ortholog set. Of these 31237 contigs, 88.98% had a significant hits against the UniProtKB/SwissProt database.

2.2 IS THERE A SPECIFIC DESICCATION RESPONSE?

We identified a total of 1853 transcripts ($p < 0.1$, 5.9 % of all transcripts) as differentially expressed between water and desiccation treatments at at least one time point, of which 1278 (70.0 % of all DEGs) could be annotated. There were a total of 1320 differentially expressed genes between water and saltwater treatment. There were 744 genes that were differentially expressed in both desiccation and saltwater treatment (37.1 % of desiccation-specific DEGs, 51.2 % of saltwater-specific DEGs).

The transcriptional response to desiccation measured as number of significantly differentially expressed genes was strongest at t1: stressor short, decreased upon longer air exposure and was indistinguishable from baseline levels of DEGs during recovery (Figure 5). The transcriptional response to saltwater was only distinguishable from the baseline levels of DEGs at t2: stressor long (Figure 5). Details of the expression patterns are presented below.

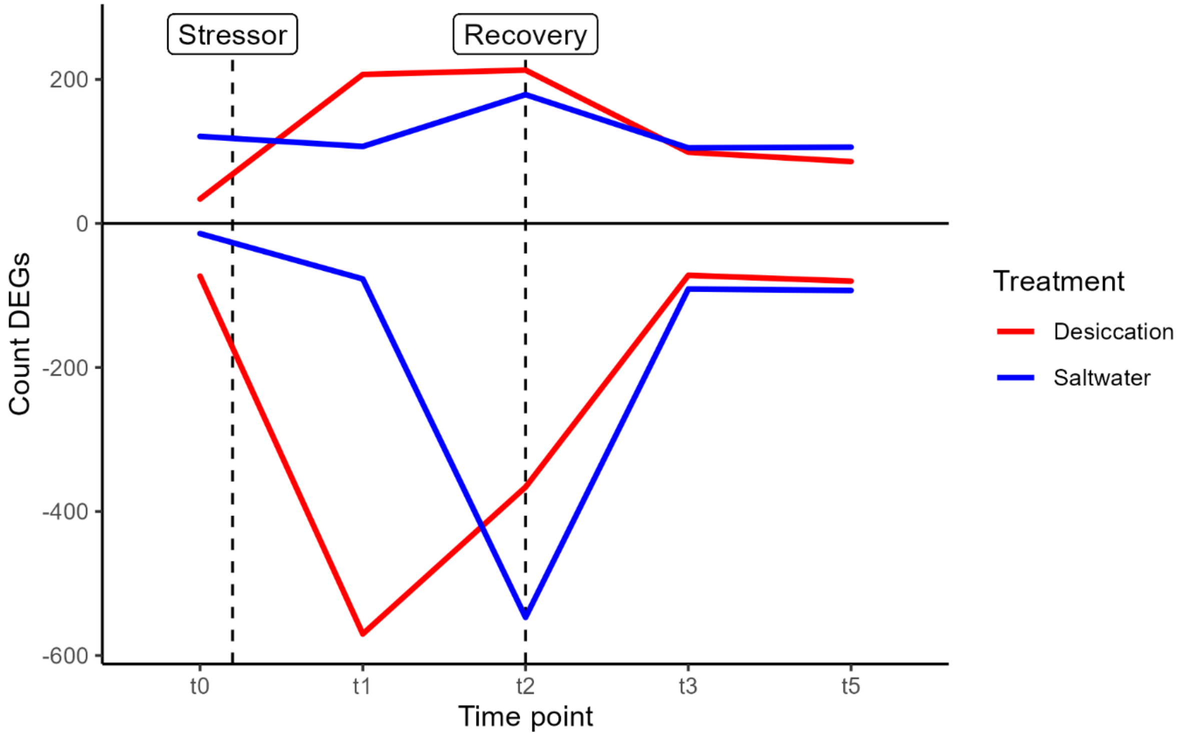


Figure 5 Patterns of differential expression between round goby eggs exposed to desiccation (red) or saltwater (blue) compared to control eggs continuously kept in water. t0 = baseline differential expression before treatment. Afterwards, eggs were submitted to treatments ("Stressor"). After 3 h of stress exposure (t2), stress-exposed eggs were re-submerged in water ("Recovery"). Positive counts represent upregulated DEGs, negative counts represent downregulated DEGs. t4 is not shown, because of the reduced sample size of n = 2 at that time point.

Baseline: There were a total of 107 DEGs between control and desiccation exposed eggs already before we started the treatment, of which 34 were upregulated and 73 were downregulated (Figure 5). There were 135 DEGs in the samples designated for the saltwater treatment, of which 121 were upregulated and 14 were downregulated (Figure 5). Since we chose less conservative settings for the identification of DEGs, we expected an inflated number of false positives. The numbers of DEGs that appear at t0: baseline can therefore serve as a measure for the number of false positives we can expect in our samples during treatment.

Expression patterns during treatment: During the time of desiccation, a total of 1270 genes were differentially expressed compared to control samples. The number of DEGs in the desiccation treatment at t1: stressor short (777 DEGs) was higher than at t2: stressor long (579 DEGs). Thereby, especially the number of down-regulated genes decreased (t1: 207 DEGs, t2: 213 DEGs), while differential expression levels of up-regulated genes remained similar (t1: 570 DEGs, t2: 366 DEGs, Figure 5). There were only 21 genes that were differentially expressed at both time points (Figure 6 A).

During the time of saltwater exposure, a total of 886 geneswere differentially expressed compared to control samples. The number of DEGs in the saltwater treatment was lower at t1: stressor short (184

DEGs, 107 upregulated and 77 downregulated) than at t2: stressor long (726 DEGs, 179 up-regulated and 547 down-regulated, Figure 5). There were only 24 genes that were differentially expressed at both time points during saltwater exposure (Figure 6 B).

At time point t1: stressor short, 95 genes were differentially expressed in desiccation as well as saltwater treatments (12.2 % of desiccation-DEGs, 51.1 % of saltwater-DEGs, Figure 7 A). 682 DEGs (87.8%) were therefore specific to the desiccation treatment after 1.5 h of air exposure (Figure 7 A). There were proportionally less upregulated DEGs specific to the desiccation response (57.0 %) than downregulated DEGs (87.8 %). At time point t2: stressor long, 334 genes were differentially expressed in both treatments (67.7% of desiccation-DEGs, 46.0% of saltwater-DEGs, Figure 7 B). Only 180 DEGs (32.3%) were specific to desiccation treatment after 3 h of air exposure (Figure 7 B). There were 46 DEGs shared between desiccation treatment at t1: stressor short and saltwater treatment at t2: stressor long. The molecular response to saltwater after 3 h is therefore not a delayed, but similar response as the response to desiccation after 1.5 h.

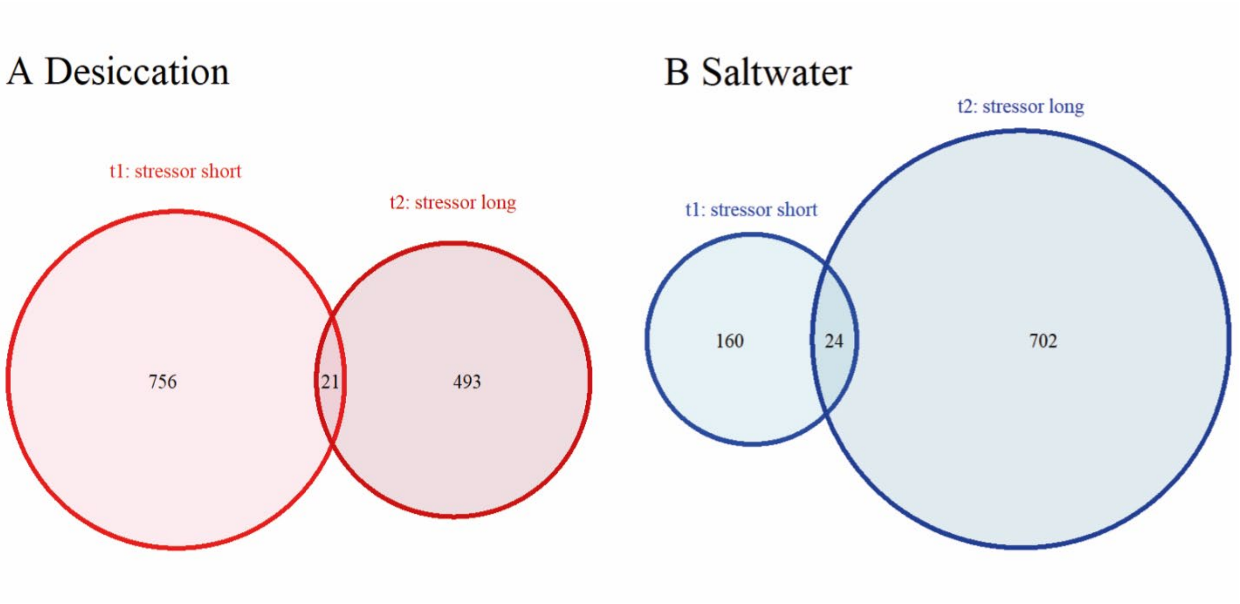


Figure 6 Number of DEGs at two time points during desiccation and saltwater (t1: stressor short = 1.5 h, t2: stressor long = 3 h). ***A*** *Transcripts with different expression levels compared to control samples when exposed to desiccation.* ***B*** *Transcripts with different expression levels compared to control samples when exposed to saltwater. The numbers in the centre of the circle represent the number of transcripts uniquely differentially expressed at one o the two time points. The numbers in the intersection represent transcripts that are differentially expressed at both time points. Circle sizes are scaled to count within* ***A*** *and* ***B****, but not across* ***A*** *and* ***B****.*

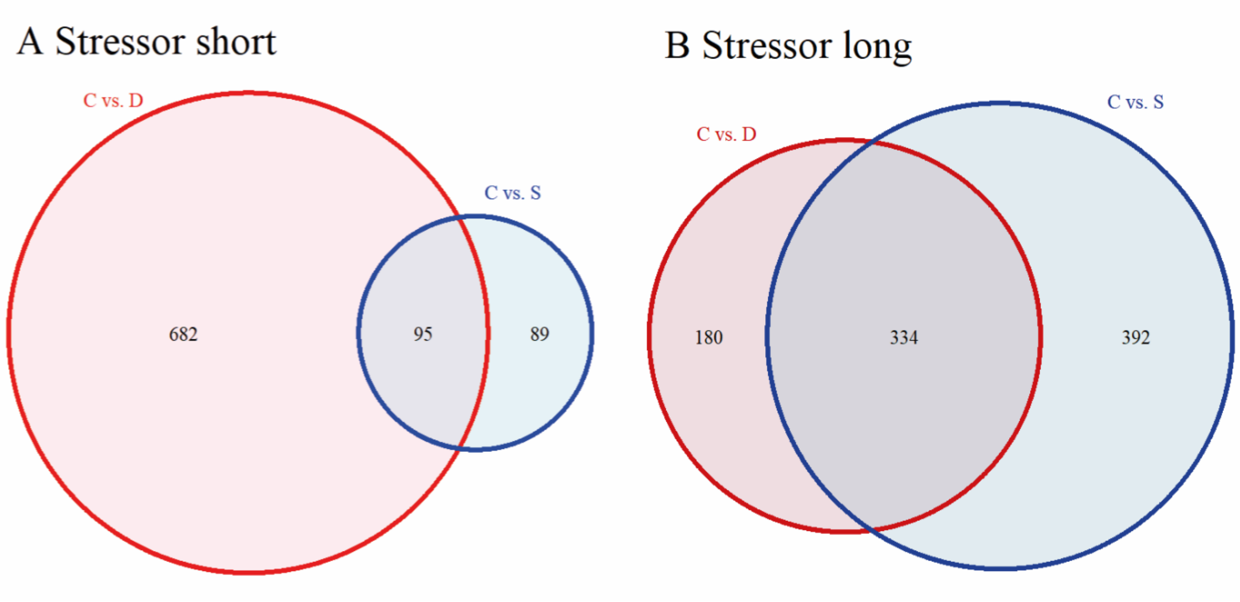


Figure 7 Number of DEGs in samples exposed to desiccation (C vs. D) and between samples exposed to saltwater (C vs. S) at two time points during treatment (stressor short = 1.5 h, stressor long = 3 h). ***A*** *Transcripts with different expression levels in the two treatments compared to control samples at time point t1: stressor short.* ***B*** *Transcripts with different expression levels in the two treatments compared to control samples at time point t2: stressor long. The numbers in the centre of the circle represent the number of transcripts uniquely differentially expressed in one of the two treatments. The numbers in the intersection represent transcripts that are differentially expressed in both treatments. Circle sizes are scaled to count within* ***A*** *and* ***B****, but not across* ***A*** *and* ***B****.*

Expression patterns during recovery: Differential expression patterns during recovery are considerably less strong that during exposure to osmotic stress and are very close to baseline differential expression levels (Figure 5). Already at time point t3: recovery short, there were only 171 DEGS (99 upregulated, 72 downregulated) in the desiccation treatment and 196 DEGs (105 upregulated, 91 downregulated) in the saltwater treatment (Figure 5). 92 genes were differentially expressed in both desiccation and saltwater treatments (53.8% of desiccation-DEGs, 46.9% of saltwater-DEGs). The results of t4: stressor long are not considered here, because we could not get samples from one of the clutches at this time point and therefore only have a sample size of n = 2. The results would therefore statistically particularly unreliable. At time point t5: recovery end, there were 166 DEGs in the desiccation treatment (86 upregulated, 80 downregulated) and 199 DEGs in the saltwater treatment (106 upregulated, 93 downregulated, Figure 5). 84 of these DEGs were shared between desiccation and saltwater treatment (50.6% of desiccation-DEGs, 42.2% of saltwater DEGs).

2.3 GENE ONTOLOGY (GO) ENRICHMENT OF THE TRANSCRIPTIONAL RESPONSE TO DESICCATION

We only conducted a detailed analysis of the functional enrichment for the time points t1: stressor short and t2: stressor long, because the number of differentially expressed genes during recovery was almost as low as at time point t0: baseline, i.e. before the eggs were submitted to the treatment.

At time point t1: stressor short, upregulated genes in the desiccation treatment were enriched in biosynthetic processes, response to stimuli or stress, regulation of transcription, and cell growth (Figure 8 A). At time point t2: stressor long, upregulated genes were enriched in transcriptional and translational biological processes (Figure 8 B). Additionally, the cellular response to DNA damage stimulus and biological processes involved in cell cycle processes were enriched (Figure 8 B). Significant GO terms for downregulated transcripts at time point t1: stressor short included regulation of responses to stress or stimuli and cell repair functions (regulation of autophagy and apoptosis and signalling pathways involved in answering to oxidative and other stress), translation and protein metabolism (especially catabolic processes), embryonic development, cell cycle and cell differentiation, and cytoskeleton reorganization (Figure 9 A). Significant GO terms for downregulated genes included transcriptional and translational processes and protein metabolism (Figure 9 B).

In the saltwater treatment, upregulated genes at time point t1: stressor short were enriched in transcription and translation processes and metal ion binding (Figure 10 A). Upregulated genes at time point t2: stressor long were only enriched in DNA binding (Figure 10 B). Significant GO terms for downregulated transcripts in the saltwater treatment at time point t1: stressor short were related to protein catabolic processes (A). Downregulated genes in the saltwater treatment at time point t2: stressor long were enriched in osmoregulation processes, metabolic processes, responses to stress or other stimuli and cell proliferation (B).

There was minimal overlap between the enriched GO terms in the desiccation and saltwater treatments at both time points during stress exposure. At time point t1: stressor short, only organonitrogen compound catabolic process (GO:1901565, downregulated) was enriched in both treatments. Interestingly, at time point t2: stressor long, there were no overlapping GO terms despite the high overlap of DEGs. The GO term DNA binding (GO:0003677, upregulated) was enriched in both desiccation (stressor short) and saltwater (stressor long).

In the discussion, we detail the biological significance of three exemplary groups of GO terms and genes involved in 1) oxidative stress response, 2) protein metabolism, 3) embryonic development and cell cycle. The grouping of GO terms and genes in the three categories are presented in Supplementary table 1.

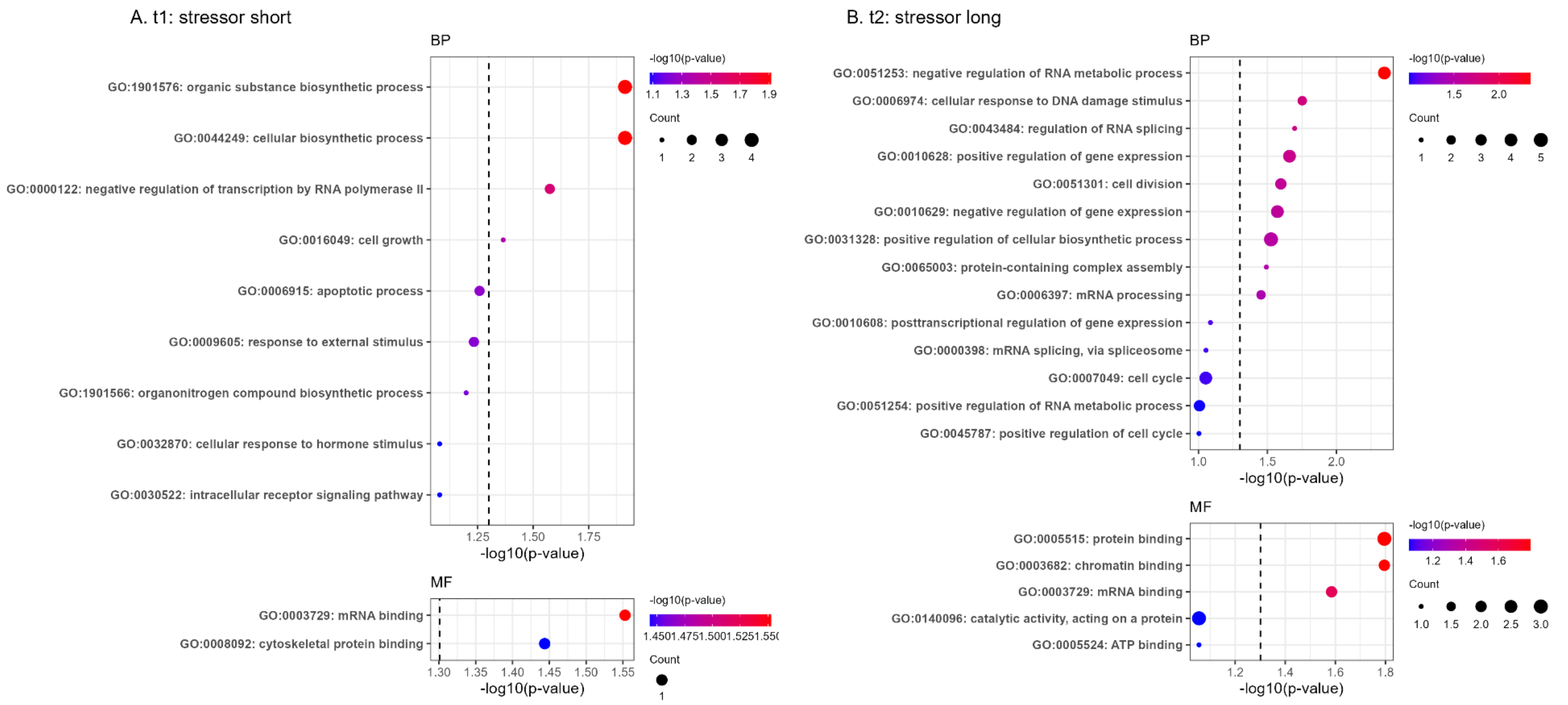


*Figure 8 GO enrichment results of up-regulated transcripts in desiccation vs. control at time point t1: stressor short (**A**) and t2: stressor long (**B**). Enriched GO terms belong to the categories BP = Biological process or MF = Molecular functions. X-axis and color scale represent the –log10(p-value) of Kolmogorov-Smirnov tests of GO terms with p < 0.1. Size represents the number of significant genes in each category. Vertical dashed line represents a p-value of 0.05.*

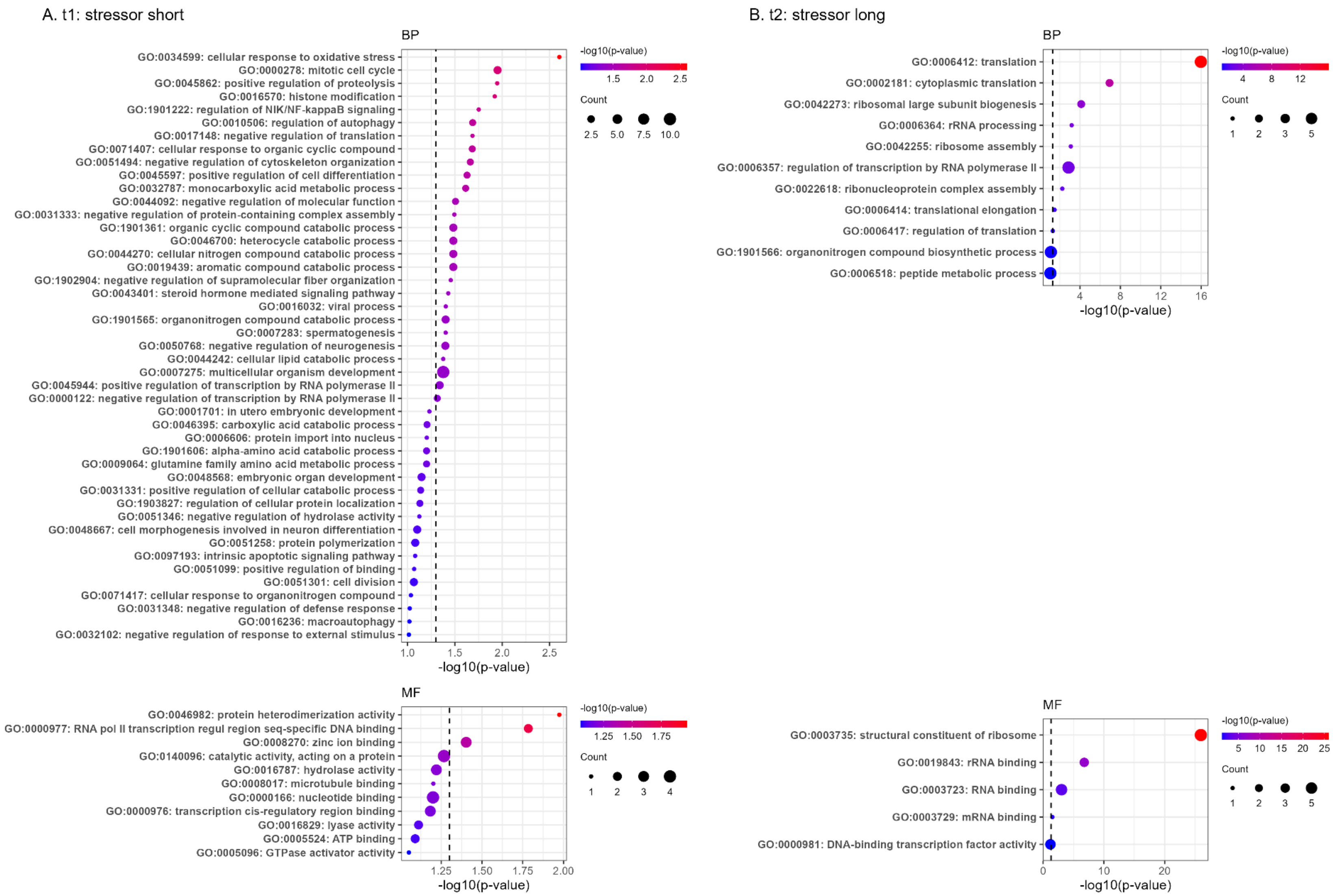


*Figure 9 GO enrichment results of down-regulated transcripts in desiccation vs. control at time point t1: stressor short (**A**) and t2: stressor long (**B**). Enriched GO terms belong to the categories BP = Biological process or MF = Molecular functions. X-axis and colour scale represent the –log10(p-value) of Kolmogorov-Smirnov tests of GO terms with p < 0.1. Size represents the number of significant genes in each category. Vertical dashed line represents a p-value of 0.05.*

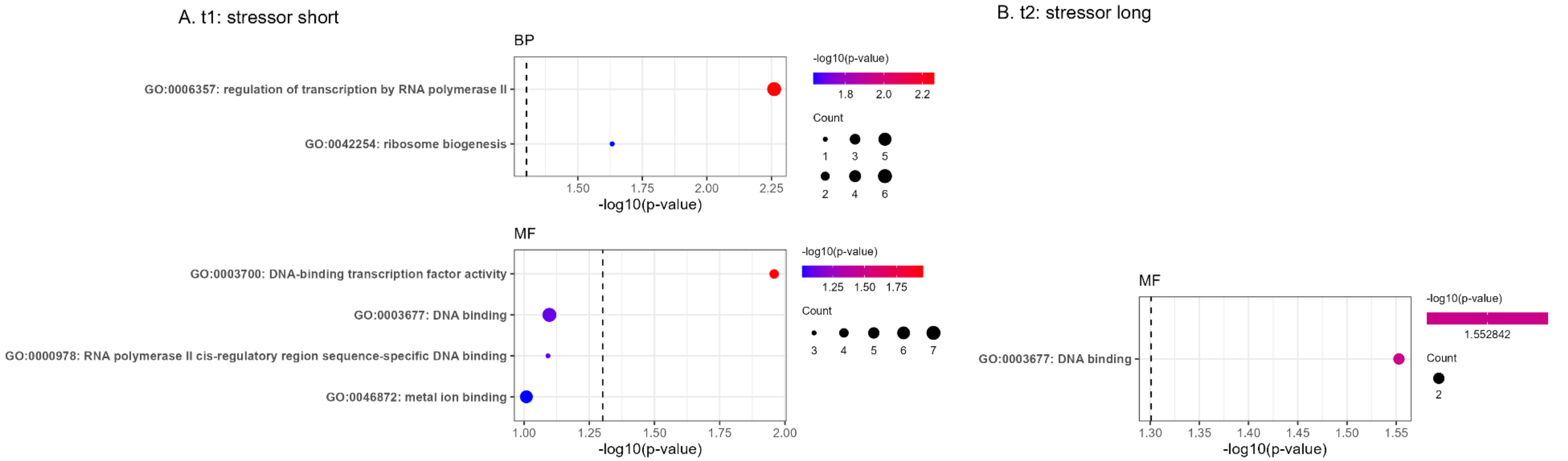


*Figure 10 GO enrichment results of up-regulated transcripts in saltwater vs. control at time point t1: stressor short (**A**) and t2: stressor long (**B**). Enriched GO terms belong to the categories BP = Biological process or MF = Molecular functions. X-axis and colour scale represent the –log10(p-value) of Kolmogorov-Smirnov tests of GO terms with p < 0.1. Size represents the number of significant genes in each category. Vertical dashed line represents a p-value of 0.05.*

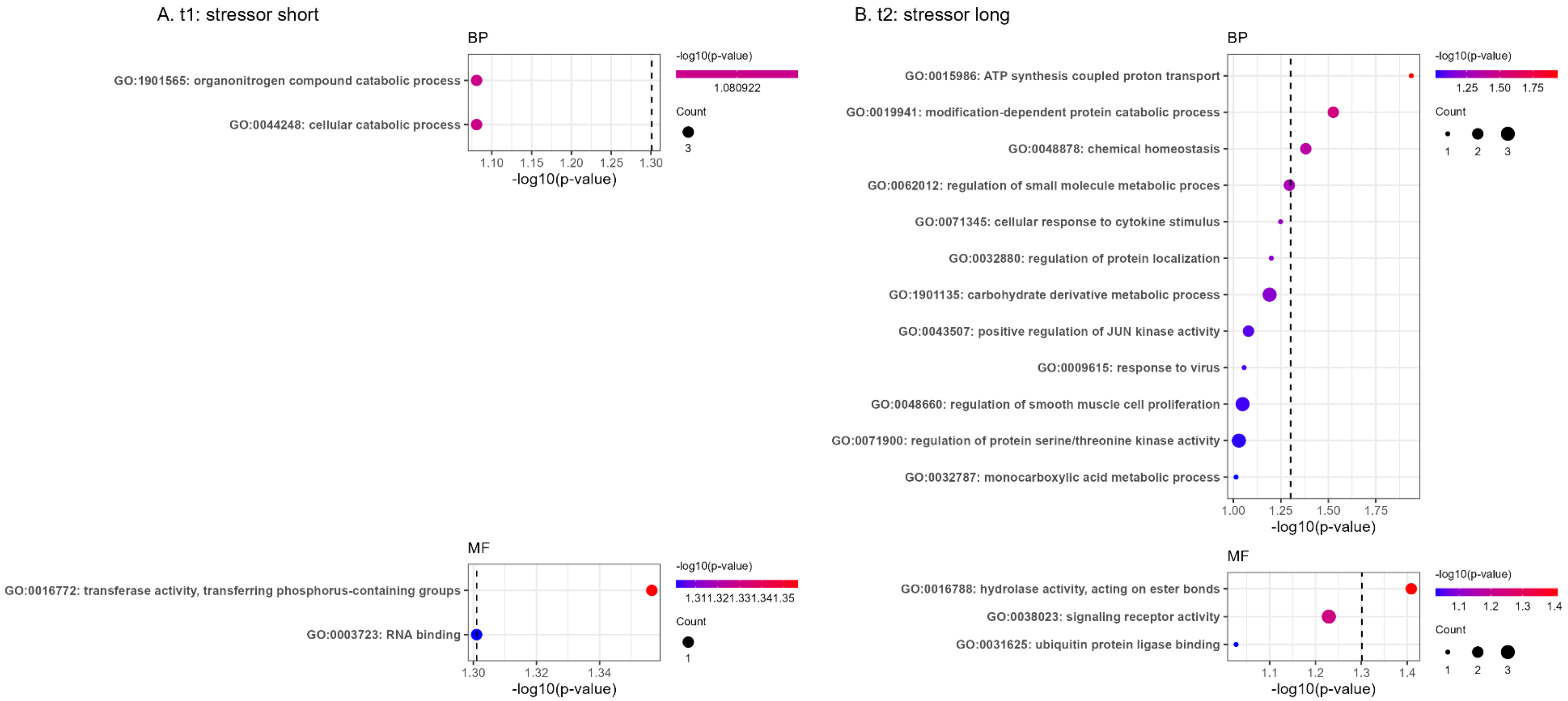


*Figure 11 GO enrichment results of down-regulated transcripts in saltwater vs. control at time point t1: stressor short (**A**) and t2: stressor long (**B**). Enriched GO terms belong to the categories BP = Biological process or MF = Molecular functions. X-axis and colour scale represent the –log10(p-value) of Kolmogorov-Smirnov tests of GO terms with p < 0.1. Size represents the number of significant genes in each category. Vertical dashed line represents a p-value of 0.05.*

DISCUSSION

In this study, we demonstrate a remarkable tolerance for desiccation in a fish without known adaptations to prolonged air exposure. The transcriptional response to desiccation is strongest shortly after the onset of air exposure and is reduced and changed after several hours. Differential gene expression in the desiccation treatment is not comparable to a different osmotic stressor (saltwater) shortly after onset of stress exposure, but becomes more similar after several hours. However, the biological processes and molecular functions enriched in both treatments are notably different from each other, despite the number of shared DEGs.

EFFECTS OF DESICCATION ON ROUND GOBY EGGS

In experiment 1, we demonstrate that the round goby can survive desiccation for up to 48 h. While Hirsch et al. (2016a) showed that round gobies can survive 24 hours of desiccation and did not find an effect of desiccation on developmental speed, their results were based on a small sample size (n = 2-3) and did not test for the upper limit of desiccation tolerance. We only found surviving eggs after 48 hours of desiccation once, but both of the clutches exposed for 36 h partly survived. It is noteworthy that the dead eggs in clutches that survived only partly were predominantly along the fringes of the clutches, indicating that not only processes within an individual egg contribute to desiccation tolerance, but also the physical properties of the clutch. The pear-shape of the eggs combined with the typically curved surfaces (e.g. mussel shells) in which round gobies breed may contribute to the ability of the clutches to retain water in the interstitial spaces between the eggs. Additionally, the composition and thickness of the egg shell could contribute to the observed desiccation tolerance (Podrabsky et al. 2001, Messaddeq et al. 2017), but little work has been published about chorion features of round goby eggs. However, eventually all eggs are exposed to air and need to individually react to that challenge.

We did not find desiccation-stimulated or delayed development speed in our first experiment. In some other fish species desiccation substantially influences their developmental speed. For example, in the beach-spawning killifish *Fundulus heteroclitus* developmental speed is increased through air exposure (Tingaud-Sequeira et al. 2009). Air exposure also leads to earlier hatching in salmonids (Wedekind and Muller 2005, Fisk et al. 2013). On the other hand, in highly adapted species of annual killifish, air exposure leads to developmental arrest and the entering of a dormant stage to survive months of desiccation in ephemeral ponds (Furness 2016, Polačik et al. 2021), while it delays development in non-annual rivulids (Varela-Lasheras and Van Dooren 2014). The absence of a change of developmental speed in round goby embryos that survived desiccation indicates that the molecular reactions towards desiccation are different from highly desiccation-adapted fish.

DIFFERENTIAL GENE EXPRESSION PATTERNS OVER TIME

Desiccation induced effects were strongest after 1.5 h, diminished over time of desiccation and were practically absent following rehydration. In desiccation-adapted killifish embryos, the number of DEGs is also highest within the first three hours after air exposure – and like in the round goby, most of the genes are down-regulated (Tingaud-Sequeira et al. 2013). The rapid transcriptome response to an environmental stressor demonstrates the adaptive ability of killifish embryos to react to desiccation. While round goby embryos are not obviously adapted to dry conditions, the similarly fast response to air exposure indicates that round gobies might be able to adapt to future conditions in which air exposure could occur more regularly. Potentially, the ability to tolerate a certain level of desiccation has its origins in the historical fluctuations in water levels within the Ponto-Caspian basin during the Pleistocene, during which adaptive processes lead to the split of round goby into two subspecies (*N. m. melanostomus* in the Black Sea and *N. m. affinis* in the Caspian Sea (Reid and Orlova 2002, Neilson and Stepien 2009).

The transcriptional response towards desiccation differed from responses to saltwater exposure after 1.5 h. After 3 h, the number of genes differentially expressed in both conditions increases. However, the GO term enrichment of DEGs in both conditions shows that the functional response towards these stressors is different. Both treatments are expected to cause some osmotic stress, as both would lead to water loss in the egg. The statistical test used for the functional enrichment analysis takes into account the significance of the individual DEGs. Probably the DEGs shared between desiccation and saltwater treatment are not of the same importance in the two treatments and are therefore not significantly enriching the same biological functions.

The strong initial response towards desiccation compared to saltwater could also indicate that desiccation stress is recognized earlier than exposure to saltwater and that the response towards saltwater is delayed, but similar. However, we did not observe high similarities between the early desiccation response and the late saltwater response. We therefore conclude that the two stressors are experienced and countered specifically, not by a general cellular stress-response machinery. Given the scope of our study, we focus on the desiccation-specific molecular response observed in round goby embryos in the hereafter.

FUNCTIONAL ENRICHMENT OF RESPONSE TO DESICCATION

As a detailed discussion of all significant genes and GO terms was beyond the scope of this study, we discuss three exemplary groups of genes involved in 1) oxidative stress response, 2) protein metabolism, 3) embryonic development and cell cycle and detail their potential biological significance in reacting to desiccation.

1) Oxidative stress response: Desiccation stress leads to the accumulation of reactive oxygen species (ROS), which can lead to oxidative damage caused by water stress in aquatic organisms (França et al. 2007, Zajic and Podrabsky 2020). A variety of processes can be impaired by free radical formation, including DNA damage or denaturation of proteins, eventually affecting the overall metabolism (Hansen et al. 2006). During air exposure, biological processes involved in responses to stress (especially: oxidative stress), apoptosis (programmed cell death), and autophagy were functionally enriched. One candidate gene for the enrichment of the cellular response to oxidative stress and macroautophagy after 1.5 h was the downregulated SIRT2. SIRT2 can act as a negative regulator of oxidative stress, its downregulation under desiccation conditions therefore results in a de-facto upregulation of the biological stress response by prolonging mitotic arrest and preventing cells from apoptosis (Lynn et al. 2008, Inoue et al. 2009). Apoptotic processes were further regulated at 1.5 h by the reduced expression of the gene LATS1 and by the enhanced expression of the gene RNF216. LATS1 is a serine/threonine-specific protein kinase that promotes apoptosis and autophagy (Yang et al. 2004, Hao et al. 2008), its downregulation therefore leads to reduced apoptosis. RNF216 on the other hand down-regulates activation of NF-κB signalling, which protects cells from apoptosis through oxidative stress (Morgan and Liu 2011). NF-κB signalling was further downregulated by the gene AGO3, which is responsible for RNA-mediated gene silencing. In total, there is evidence for enhanced as well as reduced apoptosis, painting a mixed picture of the cellular response toward desiccation stress. It is possible that some cells are damaged enough to commit to programmed cell death and autophagy, while others enhance mechanisms protecting them from apoptosis through oxidative stress, making both processes detectable in our pooled samples. Suppressing programmed cell death through various pathways was found in several organisms and might be a conserved strategy to counteract desiccation damage, as an accumulation of dead cells could lead to tissue damages (Chen et al. 2009, Rodríguez-Porrata et al. 2012, Tingaud-Sequeira et al. 2013, Thompson et al. 2017). On the other hand, apoptosis is a process that prevents cells that suffered severe DNA damage from uncontrolled proliferation and becoming cancerous, and is therefore an important mechanism to keep organisms healthy. A mixture of cells preventing apoptosis and committing to it is therefore a plausible reaction of the entire embryo.

Other mechanisms likely contributing to a protective cellular response were responses to hormone stimulus and intracellular receptor signalling pathways, which were driven for example by the epigenetic regulator KMT2D, a promotor of DNA repair processes (Shinsky et al. 2015).

The stress response to desiccation after 3 h mainly consisted of reactions to DNA damage, for example mediated by differential expression of the serine/threonine-protein kinase DYRK2 and EPC1 as component of the NuA4 histone acetyltransferase complex. Both genes are known to be involved in DNA repair processes (Doyon et al. 2004, Taira et al. 2007, Yoshida 2008)

2) Protein metabolism: After 1.5 h of desiccation, we observed the downregulation of a set of genes involved in protein catabolic processes and an upregulation of biosynthetic processes. Catabolic processes lead to the breakdown of compounds, thereby releasing energy that can be used by the cell in other processes. Two downregulated HAL-genes played a role in the enrichment of catabolic processes, which are important in the pathway of L-histidine degradation into L-glutamate, which can increase oxidative stress (Babu and Bawari 1997, Amonpatumrat et al. 2008). The reduction of catalytic processes could therefore alleviate oxidative stress. Furthermore, instead of utilizing the energy that catabolic processes could deliver, round goby embryo cells inhibit the catabolism of compounds and modifies or even synthesizes new compounds. A restructuring of cellular components could be a way of counteracting desiccation stress. Especially organonitrogen compounds seem to play a role in the desiccation response, as their biosynthesis is enhanced and their catabolism. However, it remains unclear which organonitrogen compounds are influenced and which role they might play in the reaction to desiccation.

After 3 h of desiccation, most enriched processes are related to transcription or translation. This indicates that the cell undergoes changes in the protein composition. Ribosome biogenesis is decreased by the downregulation of ribosome components SRSF11, RPLP2, RPL3, RPL5, RPL7, RPL8, and RPS8, probably because it is a major consumer of cellular energy. An inhibited ribosome biogenesis is also involved in the stabilization of tumor-supressor p53, which is also a protective mechanism of the cell (Sloan et al. 2013).

3) Embryonic development and cell cycle: Without signs of adjusted developmental speed even after 24 h of air exposure in the first experiment, we did not expect to find biological processes involved in embryonic development to be significantly altered during desiccation. Finding differential expression of genes involved in embryonic development and cell cycle in our second experiment came therefore as a surprise.

The downregulation of a set of genes involved with mitotic cell cycle, cell differentiation and morphogenesis indicates that embryonic development is slowed down 1.5 h after exposure to desiccation. Especially the development of the nervous system is affected by the downregulation of genes including the GTPase SEPT1 (involved in the formation of the neural tube, Zhai et al. 2014), the tubulin-specific chaperone TBCD (involved in neuron biogenesis, Miyake et al. 2016), or the genes regulating the NF-κB signalling pathway (regulation of neurogenesis, Zhang and Hu 2012). A slow-down of embryonic development indicates that the strategy of round goby embryos to avoid desiccation damage more in the direction of developmental arrest related to that of annual and non-annual killifish (Podrabsky and Culpepper 2012, Varela-Lasheras and Van Dooren 2014, Thompson et al. 2017) than in the direction of early hatching as e.g. in salmonids (Wedekind and Muller 2005).

Although these processes of embryonic development are downregulated under desiccation stress, there is no effect on the onset of eyespot development or hatching. Already after 3 h of desiccation, cell cycle and cell division processes are enhanced, indicating that developmental deceleration only happens during the initial response phase, when the cells are expressing most stress-related genes. For example, the phosphatase CDC14A is required for centrosome separation and productive cytokinesis during cell division and is upregulated at 3 h desiccation. Additionally, the quick return to baseline differential expression levels after rehydration indicates, that putative effects on development are reversed.

LIMITATIONS

The molecular part of this study is based on samples collected from the field. The inherent variation that comes with these non-standardized samples of unknown genetic background and past environmental influences makes the detection of subtle changes in RNA expression patterns difficult and introduces some random variation in differential expression patterns that might bias the results. This limitation is enhanced by the small sample size (n = 3) of our experiment. We initially aimed for more clutches for the experiment, but the number of clutches fitting our pre-defined criteria of inclusion that we found in the spawning traps was limited. Additionally, one of the samples was slightly further in its development (early epiboly instead of 1000-cell stage). After Adrian-Kalchhauser et al. (2018), expression patterns during of pre-somite developmental stages are comparable. However, it turned out that the clutch that was in early epiboly stage showed different expression patterns for many genes. While it is interesting to observe developmental differences in the reaction towards environmental stressors as they have been shown in the killifish *Fundulus heteroclitus* (Chuaypanang et al. 2013), we did not have the experimental set-up or statistical power to investigate them further.

Another limitation of the study is the analysis of time course data by comparing the effect of treatment within, but not among the time points. Possible effects of autocorrelation are therefore not taken into account. . The available tools to analyse a set-up of a time course including three experimental groups are limited and not well tested yet. This is why some authors recommend our way of testing time course data including experimental conditions as the to-date most robust method (Robinson et al. 2009, Love et al. 2014, Ritchie et al. 2015, Varoquaux and Purdom 2020).

CONCLUSIONS

This study shows that round goby eggs can withstand desiccation for up to 48 hours without showing developmental differences to continuously hydrated counterparts. The basis for this remarkably desiccation tolerance is partly explained by a specific molecular reaction shortly after the onset of air exposure. The molecular response includes the onset of a diverse set of cellular protective mechanisms including responses to oxidative stress, changes in protein metabolism, and developmental processes. Putative effects on development seem to be reversed when the eggs are rehydrated. The possibility of round gobies laying their eggs on boat hulls combined with the ability to react to and withstand the challenge of desiccation might contribute to the spread of round gobies to new waterbodies. Additionally, desiccation tolerance may provide the invasive round gobies with an advantage over native fish in a future with decreasing and more fluctuating water levels. Further studies using a larger and more controlled dataset may provide a more comprehensive and in-depth analysis to further our understanding of fish embryo desiccation response. The present study lays the foundation for identifying fish species and genotypes with the best chances to survive under harsh environmental conditions in the future.

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Table S 1 Grouping of exemplary groups of GO terms and genes involved in the response of round goby embryos to desiccation. Oxidative stress response: response of the cell to reactive oxygen species and DNA damage. Protein metabolism: biosynthesis and breakdown of proteins and amino acids. Embryonic development and cell cycle: processes involving cell division, cell differentiation and development of the embryo. Candidate genes are those genes that significantly contributed to the enrichment of a GO term.

Functional group	**GO term**	**Description**	**Candidate gene names | proteins**	
Oxidative stress response	GO:0006915	apoptotic process	E2F6	Transcription factor
	GO:0009605	response to external stimulus	RNF216	Ring finger protein 216
	GO:0032870	cellular response to hormone stimulus	-	adhesive plaque matrix protein-like isoform
	GO:0030522	intracellular receptor signaling pathway	MASP2	Mannan-binding lectin serine protease
	GO:0034599	cellular response to oxidative stress	KMT2D	histone-lysine N-methyltransferase 2D
	GO:1901222	regulation of NIK/NF-kappaB signaling	AGO3	Argonaute RISC catalytic component 3
	GO:0071407	cellular response to organic cyclic compound	SIRT2	Sirtuin 2
	GO:0010506	regulation of autophagy	LATS1	Large tumor suppressor homolog 1
	GO:0016236	macroautophagy	DYRK2	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2)
	GO:0097193	intrinsic apoptotic signaling pathway	SET	Su(var)3-9, Enhancer-of-zeste, Trithorax domain
	GO:0071417	cellular response to organonitrogen compound	AR	androgen receptor
	GO:0031348	negative regulation of defense response		
	GO:0032102	negative regulation of response to external stimulus		
	GO:0006974	cellular response to DNA damage stimulus		

Protein metabolism	GO:1901576 organic substance biosynthetic process GO:0044249 cellular biosynthetic process GO:1901566 organonitrogen compound biosynthetic process GO:0003729 mRNA binding GO:0019439 aromatic compound catabolic process GO:0009064 glutamine family amino acid metabolic process GO:0017148 negative regulation of translation GO:0031331 positive regulation of cellular catabolic process GO:0031333 negative regulation of protein-containing complex assembly GO:0032787 monocarboxylic acid metabolic process GO:0044242 cellular lipid catabolic process GO:0044270 cellular nitrogen compound catabolic process GO:0045862 positive regulation of proteolysis GO:0046395 carboxylic acid catabolic process GO:0046700 heterocycle catabolic process GO:0046982 protein heterodimerization activity	E2F6 Transcription factor. EIF4A1 Eukaryotic translation initiation factor 4A, Isoform 1B. EIF4G2 Eukaryotic translation initiation factor 4, gamma - adhesive plaque matrix protein-like isoform KMT2D histone-lysine N-methyltransferase 2D. RBMX RNA binding motif protein, X-linked EIF4G2 Eukaryotic translation initiation factor 4 RPL3 60S ribosomal protein L3 RPL5 60S ribosomal protein L5 RPL7 60S ribosomal protein L7 RPL8 60S ribosomal protein L8 RPS8 40S ribosomal protein RPLP2 60S acidic ribosomal protein P2 HAL Histidine ammonia-lyase AGO3 Argonaute RISC catalytic component 3 LATS1 Large tumor suppressor homolog 1 SIRT2 Sirtuin 2

	GO:0051258 protein polymerization GO:0051346 negative regulation of hydrolase activity GO:0140096 catalytic activity, acting on a protein GO:1901565 organonitrogen compound catabolic process GO:1901361 organic cyclic compound catabolic process GO:1901606 alpha-amino acid catabolic process GO:0031328 positive regulation of cellular biosynthetic process GO:0010608 posttranscriptional regulation of gene expression GO:0005515 protein binding GO:0065003 protein-containing complex assembly GO:0002181 cytoplasmic translation GO:0006518 peptide metabolic process GO:0006417 regulation of translation GO:0022618 ribonucleoprotein complex assembly GO:0042273 ribosomal large subunit biogenesis GO:0042255 ribosome assembly GO:0003723 RNA binding GO:0019843 rRNA binding	TBCD Tubulin-specific chaperone d. CENPA Centromere kinetochore component CENP-T histone fold CCDC53 Coiled-coil domain containing 53 FKBP4 FK506 binding protein 4 DYRK2 Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2) CDC14A CDC14 cell division cycle 14 homolog A SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) Domain MARK3 Microtubule associated serine threonine kinase 3 CCNT1 Cyclin-T1 EPC1 Enhancer of polycomb homolog PPP1R15B Protein phosphatase 1, regulatory subunit 15B - Zinc-finger of C2H2 type NR6A1 Nuclear receptor subfamily 6, group A, member

	GO:0006364 rRNA processing GO:0006412 translation GO:0006414 translational elongation	
Embryonic development and cell cycle	GO:0016049 cell growth GO:0048667 cell morphogenesis involved in neuron differentiation GO:0050768 negative regulation of neurogenesis GO:0000166 nucleotide binding GO:0001701 in utero embryonic development GO:0007275 multicellular organism development GO:0007283 spermatogenesis GO:0045597 positive regulation of cell differentiation GO:0048568 embryonic organ development GO:0051301 cell division GO:0000278 mitotic cell cycle GO:1903827 regulation of cellular protein localization GO:0007049 cell cycle GO:0003682 chromatin binding GO:0045787 positive regulation of cell cycle	KMT2D histone-lysine N-methyltransferase 2D. E2F8 Transcription factor SIRT2 Sirtuin 2 MARK3 Microtubule associated serine threonine kinase 3 LATS1 Large tumor suppressor homolog 1 SEPT6 Septin-6 TBCD Tubulin-specific chaperone d. ANKH Progressive ankylosis protein homolog COBLL1 Cordon-bleu WH2 repeat protein-like 1 ZMYM4 Zinc finger MYM-type protein CLUAP1 Clusterin associated protein 1 FKBP4 FK506 binding protein 4 CDC14A CDC14 cell division cycle 14 homolog A CCNT1 Cyclin-T1 SET (Su(var)3-9, Enhancer-of-zeste, Trithorax)

		domain AR androgen receptor DYRK2 Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2) EIF4G2 Eukaryotic translation initiation factor 4, gamma TBCD Tubulin-specific chaperone d. RBMX RNA binding motif protein, X-linked

PAPER IV

Invasive goby larvae: First evidence as stowaways in small watercraft motors

Management of Biological Invasions (2022) Volume 13 **Article in press**

CORRECTED PROOF

Short Communication

Invasive goby larvae: first evidence as stowaways in small watercraft motors

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Abstract

Aquatic invasive species (AIS) are a major threat to aquatic ecosystems worldwide. Despite management efforts, human assisted dispersal continues to distribute AIS within and across waterbodies. An understudied translocation vector for AIS, especially for invasive fish, are the cooling systems of small watercraft motors. Here, we investigate the contents of boat motor cooling systems for the presence of invasive goby larvae in a collaboration with local boat owners. Because of the exclusively nocturnal drift activity of goby larvae, to collect samples we drove boats in the first hours after sunset. For an estimate of the translocation potential, we quantified drift density of goby larvae as well as boat traffic after sunset. We found a goby larva in a boat motor once in 30 boat drives of 1–2 hours duration each. Peak drift densities of goby larvae were 2.5 per 100 m^3, which is comparable to previously reported data. Recreational boats were active after sunset throughout the reproductive season of invasive gobies and are therefore a realistic translocation vector for goby larvae. Additionally, evidence of fish and other animals inside boat motor cooling systems, gathered from online boating forums, demonstrates the potential of AIS transport in small watercraft. Translocation inside motors is especially likely for in-water transport of boats, which should be a management focus in interconnected aquatic systems.

Key words: anthropogenic transport, biosecurity, invasive fish, *Neogobius melanostomus*, non-indigenous species, translocation, vector

Introduction

The role of recreational boats in the translocation of aquatic invasive species (AIS) has been well established for plants, invertebrates and microbes (Ashton et al. 2014; Clarke Murray et al. 2011). Most studies focus on biofouling organisms as potential hitchhikers on the outside of recreational boats and on boat trailers (Rothlisberger et al. 2010; Ulman et al. 2019). Residual waters on recreational boats are of concern as well, because of their vector potential for mobile species – for example, Campbell et al. (2016) and Darbyson et al. (2009) confirmed the presence of AIS in bilge waters. However, to fully estimate and manage the risk of translocation by small watercraft, investigating motor cooling water systems in addition to bilge water or other standing water compartments is important. Bilge water originates from passive processes (water spilling on the deck and draining into the bilge), while cooling water is actively sucked into the motor from the water column. The potential for uptake and survival of propagules should therefore differ between the two types of residual waters. Problematically, the insides of boat motors are often hard to access and therefore their potential to harbour AIS has been poorly characterized. While some studies have investigated the residual cooling water for AIS (Johnson et al. 2001; Minchin et al. 2006; Montz and Hirsch 2016), all these studies have taken their samples when the boats were out of the water. This sampling strategy might be realistic if one only focuses on overland-transport, but it neglects potential translocation within a connected system of waterbodies, or in a marine system.

Transport of small watercraft within systems of interconnected waterbodies is an important secondary transport mechanism, as it can help AIS to overcome migration barriers like dams or waterfalls via locks or boat lifts (Rahel 2007; Kelly et al. 2012). Organisms inside of cooling systems of boat motors might have increased chances of survival than those in standing waters, because aeration and temperature could be more favourable in cooling systems while the motor is still submerged, than they are in small volumes of residual waters during overland transport (Havel and Stelzleni-Schwent 2000; Johnson et al. 2001). However, sampling cooling systems while the boat is inside of the water can be challenging, because most openings for draining water are below the water surface or require tools to reach, which in turn might cause hesitance of boat owners to grant access to the cooling systems.

The round goby *Neogobius melanostomus* (Pallas, 1814) is one of the most prominent aquatic invasive fish in Europe and North America. Inside of navigable waters, ballast water transport of commercial ships is considered its main translocation mechanism (Hensler and Jude 2007; Kotta et al. 2016), but it also continues to spread in waters without commercial shipping (Bronnenhuber et al. 2011). Active upstream migration of fish is often prevented or hindered by dams and weirs, even if there are fish passes around them (Rahel 2007). For bottom-dwelling fish like the round goby, high flow rates within upstream fish passes might make a passage unlikely (Wiegleb et al. 2020). Secondary transport mechanisms are therefore probable to promote their dispersal in waters without commercial shipping (Bronnenhuber et al. 2011). Round gobies have pelagic larval and juvenile stages, which are present in the water column throughout the reproductive season of the gobies from dusk until dawn (Borcherding et al. 2016; Ramler et al. 2016). The typical size of a round goby larva during their drifting stages is 6–10 mm (Borcherding et al. 2016; Ramler et al. 2016), the gape width in that size range is 0.5–1 mm (Olson and Janssen 2017). The drifting life stages are considered propagules for translocation via ballast water of commercial ships (Hensler and Jude 2007). The typical time of larval drift is between April and August and overlaps with the peak boating season in

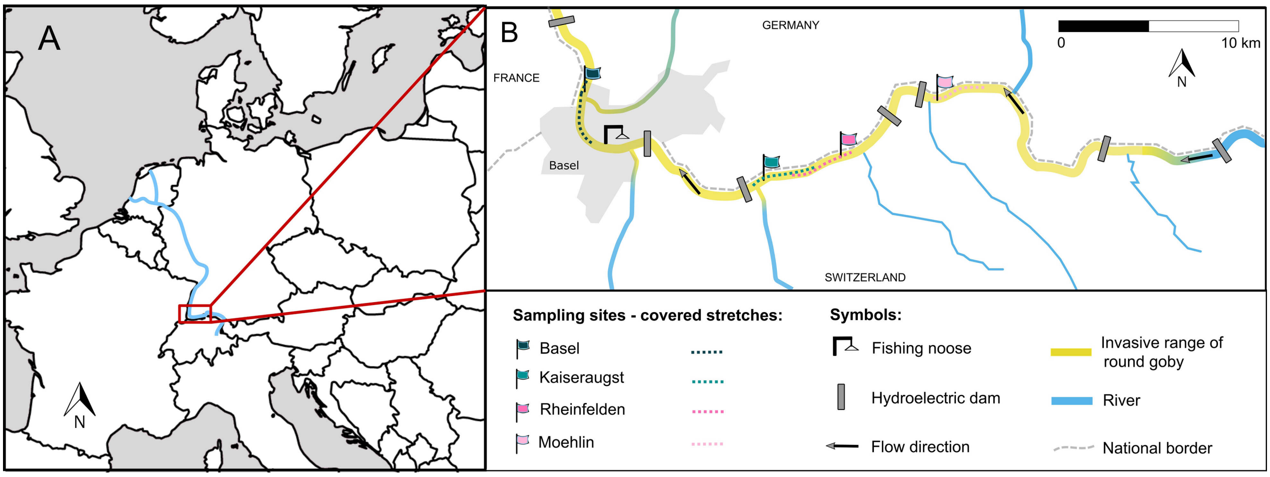

Figure 1. Map of the study locations. A: Europe showing the river Rhine (blue) and the location of the studied area (red box). B: The river Rhine and its tributaries in the study area.

Europe (Hirsch et al. 2016). The possibility of goby larvae transport in residual waters of recreational boats, however, is so far unexplored, and it is unclear how much recreational boat traffic happens during the high-risk hours after sunset.

Here, we investigate the role of motor cooling water of recreational boats in assisted dispersal of invasive ponto-caspian gobies. To study the spatial and temporal overlap with vector activity and propagule, we examine boat motor contents after drives after sunset in the river Rhine. Additionally, we determine drift densities of goby larvae at the water surface to investigate the potential for uptake into boat cooling systems. We sampled boat motors in close collaboration with local boat owners, enabling us to access the understudied cooling systems, and resulting in a mutually informative exchange of knowledge and experiences about invasive species translocation that furthered our understanding of boater behaviour.

Materials and methods

Study location

Our study took place at four locations along the High Rhine between Ryburg-Moehlin (km 144) and Basel (km 170, Figure 1). Round goby populations are established at all locations investigated.

Contact to boat owners

We sent an inquiry to 33 contacts from a stakeholder network that our working group (Program Man-Society-Environment, University of Basel) has established in the context of the ponto-caspian goby invasion in the High Rhine area since 2012 (e.g. N'Guyen et al. 2016). The inquiry asked if they a) had a suitable boat available, and b) would be willing to either lend us the boat, or drive with us (complete inquiry presented in Supplementary material Appendix 1). We defined a suitable boat as a boat with either an outboard or an inboard motor/stern drive with a minimum power of

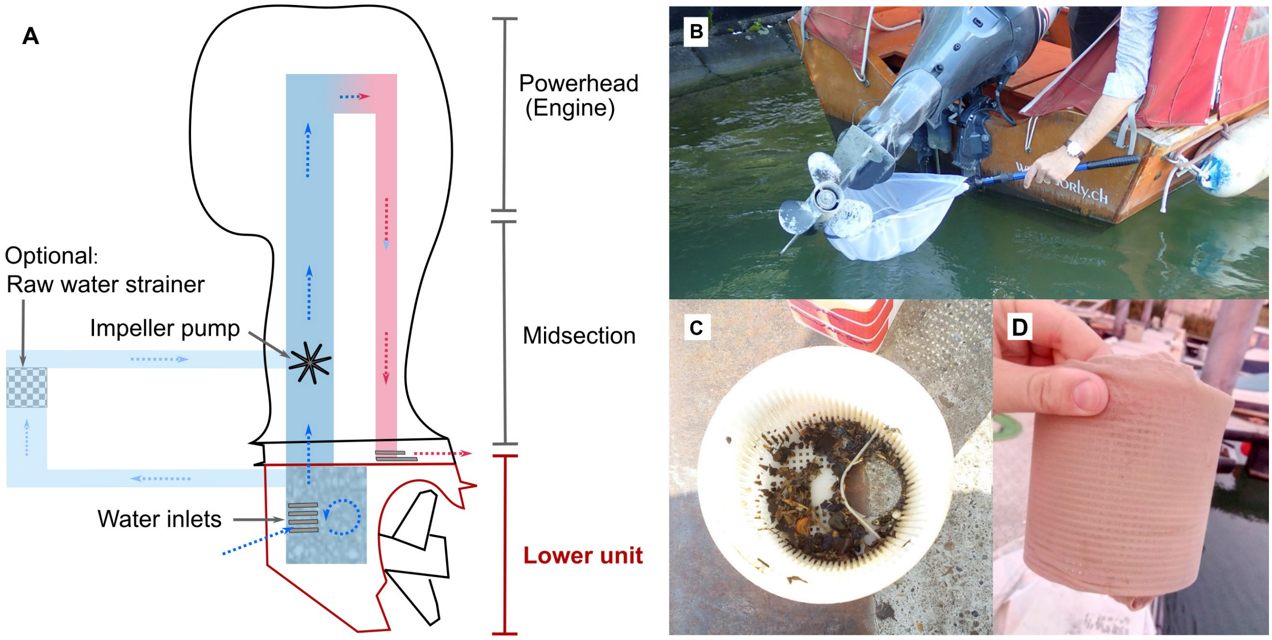

Figure 2. Boat motor water circuits and sampling. A: Simplified schematic drawing of an outboard boat motor and the water flow through the cooling system. Arrows represent flow direction. In the cavity of the lower unit (shaded), sediment and organisms accumulate if left under water. For inboard motors/stern drives (optional circuit, light blue), the engine is located inside the boat and the water circuits are therefore longer and hold more water. Additionally, they optionally include a raw water strainer to filter larger solids from the cooling water before they reach the impeller pump and the engine. B: Sampling of an outboard motor in the water using a landing net. C: Raw water strainer with contents (mesh size 1 mm). D: Additional fine-meshed filter material wrapped around the strainer for boat drives in this study. Photographs by K. Bussmann, Program MGU, University of Basel.

50–75 HP, as those motors have large enough water inlets to take up particles in the size range of drifting goby larvae (6–10 mm, Borcherding et al. 2016; Ramler et al. 2016), as well as sizeable residual water volumes.

Sampling of boat motor cooling systems and control sampling

Five boat owners were chosen at four locations (specifications in Table S1). We conducted 30 drives in total: 14 drives in Basel, five drives in Kaiseraugst, three in Möhlin, and eight in Rheinfelden (Figure 1, Table S1). All sample drives were undertaken after sunset over the reproductive season of round gobies (June–August 2020). In August, sampling only took place in Basel. In total, there were 43 hours of engine running time covering a distance of 202 km (Table S1). After clearing the initial contents of the boat motor residual waters, we drove the boat for 1–2 h on the river with speeds < 10 km/h above water. We drove the boat up- as well as downstream, while keeping the boat close (< 10 m) to shore. According to the boat owners, this drive profile is realistic for boaters enjoying sunset-drives, or anglers on night time fishing-trips.

To collect the samples, for outboard motors, the boat owners lifted the motor slowly, while we caught the contents exiting the water inlets using a landing net (mesh size 500 µm, Figure 2B). The filtered sample was then preserved in 100% ethanol. For boats with a sterndrive or an inboard motor and a raw water strainer, we opened the strainer before the drive and collected the content (Figure 2C). We installed a second layer of filter by wrapping a stocking around the raw water strainer (mesh size 75 µm, Figure 2D), as smaller goby larvae might be flushed through the raw water strainer (mesh size 1 mm). After each drive, we collected the contents of the raw water strainer, and then removed the stocking and emptied it in a sampling vial filled with 100% ethanol.

Additionally, we quantified the drift density of goby larvae and native fish larvae in the upper water layers during the boat drives, assuming that the likelihood of a boat motor to take up goby larvae is dependent on their occurrence per volume of water. To catch goby larvae drifting in the upper water layers, we used a plankton net (manta trawl, HYDRO-BIOS Apparate Bau GmbH, Altenholz, Germany), which was towed along the water surface 15 m behind the boat (Figure S1A, B). The manta trawl consisted of a metal frame with a mouth opening of 30 × 15 cm, two lifting bodies attached on both sides of the frame to keep the trawl at the surface, a net length of 2 m, a mesh size of 300 µm, and a removable soft net bucket to empty the contents (Figure S1A, B). A flow meter was attached on the inside of the metal frame to collect data on the amount of water filtered during each drive. After each drive, we removed the net bucket at the lower end of the plankton net and preserved the contents in sample containers (100 ml), filled with 100% ethanol.

Furthermore, we wanted to find out if goby larvae are also present in the shallow waters next to shore (< 5 m distance to shore). This information is important, because the shoreline is often the part of the river in which boats stay for longer times to load/unload the boat, or warm up the engine. We sampled shallow waters using a so-called “fishing-noose”, a traditional local fishing device equipped with a fishing net, which is lowered into the water parallel to the riverbed (Figure S1 C, D). We installed a plankton net with a mesh size of 650 µm and dimensions of 3.5 × 3.5 m on the noose. We lowered the net into the water at sunset until it just touched the river bottom (depth 0.5–1.5 m) and pulled it to the shore after half an hour to search for fish and fish larvae. We euthanized all goby larvae and stored them in 100% ethanol. We repeated this procedure four times every night starting at sunset with an interval of 30 minutes each. In total, we used the fishing noose on 19 nights across the sampling season.

Quantification of boat traffic after sunset

To evaluate whether the uptake of goby larvae into boat motors after sunset was a realistic option, we collected data on boat traffic at the same time as when our sampling took place. Starting on June 24th, we recorded the presence of other boats active on the sampled stretch of the Rhine during sampling. On some dates, we were not able to count boats because of poor visibility.

Analysis of samples

In the lab, we searched all samples for fish larvae. For this, we removed all larger debris from the sample and spread out the remaining material in a thin layer, which was then systematically searched from top left to bottom right using spring steel tweezers to remove fish larvae. Each fish larva was identified under a dissecting microscope as goby larva or native fish larva and counted. Gobies were identified by the presence of a fused ventral fin, a trait that no native species exhibits (Kottelat and Freyhof 2007; Ramler et al. 2014).

We calculated the drift density per 100 m^3 of goby larvae and native fish caught in the manta trawl using the formula $DD = n*100/ r*0.3*A$, where DD = drift density, n = number of larvae caught, r = number of revolutions of the flowmeter, A = area of net opening (constant: $0.3 * 0.1 = 0.03$; 0.1 m was the average estimated depth to which the net opening was submerged).

We counted all goby larvae caught close to shore with the fishing noose and calculated an point abundance estimate in larvae per 100 m^3 using the formula $A_P = n*100/l*w*d$, A_P = abundance at the time of the pull, l = length of the net, w = width of the net, and d = depth of the net below water surface.

Collection of anecdotal evidence for organisms in cooling systems

To further support our finding of fish in boat motor cooling systems and to increase the geographical scope with potentially available anecdotal evidence from international boat owners, we conducted an online search using the search engine Google. On 29. January 2021, we entered the keywords “raw water strainer” OR “outboard motor lower unit” AND “fish” OR “animal” OR “critter” OR “crab” OR “shrimp”. Within the results, we focused on boating forums or grey literature in the area of aquatic invasive species, and compiled reports, pictures and anecdotes of live and dead animals in water circuits in a supplementary document (Appendix 2).

Results

Goby larvae in boat motor cooling systems

We detected one goby larva in a raw water strainer sample from a sterndrive once (sample taken after a drive on August 19th in Basel). The goby larva caught measured 7 mm total length (Figure 3). It was located in the fine-meshed filter, indicating that it moved through the raw water strainer. The goby larva looked externally unharmed when investigated under a dissection microscope. We did not find any other fish or fish larvae inside the boat motors. However, we found invertebrates inside the motors, including invasive species like the killer shrimp *Dikerogammarus villosus* (Sowinsky, 1894), zebra mussel shells (*Dreissena polymorpha* Pallas, 1771), or the freshwater shrimp *Atyaephyra desmarestii* (Millet, 1831). The latter two species were both found in the motors after the respective boat had been to rivers in France. Given the scope of the paper, we did not identify and count the invertebrates in detail.

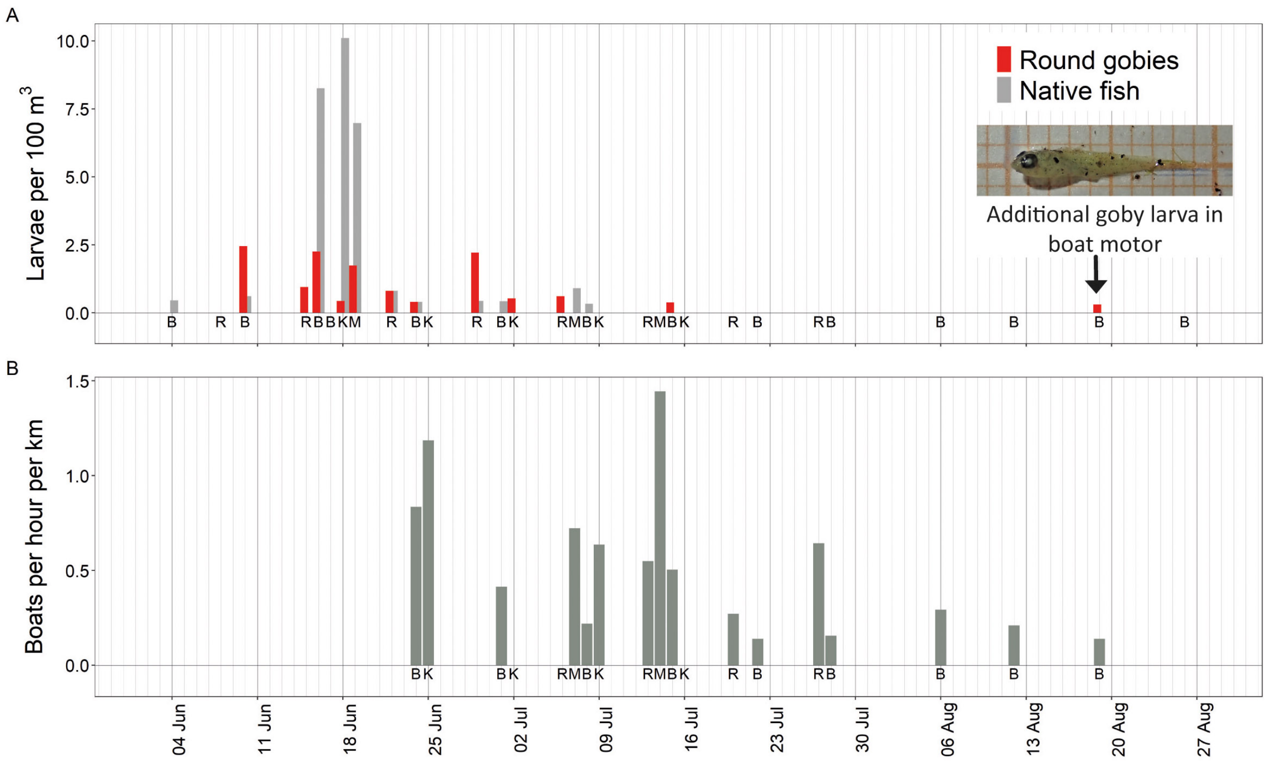

Figure 3. Results of invasive goby larvae sampling and boating activity after sunset. A: Drift density of fish larvae caught in the plankton net towed behind recreational boats after sunset at different locations along the river Rhine (B = Basel, R = Rheinfelden, K = Kaiseraugst, M = Moehlin). Boat drives only took place on dates that are indicated with a study location. Round goby larvae are presented in red, native fish larvae (species not identified) are presented in grey. On 19th August, an additional goby larva was caught in a boat motor. B: Number of recreational boats active during the 1–2 h after sunset per kilometre at different locations along the river Rhine. Quantification of boats only took place on those dates indicated with a study location. Vertical grid lines represent days. A value of zero on a date at which a sampling took place means that no fish larvae were caught on that date, respectively no boats were seen.

Quantification of propagule density and vector activity

The plankton net, which was towed behind the boat, filtered a total of 6050 m^3 of water and caught 50 larvae of native fish and 23 larvae of invasive gobies in total. Native fish larvae in the surface waters were present predominantly in June and were not present in the plankton net after mid-July, while goby larvae occurred from early June until late August (Figure 3A). For gobies, the number of larvae caught was higher in June than in July or August. Goby larvae were present in samples of all four locations, and none of the locations stood out with particularly high or low catch numbers. We only caught goby larvae with the fishing noose between mid-June and mid-July, reflecting the peak of larval drift. In those nights, the absolute number of goby larvae caught per night was between 1 and 3 (0.5–1 per pull of the noose, or 5.5–11.1 per 100 m^3).

During all but two drives on which we quantified boat traffic, there were other boats present and active after sunset (Figure 3B). Identifiable categories of boat rides were pleasure rides (people eating/drinking), water sports (swimming, water ski, wakeboarding), or angling trips (fishing rods visible).

Collection of anecdotal evidence for organisms in cooling systems

We found eleven websites which documented evidence of fish and other creatures inside of boat cooling systems and other boat compartments. Especially in boat forums there were often several reports by different users reporting findings, so that the total number of reports exceeds that of the number of websites found: There were three reports of live fish, five reports of dead fish, and eight reports of fish for which it was unclear whether they were alive or dead. Additionally, we found reports of live and dead other animals inside of cooling systems, among those were crabs, shrimp, jellyfish and even a snake. The complete results of the online search are presented in Appendix 1.

Discussion

The presence of a goby larva in the cooling system of a boat motor is an important proof of principle for our hypothesis: that invasive gobies can be translocated by small watercraft via motor cooling systems. Invasive goby translocation by small watercraft has long been assumed but to date has lacked empirical evidence (Ahnelt et al. 1998; Moskal'kova 1996; Bussmann and Burkhardt-Holm 2020). Moreover, the only mode considered for goby translocation via recreational boats so far is transport of their eggs on boat hulls similar to biofouling organisms (Adrian-Kalchhauser et al. 2017). Goby larvae as propagules transported by recreational boats have, to the best of our knowledge, never been considered in the scientific literature.

The density of round goby larvae in the uppermost layers of water in the sampled area of the river Rhine between June and August 2020 was never higher than 2.5 individuals per 100 m^3, which is in a similar range as found in some other drift-net studies (Hensler and Jude 2007; O'Brien et al. 2019). However, the long duration of round goby reproductive season makes the pick-up and viable transport and release of larvae within recreational boat engines possible at any point of time during the European summer months. Additionally, the density of round goby larvae can be much higher and vary between locations and years (Borcherding et al. 2016; Hayden and Miner 2009). Studies of larval drift at different depths indicate that larval densities can be higher in deeper water layers (depth > 2 m), while the uppermost layers hold most larvae of the smallest size class (< 9 mm), which might be especially prone for uptake by small watercraft motors (Hayden and Miner 2009; Juza et al. 2016). Data collected with the fishing noose shows that goby larvae are present in shallow waters along the shore of rivers. The abundances of goby larvae can reach numbers as high as 11 larvae per 100 m^3 when point sampling. This has ramifications for assessing the probability of larval uptake by boats: boats often remain running for a long time to warm up the engine or load/unload the boat close to the shoreline, which might increase uptake probability.

The observed numbers of recreational boats active after sunset confirm that the intake of goby larvae is not just a hypothetical risk. Angling in particular is a popular night-time activity for boat owners in the area (personal communication of various boat club members and anglers). Even if they would not move their boat to a new location during the night, fish larvae caught inside the cooling systems could stay inside and only move out after a relocation on a day afterwards. This connection highlights how important it is to gain insight into the behavioural patterns of stakeholders, who might unintentionally translocate invasive species. For example, fishing trips can be used to infer invasion risk of round goby released as baitfish (Drake and Mandrak 2014). Our study demonstrates how invasive species could be translocated not only as baitfish but also within motor engines of fishing boats. This information can inform and improve risk models evaluating translocation probabilities (e.g. Acosta and Forrest 2009; Parretti et al. 2020).

Limitations

We cannot be certain that the goby larva that we found in the raw water strainer was still alive in the motor, because we only found it during analysis in the laboratory. To prove the actual translocation potential of boat motors, the documentation of a live fish larva would be necessary. We have ample reason to believe that survival in motor cooling systems is possible: The goby larva we found looked externally unharmed upon examination of the sample. Fletcher et al. (2017) documented the successful transport of a living juvenile fish through an impeller bilge pump, which is a similar pumping system as used in boat motors. Furthermore, some of the collected anecdotal evidence (see Appendix 1) speaks for the possible survival of organisms in cooling water circuits and raw water strainers. Additionally, we found numerous other live organisms in the boat motor cooling systems and strainers over the course of this project, as well as during former projects looking at motor interiors (Amt für Umwelt und Energie Kanton Basel Stadt 2019).

The quantification of boat traffic during the nights in the area was circumstantial and did not include the weekend nights, when traffic is likely to be highest. While we can show that boat traffic after sunset is not an exception, we acknowledge that our data likely underestimates the true extent of after-sunset boating activities.

Minimizing translocation risk of cooling systems

The risk of taking up a round goby larvae at surface drift densities as determined in our study might seem low, as we only found a single goby larvae in one out of 30 drives. However, the uptake risk of an individual boat is determined by multiple factors like motor type and size, motor running time, and speed. For example, a standard impeller pump for a boat motor up to 400 HP nominally pumps 80 l/min at 1500 rpm (4.8 m^3 per hour), while its maximum pumping capacity is 102 l/min (6.12 m^3 per hour). If driven for 2 h during peak drifting season (2.5 goby larvae per 100 m^3), a boat would take up 0.24–0.31 goby larvae. While these numbers are low for any individual boat, the probability of uptake can become substantial if taken cumulatively across all boats moved from invaded to uninvaded areas. For example, in the year 2013, there were between 10 and 5200 boat transports per weir in the Rhine upstream of the current invasion front (Figure 1), with the highest numbers at a weir very close to the ecologically and economically valuable Lake Constance (Hirsch et al. 2016).

Measures against the translocation of invasive species in the inside of boat motors and residual waters could include: motor flushing devices ("muffs") as mandatory boat equipment, check-points with information and instructions for boats at harbours or locks, or (mobile) boat cleaning stations (Horvath 2008). We forwarded the outcomes of this study to relevant authorities in Switzerland, resulting e.g. in adjustments in a newly launched information campaign about translocation risks of recreational boats and measures for prevention to specifically include the insides of boat motors (AWEL Zuerich 2020).

Conclusions

The residual water in the insides of boat motors present an understudied potential for secondary translocations AIS. The finding of invasive goby larvae inside the cooling water system of a recreational boat motor is important to consider in order to develop effective measures against translocation of AIS, especially during in-water transport of boats. Anecdotal evidence suggests the repeated occurrence and viability of organisms inside the cooling systems. In addition, management recommendations for boat cleaning often neglect to include flushing of cooling systems. However, there are simple methods to avoid unintentional translocation of AIS in cooling systems: tilting the motor to remove most residual water whenever stationary, flushing the motor with hot water, and educating boat owners about the hidden organisms inside the motors and effective measures for the different types of motors could help preventing the further spread of AIS, especially across biogeographical barriers.

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Author's contribution

KB: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation; writing - original draft, writing – review and editing. PEH: research conceptualization, sample design and methodology, writing – review and editing. PBH: research conceptualization, sample design and methodology, funding provision, writing – review and editing.

Ethics and permits

Ethics approval was not required according to Swiss law. The offices for environment and energy of the cantons Basel-Stadt and Aargau granted permits for using the manta trawl and fishing noose to catch fish.

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Supplementary material

The following supplementary material is available for this article:

Appendix 1. Inquiry sent to boat owners to ask for collaboration on the project.
Appendix 2. Anecdotal evidence for organisms in boat motors from online boat forums.
Figure S1. Figure showing control sampling methods.
Table S1. Data on sampling locations, sampling dates, and boat types used for sampling.

Appendix 1: Inquiry sent to boat owners to ask for collaboration on the project.

English translation:

Dear friends of the goby-team,

The goby-season is starting soon and we urgently ask for your help:

Do you have a boat that you could lend us, or conduct boat drives with us for a research project? Or do you know boat owners who we can address with this inquiry?

What are we looking for?

- Boat with a **stern drive/inboard motor** with an easily accessible **raw water strainer** that can be emptied by hand.
- Boat with **outboard motor** (at least **50-75 HP)**
- **Outboard motor** has to be fixed on the boat in a way that it can be lifted completely **out of the water.**

When?

- Possible time frame: Mid May – End August
- Due to coronavirus – regulations: Start as soon as it is legal.
- Drives in the evening, after sunset.

Where?

…in the area of goby distribution (Rhine an tributaries, downstream of Bad Säckingen until ca. Freiburg)

How?

We have boat driving licenses and are able to drive independently; or we join you on the boat.

Why?

We found that residual waters (e.g. inside the motor or the bilge) can harbour non-native animals and plant. We suspect that also larvae of gobies can get into residual waters of boats. To test this, we will conduct boat drives **after sunset once or twice a week in the coming weeks** (goby larvae swim to the surface at nights and therefore get into the range relevant for boats). If you are interested, we conduct these drives together and we tell you all about the project and the gobies.

Of course we cover all arising costs – and you can be sure to get an appropriate thank you.

Please contact us if you would make your boat available to us, as well as for any questions you have about the project. We are looking very forward to hearing from you and find out more about the gobies and their distribution with you!

Best regards and stay healthy!

Original version:

Liebe Freunde des Grundel-Teams,

Die Grundelsaison beginnt in Kürze und wir bitten Sie dringend um Ihre Hilfe:

Haben Sie ein Boot und können uns dieses ausleihen oder mit uns Fahrten für ein Forschungsprojekt durchführen? Oder kennen Sie Bootsbesitzer, an die wir uns mit dieser Bitte wenden könnten?

Was genau suchen wir?

- Boot mit **Z-Antrieb/Inbordmotor**, dieser hat leicht zugänglichen **Filter**, den man von Hand leeren kann.
- **Boot mit Aussenbordmotor** (mind. **50-75 PS)**
- **Aussenbordmotor** so am Boot angebracht, dass man ihn beim Hochklappen komplett **aus dem Wasser heben** kann.

Wann?

- Möglicher Zeitraum: Mitte Mai – Ende August
- Wegen Einschränkung durch Corona-Regelungen: Beginn sobald es zulässig ist.
- Fahrten Abends ab der Dämmerung

Wo?

.... im Verbreitungsgebiet der Grundeln (Rhein und Nebengewässer, ab Bad Säckingen abwärts bis ca. Freiburg)

Wie?

Wir verfügen über Bootsführerscheine und können eigenständig Fahrten durchzuführen, oder kommen zu Ihnen an Bord.

Wieso?

Wir haben festgestellt, dass Wasserüberstände (z.B. im Motor oder der Bilge) nicht-einheimische Tiere und Pflanzen beherbergen können. Wir vermuten, dass auch Grundellarven in die Wasserüberstände in Booten gelangen können. Um dies zu testen, werden wir in den kommenden Wochen **ein- bis zweimal jede Woche ab der Dämmerung Bootsfahrten durchführen** (Grundellarven schwimmen abends und nachts an der Wasseroberfläche und kommen somit in den Bereich, der für Boote relevant ist). Wenn Sie interessiert sind, machen wir diese Fahrten gemeinsam und erzählen Ihnen alles über das Projekt und die Grundeln.

Alle anfallenden Kosten übernehmen wir selbstverständlich - und ein entsprechendes Dankeschön ist Ihnen natürlich auch sicher.

Kontaktieren Sie uns bitte falls Sie Ihr Boot zur Verfügung stellen würden und bei allen Fragen, die Sie zum Projekt haben. Wir freuen uns schon sehr, von Ihnen zu hören und gemeinsam mehr über die Grundeln und ihre Verbreitung herauszufinden!

Beste Grüsse und bleiben Sie gesund!

Appendix 2: Anecdotal evidence for organisms in residual waters of recreational boats

This document contains evidence for fish and other organisms in boat motor cooling systems, particularly in raw water strainers. It is the result of an online search using the search engine Google on 29. January 2021. We entered the keywords “raw water strainer” OR “outboard motor lower unit” AND “fish” OR “animal” OR “critter” OR “crab” OR “shrimp” and focused on boating forums or grey literature in the area of aquatic invasive species. We obtained 8 340 000 hits and looked at the first 100 of them in detail. We scanned the 50 hits beyond the first 100 hits, but we deemed the results of further hits to become progressively less relevant and we did not find any new results of interest.

We extracted documents and forum entries that report animals inside cooling systems and compiled them below. The actual documentations of the findings are marked in bold font. We anonymised the posts to ensure privacy of the original authors.

Documentations were grouped into: A) grey literature such as reports by recreational associations, B) forum and blog entries. For the latter, we provide the title of the main thread in which we found the relevant quote, and the quote itself.

A) Grey literature

Dodgshun, T. J., M. D. Taylor and B. M. Forrest (2007). Human-mediated pathways of spread for non-indigenous marine species in New Zealand, Science & Technical Pub. Department of Conservation.

Sea water contained in the keel centre cases of some types of ocean-going yachts arriving from the South Pacific islands **has occasionally been found to contain small fish** (G. Grant, Ministry of Agriculture and Forestry (MAF) Quarantine Service, pers. comm.). Although few of these vessels come to New Zealand, with only one or two visiting Whangarei each season (T. Hamilton, H & H Slipway Ltd, Whangarei, pers. comm.), they may still be a significant pathway for certain species, especially where adult life stages are transported. Similarly, overseas cruising yachts visiting New Zealand from areas outside the South Pacific could carry a variety of other organisms in their keel centre cases, which could be inadvertently released upon the vessel’s arrival. Education of inspecting staff and the owners of these vessels about the possible presence of NIMS would be worthwhile.

Vessel Questionnaire on Cooling Water Impacts by Menhaden Fish

https://www.swedishclub.com/media_upload/files/Member%20Alerts/2017/2017%20Menhaden%20questionaire_SETWAC%20%20LSHSC%20%28003%29.pdf (last accessed 05.03.2021):

Although we had a reduced Menhaden population in 2016, we should prepare for a more robust 2017 of young menhaden fish in the Gulf of Mexico and in the Sabine-Neches Ship Channel. Texas Parks and Wildlife is also projecting a heavy Menhaden season in the Sabine/Beaumont areas (see attached data sheets). **These fish can clog sea chest strainers and restrict the flow of sea water cooling, not only while transiting, but also while your vessel is alongside a berth.** Please review the attached USCG Marine Safety Information Bulletin MSIB 02-17 from Coast Guard Sector Houston-Galveston, pertaining to Loss of Vessel Propulsion and Maneuverability due to clogged sea strainers, as the Sabine Neches Ship Channel also has a very high population of Menhaden fish.

B) Forum and blog entries

Thread: “Clogged raw water intake”

Post 1: Minnow stuck in water intake
Well, I went to take the boat out and it seemed OK for a while until I increase the RPMs. It then took on the unmuffled sound like when the intake valve is closed. I checked the valve and continued. After I had gone out, 15 minutes later my high temperature alarm sounded and I shut down the engine. I checked the valve again. I figured the intake had gotten clogged and decided to go over the side and check. (The correct way would have been to shut the valve, disconnect the hose, then open the valve to see if water flowed freely.) I didn't feel anything when I put my finger in the through-hull while in the very cold water, but when I got back to the dock and diconnected the hose **I found a fish tail protruding from the fitting where a minnow had gotten sucked into the intake**. I took a bit of effort, but the fish was removed, the hose reconnected, and the engine run successfully without overheating.

Post 2: Catfish in raw water filter
I carry a 12 volt tire inflator with an adaptor hose and tire valve clamped in it. Twice I've used it to clear an obstruction.
Close valve; remove raw water hose; open valve--no water fountain, close valve; install adaptor hose; attach pump; fire up pump and at about 80 pounds, open ball valve--acts like a torpedo shot; replace raw water hose and get back under way.
Last year the AC shut down on a hot day. **Found a baby catfish got sucked into the raw water cooling hose and got trapped in the filter.**

Post 3: Small, alive fish in raw water strainer

I also installed an aft facing slotted thru hull skimmer on my 2000 H340 and have not had a blocked intake in many years. **However, every year, when I check my raw water strainer in mid-season, I find a small fish alive and well inside. I guess he/she swims in when very small and lives and grows inside the strainer.** Catch and release!

Thread "How important are raw water strainers?"

Post 1: Live fish and other animals in raw water strainers

I have cleaned **weed, live squid, plastic, live fish, jellies, unidentifiable stuff...**you name it **from strainer baskets.** Nuff said.

Thread: "Raw water intake strainer"

Post: Live fish in raw water strainer

I pull mine out after each outing, **found a little live fish in the strainer on the weekend!!**

Thread: "Fish Blocking Raw Water Intake"

Post 1: Fish blocking water intake in front of raw water strainer

Sharing an unanticipated sailing adventure, or perhaps they are all like that. I was moving my Pearson 33 on Chesapeake Bay this weekend and arrived early to get organized. Since it was a cold morning, I started the engine to warm up, of course checking the exhaust for cooling water. As I was about to leave, fortunately checked the exhaust again and saw no water coming out. There was a bit of seaweed in the raw water strainer but nothing that would create a blockage. But I could feel what felt like a complete blockage by something rubbery where the raw water came into the strainer. To make a long story short, after multiple efforts to clear the obstruction without success, had to take apart the hose and strainer to figure out what it was**. It was a small fish that had been sucked into the water intake and lodged just in front of the strainer where it was totally blocking the intake.** After removing the fish, everything back to normal and an uneventful trip, albeit several hours later than planned.

Lessons learned: 1. Checking the exhaust regularly paid off; if I hadn't noticed until after leaving the dock it would have been a much bigger problem as I was single-handing and would have had to get a tow. 2. Allowing lots of extra time allowed for 2 hours of fish extraction and still plenty of time to get to the destination. 3. Just when you think you have everything covered and thought through, a fish swims in your engine intake.

Post 2: Herring in raw water intake

Yep, likely a **herring (menhaden). I had one lodge in my A/C raw water intake last year.** When I pulled he hose off the thru hull it shook out. It had been in here awhile because the fins were cropped and stunted. Keep an eye on your discharge water.

Post 3: Fish in residual water

Not all fish make it into the hose. I was getting ready to clean a hull and I always take pictures of the hull beforehand so that the owners know how well their paint is holding up. I told the owner of this boat to cancel her A/C service call.

[Picture of fish, supposedly dead, in residual water]

Yep, he's stuck up in there.

Post 4: Dead fish in water intake

Our engine through hull fitting has a screen on it, but the head intake does not. A few years ago, after sitting idle for some time, I used the aft head to bring in sea water to flush, but nothing would come in but a little black water **with pieces of some rotted stinking creature**. In order to clear the clog I used a fitting to connect a municipal water hose to the intake line. Blew it right out, whatever it was; **likely a dead fish.**

Post 5: Jellyfish in water intake in front of raw water strainer

I once **had seaweed with jellyfish up against it just against the strainer**. Not too easy to get it out of the tubing.

Post 6: Fish in raw water strainer

Yep, been there.......

[Picture of raw water strainer with fish inside.]

Post 7: Snake in water intake

Aft head intake clogged. closed the seacock and pulled the pump apart**. found a foot long snake in there! and it was tail to the pump**. either the suction of the pump sucked it in tail first or it managed to get turned around before drowning.

Post 8: Fish parts in water intake

Had an overheat problem on a raw water cooled 2 QM 20. **turned out to be fresh fish parts in the thermostat.. The impeller in the pump had beat him up pretty badly.** Boat had no raw water strainer installed.

Post 9: Fish in water intake

Why not add an external strainer**? I added one to my AC intake after sucking up a fish.**

Thread "Fish in raw water intake"

Post 1: Fish (eel) stuck in raw water intake

Yesterday I was finally able to take the boat out for the first time this year, just to knock off the rust of my motoring skills. After about 10 minutes the engine overheating alarm comes on. I limped the boat back into the slip. (Nuts.)

After two hours which included replacing the impeller, I found the problem. **A small eel, or similar fish, was sucked up into the raw water intake on the sail drive.** I removed the 2" of fish in the pipe shown below. Opening the seacock no water flowed in. I reattached the hose, disconnected the end at the strainer and blew into the hose. I could hear air bubbling around the hull, so I could get air out though the intake. But, hooking everything up showed no water flowing. It appears the rest of fish just gets sucked back into the intake when motor starts spinning the impeller.

OK, any good ideas on how to remove the fish remains?

[Picture of a fish tail stuck in a pipe of the raw water intake of a boat motor.]

Thread: "Air conditioning raw water system maintenance"

Post 1: Live shrimps and crabs in raw water strainer

I have cleared live shrimp and crabs out the AC strainer along with lots of seaweed and other nasty things. not sure how much of the finer stuff ends up in the AC <u>cooling</u> coil. the guys around my <u>dock</u> showed me how to backflush a clogged intake hose after the compressor overheated. this usually happens on the hottest and muggiest night of the summer.

Thread: "AC Raw Water Strainer Completely Fouled with Pea Sized Oysters"

Post 1: Crabs in raw water strainer

(…) I was able to find Bromine at the walmart in (…). It was on top shelf in can with purple top. I may go buy a few more after what you stated. To the OP I wasn't running Bromine in the freshwater and found out I needed to in the saltwater. **when I pulled the strainer out to clean it I had 3 baby crabs in it.** No telling what else has went through the system. I flushed them out with phosphoric acid solution to be safe

Thread: “AC question...is there a sea-strainer?”

Post 1: Live crab in raw water strainer

I would be very surprised if there isn't a raw water strainer between the seacock and the pump. If so, that's where the biggest chance for obstruction is. **I once got a live (small) crab out of the top of our A/C raw water strainer.** Pull the filter cylinder out of the strainer and clean it. It'll probably be pretty nasty. We're on the gulf and our A/C run a lot. so in that case, you've essentially got water running thru the raw water strainer almost all the time. To determine without doubt whether there's a raw water strainer, just trace out the hose from the intake seacock to the pump.

Figure S1:

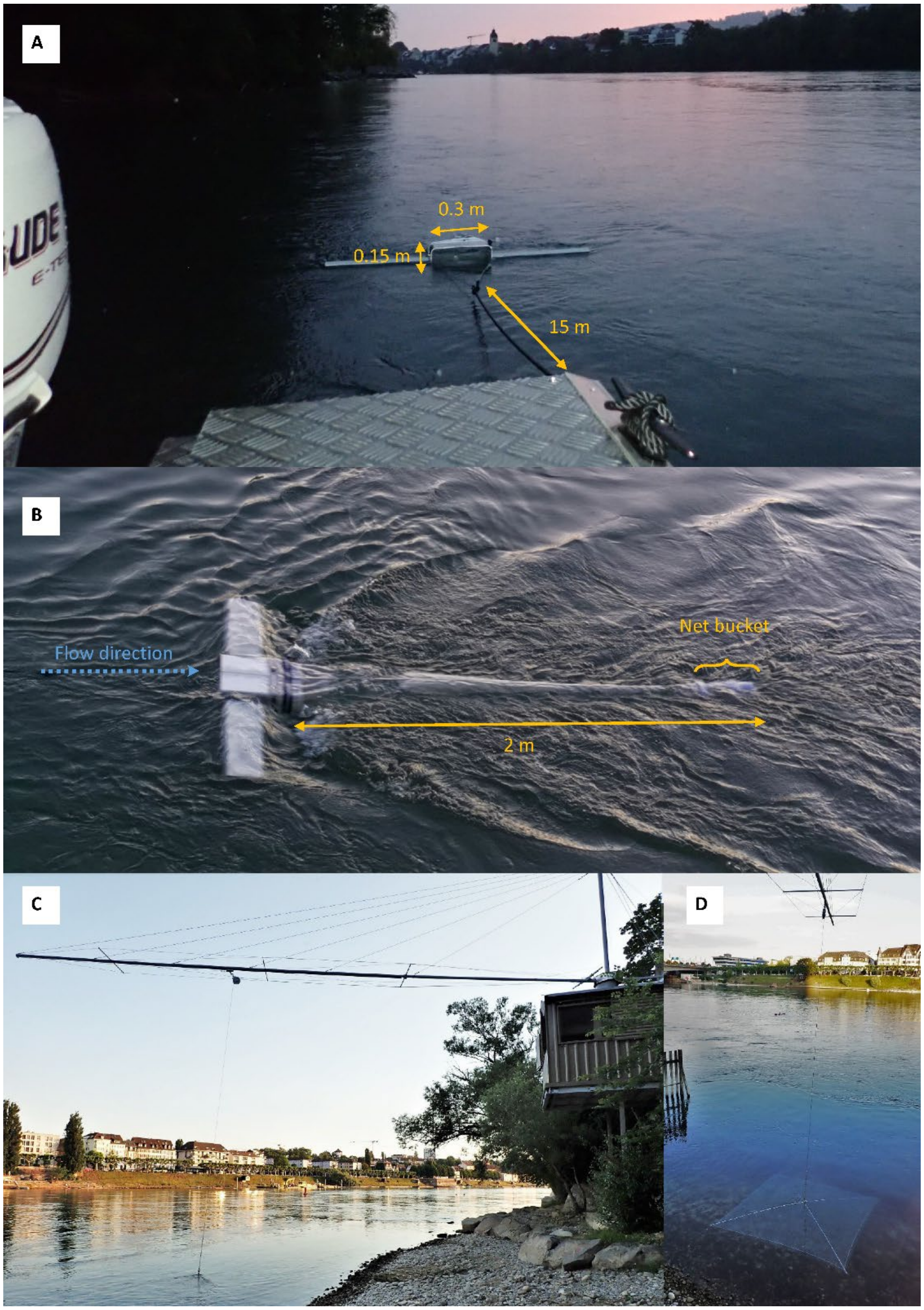

Figure S1. Control sampling methods. **A, B** Manta trawl at the water surface as it was towed behind one of the boats used for sampling. The mesh size of the plankton net was 300 µm. A mechanical flow meter was mounted in the net opening. The rope in **A** is not extended to the full 15 m. **C** Traditional fishing noose used as second, stochastic control for larval drift and **D** plankton net installed in the noose. Photographs by K. Bussmann, Program MGU, University of Basel.

Table S1:

Table S1. Data on sampling locations, dates and boats used to investigate the prevalence of round goby larvae in motor cooling systems and/or filter systems. The hour of sunset represents also the start of sampling.

Location	**Shore type**	**Flow regime**	**Boat drive, HP**	**Distance covered**	**Duration drive (h:min)**	**Sunset**	**Date**
Basel 47.5855°N, 7.5877°E	Anthropomorph: concrete walls, gravel, rip-rap	Fast flowing: Runoff June-August 629 – 1784 m^3/s	Stern drive, 250 HP	7.2 km	1:36	21:20	04. Jun
				5.4 km	1:13	21:25	10. Jun
				4.4 km	1:20	21:28	16. Jun
				7.5 km	1:00	21:28	17. Jun
				8.4 km	1:15	21:29	24. Jun
				7.7 km	1:50	21:29	01. Jul
				9.9 km	1:22	21:27	08. Jul
				8.7 km	1:10	21:22	15. Jul
				6.1 km	1:45	21:16	22. Jul
				9.6 km	1:20	21:08	28. Jul
				9.1 km	1:30	20:56	06. Aug
				11.0 km	1:18	20:46	12. Aug
				10.8 km	1:20	20:34	19. Aug
				10.1 km	1:19	20:21	26. Aug
Kaiseraugst 47.5377°N, 7.7134°E	Natural: muddy, submerged vegetation, inflow of river Ergolz, adjacent nature reserve	Slow flowing: backwater of hydroelectric dam	Outboard, 115 HP	7.5 km	1:30	21:28	18. Jun
				7.3 km	1:44	21:29	25. Jun
				6.3 km	1:10	21:28	02. Jul
				5.4 km	1:10	21:27	09. Jul
				5.1 km	1:05	21:22	16. Jul
Rheinfelden 47.5518°N, 7.7782°E	Natural & anthropomorph: concrete walls, rip-rap, gravel, submerged vegetation, rock faces	Fast flowing: Runoff June-August 636 – 1759 m^3/s	Outboard, 80 HP	3.4 km	1:37	21:23	08. Jun
				7.0 km	1:39	21:27	15. Jun
				4.1 km	1:11	21:29	22. Jun
				7.5 km	1:35	21:29	29. Jun
			Outboard, 90 HP	5.5 km	2:00	21:27	06. Jul
			Inboard, 110 HP	7.0 km	2:21	21:24	13. Jul
			Outboard, 90 HP	6.1 km	1:48	21:18	20. Jul
			Outboard, 115 HP	7.1 km	1:45	21:10	27. Jul
Möhlin 47.5808°N, 7.8401°E	Natural: gravel, mud, submerged vegetation, sandbank	Slow flowing: backwater of hydroelectric dam	Outboard, 70 HP	3.8 km	1:32	21:29	19. Jun
				3.7 km	1:30	21:27	07. Jul
				3.3 km	0:51	21:23	14. Jul

DISCUSSION

The main objective of this dissertation was to uncover mechanisms of round goby translocation via recreational boats and to derive measures to prevent it. We answered our associated research questions by conducting four studies that address relevant ecological, behavioural and molecular aspects of round goby biology that enable translocation via boats.

WHAT ARE RELEVANT PROPAGULES FOR SECONDARY TRANSPORT VIA RECREATIONAL BOATS?

To understand how translocation of invasive fish by boats is possible, it is crucial to know which life stages are relevant propagules. For round gobies in particular, eggs have long been considered potential propagules (Ahnelt et al. 1998, Borcherding et al. 2011, Kotta et al. 2016). While lacking verified proof, circumstantial evidence corroborates the possibility of round goby egg transport on boat hulls (Adrian-Kalchhauser et al. 2017). Accordingly, Paper I – III are dedicated to substantiate the plausibility of round goby eggs as propagules.

Our results uncover important aspects that enable round goby translocation via boats. We provide the first documentation of round goby presence on boat hulls (Paper I), we verify that round gobies use vertical habitats for spawning (Paper II), and we elucidate the ability of round goby eggs to withstand water withdrawal as expected during overland transport of boats (Paper III). These results should suffice to warrant attention of managers and boat owners towards round goby translocation not just via commercial, but also via recreational boats.

One limitation of the presented studies is that we did not find direct practical proof to verify the role of round goby eggs as propagules for translocation throughout the four years of studying it. Several aspects contribute to the difficulty of finding practical proof. Round gobies are cave spawners and would as such hardly lay their eggs on easily visible areas of the boat hull. Finding evidence of round goby nests inside of pipes and grates is difficult if the boat is outside of the water, and even more challenging while snorkelling or diving. Another reason for the lack of direct evidence could be that our study location is not well suited to find it. The Rhine around Basel is a fast flowing river and many marinas are built without protection from the current. While we observed round gobies on a boat hull that was exposed to mild current (described in Paper I), we never saw them on boats that moored in strong currents. Still waters or marinas with walls protecting them from strong currents might be more promising locations to document round goby nesting on boat hulls. Furthermore, marinas around Basel are small with ca. 15 – 30 boats and several kilometres distance between them, which makes efficient monitoring of a large number of boats challenging. However, our result give good indication that round goby nesting on boat hulls is plausible, so the detection of practical evidence seems to be a matter of time. The ubiquity of cameras and the increase in boat cleaning programs as preventive measures will likely lead to the documentation of round goby eggs on boats in the future.

In addition to round goby eggs, Paper IV introduced larvae as possible propagules for secondary translocation. Round goby larvae are long considered to play a role in their primary introduction, because they can be taken up into ballast water of commercial ships in great numbers (Hensler and Jude 2007, Hayden and Miner 2009, Kotta et al. 2016). However, there is to our knowledge no scientific publication to date that considers them as propagules for secondary transport. But is our detection of one larva inside a boat motor enough proof to warrant more consideration from scientists and management? We argue that it should be, because of the additional finding that boat owners find living fish and other macrofauna in their cooling systems, and because of the potentiation of its significance if extrapolated over all occasions of uptake and transport. The simple methods that could avoid this way of translocation proposed in Paper IV (emptying the residual cooling water after drives and before passing ship locks, flushing the motor whenever possible) are therefore appropriate to implement in management campaigns directed at the prevention of the spread of invasive species.

Adult specimen as propagules for translocation could pose an additional translocation risk that we did not assess here. Wonham et al. (2000) documented adult fish in ballast water tanks, but fish of this size are too big to be passively taken up even by large recreational boat motors. Adult round gobies hiding in crevices and holes is, however, not impossible and should not be dismissed as a way of translocation. For example, some creviculous species seem to be able to travel large distances hidden in suitable structures. While not described in the scientific literature yet, translocation of fish on boat hulls was documented by technical divers in Stavanger, Norway, who found many specimen of the blenny molly miller (*Scartella cristata*, Linnaeus, 1758) hiding in empty barnacle shells on an ocean going freighter that travelled from the Caribbean across the Atlantic with these fish as a stowaways (Figure 4). This documentation opens avenues for further investigations into fish translocation by boats – a topic that is far from being understood in its prevalence and significance.

Figure 4 Another way of translocation for fish: One of many specimen of a molly miller inside a barnacle shell that was attached to a commercial ship that had travelled from the Caribbean to Norway. © Rudolf Svensen

HOW IS VECTOR CONTACT ESTABLISHED?

For the development of measures against the translocation of round gobies via recreational boats, it is important to understand how vector contact is established. When this project started, nothing more than unsubstantiated reports of round goby eggs on boat hulls supported their association. In Paper I and Paper IV, we document and elaborate ways in which round gobies connect with boats.

One key aspect enabling round gobies reach boats is the use of vertical habitat. Boat hulls are vertical or angular structures, which do not resemble the horizontal rocky or sandy bottoms round gobies are commonly found on. The use of vertical harbour walls was therefore a first indication that round gobies would accept boat hulls as habitat. Additionally, in Paper I we observed round gobies moving directly from vertical walls to boat hulls, proving that harbour walls can act as beachheads for round goby translocation. The connection between the use of harbour walls as habitat and potential round goby presence on boat hulls was mentioned once before (Hensler and Jude 2007), but not given further consideration in the published literature. However, this connection is of ecological as well as practical relevance. The ecological relevance arises from the indications this behaviour has for population dynamics and invasion processes, as elaborated in Paper II. The practical relevance lies in the potential to develop measures to prevent round gobies connecting to boats. For example, treating harbour walls with antifouling paints might prevent round gobies from using them as habitat, as they would find less food organisms growing there and less structures like mussel shells to hold onto. This would also help to limit the function of harbour walls as beachhead for invasive fouling organisms (Bulleri and Airoldi 2005, Glasby et al. 2007, Dafforn et al. 2009).

The second important aspect enabling round gobies to connect with boats is larval drift. Round goby larvae ascend in the water column at night and drift in the current as a natural dispersal strategy. Larval drift thereby also contributes to closing distribution gaps between primary introduction sites in the invasive ranges of the round goby (Roche et al. 2013, Nogueira Tavares et al. 2020). While drifting at the water surface, larvae can be sucked into boat motors and travel within the cooling water systems. This mechanism is particularly relevant for boats driving during evening and night hours. In Paper IV, we observed boats active after sunset throughout the summer. For instance, angling during the night is popular among fishers targeting nocturnally active fish like common carp or catfish (Klefoth et al. 2013, Hyman et al. 2017, Žák 2021). In general, larger boats pose a greater risk, as their motors hold more cooling water, have a higher cooling water throughput and protrude deeper into the water. These factors increase the probability of uptake and survival of fish larvae, so that even low densities of drifting larvae can contribute to translocation. A controlled and standardized sampling regime that quantifies the uptake and survival of fish larvae into cooling systems, including a comparison of different motor types and driving profiles, would enhance our understanding of the relevance of this mode of translocation.

WHAT ARE THE BIOLOGICAL MECHANISMS ENABLING SUCCESSFUL TRANSLOCATION?

For successful translocation, propagules need to be taken up by a vector, but they also need to survive transport. The discipline of invasion biology is concerned with understanding why some species are successfully introduced and move on to becoming established invasive species (i.e. their invasiveness), while many other species do not survive uptake and transport (Marco et al. 2002, Colautti et al. 2014). Paper II and III contribute to this question by investigating mechanisms enabling round gobies to endure transport conditions.

One important feature of many successful invaders is the ability to efficiently exploit anthropogenic habitats, especially in species that are introduced unintentionally by hitchhiking on human means of transportation (Hufbauer et al. 2012, Wang et al. 2021). The round goby clearly succeeds in exploiting anthropogenic habitats, as demonstrated by their high population densities in harbours (Wiesner 2005, Vélez-Espino et al. 2010). We assume that they also inhabit anthropogenic habitats in their native range, as the original uptake of round gobies by ballast water likely occurred in harbours in the Black and Caspian Sea (Kornis et al. 2012). Our results show that the use of vertical walls by round gobies increases the availability of inhabitable space within harbours and suggest that even bigger population sizes than commonly estimated are probable. Higher abundances at the sites of uptake increase propagule pressure and therefore contribute to the invasiveness of round gobies (Blackburn et al. 2015).

Because harbour walls differ from habitats round gobies typically encounter in nature, vertical habitat use could be termed an “anthropogenically induced adaptation to invade” (Hufbauer et al. 2012), meaning that adaptations to anthropogenic habitats in their native range facilitated their establishment in harbours in their introduced range. If the preferential use of vertical habitats by a more competitive subset of the population, as demonstrated herein, leads to assortative mating, this could enhance the likelihood of survival of transport and establishment at a new location. The practical confirmation of consistent differential habitat use and its seasonal dynamics needs to be confirmed, for example by the use of mark-recapture studies or acoustic telemetry (Ray and Corkum 2001, Cunjak et al. 2005, Bergman et al. 2022). Assortative mating and potential adaptations to vertical habitat use should be confirmed by population genetics and morphological investigations to confirm the proposed mechanisms.

Another preadaptation to translocation via human transport is the high desiccation tolerance of round goby eggs. Because this tolerance likely did not evolve because of anthropogenic habitat alterations, let alone overland transport, it is rather called an exaptation (a trait that has evolved under one selection regime which is co-opted and increases fitness under a different scenario, (Gould and Vrba 1982) than a prior adaptation. The original selective pressure to develop desiccation tolerance might have been the variable water levels of the Ponto-Caspian region during the past millions of years (Reid and Orlova 2002, Neilson and Stepien 2009). This exaptation enables round goby eggs to survive conditions of translocation via trailered boats and therefore to colonize new waters. While Paper IV only begins to understand the processes involved in desiccation tolerance of round goby eggs, it shows that there distinctive molecular mechanisms contribute to their survival capabilities. Because the majority of overland boat transports happens over short distances (Buchan and Padilla 1999, De Ventura et al. 2016), withstanding water withdrawal for up to 48 hours makes the trailered boats risk vectors for round goby translocation.

WHICH MEASURES CAN PREVENT ROUND GOBY TRANSLOCATION VIA BOATS?

After uncovering the potential ways of the round goby`s connection to boats, we can make informed management recommendations to prevent their unintentional translocation:

1) Inspect and clean potential nest sites for round gobies (pipes, grates, narrow spaces etc.) before transporting a boat into a new water body.

2) Dry the boat for more than 48 hours before transporting it into a new water body.

3) If possible, avoid mooring too close to a vertical wall to prevent their beachhead function.

4) Do not attract round gobies to use the hull as habitat by allowing fouling organisms to settle (regular cleaning and/or use of antifouling paints).

5) While travelling in water into new areas (separated by a lock, sluice or boat lift), make sure to empty the cooling water of the boat before crossing the separation between stretches of water.

These measures do not only apply to the prevention of invasive goby translocation, but also contribute to stopping the spread of most other aquatic invasive species. Many of the measures are already recommended and advertised in management campaigns all over the globe (Rothlisberger et al. 2010, Anderson et al. 2015, Mohit et al. 2021). However, the notion that these measures could also prevent the spread of invasive fish might gain the campaigns more attention, as vertebrates generally attract audiences more than invertebrates (Clucas et al. 2008, Schlegel and Rupf 2010). Round gobies and ecologically similar fish species could therefore even serve as flagship species ("popular, charismatic species that serve as symbols and rallying points to stimulate conservation awareness and action", (Heywood and Watson 1995)) in future versions of information campaigns.

While proposing these recommendations to the scientific community is an important part of academic work, the applied nature of this project and cooperation with environmental offices and stakeholders made a quick practical implementation of the presented results possible. For example, a newly launched campaign to keep Swiss waters free of aquatic invasive species included our results on round goby eggs and larvae as propagules. The official boat cleaning instruction leaflets and videos prepared for the campaign were created with scientific advising of the author of this dissertation (AWEL Zuerich 2020).

CONCLUSIONS

Understanding translocation mechanisms of invasive species is crucial for the prevention of their spread. Research on secondary range expansion of fish is sparse compared to other invasive species, with spotlight studies considering for example the role of bait bucket transport (Drake and Mandrak 2014) or birds (Hirsch et al. 2018) in moving fish to uncolonized waters. The research presented here contributes to the comprehension of a mode of translocation that is underrepresented in the scientific literature: the unintentional transport of invasive fish via recreational boats. Our studies show that this way of translocation is plausible and likely to contribute to the spread of invasive gobies. Ecological, behavioural, molecular mechanisms contribute to the connection of round gobies and boats as their transport vectors.

We identified and characterized the role of early life stages of round gobies as propagules. Eggs laid in pipes, grates and other cavities on boat hulls can be transported within and across water bodies. Given the substantial probability of occurrence, we expect documented direct evidence of this mode of translocation in the future. We also documented larvae drifting in the water column at night as propagules in the context of recreational boating for the first time. This mode of translocation is not limited to invasive gobies, as larval drift is common for many fish species (Norcross and Shaw 1984, Lechner et al. 2016). Fish larvae in boat motors could therefore globally contribute to the spread of invasive fish in freshwater as well as marine environments.

Behavioural and ecological aspects of habitat use proved to be of key importance for the establishment of vector contact in the presented studies. Association with a vector is imperative for translocation. Yet, although round gobies have long been under suspicion to use boat hulls as nesting opportunities, it remained unclear why and how the bottom-dwelling fish would reach boats on the water surface. While the association between settling on anthropogenic habitats close to the surface and translocation via boats received ample attention in invertebrates (Airoldi and Bulleri 2011, Foster et al. 2016), the same association was rarely made for fish. Given that the most commonly translocated fish genera are the benthic gobies and blennies (Wonham et al. 2000), our results are also applicable and offer explanations for invasion processes beyond our study system.

The high desiccation tolerance of up to 48 hours shown by round goby eggs is a relevant trait for survival of overland transport. The molecular response to air exposure includes cellular protective mechanisms like responses to oxidative stress, changes in protein metabolism, and retardation of developmental processes. Shedding light on the molecular mechanisms of desiccation tolerance is interesting from an evolutionary perspective to understand conserved and unique aspects of fish survival under adverse conditions. However, it is also highly relevant for freshwater systems, which are vulnerable to decreasing and more fluctuating water levels in the face of climate change. The analysis of molecular processes promoting desiccation tolerance therefore opens avenues to new hypotheses and novel research around the future survival of fish species inhabiting those systems.

In conclusion, the research presented herein promotes the understanding of invasion dynamics and spread of aquatic invasive species, in particular fish. The combination of observational and experimental approaches enabled the discovery of important propagule-vector associations and expanded the scientific knowledge about mechanisms underlying successful translocations. The practical implications of this work can contribute to targeted and efficient management of aquatic species invasions, with the prevention of a further spread of round goby as an important objective.

OUTLOOK

In the future, expansion of the presented work will expand the gained knowledge around translocation mechanisms of invasive fish deepened in various ways. Because many of the covered topics received limited scientific attention so far, many questions remain unanswered, promising important future insights into invasion dynamics in general, and round gobies` success as invader in particular.

One future research avenue is to examine the relevance of differential habitat use between vertical walls and the bottom substrate further. In Paper II, we propose that more competitive individuals preferentially use harbour walls as habitats, which might therefore be the main constituents of newly introduced populations. To confirm this hypothesis, evidence for assortative mating based on habitat choice is needed. Additionally, potential differences in morphological features could reveal adaptations underlying, or resulting from, differential habitat use. The expected results will contribute to one of the main questions of invasion biology: Why are some species successful invaders, while most other species are never successfully translocated or established?

The results of Paper III also hold great potential for follow-up studies. Of particular interest are the roles of the eggshell and clutch structure in promoting desiccation tolerance. These aspects could not be sufficiently investigated within the scope of Paper III, but they likely contribute to the eggs` ability to withstand prolonged air exposure by retaining water inside of the egg, as well as in the interstitial spaces between the eggs. The expected results are on the one hand interesting in the context of translocation of an invader, but are even more relevant in the light in one of the biggest environmental challenges the planet is facing: climate change and related land use change. While current research revolves mainly around the desiccation tolerance of species adapted to dry conditions, we should strive to understand general mechanisms allowing fish to survive water withdrawal. With decreasing and more fluctuating water levels expected within the next century, desiccation is a challenge that many littoral spawning fishes may face soon. Knowledge about potential coping mechanisms of fish eggs will therefore become an increasingly relevant aspect in the prediction of which fish will be “winners” or “losers” of climate change.

From a practitioner`s perspective, the results of this work can improve future risk modelling approaches and management campaigns. Models forecasting invasion risk should include information about population densities at the site of uptake as well as survival during transport to determine propagule pressure. The presented results should be further disseminated to the public and relevant stakeholders. While some of the results are already considered in management campaigns, additional input through scientific advising in new projects will help preventing the spread of invasive species. For example, we will actively contribute these results to a new pilot project that aims to register boat transports between Swiss lakes and ensure effective cleaning of boats. Fruitful transdisciplinary collaboration is crucial to exchange knowledge and to promote invasive species prevention.

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APPENDIX

AUTHOR`S CONTRIBUTIONS

PAPER I:

KB: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation; writing - original draft, writing – review and editing.

PBH: research conceptualization, funding provision, writing – review and editing.

PAPER II:

KB: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation; writing - original draft, writing – review and editing.

PEH: research conceptualization, sample design and methodology, writing – review and editing.

ML: sample design and methodology, data analysis and interpretation.

PBH: research conceptualization, sample design and methodology, funding provision, writing – review and editing.

PAPER III:

KB: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation; writing - original draft, writing – review and editing, funding provision.

JNE: data analysis and interpretation; writing - original draft, writing – review and editing.

IAK: research conceptualization, sample design and methodology, data analysis and interpretation, funding provision.

PBH: research conceptualization, funding provision.

PAPER IV:

KB: research conceptualization, sample design and methodology, investigation and data collection, data analysis and interpretation; writing - original draft, writing – review and editing.

PEH: research conceptualization, sample design and methodology, writing – review and editing.

PBH: research conceptualization, sample design and methodology, funding provision, writing – review and editing.