Boldness and physiological variation in round goby populations along their Baltic Sea invasion front

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Short running title: Round goby boldness and physiological variation

Abstract

The round goby (*Neogobius melanostomus*) is a fish native to the Ponto-Caspian region that is highly invasive through freshwater and brackish habitats in northern Europe and North America. Individual behavioural variation appears to be an important factor in their spread, for example a round goby's personality traits can influence their dispersal tendency, which may also produce variation in the behavioural composition of populations at different points along their invasion fronts. To further analyze the drivers of behavioural variation invasive round goby populations, we focused on two populations along the Baltic Sea invasion front with closely comparable physical and community characteristics. Specifically, this study measured personality within a novel environment and predator response context (i.e., boldness), and directly analyzed links between individuals' personality traits and their physiological characteristics and stress responses (i.e., blood cortisol and lactate, brain neurotransmitters). In contrast to previous findings, the more recently established population had similar activity levels but were less bold in response to a predator cue than the older population, which suggests that behavioural compositions within our study populations may be more driven by local environmental conditions rather than being a result of personality-biased dispersal. Furthermore, we found that both populations showed similar physiological stress responses, and there also appeared to be no detectable relationship between physiological parameters and behavioural responses to predator cues. Instead, body size and body condition were important factors influencing individual behavioural responses. Overall, our results reinforce the importance of boldness traits as a source of phenotypic variation in round goby populations in the Baltic Sea, but that the physiological mechanisms underpinning personality trait variation in these populations remain unclear.

Keywords: invasive, personality, risk-taking behaviour, predation, novel environment, stress

1. Introduction

Biological invasions are multi-phase processes that can have serious impacts on invaded ecosystems, particularly in marine and estuarine environments. These ecosystems are particularly exposed to invasion due to human-driven introduction pathways such shipping (Kotta et al., 2016), recreational boating, aquaculture and even aquarium trade (Williams & Grosholz, 2008). The round goby (*Neogobius melanostomus*, Pallas, 1814) is a highly successful invasive species in these coastal habitats. Most likely introduced from ballast water from the Ponto-Caspian region, the first appearance of round gobies within the Baltic Sea region was in the Gulf of Gdansk in Poland in early 1990 (Skóra & Stolarski, 1993). By the end of the decade, it became the dominant fish in shallow waters of the western part of the gulf (Sapota & Skóra, 2005). Since then, this predatory fish has spread west along the coasts of Poland, Germany, and now into Denmark. In 2009 the species was first observed in the inner Danish waters south of Zealand in Guldborgsund (Fig. 1), and since then it has spread on average 30 km yr^{-1} along the coastline of Zealand and the islands in Smålandsfarvandet (Azour et al., 2015). In the Baltic Sea, round gobies appear to eat the eggs of native species, including flounder (*Platichthys flesus*), and native gobies (Karlson et al. 2007), and can have major impacts on the abundances and composition of local benthic communities via their feeding behaviour (Kipp et al., 2012; Pennuto et al., 2018; van Deurs et al., 2021).

Personality is often defined as behavioural variation that shows consistent variation among-individuals over time (or context), measured as the relative proportion or component of among-individual variance estimated from repeated behavioural measurements (Dall et al., 2004; Sánchez-Tójar et al., 2022). Personality differences in boldness, aggressiveness, activity, and sociability have been linked to dispersal (Cote et al., 2010; Hirsch et al., 2017; Myles-Gonzalez et al., 2015; Rehage & Sih, 2004), and modelling has also suggested that greater diversity of behavioural traits within a population may greatly accelerate invasion rates (Elliott and Cornell 2012). Personality may also influence dispersal and invasion spread at multiple stages, including decisions to stay or depart, when and where to settle, and post-dispersal success (Chapple et al., 2012; Weis & Sol, 2016). In a study about western bluebirds (*Sialia Mexicana*), Duckworth (2008) demonstrated that more aggressive individuals lead dispersion at the invasive front, and after establishment they are substituted by less aggressive individuals because of their poor parental care. These differences in personality types are spread along the invasion succession and can produce population at different stages of invasions with contrasting behavioural compositions, also in the round goby (Myles-Gonzalez et al., 2015; Thorlacius, Hellström, Finn, et al., 2015). This is likely to influence how the invasive populations at different stages of an invasion impact the communities in recipient ecosystems, as personality can be linked to habitat use and foraging behaviour in individuals (Moran, Wong, et al., 2017; Patrick & Weimerskirch, 2014). Nonetheless, there is still limited data on behavioural variation within invasive populations along invasion fronts, and what underlying mechanisms can produce these behavioural differences.

Several underlying mechanisms or proximate causes might lead to personality variation, which may be linked genetic/epigenetic variation, phenotypic plasticity in response to individual-level differences in environmental/state variables, or the interaction between the two (Dewitt & Scheiner, 2004; Pigliucci, 2005; Wolf & Weissing, 2010). Differences between individuals' intrinsic states can lead to differences in behaviour. For example, (Behrens et al., 2020) found that body size was associated with boldness in round gobies where bolder fish tended to be smaller, potentially due to the metabolic cost of their behaviour that results in a slower growth rate. Body condition may similarly influence behaviour (Moran et al., 2021), where lower body condition is often associated with higher risk-taking. Also, differences in hormone levels (Niemelä & Dingemanse, 2018) and certain neurotransmitters (Ferris & Delville, 1994) can produce differences in behaviour.

Stress triggers a neuroendocrine response in vertebrates that results in the production of corticosteroids and catecholamines (Wendelaar Bonga, 1997). Cortisol is the main corticosteroid in teleost fish and is widely used as a stress marker. Among other functions, it increases energy availability through gluconeogenesis, complementing the action of catecholamines mobilizing glucose from glycogen stores. This availability of energy facilitates the necessary responses to physical adaptation to the stressor. Another parameter used to measure stress response in vertebrate is lactate. Lactate is a product of anaerobic metabolism, usually triggered when oxygen supply to tissues is in shortfall, for example during strenuous exercise/muscular activity. High swimming activity occurs often as part of the behavioural response to stress (Wells & Pankhurst, 1999), leading to an increase of lactic acid production and, thus, to a higher concentration of lactate in blood. Brain monoaminergic neurotransmitters such as dopamine and serotonin are believed to have a prominent role in the regulation/organization of the stress response of vertebrates, and seem to be at least partially responsible for the differences in stress response associated to personalities

(Crawford et al., 2010; Winberg et al., 2016). Serotonergic activity, in particular, appears consistently elevated in certain areas of the vertebrate brain during acute stress (Emerson et al., 2000; Gesto et al., 2013) and can be also affected by persistent or repeated stressors (Winberg & Thörnqvist, 2016). Importantly, both stress-response phenotypes and brain monoamines are linked to individual behavior and personality (Soares et al., 2018; Winberg & Sneddon, 2022).

The overall goal of this study is to measure differences in boldness/risk-taking behaviour and physiological stress responses of round gobies in two populations across their invasion path (Grønsund and Søvang, first recorded in 2011 and 2016, respectively) in the southeast Baltic Sea (Fig. 1). By comparing these populations, we specifically tested the following research questions:

1. *Do round gobies within these two populations show among-individual differences in boldness/risk-taking behaviour in a novel environment and/or predator response context (i.e., personality)?* We predicted that both populations would show individual differences in boldness traits, as consistent among-individual variation has commonly been found in round gobies (Myles-Gonzalez et al., 2015; Thorlacius, Hellström, Finn, et al., 2015; Thorlacius & Brodin, 2018; Behrens et al., 2020).
2. *Is there variation in boldness/risk-taking behaviour associated with (a) the population, and/or (b) the physical state (i.e., body size, body condition) of round gobies?* Some studies have shown that the populations nearer the invasive front tend to be bolder in novel environments (Myles-Gonzalez et al., 2015; Thorlacius, Hellström, Finn, et al., 2015) consistent with personality-biased dispersal favoring bolder individuals, therefore we expected to find bolder fish in the newer population (i.e. Søvang). We also expect smaller fish and fish in lower body condition to show higher levels of boldness (following Behrens et al., 2020; Moran et al., 2021).
3. *Are there (a) population differences in the physiological responses to acute stress, and (b) are physiological and behavioural responses linked?* The hormonal and neurochemical state of individuals may be important sources of variation underlying behavioural variation among individuals (Sih et al., 2015; Wolf & Weissing, 2010). Therefore we expect increased boldness/risk-taking to be negatively correlated with blood cortisol and lactate responses in individuals from both populations, and dopaminergic and serotonergic activity in the brain (Gesto et al., 2013). If we find

higher boldness/risk-taking in Søvang (as above), we also expect to find lower physiological stress responses in Søvang.

2. Methods

2.1. Fish and holding conditions

Fish were collected from two sites, Grønsund (54.90371°N, 12.10367°E, established 2011) and Søvang (55.57146°N, 12.62579°E, established 2016) (Fig. 1). Both of which are shallow brackish coastal areas, with predominantly sandy substrate with scattered boulders, where round gobies are typically found in high abundances particularly during spring and summer periods (Azour et al., 2015). Collection occurred in May 2021 as part of a broader sampling effort to collect round gobies at numerous sites along their east-coast Danish invasion front. Fish from these two sites chosen for this experiment, as they both had similarly high abundances at sampling and both showed very low mortality during laboratory acclimation, limiting survivorship bias effects on experimental fish.

Fish were caught using a combination of passive sampling methods set overnight (i.e. double funnel fyke nets, baited box and cylinder traps) to reduce the potential for personality-biased sampling (Biro & Dingemanse, 2009; Michelangeli et al., 2016). Physical conditions measured within sites during sampling were similar (Søvang 16/5/21: 8.75ppt, 14.1 °C; Grønsund 18/5/21: 8.27ppt, 13.2 °C). Fish were transported to DTU Aqua, Lyngby, Denmark, and acclimated to laboratory conditions for 48 hours before being individually tagged with passive integrated transponder (PIT) tags (12 × 2 mm, 0.1 g, Oregon RFID Inc.), using previously published methods that have minimal effects of fish health and condition (Jørgensen et al., 2017).

Before the start of this experiment, fish were held in two large mixed-sex and mixed-population round holding tanks (3000 L) enriched with artificial eel grass and connected with a recirculating water system. Fifteen days before the behavioural experiment, experimental fish (n = 36 per population) were randomly selected and allocated to three round tanks (800 L) in groups of 24 per tank. Only males were selected for this experiment as both populations were heavily male-biased (Søvang, 90.2% male; Grønsund, 86.9% male at sampling), and there were too few females to include sex as a factor in analysis. The lack of females in goby samples is commonly found in invasion front populations, which may be results of male-biased spread and dispersal producing male-biased populations, and/or because the males are often found to be more active, facilitating their capture with passive traps (Corkum et al., 2004; Marentette et al., 2011).

The salinity in the holding tanks was 10.5 ± 0.5 ppt and the temperature was constant at 10.5 ± 0.1°C with a daily cycle of 12 hours of light and graduated increases/decreases in light intensity at dawn and dusk to simulate a natural light regime. During the whole project fish were fed every two days with dry pelleted feed (3mm Ivory Ex composite pellets, Aller Aqua, Denmark). Experimental fish were fed on the day before behavioural trials. Before the physiological sampling feeding was stopped for 48 hours before the 16 hours of isolation, to help maintain water quality in their individual holding tanks before blood and brain sampling.

2.2. Experiment A: Behavioural repeatability and responses to stress

After acclimating to their holding tanks over four weeks, all fish from one holding tank fish were assayed per day over 3 days, followed by repeat trials the following week. Individuals were selected using random sweeps through the holding tanks, and fish were not identified until after trials, so the order was blinded and assumed to be random within tank groups/trial days. The experiment started at 9:00am and every 30 minutes two fish were moved from the holding tank to individual black tanks (24 x 34 x 15.5 cm) covered with brown cardboard and aired with air stones for 2 h to standardize pre-trial handling and stress levels. After isolation, the fish were moved to the behavioural arenas using the individual tanks still covered and opened only once they are immersed in the arena water.

The behavioural arenas consisted of two opaque PET-plastic white tanks, A and B (internal dimensions, 32.25 x 49.25 cm), surrounded and separated by white polystyrene sheets to insulate the arenas from both external sound and visual inputs (Fig. 2). The experiment was performed under constant laboratory fluorescent lighting (approximately 45-50 lux within arenas at the water surface), with temperature and salinity identical to the holding tanks. One camera (a modified Logitech BRIO 4K Ultra HD webcam, Logitech, Switzerland) connected to a laptop (Logitech Capture, version 2.06.12) recorded the tanks from 1 m above. After a brief 5 min acclimatization, behaviour was recorded for a further 5 minutes to provide baseline behavioural measures (*pre-strike* period). A simulated bird strike was then performed using a bolt (5.5 cm, 23 g) suspended 80 cm above the water in the centre each tank by fishing lines (method adapted from Behrens et al., 2020). Bolts were released to hit the water surface and penetrate 5 cm into the water column, then were immediately retracted to their original positions. Behaviour was recorded for 5 minutes after the strike before the trial was terminated (*post-strike* period). After every run the arenas were rinsed with deionized water and the water was entirely replaced using freshly filtered water from the laboratory's recirculating system, to prevent contamination from the previous fish by conspecific chemical cues that could alter the stress level during the experiment (Barcellos et al., 2011).

Videos were analyzed by using the software Toxtrac (Rodriguez et al. 2018, version 2.96). Each video/arena was individually calibrated to account for any minor variation in the positions of tanks and the camera in each trial. Behavioural variables, activity, centre area use and time spent frozen (see Table 1), were collected in both the *pre-strike* and *post-strike* periods to represent boldness/risk behaviours associated with fish movement in a novel environment and predator response context respectively (Moran et al., 2021). Direct responses to the predator strike cue were quantified as the latency to freeze following the strike, and latency to resume movement after freezing (as per (Behrens et al., 2020), both of which were quantified within ToxTrac (see details in Table 1).

2.3. Experiment B: Physiological responses to stress

Physiological stress response was measured in fish a minimum of 10 days after completing behavioural trials. Groups of 12 fish were moved each day from holding tanks to individual covered black tanks (24 x 34 x 15.5cm) for 16 hours to allow cortisol concentration in the blood decrease to baseline levels. The tanks were well aerated with an air stone and covered by green tarpaulin over 80% of the tank to reduce external sounds/visual inputs and provide a low stress individual holding environment for each fish.

Fish were randomly allocated to three treatment groups: 1) *sham control*, exposed to 10 mL of unscented water (n = 12 per population); 2) *cue*, exposed to 2,5 mL of water with chemical conspecific alarm cue mixed with additional 7,5 mL of unscented water (n = 18 per population); and. 3) *baseline control*, sampled after 16 hours without the addition of any stressor (n = 3 per population). The chemical alarm cues were produced to mimic chemicals released by an injured conspecific (e.g., due to a predator attack) using a method based on Smith (1989). Specifically, one non-experimental fish from laboratory stock was euthanized with an overdose of tricaine methanesulphonate ("MS-222", Acros Organics) and placed in a clan petri dish: 25 cuts were done on each flank by using a scalpel, then the fish was rinsed with 15 mL of marine water. 2,5 mL of this solution were mixed with 7,5 mL of marine water, to be added to their individual holding tanks. A small hole in the tarpaulin allowed for the administration of *cue* treatments, while avoiding visible contact between the operator and the fish.

The *baseline control* group were sampled first, while the *cue* and *sham control* groups were sampled between 30 and 45 minutes (short exposure) and 75-90 minutes (long exposure) after the adding the cue mixtures to also allow for the analysis of exposure time on physiological responses. Each fish was then exposed to an overdose of tricaine methanesulfonate (MS-222 500 mg/L), and blood samples were collected immediately using a heparinized syringe (heparin lithium salt, Sigma-Aldrich, Denmark; diluted 1 mg/mL in 0.9 % NaCl) (Marentette et al. 2013) from the caudal vein and stored on ice. Blood samples of 0.3 mL were stored in 1.5 mL Eppendorf tubes containing 15µL of the heparin solution. The fish was then measured for weight and total and standard length ('SL') before the head was removed with a sharp knife and placed immediately in aluminum foil within plastic sample bags on dry ice. Blood samples were then centrifuged (accuSpin™ Micro 17R, Fisherbrand™ Microcentrifuges, UK) at 2500 x g for 2 minutes to obtain plasma, and both plasma and whole head samples were was stored at -80°C with the heads until the analysis.

Plasma cortisol was quantified by means of a commercial ELISA kit (ref. 402710, Neogen Europe, Ayrshire, UK). Plasma lactate was analyzed with a colorimetric kit from Sigma-Aldrich (ref. MAK064, Merck, Darmstadt, Germany). Frozen fish heads were left at room temperature for 5 min and the telencephala were dissected out from fish brains while still semi-frozen, after removing the roof of the skull with a scalpel. This brain region was selected for analysis for having a key involvement in the regulation of emotional reactivity, including stress responses in fish (Vindas et al., 2018). Telencephala were weighed and then homogenized in 400 µL of a 0.4 M perchloric acid, 0.1 mM EDTA solution, using ultrasounds (Sonopuls ultrasonic homogenizer, Bandelin, Germany). Homogenates were centrifuged (14000 x g, 4°C, 10 min), and the supernatants were immediately analyzed by means of HPLC with electrochemical detection, as described elsewhere (Alfonso et al., 2019). The levels of serotonin, dopamine, and their respective main oxidative metabolites, 5-hydroxyindoleacetic acid (5HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were quantified (for further details of the physiological variables used in the study, see Table 2).

2.4. Statistical analysis

All the analysis was performed in R (v4.1.2, R Core Team, 2013). Before behavioural analysis, distributions were assessed for normality, and transformed where necessary (see Table 1). Intraclass correlation coefficients were used to calculate the repeatability of behavioural variables (package “rptR”, v0.9.21, Stoffel et al., 2017), which represents the proportion of among-individual variance relative to total phenotypic variance for each variable (Nakagawa & Schielzeth, 2010). To assess repeatability across all experimental fish, we calculated raw repeatabilities and population-adjusted repeatabilities. Adjusted repeatability includes population as a random effect to remove influence of population-level differences on the among-individual variance component. Closely related populations may show different levels of behavioural consistency, which may be related to local environmental conditions or dispersal processes (as in Moran et al., 2017; Thorlacius, Hellström, & Brodin, 2015), therefore, repeatability was also estimated for each behavioural variable within each population. Data for four replicate trials were lost due to technical problems with video recording and/or tracking (trial 1, IDs: #447, #9772, #9888, #9955). Data for three fish were also removed from all behavioural analysis due to injuries (IDs: #485, #9765, #9924) and cataracts (#9765) found during physiological sampling, as injuries incurred during the experiment may affect their behavioural responses and cataracts are known to influence predator responses in this species (Flink et al. 2017).

Effects of population and condition on behavioural responses to the predator cue were tested using linear and generalized mixed-effect models (package “lme4”, v1.1-27.1, Bates et al. 2015). Behavioral variables included here were those that showed significant non-zero repeatability (i.e., activity, centre area use and freeze time) so represent measures of among-individual behavioural (personality) variation in these populations (see Table 3). To test for changes in behaviour in response to the predator cue, behaviour from the *pre-* and *post-strike* ‘period’ were analyzed together, where the interaction between fixed effects and period represents its effect on the behavioural response of fish to the predator cue. Fixed effects, fish length (SL), body condition and population (and their interaction with period) were included. There appeared to be a correlated between SL and Fulton’s condition factor ($r_{pearson} = -0.25$, $p = 0.036$) (Ricker, 1975), therefore a group-specific condition factor was calculated using the slope of a $\log_e$(SL) - $\log_e$(weight) regression line (based on Green, 2001). To confirm that weight-length relationship is similar across sites, we tested for a site*$\log_e$(weight) interaction.

This interaction was not significant (p-value = 0.519), so a common slope was used. Condition factor (‘*K*’) was calculated as: $K = W / SL^n * 100$, where *W* is the weight (g), *SL* is the total length (cm) and n is the slope of the joint regression line (i.e. 2.7468). Continuous fixed effect factors (i.e. *SL* and *K*) were then Z-scaled for analysis to improve the interpretability of regression coefficients within models (Schielzeth, 2010). In addition to fish ID, random effects initially included trial arena, round/order, holding tank and replicate as potential grouping factors/sources of non-independence within the trials, although arena and holding tank were removed from all final models as they explained very little or no variance, and replicate was also removed from the final centre use model. Conditional and marginal R^2 values were also calculated to estimate to total proportion of variance explained by fixed and random effects in the models, and the proportion of variance explained by fixed effects only, respectively (package ‘performance’, v0.7.0, Lüdecke et al., 2021).

Linear mixed-effect models were used to test for effects on physiological response variables (Table 5). Response variables were log transformed to approximate normality and the continuous fixed effect factors were also scaled as above. Fixed effects included body size (SL), condition factor, exposure time (i.e., as short versus long cue exposure), population and treatment (*sham control* v *cue*). A population*treatment interaction was also included to specifically test if populations responded differently to the alarm cue. Additional interactions (as in behavioural models above) were not included here to limit overparameterization of the models, particularly given the smaller number of replicates per treatment and additional factors being analyzed. The *baseline control* group was used to provide a qualitative comparison with other treatment groups only so was not analyzed in these models. Random effects initially included were holding tank and sampling day, to account for these potential sources on non-independence, but were removed if they resolved little or no variance. As above, data from injured fish were removed from analysis, and samples for two additional fish could not be included due to technical issues with sample collection and processing (#9928, #9822).

For fish that were included in both behavioural and physiological analysis, we tested for correlations between physiological responses (in *cue* treatments group only) and behavioural variables (*pre-* and *post-strike* separately, using the average score across both trials). Populations were analyzed separately. Spearman’s rank (non-parametric) correlations were used with untransformed response variables. We present correlations without adjustments for multiple comparisons, so the significance of any one single significant correlation should be interpreted cautiously, although this was not an issue with this dataset.

2.5. Animal ethics statement

Ethical permit 2017-15-0201-01282 from the Danish Animal Ethics Committee (Dyreforsøgstilsynet) and its extensions covered all experiments reported here.

3. Results

Activity, centre use and freezing time variables showed significantly non-zero repeatability associated with fish ID between behavioural trials (Table 3), and estimates were largely similar in the *pre-strike* and *post-strike* periods. This suggests behavioural variation among individuals is maintained under acute predation pressure. Behaviour was also highly correlated between *pre-* and *post-strike* periods for each for these variables (activity$_{\text{pre-post}}$, $r_{\text{spearman}} = 0.72$, $p < 0.001$, centre use$_{\text{pre-post}}$, $r_{\text{spearman}} = 0.56$, $p < 0.001$, freeze time$_{\text{pre-post}}$, $r_{\text{spearman}} = 0.73$, $p < 0.001$). In contrast, both latency to freeze and latency to recover did not show significant repeatability associated with fish ID. Within populations, activity and freezing time showed significant repeatability in the older population (Grønsund, est. 2011) but not the newer population (Søvang, est. 2016) in both periods, whereas for centre use Søvang fish showed strong repeatability relative to Grønsund fish (see Table 3).

Populations showed different activity-level responses to the predator strike (i.e., period*population interaction effect), where Søvang fish reduced their activity more in the *post-strike* period relative to the Grønsund fish (Table 4, Fig. 3a). There was no significant population effect on activity, only a trend showing that Søvang fish were slightly less active (see Fig 3a). Instead, body size (SL) was a strong predictor of activity level, where smaller fish appeared more active overall, but showed greater reductions in their activity following the predator strike relative to larger fish (i.e., length and period*length effects, Table 4, Fig. 3b). Therefore, the lack of population difference despite the apparent trend of lower activity in Søvang fish may have been a result of their slightly larger average body size than Grønsund fish [$SL_{\text{Grønsund}} = 13.10$ (s.d. 1.54); $SL_{\text{Søvang}} = 14.61$ cm (s.d. 1.61); for further details see supplementary materials S1, Fig. S1].

Fish overall reduced their use of centre areas following the predator strike (i.e., period effect, Table 4), and both condition and length influenced individual responses, with larger fish and fish in better body condition being less responsive to the predator strike (i.e., period*length, period*condition effects, Table 4). Population had no effects on centre use. Body size (SL) also showed effects on freezing time, where smaller fish spent less time frozen overall, but increased their time spent frozen following the predator strike more relative to larger fish (i.e., length and period*length effects respectively, Table 4). There were no effects of condition or population on freezing time. Note, the three repeatable variables were all strongly correlated to each other despite showing differing effects from length, condition, and population (activity-centre use, $r_{spearman} = 0.57$, $p < 0.001$; activity-freezing time, $r_{spearman} = -0.95$, $p < 0.001$; centre use-freezing time, $r_{spearman} = -0.56$, $p < 0.001$), such that more active individuals also spent less time frozen, and more time in the exposed centre area (i.e. bolder fish tended be more bold across all three variables).

Treatment, i.e., conspecific chemical alarm cue, had a significant positive effect on cortisol concentration in blood (Fig. 4), but no effect on blood lactate (Table 5). There were no significant interactions between the treatment effect and the Population, suggesting there is no difference between populations in the cortisol or lactate responses to a conspecific chemical cue. Body size and condition also had no effect on cortisol or lactate levels. Dopaminergic and serotonergic mass ratio was negatively associated with body size, i.e., dopaminergic activity was generally lower in larger fish. Similarly, body condition also had a negative effect on serotonergic mass ratio, i.e., fish in poorer body condition had higher overall serotonergic activity than higher condition fish. There was no population or interaction effects dopaminergic and serotonergic mass ratio (Table 5).

Within both populations, there were not significantly non-zero correlations between behavioural response variables, and physiological response variables in the cue treatment group, suggesting that behavioural responses were not directly linked to the measured physiological stress response parameters (for further details see supplementary materials S2, Table S1 and S2).

4. Discussion

In this study, behavioural consistency was found to be high overall across most behavioural variables measured, by the level of repeatability varied between populations. Repeatability of activity, centre use and freezing time appeared to be strong, relative to the finding of Bell et al. (2009), who showed in a meta-analysis that approximately 35% of phenotypic variation in behaviour could be attributed to among-individual differences. We found boldness in the old population to be repeatable across more contexts (i.e., activity, centre use and freezing) than the newer population. In Thorlacius et al. (2015), bolder gobies from a newly established populations showed more repeatable behaviours. This is in contrast with our results, as the old population seemed to be bolder and more consistent. Moreover in a study about the relation between personality and metabolism (and also size) in round gobies from an established population in Guldborgsund (first recorded in 2009), latency to resume movement was found to be repeatable (Behrens et al., 2020).

In contrast with our prediction, fish from Søvang (est. 2016) appeared more responsive (i.e., higher decrease in activity) to the predator strike than the fish from Grønsund (est. 2011). In Thorlacius and Brodin (2018), a positive correlation was found between activity and boldness in round gobies. In that study, the experimental design was the same as ours. Thus, fish from the more established population might be considered bolder, differently from our original prediction. In another study on round gobies, Groen et al. (2012) did not find any significant difference in boldness between old and new populations Notably, our newer population was older (5 years post-establishment) than comparable studies (i.e. 2-3 year; Myles-Gonzalez et al. 2015; Groen et al. 2012), so that difference like observed in those studies may have been lost by the time of sampling. Traits linked to dispersal like boldness may be lost over time due to differences in density between populations: lower densities lead to selection of traits that increase reproductive rate rather than dispersal tendency (Duckworth, 2008). The type of behavioural variables being considered may also be a factor leading to our contrasting results, for example only Groen et al. (2012) measured boldness as activity after a stressful event, while Myles-Gonzalez et al. (2015) measured it as the latency to emerge from a shelter.

Contrary to expectations, there was no difference between populations in their physiological stress responses. The fish did show a stress response after exposure to the cue, as demonstrated by the blood cortisol concentration increase, however, there was no effect of the cue on lactate. No significant changes in lactate level in blood suggests that no anaerobically fueled activity was triggered, probably due to the small tanks where the experiment was performed. Furthermore, the typical response to predators of round gobies is to freeze, sometimes after a quick escape.

We predicted an effect of condition on behavioural responses to predator cues, but only a small effect on centre use was found, where fish in better condition were less responsive to the predator strike. This result is in contrast with Moran et al. (2021), who showed a negative effect of nutritional condition on boldness, where fish subject to poor feeding treatments tended engage in more risk-taking behaviour than well fed fish. However, in the present study, no manipulation of the feeding regime was performed, the two populations did not show difference in condition, and variation within populations was low. Moreover, the long time spent in the holding facility could have affected the general condition of the fish, that in nature is likely to be more variable, thus the only effect we detected was small and on only one of the behaviour variables (centre use). Total length, instead, showed significant effects on every behaviour variable. Total length showed a very strong negative effect on the activity in a novel environment and a minor non-significant negative effect on the activity in a predator response context: smaller fish were more active overall, but they showed greater reduction in activity after the strike. Movement into refuge areas is a typical response of animals to a predator presence and moving into the corner areas of the arenas may reflect this behaviour given there were no sheltered areas in the arena. The negative effect of size on boldness contrasts with the results from the meta-analysis by Niemelä and Dingemanse's study (2018), as for hormone levels, Niemelä and Dingemanse found a weak positive correlation between body size and boldness traits. However, in a species-specific case the results could be different. In fact in (Behrens et al., 2020), the correlation between size and boldness in round gobies resulted to be negative. We found a small significant effect of total length was detected on centre use, both before and after the strike, where larger fish were less responsive to the predator strike, and so slightly bolder. Finally, we detected a significant effect of total length on freeze time, where smaller fish spent less time frozen overall, but more after the predator strike. From these two results, it seems like larger fish are generally less responsive to a predator strike. This could be because they were already less active in the pre-strike period, or maybe because their size makes them less vulnerable to predation. However, even if the fish closer to the invasion front are larger, no population effect was detected.

We found no correlations between physiological and behavioural variables. This is in contrast with the result from meta-analysis performed by Niemelä and Dingemanse (2018). In their study, Niemelä and Dingemanse demonstrated that stress coping variables such as metabolic rate, hormone levels, body weight and/or size were weakly correlated to aggression, boldness, exploration, and activity in animals. Notably, these are the result of a meta-analysis of many studies, and outcomes of single studies may deviate. Studies focused on single species have shown no relationship between stress coping styles (e.g., cortisol level in the blood) and behavioural responses, e.g. in rainbow trouts in acute confinement stress and novel object response (*Oncorhynchus mykiss*; Gesto, 2019; Thomson et al., 2011) and seabream exposed to air (*Sparus aurata*, famred; Höglund et al., 2020). Therefore, the belief that physiological and behaviour responses in fish are correlated is slowly fading over time. Our results suggest that this correlation is absent in round gobies too.

In summary in invasive round gobies, population differences, body size and body condition all appear to be factors influencing boldness/risk-taking behaviour, but effects are different depending on the specific behavioural measure being used to measure boldness. Fish closer to the invasion front showed less repeatable behavioural responses than the ones from the older population. Exposure to conspecific chemical cues resulted in an increase of cortisol concentration in blood, but with no differences in responses between the two populations with different invasion histories. Finally, no correlation between behavioural responses and physiological responses was detected, and only a small effect of condition on centre use was found. This study further highlights the importance of among-individual behavioural variation to the invasion process of the round goby, and specifically identifies boldness in a predator response context as a key behavioural difference in two populations along the species' Baltic Sea invasion path. Despite this, physiological variation was not identified as factors directly driving personality trait variation within or between these two populations. Therefore, the processes that underpin personality variation in the round goby, and the processes driving their ongoing invasions in northern Europe and beyond are valuable topics for future research.

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6. Author Contributions (CRediT taxonomy)

Andrea Galli: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing

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7. Data Availability Statement

All data and code used (including data processing, preparation, analysis and presentation) are available at the Open Science Framework (https://osf.io/fb8nz/, doi:10.17605/OSF.IO/FB8NZ)

8. Supporting Information

S1. Physical composition of experimental populations

- Fig. S1, condition, length and weight comparisons between populations.

S2. Correlation Matrices

- Table S1, correlation matrix, Søvang.
- Table S2, correlation matrix, Grønsund

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Tables

Table 1. Behavioural variables recorded from predator response experiments, including the period they were collected in, and the distributions/transformations used in analysis.

	Phase	**Distribution**	**Description**
Activity (mm)	*pre-strike/ post-strike*	Gaussian (*sqrt(x) transformed*)	Total distance moved by the fish during each 5 min period. Greater movement can represent increased boldness/ risk-taking.
Centre use (s)	*pre-strike/ post-strike*	Poisson	The number of seconds spent more than 5 cm from the arena's edges during each 5 min period. Spending more time in central/exposed areas can represent increased boldness/ risk-taking.
Freezing time (s)	*pre-strike/ post-strike*	Gaussian (*sqrt*(t_{max}-*x*) *transformed*)	Total time frozen during each 5 min period. Freezing event were recorded within ToxTrac as periods of greater than 3 s, where the fish's movement was below 30mm. The minimum speed to be considered mobile was 5 mm/s. More time spend frozen can represent lower boldness/ risk-taking behaviour.
Latency to first freezing (s)	*post-strike*	Binomial (1 = freeze < 9 s) 0 = freeze > 9 s)	Fish that were quick to freeze those with a latency < median latency to first freezing post-strike (i.e. approx 9s). Freezing more quickly in response to a predation cue can represent lower boldness/ risk-taking.
Latency to resume movement (s)	*post-strike*	Binomial (1 = recovery > 30 s, 0 = recovery < 30 s)	Fish that were quick to resume movement following a freezing event were those with a latency < median latency (i.e. approx. 30s). Based on our assessment of freezing responses from videos, freezing events after 20s appeared unrelated to the strike, so fish that did not freeze within 20s were also scored as 0. Resuming normal behaviour more quickly after an exposure to an acute predator cue is considered to represent higher boldness/risk-taking.

Table 2. Physiological variables taken from blood and brain samples.

	Distribution	Description
Cortisol (ng/mL)	Gaussian (*$\log_e(x)$ transformed*)	The main glucocorticoid hormone in teleost fish, produced and released upon activation of the HPI (hypothalamus-pituitary-interrenal) axis. Plasma cortisol levels are used as primary indicator of stress.
Lactate (mM)	Gaussian (*$\log_e(x)$ transformed*)	Product of tissue anaerobic metabolism, used as indicator of behavioral activation during stress.
Dopaminergic mass ratio (%)	Gaussian (*$\log_e(x)$ transformed*)	Mass ratio in the telencephalon: DOPAC/dopamine, used as an indirect indicator of dopaminergic neuron firing.
Serotonergic mass ratio (%)	Gaussian (*$\log_e(x)$ transformed*)	Mass ratio in the telencephalon: 5HIAA/serotonin, used as an indirect indicator of serotonergic neuron firing.

Table 3. Repeatability estimates as a measure of behavioural consistency for each behavioural variable measured, including: among-individual variation in both populations as a proportion of total variance ($\mathrm{ICC}_{\mathrm{Raw}}$); the proportion of among-individual variance excluding population-level variation ($\mathrm{ICC}_{\mathrm{Adjusted}}$); and estimates within the older ($\mathrm{ICC}_{\mathbf{Grønsund}}$) and more recently established ($\mathrm{ICC}_{\mathbf{Søvang}}$) populations. Values in bold represent significantly non-zero coefficients of repeatability.

Variable	**Phase**	**$\mathrm{ICC}_{\mathrm{Raw}}$**	**$\mathrm{ICC}_{\mathrm{Adjusted}}$**	**$\mathrm{ICC}_{\mathrm{Grønsund}}$**	**$\mathrm{ICC}_{\mathrm{Søvang}}$**
Activity (mm)	*pre-strike*	**0.47 [0.25, 0.61]** **($p < 0.001$)**	**0.42 [0.17, 0.63]** **($p < 0.001$)**	**0.58 [0.35, 0.75]** **($p < 0.001$)**	0.28 [0, 0.59] ($p = 0.085$)
	post-strike	**0.39 [0.2, 0.58]** **($p < 0.001$)**	**0.26 [0.06, 0.51]** **($p = 0.005$)**	**0.42 [0.11, 0.62]** **($p = 0.008$)**	0.2 [0, 0.47] ($p = 0.143$)
Centre use (s)	*pre-strike*	**0.54 [0.29, 0.72]** **($p < 0.001$)**	**0.54 [0.33, 0.68]** **($p < 0.001$)**	**0.36 [0, 0.59]** **($p = 0.032$)**	**0.67 [0.27, 0.81]** **($p < 0.001$)**
	post-strike	**0.59 [0.43, 0.7]** **($p < 0.001$)**	**0.57 [0.36, 0.77]** **($p < 0.001$)**	**0.34 [0.06, 0.6]** **($p = 0.035$)**	**0.74 [0.47, 0.86]** **($p < 0.001$)**
Freezing time (s)	*pre-strike*	**0.39 [0.2, 0.55]** **($p = 0.001$)**	**0.34 [0.14, 0.49]** **($p = 0.002$)**	**0.48 [0.17, 0.69]** **($p = 0.003$)**	0.21 [0, 0.5] ($p = 0.154$)
	post-strike	**0.44 [0.21, 0.61]** **($p < 0.001$)**	**0.3 [0.1, 0.5]** **($p = 0.001$)**	**0.47 [0.15, 0.67]** **($p = 0.003$)**	0.23 [0, 0.53] ($p = 0.114$)
Latency to first freezing (s)	*post-strike*	0 [0, 0.17] ($p = 0.5$)	0 [0, 0.09] ($p = 1$)	0 [0, 0.22] ($p = 1$)	0.01 [0, 0.26] ($p = 0.456$)
Latency to resume movement (s)	*post-strike*	0 [0, 0.18] ($p = 0.5$)	0 [0, 0.17] ($p = 0.5$)	0 [0, 0.21] ($p = 0.5$)	0 [0, 0.34] ($p = 0.5$)

Table 4. Effects of SL, condition factor and population of behavioural responses. Interactions between these factors and period represent the effect of these factors on the change in behaviour between the *pre-strike* and *post-strike* periods, i.e, effects on their response to the acute predator cue. Positive effects on activity represent increased movement (i.e. higher boldness), positive effects on centre area use represent increased use of the central/exposed area (i.e. higher boldness), and positive effects on freeze time represent reduced time spend frozen (i.e. higher boldness). Fixed effect factors/interactions with a non-zero effect are highlighted in bold. Conditional R^2 (R^2_{Con}) represents the proportion of variance explained by random and fixed effects within the model, and Marginal R^2 (R^2_{Mar}) represents the proportion of variance explained by the fixed effects.

Model						$\mathbf{R^2_{Con}}$	$\mathbf{R^2_{Mar}}$
- factors	*Estimate [95% CI]*	*S.E.*	*df*	*t/z*	*P*		
Activity$_{(\textit{sqrt(x)}, \text{ gaussian lmer model})}$						0.583	0.133
- *intercept*	75.48 [21.05, 25.83]	7.39	4.01	10.21	< 0.001***		
- *period*	-0.38 [-21.02, -5.84]	4.31	180.86	-0.09	0.93		
- *length*$_{\textit{Z-scaled}}$	**-13.43 [59.55, 91.4]**	**3.93**	**85.36**	**-3.42**	**< 0.001*** **		
- *condition*$_{\textit{Z-scaled}}$	-1.36 [-8.78, 8.02]	3.39	87.66	-0.4	0.689		
- *population*$_{\textit{Søvang}}$	-2.77 [-7.93, 5.19]	7.74	85.58	-0.36	0.722		
- *period*length*$_{\textit{Z-scaled}}$	**10.12 [-17.73, 12.2]**	**3.3**	**180.86**	**3.06**	**0.003** **		
- *period*condition*$_{\textit{Z-scaled}}$	2.62 [3.68, 16.57]	2.9	180.86	0.9	0.367		
- *period*population*$_{\textit{Søvang}}$	**-15.16 [-3.04, 8.28]**	**6.58**	**180.86**	**-2.3**	**0.022***		
Centre use$_{(\text{count, poisson glmer model})}$						0.984	0.038
- *intercept*	3.51 [2.89, 4.12]	0.31	n/a	11.38	< 0.001		
- *period*	**-0.37 [-0.42, -0.31]**	**0.03**	**n/a**	**-13.04**	**< 0.001*** **		
- *length*$_{\textit{Z-scaled}}$	0.03 [-0.42, 0.49]	0.23	n/a	0.14	0.888		
- *condition*$_{\textit{Z-scaled}}$	-0.12 [-0.52, 0.27]	0.2	n/a	-0.62	0.535		
- *population*$_{\textit{Søvang}}$	-0.59 [-1.51, 0.3]	0.45	n/a	-1.31	0.191		
- *period*length*$_{\textit{Z-scaled}}$	**0.05 [0.01, 0.09]**	**0.02**	**n/a**	**2.49**	**0.013***		
- *period*condition*$_{\textit{Z-scaled}}$	**0.07 [0.03, 0.11]**	**0.02**	**n/a**	**3.61**	**< 0.001*** **		
- *period*population*$_{\textit{Søvang}}$	0.03 [-0.05, 0.12]	0.04	n/a	0.7	0.481		
Freeze time$_{(\text{sqrt(tmax-x), gaussian lmer model})}$						0.591	0.121
- *intercept*	9.93 [2.86, 3.51]	1.15	2.64	8.65	0.005**		
- *period*	-0.08 [-2.6, -0.53]	0.59	181.99	-0.13	0.897		
- *length*$_{\textit{Z-scaled}}$	**-1.56 [7.37, 12.48]**	**0.54**	**85.62**	**-2.92**	**0.004** **		
- *condition*$_{\textit{Z-scaled}}$	-0.3 [-1.22, 1.07]	0.46	87.9	-0.65	0.52		
- *population*$_{\textit{Søvang}}$	-0.72 [-1.2, 0.6]	1.06	85.83	-0.68	0.497		
- *period*length*$_{\textit{Z-scaled}}$	**1.02 [-2.77, 1.32]**	**0.45**	**181.99**	**2.27**	**0.024***		
- *period*condition*$_{\textit{Z-scaled}}$	0.58 [0.14, 1.9]	0.39	181.99	1.46	0.146		
- *period*population*$_{\textit{Søvang}}$	-1.67 [-0.19, 1.34]	0.89	181.99	-1.86	0.064		

Table 5. Effects of fish length, condition factor, population and alarm cue treatment on physiological variables. Interactions between population and treatment are used to test if the response to an alarm cue (i.e. *sham control* versus *cue* groups) differs between populations (i.e. Grønsund and Søvang). Fixed effect factors/interactions with a non-zero effect are highlighted in bold. Conditional R^2 (R$^2_{\text{Con}}$) reprsents the proportion of variance explained by random and fixed effects within the model, and Marginal R^2 (R$^2_{\text{Mar}}$) represents the proportion of variance explained by the fixed efffects.

Model *- factors*	*Estimate [95% CI]*	*S.E.*	*df*	*t/z*	*P*	**R$^2_{\text{Con}}$**	**R$^2_{\text{Mar}}$**
Cortisol$_{(log(x),\ \text{gaussian lmer model})}$						0.223	0.132
- intercept	2.9 [2.4, 3.4]	0.27	5.95	10.56	< 0.001***		
- treatment	**0.51 [0.06, 0.95]**	**0.23**	**43.83**	**2.16**	**0.036***		
- length$_{Z\text{-scaled}}$	0.01 [-0.26, 0.27]	0.14	45.48	0.05	0.958		
- condition$_{Z\text{-scaled}}$	-0.07 [-0.31, 0.16]	0.12	46.74	-0.58	0.562		
- exposure time	-0.2 [-0.66, 0.27]	0.24	44.31	-0.81	0.42		
- population$_{Søvang}$	-0.34 [-1.01, 0.35]	0.37	4.8	-0.92	0.403		
*- treatment*population*$_{Søvang}$	-0.25 [-0.91, 0.43]	0.35	43.95	-0.71	0.481		
Lactate$_{(log(x),\ \text{gaussian lmer model})}$						0.085	0.081
- intercept	-1.82 [-2.08, -1.57]	0.14	14.54	-13.18	< 0.001***		
- treatment	-0.07 [-0.35, 0.21]	0.15	46.05	-0.46	0.644		
- length$_{Z\text{-scaled}}$	0.09 [-0.07, 0.26]	0.09	46.8	1.05	0.3		
- condition$_{Z\text{-scaled}}$	-0.07 [-0.22, 0.08]	0.08	47.99	-0.9	0.372		
- exposure time	0.27 [-0.03, 0.56]	0.16	46.58	1.7	0.095		
- population$_{Søvang}$	-0.15 [-0.47, 0.18]	0.18	46.26	-0.83	0.41		
*- treatment*population*$_{Søvang}$	-0.01 [-0.43, 0.41]	0.23	46.12	-0.03	0.974		
Dop. mass ratio$_{log(x),\ \text{gaussian lmer model})}$						0.274	0.155
- intercept	1.39 [1.09, 1.7]	0.17	5.75	8.38	< 0.001***		
- treatment	-0.05 [-0.3, 0.2]	0.13	43.97	-0.41	0.681		
- length$_{Z\text{-scaled}}$	**-0.21 [-0.36, -0.06]**	**0.08**	**45.32**	**-2.73**	**0.009****		
- condition$_{Z\text{-scaled}}$	-0.06 [-0.19, 0.08]	0.07	46.4	-0.84	0.402		
- exposure time	-0.22 [-0.48, 0.04]	0.14	44.36	-1.63	0.109		
- population$_{Søvang}$	0.29 [-0.12, 0.71]	0.22	4.79	1.3	0.254		
*- treatment*population*$_{Søvang}$	-0.06 [-0.44, 0.31]	0.2	44.06	-0.3	0.766		
Ser. mass ratio$_{log(x),\ \text{gaussian lmer model})}$						0.328	0.302
- intercept	2.95 [2.84, 3.06]	0.06	7.37	47.98	< 0.001***		
- treatment	0.03 [-0.09, 0.15]	0.06	43.96	0.48	0.632		
- length$_{Z\text{-scaled}}$	**-0.12 [-0.19, -0.05]**	**0.04**	**46.26**	**-3.31**	**0.002****		
- condition$_{Z\text{-scaled}}$	**-0.09 [-0.15, -0.03]**	**0.03**	**47.67**	**-2.83**	**0.007****		
- exposure time	0.03 [-0.09, 0.15]	0.06	44.65	0.52	0.605		
- population$_{Søvang}$	-0.03 [-0.18, 0.12]	0.08	5.46	-0.4	0.708		
*- treatment*population*$_{Søvang}$	-0.04 [-0.21, 0.14]	0.09	44.12	-0.41	0.687		

Figure Legends

Figure 1. Study sites in on the eastern coast of Denmark, and inset, within the broader south-east Baltic Sea region. Round gobies were first recorded in the areas around Grønsund (pink) and Søvang (green) in approximately 2011 and 2016, respectively, 10 and 5 years prior to sampling. Grønsund is located directly north of Guldborgsund, the first area of inner Danish waters invaded by round gobies in 2009. Søvang is located just south of Copenhagen, nearer to the DTU Lyngby laboratory in northern Copenhagen. At present, DTU Lyngby is approximately level with the northern edge of the round goby's invasion front up the eastern coast of Denmark.

Figure 2. Behavioural arenas (a) aerial view, and (b) front view. In (a), the grey area represents the 5cm edge zone used to measure centre area use behaviour, and the parallel lines across the arenas represent the clear acrylic tube used to suspend the bolt above the centre of the arena. The fishing line runs through the length of the tube and can be released and retracted by the observer. The bolt-release mechanism sits on top of a frame used to hold the polystyrene walls around the arena (dotted lines), and to hold the camera in position, so that the bolt drops approximately 80cm to break the water surface before retraction.

Figure 3. Activity response in Experiment 1, associated with (a) population, and (b) SL. In (a) fish from the newer site Søvang (est. 2016) appear to reduce their activity levels following the predator cue more than Grønsund (est. 2011) fish. In (b) smaller fish were more active overall but reduced their activity levels more relative to larger fish following the predator cue. In boxplots horizontal bars, boxes and vertical lines represent the median, interquartile range, non-outlier range, respectively.

Figure 4. Blood cortisol response to a chemical alarm cue. The dotted line represents the baseline measurements for cortisol in the *baseline control* treatment group. Concentrations are significantly higher in the *cue* treatment group, but there is no difference between populations. In boxplots horizontal bars, boxes and vertical lines represent the median, interquartile range, non-outlier range, respectively.

Figures

Figure 1

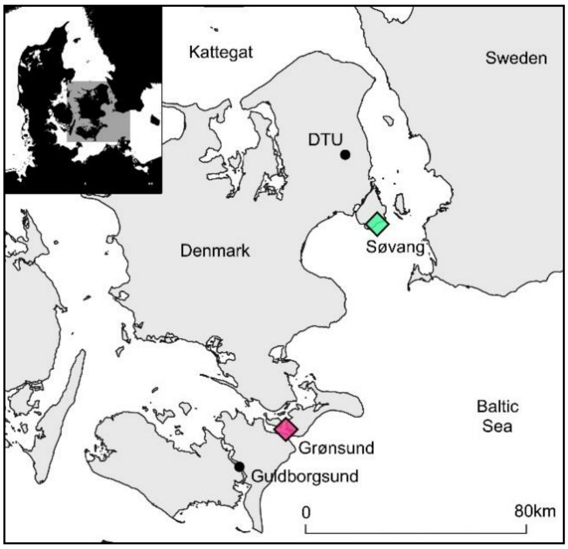
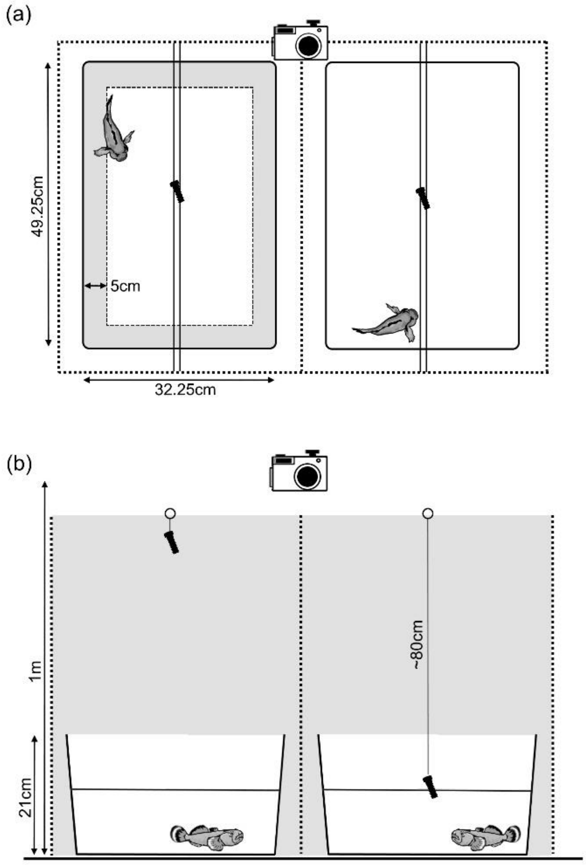


Figure 2



796 *Figure 3*

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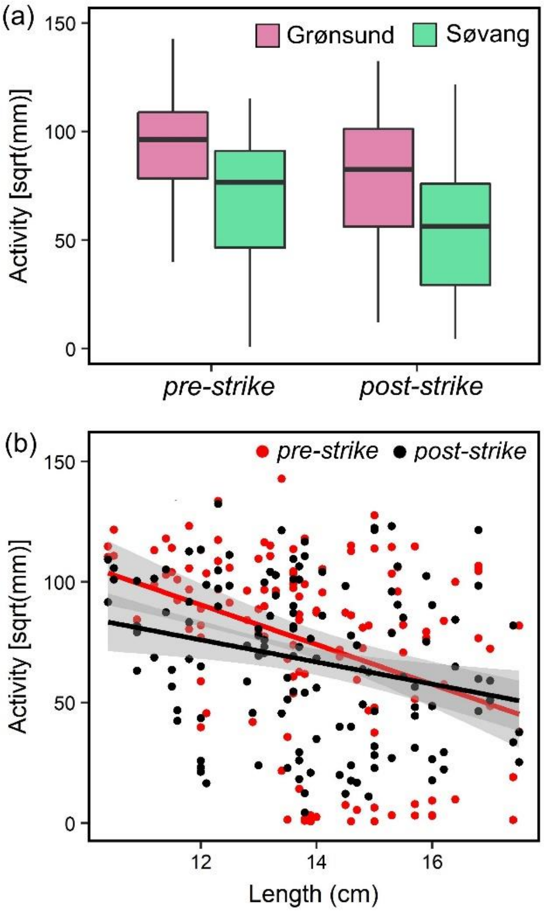
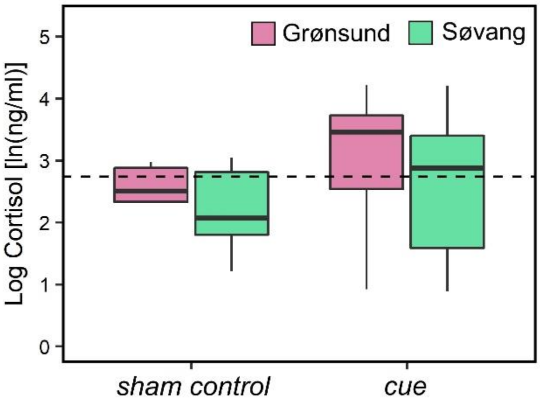


Figure 4


Grønsund
Søvang
Log Cortisol [ln(ng/ml)]
0
1
2
3
4
5
sham control
cue