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Developing species specific Heat Shock Protein (HSP) sequences for the invasive round goby to assess their environmental stress in the Baltic Sea and further spread to the North Sea

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Introduction

The round goby (*Neogobius melanostomus*) has been a very successful invader in both the Baltic Sea and Laurentian Great lakes as well as within the major rivers in Europe (Kornis et al. 2012). Within the Baltic Sea range, the species has been detected in most sub-regions including the most northern parts (Kotta et al. 2016). The wide invasive range, representing variable abiotic conditions, thus proves that the species is thriving in both fresh and brackish waters. There are speculations, however, that further range expansion of the round goby into the Danish straits and the west coast of Sweden may be limited by the prevailing high saline environment. Despite the fact that the round goby is capable of tolerating a wide range of abiotic conditions, including relatively saline water (Charlebois et al. 1997, Kornis et al. 2012), the suboptimal conditions may result in decreased individual growth or low reproduction potential in the high salinity areas.

Recent salinity tolerance experiments revealed that physiological performance was reduced at the highest salinities (30, and to some extent 25 PSU), as was survival, thus indicating that salinity may act as a barrier for further northward dispersal within the salinity gradient between the brackish Baltic Sea and the full oceanic North Sea (Behrens et al 2017). We hypothesize that this increased physiological stress can be measured by quantifying production of stress-induced proteins.

In fish there are a number of stress responses (or indicators) that can be broadly categorized as primary, secondary and tertiary. Primary indicators involve hormonal responses such as impacts in serum hormone levels (cortisol and adrenaline). Secondary indicators include more metabolic changes such as in glucose metabolism and hematological parameters. Secondary indicators also include changes in the expression of several proteins including heat shock proteins (HSP's). Tertiary responses include more severe responses, including cell necrosis (see Deane and Woo 2011 and references therein). These responses are not mutually exclusive but indicate that changes in HSPs are good indicators of stress and therefore useful as biomarkers (Deane and Woo 2011).

Many HSP's are expressed in cells constitutively, but when encountering stress and disruption in normal cellular processes, cells can amplify the production of a group of proteins belonging to the heat shock protein (HSP) families. The ultimate aim of this response is to maintain homeostasis within the cells. Members of these different HSP families are grouped according to their molecular size and they perform varying roles in the cell. Of the HSP families, HSP70 has been most widely studied as a biomarker of stress as they are necessary for translocation and protein folding and demonstrated to be upregulated in fish during stress. The HSP70 family is coded by two different genes, a constitutive type

(hsc70) and an inducible type (hsp70), and both of these genes encode proteins that play key roles in the cell as molecular chaperones. In cells, hsc70 remains unchanged or slightly upregulated upon exposure to stress whereas hsp70 is highly induced. Furthermore, HSP 70 regulation has been linked to salinity stress in fish (Tine et al. 2010) and the genes (hsp70 and hsc70) are well known (Diane and Woo 2005).

Measuring HSP70 expression in fish requires knowledge on the species-specific sequences of the genes in question, which are currently lacking for the round goby. In this study, we proposed to identify the species-specific sequences of HSP70 genes in round goby, which will allow assessing how salinity stress impacts round gobies at cellular levels.

Material and methods

Developing primers

Based on other similar studies, we chose to experiment with candidate gene approach (sensu Larsen et al. 2008). First we identified whether HSP 70 sequences would be available for closely related species. The open access databases (such as ncbi and uniprot) were searched for available sequences for 1) Gobius-species, or 2) any goby species, or 3) any perciformes species. The available sequences for gobiids were prioritized in primer selection. The HSP 70 sequences were obtained for the flounder (*Platichthys flesus*), the black goby (*Gobius niger*) and leopard grouper (*Mycteroperca rosacea*). For assessing potential increase in HSP 70 production, a reference gene that does not react to stress was also needed. One of the most commonly used genes for this purpose is the EF1 (elongation factor alpha 1). Sequences for EF1 were available for the flounder (*Platichthys flesus*), perch (*Perca fluviatilis*) and goby (*Pomatochistus* sp.). The obtained sequences were copied in FASTA format and imported into BioEdit 7.1.9 Sequence Alignment Editor (Hall 2013). The sequences were then aligned in the software using the ClustalW Multiple Alignment tool and the most conserved regions (e.g., sequences were similar for all the species) were chosen to be primers. Primers were chosen to be 18-21 bp in length and contain similar amounts of G-C pairs (resulting in similar annealing temperatures). The chosen primers were tested using ncbi's Primer Blast tool to assure for functional primers. Three best primer pairs for both HSP 70 and EF1 were tested with single tube qPCR using mRNA extracted from round gobies.

Testing the primers

The tissues for primer testing was extracted from two round gobies caught in Helsinki, Finland on December 27th. The gobies were both males and approximately 11 cm in size (TL). Tissue was extracted from brain, muscle and liver and each tissue sample was divided into 2-3 eppendorf tubes for mRNA extraction. The mRNA was extracted using Qiagen RNeasy mini Kit following the instructions carefully. The resulting RNA was stored in RNase free water and -20 C. According to Nanodrop analysis (spectrophotometry), the most RNA was yielded from brain tissue and the yield varied greatly among the other tissue types (muscle and liver). The concentration of the mRNA in the samples was needed for the cDNA reactions.

For testing of the functionality of the chosen primers with qPCR, the mRNA had to be converted to cDNA. This was done using Affinity Script qPCR cDNA synthesis kit and following the instructions carefully. The kit provides two sets of primers (Oligo dT and random) for the reaction and both primers were tested in the synthesis.

The qPCR was conducted for all samples using BioRad iQ SYBR Green super mix and the cycle was chosen based on other similar qPCR studies (95 C for 3 min, 95 C for 10 sec, 58 C for 20 sec and 72 C for 30 sec). The cycle was repeated 40 times. After the reaction, melt curves were produced for detecting the specificity of the reaction.

Measuring HSP70 production in different salinities

For the real life application of the method, the round gobies were exposed to three different salinities in experimental conditions: freshwater, 10 and 30. For the experiment, round gobies (approximately 120 fish) were caught with fyke nets in the brackish water estuary Guldborgsund (salinity 10 PSU) in the western Baltic Sea. They were transported to DTU Aqua, Denmark, and held in freshwater (FW; 0 PSU) for 4 weeks until the experimental acclimation began. Fish were fed to satiation three times a week with small commercial high-nutrition fish feed pellets on which they fed well after 1-2 weeks of customization. Altogether 60 adult fish of comparable size were allocated into two separate acclimation tanks containing 200 L 10 PSU water, with 30 fish in each tank. 30 fish of comparable size were kept in a separate tank with FW. After one week at 10 PSU, the salinity in one of the tanks was increased gradually by 5 PSU per week to obtain a treatment salinity of 30 PSU (i.e taking a four-week period). All fish were subsequently kept at their treatment salinity (0, 10 and 30 PSU) for an additional four weeks.

At termination of the experiment, duplicates of gill and liver samples were rapidly dissected from 12 euthanized fish (overdose of MS-222) from each salinity treatment. Samples were snap frozen in liquid nitrogen and subsequently stored at -80°C before RNA extraction took place following the same protocol as described above.

Results and Discussion

Testing the primers

The results from qPCR run show that one primer pair for each gene functioned well and resulted in clear product (Figure 1). The two other primers resulted in significantly higher primer-dimer production (where primer begins to copy the primer instead of the target sequence) and their usability may be low. In other words, we were able to identify functional primers for HSP70 gene as well as reference gene EF1 that can be used in assessing physiological stress in the round goby. The melt curves for the best performing primers peak only at one temperature indicating that only one product was obtained for each primer (good specificity). The darker green lines are control samples showing primer multiplication (Figure 2).

The performance of the primers was improved further by modifying the PCR cycle in the further application. In the testing phase we used a “standard cycle” and average annealing temperature (58 C) across all primers. However, the optimum annealing temperature for each primer would have been different: for HSP70 _1 primer pair the optimum annealing temperature would have been 57.3 C and for EF1_1 61.8 C. Especially for the latter primer pair the temperature was very much lower than the optimum, which probably impacted the results.

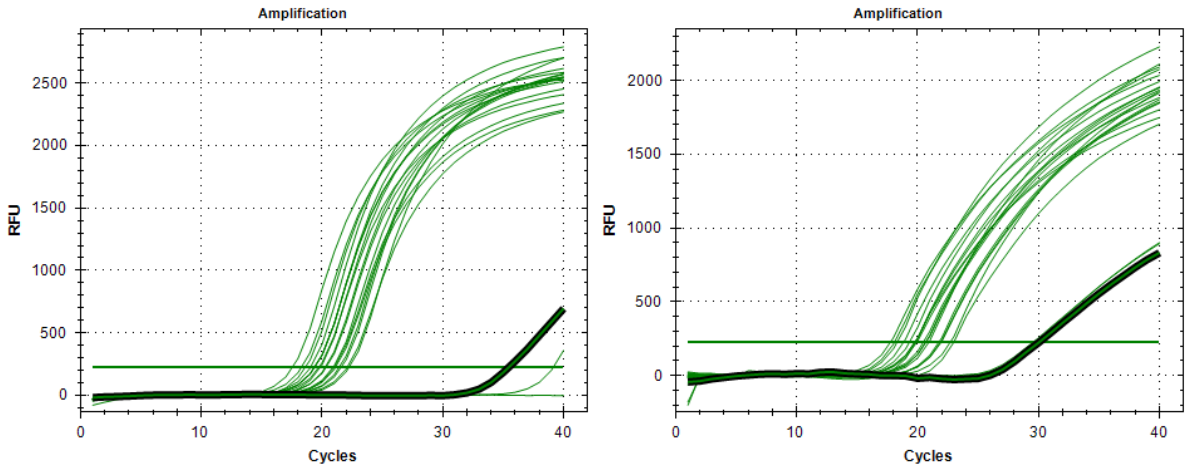

Figure 1 The qPCR yield for the primers HSP 70_1 (left) and EF1_1 (right). Darker green lines are control

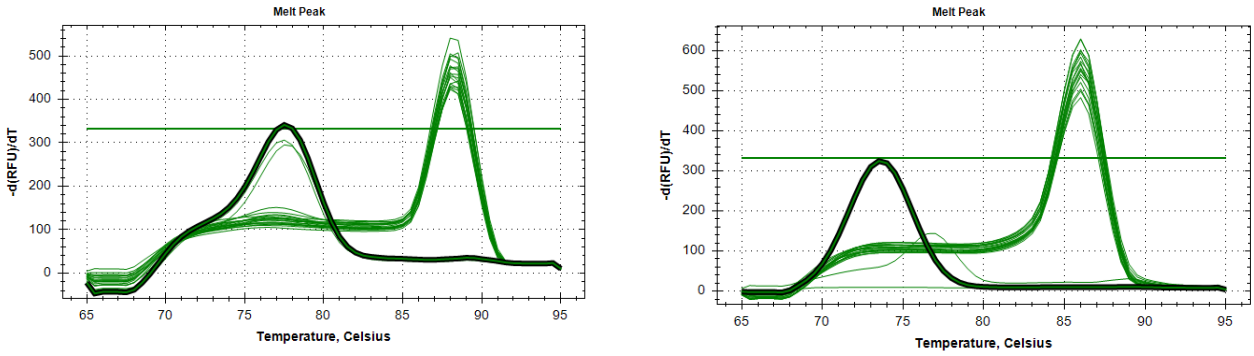

Figure 2 Melt curves indicating specificity of the qPCR product for HSP70_1 (left) and EF1_1 (right) primers. Darker green lines are control samples.

HSP 70 activity in different salinities

The samples obtained from the salinity exposure experiments were analyzed in a similar manner as described above. The qPCR cycle was optimized for each primer pair therefore resulting in improved efficiency. The results show that the round gobies had in fact elevated HSP 70 production when exposed to fresh water and lower HSP 70 production when in salinity treatments. Based on the results, the optimal (e.g., least stressful) salinity for the round gobies was be 10, which is the salinity the fish was caught. Similar results have been observed in lake trout, where the rearing salinity has great impact on the salinity tolerance in their young (Kissinger et al. 2017). Surprisingly, high salinity (30) appeared to stress the fish less than pure freshwater contrary to our expectations (Fig. 3). This may imply that the round gobies acclimated to moderate salinities (10) in the wild are capable for adapting to higher salinities as well. Behrens et al. (2017) also showed that survival in salinity 30 can be quite high (60%), although fish showed decreased aerobic scope (related to fitness-related parameters) potentially impacting their fitness in the wild. Furthermore, we only measured the HSP 70 production (indication of stress) in adult fish. Further spread to the North Sea will probably be determined by combination of the tolerance of the most vulnerable life stages and the biotic interactions (e.g., competition/predation) in the receiving habitats.

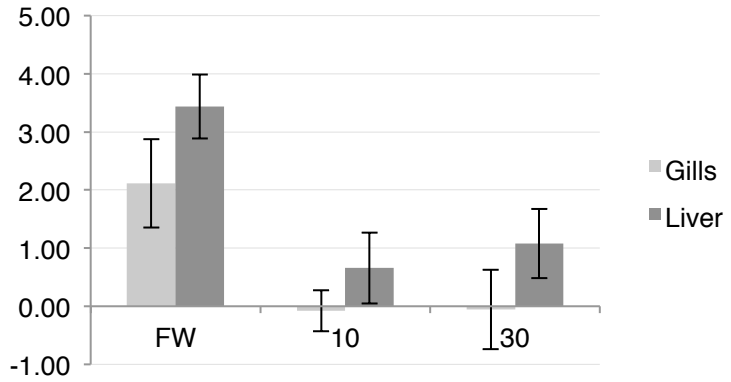

Figure 3. The HSP 70 production related to housekeeping gene (EF 1) production under different salinity treatments.

Conclusions

1) The candidate gene approach resulted in functional primers for measuring salinity related stress in terms of HSP 70 production in the invasive round goby
2) Further testing of HSP 70 production of fish exposed to different salinities indicate that the fish from brackish conditions are less stressed in high salinity treatment (30) than in pure freshwater
3) Measurements of HSP 70 expression in the round goby show that the round gobies from moderate salinities are not very stressed in oceanic salinities and support the hypothesis that fish acclimated to moderate salinities may be capable for expanding their range to the North Sea.

Budget and funds used

Grant (60%)	**6563.59**	**49200.00**
Travel from Helsinki to Copenhagen for meeting	179.92	1338.50
Agilent cDNA conversion kit	303.18	2255.50
Oligomer primers	204.42	1520.70
Qiagen mRNA extraction kit	378.39	2815.00
Supplies and facility fees at the Uni. of Helsinki (2016)	461.79	3435.80
Salinity experiments and RNA sampling	7427.50	55253.56
RNA extraction and cDNA conversion	1972.00	14669.81
Subtotal	10927.20	81288.87
Grant (40%)	**4409.47**	**32800.00**
Supplies at the Univ. of Helsinki (2017)	47.00	349.62
Total funds used	10974.20	81638.49
Balance	-1.14	361.51

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