



SHORT COMMUNICATION

Oocyte hydration in round goby *Neogobius melanostomus* from the Gulf of Gdańsk: another invasive strategy?

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Summary The round goby (*Neogobius melanostomus*) is one of the most abundant non-native species in the Gulf of Gdańsk that use various strategies to increase fecundity and achieve invasive success. Oocyte hydration appears to be a specific osmotic adaptation of round goby's gametes for successful fertilization and hatching. The aim of this study was to demonstrate details of oocyte final maturation and hydration during the spawning-capable phase in round goby and also compare various indices such as 17 β -estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI) among oocyte final maturation stages. E_2 fluctuated according to the development of oocyte batches. HSI did not show significant differences throughout the oocyte maturation. GSI was an inaccurate estimator of oocyte maturation in round goby. This study, for the first time, shows that hydration does accompany oocyte final maturation in this species. These findings are important to the cognition of biology and the adaptive strategies of this invasive species to environmental factors and can be essential components of the protection and preservation of native living resources in the Gulf of Gdańsk.

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The round goby (*Neogobius melanostomus*) is a well-known, non-native species in the Baltic Sea and the Laurentian Great Lakes, probably transported to both ecosystems in ballast waters of ships from the Ponto-Caspian region (Crossman et al., 1992; Skóra and Stolarski, 1993). The first individuals of round goby in the Baltic Sea were observed in the Gulf of Gdańsk in 1990 (Skóra and Stolarski, 1993). Since then, round goby has become one of the most abundant species in the western part of the Gulf of Gdańsk and has successfully colonized other regions of the Baltic Sea (Sapota, 2012). The rapid expansion of this euryhaline, benthic species has motivated widespread research on life history, morphology, reproduction, habitat preferences, environmental tolerances, parasites and environmental effects (Kornis et al., 2012). Extremely aggressive, territorial behaviour, a fast genetic divergence and high adaptation to variability in environmental factors, have made round goby one of the most invasive species in the Baltic Sea (Björklund and Almquist, 2010; Ojaveer et al., 2015). The species' varied diet and reproductive strategy also contribute to the invasion success (Kornis et al., 2012; Ojaveer et al., 2015). In the Gulf of Gdańsk, high densities of *N. melanostomus* slightly shifted the area of occurrence and foraging of native flounder (*Platichthys flesus*), viviparous eelpout (*Zoarces viviparus*) and black gobies (*Gobius niger*), which live in the same or similar habitats as intruders (Jazdzewski and Konopacka, 2002). In this area of the Baltic Sea, round goby diet consists of 60% of mussels, which can severely impact native benthic invertebrate populations and may result in the competition for food with both juvenile and adult flounder (Puntila et al., 2018). On the other hand, *N. melanostomus* may also predate on small individuals of the flounder and eggs of commercially valuable fish such as Atlantic herring (*Clupea harengus*) (Puntila et al., 2018; Wiegleb et al., 2019). In consequence, round goby as a competitor for food and habitats of native demersal fish can reduce the number of their populations and disturb biodiversity of native ecosystems.

N. melanostomus, as with many species of the family Gobiidae, is a batch spawner, which means that females can spawn several times throughout the same spawning period, releasing ovulated eggs in batches (Dinh, 2018). In the Baltic Sea, females may lay eggs from two to four times in the same spawning period (Sapota, 2012). This spawning strategy can be seen as a successful invasive strategy to increase fecundity and the survival probability of offspring by spreading out hatch times to mitigate predation risk and increase the likelihood that some offspring hatch during favourable conditions (McEvoy and McEvoy, 1992). *N. melanostomus* is a fish species that exhibits male parental care. Prior to spawning, the male of round goby excavates a nest underneath a stone or other hard substratum. During spawning, the female and male repeatedly deposit eggs or spread sperm on the nest ceiling. After spawning, the territorial male guards the nest against predators and continuously ventilates the eggs containing developing embryos (Meunier et al., 2009).

Another strategy of round goby to increase fecundity and achieve invasive success appears to be oocyte hydration as a specific osmotic adaptation of gametes for successful fertilization and hatching. It should be mentioned that this adaptive mechanism is considered as one of the crucial steps in the evolutionary history of the vertebrates (Finn et al.,

2008). Fish oocyte hydration is a unique biological process that has been described as a marked increase in oocyte volume caused by the rapid uptake of fluid by the oocyte and the coalescence of yolk spheres and/or oil droplets (Cerdà et al., 2007; Milla et al., 2006). This process is based on the interplay between protein hydrolysis and ion accumulation to create an osmotic gradient, and aquaporins to regulate water influx (Cerdà et al., 2007; Fabra et al., 2006). The pre-ovulatory oocyte hydration is an important osmotic adaptation for the development of viable gametes when hypo-osmotic eggs are released into the hyperosmotic environment. What is more, this fluid influx ensures the physiological hydration of the embryos prior to the development of osmoregulatory organs (gill, kidney and gut) and maintains homeostasis (Wright and Fyhn, 2001). In the Gulf of Gdańsk, salinity varies from 7 to 12 PSU as a result of Vistula River water inflow and irregular inflows of saline water from the North Sea (Sztobryn et al., 1997). Therefore, euryhaline species such as round goby and their eggs can be exposed to different osmoregulatory challenges, on account of salinity changes in their habitats (Behrens et al., 2017). The adaptive strategy of euryhaline teleosts to the environmental salinity enables a switch from plasma hyperosmoregulation (environmental salinity <9 PSU) to plasma hypo-osmoregulation (environmental salinity >9 PSU) (Kültz, 2015). In turn, eggs' hydration of these teleosts protects against water loss to the hyperosmotic environment and against environmental ions diffusion into eggs, and the relatively low water content and low vitelline membrane permeability protect eggs against water influx and a continuous ion loss in the hypo-osmotic environment (Wright and Fyhn, 2001).

Although knowledge about *N. melanostomus*' reproductive cycle seems to be extensive, the presence of hydrated oocytes in round goby was only mentioned by Tomczak and Sapota (2006). However, so far, to the authors' knowledge, the histological analysis of oocyte hydration has not been presented and described in this species. The objective of this study was to demonstrate details of oocyte final maturation and hydration during the spawning-capable phase in invasive *N. melanostomus* and also compare various indices such as 17 β -estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI) among oocyte final maturation stages. In female fish, E_2 is responsible for the regulation of oocyte growth within the gonads and supports the hepatic synthesis and secretion of vitellogenin (Lubzens et al., 2010). Moreover, changes in the circulating E_2 reflect well the oocyte batch recruitment cycle in batch spawners (Mandich et al., 2004). HSI is a measure of the energy reserves of an animal, especially in fish. GSI is used to measure sexual maturity in relation to the ovaries' development.

Adult females of *N. melanostomus* ($n = 50$) were caught in the Gulf of Gdańsk (Gdynia, Poland) during the spawning-capable phase (April–August). Fish were held in tanks with aerated water of salinity 8 ± 0.5 PSU (\pm S.E.), at the Institute of Oceanology Polish Academy of Sciences (Sopot, Poland) for one week before the experiments. Fish were maintained under a lighting regime and temperature according to conditions mimicking the natural environment. Fish were fed frozen mussels *ad libitum*. All experiments on fish complied with the EC Directive 2010/63/EU for animal experiments and with the guidelines (Resolution No 19/2012) of the Local Ethics Committee on Animal Experimentation. At the time of

sampling, the fish were anaesthetized in bicarbonate-buffered MS-222 water solution (50 mg L^{-1}) (Sigma-Aldrich) and blood samples were collected by cardiac puncture. Blood samples were centrifuged at 3000 g for 10 min and stored at -70°C prior to E_2 analysis. After transection of the spinal cord, their gonads and livers were collected, examined morphologically and then the ovaries underwent histological analysis. GSI was calculated as gonad weight/body weight $\times 100$. HSI was calculated as liver weight/body weight $\times 100$. The maturity stage of the ovary was determined according to the scale described by Tomczak and Sapota (2006). The diameter of fresh oocytes was measured for each ovary under a stereomicroscope (MSZ800/100T; mikroLAB) connected to a digital colour camera (HDCE-X3; mikroLAB) using Scopelimage 9.0 imaging software (Novel Optics). For histological analysis, ovaries were fixed in 4% buffered formalin, dehydrated, embedded in paraffin, cross-sectioned at $6 \mu\text{m}$ slices and stained using the standard haematoxylin and eosin protocol. Slides were examined with a light microscope (Leica DM500; Leica Microsystems GmbH) connected to a digital colour camera (Leica ICC50 HD; Leica Microsystems GmbH). The developmental stage of ovaries was determined according to the terminology proposed by Brown-Peterson et al. (2011). E_2 levels were determined using a Spectria Estradiol radioimmunoassay kit (Orion Diagnostica) according to the method described previously by Kalamarz-Kubiak et al. (2017). The radioactivity in each tube was measured for 1 min using a gamma counter (Wallac Wizard 1470; PerkinElmer Life Science). The detection limit of the assay was 38 pmol L^{-1} . The intra-assay coefficient of variation was 6.5% . The interassay variation was not determined because all samples were measured in the same assay. Statistical analysis was performed using STATISTICA 7.1 software (StatSoft). A one-way ANOVA followed by Spjotvoll and Stoline's *post hoc* test for unequal numbers of cases was used to compare the three indices (E_2 , GSI and HSI) among different stages of oocyte maturation. $P < 0.05$ was considered significant. The results of the statistical analysis are presented in Fig. 1.

In this study, the following stages of oocyte final maturation and ovulation in *N. melanostomus* were identified using

histological analysis: 1 – germinal vesicle migration (GVM); 2 – late germinal vesicle migration; early hydration (late GVM; early HO); 3 – germinal vesicle breakdown; middle hydration (GVBD; middle HO); 4 – final hydration (final HO); 5 – ovulation; post-ovulatory follicles (OV; POFs) (Fig. 2). It is apparent that as in other fish species, the oocyte maturation in *N. melanostomus* begins with GVM to the animal pole. During the GVM stage in this species, the nucleus (germinal vesicle; GV) is situated between the centre of the oocyte and half-way to the animal pole and oil droplets fuse into single oil globules while yolk globules begin to fuse to each other at the vegetal pole of the oocyte (ST-1) (Fig. 2). In *N. melanostomus* in the present study, oocyte hydration is observed precisely during late GVM, when oil vesicles are coalescing and yolk granules fuse into plates (ST-2) (Fig. 2). It has been shown that hydration in different fish species, including batch spawners, may occur during different stages of final oocyte maturation but is most pronounced between the start of final maturation and GVBD (Fabra et al., 2006; Skoblina, 2010). During the GVBD stage in *N. melanostomus*, the nucleus is disintegrated, hydration is continued and yolk is fused into a homogeneous mass but the cytoplasm and the cortical alveoli are restricted to a thin peripheral layer (ST-3) (Fig. 2). The final hydration in this species is characterized by fully clear ovoplasm and whole fusion and coalescence of the yolk mass that becomes homogeneous and fluid (ST-4) (Fig. 2), as has often been noted in the literature (for reviews: Fabra et al., 2006; Kjesbu et al., 1996; Lubzens et al., 2017). According to Brown-Peterson et al. (2011), the ovulation stage is characterized by the presence of POFs, not hydrated oocytes (ST-5) (Fig. 2).

It should be mentioned here that not only histology is suitable for determining the reproductive maturity of round goby and stage of gonadal development but also the macroscopic analysis of gonadal morphology is a common and sufficient method for the general differentiation of gonadal maturity stages (Ferreri et al., 2016; Tomczak and Sapota, 2006; Tomkiewicz et al., 2003).

It has been indicated that hydration of oocytes during maturation occurs in a less pronounced way in fish that produce demersal eggs or not floating ones, like *N. melanostomus* than

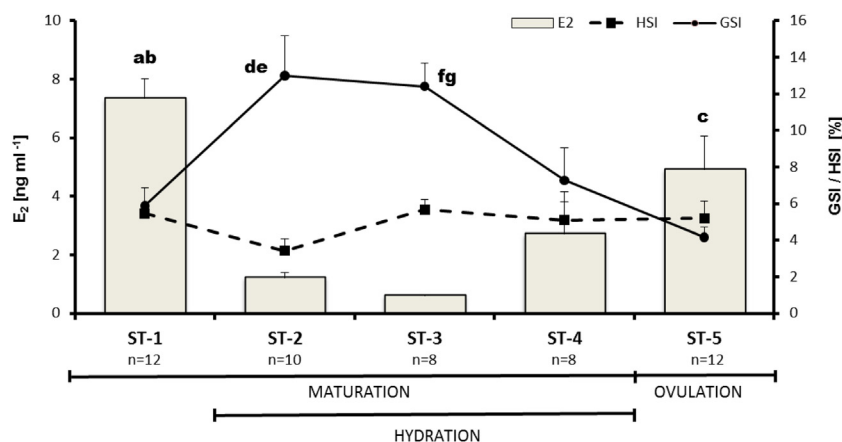


Figure 1 Plasma E_2 concentration and values of GSI and HSI during different stages of final maturation and ovulation in *Neogobius melanostomus*. n – number of individuals. Lowercase letters above bars and lines indicate significantly different values. E_2 : a, $P < 0.001$ vs ST-2 and ST-3; b, $P < 0.05$ vs ST-4; c, $P < 0.05$ vs ST-2 and ST-3. I_G : d, $P < 0.001$ vs ST-1 and ST-5; e, g, $P < 0.01$ vs ST-4.

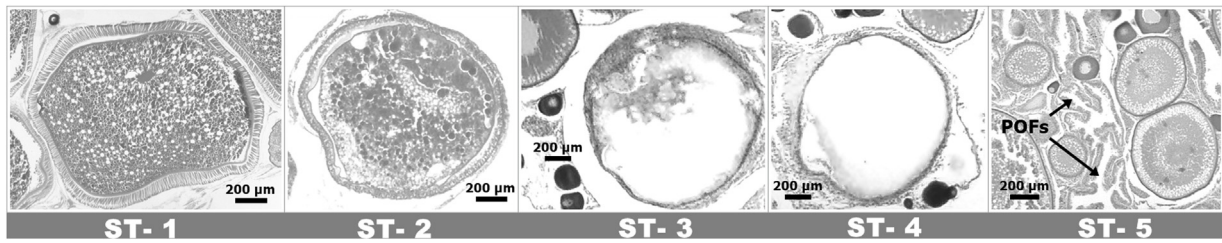


Figure 2 Transverse sections of *Neogobius melanostomus* ovaries illustrating stages of final oocyte maturation and ovulation: ST-1 – germinal vesicle migration (GVM); ST-2 – late germinal vesicle migration; early hydration (late GVM; early HO); ST-3 – germinal vesicle breakdown; middle hydration (GVBD; middle HO); ST-4 – final hydration (final HO); ST-5 – ovulation; post-ovulatory follicles (OV; POFs).

in species that have pelagic or floating eggs. The changes in the oocyte volume range from slight (1.0- to 3.0-fold) in most freshwater and euryhaline fish to several folds in marine species (3.1- to 8.4-fold) (Cerdà et al., 2007; Lubzens et al., 2017). In the present study, the diameter of not transparent oocytes, which presented GVM, was 2.324 ± 0.02 mm (\pm S.E.). The diameter of oocytes exhibiting hydration was 2.452 ± 0.02 mm (\pm S.E.) for semi-transparent oocytes and 2.987 ± 0.03 mm (\pm S.E.) for transparent oocytes. So, the 1.2-fold changes in the volume of oocytes undergoing hydration in *N. melanostomus* are in the ranges of euryhaline, benthophil fish.

In batch spawners, the circulating level of E_2 may fluctuate according to the oocyte batch recruitment cycle. However, if the time of spawning intervals is relatively short, E_2 may remain at a relatively high level throughout the whole spawning season (Mandich et al., 2004; Methven et al., 1992). In the present study, fluctuations in *N. melanostomus* plasma E_2 levels were observed in the intervals between batches of oocyte development that may indicate that one batch of oocytes starts to develop further well before the previous one has been released from the ovary. What is more, in *N. melanostomus*, a considerable statistically significant decline in E_2 level was noticed in the late GVM stage (ST-2; $P < 0.001$) and GVBD stage (ST-3; $P < 0.001$) followed by a renewed statistically significant increase of this hormone until oocyte ovulation (ST-5; $P < 0.05$) (Fig. 1). Similar fluctuations in plasma E_2 levels during the maturation process have been found in other batch spawners such as Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), Striped trumpeter (*Latris lineata*) and Eurasian perch (*Perca fluviatilis*) (Hutchinson, 1995; Kjesbu et al., 1996; Methven et al., 1992; Migaud et al., 2003).

In batch spawners, the recruitment of vitellogenic follicles and intense hepatocyte activity seems to be a continuous process during the whole spawning season, as was found in northern anchovy (*Engraulis mordax*), sand soles (*Solea lascaris* and *Solea impar*), snubnose darter (*Etheostoma rafinesquei*) and Atlantic croaker (*Micropogonias undulatus*) (Barbieri et al., 1994; Deniel et al., 1989; Hunter and Macewicz, 1985; Weddle and Burr, 1991). Therefore, the HSI value remains relatively stable during the main spawning season (Nunes et al., 2011). So, it was not surprising that in *N. melanostomus* in the present study, HSI values did not show significant differences throughout the final maturation and ovulation stages (Fig. 1).

Until recently, a widely used method for determining the reproductive status in *N. melanostomus* was to calculate the GSI value and regard any females with GSI greater than 8% to be mature and those less than 8% to be immature (Young et al., 2010). Indeed, if GSI reaches the maximum value together with significant gonad growth during the spawning season, it may be a useful indicator for division into reproductive stages (Kagawa, 2013). On the other hand, it should be noticed that GSI may be an inaccurate indicator of gonadal development because it assumes isometry between gonad mass and somatic mass and also consistency between total gonad mass and gonad developmental stage (DeVlaming et al., 1982). In addition, it has been demonstrated that spawning-capable *N. melanostomus* females may possess GSI values below 8% (~4–5%), thus the commonly used 8% cut-off may result in an incorrect classification of the reproductive stage (Zeyl et al., 2014). In this study, GSI values were below 8% in stages 1 (early GVM), 4 (final HO) and 5 (OV) while histological analysis indicated that all females were spawning-capable (Fig. 1). In multiple-spawning fish, GSI seems to be a less reliable estimator of fish maturity, which does not provide an accurate estimation of ovarian maturity or differentiation between females in a spawning-capable stage and those in a post-spawning stage (Zeyl et al., 2014).

In conclusion, this is the first histological report demonstrating that hydration does accompany oocyte final maturation in *N. melanostomus*. Moreover, it was shown that E_2 fluctuated according to the development of oocyte batches in this species. HSI did not show significant differences throughout the examined stages. However, GSI appeared to be an inaccurate estimator of oocyte maturation in *N. melanostomus*. The cognition of biology of invasive fish species such as round goby, their reproductive strategies and adaptation processes to environmental factors are important components of the protection and preservation of native living resources in the Gulf of Gdańsk. Therefore, further studies will be required to elucidate the possible mechanism of hydration in *N. melanostomus*.

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