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SHORT COMMUNICATION

First report on intersex in invasive round goby *Neogobius melanostomus* from the Baltic Sea (Gulf of Gdańsk, Poland)☆

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Summary This study is the first to report the presence of intersex in invasive round goby *Neogobius melanostomus* inhabiting the Baltic Sea. The discovery was made in the area of two harbours of the Gulf of Gdańsk (Poland). Macro- and microscopic male gonad analysis revealed the presence of female gametes in testes (testis-ova) of single specimens. In addition, a female-like urogenital papilla was observed in one of the intersex fish. These findings might be due to the exposure to estrogenic endocrine disrupting compounds (EDCs) however the occurrence of single intersex individuals as a baseline level in investigated population is also possible. In the future, more comprehensive research in the other areas of the Gulf of Gdańsk needs to be carried out in order to determine the extent and to better understand the cause of the observed phenomenon.

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1. Introduction

The intersex is an anomaly defined as a simultaneous occurrence of both male and female gonadal tissue within the same individual of a gonochoristic (separate-sex) species (Tyler and Jobling, 2008). Over the last two decades in various wild populations of these teleosts increased prevalence of the phenomenon has been identified worldwide and it has been associated with the presence of natural and synthetic endocrine disrupting compounds (EDCs) reaching aquatic ecosystems with effluents of various origin (Bahamonde et al., 2013). The most frequently observed type of intersex is testis-ova, where female gametes are distributed

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throughout the male gonadal tissue (Getsfrid et al., 2004). This phenomenon is believed to be a consequence of endocrine disruption caused, most commonly, by estrogenic EDCs (Bahamonde et al., 2013). Nevertheless, there is evidence that in some of these species, due to natural variability, intersex might also occur spontaneously at very low levels (Bernet et al., 2009).

The first intersex gonochoristic fish in the Baltic Sea were reported by Kristofferson and Pekkarinen (1975) in male eelpout *Zoarces viviparus* (L. 1758) from the Gulf of Finland where about 20% of the testes contained female gametes. Nowadays, intersex of *Z. viviparus* is used as an indicator of the impact of EDCs on coastal marine ecosystems of several Baltic Sea countries (Förlin, 2012; Hedman et al., 2011). Presence of oocytes in testes was also reported in three-spined stickleback *Gasterosteus aculeatus* (L. 1758) caught in Sweden, however, it concerned single individuals out of hundreds (Borg and Van den Hurk, 1983; Pettersson et al., 2007).

The round goby *Neogobius melanostomus* (Pallas 1811) is a batch spawning gonochorist (Moiseeva, 1983) native to the Ponto-Caspian region (Berg, 1949). The first *N. melanostomus* in the Baltic Sea was found near the Hel Harbour (Gulf of Gdańsk, Poland) in 1990 (Skóra and Stolarski, 1993). Since then this invasive bottom-dwelling fish has become one of the most abundant species in shallow coastal waters of the western part of the Gulf of Gdańsk and has spread to other regions of the Baltic Sea (Sapota, 2012).

The Gulf of Gdańsk is one of the most anthropogenically affected Polish and Baltic Sea coastal areas, due the activity of various industries, municipal discharges and inflows from polluted rivers (Andrulewicz and Witek, 2002; HELCOM, 2010). In its ecosystem EDCs, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethanes (DDTs) or phenol derivatives, some of which are known to be estrogenic (Pait and Nelson, 2002), have been identified (Pazdro, 2004; Reindl et al., 2013; Staniszewska and Falkowska, 2011; Staniszewska et al., 2014). Nevertheless, no studies concerning the presence of intersex fish has been carried out and there are no reports on this phenomenon in this area.

This communication provides the first evidence of intersex in wild population of invasive *N. melanostomus* from the Baltic Sea inhabiting shallow waters of the Gulf of Gdańsk. The discovery was made during studies aimed, among others, at collecting gonads of *N. melanostomus* in order to examine its stage of maturity.

2. Material and methods

Mature *N. melanostomus* of both sexes were caught at two stations in shallow waters of the Gulf of Gdańsk (southern Baltic Sea) using fishing rod in Gdynia Harbour (54°32′01.60″N, 18°32′52.39″E) in April 2007 and fyke nets near Hel Harbour (54°36′04.17″N, 18°47′56.06″E) in July 2007, October 2011 and July 2012 (Table 1). Males were distinguished from females on the basis of urogenital papilla morphology (Juszczyk, 1975). Fish anaesthetized with MS-222 (0.1 g l^{-1}) were sacrificed by severing the spinal cord. Before dissection and macroscopic examination, the gonads were first photographed inside the body cavity, capturing the urogenital papilla at the same time (Fig. 1a–c). Sampled gonads, a randomly selected gonad half of each fish, were preserved in 4% neutral buffered formalin and embedded in paraffin using standard techniques. Embedded tissues, of all collected fish, were cross-sectioned at 6 μm slices using Leica RM2245 microtome and stained with haematoxylin and eosin. Testes were sectioned throughout by obtaining sections from many areas of the gonad, spaced at least 30 μm apart. Whereas, sections of each ovary were acquired from three areas (proximal, middle and distal). Slides from each fish gonad were microscopically examined on a Nikon Eclipse 80i microscope in order to identify its stage of development and photographed using Nikon DS-Fi1 digital camera coupled with the microscope. Ovaries were classified on the basis of the most mature oocytes in the gonads. In case of presence of oocytes in the testis (testis-ova) the severity of the anomalies was described and additional photographs were taken.

All procedures were approved by the Ethics Committee (Resolution No: 29/2008, 33/2010 and 2/2012) given by the 3rd Local Ethics Committee for Animal Experiments in Gdańsk.

3. Results

Gonads of males sampled in 2007 were normally appearing testes without macroscopically visible structural anomalies. However, microscopic analysis revealed presence of testis-ova in two individuals (Table 1 and Fig. 2a). Among males collected in 2011 and 2012 oocyte-like, round-shaped structures were macroscopically identified in three individuals (Table 1 and Fig. 1b). Microscopic examination confirmed the intersex condition in these fish (Fig. 2b). In addition, a

Table 1 Number of *Negobius melanostomus* collected at sampling stations and identified intersex.

Station	Date	Collected fish [n]	Females [n]	Males [n]	Intersex [n]
Gdynia	April 2007	25	8	17	1 (5.9)[a]
Hel	July 2007	16	3	13	1 (7.7)
	October 2011	42	15	27	2 (7.4)
	July 2012	22	7	15	1 (6.7)
Total		105	33	72	5 (6.9)[b]

n — number of fish.
[a] In brackets, prevalence of intersex in percent among collected males.
[b] In bracket, mean prevalence of intersex in percent.

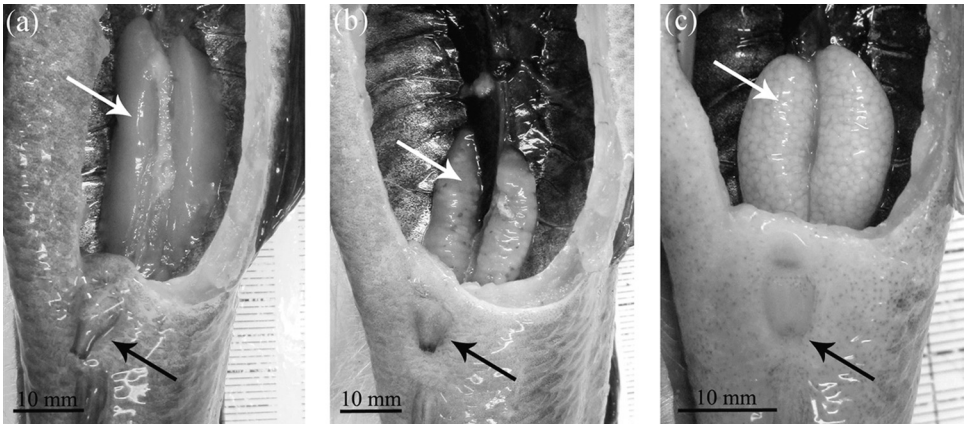

Figure 1 Photographs of gonads (indicated by white arrows) and urogenital papillae (indicated by black arrows) of *Neogobius melanostomus*. (a) Male with normal testis and pointed papilla. (b) Intersex individual with oocytes visible in testicular tissue and female-like papilla. (c) Female with normal ovary and broad papilla.

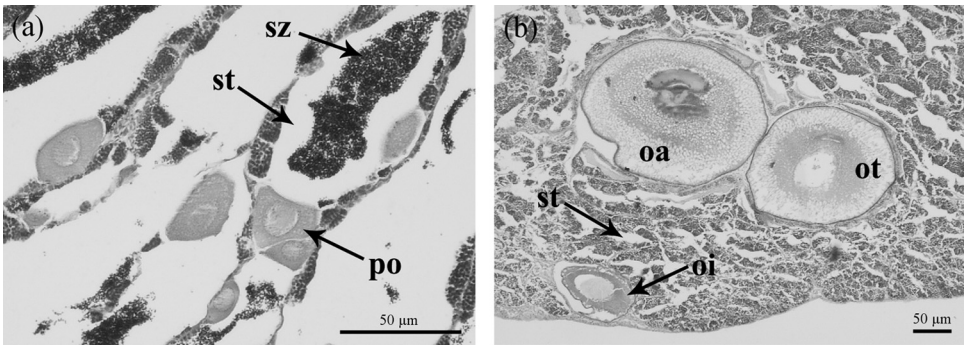

Figure 2 Gonadal histology of intersex *Neogobius melanostomus* individuals. (a) Testis-ova in male showing primary oocytes (po) situated along the walls of seminiferous tubules (st) partially filled with diluted spermatozoa (sz). Testicular tissue is at sperm releasing stage. Magnification 10×. (b) Testis-ova in male showing oocytes at initial (oi), intermediate (ot) and advanced (oa) vacuolization of the cytoplasm. Testicular tissue is at intensive spermatogenesis. Magnification 4×.

female-like urogenital papilla was observed in one of two intersex fish caught in 2011 (Fig. 1). All other fish appeared to be males with normal testes and papillae.

In general, five of 72 examined males of *N. melanostomus* (6.9% of males), collected at Gdynia and Hel stations, showed the presence of oocytes in gonads. In each examined groups single intersex fish (one or two individuals) were present (Table 1). The severity of intersex characteristics varied between individuals. In two intersex fish caught in April 2007 (Gdynia) and July 2012 (Hel) just one oocyte was found in each testicular tissue undergoing intensive spermatogenesis. Oocytes were situated distally within the testis-ova, and were in previtellogenic stage (primary oocytes) and advanced vacuolization stage, respectively. In other intersex individuals oocytes were scattered throughout the gonad. In an intersex caught in Hel in July 2007 numerous primary oocytes were observed. They were located along the walls of seminiferous tubules in the sperm releasing testicular tissue (Fig. 2a). In intersex males collected in October 2011 many oocytes during initial, intermediate and advanced vacuolization of the cytoplasm were identified. Female gametes were fixed in the testicular tissue undergoing intensive spermatogenesis (Fig. 2b). Testicular part of all testes-ova had normally appearing seminiferous structures (Fig. 2a and b) which were similar to those of normal males. The oocytes found in the gonads of intersex individuals caught in 2007 were in previtellogenic stage (Fig. 2a), while in normal females, the following stages of gonad development were present: intermediate or advanced vitellogenic stages found in April and post-ovulatory or initial vacuolization stages found in July 2007. In gonads of intersex, in October 2011 and July 2012, oocytes in various stages of vacuolization were observed (Fig. 2b). Whereas, in normal females, in 2011 and 2012, advanced vitellogenic and vacuolization stages of gonad development were found respectively.

4. Discussion

This paper is the first report on the presence of intersex in the invasive *N. melanostomus* from the Baltic Sea as well as intersex fish in Polish coastal waters. Moreover, it is also the first evidence of the anomaly in the investigated species in Europe. The discovery was made during examination of samples collected, among others, in order to examine gametogenic stages of *N. melanostomus*. Fish were collected at two stations of the shallow waters of the Gulf of Gdańsk: one

located in Gdynia Harbour and second in the vicinity of Hel Harbour. The phenomenon of intersex was identified in single individuals in each group of *N. melanostomus* sampled at both stations. Intersex individuals constituted 5.9% at Gdynia and from 6.7 to 7.7% of males at Hel station. In intersex sampled at both stations in 2007 primary oocytes located within normally appearing seminiferous structure of testicular tissue were revealed. However, in 2011 and 2012 severity of the anomalies in gobies from Hel station has slightly increased and oocytes in advanced cytoplasm vacuolization were identified. Oocytes found in majority of intersex gonads did not correspond to the reproductive cycle of normal females and were usually at lower stage of maturity. Only oocyte, undergoing advanced vacuolization, found in intersex caught in July 2012 matched the stage of gonad development in normal females. Additionally, in 2011, feminization of secondary sexual characteristics, i.e. female-like urogenital papilla, occurred in one of the intersex individuals.

The investigated stations were situated in the Gulf of Gdańsk which is one of the most contaminated Polish coastal areas (Andrulewicz and Witek, 2002; HELCOM, 2010). Gdynia Harbour is the 3rd biggest merchant port of Poland with active shipyards as well as navy, fishing and tourist fleet. In its sediments, in years preceding collection of fish in this study, EDCs such as PCBs, PAHs and DDTs, some of which are known to be estrogenic (Pait and Nelson, 2002), have been identified, usually at relatively low levels not exceeding limit values obligatory in Poland (Falandysz et al., 2006; Ministry of Environment, 2002; Port of Gdynia Authority S.A., 2003—2006). The only cases of exceeding those limits were reported for some PAHs in single samples collected at different locations of the Harbour in 2003 and 2005 (Port of Gdynia Authority S.A., 2003—2006). Hel Harbour is a base for local fishing and tourist fleet, neighbouring with military port in Hel. There is no data for this inshore area on concentrations of EDCs in sediments, however at sites farther away from the shore relatively low levels of PAHs were measured (Lubecki and Kowalewska, 2010), which might indicates presence of those compounds in the shallow zone as well. Even though some EDCs were identified in the Gulf of Gdańsk, there are no constant monitoring programmes for these contaminations. Moreover, almost each research that has been taken in order to investigate EDCs considered different sampling stations which makes it impossible to accurately evaluate their variations. As only two stations, that might be considered contaminated, were investigated in this work, in the future, less polluted reference sites should be studied. On the basis of research concerning concentrations of PCBs, PAHs and DDTs in the Gulf of Gdańsk (Lubecki and Kowalewska, 2010; Pazdro, 2004) these sites might be situated in the vicinity of Sopot (in the inner part of the Gulf) and at the outer side of the Hel Peninsula (at the open sea shoreline, e.g. near Władysławowo).

There are number of studies reporting increased occurrence of intersex in gonochoristic populations of fish as a result of exposure to EDCs. However, there is evidence that in some of these species low levels of intersex might also occur spontaneously (Bahamonde et al., 2013). *N. melanostomus* is a strict gonochorist (Moiseeva, 1983), and there are no reports on naturally occurring rates of spontaneous intersex in this species. However, presence of intersex individuals and altered secondary sexual characteristics, as an effect of exposure to EDCs, had been previously found in *N. melanostomus* at heavily polluted sites of Hamilton Harbour in Lake Ontario (Canada), where it was also shown as one of the most sensitive species to endocrine disruptions (Marentette et al., 2010). Intersex was first identified in 12.9% of males at the most contaminated site of the Harbour (Marentette et al., 2010). The phenomenon has been confirmed by Bowley et al. (2010) who had reported 10% of intersex at the same site and found individuals with testis-ova at other contaminated sites of the Harbour. In most of Canadian intersex oocytes were in previtellogenic or vacuolization stages. Whereas, single individuals, from the most contaminated site, showed advanced stages of oocytes development, i.e. late vitellogenic ova (Bowley et al., 2010; Marentette et al., 2010) and some of them did not show development of seminiferous lobules (Marentette et al., 2010). In both studies feminization of urogenital papilla has been shown to be a useful indicator of exposure to EDCs as it was reported only in males collected at contaminated sites, while at less polluted and cleaner sites, chosen as reference sites, no urogenital papilla changes nor intersex in males were observed. Since PAHs and PCBs were the major contaminants in sediments at sites, where endocrine disruptions in *N. melanostomus* were identified, they are thought to be one of the most likely agents responsible for the observed disruptions (Bowley et al., 2010; Marentette et al., 2010).

In the Baltic Sea, as particularly susceptible to develop intersex in contaminated environment turned out to be *Z. viviparus*, which since over a decade has been used in research concerning the impact of EDCs in coastal waters of such countries as Germany, Denmark or Sweden (Förlin, 2012; Gercken and Sordyl, 2002; Gercken and Sundt, 2007; Strand et al., 2009). Nevertheless, there were no reports or studies concerning the presence of intersex in *Z. viviparus*, nor in any other fish species, in the Gulf of Gdańsk. If more comprehensive research indicated that the phenomenon of intersex in *N. melanostomus* from the Gulf of Gdańsk is a response to EDCs, *N. melanostomus* could be suggested as a sentinel species in endocrine disruption research, not only in the Gulf but also in other regions of the Baltic Sea invaded by this species.

In conclusion, this is the first report of intersex in the invasive *N. melanostomus* from the Baltic Sea as well as intersex fish in Polish coastal waters. The occurrence of intersex individuals and feminization of secondary sexual characteristics might indicate that *N. melanostomus* inhabiting coastal waters of the Gulf of Gdańsk was exposed to estrogenic EDCs. However, as only two stations were studied and intersex was observed in single individuals, which might suggest occurrence of spontaneous intersex, an extended study need to be carried out in order to determine the range of the occurrence and the baseline levels of *N. melanostomus* intersex in the Gulf. Investigations are also necessary to better characterize possible endocrine disrupters at the investigated stations and other areas of the Gulf of Gdańsk. Moreover, if it is shown that the occurrence of intersex is the result of exposure to EDCs, *N. melanostomus* could be proposed as a new sentinel species in the Baltic Sea.

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