SUMMARY

Endocrine aspects of round goby (*Neogobius melanostomus*) reproductive physiology with particular emphasis on disruptions caused by exogenous 17β-estradiol

The endocrine system of fish, as in other vertebrates, is a complex network of relationships between hormones produced by endocrine glands, such as the hypothalamus, pituitary gland, pineal gland, thyroid gland and gonads. The endocrine system regulates and coordinates the functioning of tissues and organs, and maintains the body's internal balance (homeostasis), in order to ensure the proper course of life processes, including those related to reproduction. One of the key hormones that ensure the proper course of fish reproductive processes are: melatonin (Mel), thyroxine (T_4), 17β-estradiol (E_2) and 11-ketotestosterone (11-KT). In fish reproduction, melatonin, produced mainly by the pineal gland, has been reported as an important hormone in the regulation of occurrence of spawning, and thus in the regulation of gametogenic and steroidogenic functions of the gonads. These actions of Mel occur as a result of effects on the brain–pituitary–gonad axis. This hormone synchronizes different biological rhythms with other physiological processes and behavioural changes involved in fish reproduction. Moreover, Mel can also protect cells from oxidative stress in the processes of maturation and regression of gonads in an annual cycle. Whereas thyroxine produced by the thyroid gland has a wide-ranging effect on gonadal development and maturation, stimulating and maintaining these processes during the fish reproductive cycle. In female fish, T_4 stimulates the development of previtellogenic oocytes and early oocyte vitellogenesis and up-regulates hepatic synthesis of vitellogenin – the protein sequestered by growing oocytes that induces final oocyte maturation and ovulation. In male fish, T_4 modulates the actions of many factors that are important for maintaining spermatogenesis and spermiation. 17β-estradiol is the primary female sex hormone responsible for the development and regulation of the female reproductive system and secondary sex characteristics in fish. It is synthesized in gonads and in the brain and it has an important role in the regulation of oocyte growth, the hepatic synthesis and secretion of vitellogenin. This hormone is also synthesized by Leydig and Sertoli cells in fish testes and affects the regulation of spermatogenesis, especially spermatogonial proliferation. In turn, 11-ketotestosterone, produced mainly by the gonads, is the primary androgen, stimulating spermatogenesis and regulating growth and development of testes, as well as secondary sexual characteristics and reproductive behaviour in most male fish. In female fish, the function of 11-KT is not fully understood but the results of the few studies conducted so far have shown that it may affect the growth of previtellogenic oocytes and contributes to oocyte growth.

The proper functioning of the endocrine system of fish may be, however, disturbed by various chemical compounds that enter an aquatic environment with municipal, agricultural and industrial sewage. This can result in adverse changes related to, among others, fish behaviour, reproduction and development. These types of disorders are caused by endocrine disrupting compounds (EDCs), defined as an exogenous substance or mixture of substances that changes functions of the endocrine system and provokes adverse effects in an intact organism, its progeny or populations. Aquatic wildlife could be exposed to both natural and synthetic EDCs. Among natural EDCs there are estrogenic compounds that include steroid hormones, such as: estrone (E_1), 17β-estradiol (E_2), 17α-estradiol (17α-E_2) and estriol (E_3). Among these compounds, E_2 has the highest estrogenic potential. In the aquatic environment, this hormone comes mainly from municipal and agricultural sewage and it has been identified as one of the main components responsible for the estrogenicity of sewage treatment effluents. The presence of E_2 has been reported in waste and surface waters and marine sediments in many countries, with levels ranging from a few tenths of a nanogram per litre to a few thousands of a microgram per litre. The highest and supraphysiological concentrations of E_2 have been reported in coastal zones exposed to discharges from large urban agglomerations and runoff of waste from animal husbandry. Despite the great interest in the effects of estrogenic compounds on organisms, so far there has been very little research on E_2 in the Baltic Sea, and the available data, mainly on surface waters, do not fully reflect the conditions in pelagic or bottom fish habitats.

The results of studies conducted so far on the exogenous effect of E_2 on fish have shown that it may alter plasma steroid hormone levels and reduce egg production, as well as decrease egg viability and hatchability. Exogenous E_2 may also reduce sperm count and viability, thus causing reproductive failure and fish population decline in a given body of water. What is more, exogenous E_2 may provoke changes in the structure of the gonads, causing the appearance of intersex individuals, which in turn can lead to disturbances in the sex structure of fish populations. Intersex condition is an anomaly defined as the simultaneous presence of male and female gonadal tissue in a gonochoristic (fixed-sex) species. Exogenous E_2 may also impair sexual behaviour and regress sexual characteristics in males. The doses of E_2 used in the experiments carried out so far have ranged from a few nanograms to several hundred micrograms per litre of water, and from a few to several hundred milligrams per kilogram of body weight in

feed. In the natural environment, fish are exposed to fluctuating levels of contaminants, which on the one hand is caused by an irregular inputs of contaminants, and on the other hand, due to natural, diurnal or seasonal migrations linked to changes in feeding grounds or reproduction. Unfortunately, in most of the experiments, the effects of exogenous E_2 on fish have been studied during short or long, but constant, exposures by administering the hormone to the water or via feed, which did not fully reflect environmental conditions. However, the results of the few studies that have been carried out using short-term, intermittent exposure of fish to exogenous E_2 allow drawing the conclusion that exposure of this type may cause a significantly greater endocrine response in the fish than continuous exposure.

One of the gonochoristic fish species that has been found to be particularly sensitive to the effects of EDCs and have a greater predisposition, compared to other fish species, to the development of intersex individuals and feminization of secondary sexual characteristics is the round goby (*Neogobius melanostomus*). This species is a bottom-dwelling fish inhabiting mainly coastal waters, where it prefers regions with a hard substrate. The round goby, which occurs naturally in the Pontic–Caspian region, has successfully colonized the Baltic Sea. The first specimens of this non-native species were recorded in the Gulf of Gdańsk in 1990. Since then, *N. melanostomus* has become one of the most abundant fish species in the coastal areas of the western part of the Gulf of Gdańsk, also inhabiting areas along the entire Polish coast, including estuaries of rivers, lagoons and coastal lakes. The success of round goby expansion in the Baltic Sea is due to its high adaptability to changing environmental factors such as temperature and salinity, and to a varied diet. However, most of all, this success is associated with the round goby's reproductive strategy based on batch-spawning (i.e., the females may spawn several times during one reproductive period) and on the males caring for the deposited eggs, which ensures high reproductive efficiency.

Although considerable research relating to the biology of *N. melanostomus* has been conducted and the species has been shown to be sensitive to EDCs, the literature lacks thorough knowledge on the hormonal regulation of the reproductive cycle of this species, as well as studies on disturbance of reproductive physiology caused by EDCs. The existing literature on the reproductive physiology of the round goby is limited to a few fragmentary studies focusing only on steroid hormones and gonadal histology of single specimens. Moreover, so far, no studies have been conducted on the

concentrations of other hormones, such as Mel and T_4, in this species, which, apart from steroid hormones, play an extremely important role in the reproductive cycle of fish. The literature also lacks comprehensive information on the influence of exogenous E_2 as a natural EDC on the reproductive physiology of *N. melanostomus* and other fish species. Moreover, despite the numerous occurrences in the coastal zone of the Baltic Sea, where higher concentrations of various types of pollutants are recorded, no intersex individuals of this species have been found so far. Given the above, in this doctoral dissertation, the aim of the research undertaken was to supplement and broaden the knowledge on endocrine aspects of the reproductive physiology of mature *N. melanostomus* individuals, including disorders that may result from exposure to estrogenic EDCs, with particular emphasis on disruptions caused by exogenous E_2. In order to achieve this goal, the following research tasks were set: (1) determining whether intersex individuals are present in the population of round goby from the Gulf of Gdańsk (**ARTICLE I**); (2) determining Mel and T_4 concentrations in plasma, E_2 and 11-KT concentrations in plasma and gonads, and the stage of gonadal development in both sexes of round goby, in four phases of the reproductive cycle: pre-spawning, spawning, late-spawning and non-spawning (**ARTICLE II**); and (3) determination of the effect of short-term intermittent exposure to a supraphysiological dose of exogenous, waterborne E_2 on Mel and T_4 concentrations in plasma, E_2 and 11-KT concentrations in plasma and gonads, and on the stage of gonadal development in both sexes of round goby, in the four phases of the reproductive cycle mentioned above (**ARTICLE III**). The reproductive phases were chosen for the research by taking into account the fact that processes occurring at the time have a significant impact on the spawning potential and population parameters, and consequently on the volume of fish stocks, which may be of great importance especially for species targeted in fisheries and those used in aquaculture.

The presence of intersex individuals in the population of *N. melanostomus* in the Gulf of Gdańsk was determined on the basis of macroscopic and histological analyses of gonads carried out on material collected in 2007 ($n = 25$) in the area of Gdynia Harbour and in the years 2007–2012 ($n = 80$) in the vicinity of Hel Harbour (**ARTICLE I**). Studies aimed at determining the concentrations of Mel and T_4 in plasma, E_2 and 11-KT in plasma and gonads, and the stage of gonadal development in both sexes of *N. melanostomus* in four phases of the reproductive cycle: pre-spawning, spawning, late-spawning and non-spawning, were carried out on material obtained in

2011–2014 (n = 84) in the vicinity of Hel Harbour. The concentrations of Mel, T_4 and E_2 were determined by radioimmunoassay (RIA), whereas 11-KT was quantified using an enzyme immunoassay (EIA). The stage of gonad maturity was determined on the basis of histological analysis (**ARTICLE II**). Whereas, in order to determine the effect of exogenous E_2 on the concentrations of selected hormones and on the stage of gonadal development in both sexes of *N. melanostomus*, in the four above mentioned phases of the reproductive cycle, an experiment based on exposure of fish to a supraphysiological E_2 concentration (200 μg/L; nominal dose of E_2) through short-term intermittent baths was carried out. In each studied phase, four groups of fish were distinguished: three control groups (stationary, subjected to baths in sea water, and subjected to baths in sea water supplemented with ethanol that was used as a solvent for E_2) and one group exposed to exogenous E_2. Each group consisted of 21 individuals (14 males and 7 females). The stationary group was not subjected to any baths (control values obtained under **ARTICLE II**). The other two control groups and the group exposed to E_2 were subjected to four series of short-term baths with 5-day intervals during the 27 days of the experiment. Each series consisted of 2-hour baths repeated for three consecutive days. In order to control the experimental conditions, water parameters (temperature, salinity, pH, dissolved oxygen, concentrations of nitrates, nitrites, phosphates, ammonia and ammonium ions) as well as the concentration of E_2 in the water were measured at the beginning and at the end of each bath. The method of administration of E_2 (water baths), the supraphysiological dose of E_2 and the bathing schedule used in this experiment were selected based on the results of previous experimental and environmental studies described in the literature. The research was carried out in 2011–2014 on material (n = 336) obtained in the area of Hel Harbour. The concentrations of Mel, T_4 and E_2 were determined by RIA whereas 11-KT was measured using an EIA test. In addition to hormone analyses, gonadal histology was performed and gonadosomatic (GSI) and hepatosomatic (HSI) indexes were determined (**ARTICLE III**).

The results of the conducted research indicated the presence of intersex individuals in the population of *N. melanostomus* inhabiting the Gulf of Gdańsk. The phenomenon of intersexuality was identified in single individuals, both in Gdynia Harbour and in the area of Hel Harbour, which in these locations constituted 5.9% and 6.7% to 7.7% of males, respectively. In the gonads of intersex individuals caught in both locations in 2007, previtellogenic oocytes were found to be present in the tissue of

the seminiferous tubules. On the other hand, in the years 2011–2012, among males of round goby caught in Hel, intersex individuals were observed with gonads in which oocytes at the stage of advanced vitellogenesis were identified. Moreover, in 2011, in one of the intersex individuals, feminization of secondary sexual characteristics was observed, i.e. individuals having female-like urogenital papilla. The presence of intersex individuals showing changes in the structure of the gonads, as well as the feminization of secondary sexual characteristics, suggest that the round goby population living in the coastal waters of the Gulf of Gdańsk has been exposed to estrogenic EDCs. The observed anomalies and the lack of literature on the occurrence of intersex individuals in populations of other fish species living in Polish coastal waters seem to confirm the sensitivity of *N. melanostomus* to pollutants causing endocrine disorders (**ARTICLE I**).

The results obtained in this study showed that in all the phases of the study, in both sexes of round goby, the differences in concentrations of Mel in plasma were not statistically significant, except for the spawning phase, where significantly higher values were observed in males. Nevertheless, in both sexes the highest concentrations of Mel in plasma were noted in the spawning and non-spawning phases. It is known that in the spawning phase, Mel is necessary for the proper course of processes in the maturation of reproductive cells, while in the non-spawning phase its high level activates processes leading to the regression of gonads. It therefore appears that Mel may have important functions in determining a time frame for spawning in both females and males of *N. melanostomus*. In both sexes of round goby, plasma T_4 concentrations did not differ significantly in all the studied phases of the reproductive cycle, except in the non-spawning phase, where a significantly higher T_4 concentration was observed in males. The concentrations of E_2 in plasma and gonads of females were significantly higher than the concentrations of this hormone in males in all the studied phases. Interestingly, there was a similar tendency of changes in concentrations of T_4 in plasma and E_2 in plasma and gonads, with the increase recorded in the pre-spawning and non-spawning phases. This suggests that these hormones are integral in affecting the gonadal tissues of both sexes of round goby during the active spawning phase, and also the timing of initiation of spawning (stimulation of gonad development in the pre-spawning phase). High concentrations of these hormones in the non-spawning phase may be associated with, inter alia, the ongoing steroidogenesis in atretic vitellogenic oocytes in females, and with inhibition of spermatogenesis processes in males. The highest concentrations of 11-KT were observed in the plasma and gonads of males during the

spawning phase, which seems to indicate the importance of this hormone in the process of active spermatogenesis in the developing testes. In females, however, there were no significant changes in the concentrations of 11-KT in plasma and gonads during the studied reproductive phases. This is most likely related to the stimulating role of 11-KT in the growth of previtellogenic oocytes, which were present in the ovaries in similar quantities during all reproductive phases. Concentrations of 11-KT in both plasma and gonads of females were significantly lower than in males in all the studied phases of the reproductive cycle, which is typical for most fish species (**ARTICLE II**).

The presented studies showed that the natural profile of Mel plasma concentrations in both sexes of round goby, with the highest values in the spawning and non-spawning phases, did not change under the influence of short-term exposure to a supraphysiological dose of E_2, which allows the conclusion that the role of Mel in determining the spawning time frame is stable. It should be noted that the observed stable Mel levels may also protect against severe histopathological changes in gonads that could result from E_2 exposure. Exposure to E_2 caused a statistically significant increase in plasma T_4 concentration in both sexes of *N. melanostomus* in all investigated phases, except the pre-spawning phase. It is known that estrogens could affect the thyroid gland, either directly by their stimulatory effect on thyroid peroxidase activity, thyroid epithelial height, thyroidal RNA content and T_4 level, or indirectly through stimulation of thyroid-stimulating hormone (TSH) production in the pituitary. However, it should be mentioned that E_2 has been shown to have either an inhibitory or no effect on the thyroid gland in fish. In round goby females, exposure to E_2 significantly increased plasma and ovarian E_2 concentrations, independently of the investigated phases. It has been shown that exogenous E_2 may influence the hypothalamic–pituitary–gonadal (HPG) axis primarily by modulating steroid synthesis or by regulatory feedback mechanisms. The reverse reaction occurred in the case of 11-KT, where exogenous E_2 caused a significant decrease in the concentration of this hormone both in plasma and ovaries of *N. melanostomus* females in all the studied phases of the reproductive cycle. Unfortunately, the literature lacks any information on the effect of E_2 on the concentration of 11-KT in female fish. The studies conducted here are therefore the first on this topic and allow it to be assumed that the reduction of 11-KT concentration in gonads may be a result of down-regulation of steroidogenic enzyme gene expression. In round goby males, similarly to females, exposure to exogenous E_2 statistically significantly increased plasma E_2 concentration during all investigated phases. The

stimulatory effect on gonadal concentrations of E_2 was observed in all investigated phases except in the non-spawning phase, where exposure to exogenous E_2 reduced native E_2 concentration in testes. Furthermore, the decrease in plasma and testes 11-KT concentrations in response to exogenous E_2 was observed independently of the investigated phases. We can distinguish two potential mechanisms involved in estrogen's exogenous effect in fish males. First, when E_2 induces classic biomarkers of estrogen responses such as hepatic estrogen receptor 1 (ER1) expression and plasma vitellogenin, and second, when E_2 impacts the HPG axis through changing steroidogenic enzyme expression profiles potentially involved in the exogenous effects of estrogens in male fish. However, it is possible that a direct action of E_2 on the testes, independently of gonadotropins stimulation, may occur in round goby males in the reproductive cycle, except in the non-spawning phase. In both sexes of round goby, the short exposure to a supraphysiological dose of E_2 also caused changes in gonad histology, except in the pre-spawning phase, where in females' as well as in males' exposure to E_2 seemed to have no effect on gonad development. It is likely that gonads are not sensitive to an additional dose of E_2 when endogenous E_2 concentrations in plasma and gonads are very high or exposure time is not sufficient to provoke histological changes. In round goby females, in other phases, exogenous E_2 has accelerated oocyte development, ovulation and regression processes, whereas in males, exposure to E_2, influenced the testes by inhibiting spermatogenesis and inducing spermatogonial atresia. Probably, T_4 and sex steroids are integral in affecting round goby gonads by causing the changes described above in response to exogenous E_2. It seems that hormonal changes provoked by E_2 exposure cause greater disturbances in the reproduction of males than in that of females. In males, exogenous E_2 may lead to a shorter spawning period and fertility disturbances. Meanwhile, in females, the acceleration in oocyte development, ovulation and regression may paradoxically enhance their reproductive potential. Interestingly, in all investigated phases, both in females and males, no effects of exogenous E_2 on GSI and HSI were observed, except in the non-spawning phase. In this phase, in both sexes only a significant decrease in GSI values was noticed, most likely due to progressive gonadal regression (**ARTICLE III**).

This dissertation provides new information on multiple aspects of the reproductive physiology of *N. melanostomus*, thereby constituting a significant supplement to the existing knowledge. The finding of the presence of intersex

individuals in the round goby population is the first of its kind, both for this species in the Gulf of Gdańsk and in the entire Baltic Sea. It is also the first time that the hormonal regulation of the reproductive process in this species has been studied in such a comprehensive way, and the results obtained showed a high sensitivity of the round goby to endocrine disorders caused by short-term exposure to E_2. This feature and the fact that it is a widely distributed and abundant species makes *N. melanostomus* appear to be a good model organism for the study of endocrine disorders caused by estrogenic EDCs, which can be used in the monitoring of environmental pollution. On the other hand, given it is an alien species that is recognized in many countries around the world as highly invasive and requiring special observation and research, the results obtained in this dissertation may also be useful in developing strategies for the management of round goby populations. In the context of being an invasive alien species, paradoxically, any disturbance leading to a reduction in its reproductive efficiency seems to be beneficial as it helps to eliminate it from the environment.