Concentrations of melatonin, thyroxine, 17β-estradiol and 11-ketotestosterone in round goby (*Neogobius melanostomus*) in different phases of the reproductive cycle

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ABSTRACT

The aim of this study was to determine changes in concentrations of melatonin (Mel) and thyroxine (T_4) in plasma, and 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) in plasma and gonads of female and male round gobies (*Neogobius melanostomus*) from the Southern Baltic Sea in four phases of the reproductive cycle classified as pre-spawning, spawning, late spawning and non-spawning periods. The concentrations of Mel, T_4 and E_2 were determined by radio-immunoassay (RIA) whereas 11-KT was quantified using an enzyme immunoassay (EIA). The maturity stage of gonads was determined using histological analyses. The pattern of changes in Mel concentrations of females and males was similar with the greatest concentrations in the spawning and non-spawning phases. In both sexes, there was a similar tendency of change in concentrations of T_4 and E_2 with the increase being in the pre-spawning and non-spawning phases. The greatest concentrations of 11-KT were observed in the plasma and gonads of males during the spawning phase. In females, there were no changes in 11-KT concentrations either in plasma or gonads during all phases where quantifications occurred. This is the first study for determination of the pattern of changes in Mel and T_4 concentrations as well as gonadal steroids E_2 and 11-KT, supported by histological analysis of gonads, in batch spawning fish during the reproductive cycle.

1. Introduction

Since the 1990s, the round goby (*Neogobius melanostomus*) became a significant invader in several regions of Europe (including the Baltic Sea) and in the Laurentian Great Lakes of North America (Kornis et al., 2012). In the Baltic Sea, this batch spawning species is most abundant in the Gulf of Gdańsk, where the spawning season lasts from the beginning of May to the end of September and females may lay eggs from two to four times during the season (Sapota, 2012).

Although considerable research on the biology of round goby has been conducted (Kornis et al., 2012), the hormonal regulation of the reproductive cycle of this species has received little attention. It is well recognized that sex steroids have important functions in fish reproduction, and there are also other hormones such as melatonin (Mel) and thyroxine (T_4) that are essential for regulation of reproductive cycle (Borg, 1994; Falcón et al., 2010; Leatherland, 1994; Lubzens et al., 2010). To the authors' knowledge, there is no study on Mel and T_4 in round goby. In fish reproduction, Mel has been reported to be an important hormone in regulation of occurrence of spawning, gametogenic and steroidogenic functions of gonads (Popek et al., 1991; Sokołowska et al., 2004; Maitra

et al., 2006). These actions of Mel occur as a result of effects at the brain – pituitary – gonad axis (Falcón et al., 2007; Popek et al., 2010; Maitra et al., 2013). This hormone transduces seasonal temperature and photoperiod signals that are perceived *via* sensory neurons that have an important role in synchronizing the stage of different biological rhythms also involved in reproduction (Ekström and Meissl, 1989; Falcón et al., 2010; Maitra et al., 2013). Melatonin can also protect cells from oxidative stress in the processes of the maturation and regression of gonads in an annual cycle (Bromage et al., 2001; Bayarri et al., 2004; Falcón et al., 2010; Maitra et al., 2013, 2015; Alvarado et al., 2015; Maitra and Hasan, 2016). In fish, T_4 has wide-ranging effects on gonadal development and maturation, stimulating and maintaining the functions during the reproductive cycle (Biswas et al., 2006; Swapna and Senthilkumaran, 2007). In females, T_4 stimulates the development of previtellogenic oocytes and early oocyte vitellogenesis (Wiegand, 1982; Cyr and Eales, 1988), up-regulates hepatic synthesis of vitellogenin - the protein sequestered by growing oocytes (Monteverdi and Giulio, 2000) that induces final oocyte maturation and ovulation (Hurlburt, 1977; Detlaff and Davydova, 1979). In males, T_4 modulates the actions of many factors that are important for maintaining spermatogenesis and spermiation (Sower and Schreck, 1982; Leatherland, 1994; Swapna and Senthilkumaran, 2007; Flood et al., 2013).

Furthermore, there are only a few studies focused on the sex steroids in round goby. There have been results reported describing the 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) concentrations in non-reproductive and reproductive males and females and two different male reproductive morphs, namely: parental and sneaker males (Marentette et al., 2009; Bowley et al., 2010; Zeyl et al., 2014). In female fish, E_2, which is produced mainly by the ovarian follicles, has an important role in supporting the hepatic synthesis and secretion of vitellogenin (Lubzens et al., 2010). There is also an essential function of E_2 in male reproduction (Borg, 1994; Schulz et al., 2010). There is synthesis of E_2 by Leydig and Sertoli cells in fish testes (Janz and Weber, 2000) and this hormone affects the regulation of spermatogenesis, especially spermatogonial proliferation (Schulz et al., 2010). In most teleosts males, 11-KT is the primary androgen, stimulating spermatogenesis and regulating growth and development of testis as well as secondary sexual characteristics and reproductive behaviour (Borg, 1994; Schulz et al., 2010). The function of 11-KT in females is not fully understood but *in vivo* and *in vitro* studies of short-finned eel (*Anguilla australis*; Rohr et al., 2001; Lokman et al., 2007) have reported that 11-KT contributes in oocyte growth.

The objective of the present study was to investigate the changes in Mel, T_4, E_2 and 11-KT in four phases of the reproductive cycle of round goby characterized as the pre-spawning, spawning, late spawning and non-spawning phases. In addition to analyses of hormones, there were histological studies of gonads as a part of this study.

2. Materials and methods

2.1. Fish

Adult round gobies of both sexes ($n = 84$) were caught using fyke nets, near Hel Harbour (54° 36′ 04.17″N, 18° 47′ 56.06″E) (Gulf of Gdańsk, southern Baltic Sea) in March, April, July and October. After capture, the fish were transferred to the Marine Station (Institute of Oceanography, University of Gdańsk, Poland) in Hel and were sexed by examination of the urogenital papilla. Each group of 21 individuals of similar size consisted of 14 males and seven females and were acclimated to the tank conditions for 6 weeks. During this time, fish were maintained in natural photoperiodic and temperature conditions (Table 1) in a 2640 L outdoor tank with an open circuit water system providing constant flow through of fresh brackish water (˜7% salt) from the Gulf of Gdańsk. The temperature in the outdoor tank was measured using a portable meter CC-401 (Elmetron, Poland) every day at noon. The measurements were taken in the middle of the tank water column. In each tank, devices were inserted that facilitated fish seclusion that were made from PVC pipes so as to mimic natural living conditions of round goby. Fish were fed once daily with fresh fish meat (herring), frozen mussels or shrimps at 3:00 p.m. After the acclimatisation, fish were anaesthetized in MS-222 (tricaine methane-sulfonate) water buffered solution (0.1 g/L; Sigma-Aldrich, USA). Sampling occurred between 10:00 a.m. and 2:00 p.m. The length of each individual was measured and weighing occurred. Blood samples were collected by cardiac puncture, using a heparinized syringe. The plasma samples were centrifuged at 3000 *g* for 10 min at 4 °C and frozen at −70 °C until analyses. After blood sampling, fish were euthanized by transection of the spinal cord. Gonads were collected and one gonad of each individual was frozen at −70 °C and stored until steroid analysis while the second gonad was preserved in 4% buffered formalin for histology. There were histological analyses of ovaries and testes for characterization of sexual maturity stage because the gonadosomatic index (GSI) is apparently a less reliable assessment of stage of sexual maturation in batch spawners (Guerriero, 2007; Zeyl et al., 2014)

Table 1
Average day length and range of water temperature during reproductive phases.

Phase	Day length [h]	Water temperature [°C]
Pre-spawning	13	4.8 ± 0.5
Spawning	15	11.5 ± 2.5
Late spawning	14	19.6 ± 1.2
Non-spawning	10	11.0 ± 1.3

2.2. Analytical methods

2.2.1. Plasma analysis

Plasma Mel was assayed using a total melatonin radioimmunoassay (RIA) kit (IBL International, Germany), after conducting an extraction procedure using the previously reported method of Kulczykowska et al. (2007). The solid phase extraction was conducted using a 10 μm Octadecyl C18 Speedisk Column, (J.T. Baker, USA). Eluates obtained after extraction were dried and then stored at −70 °C until analysis. Before the RIA procedure, dried samples were re-suspended in Dulbecco's Phosphate Buffered Saline containing 0.01% Thimerosal (Sigma-Aldrich, USA). The labelled Mel with iodine-125 (^{125}I) was used as a tracer for this analysis. The assay was performed using polyclonal rabbit anti-Mel first antibodies and goat anti-rabbit second antibodies (double antibody method). The detection limit was 3.0 pg/mL of plasma. The intra-assay coefficients of variation were 6.5%.

There was quantification of plasma T_4 using a total thyroxine RIA-gnost kit (Cisbio Bioassays, France) without extraction procedures being conducted the method previously reported by Kulczykowska et al. (2007). The assay was performed using tubes coated with murine anti-T4 monoclonal antibodies and 8-aniline-1-sulfonic acid as a displacement reagent. The T_4 labelled with ^{125}I was used as a tracer for RIA. The detection limit was 1.1 ng/mL of plasma. The intra-assay coefficients of variation were 5.6%.

Plasma E_2 was measured using the ESTR-CTRIA RIA kit (Cisbio Bioassays, France) without conducting extraction procedures according to the manufacturer's protocol validated in our laboratory (Kalamarz-Kubiak et al., 2017). Iodinated E_2 with ^{125}I was used as a tracer. The assay was conducted in RIA tubes pre-coated with polyclonal anti-rabbit antiserum according to the kit manufacturer's instructions. The detection limit of the assay was 4.25 pg/mL. The intra-assay coefficient of variation was 2.1%.

Plasma 11-KT was quantified using the competitive enzyme immunoassay (EIA) Cayman kit (Ann Arbor, USA) with extraction procedures occurring before conducting the assay following the methods previously reported by Sokołowska et al. (2013). Before analysis, extraction of plasma samples (100 μL) was conducted using ethyl acetate/hexane (50:50 v/v) at 4 × the sample volume utilizing the method recommended in the EIA kit protocol with slight modifications. Dried extracts were stored at −20 °C until further analysis. The recovery of extraction was between 98% and 115%. Extracts were dissolved in 2 mL of EIA buffer and 50 μL of the diluted samples were used for the EIA analysis. The 11-KT–acetylcholinesterase conjugate was used as a tracer. The assay was performed in the pre-coated microplate with mouse anti-rabbit IgG using rabbit anti-11KT antibodies. The microplate was read at 412 nm using a Sunrise Absorbance Reader (Tecan, Austria). All samples were assayed in duplicate. The detection limit of the assay was 0.7 pg/mL. The intra-assay coefficient of variation was 0.6%. For the RIA, all samples were assayed in duplicate and counted for 1 min in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Science, USA). The inter-assay variations for plasma analysis were not determined because all samples were measured in the same assay

2.2.2. Analysis in gonads

Each gonad was sonicated in 0.5 mL of phosphate buffer (0.05 M, pH 7.4) supplemented with sodium azide (NaN$_3$) using a Microson™ XL 2000 (USA). Sonicated samples were centrifuged at 20,000 g for 20 min at 4 °C and the supernatants stored at −70 °C prior to the quantification of E_2 and 11-KT concentrations.

The E_2 concentrations in gonads' extracts were measured using ESTR-CTRIA RIA kit (Cisbio Bioassays, France) according to the method by Kulczykowska et al. (2015). Gonad supernatants (200 μL) were extracted with 1.6 mL of diethyl ether using the modified methods of Mori and Kano (1984). The recovery rate of the extraction was between 86% and 109%. Dried extracts were stored at −20 °C prior to analysis. Extracts were dissolved in phosphate buffer (0.05 M, pH 7.4) supplemented with NaN$_3$ and samples of 100 mL were taken for RIA analysis. Further analysis was performed according to the method described for plasma E_2 quantifications (see paragraph 2.2.1.). The detection limit of the assay was 4.25 pg/mL. The intra-assay coefficient of variation was 6.5%.

The 11-KT concentrations in gonads' extracts were determined by EIA using Cayman kit (Ann Arbor, USA) with the extraction procedure described by Kulczykowska et al. (2015). Gonad supernatants (250 μL) were extracted with 1 mL of ethyl acetate/hexane (50:50 v/v) according to the method recommended in the EIA kit protocol with slight modifications. The recovery of extraction was between 98% and 115%. Dried extracts were stored at −20 °C until further analysis. Extracts were dissolved in 0.5 mL of EIA buffer and 50 μL of the diluted samples were used for the EIA analysis. Further analysis was performed using the methods described for plasma 11-KT quantification (see Section 2.2.1). The detection limit of the assay was 1.092 pg/mL. The intra-assay coefficient of variation was 0.8%. The inter-assay variations for gonads analysis were not determined because there were quantifications for all samples in the same assay.

2.3. Histological analysis of gonads

Gonads were stored in 4% buffered formalin then dehydrated in graded ethanol and embedded in paraffin using standard histological techniques. Sections of 6 μm were cut using a Leica RM2245 (Germany) microtome and stained with haematoxylin and eosin. Slides from each gonad were examined using a Leica HI1210 light microscope (Leica Microsystems, Germany). The developmental stage of gonads was adapted according to Rocha and Rocha (2006) and Brown-Peterson et al. (2011).

2.4. Statistical analysis

Statistical analyses of data were conducted using STATISTICA 13 software. The data were expressed as means ± SEM. Significance was accepted at $P < 0.05$. For multiple comparisons of hormone concentrations (MEL, T_4, E_2 and 11-KT), analysis of variance (one-way ANOVA) was performed followed by *post hoc* tests (Spjotvoll and Stoline's test for unequal numbers of cases or LSD
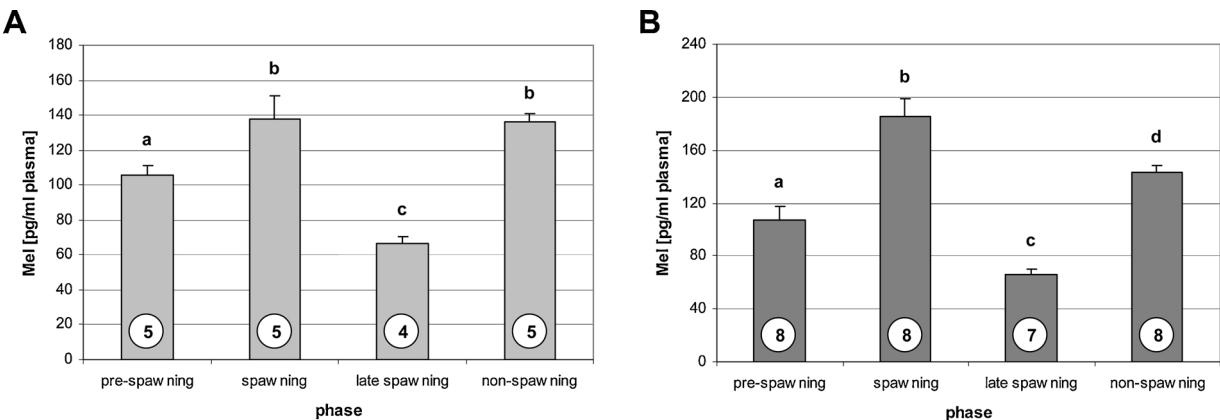
Fig. 1. Concentration of plasma melatonin in round goby females (A) and males (B) in different phases of the reproductive cycle; Data are presented as mean ± SEM, numbers of individuals are given in circles; Different superscripts indicate statistical differences among phases ($P < 0.05$).

test, and NIR test; as required). For comparisons of all hormone concentrations in plasma and gonads between males and females, Student's t-test was used.

2.5. Ethical note

All procedures performed in this study complied with the EC Directive 2010/63/EU for animal experiments and with the guidelines of the Local Ethics Committee on Animal Experimentation (Resolution No. 33/2010).

3. Results

3.1. Analysis of hormones

The pattern of changes in plasma Mel concentrations was similar in both sexes (Fig. 1). In females, there were the greatest concentrations of Mel in the spawning and non-spawning phases (Fig. 1). In males, the greatest concentration of Mel was during the spawning phase (Fig. 1). The differences in Mel concentration between females and males were observed only in the spawning phase ($P < 0.05$; Table 2).

The concentrations of plasma T_4 was of a similar pattern during the various reproductive phases in females and males (Fig. 2). In the non-spawning phase, in both sexes, there were the greatest concentrations of T_4 (Fig. 2). There were greater concentrations of T_4 in males and females in the pre-spawning phase (Fig. 2). In both sexes, there were the least concentrations of T_4 in the late spawning phase (Fig. 2A–B). There were differences in T_4 between females and males only during the non-spawning phase ($P < 0.05$; Table 2).

The patterns of E_2 changes in plasma and gonads were similar in both sexes (Fig. 3A–D). In females, there were the greatest concentrations of plasma E_2 in the pre-spawning and non-spawning phases (Fig. 3A). In ovaries, there was the greatest concentration of E_2 in the pre-spawning phase (Fig. 3B). In males, there were the greatest E_2 concentrations in plasma and gonads in the pre-spawning and non-spawning phases (Fig. 3C–D). The concentrations of E_2 in plasma and gonads of females were greater than those in males in all reproductive phases (Fig. 3A–D, Table 2).

Table 2
Statistical differences (P-values) in hormones between males and females of round goby in different phases of the reproductive cycle; significance was accepted at $P < 0.05$.

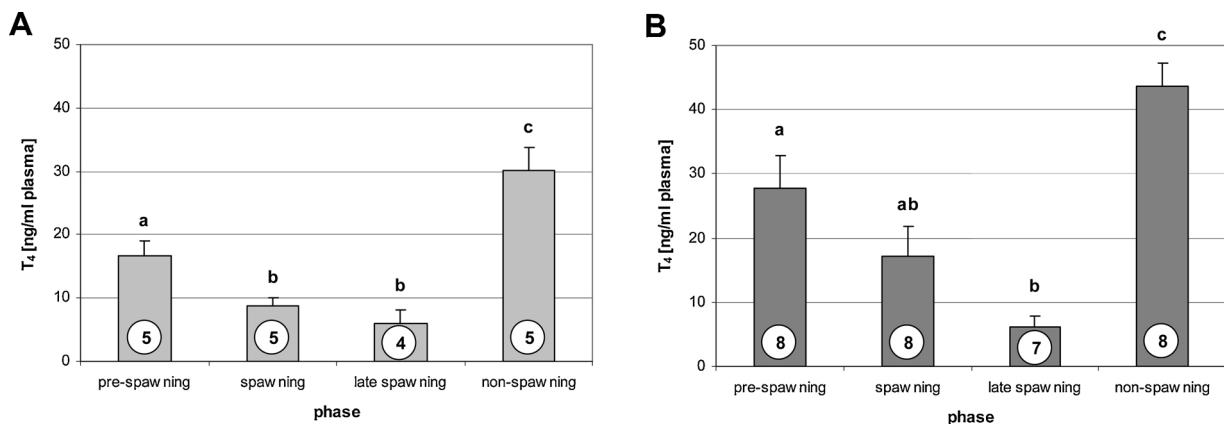|	Phase			
	pre-spawning	spawning	late spawning	non-spawning
	P			
Plasma				
melatonin	0.88	< 0.05	0.93	0.32
thyroxine	0.20	0.13	0.94	< 0.05
17β-estradiol	< 0.001	< 0.001	< 0.01	< 0.001
11-ketotestosterone	< 0.001	< 0.001	< 0.05	< 0.01
Gonads				
17β-estradiol	< 0.001	< 0.001	< 0.01	< 0.01
11-ketotestosterone	< 0.01	< 0.001	< 0.05	< 0.001

Fig. 2. Concentration of plasma thyroxine in round goby females (A) and males (B) in different phases of the reproductive cycle; Data are presented as mean ± SEM, numbers of individuals are given in circles; Different superscripts indicate statistical differences among phases ($P < 0.05$).
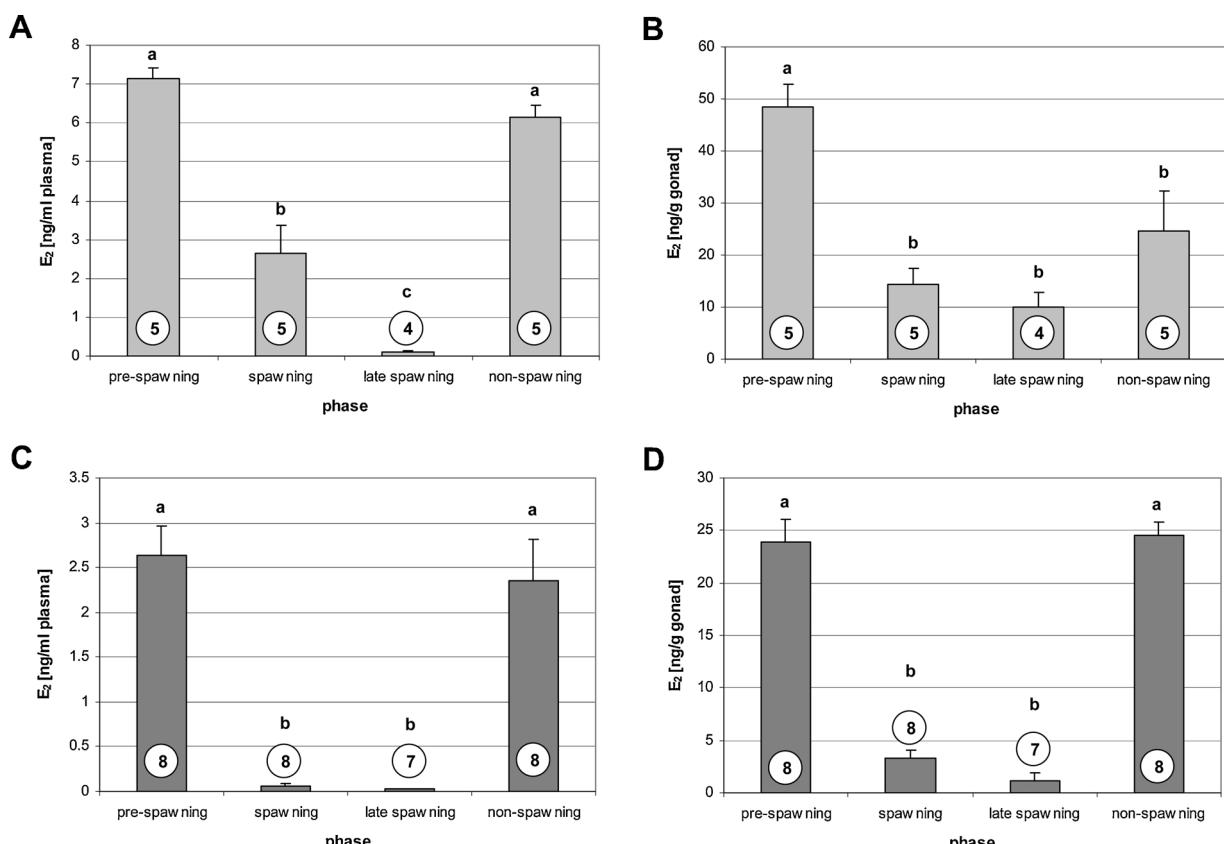

Fig. 3. Concentrations of 17β-estradiol in plasma and gonads of round goby females (A–B) and males (C–D) in different phases of the reproductive cycle Data are presented as mean ± SEM, numbers of individuals are given in circles; Different superscripts indicate statistical differences among phases ($P < 0.05$).

In females, 11-KT concentrations in plasma and gonads did not differ during the various reproductive phases (Fig. 4A–B). In males, there was a similar pattern of 11-KT in plasma and gonads with the greatest concentrations during spawning (Fig. 4C–D). The concentrations of 11-KT in plasma and gonads of males were greater than those of females during all reproductive phases (Fig. 3A–D; Table 2).
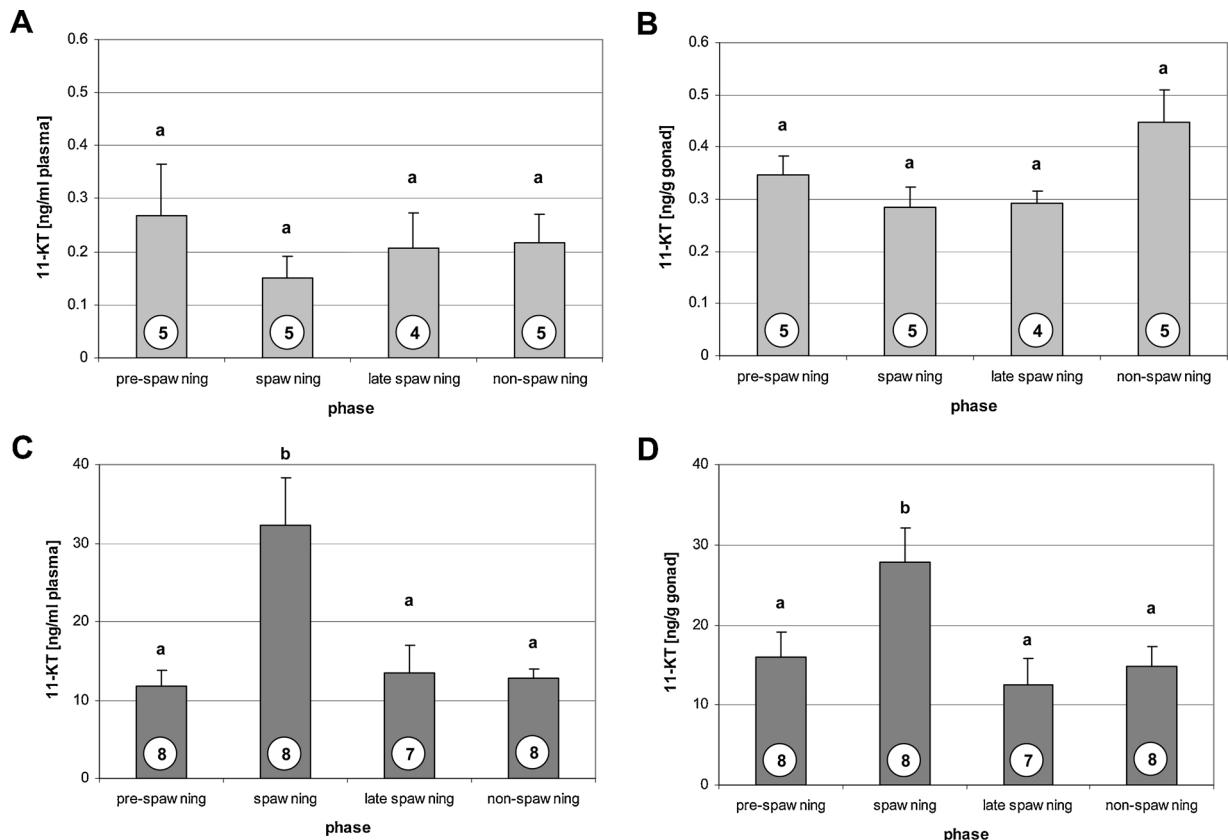
Fig. 4. Concentrations of 11-ketotestosterone in plasma nad gonads of round goby females (A–B) and males (C–D) in different phases of the reproductive cycle. Data are presented as mean ± SEM, numbers of individuals are given in circles; Different superscripts indicate statistical differences among phases ($P < 0.05$).

3.2. Histological analysis

On the basis of histological analysis of ovaries and testes, the following phases of the reproductive cycle in round goby were distinguished: pre-spawning, spawning, late spawning and non-spawning.

Ovaries of round gobies collected during the pre-spawning phase contained mostly follicles at the late stage of vitellogenesis (VTG), before there was germinal vesicle migration (GVM), and oocytes present at the previtellogenic stage (Fig. 5A). Ovaries collected in the spawning phase were characterized by the large number of oocytes at the early stage of GVM. The presence of postovulatory follicles (POFs) indicated these individuals were in the active spawning stage at the time of tissue collection. The ovaries also contained the developing batches of oocytes at the early stage of vitellogenesis (eVTG) and previtellogenic stage (PG), which is characteristic for batch spawners (Fig. 5C). In the late spawning phase, there were large numbers of POFs in the ovaries. Oocytes during GVM, eVTG and PG were also detected (Fig. 5E). During non-spawning phase, ovaries were in regression and contained mostly vitellogenic oocytes in the early atresia stage (A). In this phase, oocytes at the eVTG and PG stages were also present (Fig. 5G).

In the pre-spawning phase, maturing testes contained spermatogonia (SG) - an undifferentiated germ cell (Fig. 5B). In the spawning phase, testes were undergoing active spermatogenesis as indicated by the presence of large numbers of spermatozoa (SZ). Spermatogonial cells, SG and spermatocytes (SC), were also clearly visible during this phase (Fig. 5D). In the late spawning phase, testes were fully matured, filled with SZ (Fig. 5F). In the non-spawning phase, testes were in regression and SGs were the dominant cells. It was also noted that there were residual SZ in the lumen of narrowing seminiferous tubules (ST; Fig. 5H).

4. Discussion

The present study is the first attempt to document the changes in concentrations of plasma Mel and T_4 and plasma and gonadal E_2 and 11-KT as well as with histology of gonads in both sexes of round goby during four different phases of the reproductive cycle: pre-spawning, spawning, late spawning and non-spawning.
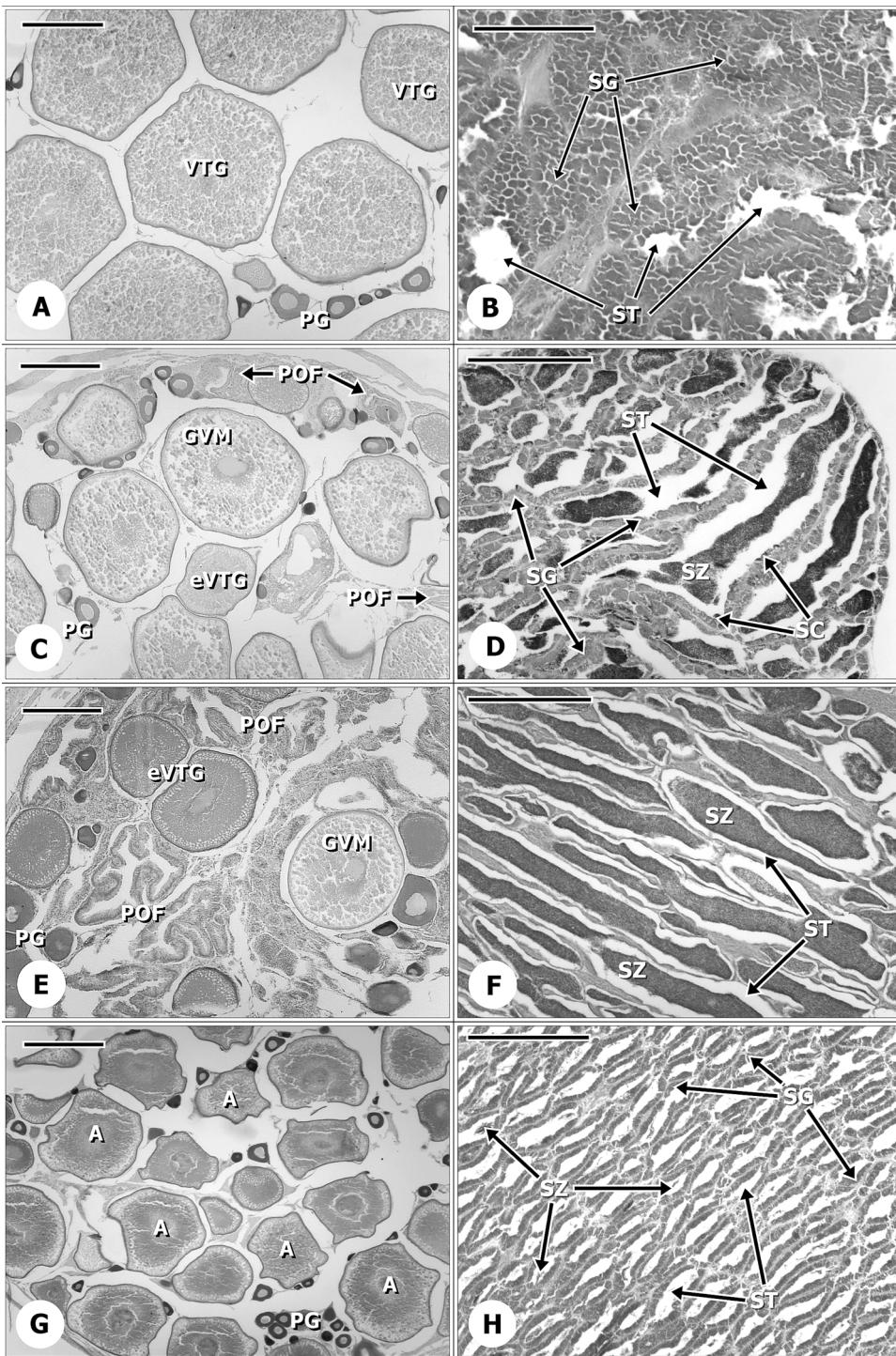
Fig. 5. Structures of ovaries and testes in round goby in different phases of the reproductive cycle. Pre-spawning phase: (A) Ovary with developing oocytes in the late stage of vitellogenesis (VTG); (B) Maturing testis with the dominance of spermatogonia (SG). Spawning phase: (C) Partially spent ovary (postovulatory follicles; POFs) with the next developing batches of oocytes in the early stage of maturation (germinal vesicle migration; GVM), early stage of vitellogenesis (eVTG) and previtellogenic stage (PG); (D) Testis under active spermatogenesis with visible spermatocytes (SC) and spermatozoa (SZ) in seminiferous tubules (ST). Late spawning phase: (E) Ovary with the dominance of POFs and the presence of GVM, eVTG and PG; (F) Fully developed testis with seminiferous tubules (ST) filled with SZ. Non-spawning phase: (G) Regressing ovary with the dominance of atretic vitellogenic oocytes (A); (H) regressing testis with the dominance of SG and residual SZ in the lumen of ST. Scale bars correspond to 500 μm.

4.1. Mel

In the present study, the plasma Mel concentrations were of a similar pattern in round goby females and males throughout the four phases where there were assessments. There were similar trends in the batch spawning common carp (*Cyprinus carpio*; Kezuka et al., 1988) and single spawning pacu (*Piaractus mesopotamicus*; Iseki et al., 2000). In round goby females, there were greater concentrations of plasma Mel in the pre-spawning phase. These findings are consistent with those of Maitra et al. (2013), where there was a greater concentration of Mel during the pre-spawning phase in the single spawning major carp (*Catla catla*) females. There were the greatest concentrations of Mel in the present study during the spawning phase of round goby females. At this phase, Mel is necessary for maturation processes and to protect the oocytes from free radicals during periods of rapid cell proliferation as has been reported to occur in the batch spawning mummichog (*Fundulus heteroclitus*; Lombardo et al., 2012) and zebrafish (*Danio rerio*; Carnevali et al., 2011) as well as in the single spawning major carp (Maitra and Hasan, 2016; Mondal et al., 2017). In the late-spawning phase, there were the least Mel concentrations in the present study, and the POFs were in predominant numbers compared with the numbers of oocytes that were in relatively greater numbers during other stages of development. The subsequent increase of Mel concentrations occurred in the non-spawning phase while there was ovarian regression occurring. There have been similar previous observations in the batch spawning three-spined stickleback (*Gasterosteus aculeatus*; Sokołowska et al., 2004), where there was an inhibitory effect on gonads. It resulted in gonadal regression that was attributed to having occurred because of the relatively greater Mel concentrations during this phase. Furthermore, during this phase, there was follicular atresia with the production of free radicals being a potential cause for the increased concentrations of Mel and other antioxidants as previously reported (Maitra and Hasan, 2016; Mondal et al., 2017) in major carp.

In round goby males of the present study, there was a greater concentration of Mel in the pre-spawning phase when testes were undergoing early maturation. Data from the present study are consistent with previous findings when there was study of the batch spawner three-spined stickleback (Borg and Ekström, 1981) and single spawner masu salmon (*Oncorhynchus masou*; Amano et al., 2000), where Mel stimulated gonadal development during the spring of the year. In round goby males, there was the greatest Mel plasma concentration in the spawning phase. Results of studies conducted in fish indicate that relatively greater Mel concentrations enhance the maturation of testes and sperm quality in a single spawning broadhead catfish (*Clarias macrocephalus*; Aripin et al., 2014). Therefore, the greater concentrations of Mel, in round goby males may also function as a defense against free radicals during the period of rapid cell proliferation that accompanies spermiogenesis, as it was presented in mammals (Gavella and Lipovac, 2000; Shang et al., 2004). In the present study, in the late spawning phase, when testes were completely matured, there was the least concentration of Mel. The decrease in Mel concentration at the end of spermiogenesis has also been reported in the three-spined stickleback (Sokołowska et al., 2004). There was a second increase in plasma Mel concentration in round goby males during the non-spawning phase when testes were undergoing regression. There has been a similar increase in Mel concentration observed after spawning in three-spined stickleback (Sokołowska et al., 2004) and in single spawning major carp (Bhattacharya et al., 2007). It has been suggested that in fish, relatively greater Mel concentrations may have an inhibitory effect on gonadotropins and induce regression of gonads (Fenwick, 1970; Maitra and Hasan, 2016). Probably, the increase in Mel concentration during this reproductive phase was also the result of free radical production during the regression of testicular tissue (Maitra and Hasan, 2016).

4.2. T$_4$

In the pre-spawning phase, there was a relatively greater concentration of T$_4$ in plasma of round goby females. Greater plasma concentrations of T$_4$ have also been reported during the pre-spawning phase in batch spawning of female goldfish (*Carassius auratus*; Hurlburt, 1977), plaice (*Pleuronectes platessa*; Osborn and Simpson, 1978), common dentex (*Dentex dentex*; Pavlidis et al., 2000) and Atlantic cod (Comeau and Campana, 2006). There have been reports that T$_4$ increases the growth rate of ovarian follicles and the number of vitellogenic follicles in this phase (Sage, 1973; Cyr and Eales, 1988; Legler et al., 2000; Supriya et al., 2005). In the spawning and late spawning phase, concentrations of T$_4$ in plasma of round goby females were basal. There have been similar reports of basal plasma T$_4$ during this reproductive phase in the batch spawning plaice (Osborn and Simpson, 1978), common dentex (Pavlidis et al., 2000) and Atlantic cod (Comeau and Campana, 2006). There was the greatest concentration of plasma T$_4$ in round goby females during non-spawning phase when the ovaries contained primarily vitellogenic oocytes undergoing atresia. There has been a similar post-reproductive surge of T$_4$ observed in such batch spawners as plaice (Osborn and Simpson, 1978), common dentex (Pavlidis et al., 2000) and Atlantic cod (Comeau and Campana, 2006). It was reported that the increase of T$_4$ in fish occurring after spawning might be associated with the function of T$_4$ in enhancing development of previtellogenic oocytes still persisting in regressing ovaries (Pavlidis et al., 2000) and also with ongoing steroidogenesis in the atretic vitellogenic oocytes (Cyr and Eales, 1988; Rinchard et al., 1993).

In males of round goby, there was a similar trend in changes of plasma T$_4$ concentration as in females in the present study. The decreasing concentrations of T$_4$ after the pre-spawning phase, and during the spawning to late spawning phases is a pattern that is consistent with progress in spermatogenesis. There have been reports of similar results for other species such as the batch spawning plaice (Osborn and Simpson, 1978) and striped bass (*Morone saxatilis*; Mylonas et al., 1997) or single spawning brown bullhead (*Ictalurus nebulosus*; Burke and Leatherland, 1983). In the present study, there was the greatest concentration of plasma T$_4$ during the non-spawning phase when testes were undergoing regression. There was also a similar increase in T$_4$ concentrations during this reproductive phase in plaice (Osborn and Simpson, 1978). Furthermore, Habibi et al. (2012) have reported that T$_4$ inhibits steroidogenesis in the batch spawning goldfish.

4.3. E$_2$

In the pre-spawning phase of the present study, when a large population of the oocytes commenced active vitellogenesis, there were the greatest concentration of E$_2$ in plasma and gonads of round goby females. There were relatively greater plasma E$_2$ concentrations during the pre-spawning phase in batch spawning females of the goldfish (Kagawa et al., 1983a), Atlantic halibut (*Hippoglossus hippoglossus*; Methven et al., 1992), tench (*Tinca tinca*; Pinillos et al., 2003), chub (*Leuciscus cephalus*; Guerriero, 2007), kutum (*Rutilus frisii kutum*; Sabet et al., 2009) and river catfish (*Hemibagrus nemurus*; Adebiyi et al., 2013). Furthermore, there have been *in vivo* and *in vitro* studies reported describing a relatively greater amount of E$_2$ production by vitellogenic oocytes during the pre-spawning phase in females of the batch spawning tilapia (*Oreochromis mossambicus*; Cornish, 1998), goldfish (Kagawa et al., 1984) and single spawning rainbow trout (*Oncorhynchus mykiss*; van Bohemen and Lambert, 1981). In the spawning phase, E$_2$ concentrations were less in plasma and ovaries of round goby. There are many batch spawning fish species such as the goldfish (Kagawa et al., 1983a), sea bass (Prat et al., 1990), gudgeon (Rinchard et al., 1993), Jundiá (*Rhamdia quelen*; Barcellos et al., 2001) and gilthead seabream (*Sparus aurata*; Kadmon et al., 1985) in which plasma E$_2$ concentration is basal during spawning. In round goby, as well as in other batch spawners during the spawning phase, besides oocytes at early stages of vitellogenesis and maturation, the ovaries contain POFs (Kagawa et al., 1984; Rinchard et al., 1993; Barcellos et al., 2001). The POFs, like other oocytes occurring in ovaries during this phase, produce also a very small amount of E$_2$ (Kagawa et al., 1983b), which could explain the relatively lesser E$_2$ concentrations in gonads. In the late spawning phase, there were minimal concentrations of E$_2$ in plasma and there were relatively lesser concentrations in ovaries during this reproductive phase in female round goby. At the end of spawning, there were relatively lesser concentrations of E$_2$ in other batch spawning teleosts, such as the common dentex (Pavlidis et al., 2000), tench (Pinillos et al., 2003), chub (Guerriero, 2007) and mahseer (*Tor tambroides*; Ismail et al., 2011). Further, the basal level concentration of E$_2$ in round goby ovaries when there was a predominance of POFs is consistent with results previously reported by Kagawa et al. (1983b) where there were minimal concentrations of E$_2$ in ovaries following ovulation in the single spawning amago salmon (*Oncorhynchus rhodurus*). During the non-spawning phase in round goby, there was the second greatest concentration of E$_2$ in plasma and greater concentrations of E$_2$ in regressed ovaries as compared with the other reproductive phases in the present study. Similarly, during regression of ovaries, there were greater concentrations of E$_2$ in the plasma of the batch spawning goldfish (Khoo, 1975) and gudgeon (Rinchard et al., 1993). Furthermore, Rinchard et al. (1993) postulated that during regression, atretic vitellogenic oocytes continue to undergo steroidogenesis, which seems to be a sufficient explanation for this observation in the present study.

In round goby males, there were the greatest concentrations of E$_2$ in plasma and testes in the pre-spawning and non-spawning phases. Relatively greater concentrations of E$_2$ have been observed in plasma of males of the single spawning Japanese huchen (*Hucho perryi*) (Amer et al., 2001) and the batch spawning gilthead seabream (Chaves-Pozo et al., 2007). The greatest concentration of E$_2$ in testes of round goby in the pre-spawning phase appears to be associated with the function of this hormone in the early stages of spermatogenesis. Miura et al. (1999) have reported that there are functions of E$_2$ in the stimulation of spermatogonial stem cell division in the Japanese eel (*Anguilla japonica*). Whereas, the greatest concentration of E$_2$ observed in testes of the non-spawning round goby is probably associated with the suppression of spermatogenesis. The E$_2$ inhibits spermatogonial proliferation and induces spermatogonial atresia (Amer et al., 2001; Chaves-Pozo et al., 2005, 2007; Basak et al., 2016). There were lesser concentrations of E$_2$ in plasma and gonads of round goby males in spawning and late-spawning phases. There were lesser concentrations of E$_2$ during the spawning season in the serum of the single spawner Japanese huchen (Amer et al., 2001) and in testes in the Japanese eel (Miura et al., 1999; Miura and Miura, 2001). The role of E$_2$ in spermatogenesis appeared to be less during spawning than in other reproductive phases.

4.4. 11-KT

During the reproductive cycle in round goby females, concentrations of 11-KT in plasma and gonads were less than in males which is consistent with 11-KT being a male-specific hormone in most of the fish species (Borg, 1994; Schulz et al., 2010). Furthermore, the concentrations of 11-KT in plasma and ovaries did not differ during reproductive phases in the present study. There is little information available regarding the functions of 11-KT in the reproductive cycle of female fish. Results from *in vitro* studies in females of the short-finned eel (Lokman et al., 2007), Atlantic cod (Kortner et al., 2008, 2009) and Japanese eel (Endo et al., 2011), however, indicate that 11-KT is one of the factors stimulating the growth of previtellogenic oocytes. In the present study, the previtellogenic oocytes were present in ovaries in similar quantities during all reproductive phases that coincide with the aligned concentrations of 11-KT during the same reproductive phases.

In round goby males, there were the greatest concentrations of 11-KT during the spawning phase in both plasma and testes. The concentrations of 11-KT were associated with the process of active spermatogenesis in the developing testes. These results are consistent with several reports in which concentrations of 11-KT are greatest at the beginning of male spawning and decrease with the advancement of sperm production and testes regression (Borg, 1994; Schulz et al., 2010). For example, a similar pattern was described in batch spawning pejerrey (Elisio et al., 2015) and Atlantic cod (Dahle et al., 2003).

5. Conclusions

There were the greatest Mel concentrations in the spawning and non-spawning phases in the present study, therefore, this hormone appears to have important functions in determining a time frame for spawning in round goby females and males. The greatest concentrations of T$_4$ and E$_2$ occurred in the pre-spawning and non-spawning phase, in both females and males, indicating

these hormones are integral in affecting the tissues of the round goby during the active spawning phase and also the timing of initiation of spawning (stimulation of gonad development in pre-spawning phase). The greatest concentrations of 11-KT in males was during the spawning phase and, therefore, this hormone appears to be functional in spermatogenesis in the developing testes. In females, however, there were no changes in 11-KT concentrations during the various reproductive phases. This finding is most likely related to the stimulatory role of 11-KT in the growth of previtellogenic oocytes, which were present in ovaries in similar quantities during all reproductive phases.

Conflict of interests

There is no conflict of interests.

Acknowledgments

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