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Changes in brain arginine vasotocin, isotocin, plasma 11-ketotestosterone and cortisol in round goby, *Neogobius melanostomus*, males subjected to overcrowding stress during the breeding season

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ABSTRACT

In natural spawning grounds, breeding round goby, *Neogobius melanostomus*, males are exposed to various social stimuli, including high density of same-sex competitors and separation from females. We hypothesize that breeding males subjected to overcrowding in the wild experience high stress that affects their socio-sexual behavior and their relationships among conspecifics. We designed an experiment to mimic natural stimulation when highly aggregated breeding males are subjected to same-sex opponents. Males were sampled sequentially from experimental tank stocked at decreasing fish densities of 15 fish/m^2, 9 fish/m^2 and 4 fish/m^2. We studied the effects of overcrowding on male behavior and selected hormones, brain arginine vasotocin (AVT) and isotocin (IT) and plasma 11-ketotestosterone (11-KT) and cortisol as these are known to play roles in reproduction and related social interactions. The highest brain AVT and plasma cortisol levels were measured in non-aggressive males kept in the overcrowded group of 15 fish/m^2. IT level was elevated in fish kept at the lower density of 9 fish/m^2, and at which the males began to display territoriality and aggression. The plasma level of 11-KT was similar in all the males. Brain AVT and IT and plasma cortisol along with behavioral observations can be applied as species-specific indicators of the well-being of round goby males.
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1. Introduction

Fish experience different types of stress throughout their life cycles; these include high density, confinement, sex-separation, food-deprivation, disturbance, and the threat of intruders. The sexually active round goby (*Neogobius melanostomus*) is a cavity-spawning fish that exhibits complex socio-sexual behaviors, including territoriality, nesting, social hierarchy, and aggression. During the breeding season in the wild, the round goby occurs in large aggregations in spawning grounds with densities at preferred rocky sites ranging from 3 to 20 fish/m^2 (Charlebois et al., 1997; Ray and Corkum, 2001; Sapota, 2005; Sapota and Skóra, 2005). Since the round goby is a batch spawner, the males are periodically separated from the females when they migrate offshore to feed in deeper waters. Thus, round goby males are exposed to social cues that are stress-evoked, including high densities, temporary separation from females, deficient space and nesting refuge, and the threat of same-sex opponents. High density-dependent stress has a strong impact on relationships between individuals, sexual selection, and consequently, breeding success (Lehtonen and Lindstrom, 2008; Kaspersson et al., 2010). Thus, it can be assumed that males that are subjected to overcrowding are unable to establish territories or select nesting sites. We studied the effects of overcrowding on behavior and hormone levels in sexually active round goby males exposed to same-sex conspecifics under controlled laboratory conditions; the focus was on the following hormones: arginine vasotocin (AVT), isotocin (IT), 11-ketotestosterone (11-KT), and cortisol, which are known to affect social, aggressive and male-typical behavior in fish during the spawning season (Mommsen et al., 1999; Páll et al., 2002a,b; Almeida et al., 2012; Kleszczyńska et al., 2012).

AVT and IT are nonapeptides synthesized in the neurosecretory neurons within the preoptic nucleus and stored in the neurohypophysis (Saito et al., 2004). Both of these neurohormones play roles in a variety of reproductive behaviors, and related social interactions such as nest-building, courtship, parental care, spawning reflex, vocal activity, social hierarchy, and competition for mates and nesting (Pickford and Strecker, 1977; Goodson and Bass, 2000a,b; Grober et al., 2002; Salek et al., 2002; Ripley and Foran, 2010; Almeida et al., 2012; Kleszczyńska et al., 2012). Some studies have also demonstrated that AVT induces the aggressive behavior (Goodson et al., 2004; Larson et al., 2006; Backström and Windberg, 2009) associated with reproduction in territorial fish. There is evidence of changes in AVT and IT concentrations in fish exposed to different kinds of stress, i.e., high density and food deprivation (Mancera et al., 2008; Kulczykowska et al., 2009;

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Kleszczyńska and Kulczykowska, 2013), confinement (Gilchriest et al., 2000; Balment et al., 2006), osmotic and disturbance (Kulczykowska, 2007; Kagawa, 2008). It is generally accepted that 11-KT, an important teleost androgen, is effective in stimulating secondary sexual characters and male reproductive behavior, i.e., nuptial coloration and courtship during breeding (Cardwell and Liley, 1991; Borg, 1994; Páll et al., 2002a,b). Furthermore, 11-KT is of high importance also in the reproductive round goby males: parental males which exhibit secondary sexual characteristics has higher plasma 11-KT concentration than non-parental sneaker males (Marentette et al., 2009). It is postulated that 11-KT which plays an instrumental role in male behaviors also impacts the main neuropeptides of hypothalamo-neurohypophysial systems – AVT in fishes (Goodson and Bass, 2001). Cortisol, the main corticosteroid in fish, is an essential component of the stress response, including overcrowding stress in fish (Mommsen et al., 1999; Ramsay et al., 2006).

This experiment was designed to mimic the stress stimulation of round gobies in the wild where highly aggregated breeding males are separated from females. Brain AVT and IT, and plasma 11-KT and cortisol concentrations together with aggression levels were determined in males kept at densities of 15 fish/m^2, 9 fish/m^2 and 4 fish/m^2.

2. Materials and methods

2.1. Fish sampling and experimental protocols

Round goby males were caught in the coastal zone of the Gulf of Gdańsk in Hel harbor (southern Baltic Sea) with a fyke net to depths of 5 m. The fish (n = 61, 125–235 mm body length) were kept outdoors in a 2400-L experimental housing tank (2 m × 2 m × 0.6 m). The fish were acclimatized for one week prior to experiment. The tank was equipped with an open circuit brackish water system. The fish were exposed to the natural photoperiod (15 L:9D) and temperatures of 17–20 °C. The fish were fed *ad libitum* with frozen herring *Clupea harengus* and *Mytilus edulis trossulus* daily, at 15:00 h. Fish behavior was observed daily every 2 h from 09:00 h to 19:00 h for 30 min.

The fish were sampled randomly from the experimental tank three times at 7-day intervals as follows: after 7 days (25 males were taken), after 14 days (20 males were taken) and after 21 days of experiment (16 males were taken) (Table 1). Thus, the males were kept in the same housing tank in a sequence of decreasing density from 15 fish/m^2 to 9 fish/m^2 and then 4 fish/m^2. This sampling protocol gave the opportunity to observe changing behavior in males coming from the same (experimental) fish community. Noteworthy, sequential fish sampling from one initial group imitates the situation in nature where male density decreases over the course of the breeding season. In the wild, round goby male abundance at spawning grounds is the highest at the beginning of breeding (Sapota and Skóra, 2005), but it gradually declines because of male–male territorial competitions and formation of a dominance hierarchy. In addition, fish housed in the same tank avoided disturbance stress and tank-changing effect.

The tank used in the current experiment had no shelters so that conditions were similar for all the fish. The males failed to establish typical territories, including nesting sites. Thus, "territory" was regarded as the few centimeters of home range where the "resident" male exhibited aggression towards approaching conspecifics. Levels of aggression were measured based on the numbers and types of responses: approach - slow or advancing movements towards another fish; chase - quick or darting movements towards another fish; hit - the "resident" male butts or bites another fish. This methodology is recommended for aggression measurement in nest-guarding round goby males by Wickett and Corkum (1998). We propose the following three-point scale of aggression:

level 1 – the majority of males exhibit quiescent behavior, aggression is sporadic, approaches below 20 times, chases below 10 times, hits below 5 times per hour;
level 2 – the majority of males exhibit enhanced aggressive behavior, approaches from 20 to 50 times, chases from 10 to 20 times, hits from 5 to 10 times per hour;
level 3 – the majority of males exhibit advanced aggressive behavior, frequent offensive interactions, approaches over 50 times, chases over 20 times, hits over 10 times per hour.

No mortality was observed during the experiment.

Table 1
Experiment schedule.

Duration of experiment	7 days	14 days	21 days
Number of males in experimental group	61	36	16
Fish density	15 fish/m^2	9 fish/m^2	4 fish/m^2
Number of sampled males	25	20	16

2.2. Analytical methods

2.2.1. Brain AVT and IT assay

The fish were killed by severing the spinal cord and the whole brains for AVT and IT measurement were removed, immediately frozen and stored at −70 °C until HPLC analysis. Frozen brains were defrosted, weighed (50–176 mg) and sonicated separately in 1 mL distilled water using Microson™ XL 2000. Glacial acetic acid (2.5 μL) was added to homogenates, and the mixtures were placed in a boiling water bath for 3.5 min. The extracts were cooled on ice, and then centrifuged at 17 970 rcf for 20 min at 4 °C. AVT and IT were extracted by solid-phase extraction method (SPE) using C_{18} Bakerbond cartridges (J.T. Baker, Phillipsburg, NJ, USA; 100 mg/mL). The supernatants were decanted and loaded onto previously equilibrated (3 mL methanol, 3 mL distilled water) SPE columns. After washing by-products with water (1 mL) and methanol (5%, 1 mL), peptides were eluted with mixture of ethanol:6 N HCl (2000:1, 2 × 1 mL). Eluent was evaporated, and the residue was dissolved in 80 μL of 0.1% TFA in acetonitrile: H_2O (3:1). Next, the derivatization of peptides was applied according to Gozdowska and Kulczykowska (2004) with modifications. Briefly, 40 μL phosphatic buffer (0.2 M, pH 9.0) was added to 40 μL of sample and mixed. The derivatization was performed using 2 μL NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole; 30 mg/mL in acetonitrile) in 60 °C for 3 min, and then the reaction mixture was immediately cooled on ice. Next, 20 μL HCl (1 M) was added and 50 μL of the mixture was injected directly to HPLC (Shimadzu HPLC Prominence System). Chromatographic analysis was performed according to Gozdowska and Kulczykowska (2004). Briefly, HPLC separation was done on an Ultrasphere ODS column (Beckman, 250 × 4.6 mm, 5 μm particle) connected with precolumn (ODS, Beckman, 45 × 4.6 mm, 5 μm particle, the mobile phase consisted of solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in acetonitrile: H_2O (3:1)). A linear gradient was 48–80% of solvent B in 20 min. The column temperature was set at 20 °C and flow rate at 1 mL/min. Fluorescence detection was carried out at 530 nm with excitation at 470 nm. Recovery of peptides was in the range 89–93% for AVT and IT. The detection limit was determined to be 100 fmol/mL. Intra-day repeatability expressed as relative standard deviation was 2–4.5% and 5.3–8.2% for AVT and IT, respectively; inter-day precision was in the range 2.5–5.5% and 5.5–8.5% for AVT and IT, respectively.

2.2.2. Plasma 11-KT assay

Blood samples for 11-KT were collected by cardiac puncture. Plasma was separated by centrifugation in heparinized tubes at 20 630 rcf for 10 min and stored at −70 °C until analysis. Plasma 11-KT concentration was determined by competitive enzyme immunoassay (EIA) with preceding extraction procedure. The assay was performed using a Cayman EIA kit (Ann Arbor, MI, USA). Extraction of plasma samples

(100 μL) was performed with ethyl actetae/hexane (50:50 v/v) in 4 × the sample volume according to the method recommended by producer. Then, the samples were frozen which allowed the separation of layers. The ethyl acetate/hexane layer was transferred to a clean glass tube and evaporated under a gentle stream of nitrogen. This step was repeated three times. Dried extracts were stored at −20 °C until further analysis. The recovery of extraction was found in the range 98 to 115%. 11-KT-acetylocholinesterase conjugate was used as tracer. Extracts were dissolved in 2 mL of EIA buffer and 50 μL of the diluted samples were used for the EIA analysis. The standard curve was prepared from 11-KT standard (10 ng/mL) by dilution method. The standard curve consisted of eight standards of the following concentrations: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 pg/mL. After the addition of tracer and specific anti-11-KT antibody, microplate containing samples, blanks, non-specific binding (NSB), Bo and the standard curve, was gently shaken for 15 min, and then incubated for 18 h at 4 °C. After incubation, plate was rinsed with wash buffer using strip-washer for microplate HydroFlex (Tecan, Austria). Optimal color was obtained by shaking with Ellman's reagent in the dark for 90 min. The plate was read at 412 nm using Sunrise Absorbance Reader (Tecan). All samples were assayed in duplicate. The detection limit of assay was 1.3 pg/mL. The intra-assay coefficient of variation was 0.8%. The inter-assay variation was not determined, because all samples were measured in the same assay.

2.2.3. Plasma cortisol assay

Blood samples for cortisol were collected by cardiac puncture. Plasma was separated by centrifugation in heparinized tubes at 20 630 rcf for 10 min and stored at −70 °C. Plasma cortisol concentration was determined using EIA with preceding extraction procedure. The assay was performed using Cayman's EIA kit. Plasma samples were acetated with 3 M HCl to pH 1.5–3. Extraction of plasma samples (100 μL) was performed with methylene chloride according to the method recommended by producer. Then, the samples were frozen which allowed the separation of layers. The methylene chloride layer was transported to a clean glass tube and evaporated under a gentle stream of nitrogen. This step was repeated three times. Dried extracts were stored at −20 °C until further analysis. The recovery of extraction was found in the range 89 to 110%. Extracts were dissolved in 2 mL of EIA buffer and 50 μL of the diluted samples were used for the EIA analysis. The standard curve was prepared from cortisol standard (400 ng/mL) by dilution method. The standard curve consisted of ten standards with the following concentrations: 20, 10, 4, 1.6, 0.64, 0.256, 0.102, 0.041, 0.0164, 0.0066 ng/mL. After the addition of tracer and specific anti-cortisol antibody, microplate containing samples, blanks, NSB, Bo and the standard curve, was gently shaken for 15 min, and then incubated overnight at 4 °C. After rinsing the plate, optimal color was obtained by shaking with Ellman's reagent in the dark for 60 min. The plate was read at 412 nm using Sunrise Absorbance Reader (Tecan). All samples were assayed in duplicate. The detection limit of assay was 0.011 ng/mL. The intra-assay coefficient of variation was 0.9%. The inter-assay variation was not determined, because all samples were measured in the same assay.

2.3. Statistical analysis

Values are presented as means ± standard error of the mean (S.E.M.). Normality (W Shapiro-Wilk's test) and homogeneity (Levene's test) of variances were assessed prior to the use of statistical analysis. Statistical differences between number of males showing various levels of aggression were measured by multiple comparisons of the analysis of variance (one-way ANOVA). Significance was taken at $P < 0.001$. Due to non-normal distribution and non-homogenous variances of part of hormonal data (AVT data: W Shapiro-Wilk test $P < 0.001$; cortisol data: W Shapiro-Wilk test $P < 0.001$, Levene's test $P < 0.001$) and unequal sample sizes, a non-parametric tests were applied. Data of AVT, IT, 11-KT and cortisol were compared with respect to density by Kruskal-Wallis's ANOVA rank test followed by a Mann–Whitney's *U*-test. Significance was considered at $P < 0.05$. Correlation between hormone levels and fish stocking densities and levels of aggression, and between fish stocking densities and levels of aggression was assessed by Spearman's rank correlation analysis at a significance level of $P < 0.05$. Statistical analyses were carried out using Statistica 8.1 software (Sokal and Rohlf, 1995).

3. Results

3.1. Variation in hormone levels

The experimental treatment caused marked changes in brain AVT and IT concentrations (Kruskal–Wallis test, $P < 0.001$) (Fig. 1). The highest brain AVT level was measured in males kept at the highest density of 15 fish/m^2 (Mann–Whitney *U*-test, $P < 0.001$). Brain AVT concentration decreased along with decreasing fish density (Spearman's rank correlation, $n = 59$, $R = 0.707240$, $P < 0.001$). The brain IT level was the highest at a stocking density of 9 fish/m^2 (Mann–Whitney *U*-test, $P < 0.001$).

The plasma concentration of 11-KT was not influenced by stocking density (Kruskal–Wallis test, $P = 0.9352$).

The experimental conditions affected markedly the plasma cortisol concentration (Kruskal–Wallis test, $P < 0.001$). The highest plasma cortisol level was measured at the density of 15 fish/m^2 (Mann–Whitney *U*-test, $P < 0.001$). Plasma cortisol dropped along with fish density (Spearman's rank correlation, $n = 31$, $R = 0.799726$, $P < 0.001$).

3.2. Behavioral changes

The number and percentage of round goby males exhibiting aggression at various levels after exposure to different stocking densities are presented in Table 2. At the density of 15 fish/m^2, the majority of fish were quiescent, inactive, behaved like subordinates, and only sporadically showed aggressiveness (level 1). Only a few males exhibited more frequent approaches and chases (level 2). At the lower stocking densities of 9 and 4 fish/m^2, the fish were more active and demonstrated distinct aggression, including frequent attacks on approaching intruders (level 3). Round goby male aggression increased as fish density decreased (Spearman's rank correlation, $R = -0.720680$, $P < 0.001$) and correlated negatively with brain AVT and plasma cortisol concentrations (Spearman's rank correlation, $R = -0.681796$, $P < 0.001$ and $R = -0.746108$, $P < 0.001$, respectively).

4. Discussion

Fish experience different types of stress during their life cycle, i.e., confinement, high density, sex-separation, or disturbance. Different fish species exhibit various behavioral responses and divergent coping strategies towards stressors (Koolhaas et al., 1999; Øverli et al., 2007; Martins et al., 2012). In the wild, at the onset of breeding, round gobies are exposed to a high density of same-sex conspecifics aggregated in the spawning grounds (Ray and Corkum, 2001; Sapota and Skóra, 2005). The density of round gobies during breeding in the wild ranges from 5 to 9 fish/m^2 (Ray and Corkum, 2001), and the preferred distance between territorial males is about 1 m (Sapota, 2005). Thus, in our study, same-sex group of 15 males/m^2 is considered as overcrowding.

The highest brain AVT concentration was observed in overcrowded males. In three-spined stickleback males (*Gasterosteus aculeatus*) which also exhibits territorial and nesting behavior during breeding, the brain AVT level is also elevated after exposure to high density of conspecifics (Kleszczyńska and Kulczykowska, 2013). Also, plasma AVT level is enhanced in immature gilthead sea bream (*Sparus auratus*) exposed to high density (Mancera et al., 2008; Kulczykowska et al.,

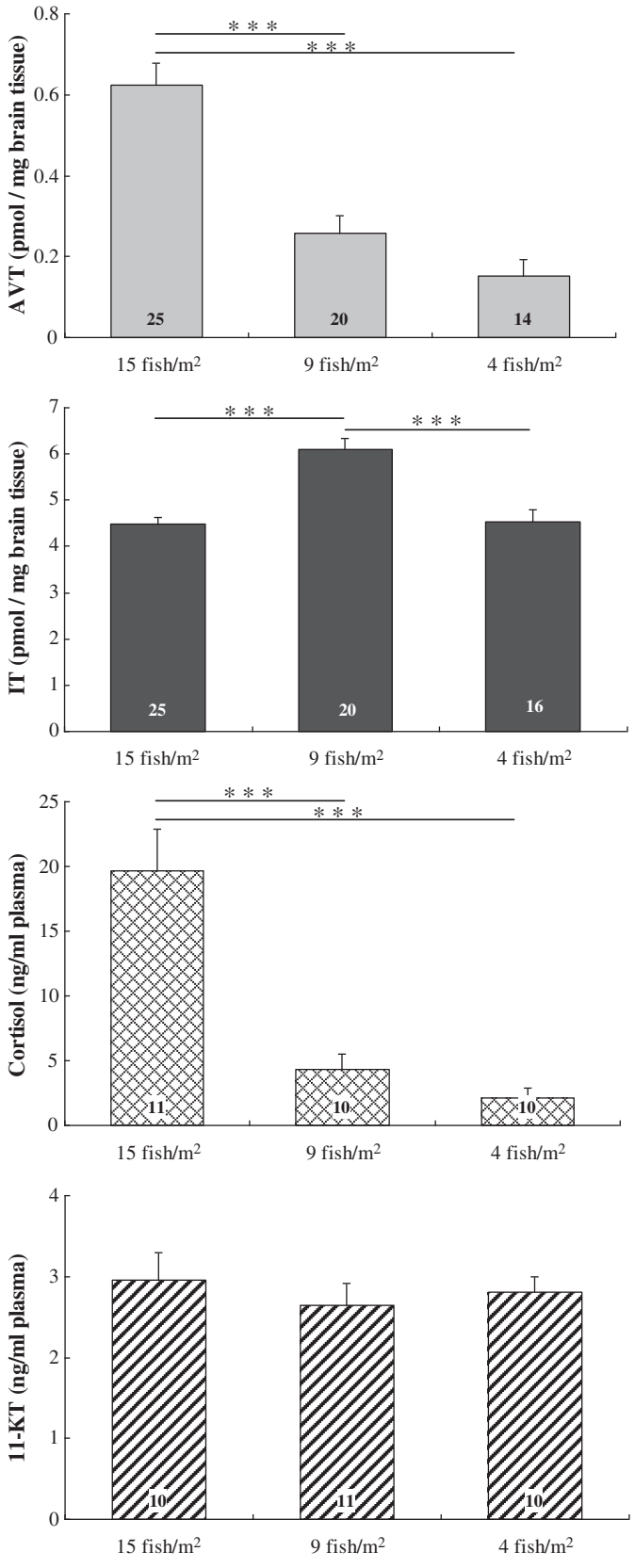

Fig. 1. Changes in brain concentration of AVT and IT, and plasma concentration of cortisol and 11-KT in males of the round goby *N. melanostomus* after exposure to densities of 15 fish/m^2, 9 fish/m^2 and 4 fish/m^2. Number of sampled males is given in the bars. The values are presented as means ± S.E.M. Significant differences are indicated as ***$P < 0.001$.

Table 2
Round goby males on particular levels of aggression after exposure to different densities. Significant differences are at the $P < 0.001$ level.

Fish density	15 fish/m^2		9 fish/m^2		4 fish/m^2	
Aggression	Number of males	%	Number of males	%	Number of males	%
Level 1	33	54	4	10	-	-
Level 2	28	46	12	35	3	21
Level 3	-	-	20	55	13	79
Total	61	100	36	100	16	100

2009). Similarly to high density-dependent effects, confinement and restraint also stimulate brain AVT in fish. Bond et al. (2007) demonstrate the elevated expression of pro-AVT mRNA in magnocellular neurons of the preoptic area (POA) in flounder (*Platichthys flesus*) exposed to acute restraint stress. A significantly higher level of AVT mRNA is also observed in the forebrain of rainbow trout (*Oncorhynchus mykiss*) in response to confinement stress (Gilchriest et al., 2000; Backström et al., 2011). As was described in Introduction, overcrowding is stressful for breeding round goby males. We reveal that plasma cortisol level was the highest in high density group of 15 fish/m^2, which confirms that overcrowded males experienced severe stress at the beginning of breeding. Apparently, round gobies present a reactive coping style characterized by the high production of corticosteroid hormones in response to severe stress (Koolhaas et al., 1999; Øverli et al., 2007).

In our study, chronic overcrowding stress that caused increase of brain AVT corresponded with fish passivity without aggression. The present results are in accordance with the findings from studies by Backström and Windberg (2009) where intracerebroventricularly administered AVT inhibits aggression in juvenile rainbow trout. Also, exogenous AVT is known to decrease the number of chases towards individuals by territorial bluehead wrasse males (Semsar et al., 2001). The number of AVT-producing neurons in the POA is higher in subordinate, quiescent clown anemonefish (*Amphiprion ocellaris*) males (Iwata et al., 2010). AVT also inhibits aggressive behavior in some bird species. Treatment with AVT attenuates crowding-induced aggression in starlings (Nephew et al., 2005), and it reduces aggression in territorial species such as field sparrows (Goodson, 1998).

It is noteworthy that non-aggressive round goby males with high AVT in the overcrowded group displayed the lowest levels of social approach. This effect of a high AVT level on behavior corresponds with data from a study by Thompson and Walton (2004), in which centrally administrated AVT inhibits approach responses toward same-sex conspecifics in male goldfish. Moreover, in studies of male goldfish, Thompson and Walton (2004) and Walton et al. (2010) show that inhibition of approach occurs with an absence of aggression.

In the groups of round goby males of decreasing densities, 9 and 4 fish/m^2, we observed lower AVT concentration together with increasing aggressiveness and territoriality that are signs of the formation of a dominance hierarchy. Apparently, social spacing enables to demonstrate aggressive responses in dominants and then induces avoidance and withdrawals in subordinates. Iwata et al. (2010) show that the number of AVT-ir neurons in the POA of clown anemonefish declines as a social hierarchy forms in this species. Zebra finch males also exhibit aggressive competition and have a small number of AVT-ir neurons (Goodson et al., 2009). On the other hand, fish aggressiveness can correspond with high concentrations of brain AVT. For example, aggressive dominant three-spined stickleback males that take care of eggs demonstrate the highest brain concentration of AVT (Kleszczyńska et al., 2012). Also, aggressive dominant zebrafish (*Danio rerio*) and plainfin midshipman males that guard eggs in nests have larger AVT-ir neurons in the POA than do male subordinates or those without nests (Foran and Bass, 1998; Larson et al., 2006). Greenwood et al. (2008) show that aggressive, territorial dominant African cichlid fish (*Astatotilapia burtoni*) males exhibit higher

levels of AVT mRNA expression in the whole brain than do non-territorial subordinate males. It appears that the link between AVT and aggressiveness is species-specific.

The role of IT in round goby males seems to be opposite to AVT action during breeding season. The highest IT concentration was measured in aggressive males kept at a density of 9 fish/m^2. Aggressiveness in this fish species is a sign of readiness for breeding. We hypothesize that an increased level of IT is a signal that triggers male reproductive behavior. Overcrowding at the beginning of the breeding season inhibits this behavior. A higher brain IT level in aggressive, dominant three-spined stickleback males that court females and defend territory has been reported by Kleszczyńska et al. (2012). Moreover, studies performed in goldfish reveal that IT stimulates social approach behavior towards conspecifics (Thompson and Walton, 2004). O'Connell et al. (2012) demonstrate that IT promotes reproductive behavior in aggressive paternal cichlid fish (*Amatitlania nigrofasciata*) males. Furthermore, administered OT, a mammalian homologue of IT, promotes sexual activity in African catfish (Viveiros et al., 2003). The highest IT level in males that exhibit aggression and territoriality and the highest concentration of AVT in non-aggressive males presented in our study indicate the different roles of AVT and IT in round goby males during reproduction. Thompson and Walton (2004) also report that AVT and IT induce the opposite effects on social behavior in male goldfish: AVT reduces social approach while IT induces it.

The plasma 11-KT in the round goby males during the experiment did not change irrespectively of social conditions or increasing aggression and territoriality. Similarly, it has been shown that the escalation of male aggressiveness in cichlid fish is independent of plasma 11-KT (Oliveira et al., 2005; O'Connell et al., 2012). Oliveira et al. (2001) demonstrate that exogenous 11-KT does not affect the behavior of peacock blenny (*Salaria pavo*) sneaker males. Moreover, gonadectomy does not influence aggression or breeding behavior in male bluehead wrasse (*Thalassoma bifasciatum*) (Godwin et al., 1996, 2000). On the other hand, aggressive and territorial stoplight parrotfish (*Sparisoma viride*) and demoiselle fish (*Chromis dispilus*) males have higher levels of plasma 11-KT than do non-aggressive males without territory (Cardwell and Liley, 1991; Pankhurst and Barnett, 1993). Thus, behavioral alterations during breeding may or may not be linked with plasma 11-KT depending on species.

In the current study, we also considered the relationship between plasma 11-KT and brain AVT, because Goodson and Bass (2001) demonstrated that androgen inhibitors or castration decreased forebrain AVT-ir, AVT mRNA level, and AVT receptor expression, whereas androgen treatment reversed these effects. In round goby males, brain AVT concentrations changed despite the constant plasma 11-KT level.

In the peacock blenny (*Salaria pavo*), exogenous 11-KT does not affect the number of AVT preoptic neurons (Oliveira et al., 2001). Gonadectomy also has no effect on the AVT mRNA level in bluehead wrasse (Perry and Grober, 2003; Semsar and Godwin, 2003). Although in round goby brain, AVT is not affected by plasma 11-KT, we cannot exclude the possibility that 11-KT produced in the brain can influence AVT production locally. It is well established that androgens, estrogens, and progesterone are synthesized *de novo* in the central nervous system of teleosts (Diotel et al., 2011).

5. Conclusions

We observed the highest AVT and cortisol levels in overcrowded, non-aggressive males. On the other hand, the IT level was elevated as overcrowding was eased and the males were aggressive, which is usually a sign of breeding readiness. Brain AVT and IT, plasma cortisol, together with behavioral observations can be used as species-specific indicators of round goby male well-being. The role of AVT and IT as hormonal markers of fish condition and welfare indicators has been suggested previously (Kulczykowska et al., 2010).

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