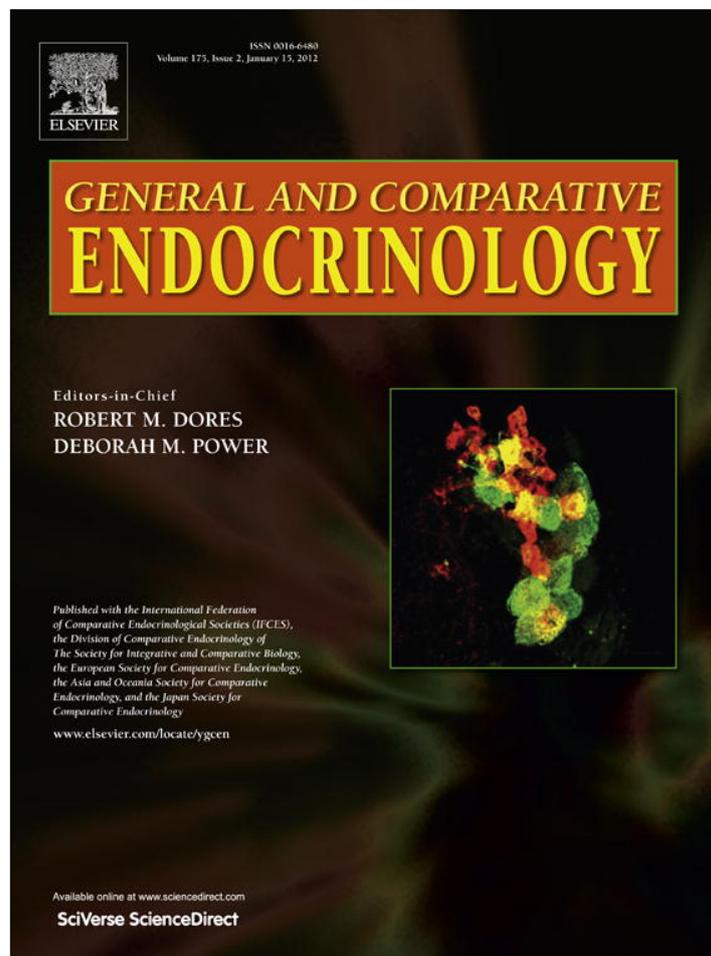


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Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of three-spined stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Arginine vasotocin (AVT) and isotocin (IT) are fish nonapeptides synthesized in separate hypothalamic neurons from where they are transported to the neurohypophysis for storage and release into circulation. AVT is known to modulate aggression, courtship and parental care or social communication in many species, including fish, amphibians and birds. In this paper we examined a link between the level of AVT and IT in the brain and particular reproductive behavior in males of three-spined stickleback (*Gasterosteus aculeatus*). AVT and IT levels in whole brain of males of three-spined stickleback vary depending on specific breeding behavior of the individuals and their social status. These studies have shown the highest AVT levels in aggressive males that took care of the eggs. Brain AVT concentrations are also increased in nuptial colored subordinate males that fight to change their social status. On the other hand, IT is significantly higher in aggressive dominant males that defend their territory. IT may be also involved in courtship in three-spined stickleback. These findings highlight the importance of determination of “free”, bioavailable neuropeptides’ level in behavioral studies.

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1. Introduction

The hypothalamic neuropeptide, arginine vasotocin (AVT), fish homologue of mammalian arginine vasopressin (AVP), is well known for its effect on reproductive and social behavior in a number of vertebrates [2,15,17,23,41,46]. In teleosts, AVT has been reported to influence social behaviors related to reproduction, including courtship in white perch (*Morone americana*) [40] and peacock blenny (*Salarias pavo*) [9,20], aggression in bluehead wrasse (*Thalassoma bifasciatum*) [43] and African cichlid fish (*Astatotilapia burtoni*) [19], vocalization in brown ghost knife fish (*Apteronotus leptorhynchus*) [3] and plainfin midshipman fish (*Porichthys notatus*) [14], and spawning reflex in the killifish (*Fundulus heteroclitus*) [25]. Moreover, measurement of AVT level in whole brain in two species of pipefishes (*Syngnathus fuscus* and *Syngnathus floridae*) revealed relationship between AVT and paternal brooding [38]. So far, isotocin (IT), teleostean homologue of mammalian oxytocin (OT), has received little study in terms of regulation of sexual behavior, however, investigation performed on *Lythrypnus dalli* has demonstrated that this neurohormone may be also involved in modulation of reproductive behavior in fish [6].

Nonapeptides, AVT and IT are synthesized in separate hypothalamic magnocellular and parvocellular neurons of the preoptic area (POA) from where they are transported to the neurohypophysis for storage and release to circulation [35,39]. Vasotocinergic and isotocinergic fibers are widely distributed in different extrahypothalamic areas, which suggest that both neuropeptides act centrally as neurotransmitters and neuromodulators [21]. It has been shown that intracerebroventricular (ICV) infusion of AVT inhibits dominance ability in rainbow trout (*Onchorhynchus mykiss*) [1] and centrally administered AVT and IT affect social behavior in goldfish [49,50]. Moreover, in male white perch, centrally injected AVT strongly stimulates courtship behavior whereas administered peripherally shows no effect [40].

Synthesis of AVT and IT is regulated independently, therefore both neurohormones may play distinct roles in reproductive behavior in fish [39]. In male goldfish (*Carassius auratus*), centrally infused AVT and IT have opposite effects on social behavior: AVT inhibits social approach while IT stimulates it [49]. In plainfin midshipman, AVT inhibits sound production in type I males that defend territories and acoustically court females, whereas IT does it in non-territorial, type II males and in females [14]. Moreover, action of AVT and IT in regulation of reproductive behavior may depend on the social status of fish. In bluehead wrasse, a sex-changing fish species with both territorial and non-territorial males, exogenous AVT increases aggression in non-territorial fish but decreases aggression in territorial males [43]. In birds, for

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instance, increased level of AVT is linked with increased aggression in colonial species such as zebra finch (*Taeniopygia guttata*) [16]. However, centrally administered AVT reduces aggression in territorial species such as field sparrows (*Spizella pusilla*) [13].

To the authors' knowledge, there are no studies on relationship between brain AVT and IT and breeding behavior of three-spined stickleback (*Gasterosteus aculeatus*). Stickleback, known for its well-defined reproductive behavior, is a common model-fish to study sexual behavior and social interactions. During the breeding season males manifest nuptial coloration, defend territory, build nest, court females and take care of the eggs and embryos [11].

The present study was designed to establish if there is a link between brain levels of bioactive nonapeptides, AVT and IT and specific behavior of males stickleback at particular phases of breeding. We measured AVT and IT concentrations in whole brains of males (i) at different social status, (ii) responding to their own image in the mirror, and (iii) responding to presence of females.

2. Materials and methods

2.1. Animals and experimental design

Males of three-spined stickleback (0.766–1.952 g) used in this study were caught in their breeding period, i.e. during spring, in the Vistula river (Northern Poland). Nuptial coloration and characteristic behavior, i.e. aggressiveness and territoriality proved the maturity of sticklebacks ready for reproduction. Before experiments, fish were adapted to the laboratory conditions for at least three days. Experiments were conducted in freshwater at room temperature and under natural photoperiod which was changing from 13L:11D to 16L:8D. Before each experiment, fish and tanks were shortly disinfected with 0.05% and 1% KMnO₄, respectively. Fish received frozen food (*Chironomus plumosus*) *ad libitum* every two days. At the end of each experiment, fish were anesthetized by immersion in 0.5% (v/v) 2-phenoxyethanol water solution. After sectioning the spinal cord brains (12.43–41.52 mg) were removed and stored in –70 °C until analysis.

2.2. Experiment 1

2.2.1. Changes in brain AVT and IT levels and specific reproductive behaviors of males at particular phases of breeding

Experiment was conducted between May and July. Males ($n = 180$) were kept in seven 30-liter tanks supplied with plants: Brazilian elodea (*Egeria densa*), coon's tail (*Ceratophyllum demersum*), angustifolia (*Hygrophila angustifolia*) and crystalwort (*Riccia fluitans*). Two males were introduced together into each tank. Male built the nests using crystalwort. Behavioral observations were made several times a day. The brains were collected parallelly from dominants ($n = 77$) and subordinates ($n = 65$; 12 subordinates did not survive until the end of the experiment) at different breeding phases as follows:

1. *Nest building*: Between 3 and 24 h after placing two males in a tank, one male became dominant and started building the nest. Twenty-four hours after one fish began nest building brains were removed from both males.
2. *Pre-spawning*: 2–4 days after the hierarchy was established and the dominant finished nest building brains were removed from both males. The nest was recognized as complete when male was wriggling the tunnel in it.
3. *Courtship*: After removal of the subordinate male (2–4 days after initial introduction), a female was introduced to the tank with

dominant – nest owner. Dominant fish exhibiting nuptial coloration began courting the female. Two hours later the brain was removed from the dominant male.

4. *Post-spawning*: After removal of the subordinate male (2–4 days after initial introduction), a female was introduced. 10–20 s after fertilizing the eggs the brain was removed from the dominant male.
5. *Paternal care*: After fertilizing eggs and removing the female, male started guarding the eggs. Male was alone in the tank. After three days of taking care of the nest the brain was removed from the male.

Aggression of males was assessed at each breeding phase by counting bites per minute as is recommended for three-spined stickleback [36,51]. The levels of aggression were defined as: level I: 1 to 5 bites per minute, level II: 6 to 10 bites per minute, level III: 11 and more bites per minute.

Brains were also removed from dominant ($n = 13$) and subordinate ($n = 13$) males without nests four days after setting the hierarchy.

2.3. Experiment 2

2.3.1. Changes in brain AVT and IT levels and specific behaviors of males in response to their mirror image

Experiment was conducted between April and June. Males ($n = 30$) were kept in seven 30-liter tanks supplied with the same plants as in Experiment 1. In five tanks there were mirrors at three sides. A single male was placed in each tank. Single males kept in tanks without mirrors made a control group. Behavioral observations were made several times a day. On the 7th day of the experiment, fish were captured and brains were removed from males from the tanks with mirrors ($n = 20$) and without mirrors ($n = 10$).

2.4. Experiment 3

2.4.1. Changes in brain AVT and IT levels and specific behaviors of males in experimental schemes: 1 male + 3 females and 1 male + 10 females

Experiment was carried out between April and June. Males ($n = 11$) were kept in two 300-liter tanks and seven 30-liter tanks supplied with the same plants as in Experiment 1: a single male was placed in each tank. First dorsal spine of each male was cut out to distinguish males from females (in case the males were losing nuptial coloration). Seven days after starting the experiment three females were introduced to a male in 300-liter tank and ten females were introduced to a male in 30-liter tank. It has been found that the presence of numerous females is stressful for males; males kept together with large group of females live shorter [11]. Our previous observations have indicated that gender proportion: 1 male/10 females in 30-liter tank evoke stress in males, in contrast to 1 male/3 females in 300-liter tanks. Behavioral observations were made several times a day. Two weeks from the beginning of the experiment, i.e. one week after introduction of females, fish were captured and brains were removed from males kept with three ($n = 4$) and ten females ($n = 7$).

2.5. Analysis of AVT and IT

Prior to analysis brains were defrosted, weighted and subsequently sonicated separately (Microson™ XL 2000) in 1 ml of distilled water and extracted with acetic acid (final concentration: 0.25% v/v). Then the samples were placed for 3.5 min in a boiling water bath. The extracts were cooled on ice before centrifugation (15000 rpm, 30 min, 4 °C). To clean up the samples and derivatize the peptides, the supernatants were decanted and loaded on

Table 1
Scale of aggressiveness observed in males of three-spined stickleback at particular breeding phases.

Particular breeding phases	Levels of aggression		
	Level I 1–5 bites per minute	Level II 6–10 bites per minute	Level III 11 and more bites per minute
Nest building	Pre-spawning	Paternal care	
Courtship	Post-spawning		

previously conditioned (2 ml methanol, 2 ml water) SPE speedisks cartridges (Baker Bond, C18, 20 mg, J.T. Baker, Phillipsburg, NJ, USA). Columns were washed successively with water, 4% acetic acid and one more time with water. Then the derivatization procedure was applied as described by Gozdowska et al. [18]. Briefly, the derivatization was performed using NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) in borate buffer (pH 9.5) for 20 min at room temperature. After subsequent washing by-products with water and 10% methanol derivatized peptides were eluted with mixture of ethanol:6 N hydrochloric acid (2000:1) and directly injected to HPLC system (Beckman modular HPLC system with Shimadzu spectrofluorometric detector RF-551). Chromatographic separation of peptides was carried out on an Ultrasphere ODS column (250 × 4.6 mm, 5 μm) using linear gradient system: 48–80% phase B (0.1% trifluoroacetic acid in acetonitrile:water 3:1) in phase A (0.1% trifluoroacetic acid in water) for 20 min. The column temperature was 22 °C and flow rate was 1 ml/min. Fluorescence detection was performed at 530 nm with excitation at 470 nm. Peptides were analyzed in every single brain and AVT and IT concentrations were expressed in pmol of peptide per mg of brain tissue. Recovery of peptides was in the 89–93% range for AVT and IT. The detection limit was determined as 100 fmol/ml. Intra-day repeatability expressed as relative standard deviation (RSD) was in the 2–4.5% and 5.3–8.2% range for AVT and IT, respectively; inter-day precision was in the 2.5–5.5% and 5.5–8.5% range for AVT and IT, respectively.

2.6. Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Correlation between number of bites and nonapeptides levels in dominants at all breeding phases in Experiment 1 was calculated using Pearson correlation. For multiple comparisons, the analysis of variance (one-way ANOVA) was used followed by Spjotvoll and Stoline test for unequal number of means (n). Significance was taken at P < 0.05. For multiple comparisons of non-paired samples in Experiment 1 Bonferroni correction was applied [37]. The statistical analyses of data were carried out using Statistica 7.1 software.

3. Results

3.1. Experiment 1

3.1.1. Changes in brain AVT and IT levels and specific reproductive behaviors of males at particular phases of breeding

Within 3 to 24 h after introducing two males into the tank one became dominant. Dominants manifested nuptial coloration, i.e. bright red abdomen and jowl, blue eyes and blue–green dorsum. Their nuptial coloration became more intensive upon the introduction of female. After fertilizing the eggs, the red and blue–green color of the body became less intensive. Twenty eight subordinates sported nuptial coloration but less intensive than that of dominants. None of the subordinates built the nest.

Dominants were aggressive toward subordinates biting and chasing them. Subordinates hid behind the water filter and did not feed. In search of a place for the nest, dominant was digging

many small holes in the ground and collecting stones or shells. After selection of one site it started to build the nest. It constructed the mound using a mix of sand and crystalwort floating on the surface of the water and cemented it with glue secreted from its kidney. Finally it tunneled into the mound. Immediately after introducing the female into the tank dominant started to attract it. It performed characteristic courtship dance in the form of series of zigzags: first making a sideways lunge toward the female, then one away from it. Courtship lasted from several minutes to several hours. Immediately after female laid eggs, the male entered the nest to fertilize them. It stayed in the tunnel from several seconds to about a minute. Males had 110–214 eggs in their nests. After fertilization the male chased the female away and began guarding the eggs. It ventilated them with the movements of his pectoral fins and successively disassembled the mound. Dominant defended his territory even being alone in the tank. It was very aggressive towards any object put into the tank and did not feed. Intensity of aggressive behavior of males was changing during the breeding as is presented in Table 1.

Brain AVT and IT concentrations are presented in Fig. 1. In dominants, level of AVT was the lowest in nest building phase and the highest in paternal care phase (one-way ANOVA, P < 0.001). In dominants, brain concentrations of AVT corresponded with a number of bites (Pearson's correlation, r = 0.51). The concentration of IT was the highest during courtship. The concentrations of AVT were significantly higher in dominants in pre-spawning, courtship, post-spawning phases and during paternal care than those in subordinates at corresponding stages (Spjotvoll and Stoline test, P < 0.001). The levels of IT were significantly higher in dominants in pre-spawning phase and during courtship than those in subordinates at corresponding stages (Spjotvoll and

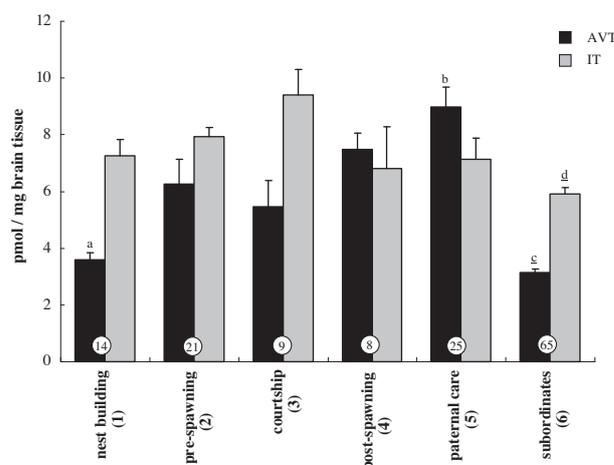


Fig. 1. AVT and IT concentrations in brains of dominant males of three-spined stickleback at particular breeding phases: nest building (1), pre-spawning (2), courtship (3), post-spawning (4), paternal care (5) and subordinate males kept with dominants (6). Number (n) of fish is given in the circles. The values are presented as means ± SEM. Statistical differences are indicated as: a: P < 0.001 vs AVT in (4) and (5); b: P < 0.05 vs AVT in (2) and (3); c: P < 0.001 vs AVT in (2), (3), (4), (5) d: P < 0.001 vs IT in (2) and (3). Statistical differences after Bonferroni correction are underlined.

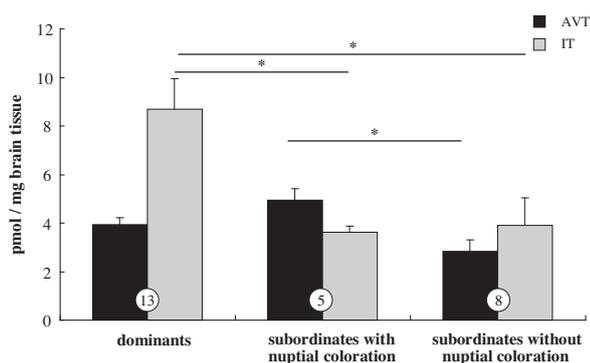


Fig. 2. AVT and IT concentrations in brains of males of three-spined stickleback without nests. Number (*n*) of fish is given in the circles. The values are presented as means ± SEM. Significant differences are indicated as **P* < 0.05.

Stoline test, *P* < 0.01). In order to make Fig. 1 comprehensible, all subordinates are presented together.

3.1.2. Changes in brain AVT and IT levels and specific reproductive behaviors of males that did not build the nests

In some of the tanks neither dominant nor subordinate built the nest. All dominants showed nuptial coloration, i.e. bright red head and abdomen and blue eyes. Five subordinates demonstrated nuptial coloration but less intensive than that of dominants. Dominants were guarding their territory chasing and biting subordinates although they did not build the nest. Five nuptial colored subordinates counter-attacked the dominant trying to change their social status, however, none of them succeeded. Dominants and subordinates intensively fed all the time.

Brain AVT and IT concentrations are presented in Fig. 2. In dominants, brain IT concentration was the highest (Spjotvoll and Stoline test, *P* < 0.05). The concentration of AVT was significantly higher in nuptial colored subordinates than in subordinates without nuptial coloration (Spjotvoll and Stoline test, *P* < 0.05).

3.2. Experiment 2

3.2.1. Changes in brain AVT and IT levels and specific behaviors of males in response to their mirror image

Sixteen males demonstrated nuptial coloration after introducing into the tanks with mirrors. Nuptial colored males were raising their dorsal and abdominal spines while swam closer to the mirror in order to fight with potential competitor. Six nuptial colored individuals built the nest. Their nuptial coloration did not change and they were aggressive towards their mirror image during all experiment. On the other hand, ten nuptial colored males without nests changed their coloration in response to their mirror image. Brightness of abdomen and eyes coloration has changed even four times a day. Changes of coloration related to behavior, i.e. brightly colored males were aggressive towards their mirror image, but losing their nuptial coloration they became calm. Four males did not show nuptial coloration and did not care about the mirror. These males intensively fed whereas males with nuptial coloration did not.

Males in control group (tanks without mirrors) neither demonstrated nuptial coloration nor built the nest.

Brain AVT and IT concentrations are presented in Fig. 3. Brain IT concentrations in males with nests were significantly higher than those in the control group (Spjotvoll and Stoline test, *P* < 0.05). AVT levels were similar in all fish (one-way ANOVA, *P* = 0.103092).

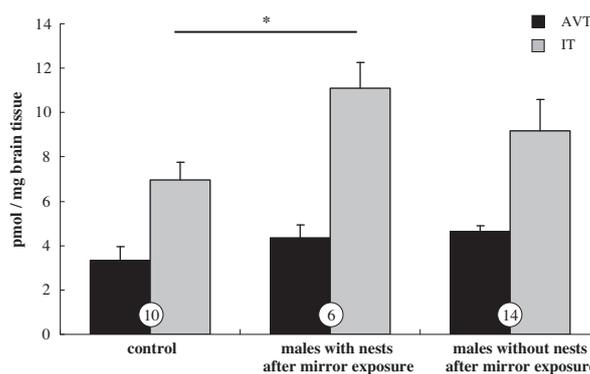


Fig. 3. AVT and IT concentrations in brains of males of three-spined stickleback with or without nests after mirror exposure. Number (*n*) of fish is given in the circles. The values are presented as means ± SEM. Significant differences are indicated as **P* < 0.05.

3.3. Experiment 3

3.3.1. Changes in brain AVT and IT levels and specific behaviors of males in experimental schemes: 1 male + 3 females and 1 male + 10 females

Before introducing three females into the tank, none of the males built the nest or showed nuptial coloration. All males intensively fed. After introducing three females, males immediately manifested nuptial coloration and started to attract females. They courted females and pretended having the nest swimming towards the corner of the tank, where some stones and shells were stored. Males did not build the nest until the end of the experiment. After five days with females in the tank, males became paler. They stayed in the corner where they simulated having the nest.

Before introducing ten females into the tank, males were calm and spent much time feeding; four individuals demonstrated nuptial coloration and built the nests. Males' behavior changed immediately upon appearance of females. The other three males showed nuptial coloration but they did not build the nests. Males without the nests hid behind the filter while individuals with ready nests were cruising close to the nests for the next two days. Then all seven males hid behind the filter.

The levels of AVT and IT were significantly higher in males kept together with three females than in those with ten females (Spjotvoll and Stoline test, *P* < 0.05 for AVT and *P* < 0.01 for IT, Fig. 4). Brain AVT and IT concentrations in males kept together with three females (Fig. 4) were comparable to those in males in pre-spawning phase in Experiment 1 (Fig. 1). Brain AVT and IT levels in males kept with ten females (Fig. 4) corresponded

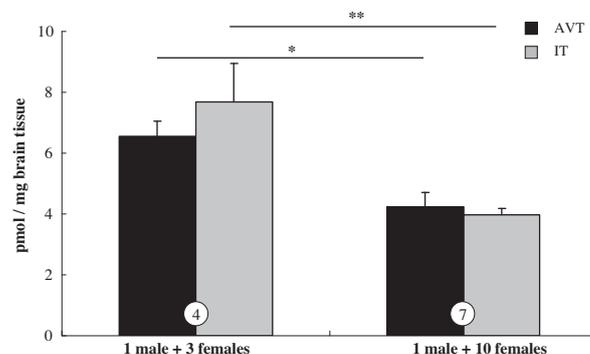


Fig. 4. AVT and IT concentrations in brains of males of three-spined stickleback while kept single with 3 or 10 females. Number (*n*) of fish is given in the circles. The values are presented as means ± SEM. Significant differences are indicated as **P* < 0.05, ***P* < 0.01.

to those in subordinates while kept with dominants without nests in Experiment 1 (Fig. 2).

4. Discussion

The present studies demonstrate for the first time the relationship between whole brain concentration of “free”, bioavailable neuropeptides, AVT and IT and aggressive behavior of male three-spined sticklebacks during breeding. AVT and IT action as neurotransmitters and/or neuromodulators in the central nervous system requires their binding to the specific receptors: only mature nonapeptides after dissociation from non-covalent complex are active at receptor level. Therefore, for the purpose of this study, we found especially important to measure the level of AVT and IT per se, instead of estimating expression of mRNA for the complex molecule, i.e. AVT and IT pre-pro-hormone, which is common practice in behavioral studies.

Males of stickleback manifest aggression in various phases of breeding, especially while defending territory or taking care of eggs. The highest brain concentration of AVT has been observed in the most aggressive males that take care of the eggs. The relationship between brain AVT level and aggression in paternal males is confirmed by positive correlation between AVT concentration and number of bites. Studies performed by Ripley and Foran [38] confirm relationship between AVT and paternal care: in two species of pipefishes, significantly higher AVT level in whole brain was observed in males during brooding than in post-brooding males. Relationship between AVT and aggression during paternal care has been also observed in the plainfin midshipman. The aggressive males that guard eggs in their nests have larger AVT-immunoreactive (AVT-ir) cells in POA compared to males without nests [12]. Larson et al. [26] have provided similar data in zebrafish (*Danio rerio*), in which dominant, aggressive males have bigger AVT-ir neurons in magnocellular preoptic area whereas subordinate males possess smaller AVT-ir cells in parvocellular POA. Size of the cells producing neurohormones may correspond with their synthesis activity. In such cases, a link between AVT and aggression of paternal males in these fish species could be suggested. In mammals, there are evidence for relationship between brain arginine vasopressin (counterpart of AVT) and aggressive behavior of males during reproduction. Bester-Meredith et al. [5] have investigated bed nucleus of the stria terminalis (BnST) as the region that might be responsible for reproductive behavior in mammals. Monogamous male mice *Peromyscus californicus*, displaying parental care have more AVP-immunoreactive (AVP-ir) neurons in BnST than not aggressive polygamous species *Peromyscus leucopus*, which does not take care of their pups. Moreover, in male Wistar rats, AVP release within lateral septum (LS) correlated positively with inter-male aggression [52].

The role of AVT in aggressive behavior of males can be coupled with steroids' action as it is shown in other fish species, e.g. plainfin midshipman, *Sarotherodon melanotheron*, *Trichogaster trichopterus*, *Chromis dispilus* and *Oreochromis mossambicus* [24,30,34]. However, it seems that in three-spined stickleback the role of AVT in aggressive behavior is independent on steroids. Although in our studies steroids' level has not been measured, the lowest concentration of 11-ketotestosterone in parental males of three-spined stickleback in comparison with males at other breeding phases has been reported by Bell [4]. Moreover, Pall et al. [33] have demonstrated that castration of three-spined stickleback has not caused the decline of males' aggressiveness during paternal care phase and administration of androgens to castrated males taking care of the eggs does not change their behavior. Semsar and Godwin [44] have shown that the action of AVT is independent on sex hormones in bluehead wrasse: cas-

tration of males has no effect on AVT mRNA level or territorial behavior.

Potential action of AVT in aggressive behavior of paternal three-spined sticklebacks can be linked to prolactin (PRL) which intensive synthesis has been observed in aggressive males that take care of the eggs [47]. Moreover, exogenous prolactin influences males' characteristic behavior during paternal phase, i.e. fanning the nests: males do that even though there are no eggs inside [10]. *In vitro* studies, which show that AVT induces prolactin secretion from pituitary [27], may suggest AVT action via regulation of PRL release from pituitary, but further studies are needed to support this hypothesis.

Moreover, our data reveal that the pattern of changes of brain AVT and IT levels depends on social status of the fish. It has been also shown in African cichlid fish that the expression of AVT mRNA is associated with the social status of males [19]. We have observed significantly higher AVT concentration in nuptial colored subordinates that counter-attacked dominant than in calm subordinates without nuptial coloration. It suggests a link between AVT and aggression during fighting for territory and domination in the group. The studies performed in other fish species confirm, even if indirectly, the relationship between AVT and males aggression during fighting for domination. In males of zebrafish, size of AVT producing cells in parvocellular and magnocellular POA of hypothalamus depends on social status of the fish: bigger AVT-immunoreactive cells are present in aggressive males, smaller in non-aggressive individuals [26]. The role of AVT in aggression during fighting for territory has been supported by our field study of sticklebacks. We have observed significantly higher brain level of AVT in males in May than in July [18]. In May, males are at the phase of fixing their territory, i.e. they have nests and court females. Brain AVT levels in males in May correspond with those observed in aggressive, nuptial colored subordinate males that counter-attacked dominant trying to change their social status (Experiment 1). Also experiments conducted in African cichlid fish confirm the relationship between AVT and aggressive behavior of males fighting for territory: territorial, aggressive males exhibit higher level of AVT mRNA expression in posterior preoptic area than non-territorial males [19]. Furthermore, in false clown anemonefish (*Amphiprion ocellaris*), it has been shown that subordinate, aggressive individuals fighting to change social status have bigger AVT neurons in magnocellular layer of POA than dominants [22]. The behavior of anemonefish resembles the behavior of subordinate male stickleback fighting for territory.

The present data suggest that brain IT changes might be linked to aggressive behavior during defending dominant position: significantly higher concentration of this neuropeptide has been observed in the aggressive dominants, even having no nests, than in subordinates (Fig. 2). Higher level of IT has been also detected in males that are aggressive towards their mirror image. In three-spined stickleback, vision plays a vital role in the breeding and aggression [8,11]. In our study, males were kept in separate tanks to exclude potential effect of pheromones on aggressive behavior. Aggressive males manifested nuptial coloration while attacking their mirror image and IT levels were distinctly higher in the aggressive individuals with nests.

The experiment with mirrors was carried out in cichlid fish (*O. mossambicus*). In males aggressive towards their mirror image, concentrations of testosterone and 11-ketotestosterone was the same as in non-aggressive ones [31]. It suggests that in cichlid fish sex hormones are not responsible for males' aggressive behavior. We postulate that also in stickleback, IT's action, if any, may be independent on steroids, similar to that of AVT.

In our study, we have observed higher levels of IT in males that courted females. This is the first study demonstrating that IT may be involved in courtship in three-spined stickleback. Males that

courted females in experimental scheme: 1 male + 3 females had significantly higher level of IT than males that did not do it in experimental scheme: 1 male + 10 females. It should be pointed out that in experimental scheme: 1 male + 3 females males had no nests and were not aggressive, thus high level of IT is probably linked only to courtship. To authors' knowledge, there is no information about the role of IT in males' courtship in fish. Recent studies indicate only that administration of AVP/OT receptor antagonist to males of monogamous cichlid fish (*Amatitlania nigrofasciata*) results in a significant reduction in affiliative behavior toward the potential mate [29]. It has been shown that oxytocin, mammalian homologue of IT, is implicated in regulation of courtship behavior in many mammalian species, i.e. in guinea-pigs (*Cavia aperea f. porcellus*), monogamous mice *Microtus ochrogaster* and monogamous pair-bonded male tamarins (*Saguinus oedipus*) [28,48,53].

Courtship of males' stickleback can be coupled with androgens' action. Higher levels of 11-ketotestosterone are observed in males during courtship than at other breeding phases [32]. Moreover, plasma levels of 11KT and androstenedione are elevated in males that have nests and start courtship [42]. Castrated males of three-spined stickleback display no courtship whereas administration of 11-ketoandrostenedione (11KA) restores this behavior [7]. Our findings suggest that courtship in three-spined stickleback may be controlled by IT. However, a potential role of androgens in this process cannot be excluded. Studies carried out by Semsar and Godwin [45] on bluehead wrasse support the hypothesis on a link between androgens and AVT affecting courtship: simultaneous administration of 11KT and AVT induces more active courtship than in case of AVT administered alone. To date, to the authors' knowledge, there are no data on interactions between sex hormones and IT.

5. Conclusions

There are the first studies on the relationship between brain levels of "free", bioactive neuropeptides, AVT and IT and reproductive behavior at particular breeding stages in three-spined stickleback. AVT and IT levels in whole brain of males of three-spined stickleback is various at specific breeding phases of the individuals and at different social status. We have found the highest AVT levels in aggressive males that took care of the eggs, thus AVT may be linked with aggression related to paternal care. The concentration of AVT was also elevated in subordinates fighting to change their social status. Brain IT level was the highest in aggressive, dominant males without nests that defended the territory and dominant position. IT concentration was also elevated in males that courted females. A different levels of AVT and IT in different breeding phases confirm the separate mechanism of the neurohormones' regulation in this species. These findings highlight the importance of determination of "free", bioavailable neuropeptides' level in behavioral studies.

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References

- [1] T. Backström, S. Winberg, Arginine-vasotocin influence on aggressive behavior and dominance in rainbow trout, *Physiol. Behav.* 96 (2009) 470–475.
- [2] A.H. Bass, P.M. Forlano, Neuroendocrine mechanisms of alternative reproductive tactics: the chemical language of reproductive and social plasticity, in: R.F. Oliveira, M. Taborsky, H.J. Brockmann (Eds.), *Alternative reproductive tactics: an integrative approach*, Cambridge University Press, Cambridge, 2008, pp. 109–131.
- [3] J. Bastian, S. Schniederjan, J. Nguyenkim, Arginine vasotocin modulates a sexually dimorphic communication behavior in the weakly electric fish *Apteronotus leptorhynchus*, *J. Exp. Biol.* 204 (2001) 1909–1923.
- [4] A.M. Bell, Effects of an endocrine disruptor on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*, *Anim. Behav.* 61 (2001) 775–780.
- [5] J.K. Bester-Meredith, L.J. Young, C.A. Marler, Species differences in paternal behavior and aggression in *Peromyscus* and their associations with vasopressin immunoreactivity and receptors, *Horm. Behav.* 36 (1999) 25–38.
- [6] M.P. Black, R.H. Reavis, M.S. Grober, Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*, *NeuroReport* 15 (2004) 185–189.
- [7] B. Borg, Androgens in teleost fishes, *Comp. Biochem. Physiol.* 09C (1994) 219–245.
- [8] V.A. Braithwaite, I. Barber, Limitations to colour – based sexual preferences in three-spined sticklebacks (*Gasterosteus aculeatus*), *Behav. Ecol. Sociobiol.* 47 (2000) 413–416.
- [9] L.A. Carneiro, R.F. Oliveira, A.V.M. Canário, M.S. Grober, The effect of arginine vasotocin on courtship behaviour in a blennioid fish with alternative reproductive tactics, *Fish Physiol. Biochem.* 28 (2003) 241–243.
- [10] A.J. De Ruiter, S.E. Wendelaar Bonga, H. Slijkhuis, B. Baggerman, The effect of prolactin on fanning behavior in the male three-spined stickleback, *Gasterosteus aculeatus* L., *Gen. Comp. Endocrinol.* 64 (1986) 273–283.
- [11] J. Fitzgerald, The reproductive behavior of the stickleback, *Scient. Amer.* 268 (1993) 50–55.
- [12] C.M. Foran, A.H. Bass, Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics, *Gen. Comp. Endocrinol.* 111 (1998) 271–282.
- [13] J.L. Goodson, Territorial aggression and dawn song are modulated by septal vasotocin and vasoactive intestinal polypeptide in male field sparrows (*Spizella pusilla*), *Horm. Behav.* 34 (1998) 67–77.
- [14] J.L. Goodson, A.H. Bass, Forebrain peptides modulate sexually polymorphic vocal circuitry, *Nature* 403 (2000) 769–772.
- [15] J.L. Goodson, A.H. Bass, Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates, *Brain Res. Rev.* 35 (2001) 246–265.
- [16] J.L. Goodson, L. Lindberg, P. Johnson, Effects of central vasotocin and mesotocin manipulations on social behavior in male and female zebra finches, *Horm. Behav.* 45 (2004) 136–143.
- [17] J.L. Goodson, J. Rinaldi, A.M. Kelly, Vasotocin neurons in the bed nucleus of the stria terminalis preferentially process social information and exhibit properties that dichotomize courting and non-courting phenotypes, *Horm. Behav.* 55 (2009) 197–202.
- [18] M. Gozdowska, A. Kleszczyńska, E. Sokołowska, E. Kulczykowska, Arginine vasotocin (AVT) and isotocin (IT) in fish brain: diurnal and seasonal variations, *Comp. Biochem. Physiol. B* 143 (2006) 330–334.
- [19] A.K. Greenwood, A.R. Wark, R.D. Fernald, H.A. Hofmann, Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish, *Proc. R. Soc. B* 275 (2008) 2393–2402.
- [20] M.S. Grober, A.A. George, K.K. Watkins, L.A. Carneiro, R.F. Oliveira, Forebrain AVT and courtship in a fish with male alternative reproductive tactics, *Brain Res. Bull.* 57 (2002) 423–425.
- [21] B.I. Holmqvist, P. Ekström, Hypophysiotropic systems in the brain of the Atlantic salmon. Neuronal innervation of the pituitary and the origin of pituitary dopamine and nonapeptides identified by means of combined carbocyanine tract tracing and immunocytochemistry, *J. Chem. Neuroanat.* 8 (1995) 125–145.
- [22] E. Iwata, Y. Nagai, H. Sasaki, Social rank modulates brain arginine vasotocin immunoreactivity in false clown anemonefish (*Amphiprion ocellaris*), *Fish Physiol. Biochem.* 36 (2010) 337–345.
- [23] N.M. Kime, T.K. Whitney, M.J. Ryan, A.S. Rand, C.A. Marler, Treatment with arginine vasotocin alters mating calls and decreases call attractiveness in male túngara frogs, *Gen. Comp. Endocrinol.* 165 (2010) 221–228.
- [24] R. Knapp, J.C. Wingfield, A.H. Bass, Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*), *Horm. Behav.* 35 (1999) 81–89.
- [25] W.R. Knight, J.N. Knight, Telencephalon removal does not disrupt the vasotocin-induced spawning reflex in killifish, *Fundulus heteroclitus*, *J. Exp. Zool.* 276 (1996) 296–300.
- [26] E.T. Larson, D.M. O'Malley, R.H. Melloni, Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish, *Behav. Brain Res.* 167 (2006) 94–102.
- [27] J.W. Liu, N. Ben-Jonathan, Prolactin-releasing activity of neurohypophysial hormones: structure-function relationship, *Endocrinology* 134 (1994) 114–118.
- [28] I.H. Machatschke, B. Wallner, D. Schams, J. Dittami, Social environment affects peripheral oxytocin and cortisol during stress responses in guinea-pigs, *Ethology* 110 (2004) 161–176.
- [29] R.G. Oldfield, H.A. Hofmann, Neuropeptide regulation of social behavior in a monogamous cichlid fish, *Physiol. Behav.* 102 (2011) 296–303.
- [30] R.F. Oliveira, K. Hirschenhauser, L.A. Carneiro, A.V.M. Canario, Social modulation of androgen levels in male teleost fish, *Comp. Biochem. Physiol. B* 132 (2002) 203–215.
- [31] R.F. Oliveira, L.A. Carneiro, A.V.M. Canario, No hormonal response in tied fights, *Nature* 37 (2005) 207–208.

- [32] M.K. Pall, I. Mayer, B. Borg, Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus*. I. Changes in 11-ketotestosterone levels during the nesting cycle, *Horm. Behav.* 41 (2002) 377–383.
- [33] M.K. Pall, I. Mayer, B. Borg, Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus*. II. Castration and 11-ketoandrostenedione effects on courtship and parental care during the nesting cycle, *Horm. Behav.* 42 (2002) 337–344.
- [34] N.W. Pankhurst, C.W. Barnett, Relationship of population density, territorial interaction and plasma levels of gonadal steroids in spawning male demoiselles *Chromis dispilus* (Pisces: Pomacentridae), *Gen. Comp. Endocrinol.* 90 (1993) 168–176.
- [35] I.S. Parhar, H. Tosaki, Y. Sakuma, M. Kobayashi, Sex differences in the brain of goldfish: gonadotropin-releasing hormone and vasotocinergic neurons, *Neuroscience* 104 (2001) 1099–1110.
- [36] H. Peeke, E. Wyers, M. Herz, Waning of the aggressive response to male models in the three-spined stickleback (*Gasterosteus aculeatus* L.), *Anim. Behav.* 17 (1969) 224–228.
- [37] W.R. Rice, Analyzing tables of statistical tests, *Evolution* 43 (1989) 223–225.
- [38] J.L. Ripley, C.M. Foran, Quantification of whole brain arginine vasotocin for two *Syngnathus* pipefishes: elevated concentrations correlated with paternal brooding, *Fish Physiol. Biochem.* 36 (2010) 867–874.
- [39] D. Saito, M. Komatsuda, A. Urano, Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout; central and neurohypophysial projections of single neurons, *Neuroscience* 124 (2004) 973–984.
- [40] S.J. Salek, C.V. Sullivan, J. Godwin, Arginine vasotocin effects on courtship behavior in male white perch (*Morone americana*), *Behav. Brain Res.* 133 (2002) 177–183.
- [41] N. Santangelo, A.H. Bass, New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish, *Proc. R. Soc.* 273 (2006) 3085–3092.
- [42] M. Sebire, I. Katsiadaki, A.P. Scott, Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback (*Gasterosteus aculeatus*, *Gen. Comp. Endocrinol.* 152 (2007) 30–38.
- [43] K. Semsar, F.L.M. Kandel, J. Godwin, Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse, *Horm. Behav.* 40 (2001) 21–31.
- [44] K. Semsar, J. Godwin, Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish, *J. Neurosci.* 23 (2003) 4386–4393.
- [45] K. Semsar, J. Godwin, Multiple mechanisms of phenotype development in the bluehead wrasse, *Horm. Behav.* 5 (2004) 345–353.
- [46] K.B. Sewall, E.C. Dankoski, K.W. Sockman, Song environment affects singing effort and vasotocin immunoreactivity in the forebrain of male Lincoln's sparrows, *Horm. Behav.* 58 (2010) 544–553.
- [47] H. Slijkhuis, A.J. De Ruiter, B. Baggerman, S.E. Wendelaar Bonga, Parental fanning behavior and prolactin cell activity in the male three-spined stickleback *Gasterosteus aculeatus* L., *Gen. Comp. Endocrinol.* 54 (1984) 297–307.
- [48] C.T. Snowdon, B.A. Pieper, C.Y. Boe, K.A. Cronin, A.V. Kurian, T.E. Ziegler, Variation in oxytocin is related to variation in affiliative behavior in monogamous, pairbonded tamarins, *Horm. Behav.* 58 (2010) 614–618.
- [49] R.R. Thompson, J.C. Walton, Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*), *Behav. Neurosci.* 118 (2004) 620–626.
- [50] J.C. Walton, B. Waxman, K. Hoffbuhr, M. Kennedy, E. Beth, J. Scangos, R.R. Thompson, Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic, *Neuropharmacology* 58 (2010) 126–134.
- [51] R.J. Wootton, Measures of the aggression of parental male three-spined sticklebacks, *Behaviour* 40 (1971) 228–261.
- [52] A.H. Veenema, D.I. Beiderbeck, M. Lukas, I.D. Neumann, Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression, *Horm. Behav.* 58 (2010) 273–281.
- [53] L.J. Young, M.M. Lim, B. Gingrich, T.R. Insel, Cellular mechanisms of social attachment, *Horm. Behav.* 40 (2001) 133–138.