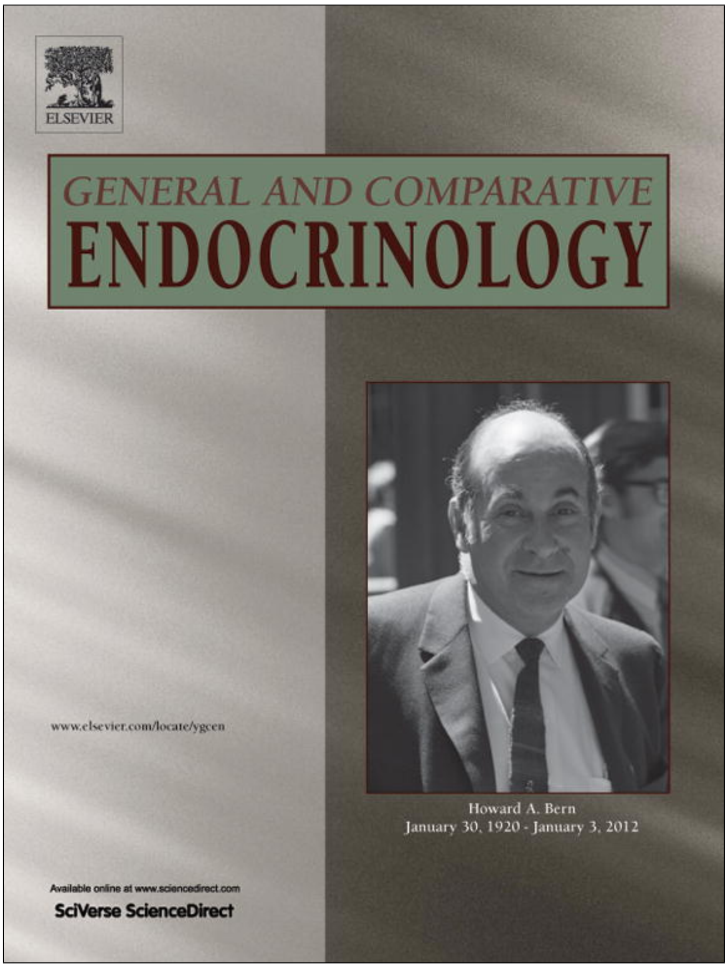

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Short Communication

Stocking density influences brain arginine vasotocin (AVT) and isotocin (IT) levels in males and females of three-spined stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Arginine vasotocin (AVT) and isotocin (IT) are fish neurohormones produced in separate parvocellular and magnocellular preoptic neurons of Teleostei. Apart from well-established peripheral action as hormones they are important neurotransmitters in central nervous system in fish. In the present study, we examined an influence of stocking density on whole brain AVT and IT concentrations in males and females of three-spined stickleback (*Gasterosteus aculeatus*). In males, the highest AVT levels have been found at stocking densities of 10 and 30 individuals per 30-l tank. On the other hand, in females, AVT concentration was significantly higher in those kept alone. Brain IT concentrations significantly increased along with stocking density only in females and did not change in males. The sex-dependent responses indicate a different stimulation of AVT and IT neurons in males and females. Consequently, roles of the neurohormones in males and females exposed to stress of overcrowding must be different.

1. Introduction

Neurohormones, arginine vasotocin (AVT) and isotocin (IT), produced in separate parvocellular and magnocellular neurons in the preoptic area (POA) in Teleostei, are well known regulators of reproductive processes and social and sexual behaviors such as mating, courtship, parental care, territoriality and aggression [4,12,19]. Both neuropeptides probably play also a role in stress response in fish: elevated expression of hypothalamic AVT mRNA and increased plasma IT and AVT concentrations were found in fish subjected to different type of stress, e.g. confinement, disturbance, high-density, food deprivation or osmotic [14–16,20]. In our previous studies in stickleback we showed for the first time that levels of bioavailable AVT and IT in whole brain of males depends on specific breeding behavior of the individuals and their social status. The highest AVT levels were found in aggressive males that took care of the eggs and in subordinate males that fought to change their social status [13]. Neuropeptides may also affect the hypothalamic-pituitary-interrenal (HPI) stress axis. Fryer and Leung [10] have demonstrated in goldfish (*Carassius auratus*), that AVT and IT stimulate cortisol secretion. Moreover, AVT and corticotropin releasing hormone (CRH) synergize to stimulate the release of adrenocorticotropic hormone (ACTH) in rainbow trout (*Oncorhynchus mykiss*) [3]. However, in goldfish, effects of AVT, IT and CRH on ACTH release are additive [9].

The goal of this study was to examine an influence of stocking density on whole brain AVT and IT concentrations in males and females of three-spined stickleback (*Gasterosteus aculeatus*). In this study, we measured concentrations of bioavailable neuropeptides, dissociated from non-covalent complex that are active as neurotransmitters or neuromodulators in the central nervous system of the fishes.

2. Materials and methods

2.1. Animals and experimental design

The three-spined sticklebacks of both sexes (0.573–2.709 g) used in this study were caught in the breeding season in the Vistula river (Northern Poland). Before experimentation, fish were adapted to the laboratory conditions for five days. Experiment was carried out between June and July in freshwater, at room temperature and under natural photoperiod 17L:7D. Before experiment, fish and tanks were shortly disinfected with 0.05% and 1% $KMnO_4$, respectively. Experiment was conducted simultaneously in six 30-l tanks provided with crystalwort (*Riccia fluitans*). Fish received frozen food (*Chironomus plumosus*) *ad libitum* every two days at the same time of the day. Males and females were kept separately at stocking densities of 1, 10, and 30 fish per 30-l tank for 7 days. It corresponds with that expressed as body mass per tank: 1 male – approx. 1.0 g/tank, 10 males – 9.3 g/tank, 30 males – 32.8 g/tank, 1 female – approx. 1.7 g/tank, 10 females – 16.4 g/tank, 30 females – 48.5 g/tank. Behavioral observations were made several times a day. On the 7th day fish were anesthetized by immersion in 0.5% (v/v) 2-phenoxyethanol water solution. After sectioning the spinal cord brains were removed, immediately frozen and stored in −70 °C until the analysis.

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2.2. Analysis of AVT and IT

Analysis of AVT and IT was performed according to procedure described by Kleszczyńska et al. [13]. Shortly, after weighting, sonicating and centrifuging the brains, AVT and IT were extracted from the supernatants by solid-phase extraction method (SPE) on SPE speedisks columns (Baker Bond, C18, 20 mg, J.T. Baker, Phillipsburg, NJ, USA) combined with derivatization of the peptides. The derivatization was carried on using NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) in borate buffer (pH 9.5) for 20 min at room temperature. The derivatized neurohormones were injected onto HPLC system (Beckman modular HPLC system) supplied with Ultrasphere ODS analytical column (250×4.6 mm, 5 μm). The detection of the neuropeptides was performed using Shimadzu spectrofluorometric detector RF 551 with excitation at 460 nm and emission at 570 nm. The concentration of the peptides was expressed in pmol per mg of brain. Recovery of peptides was in the 89–93% range for AVT and IT. The detection limit was 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.

2.3. Statistical analysis

The results are expressed as means ± standard error of the mean (SEM). For multiple comparisons, the analysis of variance (Spjotvoll and Stoline test) for unequal number of means (n) was performed. Student's t-test was used to identify significant differences between two means for non-paired sample studies. Differences were considered significant at $P < 0.05$. The statistical analyses of data were carried out using Statistica 7.1 software.

3. Results

Single males in the tank neither showed nuptial coloration nor built the nest, all of them intensively fed. Among 10 males kept together in the tank, 3 demonstrated nuptial coloration two days after beginning the experiment. On the third day one of nuptial colored males started building the nest that was ready within two next days. The nest owner obviously dominated: it guarded the nest and was attacking the other males, which were in hiding behind the filter. Males did not feed except the dominant. Among 30 males placed together in the tank, 7 manifested nuptial coloration on the second day of the experiment. The males stayed behind the water filter for most of the time, some of them fought. None of 30 males fed, built the nest or dominated. All males survived the experiment.

Single females in the tank were calm and intensively fed. Each of 10 females kept together in the tank tried to separate from the group. Five females hid themselves behind the water filter and stayed there for the whole experiment. Some of the females fought, but only occasionally. No female fed. Thirty females kept together in the tank were under apparent stress from the beginning to the end of experiment. Twelve females hid themselves behind the water filter, all the rest tried to hide there, but eventually swam away and kept near the water surface for most of the time. After four days females were utterly exhausted. Six females from this group did not survive the experiment.

Brain AVT and IT concentrations are presented in Fig. 1. AVT concentration was significantly higher in males kept at densities of 10 (Spjotvoll and Stoline test, $P < 0.05$) and 30 (Spjotvoll and Stoline test, $P < 0.01$) than that in single males. On the other hand, AVT level was significantly higher in single females than in females kept at densities of 10 and 30 (Spjotvoll and Stoline test, $P < 0.001$). In females IT increased along with stocking density and did not vary in males.

4. Discussion

During breeding season stickleback males compete to establish territories and do not tolerate the presence of other fishes. At that time dominants are aggressive toward subordinates. In our study, only in the group of 10 fish the hierarchy was established and the dominant build the nest. Observations of sticklebacks on their spawning grounds revealed that in area of 30 square meters 30 males can build the nests [7]. In this study, fish were placed in tanks of only 0.1 m^2.

Males kept in tanks at densities of 1 fish/1 l and 1 fish/3 l were subjected to overcrowding which is exceptionally stressful for stickleback males during breeding. Fishes showed evident signs of stress – they did not feed, and most of them were in hiding. Pottinger et al. [18] have confirmed that overcrowding produces stress in sticklebacks: significantly higher levels of cortisol have been measured in sticklebacks at stocking density of 20 fish in 2 l tank (1 fish/0.l l) than at stocking density of 20 fish in 1000 l tank (1 fish/50 l).

In our study, we have observed significantly higher brain AVT levels in males when kept together (10 or 30 individuals) than those in single males. Brain AVT levels are similar in both groups despite the fact that in the 10 fish group the hierarchy has already been established while in the 30 fish group territorial competition and aggression has been observed. Therefore we suggest that in this study overcrowding is the main cause of increased brain AVT in males. In immature gilthead sea breams (*Sparus auratus*), plasma AVT values were also enhanced in fish kept at high density [16]. In rainbow trout, AVT transcript in parvocellular preoptic neurons increased significantly in response to acute confinement stress [11]. Also in flounder (*Platichthys flesus*), expression of AVT mRNA in magnocellular neurons was activated by the confinement stress [6]. Moreover, Backström et al. [2] demonstrated significantly higher level of AVT mRNA in the forebrain of rainbow trout subjected to 180 min of confinement in small space than that in non-disturbed controls.

In females, on the other hand, significantly higher concentrations of IT have been observed in 10 and 30 fish kept together than those in single females. Mancera et al. [16] demonstrated elevated plasma IT concentrations also in immature gilthead sea breams kept at high density. Highest brain AVT level has been observed in single females, but it does not seem to be an effect of social isolation stress, because the females did not show any symptom of stress: they were calm and intensively fed.

Thus in females of stickleback, IT, and in males, AVT, may be involved in response to the stress that results from overcrowding. The sex-dependent responses indicate a different stimulation of AVT and IT neurons in males and females that probably utilize different mechanisms when coping with stress.

There is evidence that AVT and IT in fish response to stress may be linked with release of ACTH that induces cortisol secretion [8,17]. It has been found that, *in vitro*, AVT and IT stimulate ACTH release from pituitary cells in goldfish and rainbow trout [9,17]. Furthermore, *in vivo*, AVT and IT stimulate cortisol secretion in goldfish [10]. In the green molly, AVT-immunoreactive (AVT-ir) and IT-immunoreactive (IT-ir) fibers in POA innervate corticotropin cells in pituitary which may suggest the role of both neuropeptides in ACTH secretion [5]. In rainbow trout, AVT-immunoreactive neurons and cells producing CRH are localized in the same brain region, i.e. in magnocellular preoptic area. Moreover, mRNA AVT as well as mRNA CRH expressions are increased in response to confinement stress in rainbow trout [1].

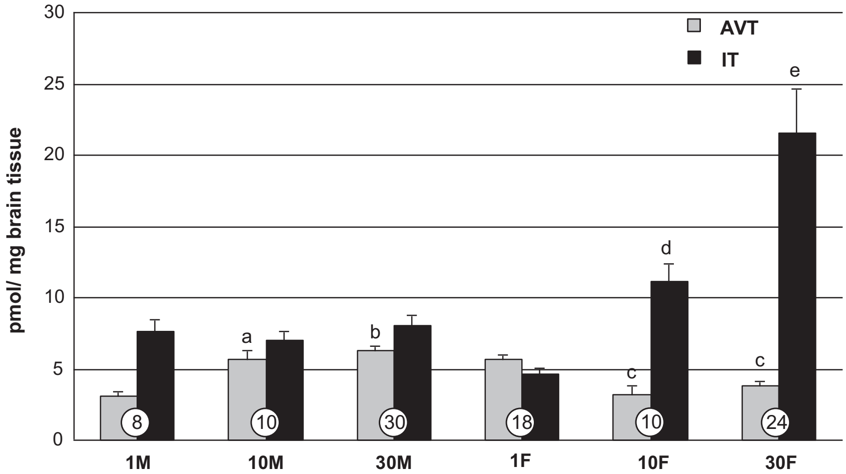

Fig. 1. AVT and IT concentrations in brains of males and females of three-spined stickleback kept in different stocking densities: 1, 10 and 30 fishes in aquarium. M – males, F – females. Number (n) of fish is given in the circles. The values are presented as means ± SEM. a: $P < 0.05$, b: $P < 0.01$ vs. AVT in 1 M group, c: $P < 0.001$ vs. AVT in 1F group, d: $P < 0.05$, e: $P < 0.01$ vs. IT in 1F group.

Response of brain AVT and IT to overcrowding that has been shown in sticklebacks together with vasotocinergic innervation of corticotropic cells in pituitary and adrenocorticotropic action of AVT and IT found in other fish species, suggest that AVT and IT may be coupled with the HPI axis in fish. However, further studies are needed to elucidate it. The three-spined stickleback seems to be a good model in AVT and IT studies. In this species, AVT and IT changes are sex-dependent and probably relates to different behavioral response to stress in males and females during breeding. In males, high density stress may be linked with aggression since they are territorial and fight to establish domination in group, but females neither establish territories nor demonstrate aggressiveness to each other [7].

Acknowledgment

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