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# Arginine vasotocin (AVT) and isotocin (IT) in fish brain: Diurnal and seasonal variations

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**Abstract**

An HPLC assay with solid-phase extraction and fluorescence derivatization was developed for measurement of arginine vasotocin (AVT) and isotocin (IT) in the neural tissues of fish. The efficiency and usefulness of the method have been verified in experiments by examination of peptides concentrations in brains of three fish species. The day–night changes in neuropeptides levels have been studied in brains of adult sea bream (*Sparus aurata*) and juvenile Atlantic salmon (*Salmo salar*). Seasonal fluctuations have been investigated in brains of three-spined sticklebacks (*Gasterosteus aculeatus*). The AVT and IT biosynthesis in brain seems to be controlled independently and probably each neuropeptide plays a different role in a circadian time-keeping system and an endocrine calendar in fish.


*Keywords:* Arginine vasotocin; Derivatization; Fish; Hormone; Hypothalamus; HPLC; Isotocin; Neurohormone; Neurohypophysis; Rhythms

## 1. Introduction

Arginine vasotocin (AVT) and isotocin (IT) are fish neuropeptides synthesized in the separate hypothalamic parvo- and magnocellular neurons of the NPO (neurosecretory preoptic nucleus) (Goossens et al., 1977; Van den Dungen et al., 1982; Duarte et al., 2001). Single AVT and IT neurons project toward both neurohypophysis and extrahypothalamic regions (Saito et al., 2004a). The peptides are closely related to mammalian vasopressin and oxytocin and have been identified in the hypothalamo-neurohypophysial system of teleosts by Acher et al. (1961, 1962) in the early sixties.

AVT and IT act as neurotransmitters and/or neuromodulators in the central nervous system of fish and both are known to play a role in modulation of reproductive processes and numerous related social behaviours (Godwin et al., 2000; Goodson and Bass, 2000). Many reports have also implicated AVT in cardiovascular activity, fluid management and interactions with other endocrine systems in teleostean fish (Fryer and Leung, 1982; Acher, 1993; Conklin et al., 1997). However, our understanding of the physiological role of AVT and especially of IT in fish is still fragmentary and needs elucidation. A shortage of methods sensitive enough to measure the peptides in a range of their physiological concentrations is a reason for that unsatisfactory state of our knowledge. A lot of difficulties have to be overcome to establish a sensitive assay for the simultaneous measurement of both hormones AVT and IT in one sample (Gozdowska and Kulczykowska, 2004). Our chromatographic assay with derivatization and fluorescent detection preceded by solid-phase extraction gives this unique opportunity.

This study was performed to verify the relevance of the developed HPLC method in experiments and to provide new data on diurnal and seasonal variations of the AVT and IT concentration in fish brain.

Data from mammals strongly suggest that neurohypophysial nonapeptide vasopressin (AVP), analogue of AVT, contributes to the circadian and seasonal time-keeping system (Hofman, 2004). Whether there is a case of AVT in fish is a matter of question, although the hypothetical role of this peptide in the circadian system in teleosts has been considered (Kulczykowska, 1995) and the marked diurnal changes in plasma AVT have been shown in rainbow trout and flounder (Kulczykowska and Stolarski, 1996; Kulczykowska et al., 2001).

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Three alternative experiments on three fish species were conducted. Diurnal variations of AVT and IT concentrations in brain tissue were studied in sea bream (*Sparus aurata*) and in juvenile Atlantic salmon (*Salmo salar*). Changes in the brain neuropeptides concentration throughout the year were investigated in three-spined sticklebacks (*Gasterosteus aculeatus*).

## 2. Material and methods

### 2.1. Analytical methods

Frozen brains were weighed quickly and sonicated separately (Microson™ XL 2000) in 1 mL distilled water, then extracted with acetic acid (final 0.25%) and placed in a boiling water bath for 3.5 min according to the procedure described by Pierson et al. (1995). The extracts were cooled on ice before centrifugation (20,000 ×*g*, 30 min, 4 °C). To clean the samples and derivatize peptides, the supernatants were decanted and then loaded into previously equilibrated (2 mL methanol, 2 mL water) spe speedisks (Baker Bond, C18, 20 mg). Columns were washed successively with 0.5 mL water, 0.5 mL acetic acid (4%) and 1 mL water. Then the derivatization procedure different from that previously described (Gozdowska and Kulczykowska, 2004) was applied. The derivatization was performed using 10 μL NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg/mL acetonitrile) in 90 μL borate buffer (pH 9.5) for 20 min at room temperature. After washing by-products with 0.5 mL water and 0.5 mL methanol (10%), derivatized peptides were eluted with mixture of ethanol: 6 N hydrochloric acid (2000:1; 0.5 mL) and directly injected to HPLC system (Beckman modular HPLC system with a Shimadzu spectrofluorometric detector RF-551). Reversed-phase HPLC analysis was performed according to a previously published method (Gozdowska and Kulczykowska, 2004). Briefly, chromatographic separation of peptides was carried out on an Ultrasphere ODS column (250×4.6 mm, 5 μm) using linear gradient system: 48–80% B (0.1% TFA in acetonitrile–water (3:1)) in A (0.1% TFA in water) for 20 min. The column temperature was 22 °C and flow rate 1 mL/min. Fluorescence detection was performed at 470 nm with emission at 530 nm. Derivatization of determined peptides is the crucial step in HPLC-FL analysis. Applying the NBD-F as a derivatizing reagent resulted in high sensitivity of vasotocin and isotocin measurement in fish plasma and allowed detection of these peptides at low physiological concentrations (Gozdowska and Kulczykowska, 2004).

### 2.2. Animals and experimental protocols

Sea bream (*S. aurata*) (300–500 g) were kept in seawater tanks at the University of Algarve (Portugal) at 19 °C under artificial light. Fish were assigned to one of two experimental groups and exposed to one of the following lighting regimes: continuous darkness (DD) or continuous light (LL). Animals were adapted for at least one week to the experimental lighting regime before experimentation. Brains were removed from decapitated fish from each group at 11:00 and 23:00 h and then stored at −70 °C.

Juvenile Atlantic salmon (approx. 40 g) from wild broodstock of the Dale anadromous strain (Southwest Norway) were kept in freshwater tanks at 8 °C under artificial light with simulated-natural photoperiod (16L:8D) at Bergen University for at least 2 weeks before experimentation. Brains for AVT and IT measurements were removed from decapitated salmon at 12:00 and 24:00 h and stored at −70 °C.

Adult three-spined sticklebacks (*G. aculeatus*) of both sexes used in this study were caught in the Vistula river (Northern Poland) in May (16L:8D), July (16L:8D) and December (8L:16D). Animals were dissected directly after catching. The brains were removed after decapitation, immediately frozen and stored at −70 °C.

### 2.3. Statistical analysis

Values are expressed as means±standard error of the mean (S.E.M.). For multiple comparisons, the analysis of variance (ANOVA) was used. Significant differences between means for paired sample studies were identified using Student's paired *t*-test. Significance was taken at $P<0.05$. Significant differences between means for non-paired sample studies were identified using Tukey's test. Significance was taken at $P<0.05$.

## 3. Results

### 3.1. Analytical step

The concentration of arginine vasotocin and isotocin in the brain tissue was measured using HPLC-FL method (Gozdowska and Kulczykowska, 2004) with significantly changed procedure of extraction and derivatization. The most important improvement was the speedisks which were applied instead of conventional spe cartridges. Moreover, the concentration of derivatizing agent was increased 15-fold. All modifications resulted in a shorter run time of analysis and more effective separation of free peptides in the neural tissue. Each sample was analysed in triplicate. Reproducibility of analysis expressed as RSD (relative standard deviation) for AVT and IT were 4.3% and 1.9%, respectively, and compared favorably with those achieved in our previous method (5.5% and 9.0%, respectively) (Gozdowska and Kulczykowska, 2004). Linearity of standard curve was $r^2=0.995$ and $r^2=0.996$ for AVT and IT, respectively. The detection limit was determined to be 10 fmol/mL.

A typical chromatogram of the AVT and IT in fish brain is shown in Fig. 1.

### 3.2. Diurnal changes

AVT and IT concentrations measured at 11:00 and at 23:00 h in brains of sea bream exposed to continuous darkness (DD) or continuous light (LL) are presented in Table 1. The AVT level in fish kept in DD displayed no day–night variation; the same applied to the IT in fish adapted to both lighting regimes. In LL adapted fish, noticeably higher concentrations of AVT were

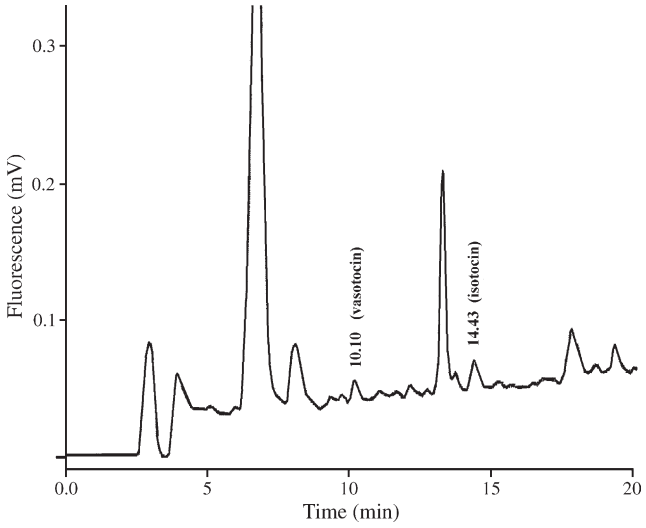


Fig. 1. Representative HPLC chromatogram of the brain tissue analyzed for AVT and IT. Samples were loaded into a C-18 column (Ultrasphere, 250×4.6 mm) under linear gradient 48–80% B in 20 min at 22 °C, at a flow rate 1 mL/min, at excitation 470 and emission 530 nm.

observed at 11:00 and higher concentrations of IT at 23:00 h, but the samples' size was too small for the statistical analysis.

The brain AVT and IT concentrations were measured at noon and at midnight in juvenile salmon kept at lighting regime of 16L : 8D (Table 2). There was no significant difference between day and night AVT content, whereas significantly higher IT levels were observed at midnight than at noon.

### 3.3. Seasonal changes

Brain AVT and IT concentrations measured in male and female three-spined sticklebacks in May, July and December are shown in Fig. 2. In both sexes, the lowest AVT concentrations were observed in December. The IT contents did not differ within three months with the exception of females in July. In females, concentrations of AVT were significantly higher than those in males in July ($P<0.001$) and in December ($P<0.01$).

In males, brain concentrations of IT in July and December were significantly higher than those of AVT measured simultaneously.

## 4. Discussion

The method we have developed is applicable for measurement of the content of neurohormones AVT and IT in the brain

Table 1
AVT and IT concentrations in brains taken at 11:00 and at 23:00 h from sea bream exposed to continuous darkness (DD) or continuous light (LL)

| | LL | LL | DD | DD |
|---|---|---|---|---|
| | 11:00 | 23:00 | 11:00 | 23:00 |
| AVT (pmol/mg brain) | 0.5±0.1 | 0.1±0.02 | 0.9±0.3 | 0.9±0.1 |
| IT (pmol/mg brain) | 0.1±0.1 | 0.3±0.20 | 0.8±0.1 | 1.0±0.3 |
| *n* | 4 | 3 | 3 | 3 |

Values are mean±S.E.M.; *n*—number of fish.

Table 2
AVT and IT concentrations in brains taken at noon and at midnight from juvenile Atlantic salmon kept at lighting regime of 16L : 8D

| | Day | Night | *P*-value |
|---|---|---|---|
| AVT (pmol/mg brain) | 0.6±0.1, *n*=7 | 0.8±0.1, *n*=7 | 0.10 |
| IT (pmol/mg brain) | 0.7±0.1, *n*=8 | 1.0±0.1, *n*=10 | 0.02 |

Values are mean±S.E.M.; *n*—number of fish; *P*—significance of difference between day and night values.

tissue of various fish species at least stickleback, sea bream and Atlantic salmon. The method was modified to enable efficient separation, detection and measurement of both neuropeptides even in a small sample, i.e. a single stickleback brain. The procedure is repeatable, sensitive and fast. Neurohypophysial peptides are known to be stored in a brain as noncovalent complexes with neurophysins (Breslow, 1993). Sonification of a brain causes a release of complexes from the tissue. Lower pH and reduced concentration of such complexes in extracts result in a cleavage between peptide and neurophysin. The assay quantifies an active fraction of the hormones in a sample as confirmed by MS analysis (Gozdowska and Kulczykowska, 2004).

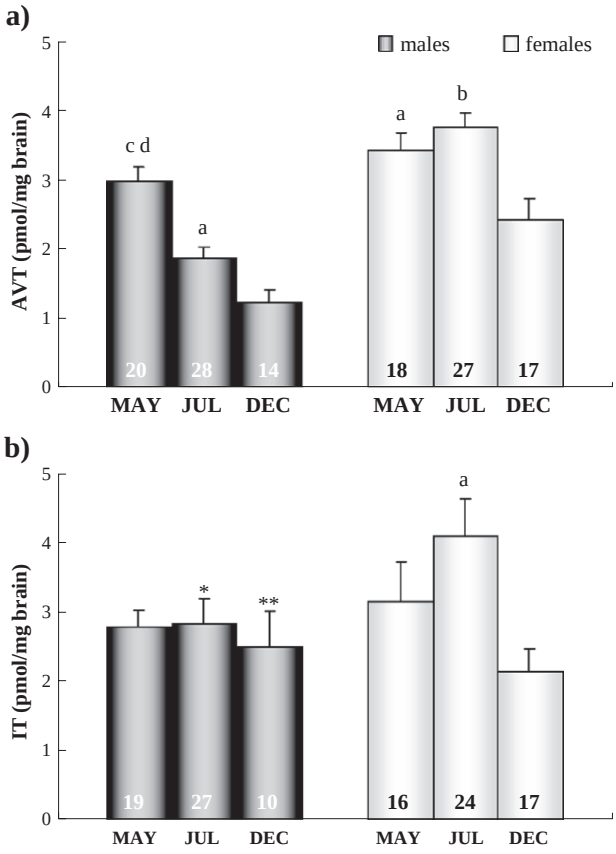


Fig. 2. (a) AVT concentrations in brains taken from male and female three-spined sticklebacks in May, July and December. Number of fish is given in the bars. a: $P<0.05$ vs. December value; b: $P<0.01$ vs. December value; c: $P<0.001$ vs. July value; d: $P<0.001$ vs. December value. (b) IT concentrations in brains taken from male and female three-spined sticklebacks in May, July and December. Number of fish is given in the bars. a: $P<0.05$ vs. December value; *: $P<0.05$ vs. males AVT value in July; **: $P<0.05$ vs. males AVT value in December.

The efficiency and usefulness of the method in research have been verified by examination of peptide concentrations in brain samples of three fish species during our experiments. It is a well-known phenomenon that hormone production in endocrine and neuroendocrine cells displays the differences within a day and a year (Hastings, 1991). The daily changes of vasopressin synthesis, with a maximum in light, is a characteristic feature of AVP neurons in the suprachiasmatic nucleus (SCN), a well-defined location of the "biological clock" in mammals (Yamase et al., 1991; Watanabe et al., 1993; Kalsbeek et al., 1995). Changes in activity of the suprachiasmatic, paraventricular and supraoptic AVP neurons result in the circadian changes in AVP concentration in the cerebrospinal fluid, where the neuropeptide is secreted (Yamase et al., 1991; Windle et al., 1992). It is generally presumed that in teleosts the preoptic area, a site of AVT and IT (analogs of mammalian AVP and oxytocin, respectively) production, is homologous with mammalian SCN. Thus, by analogy, the daily fluctuations in arginine vasotocin and/or isotocin content in the brain of fish can be expected.

In our study, no day–night differences either in AVT or IT concentration in brain of DD adapted sea bream were detected. In LL adapted fish, noticeably changes of AVT and IT levels during the day were reported, but our samples' size was too small to draw any conclusion. However, in juvenile salmon kept at artificial light with simulated-natural long-day photoperiod, a lack of difference between day and night brain AVT level was apparent, whereas a significantly higher level of IT was observed at night. This agreed with the data presented in pre-spawning chum salmon, where the hypothalamic contents of AVT mRNA did not show diurnal variation, but IT mRNA level was apparently higher at night, although not significant (Saito et al., 2004b). We can speculate herein that IT plays a role in a system of biological clock in salmonids, at least in juveniles. However, the study of a diurnal rhythm of gene transcription in the hypothalamic neurons in rainbow trout, revealed a significant diurnal change in AVT mRNA, with a peak of expression in the afternoon, and no change in IT mRNA expression in parvocellular neurons. In contrast, in magnocellular neurons, no rhythm of AVT mRNA expression was observed (Gilchriest et al., 1998). These data together strongly suggest that synthesis of neuropeptides in different neurons is regulated separately and a role of AVT and IT in a circadian time-keeping system is various.

Arginine vasopressin, hormone recognized as a component of mammalian endocrine calendar, exhibits seasonal changes in synthesis and release profiles (Hofman, 2004). Drawing an analogy with AVP, we have presumed that AVT and/or IT are components of internal calendar in fish, which control breeding. Several studies have indicated that neurohypophysial hormones are involved in reproduction in teleosts (Groves and Batten, 1986; Rodriquez and Specker, 1991; Saito et al., 2003). We studied the seasonal fluctuations of arginine vasotocin and isotocin content in brain of three-spined stickleback, a typical long-day breeder. A breeding activity, in examined fish population, extended from April to August (Sokołowska et al., 2004). Brain AVT contents in males and females in the months of their active reproduction were significantly higher than those in December. The highest brain IT content was observed in July, but only in females. The seasonal variations in expression of neurohypophysial hormone genes were observed in immature masu salmon (*Oncorhynchus masou*) (Ota et al., 1999a,b). Seasonal changes in AVT gene expression, reported even in immature males and females, were accompanied by the elevation of plasma sex steroids. The pattern of IT mRNA changes was more complicated and was different in both sexes. Sex-dependent seasonal variations in AVT and IT gene expression agreed with sexually dimorphic expression of the neurohormones genes shown in pre-spawning chum salmon (*Oncorhynchus keta*) (Ota et al., 1996). The various expression of AVT and IT genes in salmonids was proposed to be regulated by mechanisms linked to sex steroids and gonadotropin-releasing hormone (Ota et al., 1999a,b; Saito et al., 2003). The high AVT concentrations observed in both sexes of stickleback in May and July were coincident with active reproduction and probably also with high levels of sex hormones. The highest brain IT content in July in females, may suggest a distinct, specific role of this nonapeptide in reproductive cycle of females, as in the case of oxytocin, the mammalian analog of isotocin. However, in male stickleback, brain concentrations of IT in July and December were significantly higher than those of AVT, thus a physiological significance of IT in males, should not be underestimated.

The AVT mRNA differences among sexual phenotypes were described for the coral reef fish bluehead wrasse (*Thalasoma bifasciatum*), where males had AVT mRNA abundances approximately three times greater than females (Godwin et al., 2000). In contrast, we noticed meaningfully higher brain concentrations of AVT in females than in males in July and December. The different proportions of AVT to IT in both sexes in various seasons indicate the sex-dependent regulation and action of neuropeptides in stickleback. There is also a strong suggestion that stickleback reproduction is under control of the internal calendar, in which the pineal hormone melatonin plays an important role (Sokołowska et al., 2004). We propose that AVT and/or IT are components of the endocrine calendar, in which each peptide has a distinctive role.

In conclusion, our method is a useful tool for analysis of neurohormones AVT and IT in the brain tissue of various fish species. The brain neuropeptides concentration changes over the year. AVT and IT production in brain cells seems to be controlled independently and probably each neuropeptide plays a different role in a circadian time-keeping system and an endocrine calendar in fish.

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