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Diurnal Changes in Plasma Arginine Vasotocin and Isotocin in Rainbow Trout Adapted to Fresh Water and Brackish Baltic Water

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The plasma levels of arginine vasotocin (AVT) and isotocin (IT) in rainbow trout (*Oncorhynchus mykiss*) were studied to assess possible diurnal variations in neurohypophysial nonapeptides. Fish were kept under natural photoperiod and adapted to fresh and brackish Baltic water. Blood was sampled at 5:00, 11:00, 16:00, 22:30, and again at 5:00. Hormones were determined by gradient high-performance liquid chromatography preceded by solid-phase extraction. The marked diurnal changes of AVT were detected in plasma of fish adapted to both fresh and brackish waters. AVT levels (fmol/ml) were maximal at 16:00 and minimal at 5:00 (253.4 ± 35.7 and 45.5 ± 17.3, respectively). Unlike AVT, isotocin levels displayed no diurnal changes. AVT concentrations at 11:00, 16:00, and 22:30 were significantly higher than IT values measured throughout the day. Plasma AVT concentrations determined in brackish water-adapted fish at 16:00 were significantly lower than those of freshwater-adapted fish at the same time. These data suggest that synthesis and/or release of AVT and IT are controlled independently, so that these nonapeptides have different physiological roles in teleost fish. AVT might participate in circadian time-keeping system in fish.

Arginine vasotocin (AVT) and isotocin (IT) are nonapeptides in teleost fish, produced in separate hypothalamic neurosecretory neurons. The vasotocinergic and isotocinergic axons end in the neurohypophysis, where hormones are stored and released.

AVT is found in all vertebrates, but IT is restricted to teleosts. Although the teleost neurohypophysial hormones were chemically identified in the sixties (Acher *et al.*, 1964, 1968), their precise role still remains unclear. AVT seems to participate in osmoregulation, cardiovascular activity, endocrine secretion, reproduction, and probably neurotransmission and neuromodulation processes in the central nervous system in fish (Bentley, 1971; Henderson and Wales, 1974; Babiker and Rankin, 1978, 1980; Fryer and Leung, 1982; Pang *et al.*, 1983; Groves and Batten, 1986; Rodriquez and Specker, 1991; Balment *et al.*, 1993; Le-Mevel *et al.*, 1993; Amer and Brown, 1995; Oudit and Butler, 1995). Application of the new techniques to measure AVT and IT in fish plasma and tissue may improve this situation (Perrott *et al.*, 1991; Balment *et al.*, 1993; Warne *et al.*, 1994; Pierson *et al.*, 1995a,b). To date measurement of plasma isotocin concentrations is limited (Pierson *et al.*, 1995a,b).

Endocrine and neuroendocrine cells do not release their products continuously, and many display a rhythmicity (Hastings, 1991). For example the AVT content of the telencephalon and hypothalamus fluctuate within 24-hr periods in goldfish (Hontela and Lederis, 1985). However, diel changes in plasma concentrations of neurohypophyseal hormones have not been investigated. On the other hand, it is well known that the

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synthesis and secretion of AVT is very sensitive to osmotic stimuli. A few studies report variations in plasma AVT levels with respect to salinity (Perrott *et al.*, 1991; Balment *et al.*, 1993; Pierson *et al.*, 1995b).

The aim of this study was to quantitatively determine plasma AVT and IT levels of rainbow trout adapted to fresh water and brackish Baltic water and to assess possible diurnal variations. A new nonisotopic assay has been applied for the hormonal measurements.

MATERIALS AND METHODS

Animals. Rainbow trout *Onchorhynchus mykiss* (250–400 g) of both sexes were obtained from a hatchery (Institute of Inland Fisheries in Rutki, Poland). Fish were progeny of single parent spawning. In January and February animals were kept in tanks at 10–14° on a commercial trout diet under natural photoperiod (the dark period occurred between 16:00 and 8:00). Fish were adapted to fresh water (FW-adapted fish), then to brackish Baltic water (BW-adapted fish), and finally to fresh water again (FW-readapted fish).

Blood sampling. Blood samples for AVT and IT were taken from animals which had been held for at least 14 days in either FW or BW and assayed for plasma osmolality values. Blood was collected from the dorsal aorta of decapitated, unanesthetized fish. Plasma osmolality was measured using a 5500 vapor pressure osmometer (Wescor, Inc., Logan, USA). All blood samples, taken at time of sacrifice, i.e., at 5:00, 11:00, 16:00, and 22:30, were centrifuged at 1000*g* for 5 min and stored at −70° prior to analysis.

Plasma AVT and IT determination. AVT and IT were extracted from plasma by solid-phase extraction using a C_{18} Bakerbond SPE cartridge (J. T. Baker, Phillipsburg, NJ). The acidified plasma sample was aspirated through the column and washed with HPLC-grade water followed by glacial acetic acid–HPLC-grade water (4:96). The sample was eluted twice with 6 *M* HCl–absolute ethanol (1:2000). The eluate was collected, dried under air, and held at −20° prior to HPLC analysis. HPLC was performed with a Beckman modular system (Beckman Instruments, San Ramon, CA) with UV detector. Data were digitized by a Beckman 406 analog interface and processed by a Beckman analytical series System Gold data acquisition software on an IBM-compatible computer. Chromatographic separations were carried out on an Ultrasphere C_{18} column (250 × 4.6-mm i.d., 5-μm particle diameter, 80 Å pore size) connected to a precolumn (45 × 4.6-mm i.d.) filled with the same material, both obtained from Beckman Instruments. The system was run at a flow rate of 1.0 ml/min and the eluate was monitored at 215 nm. Linear gradient elution from 20 to 40% in 20 min was carried out with 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile: water (3:1). Plasma AVT and IT were identified by their retention times compared with those of standards. The retention times of AVT and IT were 8.5 and 15.0 min, respectively. The difference in times of elution protects against the coelution of these peptides. Quantitative determination of AVT and IT was performed on the basis of a standard curve. The linearity of signal responses was observed in the range of 10–500 pmol/ml (coefficient of determination $r^2 = 0.989$). To assess the precision of the assay, the retention times of plasma AVT and IT and corresponding synthetic nonapeptides in the different gradient systems and column temperatures were studied. Samples were assayed five times in the same set of experiments and in three different series. The inter- and intra-assay coefficients of variation were 17 and 10% for AVT and 15 and 12% for IT, respectively. The detection limit for both peptides was 10 pmol/ml (injected volume 10 μl). When low concentrations were being measured, 10 ml of plasma sample was required. The method has been described in detail (Kulczykowska, 1995a).

Chemicals. HPLC-grade acetonitrile, water, methanol, and TFA were purchased from J. T. Baker (Deventer, The Netherlands). Glacial acetic acid and hydrochloric acid were supplied by E. Merck (Darmstadt, Germany). Synthetic AVT and IT and ethanol were obtained from Sigma (St. Louis, MO).

Statistics. Values are presented as means ± standard error of the mean. Statistical analysis were carried out using ANOVA followed by Student's *t* test.

RESULTS

Euryhaline rainbow trout were acclimated to FW (tap running water) for at least 2 weeks prior to

experiment. Plasma osmolality was stable in a range of 310–315 mOsm/kg H_2O ($n = 6$). Transfer of FW-acclimated fish to brackish Baltic water (7‰ salt concentration, 166–170 mOsm/kg H_2O) resulted in a slight increase in plasma osmolality, which was established in a range of 327–333 mOsm/kg H_2O ($n = 6$) in fully adapted fish. In fish readapted to FW lower values of osmolality were observed: 315–320 mOsm/kg H_2O ($n = 6$).

In freshwater-adapted fish there were marked diurnal changes in plasma arginine vasotocin, but not isotocin (Fig. 1). The maximal AVT level occurred at 16:00 and the minimal at 5:00. AVT concentrations (fmol/ml) at 11:00, 16:00, and 22:30 (165.0 ± 27.3, 253.4 ± 35.7, and 146.0 ± 29.0, respectively) were significantly higher than those maintained at 5:00, i.e., 45.5 ± 17.3 and 56.7 ± 19.2 ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively). Comparison of two nonapeptide plasma levels showed that AVT concentrations measured at 11:00, 16:00, and 22:30 were significantly higher than the IT values determined at these times of day ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) (Fig. 1).

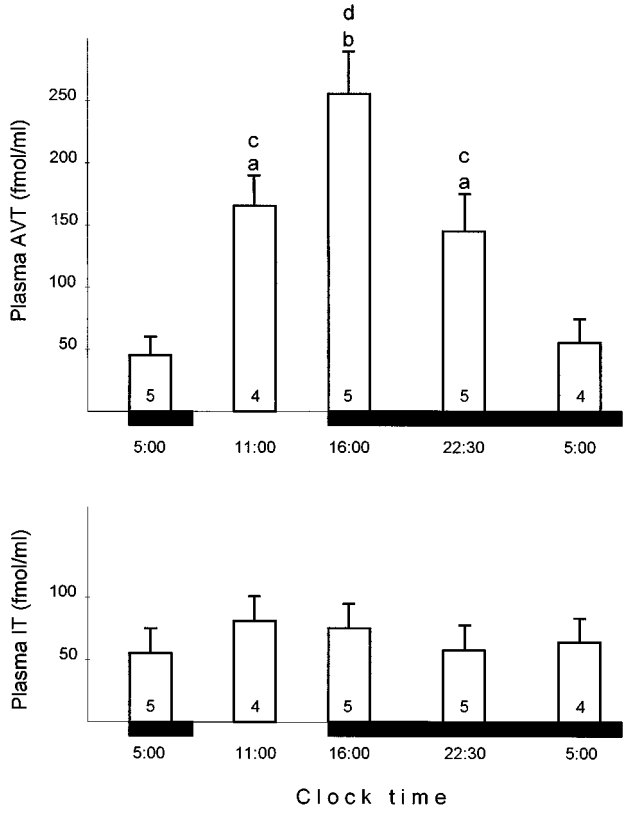

FIG. 1. Diurnal variations in plasma AVT (top) and IT (bottom) levels measured in rainbow trout adapted to fresh water. Dark bar indicates natural dark period during the experiments (January/February). Numbers of animals used are shown in the bars. a, $P < 0.05$ vs 5:00; b, $P < 0.01$ vs 5:00; c, $P < 0.05$ vs all IT values; d, $P < 0.01$ vs all IT values.

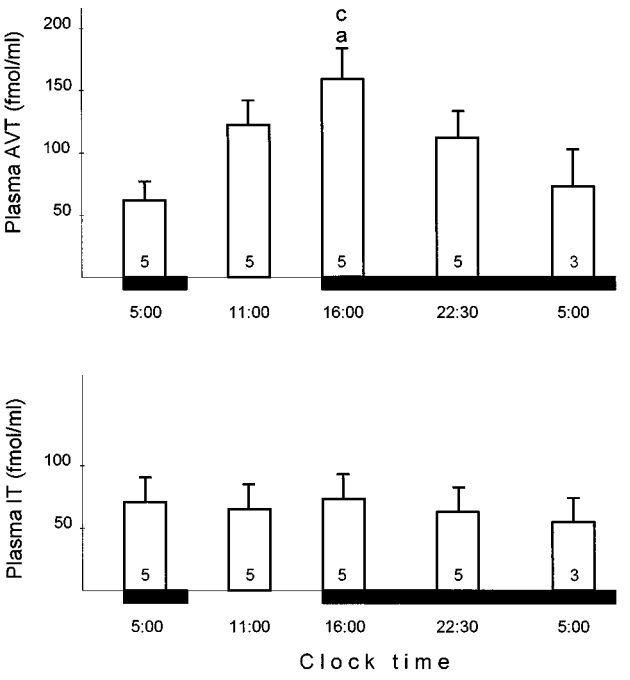

FIG. 2. Diurnal variations in plasma AVT (top) and IT (bottom) levels measured in rainbow trout adapted to brackish water. Dark bar indicates natural dark period during the experiments (January/February). Numbers of animals used are shown in the bars. a, $P < 0.05$ vs 5:00; c, $P < 0.05$ vs all IT values.

A similar pattern of fluctuation in plasma AVT also occurred in brackish water-adapted rainbow trout (Fig. 2). AVT concentrations at 16:00 were significantly higher than those measured at 5:00 ($P < 0.05$). Again there were no significant changes IT concentrations (Fig. 2).

In fish readapted to fresh water plasma AVT displayed a similar pattern of change as presented before for FW- and BW-adapted fish, with the significantly higher value at 16:00 v those measured at 5:00 ($P < 0.05$) and v IT values measured throughout the day ($P < 0.05$). The IT did not vary diurnally (Fig. 3).

Plasma AVT concentration (fmol/ml) measured in BW-adapted fish at 16:00 was significantly lower than

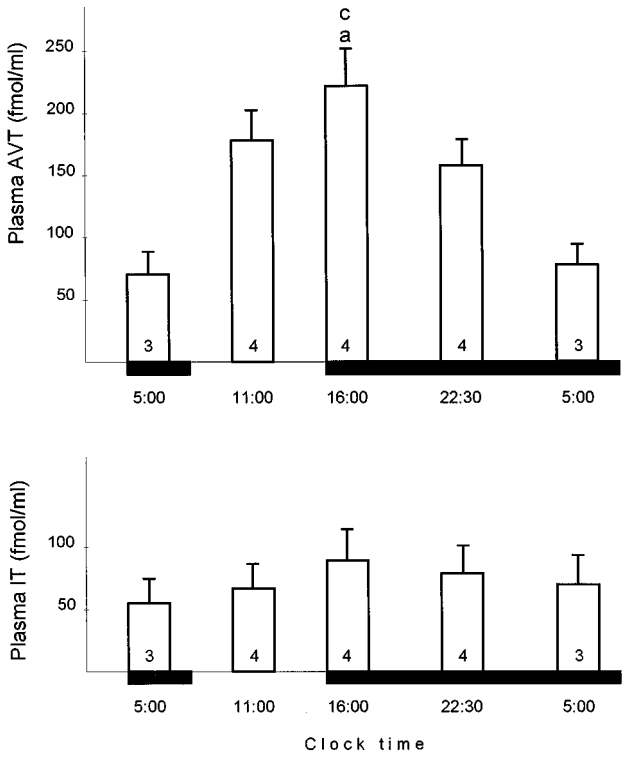

FIG. 3. Diurnal variations in plasma AVT (top) and IT (bottom) levels measured in rainbow trout readapted to fresh water. Dark bar indicates natural dark period during the experiments (January/February). Numbers of animals used are shown in the bars. a, $P < 0.05$ vs 5:00; c, $P < 0.05$ vs all IT values.

those measured in FW-adapted and FW-readapted fish at the same time (158.6 $\pm$ 24.7 vs 253.4 $\pm$ 35.7, $P < 0.05$ and 158.6 $\pm$ 24.7 vs 222.3 $\pm$ 29.3, $P < 0.05$, respectively). Unlike arginine vasotocin, isotocin levels displayed no significant differences between FW-adapted, and BW-adapted, or FW-readapted fish at any time of day.

DISCUSSION

This is the first study presenting the diurnal changes in plasma arginine vasotocin concentrations in fish. Previously, diel variations in hypothalamic and telencephalic AVT contents were reported in goldfish: higher contents were found at 22:00 than at 10:00 (Hontela and Lederis, 1985). In the present study plasma AVT levels were maximal at 16:00 (at sunset) and minimal at 5:00 (3 hr before sunrise) in all experimental groups. It is difficult to predict if the changes in AVT plasma concentration reflected the changes in synthesis, transport, and/or release of the hormone. It may be noted that a diurnal rhythm of arginine vasopressin, the mammalian counterpart of vasotocin, in plasma has been recorded: hormone concentrations were elevated during hours of light and decreased during the darkness (Greeley *et al.*, 1982; Windle *et al.*, 1992). The daily patterns of AVT plasma levels in rainbow trout thus closely match those found for vasopressin and oxytocin in the male rat by Windle *et al.* (1992) and Greeley *et al.* (1982). Moreover, a clear circadian rhythm of vasopressin (AVP) in the cerebrospinal fluid (CSF), the daily changes in AVP neuron activity, and vasopressin mRNA levels in the suprachiasmatic nucleus (SCN) in mammals were reported (Burbach *et al.*, 1988; Inouye and Shibata, 1994; Reppert and Uhl, 1987; Uhl and Reppert, 1986; Yamase *et al.*, 1991). Changes in activity in the AVP neurons, with the minimum in darkness, resulted in the circadian changes in AVP levels in the CSF, where the AVP was secreted. It is worth mentioning that a diurnal rhythm in pineal vasotocin content, with a peak during daytime and a midnight nadir, has been reported in the rat (Calb *et al.*, 1977). Together these observations suggest that nonapeptides may contribute to a circadian time-keeping system of vertebrates. It is generally presumed that in teleosts the preoptic area is the seat of the "biological clock" homologous with the mammalian SCN. Arginine vasotocin might play there analogous role with vasopressin in SCN in mammals. It is notable that the neurons in the preoptic area are immonoreactive for AVT and IT (Goossens *et al.*, 1977; Van den Dungen *et al.*, 1982), but their activities in changing photoperiod are not known. The hypothetical role of AVT in the circadian system in fish has been considered (Kulczykowska, 1995b).

It is of interest that there were no apparent diel fluctuations in plasma IT concentrations. The physiological significance of this observation is difficult to assess at present, but it may be assumed that the functions or control systems of two nonapeptides in fish differ. Isotocin level also did not change significantly following transfer of fish from fresh to brackish water or in the opposite direction. This agrees with the lack of significant differences in pro-isotocin mRNA levels between sea water (SW) and FW trout reported

by Hyodo and Urano (1991). The IT concentrations were significantly lower than those of AVT, with the exception of values detected at 5:00. For comparison, Pierson *et al.* (1995) observed in trout adapted to various salinities that, whatever the salinity, AVT concentrations were significantly higher than for IT.

It should be noted that the mean plasma AVT levels reported in rainbow trout show great variation (Perrott *et al.,* 1991; Balment *et al.,* 1993; Warne *et al.,* 1994; Pierson *et al.,* 1995a,b). Plasma AVT concentrations measured by Perrott *et al.* (1991) in FW- and SW-adapted rainbow trout were in the 10^{-10} *M* range, comparable with the present study. More recently, however, plasma AVT levels in rainbow trout have been measured at 10^{-12} *M* (Balment *et al.,* 1993; Warne *et al.,* 1994). Conversely, Pierson *et al.* (1995a,b) reported AVT plasma levels in the 10^{-8} *M* range. These differences may arise from many factors including techniques used, animal strains, or the animal's physiological state.

The present high plasma levels of AVT measured in FW-adapted and FW-readapted fish agree with those obtained in rainbow trout and flounder adapted to various salinities (Perrott *et al.,* 1991; Balment *et al.,* 1993). Similarly, the higher AVT values correspond with lower plasma osmolalities in FW animals. The significant fall in AVT plasma level from FW to ⅓ SW was observed recently in rainbow trout adapted to various salinities (Pierson *et al.,* 1995b). On the contrary, the RIA study by Warne *et al.* (1994) showed that there was no difference in plasma AVT concentration between FW- and SW-adapted trout. However, in the euryhaline flounder, plasma AVT level in FW-adapted fish was higher than in SW-adapted fish (Warne *et al.,* 1994).

It is known that the AVT content in the pituitary of FW-adapted rainbow trout temporarily acutely decreases upon transfer of fish to SW but eventually recovers to FW-adapted level (Lederis, 1964). Similar pattern of change was found in the medaka (*Oryzias latipes*) during osmotic stress (Haruta *et al.,* 1991) and in FW- and SW-adapted flounder (*Platichthys flesus*) (Perrott *et al.,* 1991). On transfer of fish in the opposite direction the pituitary AVT content temporarily increased. It should be also noted that the AVT content in rainbow trout pituitary measured recently by Pierson *et al.* (1995b) was significantly lower than IT, and the SW levels of each hormone were not different from FW values. However, Hyodo and Urano (1991) showed, in rainbow trout, that the amount of pro-vasotocin mRNA decreased in seawater and returned to initial levels after transfer of fish back into fresh water. Accordingly the lower plasma AVT levels detected in the BW-acclimated rainbow trout may reflect inhibition of AVT synthesis and/or release during BW adaptation. The increased AVT plasma concentration in FW-readapted fish may reflect the increase in AVT synthesis and/or secretion.

In conclusion, the synthesis and/or release of AVT and IT may be controlled independently and AVT may participate in circadian time-keeping system in teleosts. The present study confirms that gradient high-performance liquid chromatography preceded by a solid-phase extraction may be a useful tool for analysis of neurohypophysial peptides in plasma without the use of radioisotopes, recognizing that there are differences in sensitivities. The principal advantage is that the method is a comparatively simple nonbiological assay for both AVT and IT.

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