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Diel changes in plasma arginine vasotocin, isotocin, and melatonin in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The diel changes in plasma AVT, IT and Mel in rainbow trout (*Oncorhynchus mykiss*) were studied to assess potential relationships. Blood was sampled at 05:00, 11:00, 16:00, 22:30 and 05:00 in freshwater-adapted fish and at 22:30 in brackish water-adapted fish maintained under natural photoperiod. A few of the FW-acclimated fish were assigned to one of two experimental groups and adapted to DD or LL lighting regimes. Blood samples were taken at 11:00 and 22:30. Hormones were extracted from plasma by solid phase extraction and determined by high-performance liquid chromatography. Marked diel variations in AVT and Mel were detected in fish maintained under natural photoperiod. Plasma AVT (fmol ml^{-1}) increased during the light to reach the maximal level at the end of that phase (261.7 ± 23.1). Thereafter, AVT concentration decreased and became minimal at 05:00 (68.9 ± 11.5) 3 h before the sunrise. Plasma Mel (pmol ml^{-1}) increased between 16:00 and 22:30 when a peak value was reached (1204.0 ± 55.5). Thereafter, Mel levels decreased and were minimal after the onset of the light phase (242.8 ± 37.0). IT levels displayed no significant diel changes. Linear regression analysis indicated the negative correlation between plasma Mel and AVT for five collecting times of the daily 24 h cycle in freshwater fish and at 22:30 in brackish water fish. A similar correlation occurred at 11:00 in the DD group and at 22:30 in the LL group. To elucidate the character of the Mel-AVT relationships further studies are required.

Abbreviations: AVP – arginine vasopressin; AVT – arginine vasotocin; BW – brackish water; DD – continuous darkness; FW – fresh water; HPLC – high-performance liquid chromatography, IT – isotocin; LL – continuous light; Mel – melatonin; SPE – solid phase extraction.

Introduction

Many hormones show fluctuations that exhibit a circadian (about 24-h) pattern, thus preparing the organism for the changes in environment associated with day and night (Binkley 1993). In all vertebrates, the most pronounced and consistent circadian endocrine rhythm is that of Mel production by the pineal gland. Mel is not stored in the pineal, so once synthesized, the hormone enters the circulatory system. In all species examined, plasma Mel levels remain low during the day and are elevated throughout the night (Binkley 1988; Reiter 1991; Filadelfi and Castrucci 1996). The pineal is involved in the control of rhythmic adaptations to daily and seasonal cycles. Studies in a few teleost fish species suggest the presence of cellular circadian oscillators within the pineal organ (Iigo et al. 1991; Zachman et al. 1992a; Bolliet et al. 1996a,b; Cahill 1996; Molina-Borja et al. 1996). However, the pineal organs of rainbow (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) do not appear to contain a circadian oscillator, but Mel synthesis is directly controlled by the pattern of illumination (Gern and Greenhouse 1988; Max and Menaker 1992).

AVT and IT, closely related to mammalian AVP and oxytocin, are produced in hypothalamic neurosecretory neurons with axons ending in the neurohypophysis, where the hormones are stored and released.

Data on daily fluctuations in plasma AVT and IT concentrations in fish are scarce (Kulczykowska and Stolarski 1996). The reported daily patterns of plasma AVT levels in rainbow trout closely match those found for AVP and oxytocin in the male rat (Greenley et al. 1982; Windle et al. 1992). In mammals, a clear circadian rhythm of AVP in the cerebrospinal fluid and daily changes in AVP neurone activity and vasopressin mRNA levels in the suprachiasmatic nucleus, where the circadian oscillator is situated, are well documented (Uhl and Reppert 1986; Burbach et al. 1988; Yamase et al. 1991). The contribution of AVP to a circadian time-keeping system in mammals is beyond any question (Binkley 1988, 1993). Moreover, diurnal and seasonal rhythms in pineal AVT content have been reported in the rat (Calb et al. 1977; Prechel et al. 1983).

It has been hypothesized that the endocrine system (pineal organ-hypothalamus-neurohypophysis) coordinating physiology and behaviour in fish implies complex hormonal interactions (Kulczykowska 1995a). The present study was undertaken to investigate diel changes in plasma Mel, AVT and IT in rainbow trout and to assess potential relationships.

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss*) (250–400 g) of mixed sex were obtained from a hatchery (Institute of Inland Fisheries in Rutki, Poland). All the fish used in the study were progeny of single parent spawning. In December, January and February animals were kept in tanks at 10–14 °C on a commercial trout diet.

Experimental protocol

I. Fish were maintained under natural photoperiod slightly modified artificially (the dark period occurred between 15:00 and 08:00 h). Fish were adapted to freshwater (FW-adapted fish) and then a few of them were transferred to brackish Baltic water (BW: 169–173 mOsm kg^{-1}) and acclimated for 2 weeks (BW-adapted fish). All blood samples (5–8 ml) were taken at the time of sampling at 05:00, 11:00, 16:00, 22:30 and 05:00 h in FW-adapted fish and only at 22:30 h in BW-adapted fish. Blood samples for AVT, IT and Mel were collected from the dorsal aorta of decapitated, unanesthetized fish. Plasma was separated by centrifugation at 1000 $\times$ g for 5 min and stored at -70 °C prior to analysis. Plasma osmolality was measured using a vapor pressure osmometer (Wescor Inc., Logan, USA).

II. FW-acclimated fish were assigned to one of two experimental groups and adapted to continuous darkness (DD) or continuous light (LL) for at least 3 days before experimentation. Blood samples (5–8 ml) were taken at time of sacrifice at 11:00 and 22:30 h. Plasma was assayed for concentrations of AVT, IT and Mel.

Plasma AVT, IT and Mel analysis

AVT and IT were extracted from plasma by solid phase extraction (SPE) using C_{18} Bakerbond cartridges (J.T. Baker, Phillipsburg, NJ, USA; pore size 60 Å, particle diameter 40 μm). The acidified plasma sample was aspirated through the column, washed with HPLC-grade water followed by glacial acetic acid-HPLC-grade water (4:96). The sample was eluted twice with 6M HCl-absolute ethanol (1:2000). The eluate was collected, dried under air, and held at -20 °C prior to HPLC analysis. HPLC was performed with a Beckman modular system (Beckman Instruments, San Ramon, CA, USA) with UV detector. Data were digitized by a Beckman 406 analog interface and processed by 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 $\times$ 4.6 mm I.D., 5 μm particle diameter, 80 Å pore size) connected to a precolumn (45 $\times$ 4.6 mm I.D.) filled with the same material, both obtained from Beckman Instruments (San Ramon, CA, USA). The system was run at a flow rate of 1.0 ml min^{-1} and the eluate was monitored at 215 nm. Linear gradient elution from 20 to 40% in 20 min was carried out with 0.1% 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. Quantitative determination of AVT and IT was performed on the basis of a standard curve. The inter- and intra-assay coefficients of variation were 17% (n=15) and 10% (n=15) for AVT and 15% (n=15) and 12% (n=15) for IT, respectively. The method has been described in detail (Kulczykowska 1995b).

Mel was extracted from plasma by SPE using the same cartridge described above. The columns were conditioned with two 1 ml portions of methanol followed by two portions of HPLC-grade water. The plasma sample was aspirated through the column, washed with a portion of 1 ml of methanol-HPLC-

grade water (1:9). The sample was eluted with two portions of 300 μl of methanol. The eluate was collected, dried under air, and held at −20 °C prior to analysis. Before assay, each sample was reconstituted with 60% methanol to 100 μl and mixed well. The 20 μl samples were injected into HPLC for analysis. The separation and detection were performed with the Beckman modular system, mentioned above, connected to a Shimadzu spectrofluorometric detector RF-551. Chromatographic separations were carried out on the same Ultrasphere C_{18} column. The column temperature was maintained at 22 °C and the flow-rate at 0.6 ml min^{-1}. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. An isocratic elution system was prepared. The mobile phase was 60% HPLC-grade methanol. The detection limit was 13 pmol per ml of plasma. The inter- and intra-assay coefficients of variation were 14% (n=9) and 10% (n=9), respectively. The method has been described in detail (Kulczykowska and Iuvone 1998).

Hormones

[Arg^8]vasotocin (mol wt 1050.2, purity 97%), IT (mol wt 966.1, purity 97%) and Mel (mol wt 232.3) were obtained from Sigma Chemical (St. Louis, MO, USA).

AVT (1 mg ml^{-1}) and IT (1 mg ml^{-1}) were dissolved in HPLC-grade water and stored in stock solutions at −70 °C. Working standards and peptide solutions for injection were prepared directly before use in HPLC-grade water and 0.9% NaCl, respectively.

The stock solution of Mel (1 mg ml^{-1}) was prepared by dissolving in HPLC-grade methanol (J.T. Baker, Deventer, the Netherlands). Working standards and solutions for injection were prepared in HPLC-grade water and 0.9% NaCl, respectively, immediately before use. It is strongly recommended that Mel solutions be kept in darkness at −20 °C until use (but not longer than 2 h) to avoid decomposition during storage.

Chemicals

HPLC-grade acetonitrile, water, methanol and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Deventer, The Netherlands). Glacial acetic acid and hydrochloric acid were purchased from E.Merck (Darmstadt, Germany).

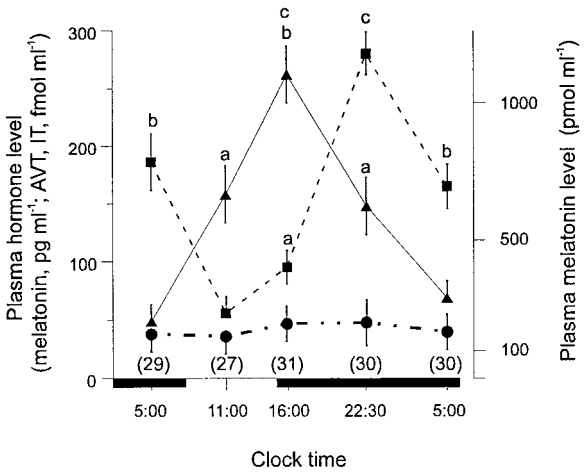

Figure 1. Time course of changes in plasma Mel (squares), AVT (triangles) and IT (circles) concentrations measured in rainbow trout adapted to fresh water. Dark bar indicates natural dark period during the experiments (December–February). Values are mean ± SEM; n is given in the brackets. AVT: (a) $p<0.05$ vs 05:00 h; (b) $p<0.01$ vs 11:00 h and 22:30 h; (c) $p<0.001$ vs 05:00 h Mel: (a) $p<0.05$ vs 11:00 h; (b) $p<0.01$ vs 11:00 h, 16:00 h and 22:30 h; (c) $p<0.001$ vs 11:00 h and 16:00 h.

Statistics

Values are presented as mean ± SEM. The statistical significance of differences between means has been assessed using Student's t-test, paired or unpaired as appropriate, with the Bonferroni correction procedure for multiple comparisons. Regression lines were fitted by the method of least squares, and the significance of the correlation coefficient was determined.

Results

Plasma osmolalities in FW-adapted and BW-adapted fish were 314 ± 5 mOsm kg^{-1} (n=30) and 342 ± 7 mOsm kg^{-1} (n=10), respectively. The values were similar to those presented earlier (Kulczykowska and Stolarski 1996).

The course of mean plasma AVT, IT and Mel concentrations measured in FW-adapted fish throughout the collecting times of the daily 24 h cycle is presented in Figure 1. Plasma AVT concentration increased during the light to reach the maximal level at the end of that phase (261.7 ± 23.1 fmol ml^{-1}; n=31). Afterwards, the AVT concentration decreased progressively and was minimal at 05:00 h, 3 h before the onset of the light phase (68.9 ± 11.5 fmol ml^{-1}; n=30). Unlike AVT, IT levels displayed no statistically significant diel changes. Plasma Mel concentration increased

between 16:00 and 22:30 h when a peak value was reached (1204.0 ± 55.5 pmol ml^{-1}; n=30). Thereafter, Mel levels decreased progressively and were minimal after the onset of the light phase (242.8 ± 37.0 pmol ml^{-1}; n=27).

Plasma AVT concentration of BW-acclimated fish at 22:30 h was significantly lower than that of FW-adapted fish at the same time (111.2 ± 18.0 fmol ml^{-1}; n=10 and 148.0 ± 21.9 fmol ml^{-1}; n=30, respectively; $p<0.05$). Conversely, plasma Mel concentration assayed simultaneously in the same fish was significantly higher in BW fish than in FW fish (1419.3 ± 61.1 fmol ml^{-1}; n=10 and 1204.0 ± 55.5 fmol ml^{-1}; n=30, respectively; $p<0.05$). The IT in the same fish did not differ significantly (BW: 42.1 ± 16.1 fmol ml^{-1}, n=10; FW: 47.8 ± 15.6 fmol ml^{-1}, n=30).

The correlations between plasma AVT and Mel concentrations for five collecting times of the daily 24 h cycle in FW-adapted fish were highly significant (05:00 h: $r = -0.86$, $p<0.001$; 11:00 h: $r = -0.69$, $p<0.01$; 16:00 h: $r = -0.88$, $p<0.001$; 22:30 h: $r = -0.67$, $p<0.05$; 05:00 h; $r = -0.82$, $p<0.01$). A similar correlation occurred at 22:30 h in BW-adapted fish ($r = -0.75$, $p<0.05$). A negative correlation was evident, although the relationships between hormones values altered throughout the day, as was presented in Figure 2a,b. When a similar analysis was performed for plasma hormones and plasma osmolality such relationships were not apparent in any of the fish group.

Plasma Mel concentrations at 22:30 h and 11:00 h in the DD group did not vary significantly (1156.1 ± 61.9 pmol ml^{-1}; n=8 and 1099.2 ± 60.9 pmol ml^{-1}; n=8, respectively). These concentrations were not significantly different from values measured at 22:30 h in the group maintained under natural photoperiod. However, both values were significantly higher ($p<0.001$) than those measured in the LL group at 22:30 and 11:00 h (245.7 ± 34.5 pmol ml^{-1}; n=7 and 229.9 ± 41.7 pmol ml^{-1}; n=7, respectively). Plasma AVT concentration at 11:00 h in the DD group was significantly lower than that measured at the same time in fish exposed to natural photoperiod (98.9 ± 18.7 fmol ml^{-1}; n=8 and 153.8 ± 24.9 fmol ml^{-1}; n=27; $p<0.01$, respectively). Plasma AVT concentration at 22:30 h in the LL group was significantly higher than that measured at the same time in fish exposed to a natural photoperiod (214.8±31.9 fmol ml^{-1}; n=7 and 148.0±21.9 fmol ml^{-1}; n=30, $p<0.05$, respectively). Negative correlations between plasma Mel and AVT

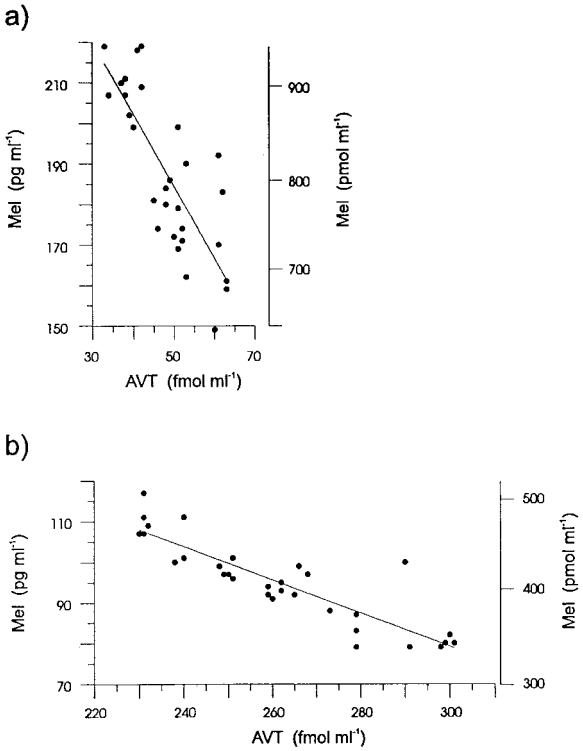

Figure 2. (a) The relationship between plasma Mel and AVT concentrations at 05:00 h. Individual values are represented and the regression line was drawn according to the equation: $y = -1.14x + 251.16$ ($r = -0.86$, $p<0.001$); n=29 (b) The relationship between plasma Mel and plasma AVT concentrations at 16:00 h. Individual values are represented and the regression line was drawn according to the equation: $y = -0.41x + 202.25$ ($r = -0.88$, $p<0.001$); n=31

concentrations occurred at 11:00 h in the DD group ($r = -0.65$, $p<0.01$) and at 22:30 h in the LL group ($r = -0.71$, $p<0.01$). Plasma IT concentrations displayed no significant differences between DD and LL groups at 11:00 h (54.7 ± 14.2 fmol ml^{-1}; n=8 and 43.8 ± 14.9 fmol ml^{-1}; n=7, respectively) and at 22:30 h (49.9 ± 14.2 fmol ml^{-1}; n=8 and 58.7 ± 19.2 fmol ml^{-1}; n=7, respectively).

Discussion

This is the first study presenting simultaneous diel changes in plasma AVT, IT and Mel in the same fish. Plasma Mel concentrations measured in this study agree with values seen in other fish: rainbow trout, carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) (Gern et al. 1978; Kezuka et al.

1988, 1992; Zachmann et al. 1992b; Randall et al. 1995). The daily fluctuation of Mel in body fluids is a common phenomenon in vertebrates, with low levels being present in the light and high levels during darkness. Light suppresses or inhibits Mel synthesis in the pineal. Therefore the circulating Mel measured in fish during the photophase or during the continuous light may be related to the Mel synthesized outside the pineal organ (Bubenik and Pang 1997).

It is noteworthy that intra-pineal oscillators driving the melatonin rhythm have been identified in all teleost fish so far examined, except for the rainbow trout (see Introduction). The lack of differences in plasma Mel concentrations at 22:30 h and at 11:00 h in fish kept under DD conditions is consistent with the prevailing opinion that the rainbow trout pineal organ as a simple photometer in terms of Mel production (see: Introduction). The variation in Mel levels between 22:30 h and 05:00 h (3 h before sunrise) observed in this study (Figure 1) could be explained by the pulsatile nature of release of Mel. However, the regulation of Mel production involving a circadian oscillator located outside the pineal organ in rainbow trout cannot be excluded, although, so far, there is no experimental evidence.

Plasma AVT concentrations measured in the present study are comparable with those reported in rainbow trout by Perrott et al. (1991) and Kulczykowska and Stolarski (1996) and are in accordance with doses producing maximum inhibition of adenylate cyclase activity in rainbow trout gills, one of the important osmoregulatory organs in fish (Guibbolini and Lahlou 1987). Diel pattern of fluctuation in plasma AVT concentrations and the lack of significant differences in plasma IT concentrations which occurred in this study, were reported earlier in a smaller group of rainbow trout assayed for neurohypophysial hormones only (Kulczykowska and Stolarski 1996).

Drawing analogies with the well-known contribution of AVP to a circadian time-keeping system in mammals, the potential relationship between Mel and AVT in fish is considered. To this date, Mel-AVT interactions in fish have not been investigated and the only data available are derived from mammals. It is established that inactivation of pineal enzymes N-acetyltransferase (NAT) and hydroxyindole-O-methylotransferase (HIOMT) is accelerated by disulfide-containing compounds, e.g., AVT (Sugden and Klein 1987; Binkley 1988). On the other hand, Mel inhibits the protein synthetic activity of the SCN in mammals (Morgan and Williams 1989) and the release of hypothalamic AVP and oxytocin *in vitro* (Yasin et al. 1993). In the study reported here the daily rhythms of Mel and AVT displayed an approximately 6 h phase shift: the peak level of Mel was preceded by 6 h by the AVT maximal value and minimal concentration of Mel appeared 6 h before the AVT peak. The high Mel concentration during the night may, directly or indirectly, inhibit the AVT synthesis or/and release. On the other hand, AVT might influence the Mel synthesis and release. Accordingly, negative correlations between plasma Mel and AVT for five collecting times of the daily 24 h cycle in FW-adapted fish and at 22:30 h in BW-adapted fish were demonstrated (Figure 2 a,b, and Results). Similar relationship occurred at 11:00 h in DD group and at 22:30 h in the LL group; the plasma AVT concentration was elevated in LL fish at 22:30 h, when the plasma Mel level was low as a result of continuous light; the plasma AVT concentration was diminished in DD fish at 11:00 h, when the plasma Mel level was high as a result of continuous darkness.

Whether the Mel-AVT correlations presented here indicate the cause-effect relationships remains still an open question. However, all these observations together provide a strong case for further study of relationship between Mel and AVT secretory patterns and potential participation of vasotocin in circadian time-keeping system in fish.

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