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Plasma Ca^{2+} concentration limits melatonin night production in two fish species

M. Gozdowska, E. Sokołowska and E. Kulczykowska*

Institute of Oceanology of Polish Academy of Sciences, Department of Marine Genetics and Biotechnology, św. Wojciecha 5 Str., 81-347 Gdynia, Poland

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In freshwater (FW) rainbow trout *Oncorhynchus mykiss* of spontaneously low plasma calcium concentrations ($[Ca^{2+}]_{pl}$), plasma melatonin at night was significantly lower than that measured in FW fish with the highest $[Ca^{2+}]_{pl}$. In brackish water adapted rainbow trout with originally high $[Ca^{2+}]_{pl}$, plasma melatonin concentration at night was elevated. In cannulated flounder *Platichthys flesus*, night plasma melatonin increases (ΔMel) corresponded to $[Ca^{2+}]_{pl}$. It is postulated that in physiological steady-state conditions, melatonin synthesis capacity is coupled to free calcium concentration in plasma of *O. mykiss* and *P. flesus*.

Key words: flounder; melatonin; *Oncorhynchus mykiss*; plasma Ca^{2+}; *Platichthys flesus*; rainbow trout.

INTRODUCTION

In fishes, as in other vertebrates, melatonin (*N*-acetyl-5-methoxytryptamine, Mel) is synthesized in the pineal organ in a rhythmic fashion with elevated levels during the night and low levels during the day (Binkley, 1988). Secretion of Mel into the circulatory system reflects the capacity of its production by the gland. In most of the fish species examined, the rhythm of Mel synthesis is controlled by endogenous intrapineal oscillators (Bolliet *et al.*, 1996). Diurnal changes in circulating Mel observed in flounder *Platichthys flesus* (L.) exposed to constant darkness also indicate the presence of an intrapineal oscillator (Kulczykowska *et al.*, 2001). Conversely, in rainbow trout *Oncorhynchus mykiss* (Walbaum) melatonin production is regulated directly by light acting on the pineal organ (Max & Menaker, 1992).

It is well established that calcium is an important component of many pineal processes as the adrenergic regulator of the gland function and in melatonin synthesis controlled by serotonin *N*-acetyltransferase (AA-NAT: arylalkylamine *N*-acetyltransferase), a key regulatory enzyme in melatonin biosynthesis (Morton & Reiter, 1991). Calcium is found to activate melatonin biosynthesis in the mammalian pineal (Morton, 1989; Morton & Reiter, 1991). The pineal of rats

*Author to whom correspondence should be addressed. Tel.: +48 58 620 8913; fax: +48 58 620 1233; email: ewakulcz@cbmpan.gdynia.pl

removed at night and perifused with calcium solution responded with increased melatonin release (Zhao & Touitou, 1993). Moreover, in both chick (Zatz & Mullen, 1988; Nikaido & Takahashi, 1996; Pablos *et al.*, 1996) and rainbow trout (Meissl *et al.*, 1996) pineal cells, removal of calcium from the extracellular medium suppressed Mel synthesis. A similar effect was observed by blocking Ca^{2+} voltage-dependent channels in chick (Harrison & Zatz, 1989) and rainbow trout pineal photoreceptor cells (Begay *et al.*, 1994). On the other hand, elevation of the extracellular Ca^{2+} level enhanced Mel production in dispersed *O. mykiss* pineal cells (Begay *et al.*, 1994). Calcium entry into *O. mykiss* pineal photoreceptor cells through calcium channels is important in maintenance of dark-induced Mel synthesis (Gasser & Gern, 1997). In the chicken retina, the activity of AA-NAT decreased rapidly when calcium channels were closed (Iuvone & Besharse, 1986; Zawilska *et al.*, 1992). In the Syrian hamster *Mesocricetus auratus* pineal glands *in vitro*, the absence of extracellular calcium reduced AA-NAT activity and prevented the induction of Mel synthesis by forskolin (Santana *et al.*, 2001). Calcium is thus thought to play a role in Mel production, but it is not known if plasma Ca^{2+}, representing an extracellular calcium reservoir, influences melatonin synthesis capacity in physiological steady-state conditions in fishes.

The aim of the present work, therefore, was to examine if there was a relationship between plasma Ca^{2+} concentration ($[Ca^{2+}]_{pl}$) and plasma Mel in two fish species, freshwater rainbow trout and seawater flounder, which represent, respectively, two different models of exogenous and endogenous hormone synthesis regulation.

MATERIALS AND METHODS

ANIMALS

Rainbow trout (250–400 g) of both sexes obtained from a hatchery (Institute of Inland Fisheries, Rutki, Poland) were progeny of a single parent spawning. Two weeks prior to the experiment, the following acclimation procedure was carried out. The fish were kept in brackish Baltic sea water (BW: 165–175 mOsm kg^{-1}) and freshwater (FW) holding tanks at 10–14° C on a commercial trout diet. Fish were maintained under natural photoperiod of 8L : 16D. Blood samples for melatonin, osmolality and electrolytes were collected from the dorsal aorta of decapitated, unanaesthetized fish at the time of sacrifice at 1100 and 2300 hours. Some of the FW-adapted fish were rapidly transferred to BW and sampled the next day at 2300 hours. Plasma was separated by centrifugation at 10 000*g* for 5 min and stored at −70° C prior to analysis.

Flounder (300–550 g) of both sexes were kept in seawater (SW) and freshwater holding tanks at the University of Manchester at 7–10° C. Fish were not fed while in captivity. The duration of the acclimation period in the laboratory was ≥1 month. Fish were kept under controlled illumination (Kulczykowska *et al.*, 2001) of 12L : 12D for at least a period of 2 days before experimentation and sampled at 0900 hours (1–1·5 h after lights on) and at 2100 hours (1–1·5 h after lights off). Blood samples for melatonin, osmolality and electrolytes were collected by needle puncture of unanaesthetized SW ($n = 8$) and FW ($n = 8$) fish. Some of the SW-adapted fish were anaesthetized in a seawater solution of MS-222 (0·1 g l^{-1} 3-aminobenzoic acid ethyl ester metanesulphonate; Sigma Chemical Co., Dorset, U.K.) and the dorsal aorta was cannulated by a polyethylene catheter (I.D. 0·58 mm, O.D., Portex Ltd, Kent, U.K.). Fish were transferred to an experimental tank and allowed to recover from the surgery for 48 h. Fish were kept under controlled

illumination of 12L : 12D ($n = 8$). Blood samples for melatonin, osmolality and electrolytes were collected from the catheter at 0900 hours (1–1·5 h after lights on) and at 2100 hours (1–1·5 h after lights off). Fish were sampled twice. A further sample ($n = 8$) were kept under controlled illumination of 16L : 12D ($n = 8$) for at least a period of 2 days before experimentation. Blood samples for melatonin, osmolality and electrolytes were collected from the catheter 1–1·5 h after lights on and 1–1·5 h after lights off. Fish were sampled twice. After blood centrifugation at 13 000*g* for 5 min plasma was rapidly frozen and stored at −70° C prior to analysis.

PLASMA ANALYSIS

Melatonin was extracted from plasma (2 ml) by solid phase extraction (SPE) using C_{18} Bakerbond cartridges (J.T. Baker, Phillipsburg, NJ, U.S.A.: pore size 60 Å, particle diameter 40 μm). The sample was eluted with methanol. Separation and detection were performed with the Beckman modular HPLC system (Beckman Instruments, San Ramon, CA, U.S.A.), connected to a Shimadzu spectrofluorometric detector RF-551. Chromatographic isocratic separations were carried out on the same 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 (San Ramon, CA, U.S.A.). The mobile phase was 60% HPLC-grade methanol. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. The detection limit was 3 pg ml^{-1} of plasma. The inter- and intra-assay coefficients of variation were 14% ($n = 9$) and 10% ($n = 9$), respectively (Kulczykowska & Iuvone, 1998). Synthetic melatonin (molecular mass 232·3) for calibration curve was obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

Plasma free Ca^{2+} ($[Ca^{2+}]_{pl}$), Na^+ and K^+ were measured by an EasyLyte ion analyser (Medica Co., Bedford, U.S.A.). Osmolality was determined by vapour pressure measurement using Wescor Vapour Pressure Osmometer (Wescor Inc., Logan, U.S.A.).

STATISTICAL ANALYSIS

Values are presented as means ± S.E. The statistical analysis of the data was performed using ANOVA followed by *t*-tests. Significance was taken at $P < 0{\cdot}05$. Melatonin night increase (ΔMel) was calculated as the difference between night and day values for individual fish.

RESULTS

Plasma osmolalities, electrolytes and Mel in *O. mykiss* adapted to FW and BW, and in fish rapidly transferred to BW are shown in Table I. Plasma osmolality, Na^+, K^+, Ca^{2+} and Mel concentrations in BW-adapted rainbow trout were significantly higher than those of FW-adapted fish. After rapid transfer of *O. mykiss* from fresh water to brackish water, however, plasma Ca^{2+} significantly increased but plasma Mel concentration did not change.

In two selected groups of FW *O. mykiss* of $[Ca^{2+}]_{pl}$ value $<1{\cdot}00$ mmol l^{-1} ($n = 9$) and $>1{\cdot}15$ mmol l^{-1} ($n = 11$), plasma Mel concentrations at night were significantly different (1115 ± 15 and 1186 ± 10 fmol ml^{-1}, respectively; $P < 0{\cdot}05$). In BW-adapted fish ($n = 32$) plasma Mel (1204 ± 10 fmol ml^{-1}) was significantly higher ($P < 0{\cdot}01$) than that in FW fish with lower $[Ca^{2+}]_{pl}$ and similar to that in FW fish with higher $[Ca^{2+}]_{pl}$. A significant positive correlation between plasma Mel and free calcium occurred in all FW fish and BW-adapted fish ($r = 0{\cdot}51$, $P < 0{\cdot}05$, $n = 82$) (Fig. 1).

Plasma osmolalities, ions and Mel in FW and SW *P. flesus* are shown in Table II. SW fish maintained their plasma osmolality and ions concentration

TABLE I. Means ± S.E. of plasma osmolality and ion and melatonin concentrations in freshwater (FW), brackish water (BW)-transferred, and BW-adapted *Oncorhynchus mykiss* at 2300 hours

	Osmolality ($mOsm\,kgH_2O^{-1}$)	Na^+ ($mmol\,l^{-1}$)	K^+ ($mmol\,l^{-1}$)	Ca^{2+} ($mmol\,l^{-1}$)	Melatonin ($fmol\,ml^{-1}$)
FW ($n = 50$)	$310{\cdot}4 \pm 3{\cdot}1$	$129{\cdot}6 \pm 2{\cdot}3$	$2{\cdot}44 \pm 0{\cdot}12$	$1{\cdot}00 \pm 0{\cdot}02$	1151 ± 12
BW-adapted ($n = 32$)	$321{\cdot}0 \pm 4{\cdot}0^{*}$	$135{\cdot}8 \pm 3{\cdot}1^{*}$	$2{\cdot}88 \pm 0{\cdot}20^{\dagger}$	$1{\cdot}15 \pm 0{\cdot}02^{\ddagger}$	$1204 \pm 10^{\ddagger}$
BW-transferred ($n = 24$)	$334{\cdot}7 \pm 3{\cdot}8^{*}$	$150{\cdot}7 \pm 2{\cdot}4^{*}$	$3{\cdot}01 \pm 0{\cdot}16^{\dagger}$	$1{\cdot}24 \pm 0{\cdot}03^{\dagger}$	1097 ± 29

$^{*}P < 0{\cdot}001$; $^{\dagger}P < 0{\cdot}01$; $^{\ddagger}P < 0{\cdot}05$ (*v.* freshwater values).

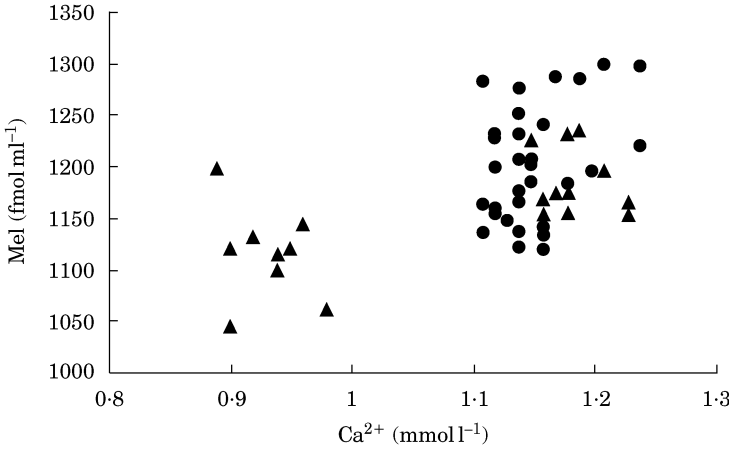

FIG. 1. Relationship between plasma Ca^{2+} and corresponding melatonin concentrations at 2300 hours in freshwater-adapted (▲) and brackish water-adapted (●) *Oncorhynchus mykiss*.

higher than those of FW fish. The Mel level at 2100 hours assayed in SW *P. flesus* was higher than that of FW fish at the same time.

In SW cannulated *P. flesus* exposed to both light and dark regimes, plasma Mel concentration at night was significantly higher than that in the day. There were no significant diurnal changes in plasma osmolality and electrolytes concentration. Results for individual fish are presented in Fig. 2. Melatonin increases (ΔMel) correlated positively with plasma calcium concentration ($r = 0{\cdot}69,\ P < 0{\cdot}05$).

DISCUSSION

Several *in vitro* studies have been performed on fishes and other vertebrates to investigate the response of the melatonin biosynthesis system to intracellular and extracellular calcium concentrations. This is the first study, however, to determine the relationship between plasma ionized calcium and the capacity of night melatonin production in fishes.

During their lifetime, fishes may experience fluctuations in surrounding calcium concentrations, which may influence circulating plasma calcium. The extracellular calcium concentration in fishes is regulated at a relatively constant physiological concentration of 2·0–3·0 mmol l^{-1} (total plasma Ca^{2+}) by many homeostatic systems (Bentley, 1998; Sasayama, 1999). If it is considered that approximately half the plasma Ca^{2+} is protein-bound (mainly to albumin), then this corresponds to a free $[Ca^{2+}]_{pl}$ of 1·0–1·5 mmol l^{-1} measured in this study.

The more pronounced increase of calcium concentration, which is observed within the first day of transfer in BW *O. mykiss*, agrees with other studies in this species (Najib & Fouchereau-Peron, 1996; Kulczykowska, 2001). This, however, does not affect plasma Mel. On the contrary, in FW-adapted fish with spontaneous low and high $[Ca^{2+}]_{pl}$, plasma Mel differs significantly (Fig. 1). Similarly, BW-adapted fish with regularly higher $[Ca^{2+}]_{pl}$ present significantly higher plasma Mel than that observed in FW fish with low $[Ca^{2+}]_{pl}$ (Fig. 1). Therefore,

TABLE II. Means ± S.E. of plasma osmolality and ion and melatonin concentrations in fresh- and seawater *Platichthys flesus* at 2100 hours

	Osmolality ($mOsm\,kgH_2O^{-1}$)	Na^+ ($mmol\,l^{-1}$)	K^+ ($mmol\,l^{-1}$)	Ca^{2+} ($mmol\,l^{-1}$)	Melatonin ($fmol\,ml^{-1}$)
FW ($n=8$)	$288{\cdot}3 \pm 2{\cdot}9$	$138{\cdot}8 \pm 1{\cdot}2$	$2{\cdot}21 \pm 0{\cdot}14$	$1{\cdot}01 \pm 0{\cdot}03$	1666 ± 62
SW ($n=8$)	$323{\cdot}6 \pm 2{\cdot}8^*$	$164{\cdot}3 \pm 1{\cdot}8^*$	$3{\cdot}14 \pm 0{\cdot}15^\dagger$	$1{\cdot}30 \pm 0{\cdot}03^\dagger$	$1804 \pm 81^\ddagger$

$^*P < 0{\cdot}001$; $^\dagger P < 0{\cdot}01$; $^\ddagger P < 0{\cdot}05$ (*v.* freshwater values).

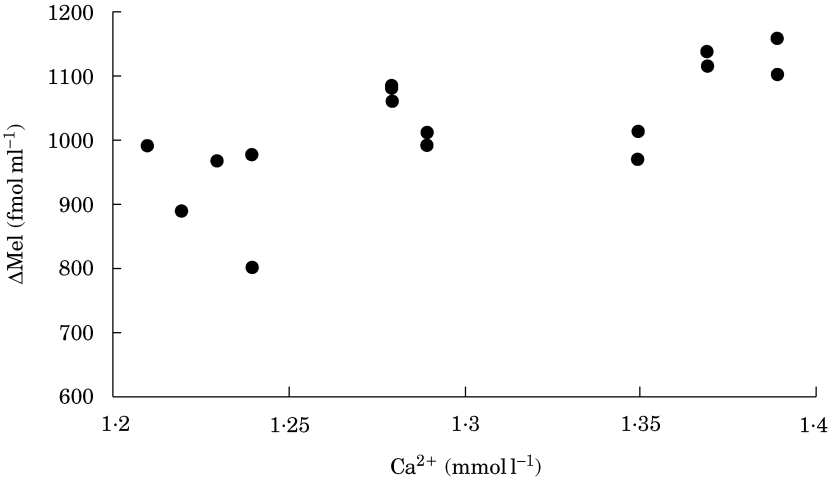

FIG. 2. Relationship between plasma Ca^{2+} and corresponding melatonin night increases (ΔMel) in chronically cannulated *Platichthys flesus*.

it seems possible that it is low free calcium that can limit Mel synthesis capacity in *O. mykiss* in physiological steady-state conditions in FW-adapted fish. Otherwise, it is plasma Mel concentration that can influence $[Ca^{2+}]_{pl}$. Yet, this is not a case here, because when the Mel level was changing during the day, plasma calcium remained stable.

It should be noted also that in SW and FW *P. flesus* plasma Mel corresponded to $[Ca^{2+}]_{pl}$: higher Mel was measured in SW flounder with higher $[Ca^{2+}]_{pl}$. Additionally, in chronically cannulated *P. flesus*, ΔMel values were correlated positively with plasma calcium concentrations measured in individual fish. $[Ca^{2+}]_{pl}$ remains stable all day. Therefore, it is not the melatonin concentration that influences $[Ca^{2+}]_{pl}$, but on the contrary, ΔMel is influenced by plasma calcium concentration.

The results provide a 'suggestive correlation' between plasma calcium and plasma Mel in both fish species, but neither mechanism nor physiological significance of this phenomenon is known at present. Changes in extracellular calcium may affect the melatonin synthesis by several mechanisms, *i.e.* induction of cAMP production and other calcium signalling pathways (Nikaido & Takahashi, 1996; Zatz, 1996; Kroeber *et al.*, 2000; Santana *et al.*, 2001). Calcium has been shown to enhance melatonin production in the rat, probably by binding to the AA-NAT and increasing its catalytic activity (Morton, 1989). Whichever intracellular mechanism is engaged in steady-state physiological condition in fishes, the melatonin synthesis capacity seems to be limited by accessibility of extracellular (plasma) calcium.

In this study, plasma osmolality, Na^+, K^+ and Mel concentrations in BW-adapted *O. mykiss* were significantly higher than those of FW-adapted fish. Similarly, the highest plasma Mel levels corresponding to the highest plasma concentrations of sodium and chloride were shown in *Oncorhynchus kisutch* (Walbaum) during seawater adaptation (Folmar & Dickhoff, 1981). Higher melatonin plasma concentrations were also measured in seawater-adapted *O. kisutch*

(Gern *et al.*, 1984) and in brackish water-adapted *O. mykiss* (Kulczykowska, 1999). It cannot be excluded that plasma important ions such as Na^+ and K^+ may directly or indirectly influence Mel production. It is also known that melatonin can exert a feedback regulation on plasma cation levels as was suggested for chickens (Pablos *et al.*, 1995), or influence electrolyte levels in rat blood (Heidrich *et al.*, 2000). All these observations together provide a case for further study of a linkage between plasma electrolytes and Mel in fishes.

In conclusion, physiological depletion of $[Ca^{2+}]_{pl}$ seems to be a factor which can limit the capacity of Mel night production in two fish species representing two different models of exogenous and endogenous hormone synthesis regulation. Further work is required to elucidate this relationship.

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