ELSEVIER

Comparative Biochemistry and Physiology Part B 137 (2004) 43–47

CBP

www.elsevier.com/locate/cbpb

Influence of exogenous thyroxine on plasma melatonin in juvenile Atlantic salmon (*Salmo salar*)

Ewa Kulczykowska[a,]*, Ewa Sokołowska[a], Børge Takvam[b], Sigurd Stefansson[b], Lars Ebbesson[b]

[a]*Department of Genetics and Marine Biotechnology, Institute of Oceanology of Polish Academy of Sciences, Sw. Wojciecha 5 Str., 81-347 Gdynia, Poland*

[b]*Department of Fisheries and Marine Biology, University of Bergen, Bergen, Norway*

Received 9 April 2003; received in revised form 1 October 2003; accepted 2 October 2003

Abstract

One of the most clearly defined endocrine changes during the parr–smolt transformation of anadromous salmonids is an increase in plasma levels of thyroid hormones. The role of pineal hormone melatonin in timing and synchronisation of smoltification is widely discussed. The effect of administration of exogenous thyroxine (T4) on plasma melatonin was investigated in juvenile Atlantic salmon (*Salmo salar*) at the early stages of parr–smolt transformation. Fish were kept in fresh water under simulated-natural photoperiod and exposed to exogenous T4. Fish were sampled at 12.00 and 24.00 h from treatment and control tanks, 2 and 14 days after treatment started. Plasma melatonin and L-thyroxine were measured using RIA and competitive enzyme immunoassay, respectively. After 2 days of T4 treatment, marked difference in plasma melatonin concentration measured at 12.00 and 24.00 h was still observed in both groups. However, 2-week exposure to T4 caused a reduction in night-time plasma melatonin level and thus, probably, inhibited melatonin related time-keeping system in juvenile salmon. Additional studies are needed to clarify the mechanism of the described phenomenon.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Anadromous salmonids; Atlantic salmon; Fish; Melatonin; Parr–smolt transformation; Pineal organ; Rhythm; Thyroxine

1. Introduction

The parr–smolt transformation of Atlantic salmon (*Salmo salar*) can be considered a synchronisation and integration of a wide range of physiological, morphological and behavioural changes, pre-adapting the juvenile salmon for entry into seawater (Hoar, 1988). The remarkable developmental transition of Atlantic salmon occurs in springtime in fish of appropriate size and given the necessary environmental signals. Photoperiod is recognised as the major long-term regulator of smoltification in Atlantic salmon (Saunders et al., 1989; Solbakken et al., 1994). The seasonal changes in day length provide the developing juvenile salmon with environmental information, entraining endogenous rhythms regulating growth and development to each other, and bringing them in step with the changing seasons (Duston and Saunders, 1990).

Considering the importance of environmental regulation, the role of the hormone melatonin in timing and synchronisation of smoltification in Atlantic salmon is widely discussed (Randall et

*Corresponding author. Tel.: +48-586-208913; fax: +48-586-201233.
E-mail address: ewakulcz@cbmpan.gdynia.pl (E. Kulczykowska).

1096-4959/04/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2003.10.001

al., 1995; Porter et al., 1998). This indole (*N*-acetyl-5-methoxytryptamine) is primarily synthesised and released by the pineal gland of vertebrates. The daily rhythm in circulating melatonin levels, with high levels occurring in the dark, is a well-known phenomenon (Reiter, 1993). Furthermore, melatonin displays annual fluctuations, which provide the organism with time-of-year information (Reiter, 1993). The role of melatonin in the mechanism controlling rhythmic adaptations to daily and seasonal cycles in fish is generally accepted (for review: Kulczykowska, 2002). Moreover, melatonin interacts with other hormones and thus adjusts fish to changes in environment (Kulczykowska, 1995, 2002).

One of the most clearly defined endocrine changes during the parr–smolt transformation of anadromous salmonids is an increase in plasma levels of thyroid hormones (Boeuf, 1993; Ebbesson et al., 2000). Thyroxine (T4) is the primary secretory product of the thyroid gland. T4 has little direct action, being mostly regarded as a precursor for triiodothyronine (T3), a biologically active form of the hormone (Leatherland, 1994). One of the best known examples of thyroid hormones effects is the initiation and regulation of amphibian metamorphosis (Atkinson, 1981). There is a large body of evidence that thyroid hormones play an important role in regulation of development, growth and reproduction in fish (Power et al., 2001). It is fully accepted that metamorphosis in flatfishes (Pleuronectiformes) is mediated by thyroid hormones (Inui and Miwa, 1985; Schreiber, 2001). Whether there is a case in the parr–smolt transformation in salmonids is still a matter of debate, because only few of the developmental changes that are considered to represent smoltification are induced by thyroid hormone administration (Boeuf, 1993; Leatherland, 1994).

The aim of the present work was to determine if administration of exogenous T4 affects plasma melatonin in juvenile Atlantic salmon at early stages of parr–smolt transformation.

2. Materials and methods

2.1. Animals and experimental conditions

Juvenile Atlantic salmon (a little over 1-year-old; mass ≈30 g), from wild broodstock of the Dale anadromous strain (Southwest Norway), were obtained in January 2001. The fish were kept in freshwater in square tanks (1 m^2) at 8 °C under artificial lighting with simulated-natural photoperiod (60°25′N), including twilight (day 2 and 14 lights on and off—04.40, 19.20 and 03.50, 20.00 h, respectively). On April 2, 20 fish were marked and transferred to a treatment tank with L-thyroxine added at a constant rate by a peristaltic pump to the constant water flow; 20 fish were kept in an identical control tank. Treatment water had a measured average thyroxine concentration of 345 ± 19 μg/l throughout the study.

2.2. Sampling

Fish for melatonin measurements were sampled at 12.00 and 24.00 h from both treatment and control tanks, 2 and 14 days after treatment started. Plasma for T4 determination was collected from individual fish at 10.00 h. The fish were starved for 16 h prior to sampling. The fish were quickly dip-netted out of the tanks and anaesthetised directly in MS222. During night samplings, fish were dip-netted and anaesthetised in darkness, with blood samples taken while the fish covered in light proof black plastic. Each fish was measured for weight (nearest 0.1 g) and fork-length (nearest millimeter). Blood was collected from the caudal vessels using heparinized syringes, and stored on ice in 1.5-ml vials until centrifuged (10 min, $3000 \times g$). The plasma was then carefully collected and stored at −80 °C.

2.3. Analytical methods

Circulating L-thyroxine levels were measured using the competitive enzyme immunoassay (EIA) method of Cerdá-Reverter et al. (1996) with a few modifications. Briefly, 30 μl of plasma was extracted with 150 μl of ice-cold methanol (99%) and centrifuged (4 °C, $1633 \times g$, 15 min). The supernatant was placed in a new vial and the pellet re-extracted twice with 60 μl ice-cold methanol and the pooled supernatant was vacuum dried (4 °C) overnight. The extracted plasma was re-constituted in EIA buffer (0.1 M phosphate buffer containing 0.15 M NaCl and 0.1% bovine serum albumin).

The assay was performed in 96-well microtiter plates pre-coated with monoclonal mouse anti-rabbit IgG antibodies (SPIBIO, France). Plates were rinsed (3×3 min) with wash buffer (0.15 M NaCl, 0.5% Tween 20). T4 (5 g; Sigma) was

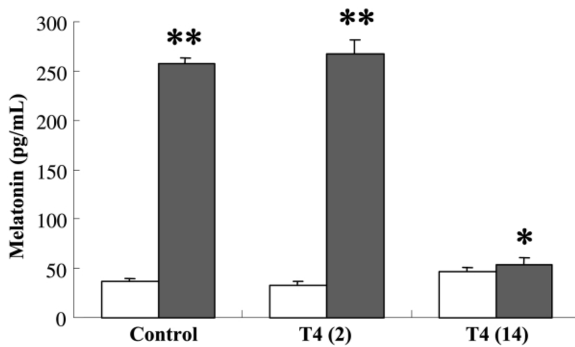

Fig. 1. Plasma melatonin concentration at 12.00 and 24.00 h in control fish and after 2 and 14 days of T4 treatment: Control, T4 (2) and T4 (14), respectively. Dark bar indicates night (24.00 h). Values are means ± S.E.M.; *$P<0.001$ vs. control and T4 (2) values; **$P<0.001$ vs. day value. The control values after 2 and 14 days are combined.

dissolved in 80 ml 0.1 M NaOH then diluted with 650 ml H_2O and stored as stock solution. A total volume of 150 μl in each well containing either 50 μl T4-AchE (SPIBIO) + 50 μl rabbit anti-L-T4 (diluted 1:10 000; Sigma) + 50 μl T4 standard (ranging from 0.039 to 20 ng/ml) or 50 μl sample. After 16-h incubation in total darkness at room temperature (22 °C) under constant agitation, unbound anti-T4 antibody, T4-tracer and standards/samples were washed off, and Ellman's reagent (SPIBIO) was added to the plates. After incubating for 10 h, the colour reaction was read at 405 nm in a temperature-controlled (25 °C) plate reader. Maximum binding (B_0; 50 μl of EIA buffer + 50 μl T4-AchE + 50 μl anti-T4 rabbit antibody) and non-specific binding (NSB; 150 μl EIA buffer) were determined on each plate. All standards and samples were run in duplicates. The EIA meets the required standards of intra- and inter-assay variation (<5%), specificity and parallelism.

Plasma melatonin was assayed using a total melatonin RIA kit (IBL, Hamburg), without preceding extraction, as described in the kit protocol. Samples were assayed in duplicate. The lowest detectable level of melatonin was 3 pg/ml. The intra- and inter-assay coefficients of variation for melatonin were 8.0 and 15.0%, respectively. The assay has been validated for fish plasma by HPLC assay (Kulczykowska and Iuvone, 1998).

2.4. Statistical analysis

Values are presented as means ± standard error of the mean (S.E.M.). The data were evaluated using ANOVA and Student's *t*-test. Differences were considered to be significant if $P<0.05$.

3. Results

After 2 days of T4 treatment, marked difference in plasma melatonin concentration measured at noon and at midnight was still observed in both groups (Fig. 1). However, after 14 days of exogenous T4 administration, the day–night difference in plasma melatonin concentration disappeared, with night-time melatonin reduced to similar levels as during light (Fig. 1). T4 treatment did not affect plasma melatonin concentrations at 12.00 h. The control day and night melatonin values did not differ after 2 and 14 days and are combined in Fig. 1. Plasma T4 levels in control and experimental fish are presented in Table 1. T4 levels in the fish continued to rise to levels approximately 10 ng/ml measured in May.

4. Discussion

The present results show for the first time that long-term administration of T4 decreases plasma melatonin concentration at night in juvenile Atlantic salmon. Since synthesis and secretion of melatonin are coupled (Reiter, 1991), the dynamic response to T4 treatment reflects the changes in synthesis capacity of the gland rather than changes in hormone release. Thus, T4 administration in 2 weeks resulted in the reduction in the ability of the pineal organ to produce melatonin at night. However, it cannot be excluded that T4 only shifted the night-time melatonin peak not actually inhibiting the hormone production. Since plasma T4 levels were beyond the physiological range, the effect can be considered as pharmacological only.

Table 1
Blood thyroxine levels in control fish, after 2 and 14 days of T4 treatment, T4 (2 days) and T4 (14 days), respectively

	T4 (2 days)	T4 (14 days)	*P*-value
Control (μg/l)	2.5 ± 0.4, *n* = 5	6.4 ± 0.6, *n* = 5	0.0002
T4-treated (μg/l)	234 ± 17, *n* = 5	251 ± 20, *n* = 5	0.6

Values are mean ± S.E.M.

The action of T4 may involve direct and/or indirect influences on melatonin plasma levels. The rate-limiting step in the synthesis of melatonin is catalysed by serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase) and one may expect this site of T4 potential inhibitory action (Zheng and Cole, 2002). Yet, T4 may influence clearance of melatonin from plasma or accelerate melatonin degradation in the pineal organ just before release. There is evidence for T4-induced increased uptake of melatonin into peripheral organs in tadpoles (Wright et al., 1997). That might also be a case in juvenile salmonids. It should also be considered that the T4 action involves suppression of TSH secretion, especially if an effect is delayed.

The reciprocal relationship between the thyroid and the pineal has been supported by morphological, biochemical and clinical findings in mammals (Rojdmark et al., 1991; Lewiński et al., 1997). The effect of alterations in thyroxine levels on nocturnal melatonin production was shown in rats and Syrian hamsters (Champney et al., 1985). Thyroidal-melatonin interactions were well described in a process of amphibian metamorphosis, where thyroid controlled the progress of metamorphosis and melatonin synchronised it with changes in environment (Wright et al., 1997; Wright and Alves, 2001; Wright, 2002a,b). Melatonin levels in the thyroid gland decreased during metamorphosis in bullfrog tadpoles. Moreover, the decrease in plasma melatonin at metamorphic climax was induced by thyroxin and was proposed to accelerate metamorphosis (Wright and Alves, 2001). Thus, an antagonistic relationship between both hormones was suggested (Wright, 2002b).

Whether this is the case in salmonids remains an open question. While in our control fish the T4 increased between days 2 and 14 in typical way of salmon (Ebbesson et al., 2000), plasma melatonin did not change. In salmonids, the increase in circulating levels of T3 and T4 associated with smoltification is a well-known phenomenon and suggests the involvement of thyroid hormones in physiological changes during transformation (Boeuf, 1993; Ebbesson et al., 2000). Yet, their exact role in this process is still difficult to specify (Leatherland, 1994). However, in flatfishes, the elevated T4 level is necessary for the completion of metamorphosis (Schreiber and Specker, 1998). Moreover, it is still not clear how melatonin itself influences development of juvenile salmonids (Porter et al., 1998). To the authors' knowledge, there is no record on plasma melatonin changes during parr–smolt transformation.

In conclusion, a 2-week exposure to high level of exogenous T4 affects plasma melatonin measured at 24.00 h. Since plasma T4 concentration in experimental fish is above its physiological level, it is risky to state that the observed effect reflects physiological events. However, it should be considered that the manipulation of thyroid hormone levels influences plasma melatonin and thus, probably, inhibits melatonin-related time-keeping system in juvenile salmon. Undoubtedly, additional studies on melatonin uptake in peripheral tissues and an effect of T4 on melatonin synthesis capacity in pineal organ are needed to clarify the mechanism of the described phenomenon.

Acknowledgments

This work was supported by the Norwegian Research Council (S.O.) and access to rearing facilities and laboratories at the University of Bergen has been supported by the Improving Human Potential Programme from the European Commission through Contract No. HPRI-CT-1999—00056 'Bergen Marine' (E.S.). Ewa Kulczykowska was supported by the Committee for Scientific Research grant (6P04C 038 16).

References

Atkinson, B.G., 1981. Hormonal control of amphibian metamorphosis. In: Gilbert, L.I., Frieden, E. (Eds.), Metamorphosis—A Problem in Developmental Biology. Plenum Press, New York, pp. 397–444.

Boeuf, G., 1993. Salmonid smolting: a pre-adaptation to the oceanic environment. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman and Hall, London, pp. 105–135.

Champney, T.H., Webb, S.M., Richardson, B.A., Reiter, R.J., 1985. Hormonal modulation of cyclic melatonin production in the pineal gland of rats and Syrian hamsters: effects of thyroidectomy or thyroxide implant. Chronobiol. Int. 2, 177–183.

Cerdá-Reverter, J.M., Zanuy, S., Carrillo, M., Kah, O., 1996. Development of enzyme immunoassays for 3,5,3′-triiodo-L-thyronine and L-thyroxine: time-course studies on the effect of food deprivation on plasma thyroid hormones in two marine teleosts, sea bass (*Dicentrarchus labrax* L.) and sea bream (*Sparus aurata* L.). Gen. Comp. Endocrinol. 103, 290–300.

Duston, J., Saunders, R.L., 1990. The entrainment role of photoperiod on hypoosmoregulatory and growth-related aspects of smolting in Atlantic salmon (*Salmo salar*). Can. J. Zool. 68, 707–715.

Ebbesson, L.O.E., Björnsson, B.Th., Stefansson, S.O., Ekström, P., 2000. Free-thyroxine during parr–smolt transformation in coho salmon, *Oncorhynchus kisutch*: comparison with total thyroxine, total triiodothyronine, and growth hormone. Fish Physiol. Biochem. 22, 45–50.

Hoar, W.C., 1988. The physiology of smolting salmonids. In: Hoar, W.S., Randall, D. (Eds.), Fish Physiology, vol. XIB. Academic Press, New York, pp. 275–343.

Inui, Y., Miwa, S., 1985. Thyroid hormone induces metamorphosis of flounder larvae. Gen. Comp. Endocrinol. 60, 450–454.

Kulczykowska, E., 1995. Arginine vasotocin–melatonin interactions in fish: a hypothesis. Rev. Fish Biol. Fisher. 5, 96–102.

Kulczykowska, E., 2002. A review of the multifunctional hormone melatonin and a new hypothesis involving osmoregulation. Rev. Fish Biol. Fish. 11, 321–330.

Kulczykowska, E., Iuvone, P.M., 1998. Highly-sensitive and specific assay of plasma melatonin using HPLC with fluorescence detection preceded by solid-phase extraction. J. Chromatogr. Sci. 36, 175–178.

Leatherland, J.F., 1994. Reflections on the thyroidology of fishes: from molecules to humankind. Guelph Ichthyol. Rev. 2, 1–67.

Lewinski, A., Wajs, E., Klencki, M., et al., 1997. Pineal–thyroid interrelationships update: 1996. PDJ Publications Limited, Westbury, New York, pp. 173–181.

Porter, M.J.R., Randall, C.F., Bromage, N.R., Thorpe, J.E., 1998. The role of melatonin and the pineal gland on development and smoltification of Atlantic salmon (*Salmo salar*) parr. Aquaculture 168, 139–155.

Power, D.M., Llewellyn, L., Faustino, M., et al., 2001. Review: thyroid hormones in growth and development of fish. Comp. Biochem. Physiol. C 130, 447–459.

Randall, C.F., Bromage, N.R., Thorpe, J.E., Miles, M.S., Muir, J.S., 1995. Melatonin rhythms in Atlantic salmon (*Salmo salar*) maintained under natural and out-of-phase photoperiod. Gen. Comp. Endocrinol. 98, 73–86.

Reiter, R.J., 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. 12, 151–180.

Reiter, R.J., 1993. The melatonin rhythm: both a clock and a calendar. Experientia 49, 654–664.

Rojdmark, S., Berg, A., Rossner, S., Wetterberg, L., 1991. Nocturnal melatonin secretion in thyroid disease and in obesity. Clin. Endocrinol. 35, 61–65.

Saunders, R.L., Specker, J.L., Komourdjian, M.P., 1989. Effects of photoperiod on growth and smolting in juvenile Atlantic salmon (*Salmo salar*). Aquaculture 82, 101–117.

Schreiber, A.M., 2001. Review: metamorphosis and early larval development of the flatfishes (Pleuronectiformes): an osmoregulatory perspectives. Comp. Biochem. Physiol. B 129, 587–595.

Schreiber, A.M., Specker, J.L., 1998. Metamorphosis in the summer flounder (*Paralichthys dentatus*): stage-specific developmental response to altered thyroid status. Gen. Comp. Endocrinol. 111, 156–166.

Solbakken, V.A., Hansen, T., Stefansson, S.O., 1994. Effects of photoperiod and temperature on growth and parr–smolt transformation in Atlantic salmon (*Salmo salar* L.) and subsequent performance in seawater. Aquaculture 121, 13–27.

Wright, M.L., 2002a. Melatonin, diel rhythms, and metamorphosis in anuran amphibians. Gen. Comp. Endocrinol. 126, 251–254.

Wright, M.L., 2002b. Melatonin in the anuran amphibian larva and its reciprocal interactions with the thyroid gland. In: Haldar, C., Singaravel, M., Maitra, S.K. (Eds.), Treatise on Pineal Gland and Melatonin. Science Publishers, Inc, Enfield (NH) USA, Plymouth, UK, pp. 199–210.

Wright, M.L., Alves, C.D., 2001. The decrease in plasma melatonin at metamorphic climax in *Rana catesbeiana* (bullfrog) tadpoles is induced by thyroxin. Comp. Biochem. Physiol. A 129, 653–663.

Wright, M.L., Pikula, A., Babski, A.M., Kuliga, K., 1997. Distribution and reciprocal interactions of ^{3}H-melatonin and ^{125}I-thyroxine in peripheral, neural, and endocrine tissues of bullfrog tadpoles. Comp. Biochem. Physiol. A 118, 691–698.

Zheng, W., Cole, P.A., 2002. Serotonin *N*-acetyltransferase: mechanism and inhibition. Curr. Med. Chem. 9, 1187–1199.