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Short-Term *In Vivo* Melatonin Activates Thyroid Axis but Deactivates Interrenal Axis in Climbing Perch (*Anabas testudineus* Bloch)

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Abstract

As a potent regulator of seasonal and circadian rhythms, melatonin is involved in many neuroendocrine functions in vertebrates including fishes. However, the interactive action of melatonin on thyroid and interrenal axes, metabolite homeostasis and ion status is less addressed in fishes. We thus analyze the plasma thyroxine (T_4), triiodothyronine (T_3) and cortisol levels and metabolite status and Na^+ and K^+ status in osmoregulatory tissues after short-term of 30 min *in vivo* exposure of melatonin (0, 0.25, 2.5, 25 ng g^{-1}) in climbing perch (*Anabas testudineus* Bloch). A rise in plasma T_4 occurred after 30 min of melatonin treatment, indicating activation of thyroid axis. On the contrary, deactivation of hypothalamo-pituitary-interenal (HPI) axis occurred due to fall in cortisol level along with decrease in plasma T_3 in the melatonin-treated fish. Significant dose-dependent increase in plasma glucose and urea were found in melatonin-treated fish. Similarly, increased plasma [Na^+] and [K^+] contents occurred in gill tissues but plasma [Na^+] and [K^+] levels remained unaffected after melatonin treatment. In kidney, melatonin treatment augmented [K^+] but decreased [Na^+] content, emphasizing a differential cation handling by melatonin. Overall, these results indicate that melatonin exerts a rapid activation of thyroid axis, but deactivates interenal axis while promoting the release of glucose and urea and tissue Na^+/K^+ ion levels in freshwater climbing perch.

Keywords: Cortisol, Fish, Interrenal Axis, Melatonin, Metabolites, Tissue Ions, Thyroid Axis, Thyroid Hormone

1. Introduction

In fishes, melatonin exerts many physiological actions including osmoregulatory and reproductive functions[1]. It shows daily and seasonal changes in plasma ion concentration and/or changes in urinary electrolyte excretion[2].Several fish osmoregulatory organs have been shown to possess receptors for melatonin and melatonin has been subsequently ascribed with a role for melatonin in hydromineral balance in fish[3]. Melatonin, as a photic signal transducer, regulates a series of physiological processes involved in locomotor activity[4], development[5], growth[6] and reproduction[7-8]. Many of the established physiological effects of melatonin are mediated via high-affinity cell membrane receptors[9]. Melatonin levels fluctuate daily with an increase during the dark period and decrease during the light period except for some cases including salmonids[10]. The melatonin dynamics of teleost fish is similar to a circadian pacemaker [11], as fish pineal organ coordinates environmental cues with biological rhythms.

Cortisol, a major glucocorticoid synthesized in the interrenal tissue of fish, plays an important role in stress response, behavior, osmoregulation, metabolism, growth,

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reproduction and immune function [12-14]. Under distinct photoperiodic conditions, circulating cortisol levels exhibit a distinct daily pattern of fluctuation[15-16], indicating a circadian rhythm[17]. Studies have shown that melatonin modulates neuroendocrine functions by targeting hypothalamic control of pituitary function[1]. It has been shown that the monoaminergic neurons in pre-optic area pass the peptidergic information to the pituitary gland through neurohypophysis (e.g. isotocin, arginine, vasotocin) or releasing factors including corticotrophin releasing hormone[7]. It is well known that the physiological response of melatonin depends on the type of melatonin receptors expressed in the pituitary glands and further to sex and/or reproductive status[1].

Similar to cortisol, thyroxine (T_4) and triiodothyronine (T_3), the principal thyroid hormones (THs), are also involved in osmoregulation and metabolism in fish[18,13,19]. These hormones have shown distinct circadian and circannual rhythms in its availability in blood depending on the varied environmental conditions[20]. Thyroid hormone production is at the maximum during summer when daylengths are long and pineal activity/melatonin production is at the minimum[21]. Studies have shown that melatonin administration inhibits the levels of THs in mammals[22] and can modify the circulating levels of THs in fish[20-21]. It is likely that the stimulatory and inhibitory action of melatonin on TH dynamics might be because of the changes in seasons, doses and the time of administration in fishes[20].

It appears that small intestine can act as a site of regulation of melatonin synthesis in fish as it provides a major source of extrapineal and extraretinal melatonin[3, 23]. The hormone synthesis in the gastrointestinal tract that probably acts as a paracrine and/or autocrine factor is stimulated by the presence of both the food and chronic stress. Melatonin, as an anti-stress molecule, is necessary for homeostatic control of energy metabolism in several vertebrate groups including fish[24]. It is also considered as an acoustic factor in diurnal and nocturnal fish species[25-26], though the effects could be attributed to a sedative action of the molecule[4]. As an anti-stress molecule, melatonin would regulate neuronal areas such as hypothalamus and hindbrain that are involved in the regulation of food intake, where glucose-sensing mechanisms are present[24]. Further, it is known that melatonin can influence the proximal tubular function and can be considered as one of the factors responsible for diurnal variations in urine monovalent ions secretion in fish [2].

Here, we examined the physiological response of an obligate air-breathing fish *Anabas testudineus* Bloch to melatonin in order to understand the short-term *in vivo* action of melatonin on THs and cortisol dynamics, metabolite status and tissue ions. To this end, we quantified the plasma T_4, T_3 and cortisol levels, plasma metabolite levels and tissue [Na^+] and [K^+] ion status in the test species treated with varied doses of melatonin.

2. Materials and Methods

2.1 Fish Holding Conditions

Tropical freshwater air-breathing fish, commonly known as climbing perch (*Anabas testudineus* Bloch), belonging to order Perciformes and family Anabantidae was used as the test species. This native teleost fish inhabiting in the backwaters of Kerala in Southern India is an obligate air-breathing fish having well equipped physiological and biochemical mechanisms to live in demanding environmental conditions[19, 27]. Adult climbing perch weighing about 35–45 g were collected from nearest water bodies and reared in large cement tanks. The fish were acclimated to laboratory conditions for two months in well water at 28 ± 1°C; pH 6.8; under natural photoperiod (12 L/12 D) and fed daily with commercial fish feed (1% body mass). Prior to experiment, fish were kept in 100 L glass aquaria for two weeks and food was withdrawn for 24 h prior to sacrifice to ensure optimum experimental conditions.

2.2 Experimental Design

Short-term dose-dependent *in vivo* action of melatonin was tested in experimental fish. The acclimated fish were divided into three groups of six each and kept in separate glass tanks (30 L). Group 1 fish that received saline injection (0.65% NaCl, *ip*) served as control. Fish in groups 2, 3 and 4 were given *ip* injection of melatonin doses at 0.25, 2.5 and 25 ng g^{-1}, respectively. All fish were sampled 30 minutes after injection. Strict care was taken to minimize stress due to injection and handling. No mortality was observed in any fish groups during the experiments.

2.3 Sampling and Analysis

Fish were netted and anesthetized in a 0.1% 2-phenoxy-ethanol solution (SRL, Mumbai) and blood samples were collected from the caudal artery using heparinized syringe fitted with #23 needle. The blood was centrifuged at 5000 × sg for 5min at 4°C and plasma was separated and stored at –20°C until analysis. Fish were then sacrificed by spinal trans-section, and the gills, kidney, intestine and liver tissues were excised.

2.4 Quantification of T_3, T_4 and Cortisol

Plasma T_3 and T_4 concentrations were measured by microwell enzyme immunoassay (EIA: magnetic solid phase) with kits (Syntron Bioresearch Inc, Carlsbad, California). The sensitivity of this method was checked earlier[28, 29]. Briefly, goat anti-mouse IgG-coated wells were treated with 50 µL standards, control and samples. After adding 100 µl T_4-HRP conjugate the wells were incubated at 37°C for 1 h. After washing, 50 µL of 0.05 M acetate buffer and TMB were added and incubated at 20°C for 15 min. The absorbance was read at 450 nm after stopping the reaction with 1N HCl. The intra-assay coefficient of variation was 7.2% and inter-assay coefficient of variation was 9.0%.

Cortisol concentrations in plasma samples were measured by competitive immunoenzymatic assay (DiaMetra, Foligno, Italy) and values were expressed as ng ml^{-1}. The sensitivity and reliability of this method was examined and the values were comparable to RIA method reported earlier[30, 28]. In brief, plasma was deproteinised with ethanol phosphate buffer (1 : 9). Plate wells coated with cortisol antibody (mouse anti-rabbit IgG) were treated with standards and diluted samples (20 µL) and incubated with 200 µL cortisol-horseradish peroxidase (HRP) conjugate at 37°C for 1 h. After washing, 100 µL Tetramethylbenzidine (TMB)–H_2O_2 was added and incubated at 20°C for 15 min in the dark. The absorbance was recorded on a plate reader (Span Autoreader 4011, New Delhi) at 450 nm after adding 0.15 mol sulphuric acid.

2.5 Plasma Metabolites and Minerals

Plasma glucose (GOD/POD test kit; Span Diagnostics Ltd., New Delhi), urea (DAM kit; Span Diagnostics Ltd., New Delhi) and lactate (PAP Fluid test; Radiant Diagnostics, New Delhi) concentrations were measured colorimetrically in a Systronic Spectrophotometer 2202 (Systronics, New Delhi) using commercial test kits. Plasma [Na^+] and [K^+] were measured ina flame photometric analyzer (Systronics 129, New Delhi) using standards of Na and K (Remedix Diagnostics, Palakkad) and values were expressed as mmol L^{-1}. Total [Na^+] and [K^+] contents in gills, kidney, intestine and liver were also determined thus, and the values are expressed in µg g^{-1} tissue weight.

2.6 Statistics

Data were collected from six animals in each group. Statistical significance among groups were tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between the groups were analyzed with the help of Graphpad Software (Graphpad Instat-3, San Digeo) and the level of significance was accepted if $P < 0.05$.

3. Results

3.1 *In Vivo* Effects of Melatonin on Plasma T_3, T_4 and Cortisol

A significant rise ($P < 0.01$) in plasma T_4 occurred after 30 min of 0.25 ng g^{-1} melatonin administration, whereas 2.5 ng g^{-1} and 25 ng g^{-1} melatonin doses failed to produce such an effect (Figure 1B). On the contrary, plasma T_3 showed significant decline ($P < 0.05$) in fish treated with all the doses of melatonin (Figure 1A). A significant decrease ($P < 0.01$) in plasma cortisol was found after medium and high doses of melatonin treatments (Figure 1C).

3.2 *In Vivo* Effects of Melatonin on Plasma Metabolites

Significant increase in plasma glucose ($P \leq 0.001$) and plasma urea ($P < 0.001$) occurred after low and medium doses of melatonin (Figure 2A, 2C). Likewise, the pattern of plasma urea level significantly increased ($P \leq 0.001$) after the tested doses of melatonin (Figure 2C). Plasma lactate, on the contrary, showed no significance after melatonin treatment (Figure 2B).

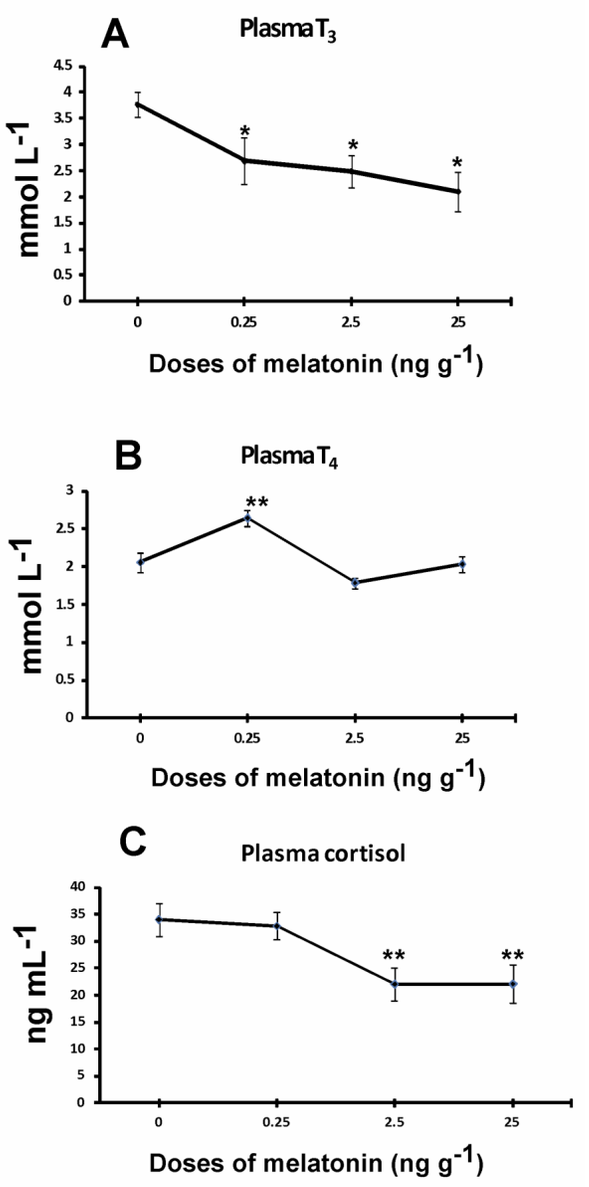

Figure 1. **(A)** Plasma T_3 **(B)** T_4 and **(C)** cortisol, levels of climbing perch after 30 min of treatment of melatonin(0, 0.25, 2.5 and 25 ng g^{-1}). Each point represents mean ± SEM of six fish. * denotes ($P < 0.05$) and ** denotes ($P < 0.01$) when compared to control.

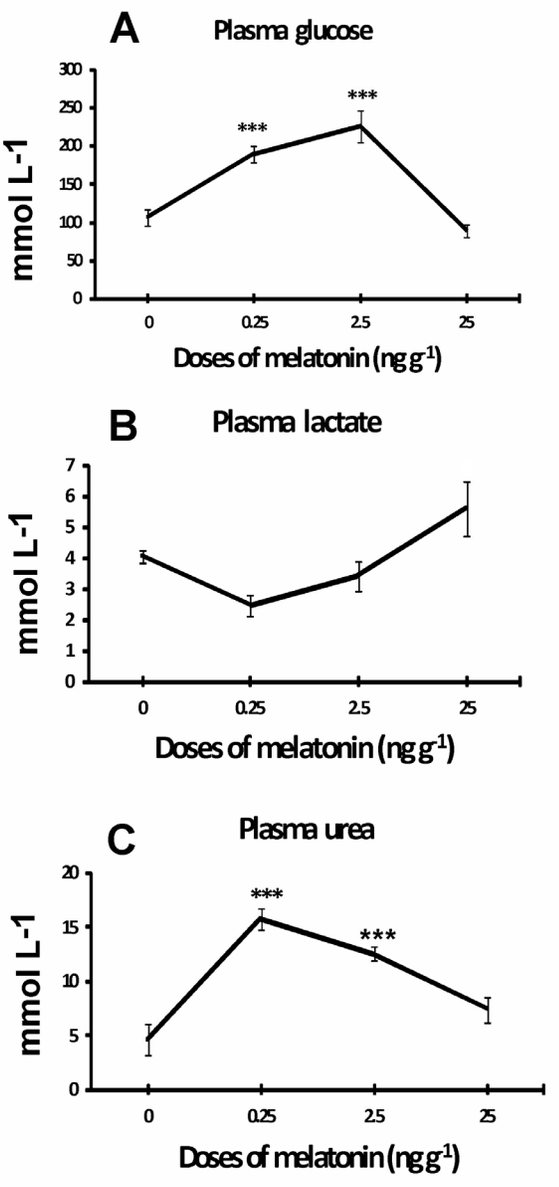

Figure 2. **(A)** Plasma glucose **(B)** lactate and **(C)** urea, contents of climbing perch after 30 min of treatment of melatonin(0, 0.25, 2.5 and 25 ng g^{-1}). Each point represents mean ± SEM of six fish. *** denotes ($P < 0.001$) when compared to control.

3.3 *In Vivo* Effects of Melatonin on Plasma and Tissue [Na^+] and [K^+] Content

Short-term melatonin administration produced significant rise in [Na^+] and [K^+] levels in the osmoregulatory tissues such as gills and kidney (Table 1 and 2). When compared with the control fish, significant rise in [Na^+] ($P \leq 0.05$) and [K^+] ($P \leq 0.01$) content were found after 30 min treatment of low dose (0.25 ng g^{-1}) of melatonin in gills (Table 1 and 2). However, in kidney, higher dose (2.5 ng g^{-1}) of melatonin produced decrease ($P \leq 0.01$) of the level of [Na^+], whereas the lower dose had little effect (Table 1). At the same time, the [K^+] content in kidney showed a significant increase ($P \leq 0.05$) after the low dose melatonin treatment (Table 2). [Na^+] and [K^+] content in intestine showed significant increase ($P < 0.05$) after the high dose (25 ng g^{-1}) of melatonin (Table 1 and 2).

Table 1. The [Na^+] content in plasma (mmol L^{-1}) and tissues (µg g^{-1}) of climbing perch after 30 min of treatment of melatonin.

Melatonin (Doses)	Plasma	Gills	Kidney	Intestine	Liver
0 (control)	137.12 ± 5.33	0.24 ± 0.16	0.59 ± 0.03	0.31 ± 0.05	0.17 ± 0.01
0.25 ng g^{-1}	139.60 ± 4.76	0.30 ± 0.02*	0.52 ± 0.01	0.24 ± 0.03	0.16 ± 0.01
2.5 ng g^{-1}	142.27 ± 4.65	0.24 ± 0.02	0.38 ± 0.03**	0.39 ± 0.03	0.16 ± 0.01
25 ng g^{-1}	141.12 ± 1.95	0.34 ± 0.02**	0.49 ± 0.04	0.49 ± 0.05*	0.16 ± 0.01

Values are mean ± SEM of six fish. * denotes ($P < 0.05$) and ** denotes ($P < 0.01$) when compared to control.

Table 2. The [K^+] content in plasma (mmol L^{-1}) and tissues (µg g^{-1}) of climbing perch after 30 min of treatment of melatonin.

Melatonin (Doses)	Plasma	Gills	Kidney	Intestine	Liver
0 (control)	4.05 ± 0.35	0.14 ± 0.02	0.45 ± 0.04	0.45 ± 0.04	0.43 ± 0.02
0.25 ng g^{-1}	2.93 ± 0.23	0.24 ± 0.01**	0.62 ± 0.03*	0.74 ± 010	0.37 ± 0.02
2.5 ng g^{-1}	3.15 ± 0.14	0.15 ± 0.01	0.47 ± 0.02	0.64 ± 0.06	0.40 ± 0.02
25 ng g^{-1}	3.30 ±0.25	0.29 ± 0.02***	0.49 ± 0.05	0.99 ± 0.14*	0.36 ± 0.03

Values are mean ± SEM of six fish. * denotes ($P < 0.05$), ** denotes ($P < 0.01$) and *** denotes ($P < 0.001$) when compared to control.

In liver, melatonin produced little effect on [Na^+] and [K^+] content (Table 1 and 2). Likewise, plasma mineral content also remained unaffected after the *in vivo* treatment of melatonin (Table 1 and 2).

4. Discussion

Melatonin has been implicated in circadian rhythm and in the regulation of varied behavioral and physiological processes[31-32]. However, the short-term impact of melatonin on TH-cortisol dynamics, and metabolite and ionic status are not yet understood in fish. In this study, short-term administration of melatonin deactivates the HPI axis as evident in the lowered plasma cortisol. This further confirms that melatonin would act as stress-lowering hormone. Similar reduction in cortisol levels has been noticed in stressed goldfish and European sea bass treated with melatonin as in humans[33-35]. In fish, the effects of melatonin are mediated through high-affinity receptors [36-38]. It has been reported that melatonin together with endogenous steroids can mediate the cyclic alterations in certain physiological processes[39]. A reduction in circulating corticosteroids after melatonin administration has been found in mammals[40] and birds[41]. Similarly, studies in fish have shown that acute melatonin administration can reduce plasma cortisol under stressful conditions[34-35].

As the end product of hypothalamo-pituitary-thyroid (HPT) axis, T_4 generally acts through the biologically active T_3, which is formed in the extra thyroidal tissues by the outer ring deiodination[42]. In contrast, we found that melatonin activates HPT axis resulting in the release of T_4, though a lowered T_3 was found in these fish. The activation of thyroid axis in melatonin-treated fish clearly indicates the sensitivity of melatonin to thyroid axis which could be either due to a direct thyrotropic action of melatonin or an alternative mechanism of lowered HPI axis that results in a lowered cortisol release. Alternatively, the increase in T_4 concentration in the fish blood may be due to a melatonin-induced inhibition of 5'–deiodinase, the enzyme responsible for accelerating peripheral deiodination of T_4 to T_3. Melatonin treatment has been reported to increase the activity of type-II thyroxine 5'-deiodinase enzyme in mammals[43]. There are several reports that support the major role of T_4 in the regulation of growth, development and reproduction which would always be associated with modified energetics and iono-osmotic homeostasis, probably as an adaptive strategy[44, 13].

In addition, the elevated plasma T_4 also points to an increased T_4 synthesis which is essential for the fish to cope up with the demand for a modified tissue ion status and osmotic balance after melatonin treatment. A rise in plasma T_4 and a decrease in T_3 during salinity exposure have been reported in rainbow trout[45] and gilthead seabream[46]. It has been further demonstrated that THs are involved in osmoregulation[47] and deiodinases found in the gills would modulate the plasma TH levels[46]. Furthermore, cortisol has also been shown to interact with the thyroid axis in fishes as in other vertebrates[13,30,46].

Melatonin has been shown to induce glucose-sensing properties in hypothalamus and in mammalian liver and muscle[48]. Similar response has been observed in rainbow trout brain areas when gluco-sensor systems are activated[49, 50]. Furthermore, melatonin treatment elicits changes in pancreatic gluco-sensor parameters in mammals such as expression of the components of the K^+ ATP channel [51]. The significant hyperglycemia in melatonin-treated fish clearly indicates the glucose-mobilizing role of melatonin in the air-breathing fish. An anti-stress action of melatonin could also be found in these fish where an inverse interaction of melatonin with HPI axis resulting in lowered plasma cortisol is indicated. Alternatively, it is also evident that modulation of glucose by melatonin would possibly be related to physiological action of melatonin in modifying the disturbed physiological processes as part of stress acclimation in fish as reported earlier[52]. This is connected with the hypothesis that melatonin would favor the recovery or ease response to rescue or correct the disturbed physiological homeostasis that could happen during stress response [52]. It is well known that glycogenolysis and subsequent hyperglycemia are the well-documented responses in fish to various stressors[19,53].

Generally, hyperglycemia, an index of stress response, has been mainly linked to the elevated cortisol and adrenaline levels[54]. In addition, a modulatory role of TH in glucose homeostasis is also well established[47,55-57]. It is likely that the rise in T_4 after melatonin treatment would favor increased oxidation of glucose indicating an elevated energy demand in these fish. This, in turn, reflects a role for melatonin in the metabolic reallocation, which is required for maintaining energy homeostasis. Interestingly, the rise in glucose and T_4 levels in this fish after melatonin treatment is in accordance with our earlier observations on the ability of THs to induce glucose production in freshwater[30,58] and marine[59] fish.

Similar to cortisol and adrenaline, THs exert major osmoregulatory and metabolic actions in fish[29,60,61], and are known for involvement in the regulation of stress response[18,19,55,62]. It shows proteogenic action in fish[58], though the catalytic actions of THs may prevail as a result of higher energy requirement[60]. It is also found that THs show permissive action with cortisol in the regulation of intermediary metabolism[13,30,60,62,63]. Further, many of the reported inconsistencies of THs actions in fish are generally explained on the basis of their synergistic and antagonistic actions with other hormones, especially cortisol and adrenaline[13,52]. It is likely that the decline of T_3 after melatonin treatment in our fish could indicate an increased metabolism of T_3or a rapid clearance of T_3 by cortisol in this fish as an adaptive strategy that the fishes have developed for fine-tuning physiological processes as indicated previously[13,56]. Similarly, adecline of plasma T_3, and not plasma T_4, has been found in confined tilapia, indicating a possibility of plasticity of TH-driven metabolism in fish [13]. The differential response resulting in an interaction of melatonin with cortisol and THs could further point to inter-hormonal interference as a mechanism of fine-tuning of physiological processes as reported earlier [13].

Most freshwater teleosts are ammonotelic and are able to excrete nitrogenous waste mainly through the gills and kidneys[64]. In contrast, air-breathers have ureogenic potential and possess more metabolic variability in their nitrogen excretion[65]. The elevated status of urea resulting in hyperuremiain melatonin-treated fish indicates an ureogenic capacity. This capacity of climbing perch further points to the ability of melatonin to take part in ammonia excretion. Similar rise in urea production has been found in perch treated T_4[47] and exposure to effluents of coconut husk retting[55] and nimbicidine[47]. In this context, it is likely that the increased T_4 level after melatonin treatment could also account for the elevated ureogenic capacity. Furthermore, the rise in T_4, along with the rise in glucose and urea after melatonin treatment in our fish clearly imply that melatonin would activate the HPT axis to perform its metabolic and nitrogen excreting functions.

The distribution and the bioavailability of cellular $[Na^+]$ and $[K^+]$ gradients are essential for an optimal cellular function. The increase of $[Na^+]$ and $[K^+]$ content of the gills and the rise in $[K^+]$ and decline in $[Na^+]$ in kidney indicate that these tissues are the sites for melatonin action in this fish. The unaffected $[Na^+]$ and $[K^+]$ gradients in the liver after the melatonin treatment imply that melatonin does not pose any threat to the liver. The $[Na^+]$ and $[K^+]$ levels in the gills, kidney and intestine after melatonin administration further indicate its role in regulation of ion transport functions in these tissues. Likewise, the unaffected plasma $[Na^+]$ and $[K^+]$ ions after melatonin treatment indicate a tight hydromineral balance in this fish. The rise in $[Na^+]$ and $[K^+]$ in gills and kidney by melatonin further point to a direct control of melatonin on the transport and bioavailabilty of these ions, indicating kidney as a target for osmoregulatory action of melatonin. A number of hormones including cortisol[66] and

THs[29] are known to regulate the transport (reabsorption or secretion) of large amount of a wide variety of substances, ions and uncharged solutes[67].

Overall, our results indicate a vital role of melatonin in fine-tuning the physiological processes where activation of thyroid axis and deactivation of interrenal axis occur, accompanied by metabolite mobilization, ureogenesis and tissue ion homeostasis in our test species.

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