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Differential daily rhythms of melatonin in the pineal gland and gut of goldfish *Carassius auratus* in response to light

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The objectives of this study were to test the effects of light on melatonin rhythms in the pineal gland and gut of goldfish *Carassius auratus* and to investigate whether melatonin function differed in these two tissues, which are photosensitive and non-photosensitive respectively. Rhythms were evaluated by measuring arylalkylamine N-acetyltransferase (*AANAT2*) and melatonin receptor 1 (MT-R1) mRNA expression and melatonin concentration in the pineal gland, gut (*in vivo*), and cell cultures of the two tissues (*in vitro*). Compared to control, pineal gland melatonin secretion was higher at night, whereas the 24-h dark and ophthalmectomy groups maintained higher *AANAT2* and MT-R1 mRNA expression during the day. Melatonin levels and *AANAT2* and MT-R1 mRNA expression in the gut were also the highest at night, but the 24-h light, dark, and ophthalmectomy groups did not significantly differ from control. Furthermore, we measured *AANAT2* and MT-R1 mRNA expression in high temperature water (30 °C) to investigate differences in the antioxidant capacity of pineal gland vs. gut melatonin. Melatonin and H_2O_2 levels, as well as *AANAT2* and MT-R1 mRNA expression, were all higher in the two tissues under thermal stress, compared with their levels at 22 °C. Taken together, our results suggest that light has no effect on melatonin patterns in the gut, which appears to exhibit its own circadian rhythm, but both gut and pineal gland melatonin exhibit similar antioxidant function.

Keywords: *AANAT2*; circadian rhythms; gut; melatonin; ophthalmectomy; pineal gland

1. Introduction

Light is one of the most important environmental factors to influence nearly all organisms (Migaud et al. 2006). Generally, invertebrates, including fish, photoreceptors in the retina process external light signals and transmits the information to the hypothalamic suprachiasmatic nucleus (SCN). From the SCN, signals are then transferred to photosensitive organs (Yoshikawa & Oishi 1998; Yamazaki et al. 1999).

A major light-dependent hormone is melatonin (N-acetyl-5-methoxytryptamine), which plays a major role in controlling fish biorhythms as a neuroendocrine communicator (Falcón et al. 2007). Besides its function as a regulator of circadian rhythms, melatonin also performs various physiological functions, such as removing free radicals, enhancing the immune system, and reducing oxidative stress in fish (Wu & Swaab 2005; Besseau et al. 2006; Kim et al. 2014). Melatonin acts by binding to melatonin

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receptors (MT-Rs), belonging to the G-protein coupled receptor superfamily and widely distributed in the vertebrate diencephalon and central nervous system (Reppert et al. 1996; Park et al. 2013). Among the MT-Rs, MT-R1 is implicated in the seasonal changes to vertebrate physiology and behavior, including those of fish, and directly affects circadian rhythms (Reppert et al. 1996).

Melatonin is secreted mainly at night by the pineal gland and retina, and regulated by arylalkylamine N-acetyltransferase (AANAT). AANAT is well known as a rate-limiting enzyme of the melatonin biosynthetic pathway (Klein et al. 2002). During night time, depolarization of light receptors results in an influx of Ca^{2+} and cyclic AMP (cAMP) into the cell, which then phosphorylates *AANAT*, increasing its intracellular amount and activity (Iuvone et al. 2005; Klein 2007).

Although melatonin is widely accepted as a major regulator of vertebrate biorhythms, the hormone has been also found in several non-photosensitive tissues (Bubenik 1980; Vakkuri et al. 1985; Bubenik & Pang 1997). One notable example is the gut, which was reported to secrete more melatonin than the pineal gland (Bubenik 2002). In addition, melatonin secretion and AANAT expression patterns in the gut appear to vary depending on the species (Velarde et al. 2010). Previous studies have shown that the melatonin-generating system in the gut and pineal gland of the major carp *Catla catla* may not be under photothermal regulation due to daily and seasonal photoperiod changes (Mukherjee et al. 2014a; 2014b). Despite these recent insights, the molecular endocrinological mechanism underlying the autonomous circadian rhythm of gut melatonin regulated by light remains poorly understood.

Previous studies have shown that melatonin acts as a direct scavenger of free radicals. Reiter et al. (2000) suggested that melatonin increases the expression and activity of antioxidants, such as superoxide dismutase, catalase (CAT), and glutathione peroxidase, and removes internally generated reactive oxygen species, including superoxide (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$). However, little is known about the role of melatonin in photosensitive and non-photosensitive tissues, such as the pineal gland and gut, under thermal and salinity stress conditions.

Goldfish *Carassius auratus* is a popular ornamental fresh water fish and an ideal model to study the mechanisms of circadian rhythms and photoresponse in any melatonin synthesizing tissue, because it maintains close contact with environmental light, due to its natural surface dwelling habit. To study the differences in the levels of *AANAT2* and MT-R1 mRNA and melatonin concentrations between the photosensitive pineal tissue and non-photosensitive gut tissue, we reared goldfish under different photoperiod conditions. In addition, we also analyzed how the absence of the retina affected the circadian rhythms of pineal gland and gut melatonin after ophthalmectomy (OP). Finally, to ascertain whether gut melatonin shared antioxidant function as pineal gland melatonin, we compared and analyzed the expression patterns of pineal gland and gut *AANAT2* and MT-R1 mRNA, as well as the levels of H_2O_2 under thermal stress conditions (temperature increased from 22 to 30 °C in daily increments of 2 °C).

2. Materials and methods

2.1. Experimental fish, OP, and conditions

For each experiment, common goldfish ($n = 720$, mean length, 8.1 ± 0.4 cm; mean mass, 16.5 ± 0.6 g) was purchased from a commercial aquarium (Choryang, Busan,

Korea) and was allowed to acclimate for 2 weeks in eleven 300-L circulation filter tanks in the laboratory. Five tanks with 60 individuals each were used for light response and thermal stress experiments, and one tank with 60 individuals for cell culture experiment. Both experiments were duplicated.

Experimental groups [control (12L:12D), continuous light (LL), continuous dark (DD), OP, and thermal stress] were exposed to light using a white fluorescent bulb (27 W, wavelength range 350–650 nm); placed 40 cm above the water surface. The light intensity at the water surface was approximately 0.96 $W \cdot m^{-2}$. Goldfish was reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatize to the experimental conditions for 24 h. Water temperature was maintained at 22 °C. All the fish was provided commercial feed daily at 0900 and 1700.

For the light response study, we used a control group (12L:12D) and three experimental groups (LL, DD, and OP). The control group was exposed to a 12-h light:12-h dark cycle (12L:12D) (lights were turned on at 0700 and turned off at 1900). The LL experimental group was exposed to 24-h light; the DD experimental group was exposed to 24-h dark; and the OP experimental group was exposed to 12L:12D.

Furthermore, the 120 fish in the OP experiment group were subjected to ophthalmectomy under anesthesia with 200 $mg \cdot L^{-1}$ tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA). An incision was made around the eye, which was then elevated using a forceps, and the optic nerve was cut to remove the eye completely as described by Martinez-Chavez and Migaud (2009). These fishes were placed in 300-L circulation filter tanks under 12L:12D period at 22 °C for one week. No mortalities were observed.

In the thermal stress experiment group, fish was reared with a 12L:12D photoperiod under a white fluorescent bulb, with lights on at 0700 and lights off 1900. Fishes were all initially exposed to 22 °C water; this temperature was maintained for the control group, but increased from 22 to 30 °C in daily increments of 2 °C for the experimental groups.

The fish was killed by spinal transection (first sampling at 1100; ZT 4) at 4-h sampling intervals (ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24) to collect the pineal gland, whole gut, and blood under dim light using an attenuated white fluorescent bulb. The fish was anesthetized with 200 $mg \cdot L^{-1}$ tricaine-methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) to minimize stress prior to blood collection. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated with centrifugation (4 °C, 1000 × *g*, 15 min) and stored at −80 °C until analysis. The pineal gland and the gut were removed from the fish, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. In vitro *culture of pineal gland and whole gut cells*

For cultures, pineal gland and whole gut tissue were quickly removed and placed in 3-mL dispersion buffer that was kept on ice for 1 min (dispersion buffer: pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, containing 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 2.5 $\mu g \cdot mL^{-1}$ fungizone; GIBCOBRL, Rockville, MD, USA). The isolated pineal gland and whole gut tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma, USA). Next, tissue samples were cut into small pieces using a single-edged razor after connective tissues and other impurities were removed. The minced tissues of the two organs were transferred to a flask and

incubated for 10 min at room temperature with slow stirring. The pineal gland and whole gut tissue mixture were filtered, and the culture medium was added (neuro basal medium, without L-glutamine, containing 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 2.5 $\mu g \cdot mL^{-1}$ fungizone and 1% fetal bovine serum, Gibco-BRL; to adjust the medium's osmolarity to goldfish plasma osmolarity, 353 mOs). The cell suspension was centrifuged at 800 × *g* for 10 min, and the cells were then resuspended in fresh culture medium. Cells from the pineal gland and gut of 60 goldfish were counted and adjusted to a final concentration of approximately 1.93×10^6 live pineal gland cells$\cdot mL^{-1}$ and 4.51×10^6 live gut cells$\cdot mL^{-1}$ (as determined by trypan blue exclusion) in supplemented media.

The pineal gland and gut cells at a concentration of approximately 1.2×10^6 cells$\cdot mL^{-1}$ were added in 24-well culture plates (SPL Life Sciences, Seoul, South Korea) and incubated at 22 °C, 100% humidity, and 5% atmospheric CO_2 in a culture chamber (HB-302L; Hanbaek Scientipic Co., Gyeonggi, Korea). Incubation started at 0700, and samples were collected at ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24. Thus, the first collection time was at the first ZT 4 (1100) to second ZT 24 (0700). During the experimental period (2 days), the pineal gland and gut cells were exposed to 12L:12D (control group), LL, and DD using a dim white fluorescent bulb placed 50 cm above the cell culture plate. After the end of incubation, a cell dissociation reagent (trypsin/EDTA) was used to degrade the adhesion between the cells and wells. Then, cell suspension was centrifuged at 800 × *g* for 10 min, and the supernatant was removed and stored at −80 °C until total RNA extraction and analysis.

2.3. *Quantitative PCR (QPCR)*

To carry out this study, we have considered the recommendations of the MIQE (Minimum Information for Publication of Quantitative Real-time PCR Experiments) (Bustin et al. 2009). Total RNA was extracted from the pineal gland and whole gut using a TRIzol kit (Gibco/BRL, USA) according to manufacturer protocol. To obtain cDNA for qPCR, reverse transcription was performed using M-MLV reverse transcriptase (Promega, USA), again following manufacturer protocol. QPCR was conducted to determine the relative expression levels of MT-R1 (GenBank accession no. AB481372), *AANAT2* (GenBank accession no. GU205782), β-actin (GenBank accession no. AB039726), and GAPDH (GenBank accession no. AY641443) mRNA in the pineal gland and whole gut. The primers used for QPCR are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignment was performed with the BLAST database to ensure primer specificity. PCR was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, USA)

Table 1. Primers used for QPCR amplification.

Genes (accession no.)	Primer	Sequences
MT-R1 (AB481372)	Forward	5′-GGT TGG CAG TAG CGA TTT-3′
	Reverse	5′-CTC ACG ACG GAA GTT CTG-3′
AANAT2 (GU205782)	Forward	5′-GAA TGT CCA CTC ACC CTT G-3′
	Reverse	5′-TCT CAT CGC TTC CTG TTC-3′
β-actin (AB039726)	Forward	5′-TTC CAG CCA TCC TTC CTA-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′
GAPDH (AY641443)	Forward	5′-GGC CGC TAC AAG GGA GAT GT-3′
	Reverse	5′-CCG GTA GAC TCG ACT ACA TA −3′

and iQ™ SYBR Green Supermix (Bio-Rad, Hercules, USA), following the manufacturer's protocol. The thermocycling profile was as follows: 95 °C for 5 min, followed by 50 cycles each of 95 °C for 20 s and 55 °C for 20 s. Experiments were duplicated with β-actin and GAPDH, and all data were expressed relative to the calculated β-actin and GAPDH threshold cycle (ΔCt) levels.

2.4. Western blot analysis

Total protein isolated from the pineal gland and whole gut of goldfish were extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., USA) following manufacturer protocol. Twenty-five micrograms of total protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad, USA). For reference, a protein ladder (Bio-Rad, USA) was also used. Samples were electrophoresed at 180 V. The gels were immediately transferred to a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA), set at 85 V for 3 min, using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% skim milk in 0.04% Tris-buffered saline with Tween (TTBS) for 45 min and subsequently washed in TTBS. The membranes were incubated with MT1 antibodies [Novus biologicals® Melatonin Receptor 1A Antibody (NBP1-28,912); dilution, 1:4000; approximately 45 kDa] for 2 h, followed by horseradish peroxidase-conjugated antirabbit IgG secondary antibodies (dilution, 1:4000; Bio-Rad, USA) for 60 min. As an internal control, incubation was performed with β-tubulin primary antibody (dilution, 1:4000; ab6046, Abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:4000; Bio-Rad) for 60 min. Bands were detected using a sensitive electrochemiluminescence (ECL) system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc™ XRS$^+$ System (Bio-Rad, USA).

2.5. Melatonin concentration level

Pineal gland and whole gut samples were homogenized and centrifuged (4 °C, 1500 × *g*, 15 min) and plasma samples were separated by centrifugation (4 °C, 1000 × *g*, 15 min). The melatonin levels of tissue and plasma were analyzed using immunoassays using Fish Melatonin ELISA kit (Catalog no. MBS013211; Mybiosource, San Diego, CA, USA). An anti-antibody that was specific to the melatonin antibody was pre-coated onto a microplate. Next, 100-μL tissue supernatant, 50-μL HRP-conjugate, and 50-μL antibody were added to each well. These were mixed and then incubated for 2 h at 37 °C. Following the last wash, any remaining Wash Buffer was aspirated or decanted off, and 50 μL each of substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which they changed from colorless to dark blue. Following incubation, 50 μL stop solution was added to each well, changing the color from blue to yellow. The optical density of the solution in each well was then determined within 10 min, using a microplate reader set to 450 nm.

2.6. H_2O_2 concentration measurement

The H_2O_2 concentrations were measured using Peroxide Detect kit (Sigma-Aldrich, St. Louis, MO). Absorbance was measured at 560 nm, and the concentration of H_2O_2

was interpolated from a standard curve. The concentrations were expressed as nmol $H_2O_2 \cdot g^{-1}$ of tissue.

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's *post hoc* test was used to compare differences in the data ($p < 0.05$). The values are expressed as the means ± standard error (SE).

3. Results

3.1. Change of pineal gland AANAT2 expression levels

In all of the experimental groups (*in vivo*), *AANAT2* mRNA expression was generally higher at night than during the day and was significantly higher at ZT20 (Figure 1). Within the DD and OP groups, *AANAT2* mRNA expression significantly decreased during the second-day cycle, compared with the first-day cycle (Figure 1(a)). When comparing across experimental and control groups, we found that both the DD and OP groups exhibited higher *AANAT2* mRNA expression than the control group. Finally, the LL group exhibited significantly lower *AANAT2* mRNA expression than the control as well as the DD and OP groups, while the latter two did not significantly differ from each other (Figure 1(a)).

The QPCR results using *in vitro* mRNA (extracted from pineal gland cell culture) generally accorded with the *in vivo* experiments. Again across all experimental groups, *AANAT2* mRNA expression was higher at night than during the day. In the DD group, *AANAT2* mRNA expression also significantly decreased in the second-day cycle compared with the first-day cycle (Figure 1(b)). Lastly, compared with control, *AANAT2* mRNA expression was significantly higher in the DD group and lower in the LL group (Figure 1(b)).

3.2. Change of whole gut AANAT2 expression levels

The results of the QPCR analysis on *in vivo* gut mRNA exhibited both similarities to, and differences from, mRNA patterns in the pineal gland. First, *AANAT2* mRNA expression was also higher at night than during the day across all experimental groups, with the ZT20 time points exhibiting significantly higher expression levels (Figure 2(a)); *in vitro* results exhibited the same pattern (Figure 2(b)). However, in contrast to results from the pineal gland, *AANAT2* mRNA expression did not differ between the first- and second-day cycles for both the DD and the OP groups (Figure 2(a)). Furthermore, *AANAT2* mRNA expression patterns did not significantly differ across any of the experimental groups, which was also the case with the *in vitro* results.

3.3. Change of pineal gland MT-R1 protein and mRNA expression levels

MT-R1 protein and mRNA expression (extracted from the pineal gland *in vivo*) were higher at night than during the day in all experimental groups, and was significantly higher at ZT20 (Figure 3(a) and (b)). In the DD and OP groups, MT-R1 mRNA expression also significantly decreased in the second-day cycle compared with the first-day cycle (Figure 3(b)). MT-R1 mRNA expression measured from *in vitro* pineal gland cells exhibited the same patterns of being higher at night and lower during the second-day cycle (Figure 3(c)).

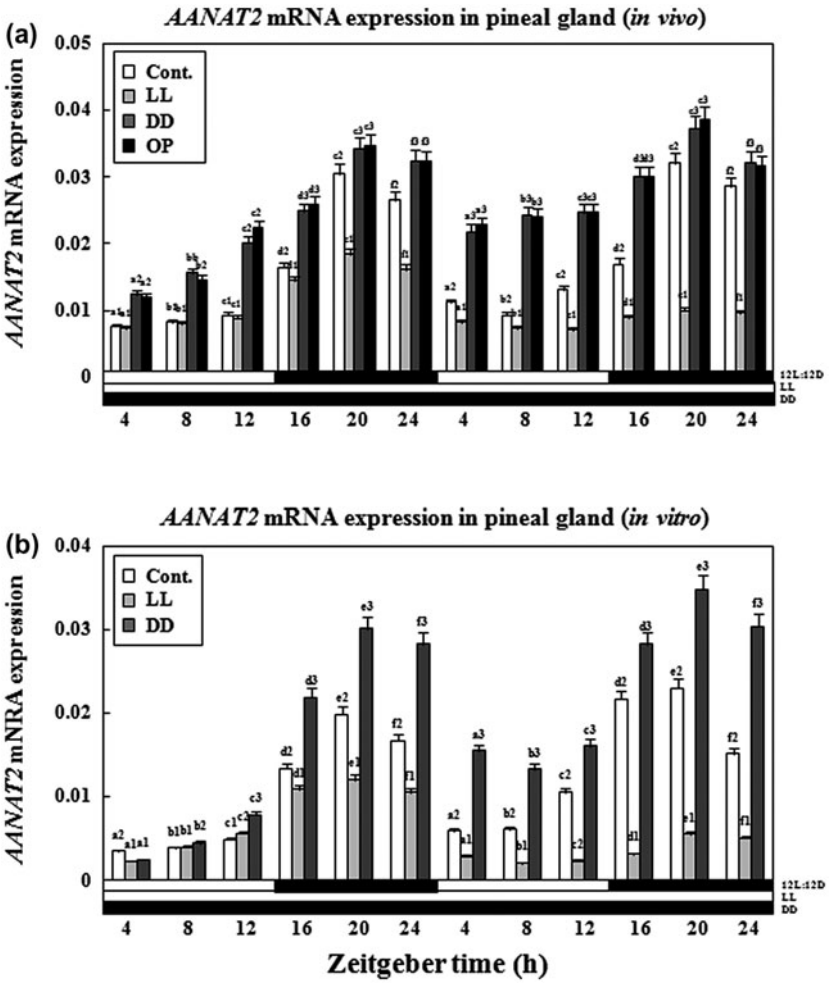

Figure 1. Changes in the expression levels of *AANAT2* mRNA (a) in the pineal gland, for 2 days using various photoperiods [12L:12L (Cont.), LL and DD] and ophthalmectomy (OP). Changes of expression levels of *AANAT2* mRNA in the cultured pineal gland cells (b) using various photoperiods [12L:12L (Cont.), LL and DD].

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are same circadian time in fish exposed to the different experimental groups ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

Compared to control, the DD and OP groups exhibited higher MT-R1 protein and mRNA expression, while the LL group exhibited significantly lower expression (Figure 3(a) and (b)). Expression levels in the LL group were also significantly lower than the DD and OP groups, which did not significantly differ from each other. The results of *in vitro* experiments again mirrored these expression patterns, with DD groups expressing significantly higher levels of MT-R1 mRNA and LL groups expressing significantly lower levels (Figure 3(c)).

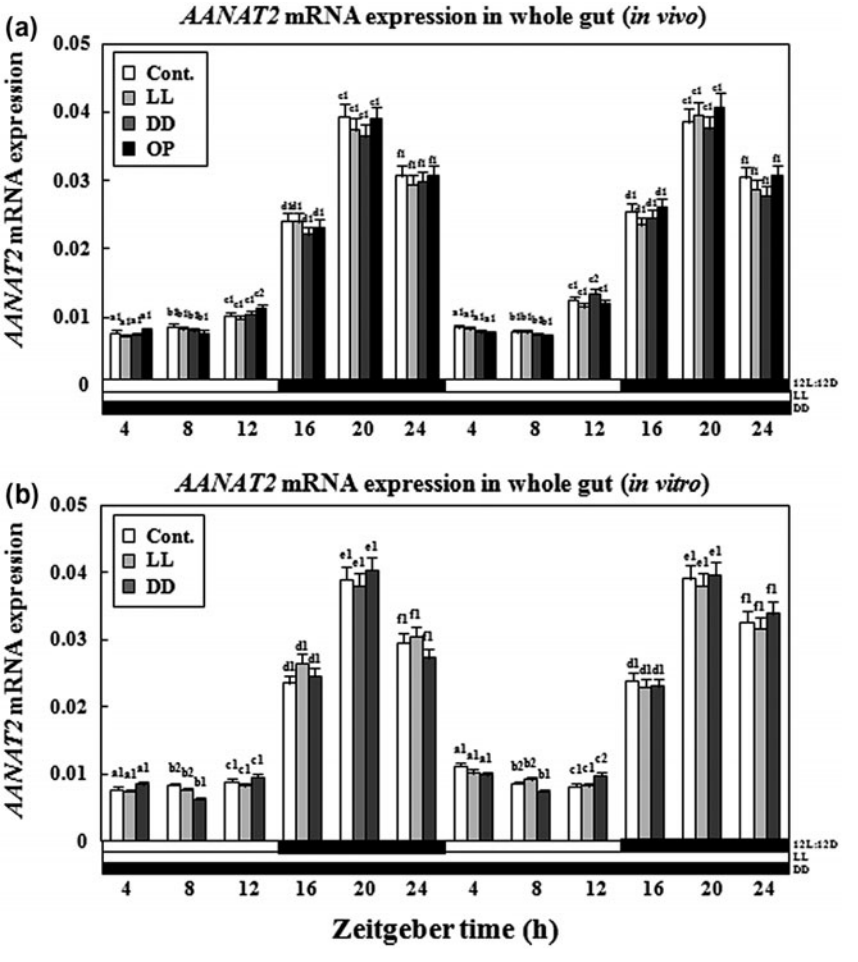

Figure 2. Changes in the expression levels of *AANAT2* mRNA (a) in the whole gut, for 2 days using various photoperiods [12L:12L (Cont.), LL and DD] and ophthalmectomy (OP). Changes of expression levels of *AANAT2* mRNA in the cultured whole gut cells (b) using various photoperiods [12L:12L (Cont.), LL and DD].

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are same circadian time in fish exposed to the different experimental groups ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

3.4. MT-R1 protein and mRNA expression levels

MT-R1 protein and mRNA expression (extracted from goldfish gut *in vivo*) were higher at night than during the day time in all experimental groups, and were significantly higher at ZT20 (Figure 4(a) and (b)), a pattern that was mirrored by the results of the *in vitro* experiments (Figure 4(c)). In contrast to the pineal gland, MT-R1 mRNA expression in the DD and OP groups did not significantly differ between the first-day cycle and the second-day cycle (Figure 4(b)). Moreover, MT-R1 protein and mRNA

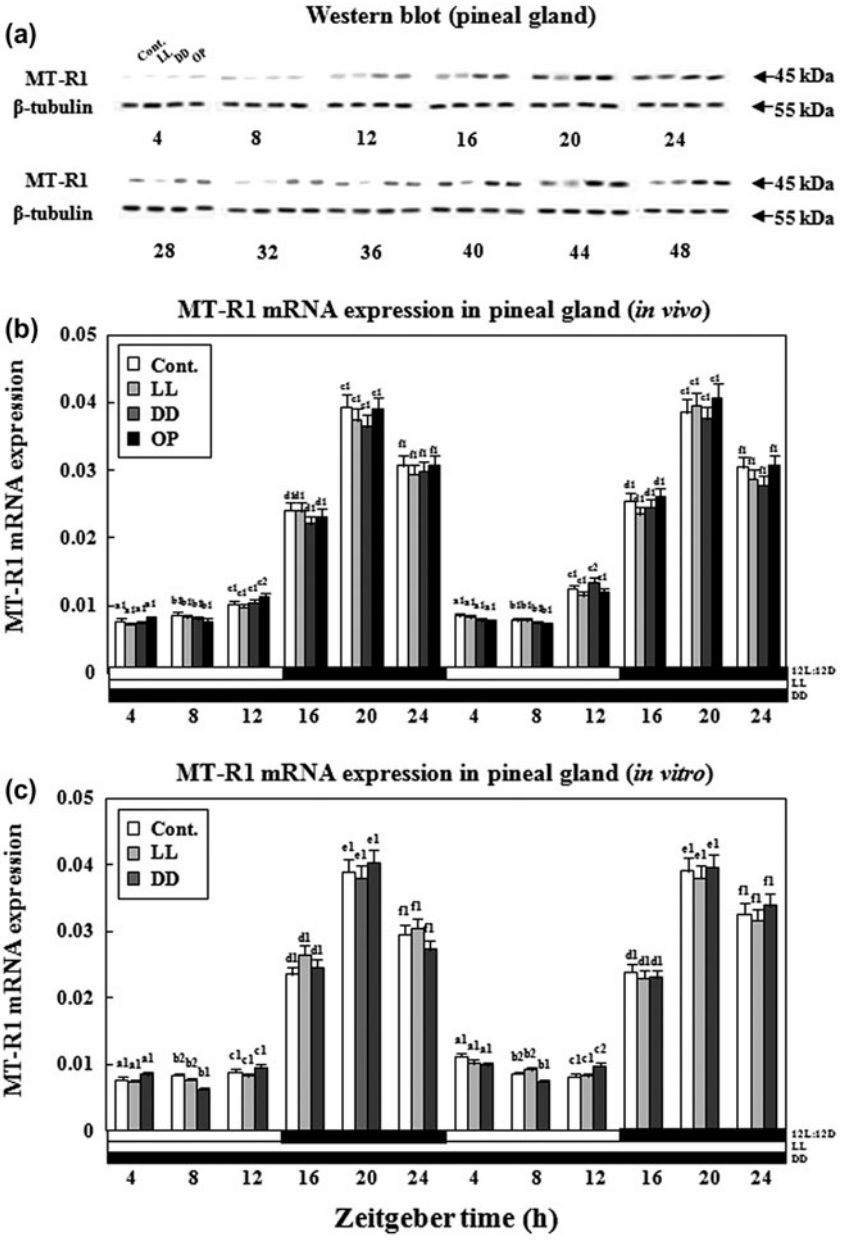

Figure 3. Changes in the expression levels of MT-R1 protein (a) and MT-R1 mRNA (b) in the pineal gland, for 2 days using various photoperiods [12L:12L (Cont.), LL and DD] and ophthalmectomy (OP). Changes of expression levels of MT-R1 mRNA in the cultured pineal gland cells (c) using various photoperiods [12L:12L (Cont.), LL and DD].

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are same circadian time in fish exposed to the different experimental groups ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

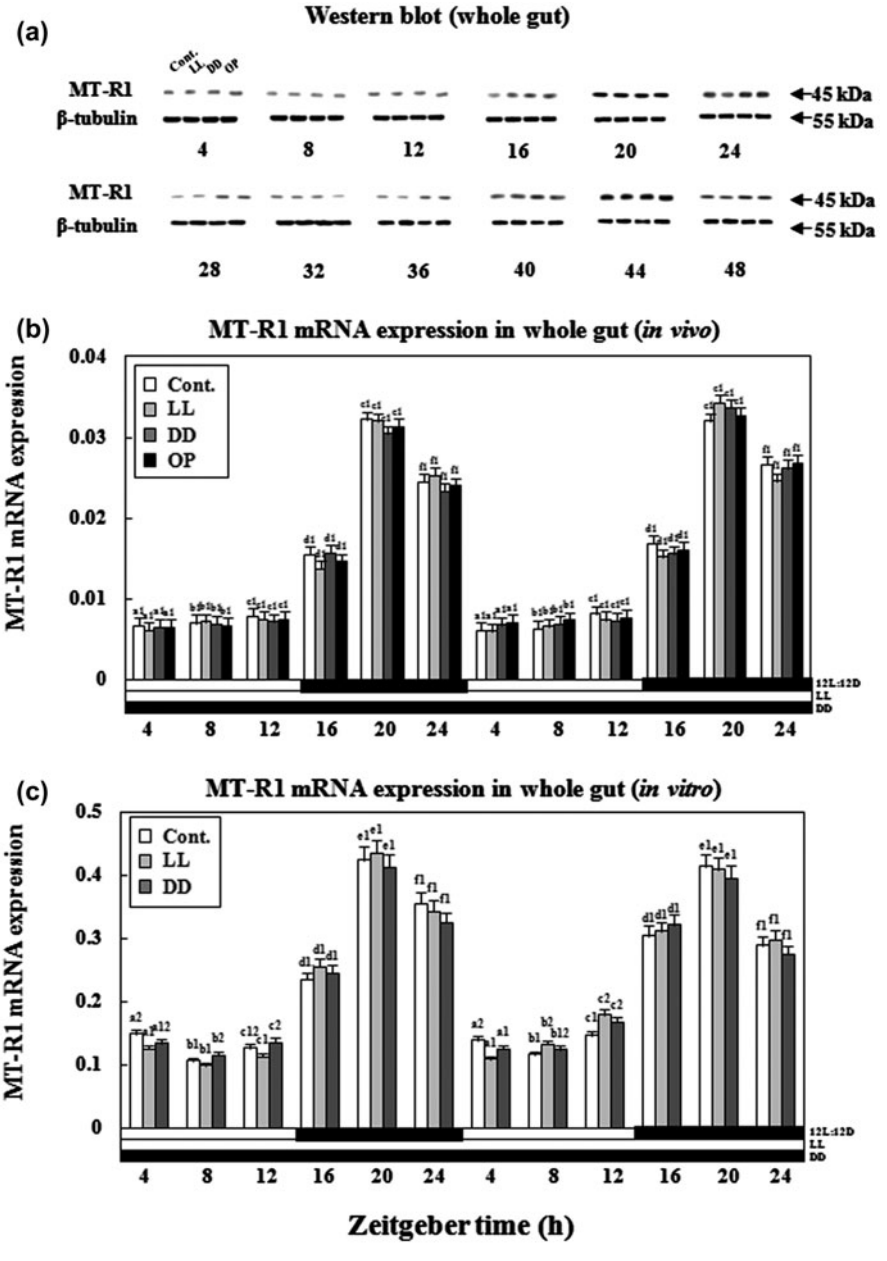

Figure 4. Changes in the expression levels of MT-R1 protein (a) and MT-R1 mRNA (b) in the whole gut, for 2 days using various photoperiods [12L:12L (Cont.), LL and DD] and ophthalmectomy (OP). Changes of expression levels of MT-R1 mRNA in the cultured whole gut cells (c) using various photoperiods [12L:12L (Cont.), LL and DD].

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are same circadian time in fish exposed to the different experimental groups ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

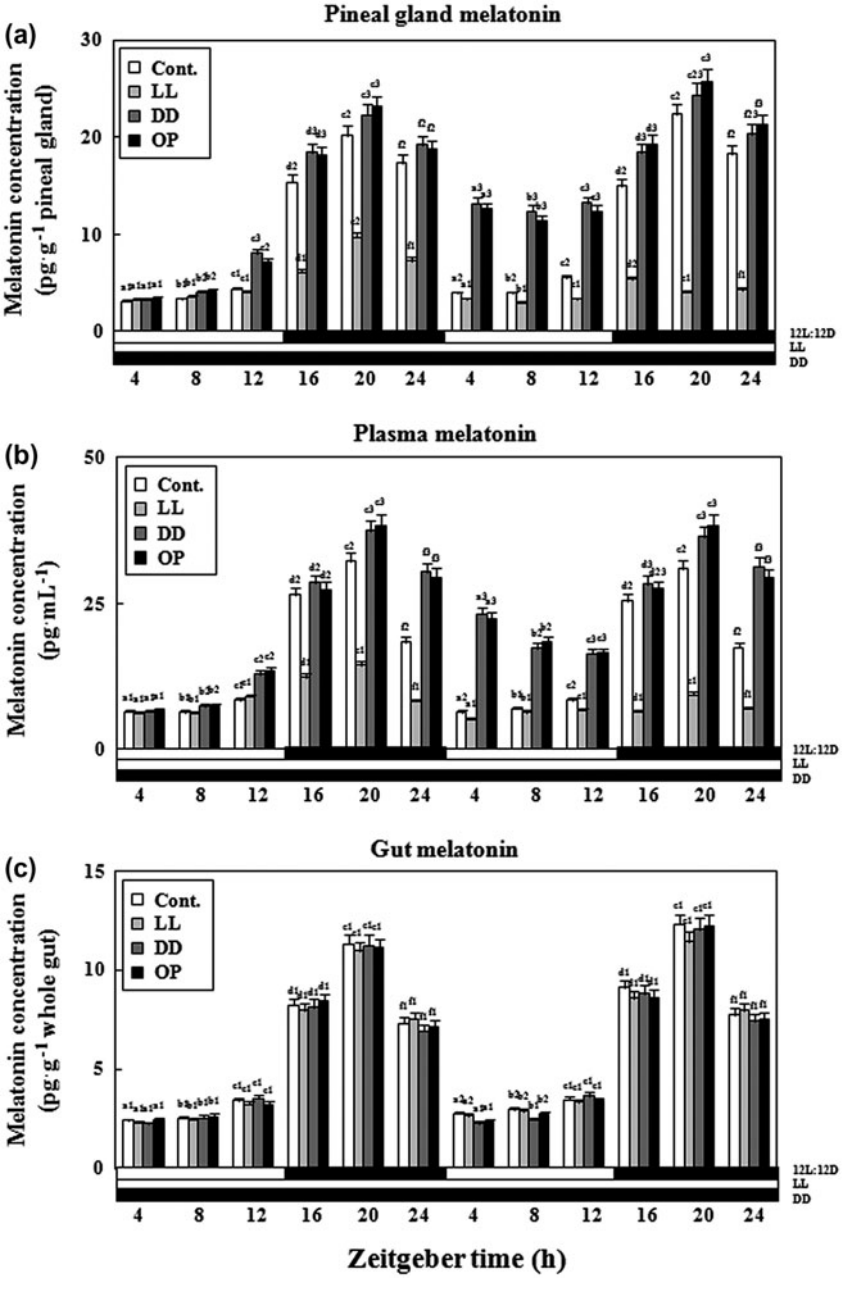

Figure 5. Changes in the levels of melatonin in the pineal gland (a) plasma (b) whole gut (c) of goldfish under various photoperiods [12L:12L (Cont.), LL and DD] and ophthalmectomy (OP) as measured by plate reader.

Notes: The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are same circadian time in fish exposed to the different experimental groups ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

expressions exhibited no significant difference across all experimental groups. The *in vitro* experiments also found no difference across the experimental groups.

3.5. Melatonin concentration

Melatonin concentrations in all tested samples (pineal gland, plasma, and gut) were significantly higher concentration at night than during the day (Figure 5). The pineal gland and plasma exhibited similar patterns, with melatonin concentrations generally lower in the LL group than in the DD and OP groups. Additionally, melatonin concentrations in the latter two groups also significantly decreased in the second-day cycle compared with

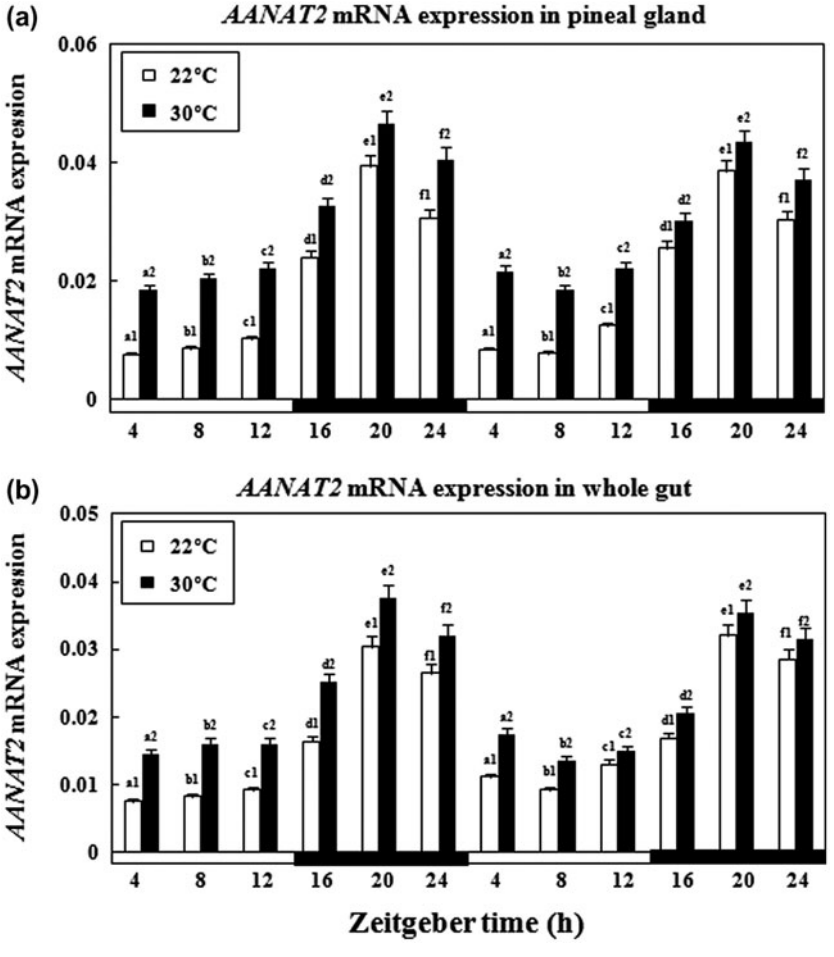

Figure 6. Changes in the levels of *AANAT2* mRNA in goldfish pineal gland (a) and in the whole gut (b) under different temperature conditions, 22 °C (control) and 30 °C (thermal stress) under the natural photoperiod (SNP).

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. Values with different characters are at same circadian time ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

the first-day cycle (Figure 5(a) and (b)). In contrast, melatonin concentrations in the gut did not significantly differ between the first-day and the second-day cycle (Figure 5(c)).

3.6. AANAT2 and MT-R1 expression in response to thermal stress

AANAT2 and MT-R1 mRNA expression in both the pineal gland and gut of the thermal stress group (30 °C) were significantly higher than expression in the control group (22 °C). For both groups, *AANAT2* and MT-R1 mRNA expression were higher at night than during the day, and were significantly higher at ZT20 (Figures 6 and 7).

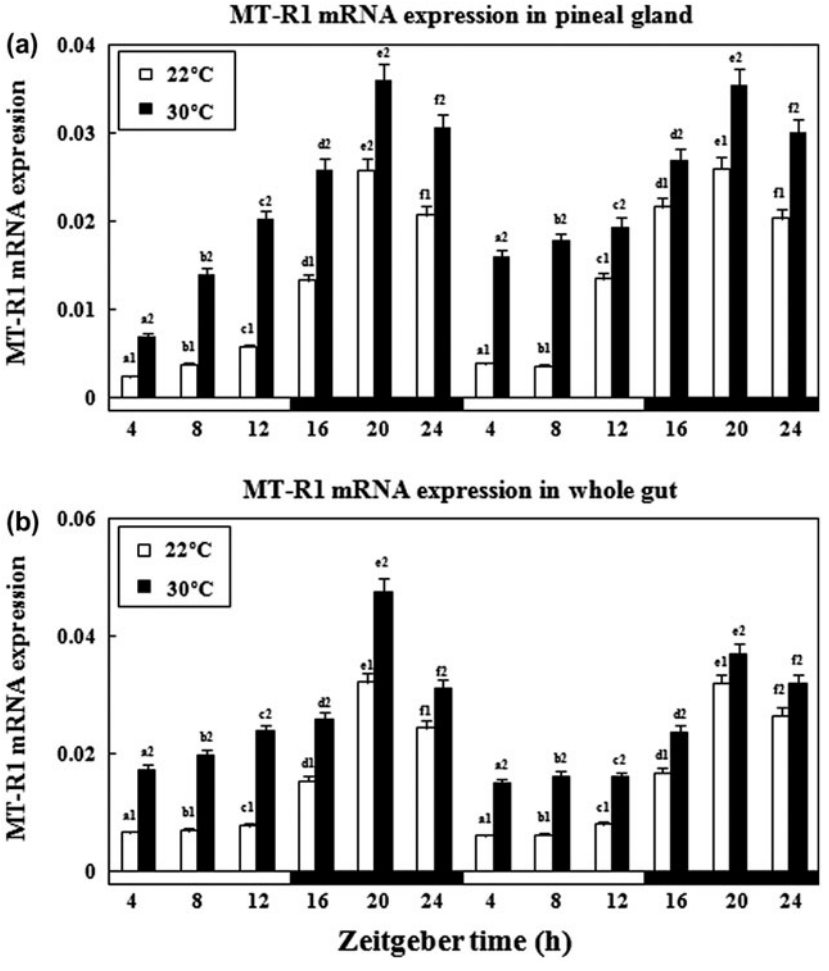

Figure 7. Changes in the levels of MT-R1 mRNA in goldfish pineal gland (a) and in the whole gut (b) under different temperature conditions, 22 °C (control) and 30 °C (thermal stress) under the natural photoperiod (12L:12D).

Notes: The expression level of each sample was normalized with respect to the β-actin and GAPDH signal and is expressed as the relative expression level. Values with different characters are at the same circadian time ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

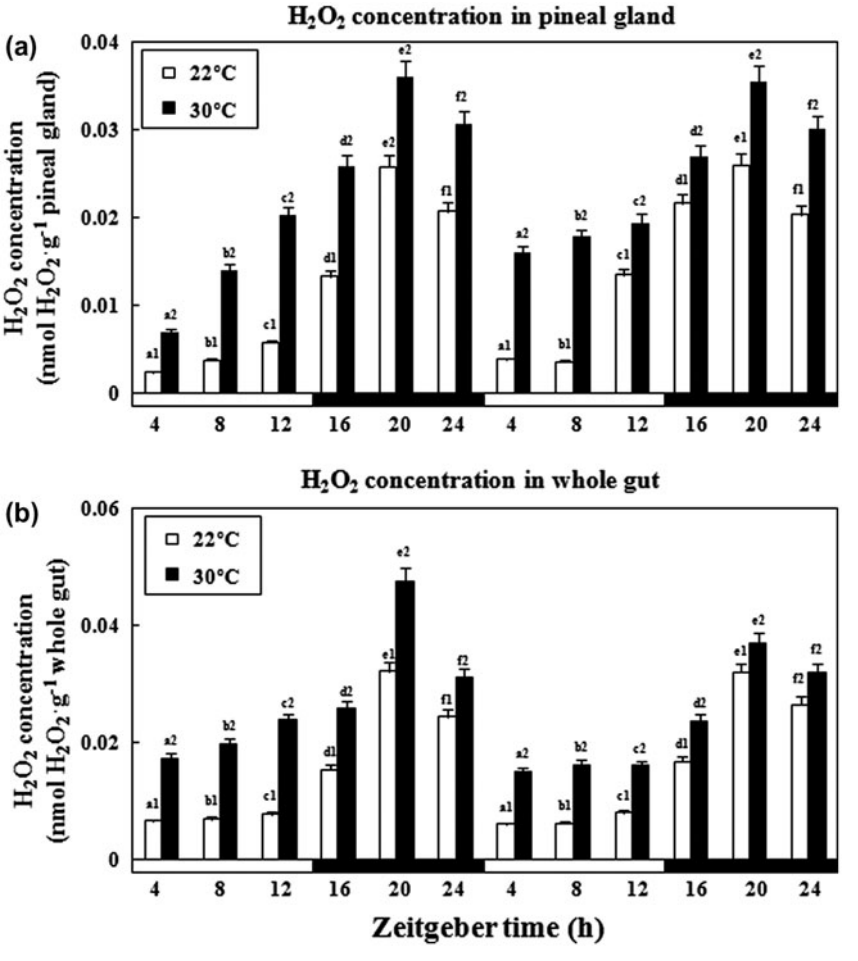

Figure 8. Changes in the levels of H_2O_2 in the pineal gland (a) and gut (b) of goldfish at 22 °C (control) and 30 °C (thermal stress), under natural photoperiod conditions (12L:12D).
Notes: Values with different characters are at the same circadian time ($p < 0.05$). Numbers indicate significant differences between and within the experimental groups ($p < 0.05$). Data are expressed as mean ± standard error (SE; $n = 5$).

3.7. H_2O_2 concentration under thermal stress conditions

H_2O_2 concentrations in the pineal gland and gut of the thermal-stress group (30 °C) were significantly higher than those of the control group (22 °C) (Figure 8). The concentrations in the pineal gland and gut did not differ significantly between the first-day and second-day cycles.

4. Discussion

In this study, we found that pineal gland and gut melatonin shared the same functions as a circadian rhythms regulator and an antioxidant, despite differences in the photosensitivity of the originating tissue. The presence or absence of light, however, did have different effects on melatonin, depending on whether it was secreted by the pineal gland (photosensitive) or the gut (non-photosensitive).

First, both *in vivo* and *in vitro* experiments confirmed that *AANAT2* and MT-R1 mRNA expression were significantly higher at night (ZT20) in the pineal gland and gut, across all experimental groups. In the pineal gland, these patterns were significantly affected by the presence or absence of light, while patterns of expression in the gut remained unaffected by light. Similar to the results of this study, Coon et al. (1999) found that *AANAT2* mRNA expression significantly decreased in the pineal gland of pike *Esox lucius* exposed to continuous light for 24 h. Moreover, Gothilf et al. (1999) also found that *AANAT2* mRNA expression significantly increased in the pineal gland of zebrafish *Danio rerio* exposed to continuous dark for 24 h. Because we also found that continuous dark conditions resulted in increased *AANAT2* and MT-R1 mRNA expression, this suggests that the constant dark environment controls melatonin synthesis through the promotion of *AANAT2* mRNA expression, resulting in higher levels of MT-R1 expression as well. Although less well studied, our results on gut melatonin secretion patterns also have some corroboration in previous research: Vakkuri et al. (1985) reported that the secretion of gut melatonin in the pigeon was unaffected by exposure to light at midnight vs. exposure to a natural photoperiod.

In this study, goldfish subjected to ophthalmectomy had similar patterns of gut *AANAT2* and MT-R1 mRNA expression compared with those exposed to continuous light or dark. We thus infer that gut melatonin possesses a circadian rhythm unaffected by light, and therefore unaffected by the presence or absence of the eye (retina). This seems logical given that the gut cannot directly receive light signals from the retina, whereas the more closely situated pineal gland is known to do so. In other words, our study suggests that the gut can regulate its own circadian rhythm, without using light signals, through the expression of *AANAT2* and MT-1R mRNA. Further supporting this conclusion, we found that the oscillation amplitude of gut *AANAT2* and MT-R1 mRNA was maintained without reference to light, or the first- and second-day circadian cycles.

In contrast, pineal gland and plasma *AANAT2* and MT-R1 mRNA were significantly lower during the second-day cycle compared with the first-day cycle (ZT4–ZT24). This decrease over time is not light sensitive, as it occurred in all of the experimental groups (LL, DD, and OP). A similar decrease in the oscillation amplitude of *AANAT2* mRNA expression and activity was found in chickens exposed to continuous light or dark conditions (Klein et al. 1997). We suggest that this pattern occurred here because the goldfish gradually adapted to either continuous light or continuous dark.

We also determined that the daily secretion pattern of melatonin in the pineal gland, plasma, and gut were similar to the observed changes in *AANAT2* and MT-R1 mRNA expression across all experimental groups. In particular, we found no difference in melatonin concentrations between the DD and OP groups, as fish in the OP condition was effectively deprived of light signals, similar to the DD condition. Additionally, melatonin daily rhythms in the pineal gland and plasma are similar to each other, but gut melatonin rhythms observed constantly across all experimental groups (LL, DD, or OP). Our findings on gut melatonin are supported by a previous study in rats, which also observed a constant daily rhythm of gut melatonin, even after a pinealectomy (Bubenik & Brown 1997). Again, these results suggest that the gut possesses its own biorhythms for melatonin secretion and the synthesis of melatonin and *AANAT2* in the gut is governed by a circadian clock and entrained by non-photic cues.

Despite differences in light sensitivity, we found that gut and pineal gland melatonin both exhibited antioxidant function. In both organs, *AANAT2* and MT-R1 mRNA expression increased in response to thermal stress, suggesting that the oxidative stress induced by high water temperatures caused an antioxidant response in the fish. Several

studies corroborate our findings. For example, Choi et al. (2014) also found that MT-R1 mRNA expression significantly increased in goldfish as an antioxidant response to increased water temperatures. In addition, a study on trout found that high water temperature appears to regulate *AANAT2* activity, thereby increasing melatonin synthesis (Benyassi et al. 2000).

In order to study the effects of oxidative stress on goldfish under thermal stress conditions, we measured the levels of H_2O_2 in the pineal gland and gut. Significantly higher H_2O_2 levels were noted in the tissues in the thermal stress groups (30 °C) than in tissues in the control groups (22 °C). These results were in agreement with those reported by Nakano et al. (2014), in which the oxidative stress on Coho salmon *Oncorhynchus kisutch* was higher under high-temperature conditions, and as a result, the levels of plasma lipid peroxides were also high. Our results suggested that the pineal gland and gut exhibit antioxidant functions under thermal stress conditions, and that the two tissues have the similar antioxidant mechanisms.

To summarize, we found that: (1) *AANAT2* expression, MT-R1 mRNA and protein expression, and melatonin concentrations in the pineal gland and plasma are controlled by light, (2) gut melatonin differs from pineal gland melatonin, possibly because it possesses an autonomous circadian rhythm, which is not affected by light, (3) the function of melatonin as an antioxidant does not differ across photosensitive (pineal gland) and non-photosensitive tissue (gut). However, because so few studies have focused on gut melatonin, further research is required before we can fully understand the function of gut melatonin and the mechanisms involved in its secretion.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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