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Daily Rhythmic Expression Patterns of Melatonin Bio-synthesizing Genes in Zebrafish (*Danio rerio*) Testis in Response to Altered Feeding Condition

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Abstract

The alternation of light (L) and darkness (D) cycle is the most important zeitgeber ("time giver") of the circadian system. Still, feeding time also acts as a potent synchronizer of the teleost circadian system. In fish, the impact of the altered photoperiodic condition is known, but the impact of altered feeding cycles in the daily rhythm of fish circadian system is largely unknown. The objective of this work was to explore how 12 hr shift in feeding time alters expression of genes concerned with melatonin synthesizing enzymes in zebrafish testis tissue. In this study, zebrafish maintained under a 12 hr light-12 hr darkness were fed at light phase (ZT03 and ZT10) in normal feeding (NF) group and another one was the altered feeding group (AF) fed at dark phase (ZT15 and 22) for 30 days. Daily rhythms of expression of genes concerned with melatonin synthesizing enzymes and circulating melatonin level were studied. The 12 hr shift in scheduled feeding induced a phase delay of 4-5 hr in the acrophases in the case of *aanat2*, 10-11 hr for *asmt* and 4 hr for *aanat1* but a slight shift seems to exist in case of *tph1*. Serum melatonin levels showed a significant daily rhythm in both condition but displayed phase delay in AF condition. Rhythmic expression of *aanat2* and peak at midnight corresponds with the high concentration of melatonin during the night. Melatonin is a multi-potent molecule; the change in the rhythmic expression of its bio-synthesizing enzyme genes through altered feeding time may lead to desynchronization in the physiology.

Keywords: Circadian Rhythm, Feeding, Melatonin, Melatonin Biosynthesis, Testis

1. Introduction

Virtually all organisms have adapted their behaviors and functions to the daily and annual variations of the external cues. The alternation of light (L) and dark (D) cycle is the most important zeitgeber ("time giver") of the circadian system. Many rhythms in most animals, from invertebrates to vertebrates, can be entrained by other external synchronizers such as temperature, food and tides[1-4]. These environmental factors are considered as 'inputs' of the circadian system, whereas the rhythms that are generated are called 'outputs' or 'overt rhythms'. Food restriction and fasting are the most frequently used approaches to study if a rhythm is affected by the disturbing/masking effect of feeding. Periodic feeding is a strong synchronizer and is also known to synchronize many rhythms[3,5]. It is also an essential external cue for the circadian system in different vertebrates[6-9]. When food delivery is fixed at the particular time every day, fish, like other animals, display Food-Anticipatory

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Activity (FAA) under an LD cycle[10,11]. Daily variations in several metabolites and hormones in a few fish are also dependent on feeding regime[6] as the feeding time also acts as a potent synchronizer in the teleost circadian's functional organisation system. It has been suggested that alteration of regular daily feeding rhythms can induce poor performance and eventually diseases and death in fish[12].

Melatonin plays a role in the regulation of food intake, including feeding behavior and dietary selection in fish[13,14], and deficient nutritional status of fish could impair pineal melatonin synthesis through inhibiting aryl-alkyl amine-N-acetyltransferase (Aanat) activity, as has been suggested in rats[15]. It is well known that the indoleamine melatonin, acts as a neuroendocrine transducer, primarily produced by the pineal gland of vertebrates and the presence of independent melatonin-synthesizing machinery in various tissues, including the ovary of Zebrafish[16] and various extra-pineal organ of carp[17] indicates the local synthesis of melatonin. The biosynthesis is conserved in all vertebrates[18,19]. It starts with tryptophan and involves four enzymatic steps, hydroxylation (tryptophan hydroxylase-1, Tph1), decarboxylation, acetylation (aryl-alkyl amine-N-acetyltransferase, Aanat) and, finally, melatonin is formed after methylation by hydroxy indole-O-methyltransferase, [Hiomt or acetylserotonin O-methyltransferase (Asmt)][20]. The activity, protein concentration, and gene expression of Aanat ensure the nocturnal increase of the melatonin[21-23]. This molecule can synchronize various physiological functions[16, 24-26]. Melatonin plays a vital role in the regulation of testicular function including testosterone secretion[27] and mRNA expression of proteins and enzymes essential for testosterone synthesis[28,29]. Although the light:dark cycle is generally accepted as the predominant entrainer of melatonin production, daily restricted food access schedules in most mammalian species result in marked phase shifts of the circadian rhythms of many biological variables, including plasma melatonin rhythm[30]. A study on the rainbow trout (*Oncorhynchus mykiss*) provided evidence that starvation impairs daily rhythms of melatonin content in the pineal by affecting the activity of melatonin-synthesizing enzymes rather than by a deficiency in substrate[31]. Plasma levels of several metabolic products and hormones with a well-established dynamic relationship with feeding may influence the levels of melatonin[9] following synthesis and/or activation of Aanat[32] in the fish gut. Furthermore, in some species, such as sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*), rainbow trout, and channel catfish (*Ictalurus punctatus*), food access has been suggested as one of the factors entraining/shifting circadian rhythms[33,34]. Daily rhythms in expression of genes concerning melatonin biosynthesis linked under different photoperiodic conditions in the ovary of zebrafish have been described, but there is a lack of information regarding the influence of altered feeding timing on the expression of genes concerning melatonin bio-synthesizing enzymes. In zebrafish, there is no such study regarding the rhythmicity of genes concerned with melatonin bio-synthesizing enzyme in the test is under different feeding regimes.

In fact, a shift in feeding time in mammals is able to shift the phase of peripheral oscillators, e.g., liver, regardless of lighting conditions[35,36]. This study was undertaken to understand the impact of 12 hr shifted feeding schedule on the daily expression of genes concerned with expression of melatonin bio-synthesizing enzymes in the testis of zebrafish. We have also studied the change in rhythmicity, phase advance, or delay in the transcriptional profile of expression of genes associated with melatonin bio-synthesizing enzymes of zebrafish testis in standard and altered laboratory feeding conditions. Further, we have also investigated if this 12 hr shifted feeding schedule would affect circulating melatonin level in 24 hr cycle.

2. Materials and Methods

2.1 Animals and Housing

Zebrafish (*Danio rerio*), 6-7-month-old, of average body length 4.0 ± 0.3 cm and weight 0.4 ± 0.15g, belonging to the F3 generation were obtained from the IBSD Zebrafish facility centre (IBSD, Imphal, Manipur). Fish were placed in aerated, recirculated clean water in 50 Litre glass aquaria (30 fish/aquarium) equipped with a biological filter (E-Jet, P.R.C). The photoperiod was set at a 12L:12D (light:dark) cycle, when light intensity at the surface water was 300 lux (household fluorescent tube), with lights on at 06:00 am and turned off at 06:00 pm by the timer (Frontier Digital Timer, Taiwan)[37]. Water quality (pH, dissolved oxygen, hardness, and other parameters) were daily checked, and the temperature was maintained at 28 ±0.5^{0}C (100 W, RS Electrical, India)[16,38]. Fish were fed twice a day at ZT03 (09 hr) and ZT10 (16 hr) with commercial floating type flake (Perfect Companion Group Co. Ltd., Thailand). Fish

care and study schedule were maintained according to the international standards[39]. Ethical clearance was obtained from the Institutional Animals Ethical Committee constituted as per the Prescriptions of the Committee for control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Experimental Designs

The zebrafish were divided into two experimental groups depending on the feeding time: Normal Feeding (NF), feed at 09 hr (ZT03) and 16 hr (ZT10); Altered Feeding (AF), 12 hr shifting of food supply i.e. feed at 21 hr (ZT15) and 04 hr (ZT22). A total of 12 tanks were used, 6 for each feeding condition. For food delivery to each feeding treatment according to their earlier said respective time, an automatic timer feeder (Food Timer, ZW-82, Guangdong Boyu Group, China) was placed in each tank, commercial floating type flake (Perfect Companion Group Co. Ltd., Thailand) was provided. Excess food was removed without disturbing the fish from each tank to make sure that food was available according to the set respective period. After 30 days of synchronization to the two (NF and AF) feeding regime group under LD, zebrafish were anasthetized and sacrificed at every 4 hr interval in 6 time points (04.00 hr/ZT22, 08.00 hr/ZT02, 168 12.00 hr/ZT06, 16.00 hr/ZT10, 20.00 hr/ZT14 and 24.00 hr/ZT18) in a 24 h daily cycle from each feeding groups[40,41]. In each time point for both feeding treatments, the whole testis was removed from five (n=5) zebrafish and stored at -80°C in TRIzol® (Ambion, Carlsbad, CA, USA) until further studies. Blood was collected for serum melatonin for Enzyme Linked Immunosorbent Assay (ELISA). Samplings at dark were carried out in dim red light[42].

2.3 RNA Extraction and cDNA Synthesis

The testis of zebrafish was homogenized with TRIzol® Reagent (Life Technologies, USA) and total RNA was isolated according to the manufacturer's instructions. The RNA pellets were dissolved in RNase-free water (DEPC water, Sigma-Aldrich, USA). The quality and concentration of RNA were determined by using a Nano Spectra (Shimadzu, Japan). Then, to remove genomic DNA contamination, 5 µg of total RNA was treated with DNA-free™ Kit" (Ambion®RNA by Life Technologies™, USA). Further, the integrity of RNA was checked by staining 28S and 18S RNA bands with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) on 0.8% agarose gel. In the next step, 1 µg of DNase treated total RNA was retrotranscribed using "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems™, USA) according to manufacturer protocol. The cDNA synthesis was carried out in "ProFlex™ Base PCR System" (Applied Biosystems®, Inc, ABI, USA) following the PCR cycling protocol: 25˚C for 10 min, followed by 37˚C for 2 hr, 85˚C for 5 min and a final incubation at 4˚C. The total 20 µL reaction volume contained 2 µL 10 x RT Buffer, 0.8 µL 10 mM dNTP Mix, 2 µl 10 x RT primer, 1 µL MultiScribe Reverse Transcriptase (50 U/µL), 1 µL of RNase Inhibitor (20 U/µL), 10 µL DNase-treated RNA to make final volume using nuclease-free water.

2.4 Quantitative Real-Time PCR

The expression level of genes was measured by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) using Jumpstart SYBR Green/ROX qPCR Master Mix (Sigma-Aldrich, USA). Real-time PCR was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Inc, ABI, USA). Primers were used from the published data[16,43,44], and synthesized from IDT, India (Table 1). The PCR reaction condition included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s. Melting curve analysis (Tm) was performed to confirm single gene amplification by designated primers, and 2% agarose gel was used to view the endpoint PCR product. Amplification was performed in 10 µL reaction volume containing forward and reversed primers, qPCR Master Mix, and cDNA. Technical triplicates were used for each sample. The relative expression of the gene was calculated by 2[-Delta Delta C(T)] method[45] using rpl13a gene as a reference[46]. It has been demonstrated by earlier that Rpl13α is more suitable as a reference gene for zebrafish tissue analysis and same has been tested in our laboratory[46,47].

2.5 Melatonin ELISA in Serum

The blood was collected according to the published protocol[48,49] and was centrifuged at 3000 × g at 4°C for 20 min. The supernatants (serum) were collected, and melatonin levels were quantified using Fish Melatonin (MT) ELISA Kit (Gen Asia, China)[42], according to the manufacturer's instruction. Absorbance was measured at 450 nm using a Multiskan spectrum reader (Thermo

Table 1. List of Primer sequences used in Quantitative Real-time PCR (RT-PCR) analysis. F, forward; R, reverse. *Accession Number is provided by the National Centre for Biotechnology Information, Bethesda, MD, USA. The primers were taken from the published data, references have been given in the text.

Gene	Primer Sequence 5'-3'	Amplicon Size	Accession Number*
tph1a	F: ACTCTATCCCTCACACGCCT R: TGTTGTCTTCACGGGAGTCG	83	NM_178306.3
aanat1	F: CTTCCGCCAGCAAGGAAAAG R: CACGGCGCACATAAGGTAGA	80	NM_200704.1
aanat2	F: CAGGGCAAAGGCTCCATCT R: CAGGCAGACAGCGCAGGT	58	NM_131411.2
asmt	F: GACCTGTTTGAAGCCCTCTACA R: ACAGATGGTCTTGTACGGTGTC	132	NM_001114909.1
rpl13a	F: TCTGGAGGACTGTTAGAGGTATGC R: AGACGGACAATCTTGAGAGCAG	148	NM_212784.1

Fisher, USA). The concentration of melatonin was presented as pg/mL of serum.

2.6 Statistical Analysis

Changes in expression of genes and distribution of the level of melatonin during different time points in the testis and serum were analyzed by one-way ANOVA (SPSS 16.0 software; Macrovision Corporation Santa Carlo, California, USA) followed by Tukey's post hoc test to compare the difference between the time points. $p<0.05$ was considered as statistically significant. Rhythm analysis was done using COSINOR PREIODOGRAM 2015 (Boise University, USA)[49] based on cosinor rhythmometry[50]. Nonlinear regression curve was fitted in data using the formula "Y = Mesor + Amplitude Cos (Frequency X + Acrophase)" with Prism software (GraphPad; GRAPHPAD Software Inc., CA, USA).

3. Results

3.1 Circadian Rhythmicity of Melatonin Synthesizing Genes under Different Feeding Regimes (NF and AF)

In this experiment, genes of four essential enzymes, namely *tph1*, *aanat1*, *aanat2*, and *asmt* were taken for the study of their expression in the testis of zebrafish in two feeding conditions. Fish were fed exclusively during the night, which is 12 hr shift in feeding schedule from the regular feeding to examine the effect of feeding time on the melatonin bio-synthesizing gene expression. Fish of altered feeding treatments (AF) exhibited phase delay from the fish of routine feeding treatments (NF) in the daily variation of expression of melatonin synthesizing genes in the testis. From cosinor analysis, it was revealed that in NF condition (where food was given in light phase or daytime), all studied genes i.e. *tph1*, *aanat1*, and *aanat2* exhibited significant 24 hr rhythms except *asmt* (Table 2). With acrophases of *aanat2* genes at the mid-dark phase (ZT17) and at the end of the dark phase for *asmt* (ZT21) (Figures 3a, 4a). In AF condition, when food was given at dark phase, *tph1*, *aanat1*, and *aanat2* lost their significant daily rhythmicity when scheduled feeding was shifted from light phase to dark phase except for *asmt* (Figure 4b). Phase delay of 4-5 hr in the acrophases in the case of *aanat2*, 10-11 hr for *asmt* and 4hr for *aanat1* but a slight shift appears to exist in *tph1* (Figures 3b,4b,1b).

3.2 Daily Rhythms of Circulating Melatonin Level Under Different Feeding Regimes (NF and AF)

Circulating melatonin displayed a significant daily rhythm in zebrafish fed under NF with robust amplitude (94.8 ng/mL) and the acrophase during the scotophase (ZT20) (Figure 5a) which is 4 hr before lights were on. Similarly, this 24 hr rhythmicity of circulating melatonin was maintained under AF condition, but the acrophase was found at the end of scotophase (ZT23) (Figure 5b). Thus, the 12 hr shift in feeding schedule induced a phase delay in the daily rhythm of melatonin level. Moreover,

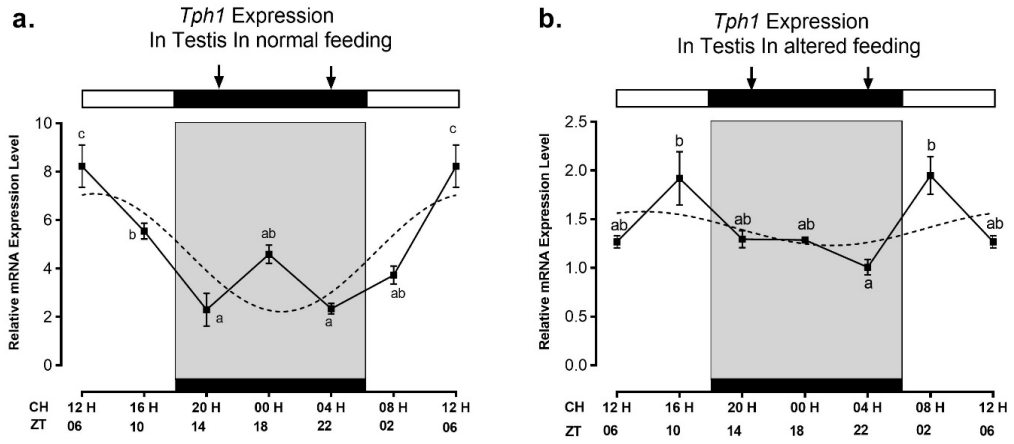

Figure 1. Daily expression profiles of *tph1* gene concerned with melatonin bio-synthesizing enzyme in **(a)** Normal feeding (NF, food was given at ZT03 and ZT10) **(b)** Altered feeding (AF, food was given at ZT15 and ZT22) in the testis of zebrafish, maintained under normal photoperiodic condition (LD). Data obtained by qRT-PCR are shown as the mean ± SEM (n=5 fish/time point) in relative units (ΔΔCt method). Different letters indicate statistically significant differences between the time points (one-way ANOVA, $p<0.05$, followed by a Tukey's post hoc test). The dashed line represents the periodic sinusoidal functions determined by cosinor analysis. The white and black bars at the top of the graphs represent light and darkness, respectively. The black arrows indicate the feeding time. CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

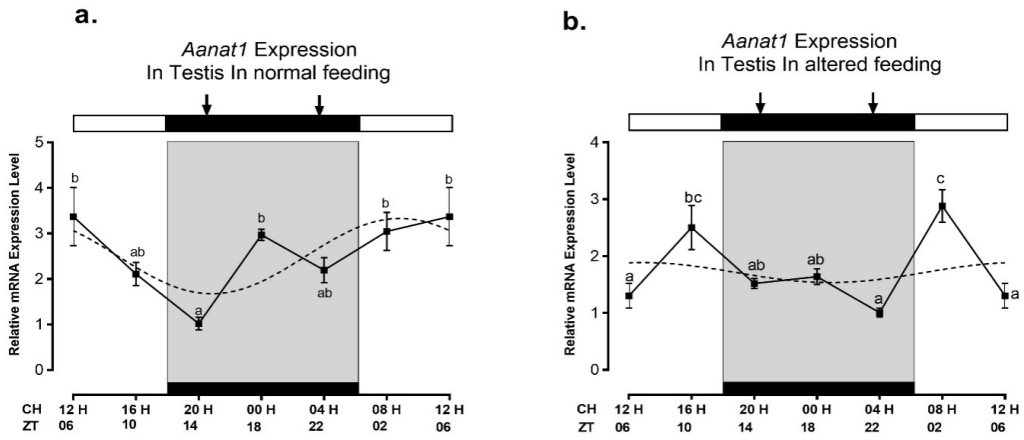

Figure 2. Daily expression profiles of *aanat1* gene concerned with melatonin bio-synthesizing enzyme in **(a)** Normal feeding (NF, food was given at ZT03 and ZT10) **(b)** Altered feeding (AF, food was given at ZT15 and ZT22) in the testis of zebrafish, maintained under normal photoperiodic condition (LD). Data obtained by qRT-PCR are shown as the mean ± SEM (n=5 fish/time point) in relative units (ΔΔCt method). Other details, as in Figure 1.

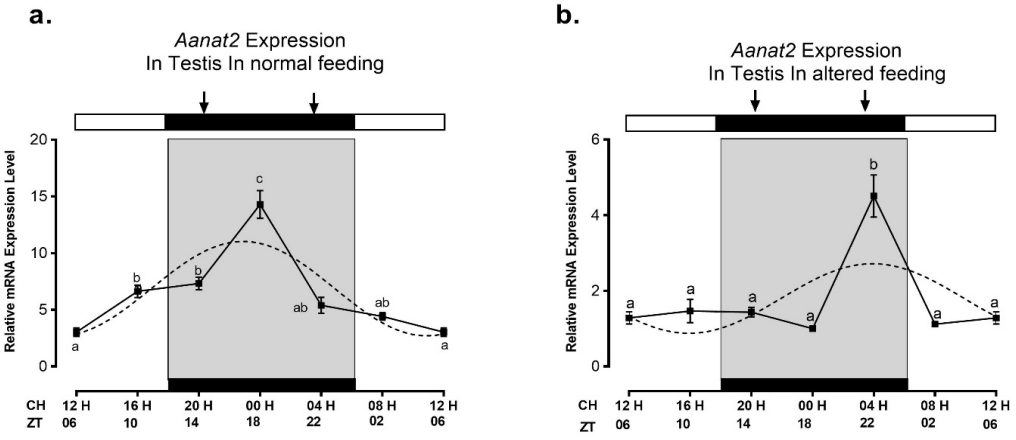

Figure 3. Daily expression profiles of *aanat1* gene concerned with melatonin bio-synthesizing enzymein **(a)** Normal feeding (NF, food was given at ZT03 and ZT10) **(b)** Altered feeding (AF, food was given at ZT15 and ZT22) in the testis of zebrafish, maintained under normal photoperiodic condition (LD). Data obtained by qRT-PCR are shown as the mean ± SEM (n=5 fish/ time point) in relative units (ΔΔCt method). Other details, as in Figure 1.

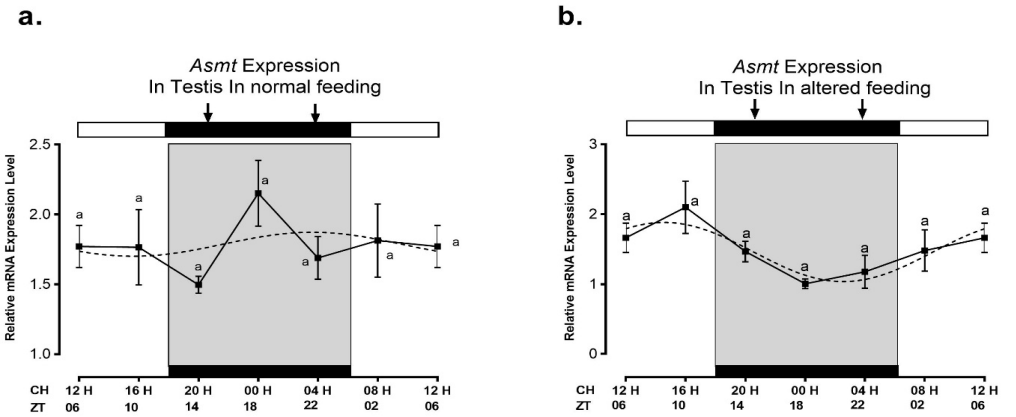

Figure 4. Daily expression profiles of *asmt1* gene concerned with melatonin bio-synthesizing enzymein **(a)** Normal feeding (NF, food was given at ZT03 and ZT10) **(b)** Altered feeding (AF, food was given at ZT15 and ZT22) in the testis of Zebrafish, maintained under normal photoperiodic condition (LD). Data obtained by qRT-PCR are shown as the mean ± SEM (n=5 fish/ time point) in relative units (ΔΔCt method). Other details, as in Figure 1.

Table 2. Cosinor analysis of genesconcerned with melatonin synthesis (*tph1, aanat1,aanat2 and asmt*) and serum melatonin under different feeding conditions in the testis of zebrafish. The table is showing the parameters defining the gene expression rhythms in testis of zebrafish with oscillation (* P < 0.05).

	Feeding conditions	Mesor	Amplitude	Acrophase	F value	*p value*	% Rhythm
Tph1	**NF**	4.45	2.04	12 h 57 min	5.97	**0.01**	44.40%
	AF	1.45	0.26	13 h 05 min	1.98	0.17	20.90%
Aanat1	**NF**	2.45	0.76	08 h 27 min	3.75	**0.04**	33.30%
	AF	1.80	0.36	12 h 24 min	1.05	0.37	12.30%
Aanat2	**NF**	6.85	4.21	22 h 54 min	12.86	**0.00**	63.20%
	AF	1.80	0.91	03 h 57 min	2.56	0.10	25.40%
Asmt	**NF**	1.75	0.09	03 h 14 min	0.27	0.76	3.60%
	AF	1.48	0.45	14 h 19 min	5.80	**0.01***	43.60%
Serum Melatonin	**NF**	304.26	94.82	02 h 16 min	14.67	**0.00***	66.20%
	AF	299.56	20.90	05 h 14 min	8.60	**0.00***	53.40%

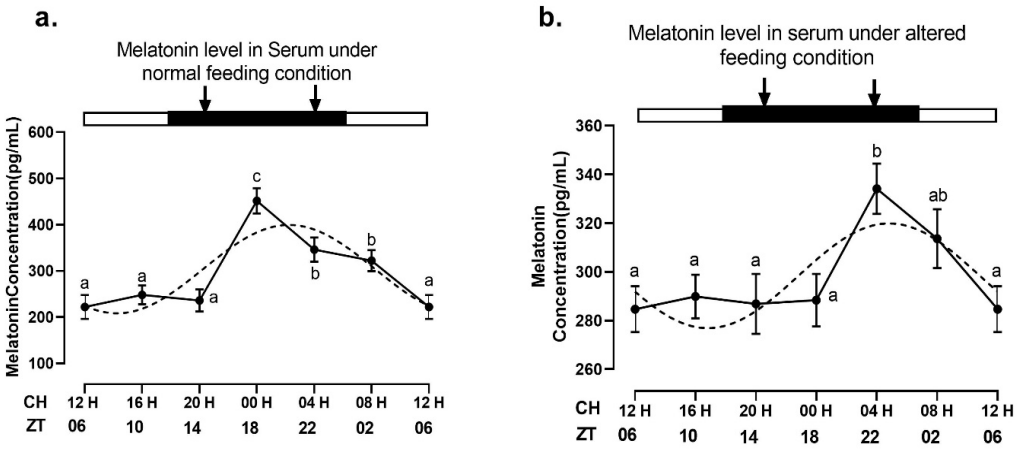

Figure 5. Daily expression profile of zebrafish serum melatonin concentration maintained under **(a)** normal feeding (NF, food was given at ZT03 and ZT10) **(b)** Altered feeding (AF, food was given at ZT15 and ZT22). Data obtained by ELISA are shown as the mean ± SEM (n=5 fish/time point) in relative units (ΔΔCt method), other details, as in Figure 1.

the fish under NF showed higher levels of melatonin (451.44 pg/mL) than observed in AF condition (334.06 pg/mL).

4. Discussion

The present study provides the first experimental data to show the effect of alteration of feeding time by a 12 hr shift on the daily profiles and rhythm features of melatonin biosynthesizing enzyme and core clock genes in the testis of zebrafish. Light entrainment and any other physical or biological factors primarily associated with the synchronization of melatonin synthesis and clock genes expression were neutralized by experimenting with ambient LD cycles [lights on at 06 hr. (ZT00) and turned off at 18 hr. (ZT12)]. Food can be a strong zeitgeber not only for circadian activity rhythms[2,51-53] but also for clock synchronization[8,35,54] in both mammals and fish. Our findings on the impact of food availability on the daily profile of genes concerned with melatonin synthesis in the testis have two consequences: i) their expression level at a particular time point in the daily cycle, and ii) their rhythmicity. The relative abundance or highest level of expression of *tph1*, *aanat2*, and *asmt* at a specific time point varied significantly with cyclic changes of food availability. In fish fed under light (NF), the acrophases of *aanat2* genes were at the mid of the dark phase (ZT17; Figure 3a) and at the end of a dark phase it was *asmt* (ZT21; Figure 4a). Moreover, the cosinor analysis of the daily expression of *tph1*, *aanat1*, and *aanat2* reveals rhythmicity,whereas *asmt* shows arrhythmicity (Table 2). This result is similar to the other peripheral organ-like ovary in zebrafish where all genes concerned with melatonin synthesis were rhythmic except *asmt* and melatonin level was high at night[16]. Our findings reveal that feeding time exerts different effects on melatonin synthesizing enzymes and clock genes expression at the peripheral level as the 12 hr shift in the feeding schedule (AF, food was given at dark phase) induced phase shift of the acrophases of the target genes. Expression of *tph1*, *aanat1*, and *aanat2* lost their significant daily rhythmicity when scheduled feeding was shifted from light phase to dark phase except for *asmt* (Figures 1b,2b,3b,4b and Table 2). The acrophase of *asmt* transcripts was opposite to the NF, i.e. it was in the light phase (ZT08), whereas *aanat2* showed phase delay, i.e. at the end of the dark phase (ZT22) (Table 2). In most mammalian species, daily restricted food access schedules result in marked phase shifts of the plasma melatonin rhythm[30]. Moreover, reduced amplitude and duration of pineal melatonin rhythms were found in food-restricted rats[55]. Our results show that the daily rhythm of genes concerned with melatonin synthesis is influenced by the time of food supply. These results support the earlier studies performed in the gut[24]. Moreover, an increase in the amplitude of nocturnal AANAT peak was found in refed fish as compared with control fed fish, which also might address a role of food availability since increased food intake naturally occurs after refeeding fasted fish[56].

Further, we tried to evaluate the level of melatonin in serum under NF conditions and compare with AF conditions. Our results demonstrate a daily melatonin rhythm in both states but induced phase delay in AF conditions (Figure 5a, b and Table 2). Similarly, our study also revealed the rhythmic expression of *Aanat2* and peak at midnight along with the high concentration of melatonin during the night indicates that the zebrafish testis might be photosensitive. The serum melatonin level under in NF condition is in support to give more emphasis on the role of *Aanat2* in maintaining melatonin rhythm in fish as proposed earlier[16,57].

Our findings indicate that feeding time can induce the displacement of the acrophases and time-lag in feeding schedule changes the pattern of expression of gened concerned with melatonin synthesis. In conclusion, the present study indicates that altered feeding time would affect melatonin synthesizing gene expression in the testis of zebrafish.

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6. Author Contributions

SDD: acquisition of data, analysis/interpretation, statistical analysis, drafting of the manuscript. GM&RKL: formatting, sampling. ZAK: critical analysis of the data,

organization of figures. HKS: planning and reviewing. AC: concept/design, manuscript preparation, and critical review of the definitive version

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8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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