Biological Rhythm Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/nbrr20

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To cite this article: Sijagurumayum Dharmajyoti Devi, Gopinath Mondal, Zeeshan Ahmad Khan, Hridip Kumar Sarma & Asamanja Chattoraj (2021): Differential gene expression and immunohistochemical localization of the key melatonin biosynthesizing enzymes in the testis of zebrafish (*Danio rerio*), Biological Rhythm Research, DOI: 10.1080/09291016.2021.1926078

To link to this article: https://doi.org/10.1080/09291016.2021.1926078

Published online: 11 May 2021.

BIOLOGICAL RHYTHM RESEARCH
https://doi.org/10.1080/09291016.2021.1926078

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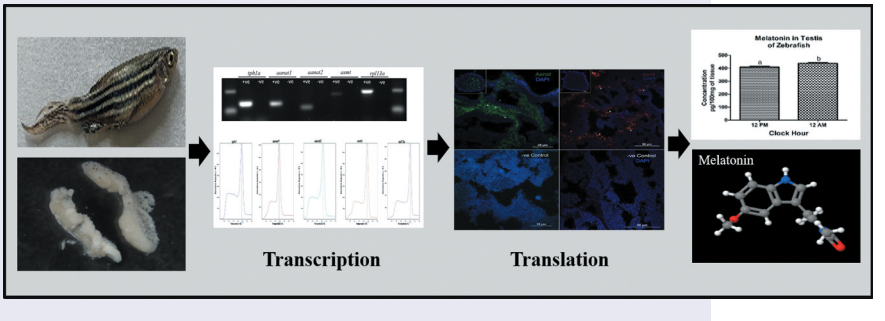
Differential gene expression and immunohistochemical localization of the key melatonin biosynthesizing enzymes in the testis of zebrafish (*Danio rerio*)

Sijagurumayum Dharmajyoti Devi[b], Gopinath Mondal[b*], Zeeshan Ahmad Khan[b*], Hridip Kumar Sarma[c] and Asamanja Chattoraj[a]

[a]Biological Rhythm Laboratory, Department of Animal Science, , Asansol, West Bengal, India; [b]Department of Biotechnology, Biological Rhythm Laboratory, Animal Resources Programme, Institute of Bioresources and Sustainable Development, Government of India, Takyelpat, Imphal, Manipur, India; [c]Department of Biotechnology, Gauhati University, Guwahati, Assam, India

ABSTRACT

This study for the first time showed the presence of melatonin biosynthesizing genes (*Tph1a, Aanat1, Aanat2 and Asmt)* in the zebrafish (*Danio rerio*) testis. Melatonin is reported to be produced mainly in the pineal organ, but there is also evidence for the presence of melatonin in several extra-pineal sites. The presence of a single gene-specific band in agarose gel electrophoresis after end-point PCR and a single peak in qRT-PCR melting curve analysis indicates that the testis of zebrafish expresses the mRNA of enzymes for melatonin biosynthesizing machinery. Furthermore, we tried to demonstrate the presence of melatonin biosynthesizing enzymes at the translational level by immunohistochemistry, which confirms the localization of both Aanat and Asmt proteins in the testis of zebrafish. Moreover, melatonin ELISA reveals a higher level in the testis than the serum at noon, but a similar concentration is observed during the midnight. Moreover, testes display a rhythmic mRNA expression of *Tph1*, *Aanat1*, and *Aanat2*, while *Asmt* is arrhythmic. These results support an almost ubiquitous biosynthesis of melatonin in the peripheral organs of zebrafish, which can be related to a local role of this hormone as an autocrine or paracrine manner.



ARTICLE HISTORY
Received 9 December 2020
Accepted 2 May 2021

KEYWORDS
Melatonin; testis; circadian rhythm; Tph; Aanat; Asmt; zebrafish

CONTACT Asamanja Chattoraj asamanja.chattoraj@gmail.com Professor Department of Animal Science, Kazi Nazrul University, Paschim Brdhaman, Asansol, West Bengal 713 340, India

*At present, working as a Postdoctoral Researcher, Bio-Clock and Aging Laboratory, Inje University, Gimhae, South Korea.

1. Introduction

Melatonin (5-methoxy-N-acetyltryptamine) is a neuroendocrine-transducing molecule that is ubiquitously distributed, functionally diverse and highly conserved (Reiter et al. 2013). The molecule is also identified as a potent regulator of circadian and circannual rhythms enabling the synchronization of physiological processes, metabolism, and behavior with observed variations under different sets of environmental conditions (Falcón et al. 2011). Moreover, melatonin is an effective antioxidant and free-radical scavenger, and its other functions include shoaling, locomotor activity, vertical migration, food intake, sedation (sleep), thermal preference, skin pigmentation, growth, reproduction, immune system and osmoregulation (Binkley et al. 1988; Ekstrom and Meissl 1997; Falcon 1999; Falcon et al. 2007, 2009, 2010; Reiter et al. 2016). Melatonin is synthesized from the amino acid tryptophan with the production of serotonin as an intermediate compound by tryptophan hydroxylase (*Tph1*). The conversion of serotonin to melatonin involves two enzymes, arylalkylamine N-acetyltransferase (*Aanat*) and acetylserotonin O-methyltransferase (*Asmt*).

In mammals, the presence of a similar homologous gene set of *Tph* is reported (Walther et al. 2003; Huang et al. 2008). The expression of these two homologous sets of *Tph* genes are tissue specific; whereby *Tph1* is exclusive to pineal organ and peripheral tissues, while *Tph2* is found in the brainstem raphe nuclei (Patel et al. 2004). In some teleosts, including zebrafish (*Danio rerio*), stickleback, and medaka, three types of *Tph* genes, namely *Tph1a, Tph1b*, and *Tph2* were reported (Lillesaar 2011). *Tph1* is mainly distributed in the pineal gland and peripheral gut, spleen, and thymus; *Tph2* is predominantly expressed in abundant neuronal tissues like the brain (Cornide-Petronio et al. 2013). Two *Asmt* genes have been reported in fish genomes, namely *Asmt* and *Asmt2* (also known as *hiomt* and *hiomt2*). *Asmt* has been identified in the brain, pineal organ, eye, retina, gastrointestinal tract, liver, muscles, gills, gonads and skin of teleost (Velarde et al. 2010; Khan et al. 2016; Muñoz-Pérez et al. 2016). It is well established that *Aanat* is the rate-limiting enzyme in the conversion of serotonin to melatonin and the daily rhythm of melatonin production is regulated by diurnal changes in *Aanat* capacity to acetylate serotonin and therefore *Aanat* has been named as "Timezyme" (Klein 2007). Incidentally, two types of *Aanat* genes (*Aanat1 and Aanat2)* have been identified in teleost fish (Begay et al. 1998; Coon et al. 1999). *Aanat1* is expressed exclusively in the retina, while *Aanat2* expression has been demonstrated in the pineal organ of several teleost species (Coon et al. 1999; Gothilf et al. 1999; Zilberman-Peled et al. 2004). Later research showed that the teleost-specific whole-genome duplication generated *Aanat1* subtypes (*Aanat1a* and *Aanat1b*). *Aanat1a* and *Aanat1b* displayed a wide and distinct distribution in the nervous system and peripheral tissues, while *Aanat2* appeared as a pineal enzyme (Paulin et al. 2015). The findings of rhythmic expression of *Aanat2* observed to be lower at midday and higher at midnight in the turbot (*Scophthalmus maximus*), zebrafish, and sole (*Solea senegalensis*) (Vuilleumier et al. 2007; Isorna et al. 2009) are in agreement with the role of *Aanat2* in timekeeping (Falcon et al. 2009). Additionally, with the use of highly sensitive antibodies raised against melatonin and by applying molecular biology tools, melatonin was also identified in other extra-pineal tissues including the gut mucosa, airway epithelium, liver, kidney, adrenals, thymus, thyroid, pancreas, ovary, carotid body, placenta, endometrium, mast cells, natural killer cells, eosinophilic leukocytes, platelets, and endothelial cells (Stefulj et al. 2001; Sanchez-Hidalgo et al. 2009).

Rhythmic production of melatonin at peripheral tissues of fish is a growing topic of interest. Following the discovery of melatonin synthesis in the pineal gland, the existence of non-pineal melatonin was related to various extra-pineal tissues, like the brain, retina, Harderian gland, gastrointestinal tract (GIT) or gut, liver, and gonads of carp (MSaHK 2015; Hasan et al. 2016). Expression of melatonin receptor proteins in different extra-pineal tissues/organs including the retina, and kidney indicated possible autocrine/paracrine functions of melatonin on the target cells (MSaHK 2015). The presence of melatonin in the ovary showed the importance of melatonin in ovarian development and maturation in mammals as well as lower vertebrates including fish (Chattoraj et al. 2005; Maitra et al. 2013; Reiter et al. 2013) as it is also known to minimize the free radical damage in the ovary by acting directly as a free radical scavenger and ultimately to improve the quality of oocytes.

The presence of melatonin biosynthesizing enzyme genes in the reproductive organs of many animal species including fish has been reported through recent advanced molecular biology techniques. The expression of mRNAs for *Aanat* and *Asmt* and their activities in protein synthesis in the ovaries (Itoh et al. 1999; Sakaguchi et al. 2013; Coelho et al. 2015; He et al. 2016) have been observed. In zebrafish, melatonin synthesizing machinery with a daily variation of melatonin in the ovary has been shown (Khan et al. 2016). In recent years, zebrafish became one of the most studied model organisms for understanding the underlying research in biology and medicine (Grunwald and Eisen 2002) and is also regarded as an ideal organism for chronobiological studies (Vatine et al. 2011). However, the information regarding the presence of melatonin biosynthesizing enzyme genes in the testis of zebrafish is lacking. In this study, we demonstrate the expression of mRNA of the melatonin biosynthesizing enzyme genes in the testis of zebrafish in a 24-hour cycle, observe day/night differences in melatonin concentration in blood serum, and testis and finally indicate tissue-specific localization of *Aanat* and *Asmt* genes by immunohistochemistry.

2. Materials and methods

A. Animals and housing

Fish care and study schedule were in agreement as per the international standards (Portaluppi et al. 2010). Ethical clearance collected from the Institutional Animals Ethical Committee constituted as per the recommendations of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Wild-type male and female zebrafish (*Danio rerio*) (2–3 months old) were obtained from North-East India and kept for 2–3 months in 50-l Glass aquaria (30 fish/aquaria) under normal photoperiod which is 12 L:12D (light: dark) for acclimatization (300 lux with standard household fluorescent tube) (Khan et al. 2018). The light was on at 06:00 am and turned off at 06:00 pm, maintained by the timer (Frontier Digital Timer, Taiwan) (Reed and Jennings 2011). The water temperature was maintained at 28 ± 0.5°C using immersion heaters (100 W, RS Electrical, India) located in each aquarium. Water was aerated and recirculated through a biological filter (E-Jet, P.R.C.). The pH, hardness, and other parameters of water were maintained under standard conditions (Westerfield 2000) at the IBSD Zebrafish facility, Imphal in Manipur, India (Khan et al. 2016). Fish were fed thrice

a day, with commercial floating type small pellets (Perfect Companion Group Co. Ltd., Thailand) in the morning (9:00 am, ZT03), midday (1:00 pm, ZT07), and live *Artemia nauplii* (Artemia cysts, Ocean Star International, USA) in the evening (5:00 pm, ZT11). The wild-type embryos were gained from pairing gravid females and males in the evening and spawning occurred in the first hour of light's on (Westerfield 2000) in the institutional zebrafish facility. These embryos were raised in the condition mentioned above and further breed to get the F2 generation. The adult male fish of 6–7 months old from F2 generation were used in all experiments when they reached a total body length of 4.5 ± 0.3 cm and body mass of 0.5 ± 0.10 g.

B. Experiment designs and sampling

The acclimatized adult male zebrafish were transferred to a new experimental tank adjusted at normal temperature (28°C) with normal photoperiod. Fish were fed thrice a day, (i) morning (9:00 am, ZT03), (ii) noon (1:00 pm, ZT07), and (iii) evening (5:00 pm, ZT11). The light was turned on at 06:00 am and off at 06:00 pm with an automated timer for normal photoperiod (LD) of 12 L-12D. The fish were kept for 2 weeks at the above-mentioned condition before sampling. The sampling time was scheduled in every 4-hour interval at six time points (ZT22, ZT02, ZT06, ZT10, ZT14, and ZT18) in a 24 h daily cycle for gene expression study in testis. During the collection of samples, adult male zebrafish were taken in 0.1% Tricane (Sigma–Aldrich, USA) solution then kept in ice for anaesthesia before they were euthanized.

At each time point, the testis from five (n = 5) fish was taken out by dissecting body cavities, then washed with PBS (pH 7.4) and quickly stored in TRIzol® (Ambion, Carlsbad, CA, USA) and frozen at −80°C before total RNA extraction. Blood was collected for serum melatonin measurement and testis tissues from three (n = 3) zebrafish were collected in [Phosphate buffer saline (PBS, Sigma–Aldrich)] for Enzyme-Linked Immunosorbent Assay (ELISA) and in [4% paraformaldehyde (PFA, Sigma–Aldrich)] for Immunohistochemistry (IHC) at two-time points (ZT06 and ZT18).

C. RNA extraction and cDNA synthesis

Total RNA was isolated from the homogenized whole testis of zebrafish with TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's instructions. Then RNA pellets were eluted in RNase-free water (DEPC water, Sigma–Aldrich, USA). RNA quality and quantity were measured by using a Nano Spectra (Shimadzu, Japan) then 5 µg of total RNA was treated with DNA-free™ Kit" (Ambion®RNA by Life Technologies™, USA) to remove genomic DNA contamination. RNA integrity was checked by staining 28S and 18S RNA bands with GelRed™Nucleic Acid Gel Stain (Biotium, USA) nucleic acid stain on 0.8% agarose gel. One microgram of DNase treated total RNA was reverse transcribed into cDNA using "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems™, USA) according to the manufacturer protocol. The cDNA synthesis was carried out in the "ProFlex™ Base PCR System" (Applied Biosystems®, Inc, ABI, USA) following the manufacturer protocol. Briefly, 20 µl reaction containing 2 µl 10X RT Buffer, 0.8 µl 10 mM dNTP Mix, 2 µl 10X RT random primer, 1 µl MultiScribe Reverse Transcriptase (50 U/µl), 1 µl of RNase Inhibitor (20 U/µl),10 µl DNase treated RNA and the final volume made up of 20 µl by

nuclease-free water. The conditions for PCR cycling for cDNA synthesis were 25°C for 10 min, followed by 37 °C for 2 hours, 85 °C for 5 min and a final incubation at 4°C.

D. Endpoint PCR

Endpoint PCR amplification was performed to confirm the presence of melatonin biosynthesizing enzyme genes in testis. PCR amplification was done with 2 µl of the earlier obtained cDNA in a total volume of 25 µl with 0.5 µl of AmpliTaq Gold® DNA Polymerase (5 U/µL) (Applied Biosystem™, USA), 2.5 µl PCR buffer II (10X) (Applied Biosystem™, USA), 0.5 µl of 10 mM dNTP Mix (Invitrogen, USA), 1.5 µl of 25 mM $Mgcl_2$ solution (Invitrogen, USA), 1 µl of each forward and reverse primer (Table 1) and the final volume made up to of 25 µl by nuclease-free water. *Rpl13a* was used as a positive control of the quality of the cDNAs obtained and similarly, a master mix without the cDNA was used for the negative control. The PCR reaction condition includes an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were separated on 2% agarose gel electrophoresis for viewing the endpoint PCR product.

E. 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). qRTPCR was carried out on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Inc, ABI, USA). Primers for this study were used from the published data (Khan et al. 2016) and synthesized from IDT, India (Table 1). The PCR reaction condition includes an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Melting curve analysis (Tm) was performed to confirm single-gene amplification by designated primers. Amplification was performed in 10 µl reaction volume containing forward and reverse primers, qPCR Master Mix, and cDNA. Technical triplicates were used for each sample. The

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

relative expression of the gene was calculated by the 2[-Delta Delta C(T)] method (Livak and Schmittgen 2001) using the Rpl13a gene as a reference (Tang et al. 2007).

F. ELISA

(a) Melatonin ELISA in serum

The blood was collected according to the published protocol (Babaei et al. 2013) and was centrifuged at 3000 × *g* at 4°C for 20 min. The supernatant (serum) was collected, and melatonin levels were quantified using Fish Melatonin (MT) ELISA Kit (Gen Asia, China) (Yumnamcha et al. 2017), according to the manufacturer's instruction. Absorbance was measured at 450 nm using a Multiskan spectrum reader (Thermo Fisher, USA). The concentration of melatonin was presented as pg per ml (pg/ml) of serum.

(b) Melatonin ELISA in testis

For melatonin quantification in the testis, 100 mg of the testis tissues were used. The tissues were sonicated and centrifuged twice at 3000 × *g* at 4°C for 20 mins until the supernatant became clear. The clear supernatant was used for the quantification of melatonin using Fish Melatonin (MT) ELISA Kit (GA-E0011FS, Gen Asia, China) (Yumnamcha et al. 2017). Absorbance was taken at 450 nm using a Multiskan spectrum reader (Thermo Fisher, USA). The concentration of melatonin in the testis was presented as pg per 100 mg of tissue.

(c) Immunohistochemistry

The presence of *Aanat* and *Asmt* in the zebrafish testis was studied by immunohistochemical staining. The above-described tissue samples for IHC were washed twice in 0.1 M PBS and immersed in PBS, containing 30% sucrose at 4°C for overnight, then embedded in Jung TISSUE FREEZING MEDIUM@ (Leica Microsystems Nussloch GmbH). Frozen testis section (8 µm) was prepared with Leica CM3050S cryostat microtome. Immunohistochemistry was conducted with some modifications as described previously. After keeping at room temperature for 15 minutes, the sections were first rinsed in PBS and then incubated in PBS containing 0.2% Triton-X100 followed by blocking in 3% BSA (dissolve in 0.1 M PBS) for 1 hour at room temperature. After overnight incubation with primary antibody rabbit polyclonal Anti-Arylalkylamine-N-acetyltransferase Antibody (1:100; GA-P6820RA, GenAsia, China) and other slides with rabbit monoclonal Anti-ASMT antibody (1:10; Ab180511, Abcam, UK). Accordingly, Alexa Fluor®488 donkey anti-rabbit IgG (H + L), (1:200, Life Technology™, USA) for Aanat and goat anti-rabbit IgG (H + L) highly cross-adsorbed Secondary Antibody, Alexa Flour™594 for Asmt (1:200, Invitrogen; USA) were used as the secondary antibody.

Then, the tissues were counterstained with 4′,6-diamidino-2-phenylindole (DAPI); (Invitrogen, USA) for nuclei staining. For negative control, a separate set of tissues was treated with secondary antibodies only. The slides were covered with Vectashield (H-1000; Vector Laboratories, Inc., Burlingame, California, USA) and observed under the Nikon A1 R HD25 confocal microscope (NIKON CORPORATION, Konan, Minato-ku, Tokyo, Japan).

G. 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 statistically significant. Rhythm analysis was done using COSINOR PREIODOGRAM 2015 (Boise University, USA) (Refinetti et al. 2007) based on cosinor rhythmometry (Nelson et al. 1979). Nonlinear regression curve was fitted in data using formula "Y = Mesor + Amplitude Cos (Frequency X + Acrophase)" with Prism software (GraphPad; GRAPHPAD Software Inc., CA, USA).

3. Result

3.1. Expression analysis of melatonin biosynthesizing enzyme genes in the whole testis by Endpoint PCR

The most widely used method for analyzing the PCR product is the use of agarose gel electrophoresis, which separates DNA products based on size and charge. It allows the determination of the presence and the size of the PCR product in the form of a band by running a predetermined set of DNA products with known sizes. RNA was isolated from the testis samples and the amplification assay was performed using gene-specific PCR. The amplified products corresponding to the *Tph1a, Aanat1, Aanat2, and Asmt* genes were obtained by running 2% agarose gel electrophoresis after completion of the endpoint PCR (Figure 1(a)). A single band was obtained in all genes using the described primers (Table 1). Moreover, there was no amplification found in the negative control (without cDNA).

3.2. Expression analyses of melatonin biosynthesizing enzyme genes in the whole testis by qRT-PCR

The ***qRT-PCR*** assay is used to further confirm the endpoint PCR results. The transcripts for the melatonin-synthesizing genes (*Tph1a, Aanat1, Aanat2, and Asmt*) obtained from the RNA isolated from the testis samples were detected by qRT-PCR. The melting curve analysis showed (Figure 1(b)) a single peak in all genes made by the primers (Table 1), no peak was observed in the – ve control. Melting peak analysis allows product identification during PCR without subsequent electrophoresis. The technique can be used to identify single-copy sequences amplified from genomic DNA or cDNA in which a single peak corresponds to one amplicon.

3.3. Immunohistological localization of melatonin synthesizing enzymes in testis

Aanat and Asmt enzyme proteins, which are involved in melatonin biosynthesizing machinery, are localized in the sections of zebrafish testis using immunohistochemistry. A detectable immunostaining signal was found for Aanat and Asmt in the testis (Figure 2). We have observed a specific green colour pigment due to binding of anti-Aanat antibody which further binds to secondary antibody labelled with green-fluorescent dye. This specified the presence of Aanat protein in the testis. Similarly, the specific red colour

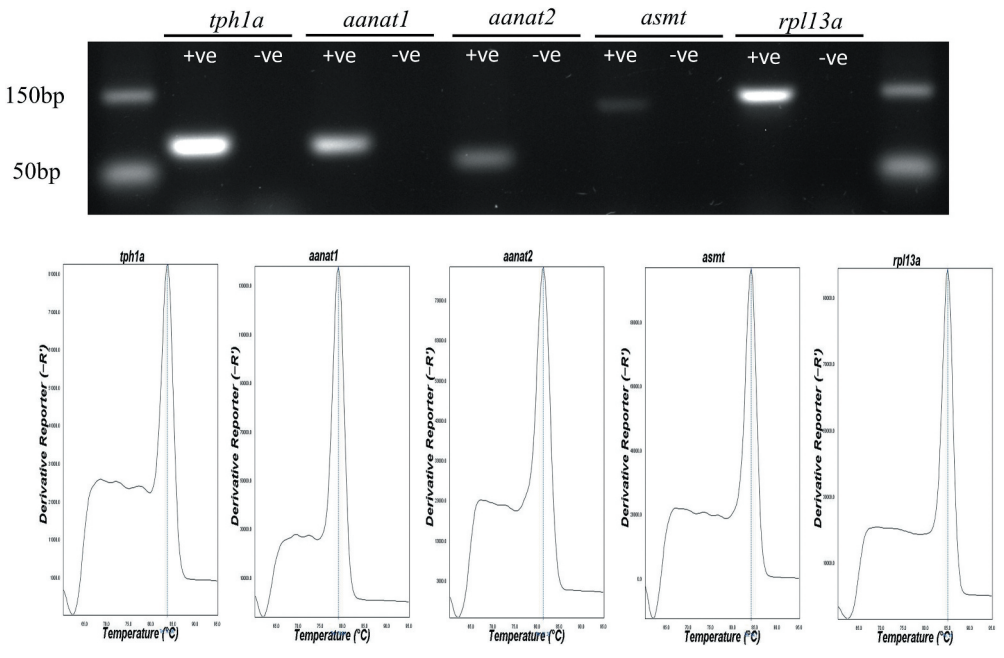


Figure 1. (a) Agarose gel electrophoresis (2%) showing the end point PCR amplified product of melatonin biosynthesizing genes obtained from the testis of zebrafish tissue. DNA-free™ Kit treated RNA was taken as template for RT-ve (as control) amplification. Representative figure from testis is displayed here. (b) Melting curve analysis of melatonin biosynthesizing (*Tph1, Aanat1, Aanat2* and *Asmt*) enzyme genes and reference gene (*Rpl13a*) are showing the accuracy of amplification of desired genes. Representative figure from testis is displayed here.

pigment was observed due to the binding of anti-Asmt antibody, which binds to the secondary antibody labelled with red colour fluorescent dye. This confirms the presence of Asmt protein in the testis of zebrafish. This indicates the presence of independent melatonin biosynthesizing machinery in the testis of zebrafish.

3.4. Daily rhythmic expression analysis of melatonin biosynthesizing enzyme genes in the whole testis

The expression levels of *Tph1a, Aanat1*, *Aanat2*, and *Asmt* in the testis of zebrafish represented a diurnal variation (Figure 3). The cosinor analysis exhibited a significant diurnal rhythmicity of *Tph1a* ($p = 0.01$), *Aanat1* ($p = 0.04$), and *Aanat2* ($p = 0.01$) in their expression under normal photoperiodic condition. However, the expression of *Asmt* ($p = 0.74$) was arrhythmic in testis (Table 2). Moreover, the acrophase of expression for *Aanat2* (ZT16) and *Asmt* (ZT21) was in the dark phase, but it is in the light phase for *Tph1* (ZT07) and *Aanat1* (ZT02) (Table 2). It is also observed that the *Aanat2* transcript recorded the maximum mesor and amplitude whereas *Asmt* was with the minimum mesor and amplitude (Table 2; Figure 3).

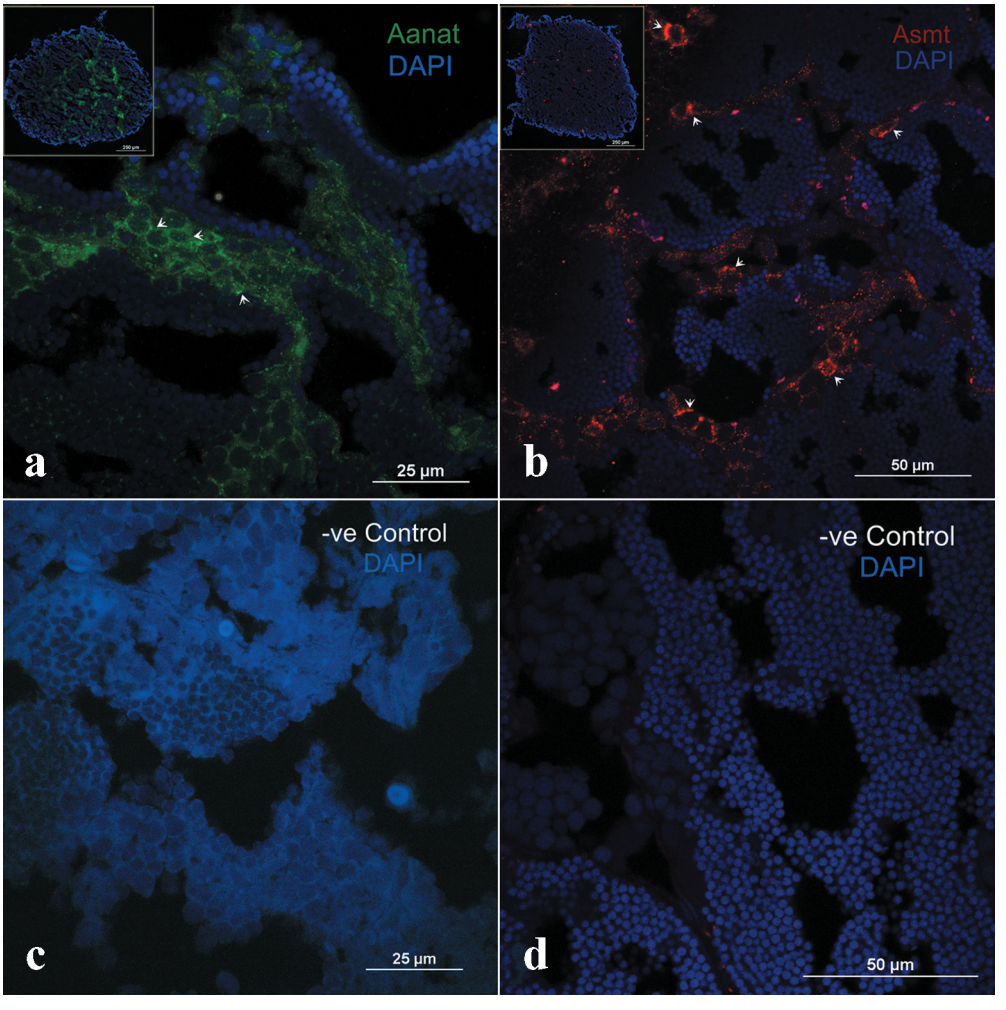


Figure 2. The section showing immunohistochemical staining. Aanat (Green) and Asmt (Red). The arrows show localization of Aanat and Asmt. A negative control for each gene lacks both immunoreactivities. These slides were labelled only with the secondary antibody. All images are merged with DAPI showing nuclei in blue. Representative images which displayed here, were taken from multiple sections of zebrafish testis.

3.5. Measurement of melatonin

1) Melatonin concentration in testis tissues

Melatonin quantification in the testis tissues was measured at two different time points (ZT06 and ZT18). The results revealed a high level of melatonin concentration at night (ZT18) (Figure 4(b)). However, the testis sample extracted during the day (ZT06) contained a low level of melatonin. These ELISA results show the presence of rhythmic synthesis of melatonin in the testis of zebrafish.

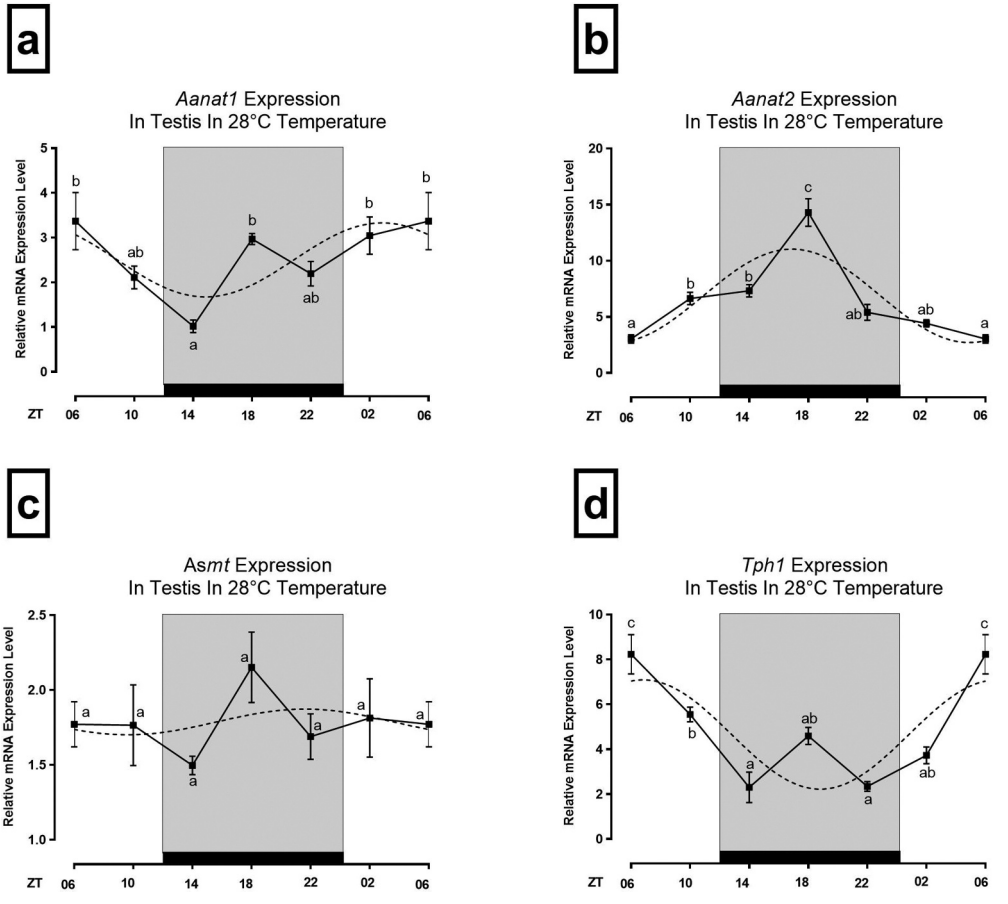


Figure 3. Transcriptional profile of melatonin synthesizing genes under LD in the whole testis of zebrafish. Expression profile of (a) *Aaant1*, (b) *Aaanat2*, (c) *Asmt and (d) Tph1*. The relative quantification values in the graph are shown as the mean ± SEM (n = 6). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($P < 0.05$). Nonlinear regression curve was fitted to all data CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

2) Melatonin concentration in serum

As expected, the samples extracted from blood serum revealed a high level of melatonin during the night as compared with the sample extracted during the daytime with a statistically significant difference between day and night (Figure 4(a)). The level of melatonin at noon (ZT06) in the serum sample was lower than the testis sample, whereas the concentration of melatonin was similar at midnight (ZT18) samples in both testis and serum. This indicates local biosynthesizing machinery in the testis of zebrafish.

4. Discussion

The present study demonstrates the presence of melatonin biosynthesizing enzyme genes (*Aanat* and *Asmt*) in the testis of Zebrafish at transcriptional as well as translational

Table 2. Cosinor analysis of melatonin genes (*Tph1, Aanat1, Aanat2 and Asmt*) in the Testis of Zebrafish (n = 5).

	Tph1	Aanat1	Aanat2	Asmt
Mesor	4.45 ± 0.40	2.45 ± 0.19	6.85 ± 0.50	1.78 ± 0.07
Amplitude	2.40 (1.315 to 3.556)	0.76 (0.2561 to 1.399)	4.21 (2.735 to 5.587)	0.09 (0.0786 to 0.1512)
Acrophase	12 h 57 min [11 h 30 min; 13 h 30 min]	08 h 27 min [7 h 15 min; 9 h 51 min]	22 h 54 min [21 h 48 min; 23 h 24 min]	03 h 14 min [2 h 90 min; 4 h 27 min]
F value	5.97	3.75	12.86	0.27
Degrees of Freedom	18	18	18	18
p value	0.01*	0.04	0.00	0.76
% Rhythm	44.40%	33.30%	63.20%	3.60%

The table is showing the parameters defining the gene expression rhythms in testis of Zebrafish with oscillation (* $P < 0.05$). The confidence interval (95%) of the amplitude values are shown inside the first brackets and the confidence interval (95%) of the acrophase values are shown inside the square brackets. Mesor are expressed as the value ± SE. Zebrafish were maintained under normal photoperiodic condition with scheduled feeding.

levels, indicating the local production of this indoleamine. Previously, our team showed the presence of independent melatonin-synthesizing machinery in various tissues, including the ovary of zebrafish (Khan et al. 2016) and various extra-pineal organs of carp (Rajiv et al. 2016), which supports the present finding in testis. Moreover, the presence of melatonin synthesizing enzyme genes has already been shown using endpoint PCR in the testis of rat (Stefulj et al. 2001), ram (Gonzalez-Arto et al. 2016), and several peripheral organs of goldfish (Velarde et al. 2010).

For the determination of the presence of Aanat and Asmt proteins, IHC was performed. Our IHC data cumulatively demonstrate that both Aanat and Asmt proteins were localized in the testis of zebrafish (Figure 2). These results are consistent with the findings of the previous studies where the presence of *Aanat* and *Asmt* was detected in rat and ram testis by IHC. However, we have not performed the localization of *Tph1* as it is not directly involved in the melatonin biosynthesizing machinery but involved in serotonin synthesis.

After confirming the presence of melatonin biosynthesizing enzyme genes at mRNA and translational level, we tried to evaluate the level of melatonin in testes and compare it with serum melatonin at noon and midnight under LD with scheduled feeding. Our results followed the classical secretion pattern of melatonin in zebrafish testis, high at midnight and low at noon. Moreover, the level of melatonin at noon in the serum sample was lower than the testis sample, whereas the concentration of melatonin in serum was comparable with the melatonin in the testis sample during the night (Figure 4). It has been reported that many extra-pineal tissues have usually higher concentrations of melatonin than plasma throughout the 24 h cycle. Similarly to our result, high melatonin concentration detected during the day in ram seminal plasma might come from the extra-pineal organs other than pineal gland (Gonzalez-Arto, Hamilton, Gallego, Gaspar-Torrubia, Aguilar, Serrano-Blesa, Abecia, Pérez-Pé, Muiño-Blanco, Cebrián-Pérez and Casao 2016, Venegas et al. 2012). Therefore, comparative analysis of melatonin in serum and testis indicates that melatonin presence in testis might be locally synthesized. The synthesis and presence of melatonin in multiple sites of the ovary and testes reflect its potential intra-, auto-, and paracrine regulation of reproductive physiology, which guarantees the quality of the egg and sperm differentiation (Acuña-Castroviejo et al. 2014).

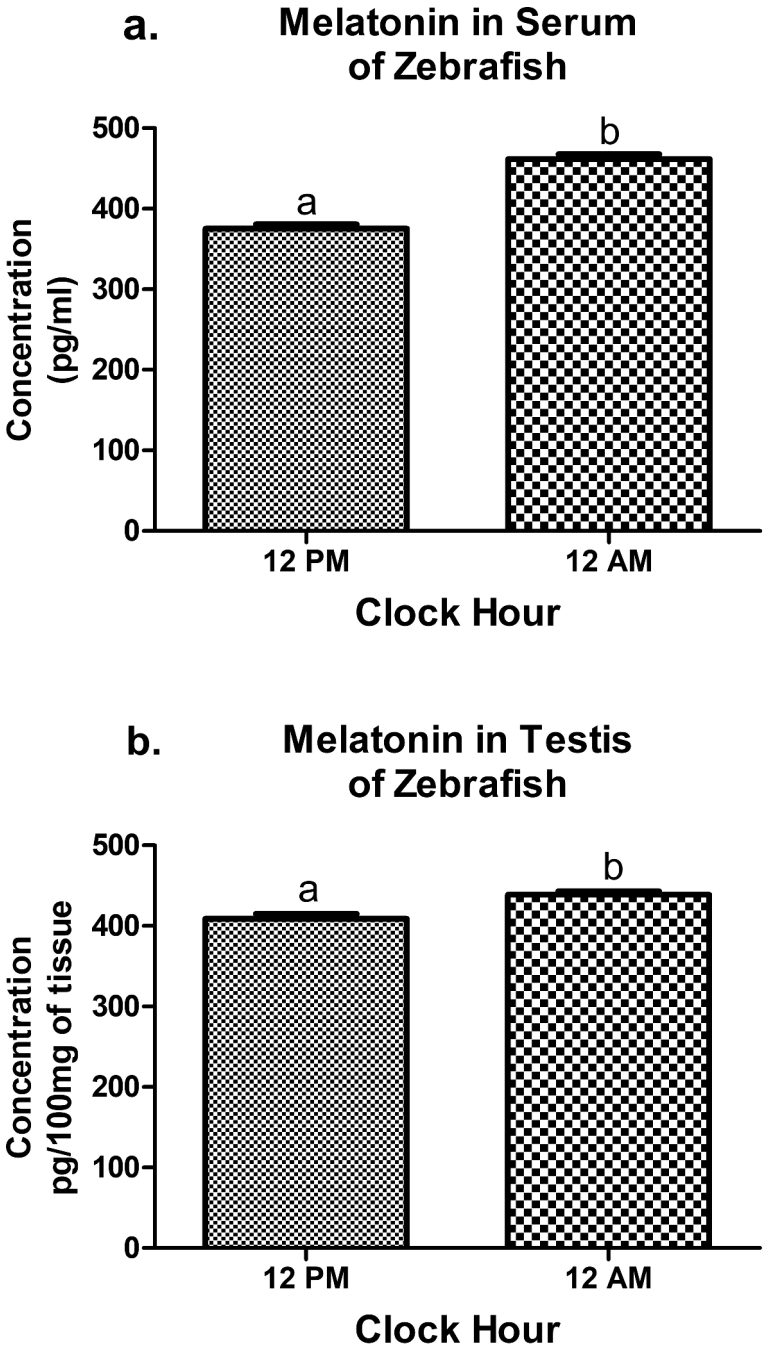


Figure 4. Level of melatonin at normal photic conditions in the blood serum and testis of zebrafish. Concentration of melatonin in normal photic conditions in the (a) blood serum and (b) testis of zebrafish (n = 6). Different letters represent a significant difference as determined by ANOVA followed by Tukey's post-hoc test ($p < 0.05$).

Further, after confirming the presence of melatonin biosynthesizing enzyme genes in testes, we have demonstrated the daily rhythmicity (4 h interval) of melatonin

biosynthesizing enzyme genes in zebrafish testis tissues in LD. One-way ANOVA of the daily rhythm of melatonin biosynthesizing enzyme genes along with cosinor analysis revealed that mRNA expression of *Tph1, Aanat1, Aanat2* are rhythmic, while *Asmt* is arrhythmic (Table 2; Figure 3). Our results on the pattern of differential expression of these genes were similar to the earlier studies performed in the ovary. The rhythmicity of *Aanat2* and its acrophase during the dark are consistent with the level of melatonin during the night. These results support the role of*Aanat2* as a rate-limiting enzyme of melatonin (Falcon et al. 2010; Khan et al. 2016). Whitmore et al. has demonstrated that every organ of zebrafish is photosensitive and generates their rhythms (Whitmore et al. 1998). 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.

Moreover, there is a growing number of evidence suggesting that melatonin plays a significant role in the maturation of gonads. Melatonin secreted in the testes could also regulate spermatogenesis by modulating the progression of germ cells to spermatozoa and enhance testosterone production by binding to its receptors in the Leydig cells (Vatine et al. 2011; González-Arto et al. 2017; Tabecka-Lonczynska et al. 2017; Deng et al. 2018). Oxidative stress is one of the most important factors contributing to poor semen quality. Intensive research has revealed that melatonin is an antioxidant molecule, which directly protects the spermatozoa from oxidative damage that could impair male fertility (Agarwal et al. 2008). Therefore, the melatonin secreted by the testes of zebrafish can have the same function as that found in higher mammals. In this scenario, zebrafish can be a good model to study the effect of different environmental cues like photoperiod, the temperature on the reproductive physiology of fish. In the context of changing environments, reproductive physiology needs to be understood properly as the oxidative damage caused by the changing environment to the gametes is an important cause of infertility.

In conclusion, our study shows that the testis of zebrafish has the machinery to synthesize melatonin, endpoint PCR, and melting curve analysis confirmed the presence of mRNA and IHC confirms the protein of melatonin biosynthesizing machinery. Furthermore, melatonin ELISA and the daily rhythm profile of melatonin biosynthesizing enzyme genes have demonstrated that testis of zebrafish might be photosensitive.

Studies in goldfish by other groups and previous studies from our lab on zebrafish and carp *catla* showed rhythmic expression of melatonin synthesizing genes and clock genes in a 24-hour cycle. However, sampling over one cycle – a single 24-hour window, is certainly not sufficient to prove the circadian rhythmicity so sampling over a 48-hour window will be needed to confirm the rhythmic expression of melatonin biosynthesizing enzyme genes.

Acknowledgments

The authors are grateful to the Directors, IBSD, India, along with all the scientists, technicians, and administrative persons for their support. The authors are appreciative of Mr. W. Rahul for his support in conducting experiments and maintenance of animals in laboratory conditions. We are also grateful to the security personnel/of the Institute for their assistance during continuous

experimentation during official holidays. Our sincere gratitude to every Indian taxpayer for their contribution towards the funding for research.

Author contributions

SDD acquisition of data, analysis/interpretation, statistical analysis, drafting of the manuscript. GM immunohistochemistry capture of image, 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

Disclosure of potential conflicts of 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.

Funding

Core grant of IBSD, Imphal, Unit of Excellence grant of Department of Biotechnology (DBT), (BT/407/NE/U-Excel/2013), Department of Science and Technology; Department of Biotechnology, Ministry of Science and Technology, India [BT/407/NE/U-Excel/2013];

ORCID

Gopinath Mondal http://orcid.org/0000-0002-9771-0767
Zeeshan Ahmad Khan http://orcid.org/0000-0002-0737-0253
Asamanja Chattoraj http://orcid.org/0000-0002-8656-7459

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