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Light, Feeding and Melatonin: An Interplay in the Appetite Regulation in the Gut of Zebrafish (*Danio rerio*)

Gopinath Mondal[1,2], Sijagurumayum Dharmajyoti Devi[1†], Zeeshan Ahmad Khan[1†], Rajendra Kumar Labala[1,3] and Asamanja Chattoraj[3*]

[1]Biological Rhythm Laboratory, Animal Resources Programme, Institute of Bioresources and Sustainable Development (Department of Biotechnology, Government of India), Takyelpat, Imphal - 795001, Manipur, India
[2]Department of Biotechnology, Gauhati University, Guwahati – 781014, Assam, India
[3]Biological Rhythm Laboratory, Department of Animal Science, Kazi Nazrul University, Paschim Bardhaman, Asansol - 713340, West Bengal, India; asamanja.chattoraj@gmail.com

Abstract

Every physiological function, including feeding and energy homeostasis is vital for animal sustainability. Melatonin is the neuroendocrine transducer of circadian photoperiod and is able to synchronize these physiological functions. The present study demonstrates the daily variation in gut melatonin and mRNA expression of appetite regulating hormone [leptin, nesfatin-1, orexin, ghrelin and ghrelin o-acetyltransferase (goat)] in relation to Gastrointestinal Somatic Index (Ga-SI) in the gut of zebrafish (*Danio rerio*), under various lighting schedule- LD (12L:12D), LL (continuous Light), and DD (continuous dark)- and scheduled feeding. The result exhibited a change in the peak level of the Ga-SI according to different photic conditions. But no change in Ga-SI was found after melatonin treatment under normal photoperiod (LD). Peak expression of anorexigenic peptide hormone genes (leptin and nesfatin-1) were found with the highest level of Ga-SI but the highest-level mRNA expression of orexigenic peptide gene hcrt was at the time of highest feeding and ghrelin with goat were after 6 hr of highest Ga-SI. These patterns changed in continuous photic conditions. This result indicates light as the critical dominant factor and, may be through melatonin, it can modulate the components of appetite regulation in this peripheral organ. This finding supports the hypothesis about the "light pollution" and its silent desynchronization of feeding behavior and related physiological functions, thereby the biodiversity and the sustainability of organisms.

Keywords: Anorexigenic, Clock, Gut, Light, Melatonin, Orexigenic

1. Introduction

Appetite regulation or balance between energy intake and expenditure is very essential for the survival of an organism. It is a complex phenomenon that involves interactions between signals from the brain and peripheral organs which show a daily pattern. Peripheral signal mainly includes satiety/hunger information (hormones) while central signals involve different neuropeptides and neurotransmitters as orexigenic or anorexigenic factors[1]. These central and peripheral signals together maintain energy balance and body weight[2]. Every vertebrate has adapted its behavior and physiological functions like feeding, growth, reproduction and osmoregulation on daily living basis[3,4]. Appetite regulation is under multifactorial control which includes different environmental synchronizers among which alternation of light and dark (photoperiod) is the main factor that

*Author for correspondence

† Equally Contributed Authors

controls the feeding. Besides, melatonin, an indoleamine, is a phototransducer or neuroendocrine transducer able to synchronize many physiological functions including feeding[5].

Melatonin is primarily produced by the pineal gland of vertebrates and its biosynthesis is conserved in all vertebrates[4,6]. Melatonin is produced rhythmically, with peaks during the darkness irrespective of the diurnal or nocturnal habit of the organism[7,8]. Although melatonin is primarily produced by the pineal, enterochromaffin cells of the mucosal membrane throughout the Gastro-Intestinal Tract (GIT) also secrete melatonin in mammals, birds, reptiles, and teleosts including goldfish[9,10]. Since the finding of melatonin production in gut of several vertebrate species and rhythmic secretion with a peak at mid-day in carp and rainbow trout gut, makes a curiosity about melatonin function in the gut of fish. Further to this, the presence of melatonin binding sites in the intestine of three different teleosts support a physiological role to gut melatonin in fish[10-14]. It has been reported that melatonin can influence the circuit for the food intake and metabolism, especially lipid metabolism at central as well as peripheral organs in zebrafish[15,16]. Moreover, after finding melatonin production by the GIT in several vertebrate species including fish, a growing number of studies have been interested in the relationship between this hormone and food intake processes[17,18].

Appetite is regulated by promoting and repressing the food intake. Two types of peptides or hormone maintain this regulation, called as anorexigenic (suppresses feeding: leptin, nesfatin-1) and orexigenic (stimulates feeding: orexin, ghrelin)[19]. Among anorexigenic peptide, leptin is a key signal for satiety. Leptin is a multifunctional hormone in fish[20] and expressed in several tissues, including liver, biliary system and intestine[21]. Leptin is known to induce decrease of food intake in goldfish[22]. An increase in the expression of *leptin* is also reported post-prandially in zebrafish[23]. Leptin inhibits food intake through upregulation of anorexigenic peptides and down regulation of orexigenic signals[24,25]. On the other hand, nesfatin-1 is a peptide synthesized from the precursor of non-esterified fatty acid or nucleobinding 2 (Nucb2) and takes part in appetite regulation or feeding[26]. In zebrafish, two isoforms of *nucb2* (*nucb2a* and *nucb2b*) exist, and both are potential precursors of nesfatin-1. The *nucb2b* is more abundant in the gut of zebrafish[27]. Food deprivation decreases the expression of *nucb2a* and *nucb2b* in the brain and gut, respectively, suggesting an anorectic effect of *nucb2b* in zebrafish[27].

Among orexigenic signals, orexin (also called hypocretins, *hcrt*) increases appetite and feeding/foraging behavior in fish[28-30] and expressed in brain and gut of fish[29,31,32]. Moreover, in fish, the peak level of expression of orexin is found before or at the time of feeding, and decreases at post-feeding[33,34]. Ghrelin is mainly produced by gastric mucosa[35-37]. It acts as an orexigenic peptide hormone and increases food intake and promotes body weight in fish, as it does in mammals[36,38]. Ghrelin-O-Acyl-Transferase (goat), also known as membrane-bound O-acyltransferase 4 (Mboat4) activates the native ghrelin peptide through acylation[39,40]. Acylated ghrelin and *goat* or *mboat4* mRNA is found to have a similar pattern of expression among mammals[41]. Goat expression is responsible for the availability of acylated ghrelin in zebrafish[36].

Changes in photoperiod can affect the circadian system which further modulates melatonin synthesis. Melatonin is involved in regulation of food intake at central as well as peripheral organs. Feeding behavior or food intake analysis in zebrafish is done by externally treating melatonin for ten days where expression of appetite-regulating peptide was demonstrated in the brain as well as in gut of zebrafish[15,16]. However, data is not available regarding the feeding under different photic conditions in zebrafish. Similarly, information on food intake, gut melatonin and transcriptional profile of the aforementioned appetite-regulatory peptides in the gut under different photic schedule is unknown in general and fish in particular. Melatonin reduces food intake, regulates energy homeostasis, modulates metabolism and prevents from obesity[42,43]. These functions performed by melatonin also involved gut through its action on the peripheral system. The use of Artificial Light At Night (ALAN) in modern society is increasing day by day and hampering melatonin production which further results in different types of metabolic disorders[44-46]. In this regard, a comparative study of the changes in the gut melatonin and daily variation of the appetite-regulating peptides related to feeding in different photic conditions are also necessary.

The present research aimed at understanding the involvement of melatonin and the expression of appetite-regulatory peptide in gut of zebrafish related to feeding, a well-accepted model for chronobiological studies under different photic conditions which are modulators of

melatonin. We have analyzed the mRNA expression level of appetite-regulating peptides or hormones along with melatonin level in the gut with the help of basic statistical methods after 48 hr of incubation at three different photic conditions (LD, LL and DD). We have also conducted the melatonin treatment experiment for short term (48 hr) incubation in LD, to observe its effect on food intake as well as appetite-regulating peptides in gut of zebrafish. This will further confirm whether the effect of photoperiod on feeding is mediate through melatonin or not in zebrafish.

2. Materials and Methods

2.1 Animals and Housing

Wild type male zebrafish (*Danio rerio;* 6-7 months old) of a total body length 4.0±0.3 cm and weight 0.4±0.15 g of the F2 generation were utilized in the current study (IBSD Zebrafish facility, IBSD, Imphal, Manipur). The fish were maintained in 50 litre glass aquaria (30 fish/aquaria) under 12 h Light:12 h Dark [12 h L: 12 h D, (300 lux; standard household LED)] at 28±0.5^{0}C using immersion heaters (100 W, RS Electrical, India). The light was on at 06:00 am and turned off at 06:00 pm, maintained by the timer (Frontier Digital Timer, Taiwan)[47]. 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 laboratory conditions[48,49]. Fish were fed thrice a day, with commercial floating type small pellets (fish meal, krill, soybean meal, flour, yeast powder, alfalfa powder, lecithin, fish oil, multivitamins and minerals) (Perfect Companion Group Co. Ltd., Thailand) in the morning (9:00 am, ZT03), and midday (1:00 pm, ZT07), and live *Artemia nauplii* (cultured from Artemia cysts, Ocean Star International, USA) in the evening (5:00 pm, ZT11). Fish care and study schedule were maintained according to the international standards[50]. Ethical clearance was obtained from the Institutional Animals Ethical Committee constituted as per the recommendations of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Experimental Design and Sampling

The zebrafish were randomly distributed into three experimental groups (60 fish/group), each group in two separate tanks (30 fish/tank): (i) regular photoperiod (12L:12D, LD); (ii) continuous light (24L, LL); and (iii) continuous dark (24D, DD)[49,51-53]. Food was given at the earlier said particular time throughout the experiment. The fish were kept for 48 hr in each experimental condition before sampling[49]. Sampling procedure for all the photic conditions was started from Clock Hour, CH48 (Day 2; ZT00) and continued upto CH72 (Day4; ZT00), with an interval of 6 hr (CH48/ZT00; CH54/ZT06; CH60/ZT12; CH66/ZT18 and CH72/ZT00)[53]. At each time point, twelve zebrafish were taken from two tanks alternatively in each group for minimal disturbance, in 0.1% Tricane (Sigma-Aldrich, USA) solution and kept on ice for anesthetization, before they were euthanized. Out of twelve fish in each time point from each experimental group, the gut from three fish were 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. Further, guts from another six fish were collected in 4% paraformaldehyde (PFA) (mass/vol) diluted in 0.1M phosphate buffer saline (PBS, pH 7.4) at 4°C for the quantification of intestinal content to derive the Gastrosomatic Index (Ga-SI)[53]. The entire intestinal content of zebrafish was considered for the quantity of feeding as they do not have any stomach[54]. The guts from the remaining three fish were collected in 0.1M phosphate buffer saline (PBS, pH 7.4) for the determination of gut melatonin by Enzyme-Linked Immuno-Sorbent Assay (ELISA)[55]. Sampling at dark was carried out in dim red light[56].

2.3 Melatonin Treatment

Two groups of male zebrafish (45 fish/group) were maintained at regular photoperiod (LD) and temperature as previously described, to observe the effect of melatonin on gut content or food intake. One group was exposed to100 nM of melatonin (Sigma Aldrich, USA) via water[15] and another group without melatonin administration (vehicle control). Fish were fed as described earlier. Treatment continued for 48 hr, and melatonin was added daily at 11.00 am (ZT05). The water of the tank was replaced exactly at interval of 24 hr (before adding the melatonin) in all experimental days. The water from the control-group was also substituted simultaneously. At the end of the study, nine fish were taken from each group at five different time points (6AM/ZT00; 12PM/ZT06; 6PM/ZT12; 12AM/ZT18; 6AM/ZT00). The gut of all the

three fishes were stored in TRIzol® (Ambion, Carlsbad, CA, USA) after washing with PBS and frozen at -80°C before total RNA extraction. Guts from remaining six fish were stored in 4% paraformaldehyde (PFA) at 4°C for the quantification of intestinal content.

2.4 RNA Extraction and cDNA Synthesis

Total RNA was isolated from the homogenized whole gut 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). The RNA quality and quantity were measured using a Nano-Spectra (Shimadzu, Japan). Then 5 µg of total RNA was treated with DNA-free™ Kit" (Ambion®RNA, Life Technologies™, USA) to remove genomic DNA contamination. The RNA integrity was checked by staining 28S and 18S RNA bands with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) on 0.8 % agarose gel. DNase treated total RNA, 1 µg, was reverse transcribed into cDNA 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 manufacturer protocol. Briefly, 20 µL reaction containing 2 µL 10xRT Buffer, 0.8 µL 10 mM dNTP Mix, 2 µL 10 x RT primer, 1 µL MultiScribe Reverse Transcriptase (50 U/µL), 1 µL RNase Inhibitor (20 U/µL), 10 µL DNase-treated RNA were taken, and final volume made up to 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 hr, 85˚C for 5 min and a final incubation at 4˚C.

2.5 Quantitative Real-Time PCR

The expression levels of genes were measured by quantitative real time-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 chosen from the published data[36,49,53,57,59], 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 the 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[60] using *rpl13a* gene as a reference[61]. It has been demonstrated by earlier that r*pl13α* is suitable as a reference gene for zebrafish tissue analysis and same has been tested in our laboratory[46,61].

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	Name of Gene	Primer Sequence 5'-3'	Amplicon Size	Accession Number*
lepa	Leptin a	F: AGCTCTCCGCTCAACCTGTA R: CAGCGGGAATCTCTGGATAA	194	NM_001128576.1
nucb2b	Nucleobindin 2b	F: TCTGTGGGCTTGTTTGGATG R: TTCTCTCTGAAATGCGGGTC	168	NM_201479.1
hcrt	Hypocretin (orexin) neuropeptide precursor	F: TCTACGAGATGCTGTGCCGAG R: CGTTTGCCAAGAGTGAGAATC	109	NM_001077392.2
ghrl	Ghrelin/obestatin prepropeptide	F:CAGAAGAGAAGCTGCTGATCCAG R:CTCCAGAAGATTCTGAAGCAC	142	NM_001083872.1
mboat4	Membrane bound O-acyltransferase domain containing 4	F:CACCCTCAGCTGTTTTACCA R:GAATCCTCCCATCGCCAAAT	120	NM_001122944.1
rpl13a	Ribosomal protein L13a	F:TCTGGAGGACTGTTAGAGGTATGC R:AGACGGACAATCTTGAGAGCAG	148	NM_212784.1

2.6 Gut Melatonin ELISA

For gut melatonin measurement, 30 mg of the gut (pooled from 3 fish) was taken from each experimental condition. Gut tissue was homogenized by sonication, followed by sequential centrifugations at 3,000 x g at 4°C for 20 min in a microcentrifuge until the supernatant became clear. The clear supernatants were carefully collected, and melatonin levels were assayed using a Fish Melatonin (MT) ELISA Kit (Gen Asia, China)[55], according to the manufacturer's instruction. Absorbance was measured at 450 nm using a Multiskan spectrum reader (Thermo Fisher, USA). The concentration of melatonin in the gut tissue is presented as pg per 100 mg (pg/100mg) of tissue.

2.7. Gastrosomatic Index (Ga-SI) Measurement

The collected GIT (in 4% PFA) were weighed (uniBloc analytical balance, Shimadzu)[53] and Ga-SI was calculated using the formula Ga-SI (%) = [Weight of gut (g) / Weight of fish (g)] x 100 (62).

2.8 Statistical Analysis

Statistical differences in the expression of genes, the quantity of gut melatonin, and gut content at different time points in every group were determined 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. The relation between the expression of genes, melatonin, and Ga-SI was analyzed by Pearson correlation at a significance level of $p< 0.05$ using SPSS 16.0 software.

3. Results

3.1 Analysis of Feeding in Different Photic Conditions (LD/LL/DD)

Changes in food intake were observed in fish during the altered photic conditions. The gut food content in terms of Gastrointestinal Somatic Index (Ga-SI) was found to be the highest at LD cycle at ZT12 (end of the light phase) and lowest at ZT00 (end of the dark period) (Figure 1a). In LL, the Ga-SI remained high throughout the day, quantitatively same as for the LD where highest Ga-SI was observed (Figure 1b). On the other hand, in DD, the Ga-SI was highest at ZT06 or start of the day and gradually decreased later during the day (Figure 1c). The Ga-SI indicated a constant high feeding in LL, but a gradual decrease was observed in DD (Figure 1b, c). The remaining food was observed in the morning for LD and DD tanks, but not in LL. These results demonstrated a rise and decline in food intake in continuous light and dark, respectively, from the normal photic state.

3.2 Gut Melatonin and Ga-SI in Different Photic Conditions (LD/LL/DD)

The gut melatonin in the regular LD cycle demonstrated a peak (Figure 2a) along with the Ga-SI. A highly significant positive coefficient of correlation between Ga-SI and gut melatonin was found in LD (Table 2A). Interestingly, a separation in the synchronization of the gut melatonin and Ga-SI was detected in LL and DD (Table 2A). Comparatively low level of melatonin was detected in the gut without any light phase peak in LL (Figure 2b) but in DD a continuously high level of melatonin was found (Figure 2c), although a higher level of melatonin was found at the start of the day in both the continuous photic conditions (Figure 2b and 2c).

Table 2A. Pearson's correlation coefficient (R) analysis of Ga-SI and gut melatonin in the gut of zebrafish in LD, LL and DD. Sign of '–' reflecting the negative correlation and without any sign is for positive correlation. ** ($p< 0.01$), * ($p< 0.05$).

Gut Melatonin			
		F-value	**P-value**
Ga-SI	LD	0.898**	0.000
	LL	-0.282	0.308
	DD	-0.260	0.349

Table 2B. Table Pearson's correlation coefficient (R) analysis of *lepa, nucb2b, hcrt* with Ga-SI in the gut of zebrafish in LD, LL and DD. Sign of '–' reflecting the negative correlation and without any sign is for positive correlation. ** ($p< 0.01$), * ($p< 0.05$).

		lepa		***nucb2b***		***hcrt***	
		F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Ga-SI	LD	0.677**	0.006	0.764**	0.001	0.808**	0.000
	LL	-0.140	0.619	0.269	0.332	0.116	0.680
	DD	-0.121	0.668	0.652**	0.008	-0.503	0.056

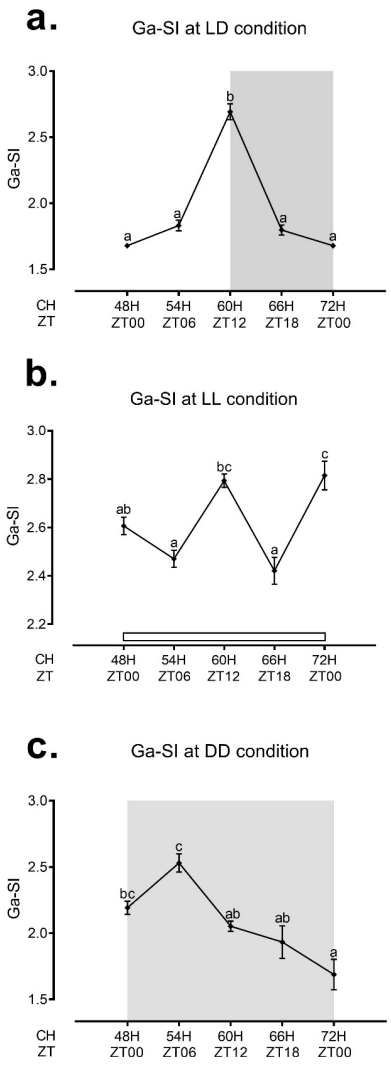

Figure 1. Ga-SI (Gastrointestinal Somatic Index) in different photic conditions (LD, LL, and DD). Ga-SI (Intestine food content relative to body mass) from 48 h to 72 h at different photoperiodic conditions **(a)** LD, **(b)** LL, and **(c)** DD. The relative quantification values in the graph are shown 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). The grey background indicates the dark phase and white background is a light phase. CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time.

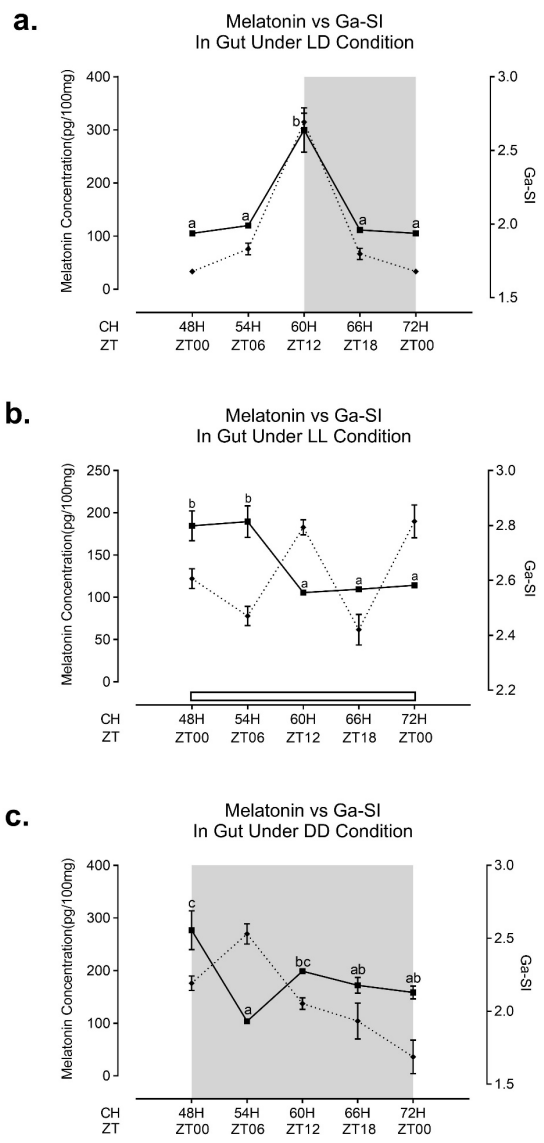

Figure 2. Level of melatonin at different photic conditions (LD, LL, and DD) in the gut of zebrafish. Melatonin profile in the gut under LD **(a)**, LL **(b)** and DD **(c)**. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference (p< 0.05). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

Table 2C. Pearson's correlation coefficient (R) analysis of *ghrl* and *mboat4* in the gut of zebrafish in LD, LL and DD. Sign of '–' reflecting the negative correlation and without any sign is for positive correlation. ** ($p< 0.01$), * ($p< 0.05$).

		mboat4	
		F-value	**P-value**
ghrl	LD	0.637*	0.011
	LL	0.979**	0.000
	DD	0.772**	0.001

3.3 Expression of Appetite-Controlling Genes in the Gut about Ga-SI Under Different Photic Conditions (LD/LL/DD)

The anorexigenic genes leptin (*lepa*), nesfatin-1 (*nucb2b*) and the orexigenic genes orexin (*hcrt*), preproghrelin (*ghrl)* and goat (*mboat4*) were considered for this study to analyze their mRNA expression concerning feeding behavior under different photic conditions in zebrafish gut. In LD condition, the highest expression of anorexigenic genes was observed when Ga-SI was the highest (at ZT12) (Figures 3a, 4a). In LL and DD this pattern of association between anorexigenic genes and Ga-SI was lost except *nucb2b* in DD where it showed a similar pattern (Figures 3b, c and 4b, c). The *hcrt* also showed the same type of expression pattern, where the peak was before or at the same time when Ga-SI was the highest (at ZT12) during the light phase (Figure 5a) in LD, and in the continuous condition the expression level was low at the start of the experiment and increased at the end of it (Figure 5b, c), but the peak of ghrl and *mboat4* were after 6 hr of the highest level of Ga-SI, which corresponds the middle of the dark period in LD (Figures 6a and 7a). However in LL the peak was at the start of the experiment, and in DD, it was at the end of the experiment (Figures 6b, c and 7b, c). Further, a significant positive coefficient of correlation between the Ga-SI and anorexigenic genes and *hcrt* was found in LD (Table 2B). Interestingly, the pattern of the relationship found in LD was abolished in LL and DD (except *nucb2b* in DD where it still showed positive correlation with Ga-SI) (Table 2B).

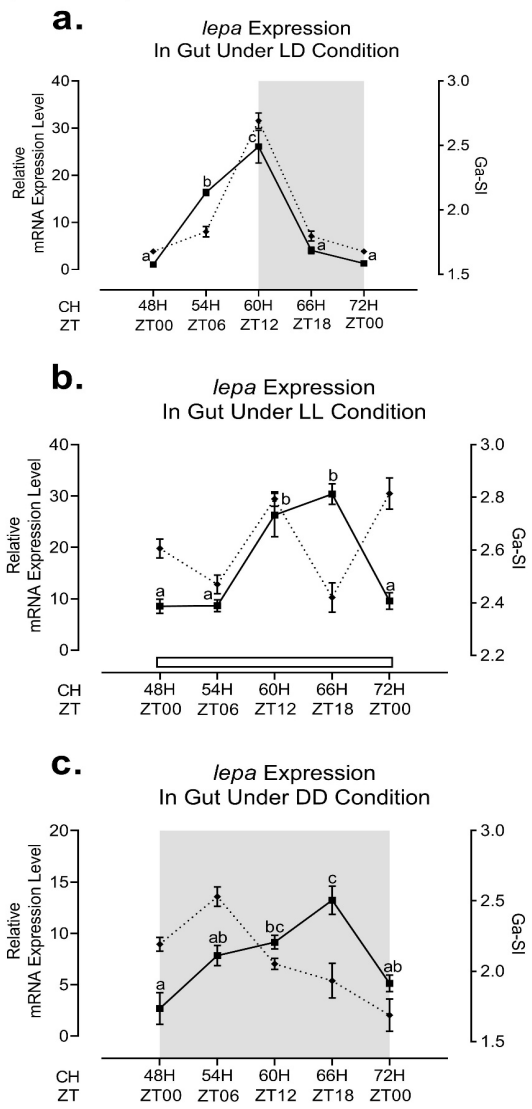

Figure 3. Transcriptional profile of leptin genes under different photoperiodic condition (LD, LL and DD) in the gut of zebrafish**.** Expression profile of *lepa* in **(a)** LD, **(b)** LL, **(c)** DD. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

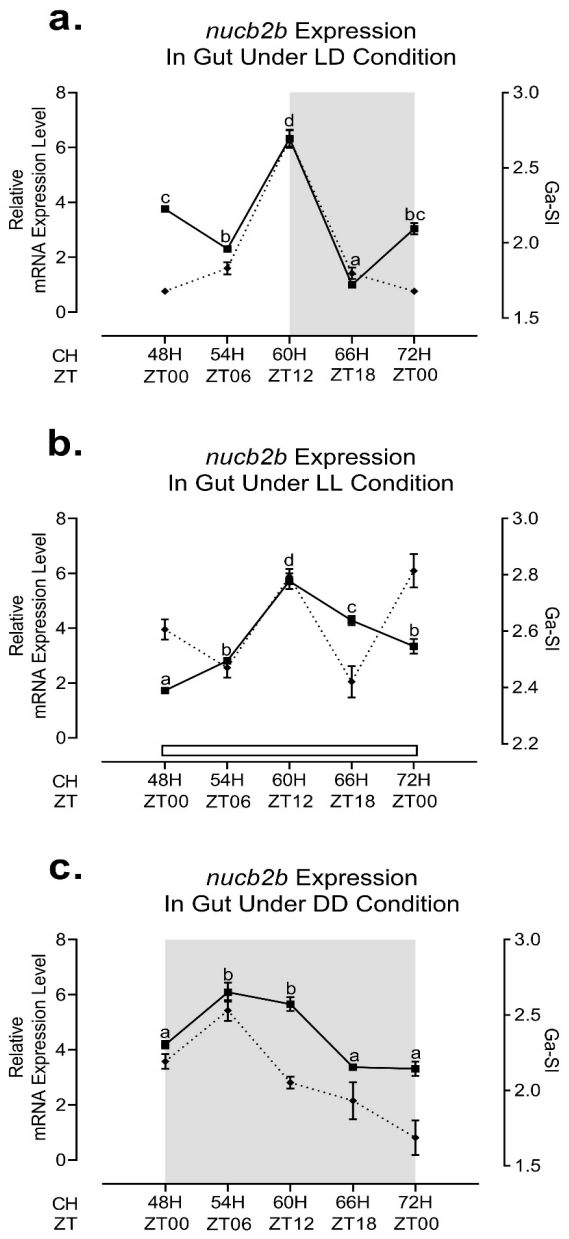

Figure 4. Transcriptional profile of nesfatin-1 genes under different photoperiodic condition (LD, LL and DD) in the gut of zebrafish**.** Expression profile of *nucb2b* in **(a)** LD, **(b)** LL, **(c)** DD. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

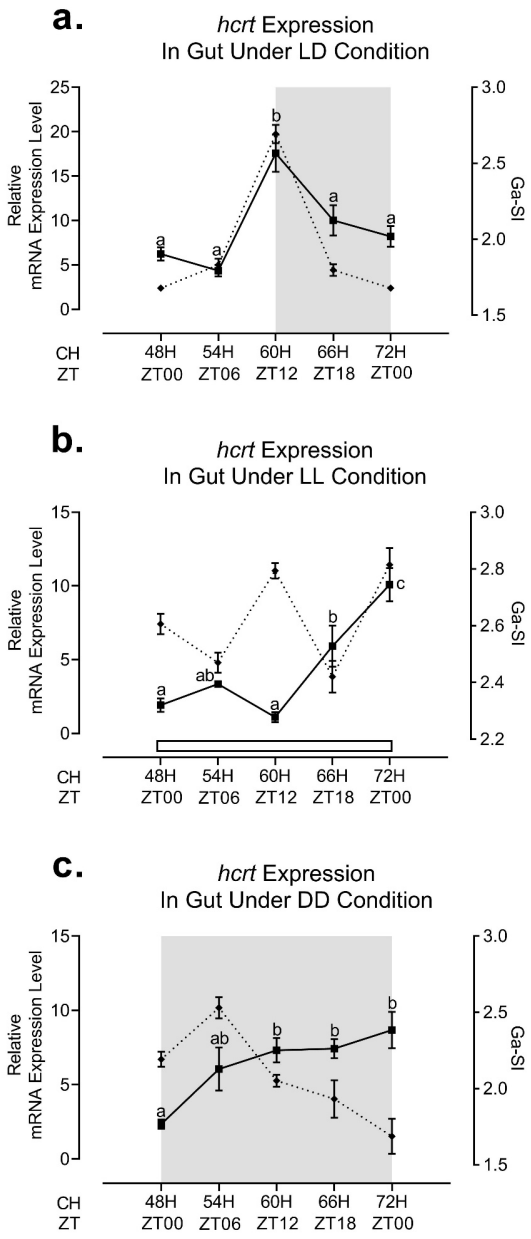

Figure 5. Transcriptional profile of orexin genes under different photoperiodic conditions (LD, LL and DD) in the gut of zebrafish. Expression profile of *hcrt* in **(a)** LD, **(b)** LL, **(c)** DD. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

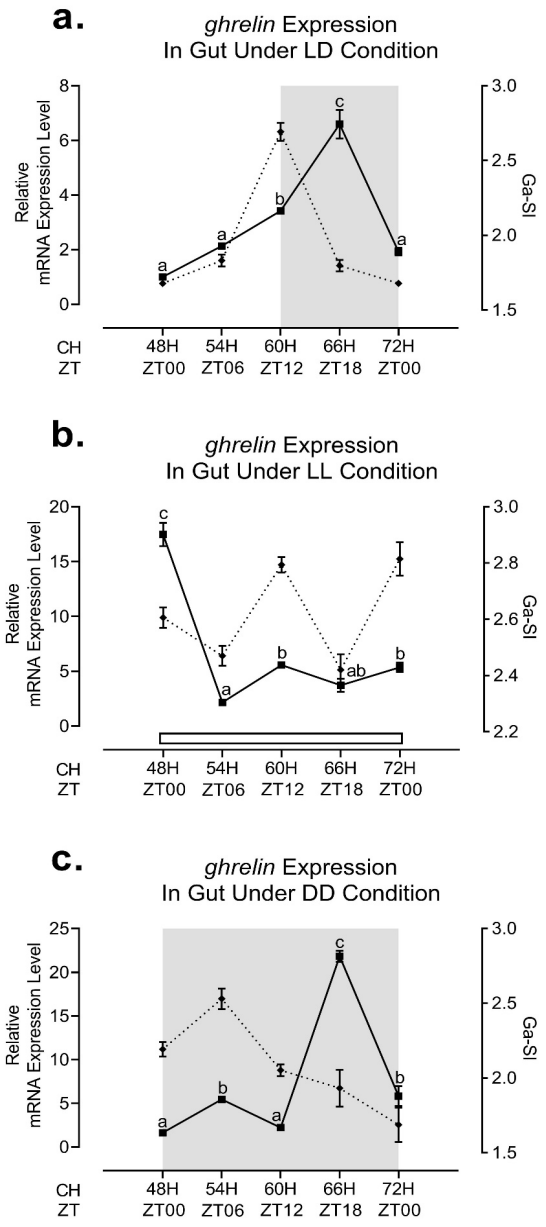

Figure 6. Transcriptional profile of preproghrelin genes under different photoperiodic condition (LD, LL and DD) in the gut of zebrafish**.** Expression profile of *ghrl* in **(a)** LD, **(b)** LL, **(c)** DD. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

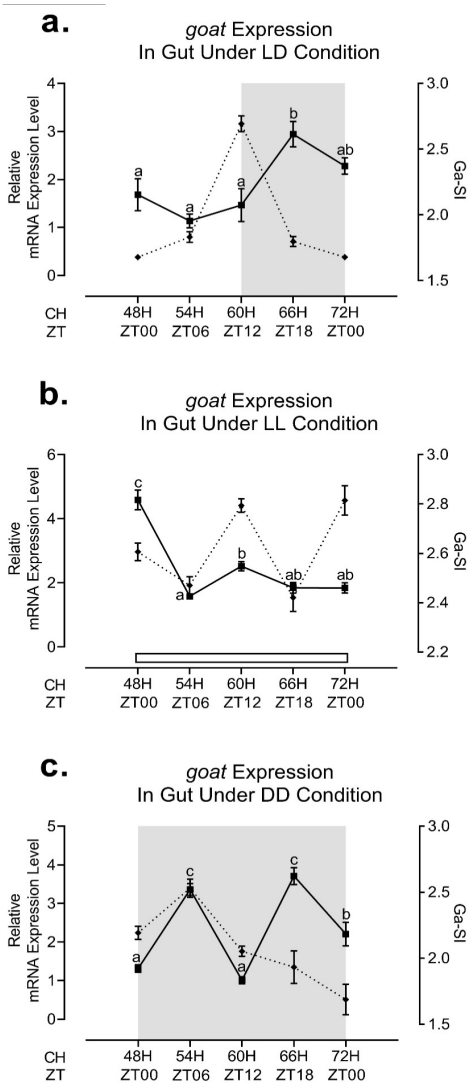

Figure 7. Transcriptional profile of goat genes under different photoperiodic condition (LD, LL and DD) in the gut of zebrafish**.** Expression profile of *mboat4* in **(a)** LD, **(b)** LL, **(c)** DD. The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value has been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

3.4 Melatonin Treatment

3.4.1 Analysis of Feeding

No significant change in Ga-SI was observed in melatonin-treated group compared to the LD group without melatonin treatment. That means, there was a high level of food intake at the end of the light phase in this treated group (Figure 8a).

3.4.2 Expression of Appetite-Controlling Genes in the Gut about Ga-SI

In the melatonin treated group under normal photoperiod, the expression pattern of appetite regulatory peptides remained the same as it was found in LD. The highest expression of *lepa, nucb2b* and *hcrt* were found at the same time when Ga-SI was high or at the end of the light phase (Figure 8b, c and d), whereas the peak expression of *ghrl* and *mboat4* were found after 6 hr of the highest Ga-SI (Figure 8e, f).

4. Discussion

In teleost, melatonin can regulate food intake and control energy homeostasis through reducing food intake[63]. Melatonin regulates these processes by activating anorexigenic and inhibiting orexigenic peptides[15]. The regulation of food intake must be governed by exogenous and endogenous factors. These factors rely on a complex neuroanatomical network between the brain and peripheral signals which allow the organisms to maintain their energy balance and body weight stability by modulating energy expenditure and energy intake[1,16]. Melatonin is involved in the vertebrate circadian clock as a neuroendocrine hormone. To that end, this hormone can synchronize many behavioral and physiological processes in relation to photoperiod. Although melatonin suppresses appetite, and has a contribution to the energy balance of vertebrates, the real role of melatonin in this process needs to be fully elucidated. In this scenario, the effect of different photoperiods on melatonin synthesis and transcriptional profile of appetite-regulating peptides with Ga-SI in the gut of any fish is scarce. This study is the first evidence of the interaction of transcriptional profile of appetite regulatory genes along with the gut melatonin concerning the feeding behavior of vertebrates in general and fish in particular under different photic conditions.

The Light Entrainable Oscillators (LEO) is known to be entrained by LD cycles, and Food-Entrainable Oscillators (FEO) are entrained by feeding-fasting cycle[64]. A few studies have focused on the connection between these two oscillators[52,65] and concluded that both LEO and FEO might be involved in the feeding entrainment. The present study indicates that light has an influence on Ga-SI or gut content. Exposure to continuous light and dark brought up a constant high and gradually low levels of Ga-SI respectively (Figure 1b, c), which indicate that light may provoke food intake in zebrafish. Study on large-mouth bass also showed that food consumption was greater under continuous light than the normal day/night cycle[66]. In mammals, increase in the photoperiod results in increase of the appetite, also reported earlier[67]. This change in feeding due to photoperiod can result in a change in the pattern of melatonin level in gut. The photic condition influences the diurnal variation and/or low level of melatonin which can affect food intake, digestive processes, and many other physiological functions in the gut of organisms[68,69]. In recent times several researchers have focused on the influence of photoperiod and melatonin on regulation of food intake in fish and mammals[69,70]. In our study we have found that the peak level of gut melatonin was found with Ga-SI, at the end of light phase and also a significant positive correlation depicted between gut melatonin and Ga-SI in LD (Figure 2a and Table 2A). A similar trend of high level melatonin was detected during the light phase or feeding period in the major carp catla[13]. In LL, a low level of melatonin was found in the gut without any peak during light phase whereas continuous high-level melatonin was observed in DD (Figure 2b, c). In our earlier publication, we have shown that keeping zebrafish in continuous light causes decrease in melatonin; increased level of melatonin was found when fish were kept in continuous dark[46,55]. This finding supports the LEO controlling melatonin synthesis, but in LD the peak level of melatonin during high Ga-SI also indicate a FEO regulating melatonin synthesis in gut of zebrafish. A detailed study is needed with regard to gut melatonin synthesis in different feeding regimes of this very popular chronobiological model.

After observation of changing in Ga-SI under different photoperiods we have checked the daily mRNA expression pattern of some appetite-regulating peptide hormones in gut of zebrafish. In this study, *lepa* expression in gut shows a daily variation with peak at the end of light phase when the Ga-SI was highest in LD

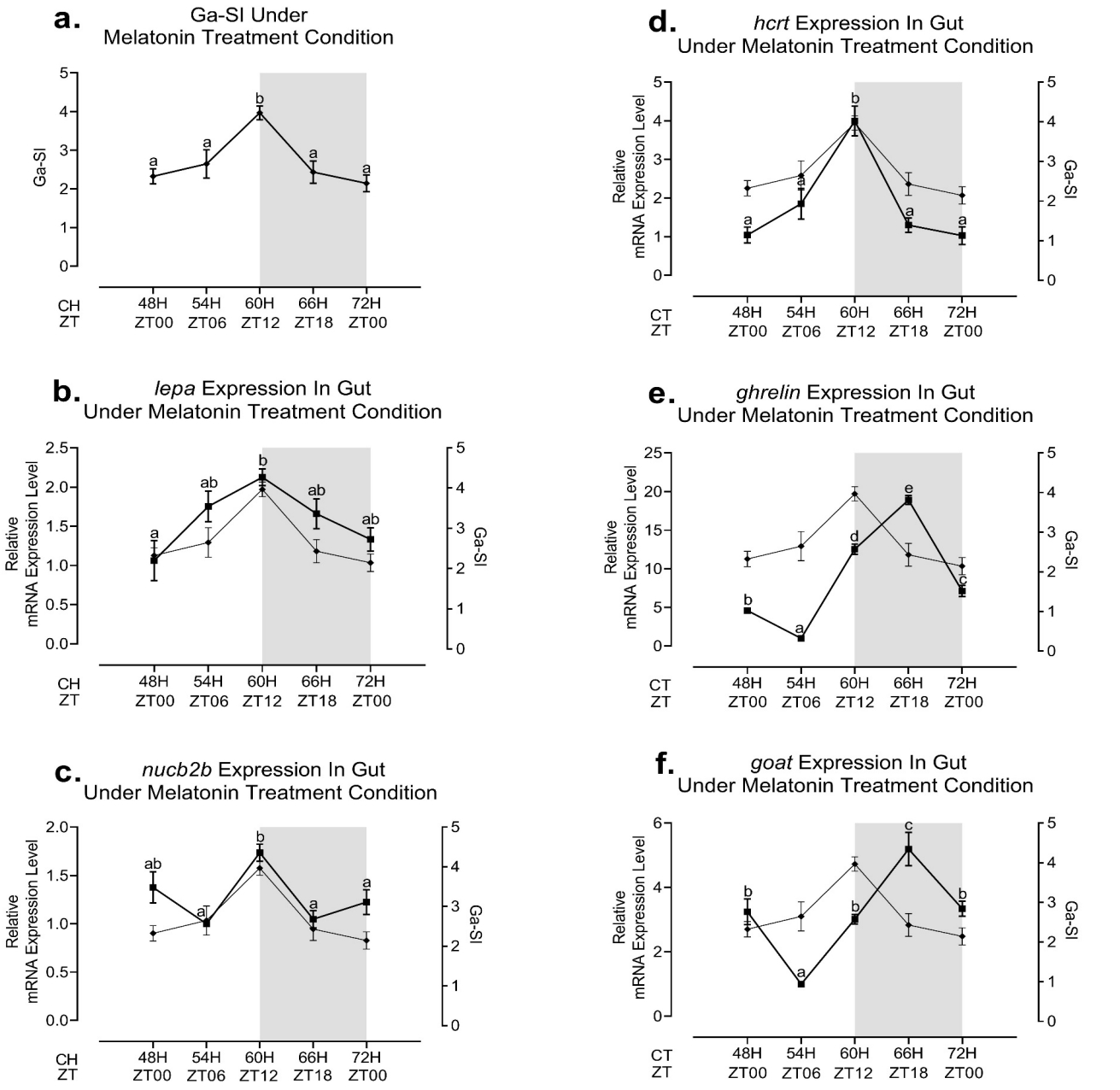

Figure 8. Ga-SI (Gastrointestinal Somatic Index) after melatonin treatment under LD. The gut food content (in term of Ga-SI) after the melatonin treatment (100nM) in zebrafish **(a).** Data are expressed as mean gut content. Results are expressed as mean ± SEM (n=6). Different letters indicate significant differences evaluated by one-way ANOVA with the Tukey post-test ($p< 0.05$). Transcriptional profile of appetite-regulating peptidegenes leptin, nesfatin-1, orexin, preproghrelin and goat under normal photoperiodic condition (LD) in the gut of zebrafish after melatonin treatment. Expression profile of **(b)** *lepa*, **(c)** *nucb2b*, (d) *hcrt*, (e) *ghrl* and (f) *goat* in the gut of zebrafish under LD after 48 hr. of incubation in melatonin treatment (100 nM). The relative quantification values in the graph are shown as the mean ± SEM (n = 3). One-way ANOVA followed by post–hoc Tukey's test of relative quantification value have been done. Group sharing common letter shows no significant difference ($p< 0.05$). The grey background indicates the dark phase and white background is a light phase. The dotted line represents intestine food content (Ga-SI). CH and ZT in the X-axis stand for Clock Hour and Zeitgeber Time, respectively.

(Figure 3a). This type of expression pattern was reported in goldfish on another peripheral organ, the liver[71]. In continuous photic condition, the expression pattern of *lepa* shows a phase shift (Figure 3b, c) as Ga-SI (increased in LL and decreased in DD) was also changed in the continuous condition where the correlation between *lepa* and Ga-SI was lost; however, a positive correlation was found in LD (Table 2B). The same type of expression pattern was found in the case of *nucb2b*, where the peak coincides with *lepa* (Figure 4a). In the gut of goldfish, the lowest level of expression in light phase and highest expression at the dark phase of *nucb2b* was reported with a daily rhythmic pattern in LD cycle[72]. Although a phase early was depicted in DD, no change was observed in LL of *nucb2b* expression pattern in the gut (Figure 4b, c). Moreover, a positive correlation between *nucb2b* and Ga-SI was found except in LL (Table 2B). *Nucb2b* shows a positive correlation with Ga-SI in the continuous dark condition, which means it can suppress the appetite in the peripheral organ where leptin doesn't engage in this type of expression in continuous dark conditions. The mRNA expression profile of these two peptide hormones in the peripheral organ of goldfish shows a rhythmic expression in LD cycle with scheduled feeding but it changed in photic manipulation or feeding changes[71,72]. This study also indicates a phase change in their expression pattern in the continuous photic condition in zebrafish gut even though the feeding was scheduled. This type of phase change in expression may be the result of disruption of the circadian system due to the changes in photic condition, which further changes the Ga-SI of zebrafish.

Orexin (orexigenic peptide, also known as hypocretins *hcrt*) acts as an orexigenic factor in both zebrafish and goldfish, found in the gut of many fishes like Atlantic cod, Orange-spotted grouper and even in human[28,30,34]. Our data in LD demonstrate that the peak expression of *hcrt* occurs before or at the time when Ga-SI is high (Figure 5a) indicating initiation of the foraging behavior for feeding. Our result supports the previous study in goldfish and orange-spotted grouper that the peak level of hypothalamic *hcrt* mRNA expression occurs prior to or at the time of scheduled meal in the active phase of fish[73] and decreases at post-feeding in cavefish[74]. This type of daily pattern was absent in continuous conditions where the expression increased and peaks were found at the end of experiments (Figure 5b, c). Finally, the positive correlation found in LD was lost in continuous conditions (Table 2B). Orexin synchronized the clock system and locomotor activity in 24 L and fasting conditions in goldfish, also it was reported that a cross-talk between orexin and feeding regulators in the central and peripheral organ is persisting[75]. In that way, any disruption of the circadian system may be responsible for the change in the expression of *hcrt* in continuous photic conditions in zebrafish gut.

Ghrelin is another major orexigenic factor in fish and is expressed in the gut which has been shown to stimulate food intake. In the present study, *ghrl* and *mboat4* mRNA expressions show daily variation, and increased after the highest Ga-SI or at the dark phase when fish was unfed. Low expression of *ghrl* and *mboat4* mRNA was observed when Ga-SI was highest during the light phase in LD (Figure 6a, 7a). The same type of daily variation with night time peak also was reported in the gut of goldfish[76]. This *ghrl* and *mboat4* mRNA expression decreased in LL (continuously taking food) and increased in DD (reduced food intake) (Figures 6b, c and 7b, c). The preproghrelin (*ghrl*) and goat (*mboat4*) mRNA expression in gut increased significantly in unfed zebrafish and decreased in fed zebrafish during regular feeding time[36]. The positive correlation found in this study between *ghrl* and *mboat4* in all photic conditions again support the acylation of preproghrelin by goat (*mboat4*) in the gut of zebrafish (Table 2C). Goat expression is responsible for the availability of acylated ghrelin in a teleost[36].

So, in this study, the phase changes or changes in daily variation in mRNA expression of the appetite-related peptide under different photic conditions in zebrafish gut may be due to disruption of the circadian system. Whether this circadian desynchronization causes changes in melatonin synthesis which further changes the Ga-SI or changes in Ga-SI causes changes in melatonin in the gut of zebrafish needs to be verified, as it is known that the circadian system and melatonin can regulate each other as well as feeding and the appetite-regulating peptide[15,43,71,76-78]. The changes in appetite regulatory peptide expression occurs due to different photic condition (because the food was scheduled) through melatonin or not in zebrafish gut is not clearly recognized. So, we set up an *in vivo* experiment of melatonin treatment in the LD cycle to confirm the effect of melatonin treatment on the expression of the mentioned appetite regulatory peptides in the gut of zebrafish. Nevertheless, no such changes were found in the expression of *lepa*, *nucb2b*, *hcrt*, *ghrl* and *mboat4* in the gut of zebrafish (Figure 8). Moreover, Ga-SI also remained unchanged in melatonin treated group,

which depicts in normal LD cycle melatonin doesn't have any role in regulating appetite-related peptides and Ga-SI.

The circadian rhythm synchronizes many processes such as food intake and metabolism to ensure the efficiency of energy homeostasis[79]. The disruption of the circadian rhythm can alter the Ga-SI and melatonin synthesis in the gut of zebrafish, as a result of which the changes in daily expression of the appetite-related peptide is shown in this study. This type of changes in Ga-SI, melatonin and appetite-regulatory peptide in the gut may be due to the disruption of circadian rhythm by photoperiod as the food was scheduled. This change in expression of the appetite-related peptide by photic conditions can be made through some tweaks in the melatonin level but in the LD cycle, melatonin itself can't change the Ga-SI and appetite regulatory peptide in the gut of zebrafish even though a daytime peak in melatonin level is reported during feeding time in the carp *Catla*[13]. So, future studies are needed regarding the detailed regulation of gut melatonin synthesis and the effect of gut melatonin on peripheral appetite signal. The circadian system plays an important role in the temporal regulation of metabolic processes, such as anabolism and catabolism, and food intake to maintain energy efficiency. The organism takes food at its respective attribute (diurnal or nocturnal); any change in the lighting schedule affects this physiological process, along with the difference in the expression of clock system, appetite regulator genes, and melatonin concentration. It is still unclear whether the disruption in circadian systems is causing the disruption in food intake patterns or vice versa but it is clear that the environmental light condition is a strong zeitgeber regulating many peripheral processes particularly in the GIT of zebrafish. Our body's energy homeostasis depends upon proper maintenance of these appetite regulatory factors. Any dysregulation of this system can cause different metabolic diseases. In this respect, zebrafish can be a good model for obesity and lifestyle disease research in chronic circadian disruption due to jet lag and shift work of modern societies.

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6. Author Contributions

GM: acquisition of data, analysis/interpretation, statistical analysis, drafting of the manuscript. ZAK: critical analysis of the data, organization of figures, preparation of the manuscript. SDD, RKL: sampling and manuscript editing. AC: concept/design, manuscript preparation, and critical review of the definitive version.

7. Conflict of Interest Statement

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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