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Daily Rhythmicity of Hepatic Rhythm, Lipid Metabolism and Immune Gene Expression of Mackerel Tuna (*Euthynnus affinis*) under Different Weather

Wenwen Wang [1,2,†], Jing Hu [1,2,†], Zhengyi Fu [1,2,3], Gang Yu [1,2] and Zhenhua Ma [1,2,3,*]

1 Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya 572018, China
2 Key Laboratory of Efficient Utilization and Processing of Marine Fishery Resources of Hainan Province, Sanya Tropical Fisheries Research Institute, Sanya 572018, China
3 College of Science and Engineering, Flinders University, Adelaide, SA 5001, Australia
* Correspondence: zhenhua.ma@scsfri.ac.cn
† These authors contributed equally to this work.

Abstract: In order to investigate the rhythmic changes in gene expression in the liver of mackerel tuna (*Euthynnus affinis*) under sunny and cloudy conditions, this experiment had four sampling times (6:00, 12:00, 18:00 and 24:00) set on sunny and cloudy days to determine the expression of their immune, metabolic and rhythmic genes. The results showed that daily rhythmicity was present within most of the rhythm genes (*CREB1*, *CLOCK*, *PER1*, *PER2*, *PER3*, *REVERBA*, *CRY2* and *BMAL1*), metabolic genes (*SIRT1* and *SREBP1*) and immune genes (*NF-kB1*, *MHC-I*, *ALT*, *IFNA3*, *ISY1*, *ARHGEF13*, *GCLM* and *GCLC*) in this study under the sunny and cloudy condition ($p < 0.05$). The expression levels of *CREB1*, *PER1*, *PER3*, *RORA*, *REVERBA*, *CRY1* and *BMAL1* within rhythm genes were significantly different ($p < 0.05$) in the same time point comparison between sunny and cloudy conditions at 6:00, 12:00, 18:00 and 24:00; metabolic genes had the expression levels of *LPL* at 6:00, 12:00, 18:00 and 24:00 in the same time point comparison ($p < 0.05$); immune genes only had significant differences in the expression levels of *IFNA3* at 6:00, 12:00, 18:00 and 24:00 ($p < 0.05$). This study has shown that rhythm, lipid metabolism and immune genes in the livers of mackerel tuna are affected by time and weather and show significant changes in expression.

Keywords: cosinor analysis; biological clock; circadian rhythm; gene expression

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1. Introduction

Due to the various changes caused by the Earth's rotation and autotransfer, all organisms have biological clocks that are autonomous endogenous timing mechanisms within the organism that regulate its adaptation to exogenous rhythmic changes in light, temperature and other environmental factors [1,2]. The daily rhythmicity of animal behavior, physiology, metabolism and immunity is controlled by biological clocks that are genetically synchronized with environmental cycles and can maintain a 24 h rhythm even in the absence of environmental cues [3]. The biorhythmic center that controls periodic changes in biological functions is called the biorhythmic pacemaker [4]. At the molecular level, biological rhythms are regulated through feedback loops formed at the level of highly conserved transcripts [5]. The biological clock consists of biological clock genes and transcription factors involved in the transcription–translation feedback loop, including *BMAL1*, *CLOCK*, period genes (*PER1*/*PER2*/*PER3*) and cryptochrome genes (*CRY1*/*CRY2*) [2,6]. These genes are not only expressed and function in the cells of the biological rhythm centers, but are also present in all tissues and cells of the organism. Therefore, transcription factors have an important role in regulating circadian rhythms [7].

The physiology, metabolism and immunity of most fish are regulated by the biological clock [8]. Additionally, the liver, the main metabolic organ for lipids, is controlled by

circadian rhythms [9]. Some studies have found that circadian rhythms exist in reptiles and birds [10,11]. Mammalian lipid metabolism also follows a circadian rhythm [12,13]. A correlation between metabolic pathways and circadian rhythms has also been found in studies of mice rhythmically oscillating [14]. Rhythmic gene expression for lipid metabolism in Atlantic bluefin tuna found in fish studies [15]. In recent years, studies have been conducted on the genetic correlation between mammalian immunity and daily rhythmicity. Studies show that mammals develop innate immune effects and daily rhythmicity during the feeding of mammals exposed to microorganisms associated with food [16]. However, there are fewer studies on the immune system and circadian rhythms in fish [17].

The mackerel tuna (*Euthynnus affinis*) is a species of tunas known as the eastern little tuna, skipjack tuna or kawakawa [18]. The production of mackerel tuna is entirely dependent on fishing, and a large proportion of mackerel tuna is 12–32 cm juveniles since the main fishing methods are seining and trawling. Although the production of mackerel tuna is still increasing, the Catch-MSY model estimates that it is currently overfished and will continue to obey the overfishing trend [19]. Therefore, from the perspective of marine resource conservation, it is necessary to carry out captive breeding of mackerel tuna. Currently, captive breeding of mackerel tuna has been reported only in Japan [18]. Our research team realized the land-based recirculating water culture of mackerel tuna [20]. Although the survival rate and better artificial domestication are guaranteed in artificial culture, there are still many problems. The situation of an unsynchronized circadian rhythm system of aquatic animals with the existing environment caused by the change of relative time under artificial culture conditions may lead to a state of stress and undesirable consequences, such as slow growth and reduction of disease resistance, which is a great obstacle to the development of artificial culture [21]. In this study, the mRNA expression levels of immune, metabolic and rhythm genes in the liver of mackerel tuna were investigated by RT-qPCR. The study aimed to elucidate the daily rhythm expression of lipid metabolism genes, immunity genes and rhythm genes in the liver of the mackerel tuna. This study is essential for this species to be cultivated in captivity to maintain its population and provides basic information to ensure the healthy, green and sustainable development of the mackerel tuna farming industry.

2. Materials and Methods

2.1. Animal

Mackerel tuna (total length: 32.38 ± 4.71 cm, weight: 1163.12 ± 284.60 g) were acclimated for more than six months in indoor ponds (8 × 5 m) with a recirculating water culture system and a natural light condition at the Tropical Aquatic Research and Development Centre, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Hainan, China. Fish were fed ad libitum once a day at 09:00 h, and miscellaneous fresh fish (4 × 2 cm pieces) were used as feed. The water quality parameters were maintained at ammonia nitrogen < 0.1 mg L^{-1}, nitrite nitrogen < 0.02 mg L^{-1}, pH 7.8, dissolved oxygen > 7.0 mg L^{-1}, and salinity 33 psu.

2.2. Sample Collection

Samples were collected in two different weather conditions, sunny and cloudy. Four sampling times (6:00, 12:00, 18:00 and 24:00) were set for one daily cycle with three parallel ponds. The three fish were taken from each parallel pond. The environmental conditions at each sampling time are shown in Table 1. Fish were deprived of feed for 24 h before the sampling was conducted. Three fish were randomly selected for sampling at each time point. Samples were collected under dim red light at night. After anesthesia with eugenol (80 mg·L^{-1}), the body length and weight were measured, and the liver tissue was quickly collected, snap-frozen in liquid nitrogen and preserved at −80 °C until use.

Table 1. Environmental indicators.

		6:00	12:00	18:00	24:00
Sunny day	Water temperature (°C)	31.8 ± 0.13	33 ± 0.21	33.6 ± 0.17	32.6 ± 0.22
	Light intensity (Lx)	2.7 ± 0.02	1116 ± 0.12	913 ± 0.04	1 ± 0.01
	DO (mg·L)	7.76 ± 0.24	7.59 ± 0.19	7.6 ± 0.2	7.61 ± 0.25
Cloudy day	Water temperature (°C)	31.6 ± 0.11	33 ± 0.16	32.9 ± 0.2	32.6 ± 0.16
	Light intensity (Lx)	1.9 ± 0.01	698 ± 0.03	192 ± 0.05	1.4 ± 0.01
	DO (mg·L)	7.62 ± 0.17	7.54 ± 0.11	7.55 ± 0.13	7.58 ± 0.09

2.3. Total RNA Extraction and cDNA Synthesis

The tissue samples were homogenized in a centrifuge tube containing 1 mL of Trizol (Invitrogen, Carlsbad, CA, USA) and extracted total tissue RNA according to the instructions. The quality and quantity of total RNA were tested using agarose gel electrophoresis and a Micro UV spectrophotometer (Biotec Biotechnology Co., Ltd., Beijing, China). Reverse transcription was performed on 1 μg of total RNA using an EasyScript® All-in-One First-Strand cDNA Synthesis SuperMix (All-Strand Biotechnology Co., Ltd., Beijing, China).

2.4. Real-Time qPCR Analysis

Primers (Table 2) were designed by Primer Premier 5 [14] software based on the gene sequence of the mackerel tuna genome (Non-public data). GAPDH was used as the housekeeping gene. The qPCR was conducted with the Real-time qPCR analysis (Analytik Jena GmbH, Jena, Germany) using SYBR Green (Tiangen Biotech Co., Ltd., Beijing, China). The 20 μL of reaction, including 10 μL of 2× RealUniversal PreMix, 0.6 μL each of forward and reverse primers (10 μM), 2 μL of cDNA template and 6.8 μL of RNase-free ddH2O. Reaction conditions were: (1) Pre-denaturation at 95 °C for 15 min; (2) Amplification reaction, denaturation at 95 °C for 10 s, annealing at 56 °C for 20 s, extension at 72 °C for 30 s and 40 cycles. There were three repetitions of each test. The dissociation curves were analyzed to ensure only specific products were obtained with no formation of primer dimers in each reaction. At the end of the reaction, the relative expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method [16]. The reaction efficiency was 90–110%, and Pearson's coefficients of determination (R^2) were >0.97.

Table 2. Relevant primer information.

Gene	Full Names of Target Genes	Primer Sequence (5′-3′)	Amplicon Size/bp
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACACTCACTCCTCCATCTTTG	100
		R: TTGCTGTAGCCGAACTCAT	
TRIM35	Tripartite motif-containing 35	F: GTCTGAAGAGCTGGTGGA	85
		R: TTACGAGGTGGTTTGTCC	
NF-kB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	F: CCCAAAGACTCCAGCATCA	117
		R: GCAGTTGTATCCCATCCTCAA	
MHC-I	Major histocompatibility complex 1	F: GCCCTCCTGCTCCTTCTT	83
		R: GGTTTGCCTCCTCCAATT	
ALT	Alanine aminotransferase	F: CAGGCTTACGGAGCAAAT	100
		R: TCGTGGTGGGATGAAGAT	
IFNA3	Interferon a3	F: GGTCTGCGTCCCTGTATT	102
		R: AGCACTGTGACCCATTCG	
ISY1	Splicing factor ISY1 homolog	F: GTTCGGATCAAAGAGTTGGG	90
		R: CAGACTGGCTGGTGTATAATGG	
ARHGEF13	A-kinase anchor protein 13	F: CCGTCAACTTCTACAAGGA	85
		R: CGCACAATGCTGCTACTC	

Table 2. *Cont.*

Gene	Full Names of Target Genes	Primer Sequence (5′-3′)	Amplicon Size/bp
SIRT1	Sirtuin 1	F: AGAAGAGGCTGCGGAAGT	99
		R: AGGCGTTTGCTGATTGGA	
GCLM	Glutamate cysteine ligase modifier subunit	F: CTGAGCGACTGGTCTTCC	93
		R: CGTGATAGCGTCTGTTGG	
GCLC	Glutamate-cysteine ligase catalytic subunit	F: CTGTTGAGAAGGGAGTGTC	87
		R: TGTTTCTGGTAAGGGTGC	
GST	Glutathione s-transferase	F: CGCCAAGAAGAACAACAT	117
		R: TCTCGAAGAGCAGGGACT	
LPL	Lipoprotein lipase	F: AGGATGCGACATACAGAACA	112
		R: GAAGAGGTGGATGGAACG	
SREBP1	Sterol regulatory element binding transcription factor 1	F: GACTGACTTGACCGTGTTC	112
		R: CTCCTCCTCTTGTTCATCCT	
CREB1	cAMP responsive element binding protein 1	F: TGCCCACTCCCATCTATC	93
		R: CTCCATCTGTGCCGTTATT	
CLOCK	Clock circadian regulator	F: TGTGGACGACCTGGAGAC	84
		R: AGGAAACGGTAGTAGCAAG	
PER1	Period 1	F: CCAAAGGCGGTTCAGTTA	144
		R: GAGGCTTCTTGTCTCCCAC	
PER2	Period 2	F: TCTAATGGAGTCGTCAGGGAG	119
		R: AGCCGCTGGTTGAAGGAT	
PER3	Period 3	F: TCATCGGACGGCATAAAG	85
		R: TGGGTGACTGGGAAATACTC	
RORA	Homo sapiens RAR-related orphan receptor A	F: CTGGATAGGGTGGGTGGAA	84
		R: CGTTGGCCCGGATTAGAG	
REV-ERBA	Nuclear receptor subfamily 1 group D member 1	F: CCTACAACCATCCCACAG	88
		R: ACCTTACATAGAAGCACCATA	
CRY1	Cryptochrome 1	F: GTGGGCAGCCTCCTCTTA	145
		R: CCGTACTTGTCTCCGTGGTC	
CRY2	Cryptochrome 2	F: CTACATGAAGCTCCGTAAGC	108
		R: CGGTCAAAGTTTGGGTTG	
BMAL1	Brain and muscle Arnt-like 1	F: CGTCCAGTGGTAATGTCA	176
		R: CATGAGTGCTTCTCCTCC	

2.5. Statistical Analysis

All data were expressed as mean ± standard deviation. The data were analyzed by SPSS 26.0 statistical software and plotted by Origin2021. The test data all conformed to Shapiro–Wilk and Chi-squared tests. A two-way ANOVA was used to test the interaction effects of different weather and times of the day and was performed using SPSS software. A one-way ANOVA was used for multiple comparisons between different sample time points on the same day, and an independent sample *t*-test was used to analyze the significant differences between the sunny and cloudy days at the same time points. The significant difference level was set as $p < 0.05$. Data were then fitted to a cosine wave to determine the presence of a significant daily rhythm. Raw data were analyzed using Acro circadian analysis programs (University of South Carolina, USA; http://www.circadian.org/softwar.html; accessed on 7 October 2022).

3. Results

3.1. Changes in Gene Expression Levels in the Mackerel Tuna Rhythm

Under the sunny condition, the expression levels of *CREB1*, *CLOCK*, *RORA*, *PER1*, *PER3* and *CRY1* were not significantly rhythmic in the liver (Table 3). The expression levels of *CREB1* and *CLOCK* were significantly higher at 18:00 than at the other time points ($p < 0.05$). The expression levels of *RORA*, *PER1* and *PER3* were significantly higher at 24:00 than at the other time points ($p < 0.05$; Figure 1a–f). The expression level of *CRY1* was significantly lower at 6:00 than at the remaining time points ($p < 0.05$, Figure 1g). The expression levels of *PER2*, *CRY2*, *REVERBA* and *BMAL1* were significantly rhythmic in the liver under the sunny condition (Table 3). The expression levels of *PER2* and *CRY2* were significantly higher at 24:00 than at the other time points ($p < 0.05$; Figure 1e,h; Table 3). The expression levels of *REVERBA* were significantly higher at 18:00 than at the other time points < 0.05; Figure 1i; Table 3). The expression levels of *BMAL1* were significantly higher at 24:00 than at the other time points ($p < 0.05$; Figure 1h; Table 3).

Table 3. Cosinor analysis board for rhythm gene expression under sunny and cloudy conditions.

Gene	Weather	Acro (*p*-Value)	Acrophase
CREB1	Sunny day	n.s.	
	Cloudy day	<0.01	12 ± 1.16
CLOCK	Sunny day	n.s.	
	Cloudy day	<0.005	6 ± 0.92
PER1	Sunny day	n.s.	
	Cloudy day	<0.001	12 ± 0.79
PER2	Sunny day	<0.001	0 ± 0.61
	Cloudy day	n.s.	
PER3	Sunny day	n.s.	
	Cloudy day	<0.05	0 ± 1.89
RORA	Sunny day	n.s.	
	Cloudy day	n.s.	
REVERBA	Sunny day	<0.001	18 ± 0.43
	Cloudy day	<0.001	12 ± 0.85
CRY1	Sunny day	n.s.	
	Cloudy day	n.s.	
CRY2	Sunny day	<0.001	0 ± 0.83
	Cloudy day	n.s.	
BMAL1	Sunny day	<0.001	12 ± 0.57
	Cloudy day	<0.005	6 ± 0.81

n.s. denotes statistical differences between the sampling points. Acrophases (circadian peak times) were calculated by a non-linear regression fit of a cosine function. Data are expressed as acrophase ± 95% confidence intervals.

Under the cloudy condition, the expression levels of *RORA*, *PER2*, *CRY1* and *CRY2* were not significantly rhythmic in the liver (Table 3). The expression levels of *CREB1*, *PER1*, *PER3* and *REVERBA* were significantly higher at 12:00 than the other three time points under the cloudy condition ($p < 0.05$). The expression levels of *CLOCK* and *BMAL1* were significantly higher at 6:00 than at the other time points ($p < 0.05$; Figure 1a,b,d,f,i,j; Table 3). The expression levels of *RORA*, *PER2*, *CRY1* and *CRY2* were significantly rhythmic in the liver under cloudy conditions (Table 3). The expression levels of *RORA*, *PER2* and *CRY2* were significantly higher at 12:00 than at the other three time points ($p < 0.05$). The expression level of *CRY1* was significantly higher at 6:00 than at the other three time points ($p < 0.05$; Figure 1c,e,g,h; Table 3).

A comparison of rhythm genes' expression levels at the same time point under different weather (sunny versus cloudy days) is shown in Figure 1. Expression levels of *CREB1*, *RORA*, *PER1*, *PER3*, *CRY1*, *REVERBA* and *BMAL1* at all the time points were significantly different between sunny and cloudy days at the same time point ($p < 0.05$; Figure 1a,c,d,f,g,i,j). Expression levels of *CLOCK* at 6:00, 18:00 and 24:00 were significantly different between sunny and cloudy days at the same time point ($p < 0.05$; Figure 1b).

The expression levels of *PER2* at 6:00, 12:00 and *PER2* at 6:00, 12:00 and 24:00 were significantly different between sunny and cloudy days at the same time point ($p < 0.05$; Figure 1e). The expression levels of *CRY2* at 12:00, 18:00 and 24:00 were significantly different between sunny and cloudy days at the same time point ($p < 0.05$; Figure 1h).

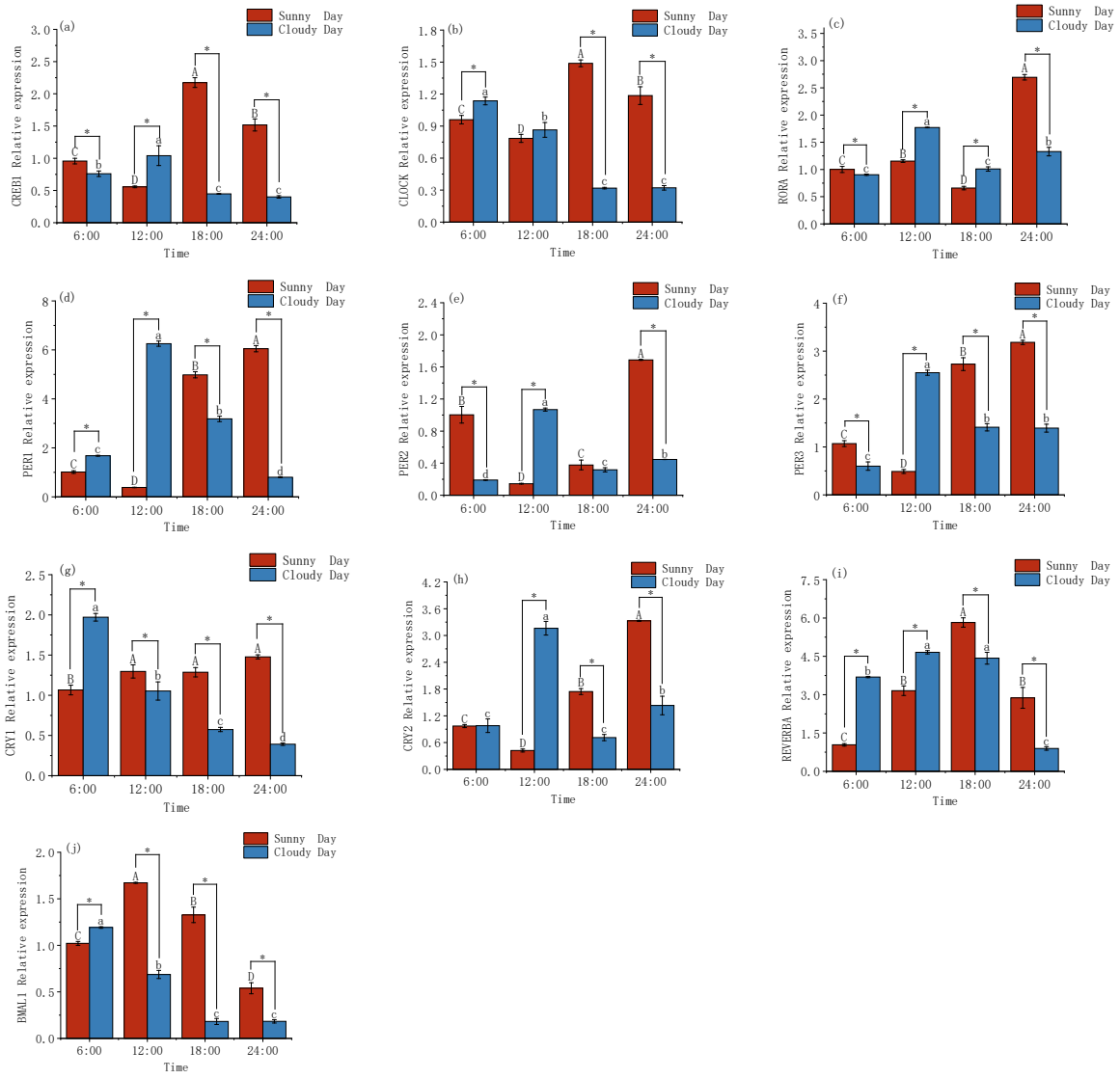

Figure 1. Expression of liver rhythm genes during 24 h in different weather in mackerel tuna. (**a**): *CREB1*; (**b**): *CLOCK*; (**c**): *RORA*; (**d**): *PER1*; (**e**): *PER2*; (**f**): *PER3*; (**g**): *CRY1*; (**h**): *CRY2*; (**i**): *REVERBA*; (**j**): *BMAL1*. Red in each graph represents sunny days, and blue represents cloudy days. The presence of different letters indicates significance by ANOVA and Tukey's tests ($p < 0.05$). * represents significant differences at the same time point ($p < 0.05$). Differences between those with different lowercase letters indicate significance ($p < 0.05$), while the opposite difference is not significant ($p > 0.05$); the same for the latter figure.

The results of the two-way analysis of different weather and time of day on mackerel tuna rhythm genes are shown in Table 4. The main effect of time and weather was significant ($p < 0.05$); there was a significant interaction between time and different weather on the level of rhythm gene expression in mackerel tuna ($p < 0.05$).

Table 4. Effects of light intensity and duration on the rhythm genes of mackerel tuna under different weather conditions.

Genes	*p*-Value		
	Time	Weather	Interactions
CREB1	<0.001	<0.001	<0.001
CLOCK	<0.001	<0.001	<0.001
RORA	<0.001	<0.001	<0.001
PER1	<0.001	<0.001	<0.001
PER2	<0.001	<0.001	<0.001
PER3	<0.001	<0.001	<0.001
CRY1	<0.001	<0.001	<0.001
CRY2	<0.001	0.339	<0.001
REVERBA	<0.001	<0.001	<0.001
BMAL1	<0.001	0.026	<0.001

Results of the two-way ANOVA with SPSS for the measured factors. When interactions in the analysis are significant ($p < 0.001$), a between-group comparison and an independent samples *t*-test at the same time point are used (the same applies below).

3.2. Changes in the Expression Levels of Lipid Metabolism in Mackerel Tuna

Under the sunny condition, the expression levels of *SIRT1*, *GST* and *LPL* were not significantly rhythmic in the liver (Table 5). The expression levels of *SIRT1* and *GST* were significantly higher at 18:00 than the other three groups at different times in sunny conditions ($p < 0.05$).

Table 5. Cosinor analysis board for metabolic genes expression under the sunny and cloudy conditions.

Gene	Weather	Acro (*p*-Value)	Acrophase
SIRT1	Sunny day	n.s.	
	Cloudy day	<0.001	18 ± 0.59
GST	Sunny day	n.s.	
	Cloudy day	n.s.	
LPL	Sunny day	n.s.	
	Cloudy day	n.s.	
SREBP1	Sunny day	<0.05	0 ± 1.29
	Cloudy day	<0.001	18 ± 0.77

n.s. denotes statistical differences between the different sampling points. Acrophases (circadian peak times) were calculated by a non-linear regression fit of a cosine function. Data are expressed as acrophase ± 95% confidence intervals.

Under the cloudy condition, the expression levels of *GST* and *LPL* were not significantly rhythmic in the liver (Table 5). The expression levels of *GST* and *LPL* were significantly higher ($p < 0.05$) than the other three groups at 18:00 under different times in the cloudy condition (Figure 2b,c; Table 5). The expression levels of *SIRT1* and *SREBP1* were significantly rhythmic in the liver under overcast conditions (Table 5).

The expression levels of metabolic genes at the same time point under different weather on sunny and cloudy days were significantly different in all four groups ($p < 0.05$). No significant differences could be seen between *GST* at 6:00 and 18:00 in the same time point comparison; *GST* expression levels were significantly higher under the cloudy condition than under the sunny condition at 12:00 ($p < 0.05$), and *GST* expression levels were significantly higher under the sunny condition than under the cloudy condition at 24:00 ($p < 0.05$; Figure 2b). The expression levels of *LPL* were significantly higher under sunny light conditions at 6:00 and 24:00 than under the cloudy condition ($p < 0.05$); the expression levels of *LPL* were significantly higher under cloudy light conditions at 12:00 and 18:00 than under cloudy light conditions ($p < 0.05$) (Figure 2c). The expression level of SREBP1 at 12:00 under the cloudy condition was significantly higher than that of the sunny condition ($p < 0.05$); the expression level of *SREBP1* at 18:00 was not significantly different in the comparison at the same time point (Figure 2d).

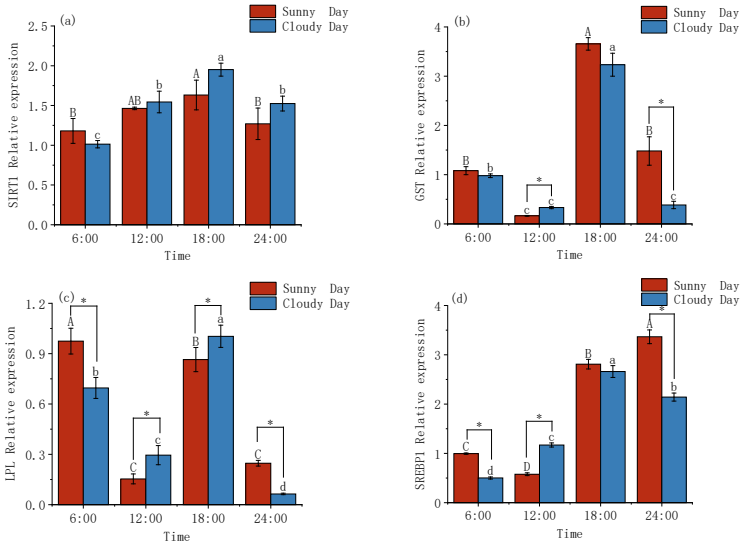

Figure 2. Expression of hepatic metabolic genes during 24 h in different weather in mackerel tuna. (**a**): *SIRT1*; (**b**): *GST*; (**c**): *LPL*; (**d**): *SREBP1*. Red in each graph represents sunny days, and blue represents cloudy days. The presence of different letters indicates significance by ANOVA and Tukey's tests ($p < 0.05$). * represents significant differences at the same time point ($p < 0.05$).

The results of the two-way analysis of different weather and time of day on metabolic genes in mackerel tuna are shown in Table 6. The main effect of time and weather was significant ($p < 0.05$); time and different weather had a significant interaction effect ($p < 0.05$) on metabolic gene expression levels in mackerel tuna.

Table 6. Effects of light intensity and duration on metabolic genes in mackerel tuna under different weather conditions.

Genes	*p*-Value		
	Time	Weather	Interactions
SIRT1	<0.001	0.035	0.022
GST	<0.001	<0.001	<0.001
LPL	<0.001	0.007	<0.001
SREBP1	<0.001	<0.001	<0.001

3.3. Changes in the Expression Levels of Immune Genes in Mackerel Tuna

Expression levels of *TRIM35* under sunny conditions were not significantly rhythmic in the liver (Table 7). The expression level of *TRIM35* at 18:00 was significantly higher ($p < 0.05$) than the other three groups at different times in sunny conditions (Figure 3a; Table 7). Expression levels of *NF-kB1*, *MHC-I*, *ALT*, *IFNA3*, *ISY1*, *ARHGEF13* and *GCLC* were significantly rhythmic in the liver under sunny conditions (Table 7).

Table 7. Cosinor analysis board for immune genes expression under sunny and cloudy conditions.

Gene	Weather	Acro (*p*-value)	Acrophase
TRIM35	Sunny day	n.s.	
	Cloudy day	n.s.	
NF-KB1	Sunny day	<0.001	18 ± 0.5
	Cloudy day	<0.01	12 ± 1.13
MHC-I	Sunny day	<0.005	18 ± 1.03
	Cloudy day	n.s.	
ALT	Sunny day	<0.005	18 ± 1.07
	Cloudy day	<0.005	12 ± 1.03

Table 7. *Cont.*

Gene	Weather	Acro (*p*-value)	Acrophase
IFNA3	Sunny day	<0.001	18 ± 0.39
	Cloudy day	n.s.	
ISY1	Sunny day	<0.001	18 ± 0.79
	Cloudy day	<0.001	18 ± 0.64
ARHGEF13	Sunny day	<0.05	18 ± 1.43
	Cloudy day	<0.005	12 ± 1.03
GCLM	Sunny day	<0.05	12 ± 1.26
	Cloudy day	<0.001	12 ± 0.75
GCLC	Sunny day	n.s.	
	Cloudy day	<0.05	0 ± 1.36

n.s. denotes statistical differences between the different sampling points. Acrophases (circadian peak times) were calculated by a non-linear regression fit of a cosine function. Data are expressed as acrophase ± 95% confidence intervals.

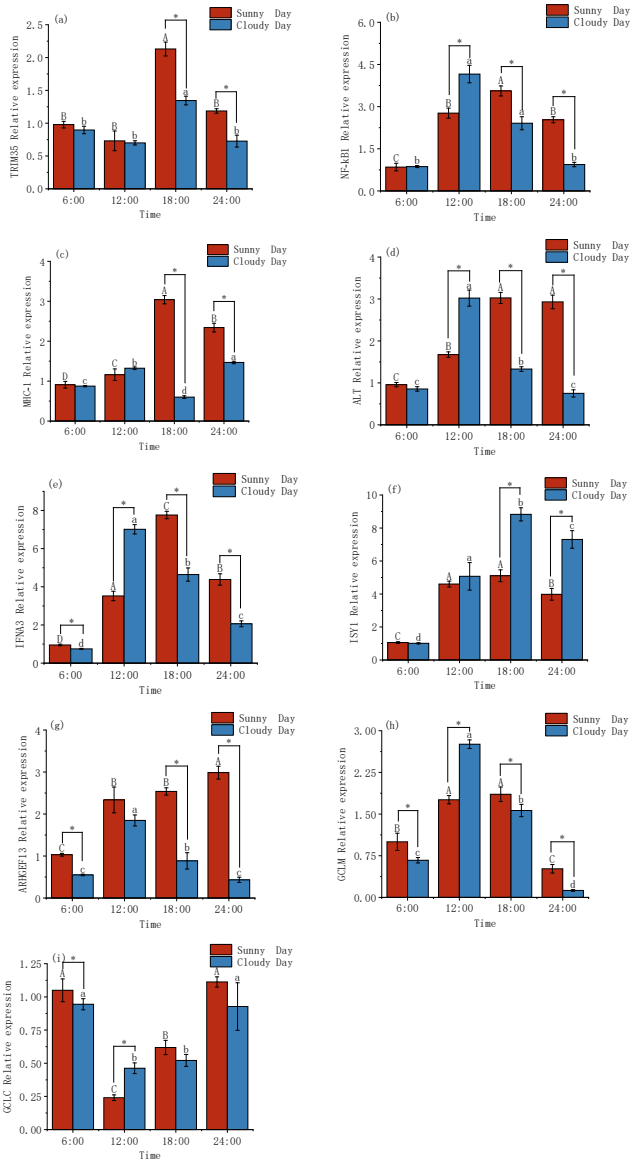

Figure 3. Expression of liver immune genes in different weather conditions in mackerel tuna over a 24 h period. (**a**): *TRIM35*; (**b**): *NF-kB1*; (**c**): *MHC-I*; (**d**): *ALT*; (**e**): *IFNA3*; (**f**): *ISY1*; (**g**): *ARHGEF13*; (**h**): *GCLM*; (**i**): *GCLC*. Red in each graph represents sunny days, and blue represents cloudy days. The presence of different letters indicates significance by ANOVA and Tukey's tests ($p < 0.05$). * represents significant differences at the same time point ($p < 0.05$).

Under the cloudy condition, the expression levels of *TRIM35*, *MHC-I* and *IFNA3* were not significantly rhythmic in the liver (Table 7). The expression levels of *TRIM35* at 18:00 were significantly higher than the other three time points at different times in the cloudy condition ($p < 0.05$), the expression levels of *MHC-I* at 24:00 were significantly higher than the other three time points in the cloudy condition ($p < 0.05$), and the expression levels of IFNA3 at 12:00 were significantly higher than the other three time points in the cloudy condition ($p < 0.05$; Figure 2a,b,e; Table 7). The expression levels of *NF-kB1*, *ALT*, *ISY1*, *ARHGEF13*, *GCLM* and *GCLC* were significantly rhythmic in the liver under overcast conditions (Table 7).

The expression levels of *TRIM35* at 6:00 and 12:00 were not significantly different between sunny and cloudy days at the same time point; the expression levels of *TRIM35* at 18:00 and 24:00 were significantly higher ($p < 0.05$) under the sunny condition than the under cloudy condition (Figure 3a). The expression levels of *NF-kB1* at 6:00 were not significantly different in the comparison at the same time point; *NF-kB1* expression levels were significantly higher under the cloudy condition at 12:00 than under the sunny conditions ($p < 0.05$); *NF-kB1* expression levels were significantly higher under the sunny condition at 18:00 and 24:00 than under the cloudy condition ($p < 0.05$) (Figure 3b). The expression levels of *MHC-I* at 6:00 and 12:00 were not significantly different in the same time point comparison; the expression levels of *MHC-I* at 18:00 and 24:00 under the sunny condition were significantly higher than those under the cloudy condition ($p < 0.05$) (Figure 3c). The expression levels of *ALT* at 6:00, 18:00 and 24:00 were significantly higher ($p < 0.05$) under the sunny condition than under the cloudy condition (Figure 3d). The expression levels of *ISY1* at 6:00 and 12:00 were not significantly different in the comparison at the same time point; the expression levels of *ISY1* at 18:00 and 24:00 under the cloudy condition were significantly higher than those under the sunny condition ($p < 0.05$; Figure 3f). The expression levels of *ARHGEF1* were significantly higher ($p < 0.05$) under the sunny condition than under the cloudy condition at 6:00, 18:00 and 24:00; the expression levels of *ARHGEF1* at 12:00 were not significantly different at the same time point comparison (Figure 3g). *GCLM* expression levels were significantly higher at 6:00, 18:00 and 12:00 in the same time point comparison between sunny and cloudy conditions ($p < 0.05$); the expression level of *GCLM* at 12:00 was significantly higher under cloudy conditions than under sunny conditions ($p < 0.05$; Figure 3h). The expression level of *GCLC* was significantly higher at 6:00 than in the same time point comparison between sunny and cloudy conditions ($p < 0.05$); The expression level of *GCLC* at 12:00 was significantly higher under cloudy conditions than under sunny conditions ($p < 0.05$; Figure 3i).

The results of the two-way analysis of different weather and time of day on immune genes in mackerel tuna are shown in Table 8. The main effects of time and weather were significant ($p < 0.05$), and there was a significant interaction between time and different weather on immune gene expression levels in mackerel tuna ($p < 0.05$).

Table 8. Effects of light intensity and duration on immune genes in mackerel tuna under different weather conditions.

Genes	*p*-Value		
	Time	Weather	Interactions
TRIM35	<0.001	<0.001	<0.001
NF-kB1	<0.001	<0.001	<0.001
MHC-I	<0.001	<0.001	<0.001
ALT	<0.001	<0.001	<0.001
IFNA3	<0.001	0.022	<0.001
ISY1	<0.001	<0.001	<0.001
ARHGEF13	<0.001	<0.001	<0.001
GCLM	<0.001	<0.001	<0.001
GCLC	<0.001	0.205	0.002

4. Discussion

4.1. Rhythmic Gene Expression Patterns

In nature, in both animals and humans, there is a 24 h circadian rhythm called the biological clock [22]. *CREB1* (cyclic adenosine monophosphate response element binding protein 1) is a protein that regulates gene transcription and can participate in cycle regulation by regulating the expression of downstream target genes [23]. In this study, the daily rhythmicity of *CREB1* was only present under the cloudy condition, and the average *CREB1* gene expression was found to be higher under the sunny condition than under the cloudy condition. The light intensity may stimulate the *CREB1* gene expression in mackerel tuna, but the regulatory mechanism remains to be investigated.

The molecular mechanism of the biological clock is primarily the existence of a cell-autonomous transcriptional–translational feedback loop in which a pair of positive regulators (*CLOCK* and *BMAL1*) form a heterodimer that activates its transcription by binding to the negative regulators (*PER* and *CRY* promoters). Subsequently, the *PER* and *CRY* proteins bind to form a complex that enters the nucleus and acts on the *CLOCK* and *BMAL1* heterodimers, thereby feeding back to repress its own transcription [24]. *BMAL1* is thought to regulate the expression of rhythmic genes throughout the liver [25]. In this study, *PER2*, *CRY2* and *BMAL1* showed daily rhythmicity under the sunny condition and *CLOCK*, *PER1*, *PER3* and *BMAL1* showed daily rhythmicity under the cloudy condition. It has been shown that the expression levels of the rhythm genes *PER1*, *PER2*, *CRY1*, *CRY2* and *BMAL1* in the liver of the Atlantic bluefin tuna (*Thunnus thynnus*, L.) exhibit daily rhythmicity [26]. Similar to the results of the present study. However, in this study, PER1 showed daily rhythmicity only under cloudy conditions, while *PER2* and *CRY2* showed daily rhythmicity only under sunny conditions, which may be due to the different changes in light intensity under the cloudy and the sunny conditions, leading to an effect of light stimulation on the circadian system of fish. It has been shown that photoperiod has an effect on core biological clock gene expression in fish [27]. In this study, the expression levels of rhythm genes were at their highest under sunny conditions at 12:00, when light intensity was the strongest of the day, reflecting that light intensity at 12:00 under sunny conditions can cause up-regulation of the expression levels of rhythm genes in mackerel tuna to some extent.

4.2. Metabolic Gene Expression Patterns

The liver is the main organ of lipid metabolism in fish and plays a huge role in the body as a hub for fat transport, influencing the breakdown and absorption of nutrients and hormonal signaling [28]. Some hepatic metabolic pathways are driven by the circadian biological clock, resulting in a circadian rhythm, and disturbances in the biological clock can cause metabolic disorders in fish. *SIRT1* is involved in a wide range of glucose and lipid metabolism pathways, as well as in the regulation of gene transcription and cellular senescence through the deacetylation of several metabolism-controlling transcription factors [29]. Current research on the *SIRT1* gene focuses on humans, mice, livestock and poultry (pigs, sheep, chickens, etc.) [30]. Little research has been reported on the *SIRT1* gene in fish. It has been found that there is a close correlation between the expression level of the *SIRT1* gene and lipid metabolism in pigs [31]. In this study, there was no daily rhythm in the expression levels of the *SIRT1* gene under sunny conditions, while there was a daily rhythm in the expression levels of *SIRT1* under cloudy conditions. This may result from the involvement of *CLOCK* and *BMAL1* in the regulation. It has been shown that *SIRT1* interacts with *BMAL1*, *PER2* and *CRY1* in the liver of mice [32]. In rainbow trout [33], *SIRT1* interacts with *BMAL1*, *PER2*, *PER3*, *CLOCK* and *BMAL1*, with *CLOCK* and *BMAL1* controlling the rhythm of *SIRT1*, which is in agreement with the results of this study.

SREBP1 plays a key role in regulating lipid homeostasis, and *REVERBA* is a powerful transcriptional repressor that plays an important role in rhythms. In this study, *SREBP1* showed a clear daily rhythm under sunny conditions, and both *REVERBA* and *SREBP1* had a daily rhythm under cloudy conditions with similar trends, but *REVERBA* peaked at 12:00 and *SREBP1* peaked at 18:00 when the rhythm genes regulate *SREBP1* expression. This

is probably due to the delayed expression levels of the *SREBP1* metabolic gene, resulting in a peak at 18:00, but *REVERBA* plays a regulatory role in *SREBP1* gene expression. It has been shown that *REVERBA* can regulate the expression of *SREBP1* in the salmon liver, that *SREBP1* exhibits daily rhythmicity, and that *REVERBA* has a regulatory effect on *SREBP1* [15], which is consistent with the results of this experimental study. In the present study, *SIRT1* and *SREBP1* showed an up-regulation trend both under sunny and cloudy conditions, and the expression level of *SIRT1* continued to increase with time. It has been shown that *LPL* is expressed in the liver of fish and can exhibit changes in lipid metabolism and deposition [34]. In this study, because of the high activity of mackerel tuna during the day under sunny or cloudy conditions, the mackerel tuna was active in the water at this time, prompting an increase in its metabolic levels and making its expression levels up-regulated during the day.

4.3. Immune Gene Expression Patterns

The TRIM family of proteins belongs to the RING family of E3 ubiquitin ligases. *NF-kB* is an important nuclear transcription factor involved in regulating the body's immune response, and abnormal regulation can lead to immune diseases, metabolic diseases, etc. The TRIM family of proteins is involved in the regulation of the NF-kB signaling pathway as E3 ubiquitin ligases [35–37]. Interferon (IFN) receptor proteins are a class of cytokines secreted by host cells that regulate the immune response. When a pathogen is present, interferon is usually released by the host cell, which is sensed by surrounding undisturbed cells and activates appropriate cellular defense mechanisms to eliminate the pathogen [38]. The composition, signaling pathways, function and evolutionary relationships of the *IFN* were extensively studied in fish many years ago [39–41]. It has been shown that *TRIM35* is a positive regulatory molecule in the natural immune signaling pathway [42]. In this study, *TRIM35* did not show daily rhythmicity under sunny conditions, but there were significant differences between groups. The expression level of *TRIM35* was up-regulated by sunny weather conditions. *NF-kB1* showed significant daily rhythmicity in both sunny and cloudy conditions. However, under sunny conditions, the expression level of NF-kB1 showed an increasing trend and started to decrease by 24:00. *IFNA3* showed daily rhythmicity only in sunny conditions. It has been shown that the expression of EcTRIM21 significantly increased the IFN promoter activity and simultaneously increased the transcriptional levels of interferon-related molecules, with a positive regulatory effect [43]. This is consistent with the results of the present experimental study.

MHC-I (major histocompatibility complex) plays an important role in adaptive immunity in vertebrates, primarily recognizing intracellular antigens and triggering adaptive immunity. This gene is currently expressed in different fish species, such as the Japanese flounder (*Paralichthys olivaceus*) [44], turbot (*Scophthalmus maximus*) [45] and rainbow trout (*Oncorhynchus mykiss*) [46]. Interferons increase the expression levels of *MHC-I* class molecules on the cell surface, and an increase in *MHC-I* molecules on the surface of virus-infected cells contributes to the delivery of antigens to T cells, causing lysis of target cells. It was found that the MHC-I protein is extremely important in immune recognition in zebrafish by positively regulating IFN immunity and inflammatory responses [47]. In the present study, both *MHC-I* and *IFNA3* were expressed in daily rhythms under sunny conditions and were positively regulated. This is consistent with the present study results.

GCL (glutamate-cysteine ligase) is composed of different gene-edited *GCLC* (glutamate cysteine ligase catalytic subunit) and *GCLM* (glutamate cysteine ligase), with *GCLC* playing all catalytic roles and being subject to feedback inhibition by *GSH* and *GCLM* having regulatory functions [48]. It has been shown that altered single nucleotide polymorphisms in the *GCLC* and *GCLM* genes can regulate gene expression processes and thus participate in various disease processes [49]. In the present study, both *GCLM* and *GCLC* were rhythmic under overcast conditions, and expression levels were simultaneously downregulated at 12:00. It has been shown that the expression of *NF-kB1* and *GCLC* are mutually regulated [50]. In the present study, *NF-kB1* expression levels were down-regulated

when *GCLC* expression levels were up-regulated, suggesting that the down-regulation of *GCLC* expression may be related to the inhibition of the signaling pathway.

5. Conclusions

In summary, the mRNA expression levels of rhythm and immune- and metabolism-related genes in the liver tissue of mackerel tuna were significantly changed under sunny and cloudy conditions, and some genes showed significant daily rhythmicity. Immune and metabolic genes can be regulated by rhythm genes and external factors through different signaling pathways and are jointly involved in regulating immunity and energy metabolism in mackerel tuna. This study provides practical implications for further understanding the regulation of lipid metabolism and immunity in mackerel tuna.

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Ethics Statement: The experiment complied with the regulations and guidelines established by the Animal Care and Use Committee of the South China Sea fisheries Research Institute, Chinese Academy of Fishery Sciences.

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