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Effects of continuous white light and 12 hours white-12 hours blue light-cycles on the expression of clock genes in diencephalon, liver, and skeletal muscle in chicks

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Abstract

The core circadian clock mechanism relies on a feedback loop comprised of clock genes, such as the brain and muscle Arnt-like 1 (Bmal1), chriptochrome 1 (Cry1), and period 3 (Per3). Exposure to the light-dark cycle synchronizes the master circadian clock in the brain, and which then synchronizes circadian clocks in peripheral tissues. Birds have long been used as a model for the investigation of circadian rhythm in human neurobiology. In the present study, we examined the effects of continuous light and the combination of white and blue light on the expression of clock genes (Bmal1, Cry1, and Per3) in the central and peripheral tissues in chicks. Seventy two day-old male chicks were weighed, allocated to three groups and maintained under three light schedules: 12 h white light-12 h dark-cycles group (control); 24 h white light group (WW group); 12 h white light-12 h blue light-cycles group (WB group). The mRNA levels of clock genes in the diencephalon were significantly different between the control and WW groups. On the other hand, the alteration in the mRNA levels of clock genes was similar between the control and WB groups. Similar phenomena were observed in the liver and skeletal muscle (*biceps femoris*). These results suggest that 12 h white-12 h blue light-cycles did not disrupt the circadian rhythm of clock gene expression in chicks.

key words: blue lighting , chicken, clock, light, white lighting

1. Introduction

The core circadian clock mechanism relies on a transcriptional-translational feedback loop comprised of clock genes, such as the brain and muscle Arnt-like 1 (Bmal1), circadian locomotor output cycles kaput (CLOCK) and their target genes period (Per) and chriptochrome (Cry): the Per and Cry proteins repress their own transcription by interfering with CLOCK-Bmal1 activity (Kohsaka and Bass, 2007; Bailey et al., 2014). Exposure to the light-dark cycle synchronizes the master circadian clock in the hypothalamic suprachiasmatic nucleus (SCN), and which synchronizes peripheral clocks through its effects on behavioral rhythms, such as feeding-fasting and rest-activity cycles, and neural and endocrine pathways (Gooley, 2016). For example, there is evidence that neuronal and/or peripheral tissue Bmal1 influences appetite regulatory systems (Fick et al., 2010; Kettner et al., 2015) and myogenesis (Chatterjee ett al., 2013). Thus, a disrupted circadian rhythm may result in detrimental effects on human health (Lucassen et al., 2016).

Birds have been represented as an excellent model for the role played by biological clocks in human neurobiology (Cassone, 2014). The expression patterns of clock genes were different between a long (16h) and short (8h) photoperiod in the SCN in the Japanese quail (Yasuo et al., 2003). The mRNA levels of Bmal1, Cry1, and Per3 showed circadian rhythm in the brain and peripheral tissues in chickens (Karaganis et al., 2009). Lines of evidence demonstrated that near or continuous light schedules disrupt the circadian rhythms of plasma hormone levels in chicks (Lauber et al., 1987; Zawilska et al., 2007). However, whether continuous light disrupts the circadian rhythm of clock gene expression in the brain and peripheral tissues in chickens has not yet been examined.

It is apparent that chickens appear calm under dark conditions. Blue light keeps chickens calmer (Prayitno et al., 1997; Parvin et al., 2014). In mammals, as illumination

decreases into the mesopic range, rod signals are known to contribute to color percepts, causing a shift in hue sensations most frequently toward blue or green (Buck, 2014). Therefore, it is possible that chickens have a similar feeling under dark and blue light conditions.

In the present study, we hypothesized that continuous light disrupts the circadian rhythms of clock genes in chicks and that 12 h blue light during the night would be effective to improve this disruption. We analyzed the effects of color of light and light schedule on the expression of clock genes in chicks. We also analyzed appetite-regulating neuropeptides such as neuropeptide Y (NPY), proopiomelanocortin (POMC), and corticotropin-releasing factor (CRF) in the diencephalon because appetite regulation is related to clock gene expression in mammals (Fick et al., 2010; 2011). Our findings suggest that 12 h white-12 h blue light-cycles did not disrupt the circadian rhythm of clock gene expression in chicks.

2. Materials and Methods

2.1. Animals and feed

Day-old male broiler chicks (ROSS 308) were purchased from a local hatchery (Ishii Co., Ltd. Tokushima, Japan). They were given free to access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulation.

2.2. Light schedule

Seventy two day-old male chicks were weighed, allocated to three groups and maintained under three light schedules: 12 h white light (400-700 nm)-12 h dark-cycles group (control); 24 h white light group (WW group); 12 h white light-12 h blue light (450-480 nm)-cycles group (WB

group). Light was turned off or changed at 6:00 and 18:00 in the control or WB groups, respectively. At 6 days of age, six birds from each group were euthanized by decapitation at 17:00, 19:00, 4:00, and 7:00. The diencephalon, liver, and skeletal muscle (*biceps femoris*) were excised and frozen immediately using liquid nitrogen for real-time PCR analysis. Diencephalon, the rostral part of the brainstem, was dissected as describe previously (Aoki et al., 2017).

2.3. Real-time PCR analysis

The total RNA was extracted from each sample using Sepasol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized using a ReverTra Ace® qPCR RT Master Mix with gDNA remover (Toyobo Co., Ltd., Osaka, Japan). Complementary DNAs of chicken Bmal1 (GenBank accession no. AF205219), Cry 1 (GenBank accession no. NM_204245), and Per 3 (GenBank accession no. NM_001289779) were amplified with the primers as follows: Bmal1 sense, 5'-CGG CTG CAT CCT CAT GTA GTA C-3'; Bmal1 antisense, 5'-CTC TGT AGG TTT CAC CCT GAT CTC A-3'; Cry 1 sense, 5'-CTC ATG GAG ACA ATC AGC AAT CAC-3'; Cry 1 antisense, 5'-CGC TAA TGC CAG TAC CAA GAG A-3'; Per 3 sense, 5'-CCT TGG TAG CAG TGG TTC TTA TGA-3'; Per 3 antisense, 5'-GCA GTT CCC ATT GGA GTC ACT T-3'. Complementary DNAs of NPY, POMC, and CRF were amplified with the primers as described previously (Saneyasu et al., 2013). As an internal standard, complementary DNA of chicken ribosomal protein S17 (RPS17) was also amplified with the primers as described previously (Saneyasu et al., 2016). The mRNA level was quantified in duplicate using an Applied Biosystems 7300 Real-Time PCR system and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan) according to the supplier's recommendations. The thermal cycle was as follows: 1 cycle at 95°C for 30 s, and 40 cycles at 95°C for 5 s and 60°C for 31 s. After the reactions, the specificity of amplifications in each sample was confirmed by dissociation analysis showing that each sample gave a single

melting peak. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT). CT was calculated for the target gene and for RPS17. For each cDNA sample, the CT for RPS17 was subtracted from the CT for the target gene to give the parameter ΔCT, thus normalizing the initial amount of RNA used. The amount of target gene mRNA was calculated as $2^{-\Delta\Delta CT}$, where ΔΔCT is the difference between the ΔCT of the two cDNA samples to be compared.

2.4. Food intake

In order to clarify the effects of different light schedules on food intake in chicks, we have carried out an additional experiment. Thirty six day-old male chicks were weighed, allocated to either control, WW, or WB group (twelve birds in each group). Two birds were maintained in the same cage (six replicates in each group). Light was turned off or changed at 6:00 and 18:00 in the control or WB groups, respectively. At 6 days of age, food intake was measured every 6 h from 12:00 to 12:00 using an electric digital balance.

2.5. Data analysis

Data were analyzed by two-way ANOVA with the main effects of time (Time) and color of light (Color). If a significant interaction was observed, the Tukey–Kramer test was performed to analyze the difference between groups. All statistical analysis was performed using a commercial software package (StatView version 5, SAS Institute, Cary, North Carolina, USA, 1998).

3. Results

Significant main effect of time was detected in the mRNA levels of all clock genes in

the diencephalon, liver, and skeletal muscle. Significant main effect of the color of light was not detected only in the mRNA levels of Bmal1 in the diencephalon and Per3 in the liver and skeletal muscle. However, significant interactions were detected in the mRNA levels of all clock genes in the diencephalon, liver, and skeletal muscle. Therefore, multiple comparison test for all possible combinations have been performed. Figure 1 shows circadian changes in the mRNA levels of clock genes (Bmal1, Cry1, and Per3) in the diencephalon in chicks. The mRNA levels of clock genes in the diencephalon were significantly different between the control and WW group (Fig. 1). On the other hand, significant difference between the control and WB groups was only detected at 4:00 (Fig. 1, Per3). The amplitude of clock gene expressions in the WW group seem to be smaller than that in other groups in the diencephalon (Fig. 1). In the liver and skeletal muscle, the mRNA levels of clock genes were significantly different between the control and WW group as well as in the diencephalon (Figs. 2 and 3). On the other hand, significant difference between the control and WB groups was only detected at 7:00 (Figs. 2 and 3, Per3). All these findings suggest that continuous white light, but not 12 h white-12 h blue light-cycles, disrupts the circadian rhythm of clock gene expression in chicks.

We also analyzed the mRNA levels of appetite-regulating peptide genes (NPY, POMC, and CRF) in the diencephalon. However, there was no significant difference between groups (Fig. 4). Therefore, we finally examined the effects of different light schedules on food intake. Significant main effect of time and significant interaction were detected in food intake. Multiple comparison test for all possible combinations showed that food intake in the WW group was significantly lower than that in the control group during lighting period, whereas it was significantly higher than that in the control group during dark period (Fig. 5). Similar tendency was observed in the WB group. There was no significant difference in food intake between the WW and WB groups. These findings clearly demonstrated that chicks can access feed under the blue lighting condition but not under the dark condition. All our findings suggest that circadian

changes of clock gene expression and food intake may not affect the mRNA levels of appetite-regulating peptides in the diencephalon in chicks, at least in these experimental conditions.

4. Discussion

The mRNA levels of Bmal1 and Cry1 in the diencephalon, liver, and skeletal muscle decreased during a dark phase, whereas the mRNA levels of Per3 increased during a light phase in the control group (Figs. 1-3). These results are in good agreement with previous studies: Bmal1, Cry1, and Per3 mRNA levels exhibited daily rhythms in the brain and liver under 12 h light-12 h dark cycles in chicks (Karaganis et al., 2009). Zeman et al. (2009) reported that the mRNA level of Bmal1 in the liver was gradually decreased during a dark phase in 4-day-old chicks. Karaganis et al. (2009) also indicated that the phases of the clock gene rhythm in the liver were delayed relative to that of the brain. In the present study, the phases of the changes of clock gene expression rhythms in the liver seem to be delayed relative to those in the diencephalon. For example, the highest value of Bmal1 mRNA level was shown at 17:00 in the diencephalon (Fig. 1), but at 20:00 in the liver (Fig. 3). Thus, the results of this study appear to be comparable with the results of similar studies in chicks.

Physical activity such as wheel-running is suggested to be a time cue that acts to synchronize clocks in skeletal muscle (Yamanaka et al., 2008; Lefta et al., 2011). Prayitno et al. (1997) reported that blue light keeps chickens calmer. In addition, it is apparent that the locomotive activity of chicks is almost abolished during dark period in chickens. It is therefore possible that the mRNA levels of clock genes show different changes in leg muscles in each group. Therefore, we analyzed the mRNA levels of clock genes in the *biceps femoris*, one of the major skeletal muscles in legs, and found that changes in the mRNA levels of clock genes showed similar between the control and WB groups (Fig. 3). It is therefore likely that physical

activity did not influence clocks in the skeletal muscle of chickens.

Our results suggest that continuous white light, but not 12 h white-12 h blue light-cycles, disrupts the circadian rhythm of clock gene expression in chicks. However, significant difference was detected in the mRNA levels of Per3 in the diencephalon, liver, and skeletal muscle at 4:00, 7:00, and 7:00, respectively (Figs. 1-3). In mammals, Per3 is associated with sleep homeostasis (Dijk and Archer, 2010; Hasan et al., 2011). In the present study, chicks can freely access to feed throughout the experimental period in the WB group, whereas food intake was almost abolished in the dark period in the control group, suggesting that sleeping time of chicks in the control group might be longer than that in the WB group. Therefore, significant differences in the mRNA levels of Per3 may be observed between the control and WB groups. Further study will be needed to clarify the role of Per3 on sleep homeostasis in chickens.

In mammals, molecular clocks within the central and peripheral tissues are influenced not only by light but also by food intake (Lefta et al., 2011). In addition, there is evidence that fasting significantly affects the mRNA levels of appetite-regulating peptides in chickens (Kameda et al., 2001; Dridi et al., 2006; Song et al., 2012). In the present study, significant differences in food intake were detected between groups (Fig. 5). However, there was no significant difference in the mRNA levels of appetite-regulating peptide genes (Fig. 4), although the mRNA levels of clock genes in the diencephalon were significantly changed in chicks (Fig. 1). Food intake was dramatically reduced under dark condition in the control group, indicating that chicks were almost fasted for 12 h. These findings suggest that 12 h of fasting and circadian changes in the expression of clock genes are not related to the mRNA levels of appetite-regulating peptides. Thus, the circadian rhythm of mRNA expression of appetite-regulating peptides would not be occurred at least in these experimental conditions.

Bmal1 mRNA peaked just before the day/night transition, approximately antiphase to the Per3 rhythm in chicks (Karaganis et al., 2009). The phases of these rhythms match clock

gene mRNA profiles reported for neural tissues of chickens (Okano et al., 2001; Yamamoto et al., 2001; Bailey et al., 2003; Chong et al., 2003). Therefore, we select the time points around the day/night transition, for the first time, to examine the effects of 12 h blue light during the night on the expression of clock genes in chicks. However, further studies will be needed to clarify the effects of blue light on the mRNA levels of clock genes at daytime and midnight.

Previous studies showed that continuous blue light does not affect food intake in chicks (Prayitno et al., 1997; Hassan et al., 2014; Seo et al., 2015). In the present study, chicks eat food under the blue light (Fig. 5). Therefore, chicken eyes may sense blue light. However, photoreceptors reside not only in the eyes but also in the pineal gland, the preoptic area, the lateral septum, and the tuberal hypothalamus, in birds (Cassone, 2014). Under a dark period, light cannot be sensed by photoreceptors in chicks, and therefore, the mRNA levels of clock genes show circadian changes. It is possible that blue light cannot be sensed by photoreceptors in these organs, as in white light in chicks.

Light schedules influence the circadian rhythm of circulating hormones, such as corticosterone (Lauber et al., 1987), and melatonin (Zawilska et al., 2007) in birds. Continuous light dramatically decreased pineal melatonin content and abolished the change of the melatonin content in the brain in chicks (Saito et al., 2005). Melatonin suppressed stress-induced elevation of plasma corticoseterone in chicks (Saito et al., 2005). It is therefore possible that hormonal changes occurred in the WB group. Thus, further studies will be needed to clarify the influence of the circadian rhythm of corticosterone and melatonin in chicks under the WB condition.

5. Conclusion

Continuous white light may disrupt the circadian rhythm of clock genes in central and peripheral tissues in chicks, whereas a 12 h white-12 h blue light schedule may be effective in

maintaining the circadian rhythm of clock genes in chicks. Our findings propose a new strategy for the development of light therapy in human health.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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Figure captions

Fig. 1 Temporal expression profiles of clock genes in the diencephalon under 12 h white light-12 h dark-cycles (control, white circle), 12 h white light-12 h blue light-cycles (WB, triangle), and continuous white light (WW, black circle). The bar at the bottom of each graph represents the dark period in the control group. Each value is the mean ± S.E.M. of six chicks in each group. The results of two-way ANOVA are shown in each graph. Difference at each time point was analyzed by Tukey-Kramer test ($P < 0.05$). The asterisk indicates statistical differences from the control group.

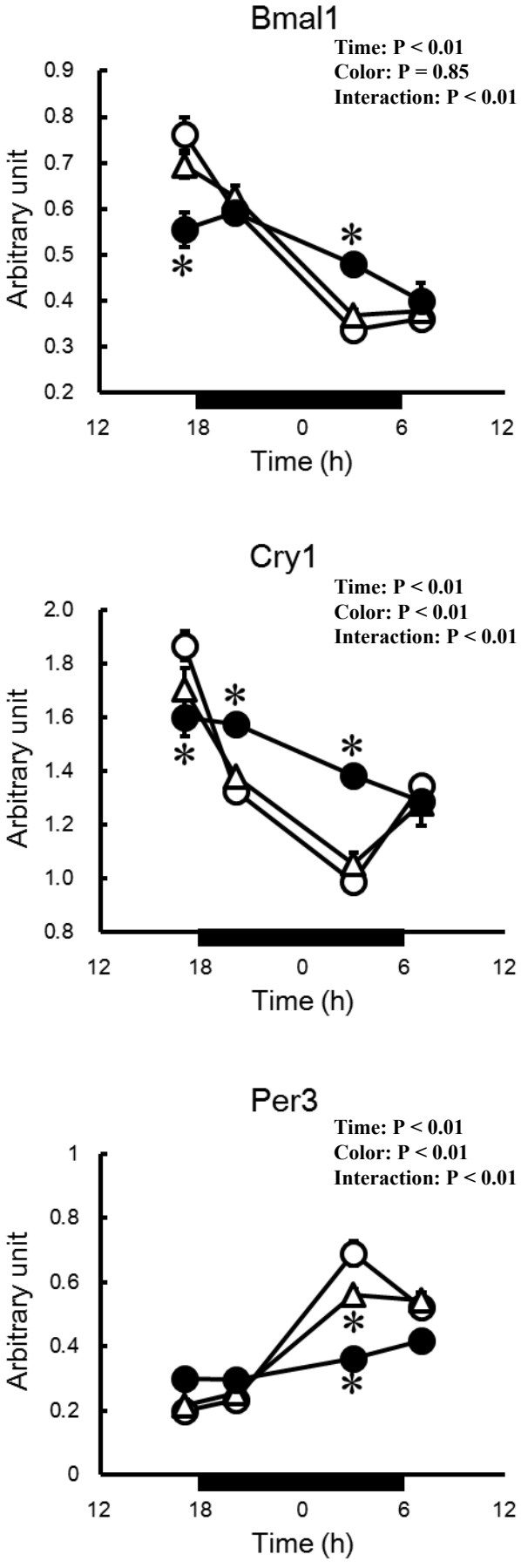
Fig. 2 Temporal expression profiles of clock genes in the liver under 12 h white light-12 h dark-cycles (control, white circle), 12 h white light-12 h blue light-cycles (WB, triangle), and continuous white light (WW, black circle). The bar at the bottom of each graph represents the dark period in the control group. Each value is the mean ± S.E.M. of six chicks in each group. The results of two-way ANOVA are shown in each graph. Difference at each time point was analyzed by Tukey-Kramer test ($P < 0.05$). The asterisk indicates statistical differences from the control group.

Fig. 3 Temporal expression profiles of clock genes in the skeletal muscle under 12 h white light-12 h dark-cycles (control, white circle), 12 h white light-12 h blue light-cycles (WB, triangle), and continuous white light (WW, black circle). The bar at the bottom of each graph represents the dark period in the control group. Each value is the mean ± S.E.M. of six chicks in each group. The results of two-way ANOVA are shown in each graph. Difference at each time point was analyzed by Tukey-Kramer test ($P < 0.05$). The asterisk indicates statistical differences from the control group.

Fig. 4 Temporal expression profiles of neuropeptide Y, proopiomelanocortin, and corticoropin-releasing factor in the diencephalon under 12 h white light-12 h dark-cycles (control, white circle), 12 h white light-12 h blue light-cycles (WB, triangle), and continuous white light (WW, black circle). The bar at the bottom of each graph represents the dark period in the control group. Each value is the mean ± S.E.M. of six chicks in each group. The results of two-way ANOVA are shown in each graph.

Fig. 5 Temporal changes of food intake under 12 h white light-12 h dark-cycles (control, white circle), 12 h white light-12 h blue light-cycles (WB, triangle), and continuous white light (WW, black circle). The bar at the bottom of each graph represents the dark period in the control group. Each value is the mean ± S.E.M. of six chicks in each group. The results of two-way ANOVA are shown in each graph. Difference at each time point was analyzed by Tukey-Kramer test ($P < 0.05$). The asterisk indicates statistical differences from the control group.

Figure 1


Bmal1
Time: P < 0.01
Color: P = 0.85
Interaction: P < 0.01
Arbitrary unit
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
12
18
0
6
12
Time (h)
Cry1
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
2.0
1.8
1.6
1.4
1.2
1.0
0.8
12
18
0
6
12
Time (h)
Per3
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
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0.8
0.6
0.4
0.2
0
12
18
0
6
12
Time (h)

Figure 2

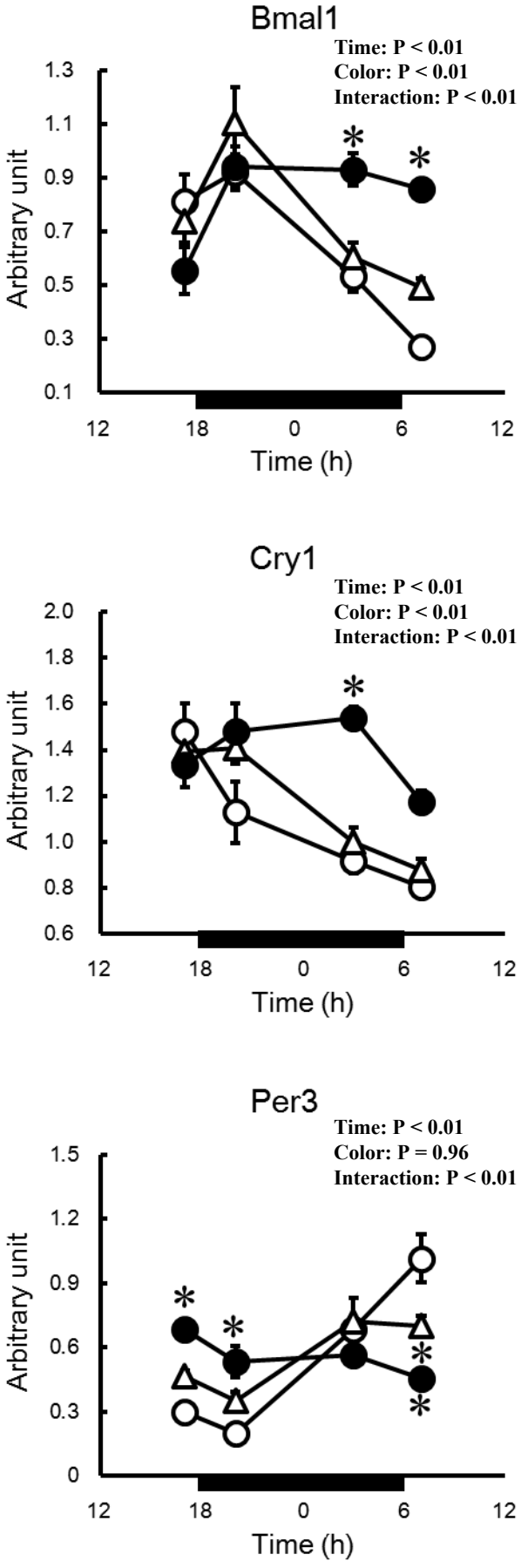

Bmal1
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
1.3
1.1
0.9
0.7
0.5
0.3
0.1
12
18
0
6
12
Time (h)
Cry1
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
12
18
0
6
12
Time (h)
Per3
Time: P < 0.01
Color: P = 0.96
Interaction: P < 0.01
Arbitrary unit
1.5
1.2
0.9
0.6
0.3
0
12
18
0
6
12
Time (h)

Figure 3

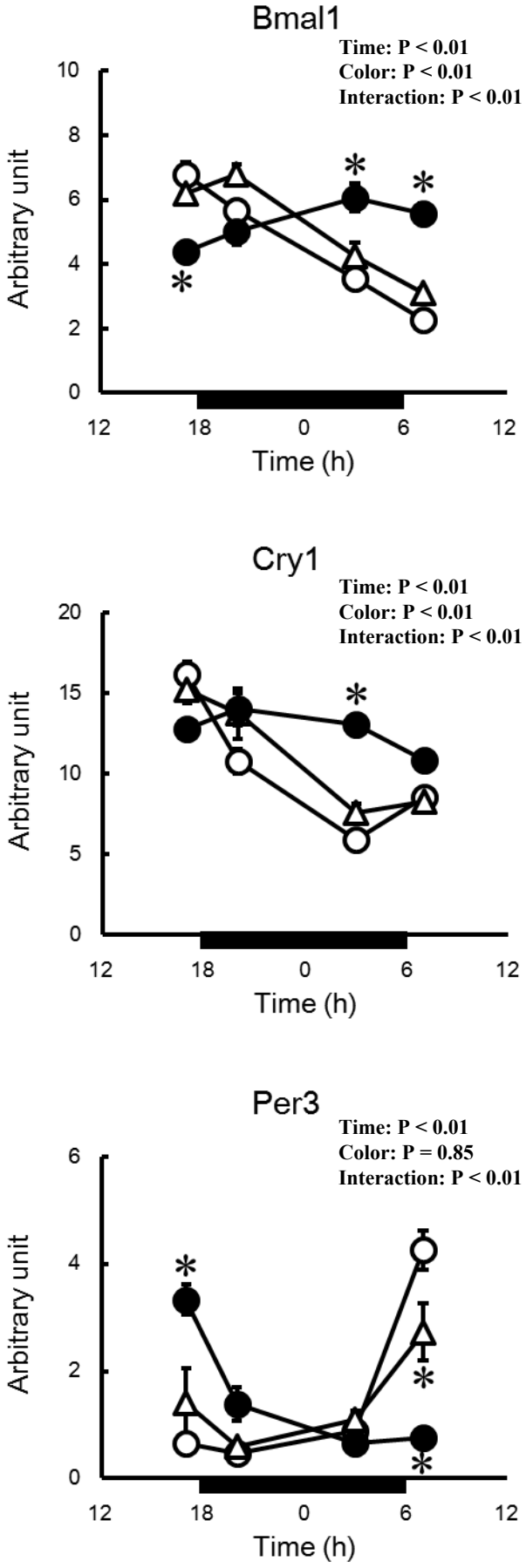

Bmal1
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
Time (h)
Cry1
Time: P < 0.01
Color: P < 0.01
Interaction: P < 0.01
Arbitrary unit
Time (h)
Per3
Time: P < 0.01
Color: P = 0.85
Interaction: P < 0.01
Arbitrary unit
Time (h)

Figure 4

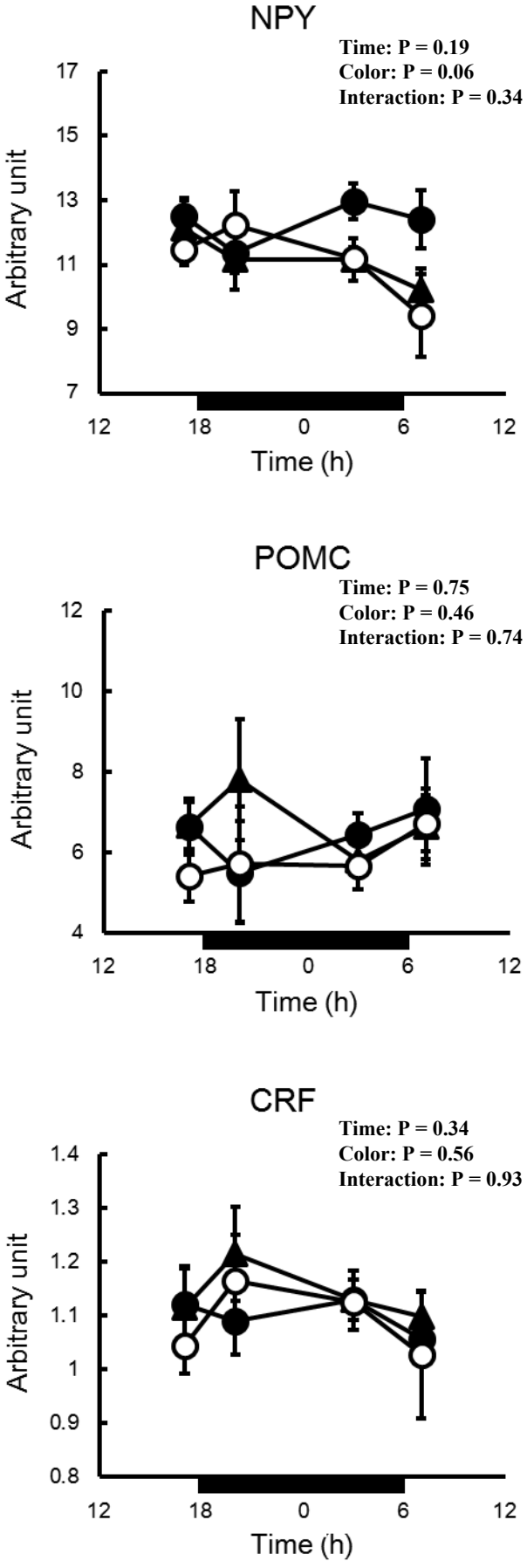
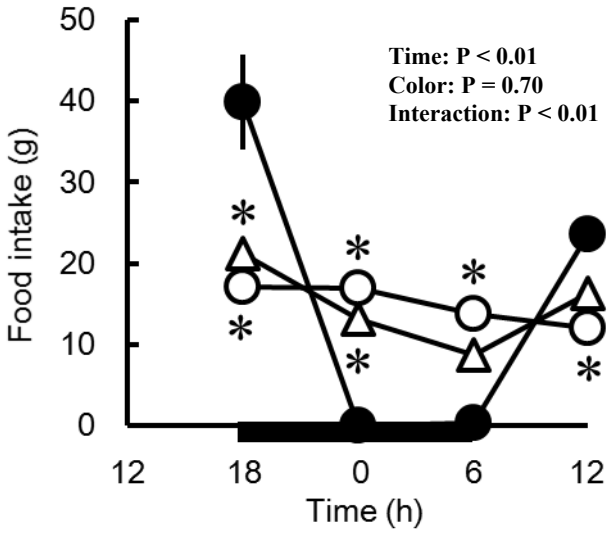

NPY
Time: P = 0.19
Color: P = 0.06
Interaction: P = 0.34
Arbitrary unit
17
15
13
11
9
7
12
18
0
6
12
Time (h)
POMC
Time: P = 0.75
Color: P = 0.46
Interaction: P = 0.74
Arbitrary unit
12
10
8
6
4
12
18
0
6
12
Time (h)
CRF
Time: P = 0.34
Color: P = 0.56
Interaction: P = 0.93
Arbitrary unit
1.4
1.3
1.2
1.1
1
0.9
0.8
12
18
0
6
12
Time (h)

Figure 5


Time: P < 0.01
Color: P = 0.70
Interaction: P < 0.01
Food intake (g)
50
40
30
20
10
0
12
18
0
6
12
Time (h)