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To cite this article: Ji Yong Choi, Na Na Kim, Young-Ung Choi & Cheol Young Choi (2017) Changes in circadian parameters of humbug damselfish, *Dascyllus aruanus* according to lunar phase shifts in Micronesia, *Biological Rhythm Research*, 48:3, 475-483, DOI: [10.1080/09291016.2016.1275395](https://doi.org/10.1080/09291016.2016.1275395)

To link to this article: <http://dx.doi.org/10.1080/09291016.2016.1275395>



Published online: 06 Jan 2017.



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Changes in circadian parameters of humbug damselfish, *Dascyllus aruanus* according to lunar phase shifts in Micronesia

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ABSTRACT

The objectives of this study were to test the nighttime effects of the lunar phase on circadian rhythm in the humbug damselfish, *Dascyllus aruanus*. We measured moonlight intensities at eight different phases across the lunar cycle. At each lunar phase, the circadian rhythm was evaluated by measuring the clock genes *cryptochrome 1* and *period 2*. In addition, we measured *arylalkylamine N-acetyltransferase 2* (*AANAT2*), melatonin and *melatonin receptor 1* (*MT-R1*). The moonlight intensity was highest at full moon and lowest during the waning crescent. Clock gene expression was highest during the full moon compared to the other phases. By contrast, the plasma concentrations of *AANAT2* and melatonin and the *MT-R1* mRNA expression were highest during the full moon phase. Our results suggest that moonlight affects circadian rhythm patterns in the humbug damselfish. There is a need to investigate potential other physiological effects of lunar phase shifts.

ARTICLE HISTORY

Received 2 December 2016
Accepted 6 December 2016

KEYWORDS

AANAT2; circadian rhythms; clock genes; humbug damselfish; lunar phase; melatonin

1. Introduction

Light is an important environmental factor that controls many physiological phenomena in fish (Jin et al. 2009). As light hits an organism's light receptors, different wavelengths may affect secretion induction or inhibition of various hormones, as well as other physiological responses including fertility, growth and behavior (Pierce et al. 2008). The origin of the light during daytime is the sun, but light also reaches the earth at nighttime from the moon that reflects light of the sun. The moon revolves around the earth every 27.3 days (Guoqing 2005) and changes phase depending on its relative position to the Earth. As a result, there is a cyclical variation both in the intensity of the moonlight that reaches the surface and in the tidal amplitude and electromagnetic fields (Leatherland et al. 1992). Changes in the lunar phase have been reported to be an important environmental factor that affects physiological changes such as reproductive cycle and strategy in fish (Ikegami et al. 2014). For example, Takemura et al. (2010) reported that the moon in tropical regions, where smaller changes in

water temperature and photoperiod are observed, is an important environmental factor causing physiological changes in fish.

Light regulates the biological rhythm through so-called circadian clocks. These clocks interact closely with many *in vivo* physiological, biochemical and behavioral functions (King & Takahashi 2000). There are a variety of clock genes, called pacemakers of the circadian rhythm, that regulate the biological rhythm in many organisms including fish (Nanako et al. 2012). Examples of such clock genes are *period2* (*Per2*) and *cryptochrome1* (*Cry1*), which are both controlled by light. *Per2* shows high expression during the day and decreased expression at night (Dunlap 1999; Kim et al. 2012). *Cry1* is also well known as a gene that is rapidly induced when stimulated by light in biorhythm oscillators (Cermakian et al. 2002; Besharse et al. 2004).

In addition to clock genes, melatonin is a well-known factor that controls biorhythm. Melatonin is a hormone that is mainly produced in the pineal gland and retina (Klein et al. 2002). Melatonin is synthesized from the neurotransmitter 5-hydroxytryptamine by the arylalkylamine N-acetyltransferase (*AANAT*) enzyme whose activity is inhibited by light and secreted into the plasma at night-time (Iuvone et al. 2005; Klein 2007). Melatonin not only functions as a neuroendocrine transporter that regulates biological rhythm (Falcón et al. 2007), but also performs a variety of physiological functions, such as antioxidant and immune enhancement in fish (Wu & Swaab 2005; Kim et al. 2014).

Melatonin functions by melatonin receptors (*MT-Rs*), which belong to the G-protein coupled receptor superfamily. *MT-Rs* are distributed throughout the vertebrate central nervous system and ganglia and play a role in controlling melatonin to perform physiological functions (Reppart et al. 1996; Park et al. 2013). Dubocovich et al. (2000) reported that there are three types of *MT-Rs* (*MT-R1*, *MT-R2* and *MT-R3*). It has been shown that *MT-R1* is mainly responsible for recognizing seasonal and environmental changes in the brain (in the hypothyseal pars tuberalis and hypothalamic suprachiasmatic nucleus) and that it plays a regulatory function of biorhythm (Reppart et al. 1996).

To investigate changes in the biological rhythm in a reef fish based on lunar phase changes, we conducted a study in a Pacific coral reef area located in the equatorial region (Chuuk Lagoon), of Micronesia. The aquatic environment in Micronesia is particularly healthy and the conditions are suitable for biological studies of aquatic life (FishBase 2002). The study area is surrounded by a wide coral reef area (fringing reefs, barrier reefs and atolls). The coastal area, which is home to over 2200 marine species, has a shallow average depth with relatively little marine pollution (Hasurmai et al. 2005). In this region, there is very limited exposure to artificial light at night, unlike in more urban areas. Artificial light contamination makes it difficult to study the effects of natural moonlight on marine life. For these reasons, Micronesia (Chuuk Lagoon) is the optimal place to investigate effects of lunar phases and moonlight intensity on fish.

In order to investigate the effect of lunar phase changes on the biological rhythm of a coral reef fish, we measured the intensity of the moonlight reaching the coastal surface in the Chuuk region. Furthermore, we measured changes in the clock genes *Per2* and *Cry1*, as well as *AANAT2*, melatonin and *MT-R1*. Measurements were taken at eight different moon phases using the humbug damselfish (*Dascyllus aruanus*) as study species.

2. Materials and methods

2.1. Experimental fish and lunar phases

This study was conducted from 24 June until 20 July 2015, on Weno island in the Chuuk Lagoon, Micronesia (7°27'N; 151°53'E). We collected 10 *D. aruanus* individuals at each lunar phase (see below), from the coast outside of the Korea South Pacific Ocean Research Center (KSORC) located on western Weno island. Fish were caught using fence and hand nets and were then kept in small sea cages [3 m (horizontal) × 3 m (vertical) × 1.5 m (height)] outside the KSORC for one day until the commencement of the experiment. Ten new fish were caught at each of the eight lunar phases. Fish were not supplied with food but had access to natural food from the sea. For our experiment, we divided one moon cycle into eight lunar phases: first quarter (June 24); waxing gibbous (June 28); full moon (July 2); waning gibbous (July 6); last quarter (July 9); waning crescent (July 13); new moon (July 16); waxing crescent (July 20). We measured the moonlight intensity (lux) at each lunar phase based on changes on the water surface of sea cage at 02:00. This was done using a PHOTO-RADIOMETER (HD 2102.1; Delta OMH CO., Caselle di Selvazzano, Italy).

Plasma and tissue samples were collected at nighttime (02:00 am) under dim light conditions maintained using an attenuated white fluorescent bulb. To minimize stress prior to blood collection, the fish were anaesthetized using 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Prior to tissue collection, the fish were killed by spinal transection. The brain tissues were removed from the fish, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. Plasma samples were separated by centrifugation (4 °C, 1000 *g* for 15 min) and stored at −80 °C until further analysis.

2.2. Quantitative polymerase chain reaction (qPCR)

We used qPCR to determine the relative expression of *Cry1* and *MT-R1* mRNA from total RNA extracted from both the brain tissue. Primers for qPCR were designed with reference to known humbug damselfish sequences: *Cry1* forward (5'-TCT ACA CCG ATG CCA CCA A-3') and reverse (5'-AGC TGG AGA AGA TCC AGA AG-3') primers; *MT-R1* forward (5'-GGT CACTCG CCT CAT CTG-3') and reverse (5'-ACG GAG TTC TTG TCG CTG TA-3') primers; and β -actin (internal control) forward (5'-TCG AGC ACG GTA TTG TGA CC-3') and reverse (5'-GAC CCA GAT CAT GTT CGA GA-3') primers. The PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR was performed as follows: 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s and annealing at 55 °C for 20 s. To confirm consistency, each experimental group was run in triplicate, whereas the internal control was run in duplicate. The efficiency of the reactions was determined by performing a qPCR. All data were expressed as the difference with respect to the corresponding β -actin-calculated cycle threshold (Δ Ct) levels. The calibrated Δ Ct value ($\Delta\Delta$ Ct) for each sample and internal control (β -actin) was calculated
$$\Delta\Delta\text{Ct} = 2^{-(\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{internal control}})}$$

2.3. Plasma parameters analysis

Plasma *Cry1*, *Per2*, *AANAT2* and melatonin concentrations were analyzed using an immunoassay technique. The ELISA kits cryptochrome 1 (Cat. no. MBS041774; Mybiosource, USA), *period circadian protein 2* (MBS108495; Mybiosource), *arylalkylamine N-acetyltransferase* (MBS021281; Mybiosource) and Fish Melatonin (MBS013211; Mybiosource) were used.

3. Results

3.1. Change in moonlight intensity

The intensity of moonlight was highest at full moon (10 lux) and lowest at the waning crescent (1.9 lux). We were not able to measure the moonlight intensity at new moon due to the weak light intensity (Table 1).

3.2. Brain *Cry1* mRNA expression

The *Cry1* mRNA expression in the brain was highest at the waxing gibbous and full moon phases that were significantly higher compared to all other lunar phases (Figure 1). The lowest expression was observed at the waning crescent (Figure 1).

Table 1. Value of the intensity (lux) of moonlight that reached the water surface in different lunar phases.

Experimental date	Lunar phase	Intensity (lux)
Jun. 24	First quarter moon	5.2
Jun. 27	Waxing gibbous	7.2
Jul. 1	Full moon	10.1**
Jul. 4	Waning gibbous	6.9
Jul. 9	Last quarter moon	4.8
Jul. 12	Waning crescent	2.9*
Jul. 16	New moon	N/A
Jul. 19	Waxing crescent	3.2

Notes: The asterisks indicate lowest (*) and highest (**) moonlight intensities, respectively. N/A: not applicable.

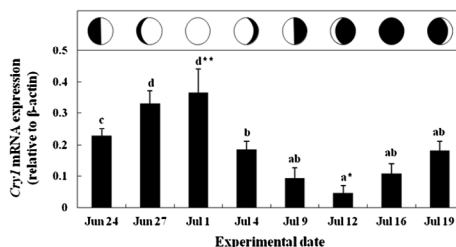


Figure 1. Change in *Cry1* mRNA expression in the humbug damselfish brain across eight lunar phases. Brain tissue was obtained from the fish at night-time (02:00 am). The lunar phases are indicated by schematic moon images: ☾, first quarter moon; ☽, waxing gibbous; ☉, full moon; ☾, waning gibbous; ☾, last quarter moon; ☾, waning crescent; ☾, new moon; and ☾, waxing crescent.

Notes: The asterisks indicate the lowest (*) and highest (**) values, respectively. Values with dissimilar letters are significantly different ($p < 0.05$), and all values are means \pm SE ($n = 10$).

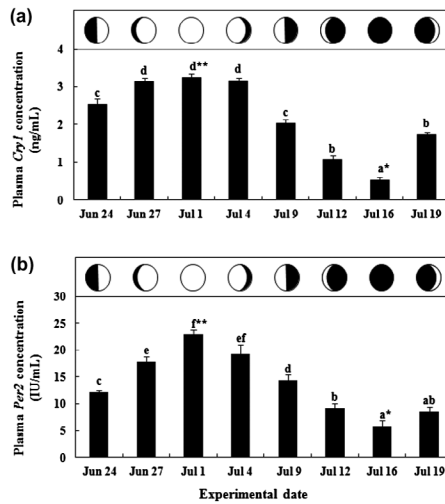


Figure 2. Changes in plasma concentrations of *Cry1* (a) and *Per2* (b) in the humbug damselfish across eight lunar phases. Plasma samples were obtained from the fish at night-time (02:00 am). The lunar phases are indicated by schematic moon images: ☾, first quarter moon; ☽, waxing gibbous; ☽, full moon; ☾, waning gibbous; ☾, last quarter moon; ☾, waning crescent; ☾, new moon; and ☽, waxing crescent. Notes: The asterisks indicate the lowest (*) and highest (**) values, respectively. Values with dissimilar letters are significantly different ($p < 0.05$), and all values are means \pm SE ($n = 10$).

3.3. Plasma *Cry1* and *Per2* concentrations

The plasma concentration of *Cry1* was highest at the waxing gibbous, the full moon and the waning gibbous compared to all other lunar phases. The concentration obtained at the new moon phase was significantly lower compared to all other lunar phases [Figure 2(a)]. The *Per2* plasma concentration exhibited similarities to both the brain *Cry1* mRNA expression and the *Cry1* plasma levels. The concentration of *Per2* was highest at the full moon (albeit not significantly different from the waning gibbous). The expression at the new moon was significantly lower compared to all other lunar phases except for the waxing crescent [Figure 2(b)].

3.4. Plasma *AANAT2* and melatonin levels

In terms of the *AANAT2* plasma concentration, we observed that it was significantly lower at the waxing gibbous and the full moon, compared to at all other lunar phases, whereas it peaked at full moon [Figure 3(a)]. The pattern observed in the plasma concentration of melatonin was similar to that observed in *AANAT2*. The melatonin levels were significantly higher at the new moon compared to at all other phases and lowest at the full moon and waning gibbous [Figure 3(b)].

3.5. Brain *MT-R1* mRNA expression

The pattern for *MT-R1* mRNA expression was the inverse of that observed for melatonin concentration: the highest expression was at the waning crescent and new moon lunar phases, whereas the lowest expression was found at the waxing gibbous and full moon (Figure 4).

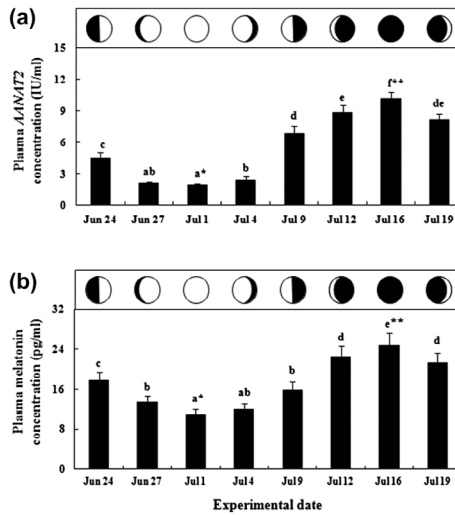


Figure 3. Changes in plasma concentration of AANAT2 (a) and melatonin (b) in the humbug damselfish across eight lunar phases. Plasma samples were obtained from the fish at night-time (02:00 am). Lunar phases are indicated by schematic moon images: ☾, first quarter moon; ☽, waxing gibbous; ☽, full moon; ☾, waning gibbous; ☾, last quarter moon; ☾, waning crescent; ☾, new moon; and ☽, waxing crescent. Notes: The asterisks indicate the lowest (*) and highest (**) values, respectively. Values with dissimilar letters are significantly different ($p < 0.05$), and all values are means \pm SE ($n = 10$).

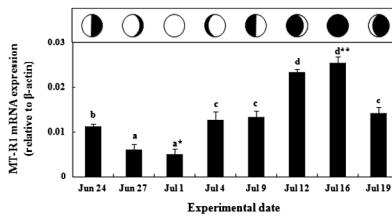


Figure 4. Change in brain *MT-R1* mRNA expression level in the humbug damselfish across eight lunar phases. Brain tissue was obtained at night-time (02:00 am). Lunar phases are indicated by schematic moon images: ☾, first quarter moon; ☽, waxing gibbous; ☽, full moon; ☾, waning gibbous; ☾, last quarter moon; ☾, waning crescent; ☾, new moon; and ☽, waxing crescent. Notes: The asterisks indicate the lowest (*) and highest (**) values, respectively. Values with dissimilar letters are significantly different ($p < 0.05$), and all values are means \pm SE ($n = 10$).

4. Discussion

To investigate the effect of moon phases and moonlight intensity on the biological rhythm of humbug damselfish, we measured changes in expression levels of genes involved in biorhythm regulation (*Cry1* and *MT-R1*) as well as plasma hormone levels (*Cry1*, *Per2*, *AANAT2* and melatonin). We found that the expression of *Cry1* in the brain and the plasma concentrations of *Cry1* and *Per2* showed similar patterns. The pattern showed a peak at full moon, whereas the lowest values were observed at the waning crescent (*Cry1* mRNA expression) or the new moon (*Cry1* and *Per2* plasma concentrations).

Sugama et al. (2008) showed similar results in another coral reef fish, the golden rabbitfish (*Siganus guttatus*), in which they found that the *Per2* mRNA expression level in the pineal

gland was approximately three times higher at the full moon phase than that observed at the new moon phase. They suggested that this was a direct effect of the moon phase change and proposed that *Per2* functions as a “circalunar oscillator”.

Studies of the correlation between *Cry1* and moonlight in aquatic organisms have been limited to only a small number of stony corals and fishes. In addition, studies of expression patterns have reported both different causes (reproductive cycle, intensity of moonlight and intrinsic clock) and different effects depending on the species (Levy et al. 2007; Fukushiro et al. 2011; Hoadley et al. 2011). For example, Levy et al. (2007) reported that the *Cry1* expression in a coral (*Acropora millepora*) was significantly increased at the new moon phase compared to at the full moon. They suggested that this was due to differences between the intensity of the moonlight and the light period.

In agreement with the previous study by Sugama et al. (2008), our study also indicated that the highest plasma *Per2* concentration occurred during the full moon phase, during which the moonlight intensity is strongest. By contrast, the lowest concentration was observed during the new moon phase. This suggests that the expression of *Per2* mRNA in humbug damselfish is influenced by moonlight intensity. The *Cry1* expression showed a similar pattern suggesting that *Cry1* expression in humbug damselfish is likely also regulated by the moonlight intensity. Our results also indicate that changes in these clock genes may be caused by moonlight, which is of weak intensity compared to sunlight.

We investigated the plasma concentrations of *AANAT2*, an enzyme that synthesizes melatonin, melatonin itself and the expression of the melatonin receptor, *MT-R1* mRNA over the course of one lunar cycle. In contrast to the expression and plasma level of the clock genes *Cry1* and *Per2*, the plasma concentrations of *AANAT2* and melatonin and the expression level of *MT-R1* mRNA were the highest during the new moon phase. In a similar study, Kashiwagi et al. (2013) reported that the expression of *AANAT1* mRNA in the retina of golden rabbitfish during the new moon phase was approximately four times higher compared to during the full moon phase. This may be due to the inhibition of *AANAT1* activity by the strong moonlight at full moon. Oliveira et al. (2010) reported that the melatonin plasma concentration in the Senegal sole, *Solea senegalensis*, kept in a shaded tank was significantly higher compared to that found in a control group that had not been reared under shade. This suggests that the activity of the melatonin synthesis system is inhibited by moonlight. Thus, a melatonin synthesis-inhibiting mechanism appears to have been activated by light. This resulted in an *MT-R1* mRNA expression level and plasma melatonin and *AANAT2* concentrations that were at their lowest around the moonlight-maximized full moon phase. This suggests that moonlight plays a role as an environmental factor that causes biological rhythm changes even at night in the absence of directly sunlight irradiation.

In conclusion, based on our results, we suggest that moonlight can induce changes in biological rhythm-related factors including melatonin secretion though the effect of clock genes in this species of fish. In particular, the expression of *Cry1* seems to be controlled by the intensity of moonlight, based on lunar phase changes, in the humbug damselfish. However, because changes in the lunar phase are likely to have complex effects on the physiological function of fish, future, more detailed studies will be needed to fully understand the mechanism involved.

Acknowledgements

This research was supported by the project 'Innovative marine production technology driven by LED-ICT convergence photo-biology', and by the project 'Development of the eco-friendly copper alloy net for antifouling and the fish farming cage'.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was funded by Ministry of Oceans and Fisheries, Korea, and by Korea Institute of Ocean Science and Technology [PO01285].

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