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Determination of the daily amount of melatonin in *Lepidurus arcticus* in natural conditions

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ABSTRACT

The role of melatonin for the biological rhythms in invertebrates has remained highly controversial. We investigated the presence and daily variation of melatonin in *Lepidurus arcticus*. Melatonin concentration was measured in a wild population of *L. arcticus* collected at 79°North during the Arctic summer. We collected $n = 24$ animals and divided them in $n = 4$ time-points: 00:00, 06:00, 12:00, 18:00 local time. At every time-point, the melatonin concentration was determined with ELISA. Our first result showed that melatonin was present in *L. arcticus*. In every sample and at every time-point, we found an amount of melatonin. As expected, statistical analysis shows that there is no difference in concentration between the time-points. For this reason, if we cannot support any results concerning any peak during the 24 h, at the same time we cannot support any daily variation. Our study is the first report on the presence of melatonin in polar freshwater crustacean, *L. arcticus*.

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Polar; melatonin; circadian; Arctic; photoperiodism

Introduction

All living organisms have “biological clocks” that regulate physiological and behavioural functions by means of rhythms similar to the geophysical rhythms of the earth. These rhythms have an evident adaptive value to enable organisms to anticipate and, hence, to prepare for predictable changes in their environment (King and Takahashi 2000). The biological clocks are synchronized with the environmental cycles, the most prominent of which is the 24-h light–dark (LD) cycle. However, in polar environments, the strength of this time cue is greatly reduced approaching summer and winter solstices, when the sun never sets or never rises (Folk et al. 2006). In general, the role of circadian clocks in extreme environments, ex. caves, the deep sea and the polar regions, has received very little attention (van Oort et al. 2005, 2007; Pasquali and Sbordoni 2014; Pasquali 2015; Pasquali et al. 2016).

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In animal physiology, many metabolic processes show daily variation that follow the photoperiodic cycle, for example, hormones like melatonin. The main function of melatonin, which has maintained a high evolutionary conservation, is to transduce the photoperiodic information from the environment to the organism, modulating physiological rhythms according to the LD cycle (Hardeland and Poeggeler 2003).

The presence of melatonin has been reported in many organisms, from bacteria to plants (Balzer and Hardeland 1991; Tilden et al. 1997; Reiter and Tan 2002), and from invertebrates to vertebrates (Gern et al. 1986; Vivien-Roels and Pévet 1993). Melatonin has different functions in the organism: energetic metabolism, enhancement of immunity, free radical scavenging and is a key component of the biological clock responsible for modulating physiological rhythms (Hardeland and Poeggeler 2003; Pandi-Perumal et al. 2006; Bentkowski et al. 2010).

In the group of crustaceans, melatonin has been shown to be involved in several physiological processes. It has been shown that melatonin modulates locomotor activity, glucose/lactate levels, neurotransmitter release, limb regeneration and the regulation of moulting (Sainath and Reddy 2010; Schwarzenberger and Wacker 2015).

Melatonin production by the pineal gland is a key aspect of vertebrate circadian organisation, but in invertebrates its role for the biological rhythms has remained highly controversial.

The first evidence of the presence of melatonin in crustaceans was reported by Vivien-Roels and Pévet (1993) by radioimmunoassay in the compound eye of the crab *Carcinus maenas*. Afterwards, other studies have identified melatonin in various species of crustaceans but never on zooplankton. But Markowska and colleagues in 2009 reported for the first time the presence of melatonin in zooplankton. By enzyme-linked immuno-sorbent assay (ELISA), they found the melatonin in *Daphnia magna* also with daily fluctuations in its concentration, that showed a high concentration during the light phase. In 2010, Bentkowski and colleagues confirmed all their previous results, and moreover, demonstrated that melatonin is involved in the regulation of vertical migration in *D. magna* with an effect that appeared more pronounced during the day.

Recently, the debate has received new impetus through the discovery that melatonin controls circadian swimming behaviour in the larvae of an annelid worm. In *Platynereis dumerilii*, melatonin plays an important role to establish a nocturnal behaviour to adjust the vertical position in response to the light. In general, the findings of this study suggest that melatonin is produced and released during the night and that melatonin release is directly controlled by light and/or by the circadian clock (Tosches et al. 2014).

The role of the clock genes and the biological clock in the melatonin synthesis has been demonstrated by Schwarzenberger and Wacker (2015) in *Daphnia pulex*. Beyond the data on the gene expression of the enzyme AANAT in the melatonin synthesis and clock genes, the melatonin concentration determined by ELISA showed a peak in the night with a concentration that increased from the beginning of the dark phase until midnight and decreased until the middle of the light phase.

In the last years, our research has been focused on characterizing circadian organization in the tadpole shrimp *Lepidurus arcticus*, a polar invertebrate representing a largely extinct lineage of arthropods (Fryer 1988). No work on the circadian biology of this fascinating group has been published so far, and behavioural and metabolic rhythmicity

of tadpole shrimps in the high Arctic is of great interest from an eco-evolutionary perspective.

Lepidurus arcticus is the ideal model to investigate issues related to circadian rhythms and food-web ecology because of its presence in melted-water pools in Svalbard (Calizza et al. 2016), and because conspecifics can also be found at a low latitude in the Arctic and sub-Arctic region. Hence, it is possible to perform comparative experiments to confront both latitudinal-behaviour and genetic aspects, and also to assess the heritability of circadian traits.

In this study, we investigated the presence of melatonin in *L. arcticus*. We also investigated to see whether the concentration of melatonin showed a daily variation.

We hypothesized that *L. arcticus* shows the presence of melatonin as in other crustaceans. We also hypothesized that there is no daily variation in melatonin concentration, in particular during the continuous light condition.

To achieve this goal, we conducted a preliminary experiment in natural conditions with continuous light during the Arctic summer when the sun never sets. Samples of *L. arcticus* were taken every 6 h from the natural pond. At every sample point, the melatonin concentration of *L. arcticus* was determined with ELISA.

Material and methods

Study area and sample collection

Lepidurus arcticus (Pallas 1793) specimens were sampled in the Solvatnet pond (78° 56' N, 11°57' E), a small shallow water body located in Ny-Ålesund on the Brøggerhalvøya peninsula on the west coast of the Spitsbergen archipelago. Samples were collected using a hand-held net (0.5-mm mesh size), adult specimens with a carapace length 8 mm were selected on site and quickly transported to the laboratory in a 5-l tank filled with filtered (0.125 mm) pond water and then immediately frozen at −80°C. Animals were collected in August 2018 at four time-points: 00:00, 06:00, 12:00 and 18:00. Continuous light is present during the summer season in the Arctic.

Permits to conduct this research were issued by the Governor of Svalbard Ref. 2014/00729-2a.512 (Fieldwork 2014, RIS-ID 10,011).

Melatonin assay

The specimens were lyophilized for at least 4 h, weight and then homogenized in a tissue grinder with PTFE pestle and glass tube at 100 rpm for 3 min in distilled water and kept in ice. The amount of water was enough to obtain about 800 µg of dry weight of tissue in 100 µl of homogenate. Following the manufacturer's instructions for the immunoassay enzyme (RE54021, IBL-Hamburg, Germany) was used. Melatonin levels were normalized on total protein, determined using the BCA method. The amount of protein was considered independent of the variable (hour of the day). The quantification was repeated three times.

Data analysis

Statistical analyses were performed with STATISTICA7.0 (StatSoft, 2004, Tulsa, OK, U.S.A.). The variation in melatonin concentration for each triplicate was analyzed by paired *t*-test. The variations in melatonin concentration between time-points were analyzed by Kruskal–Wallis non-parametric ANOVA by ranks for independent groups. The level of statistical significance was $p < 0.05$.

Results

Melatonin concentration was measured in a wild population of *L. arcticus* collected in Svalbard at 79°North. We collected $n = 24$ animals with a total length between 8–12 mm and dry weight between 0.0109–0.0829 g (Mean = 0.0244, SD = 0.0227); $n = 6$ animals were collected per each time-point: T0, T6, T12 and T18 (00:00, 06:00, 12:00 and 18:00 local time).

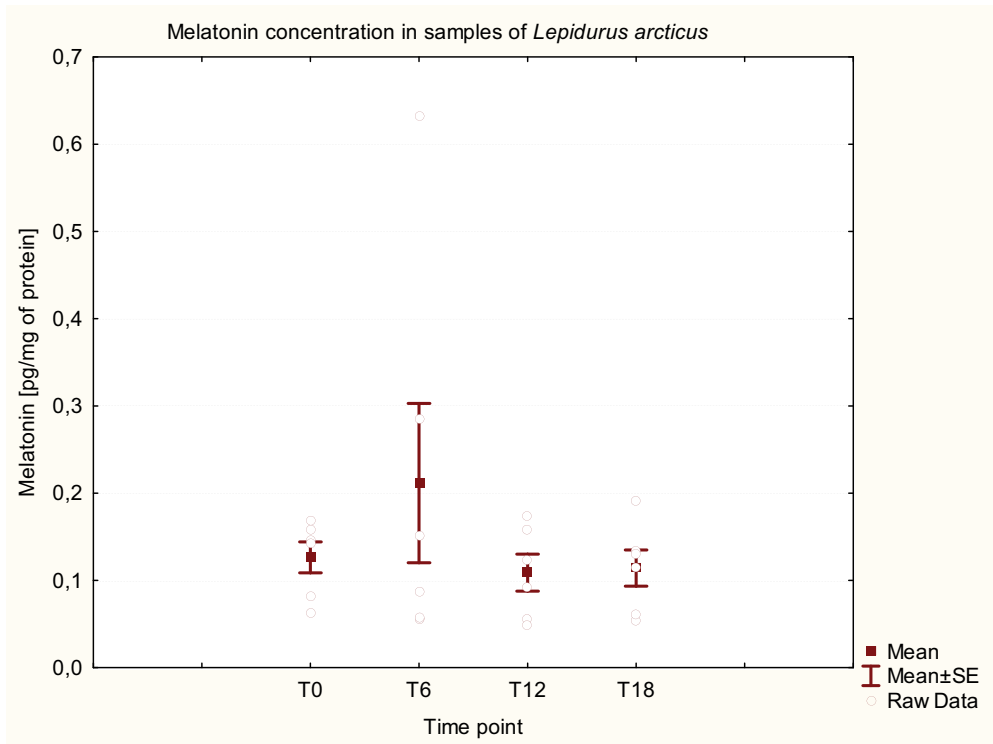
Melatonin was found present in every sample.

Measurements of the hormone concentrations were made in triplicate for each animal. Every triplicate was analyzed by paired *t*-test and no statistical differences were observed for any triplicate (result not shown). At this point, the average of the three measurements was considered as a representative value for each animal. After that, we also analyzed by paired *t*-test the difference between the animals of the same time-point and no statistical difference was observed (result not shown).

Using the ELISA immunoassay, we observed the following concentration (Mean $\pm$ SE) pg melatonin/mg of protein: T0 = 0.126 ± 0.017 ; T6 = 0.211 ± 0.091 ; T12 = 0.108 ± 0.021 ; T18 = 0.114 ± 0.020 (Table 1). Except for T6, all the value of concentration are closed as well as the standard error. Only T6 shows a high value of concentration and high value of the standard error. Checking the raw data (Graphic 1), this effect depended from a single animal, but it shows a normal weight comparable to the other animals (data not showed).

Table 1. Descriptive statistics: mean and standard error (pg melatonin/mg of protein) of melatonin for every time-point.

	Mean	SE
T0	0.126	0.017
T6	0.211	0.091
T12	0.108	0.021
T18	0.114	0.020



Graphic 1. Mean, standard error for every time point and raw data.

By inferential statistics, we verified the presence of statistical differences between the four time-points. We performed a Kruskal–Wallis ANOVA by ranks for the independent group: $H(3, N = 24) = 0.766, p = 0.85$ (Table 2), and no statistical difference was found between the four time-points (Graphic 1).

Discussion

As melatonin is strongly connected to the photoperiod, it plays an important role in animal physiology and behaviour. So, our first aim was to check the presence of melatonin in this species. The second aim was to check the presence of a daily variation.

Our first result showed clearly that melatonin was present in *L. arcticus*. In every sample and at every time-point we found an amount of melatonin. These data are in agreement with previous studies on *Daphnia magna* (Markowska et al. 2009; Bentkowski et al. 2010)

Table 2. Inferential statistics: output of Kruskal–Wallis test between the four time-points.

	Valid – N	Sum of – Ranks
T0	6	84,00000
T6	6	81,00000
T12	6	67,00000
T18	6	68,00000

Kruskal–Wallis test: $H(3, N = 24) = 0.7666667, p = 0.8574$

and *Daphnia pulex* (Schwarzenberger and Wacker 2015). These species are phylogenetic close to the Notostraca.

About the second aim, our results showed that there was not a daily variation of melatonin in *L. arcticus*. Although our result showed a high concentration at the early morning time-point in T6. The raw data showed clearly that this value depends on the single animal, an outlier but that did not differ from the other animals in weight or in other parameters.

As expected, statistical analysis did not support the presence of a difference in concentration between the time-points. For this reason, if we cannot support any results concerning any peak during the 24 h, at the same time we cannot support any daily variation.

Our study is the first report on the presence of melatonin in Notostraca and it is the first report on melatonin in the polar freshwater crustacean, *L. arcticus*. However, the absolute quantity (relative to the amount of proteins) is considerably reduced compared to that found in Cladocera living in latitudes where the biological significance of melatonin is stronger due to variations in LD cycles. This could be a pseudo loss of function associated with the under utilization of this system of circadian rhythm regulation, which relies on sunlight as a stimulus.

Our study is a preliminary study and it has some limitations. First, a continuous darkness condition in laboratory would be necessary in order to measure the melatonin in that condition. Second, an anatomic study should be useful to localize the presence of the melatonin in the *Lepidurus*'s tissue. Moreover, the number of samples, together with that of time-points during the 24 h (every hour) and of animals examined per time-point, could be increased. Lastly, it would be useful to check the amount of melatonin in the algae and in the other potential food sources present in the lake, including other animal prey on which *L. arcticus* is known to feed (Calizza et al. 2022). Indeed, *L. arcticus* has been shown able to switch from a fully predaceous diet, with *Daphnia* spp. among its preferred prey, to an omnivorous diet including lake vegetation when the stoichiometric quality of the latter increases due to nitrogen inputs in the lake from migratory bird fauna that colonizes lake catchments during summer (Calizza et al. 2022). This opens the intriguing hypothesis that changes in food consumption associated to the structure of the food web may modulate melatonin concentration in the consumer.

Further study of melatonin in polar crustaceans will make it possible to understand the role of this hormone in invertebrate and its role in biological rhythms.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

VP and AS equally involved in the conception and design, analysis and interpretation of the data; drafting of the paper, revising it critically for intellectual content; and the final approval of the version to be published. EC involved in the, analysis and interpretation of the data; drafting of the paper, revising it critically for intellectual content; and the final approval of the version to be published; and that all authors agree to be accountable for all aspects of the work.

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