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ORIGINAL PAPERS

Disrupted darkness: the impact of anthropogenic light at night on melatonin secretion of *Hermodice carunculata* (Polychaeta, Annelida)

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Abstract

Anthropogenic light at night (ALAN) can have serious impacts on marine environments. Several studies have demonstrated that ALAN disrupts melatonin production, a hormone critical for regulating circadian rhythm. In this study, the effects of ALAN on melatonin and two of its related indolamines were investigated in the annelid *Hermodice carunculata*. Specifically, melatonin, serotonin and tryptamine levels were measured every three hours over a 24 h period in the heads of the annelids maintained under constant light and a 12 h light/12 h dark photoperiod, representing control conditions. Melatonin concentration was quantified using an enzyme immunoassay, while serotonin and tryptamine were analyzed by liquid chromatography–tandem mass spectrometry. Melatonin levels in annelid heads remained relatively constant with a pronounced peak at 11:00. A similar pattern was observed under constant light, but the melatonin peak shifted to 14:00. However, serotonin and tryptamine did not exhibit any significant diurnal variations due to constant light exposure. These findings suggest that melatonin secretion in *H. carunculata* is sensitive to ALAN, whereas its related indolamines are potentially not. The disruption of *H. carunculata*'s melatonin secretion pattern may affect its night-time behavior and reproduction, highlighting the need for further studies to assess the ecological effects of ALAN on various marine invertebrates.

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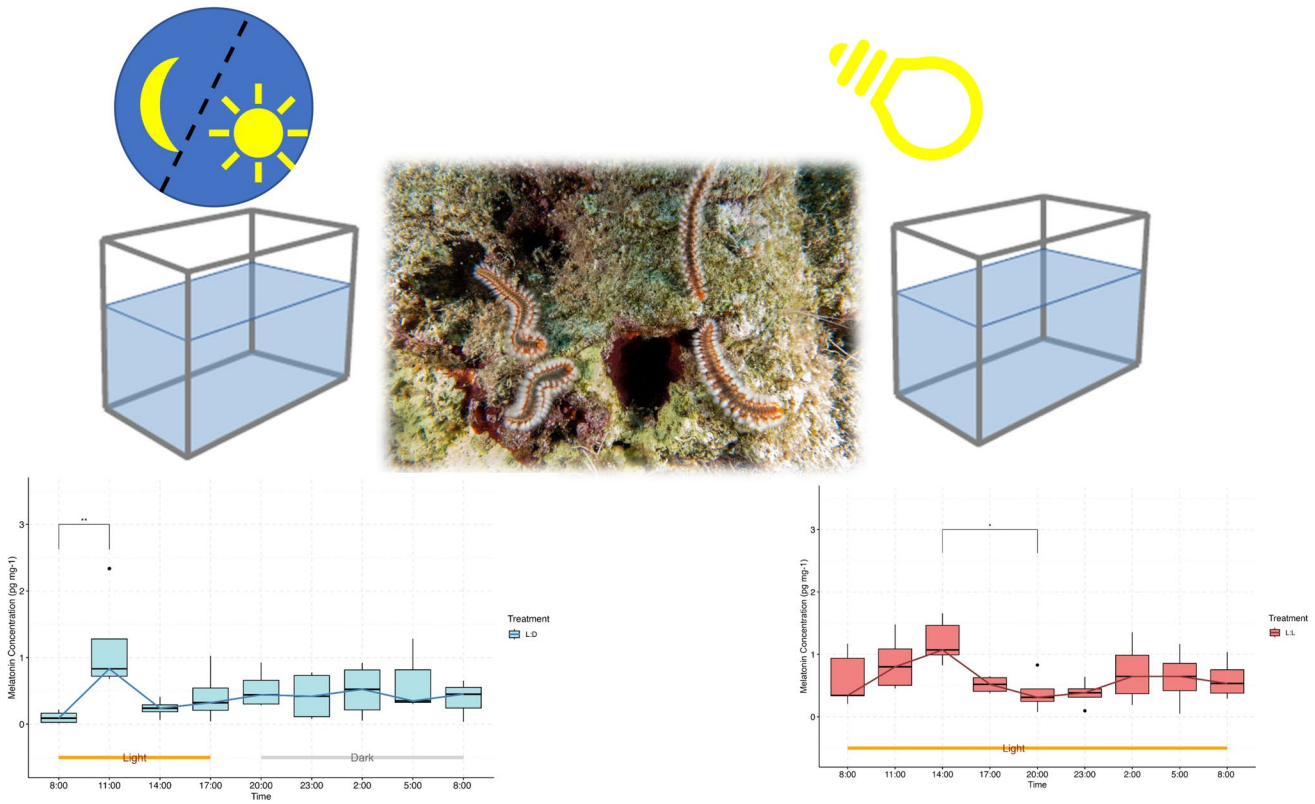
Graphical abstract



Keywords Hormones · ALAN · Invertebrates · Annelids · Circadian rhythm

1 Introduction

Coastal ecosystems and marine biodiversity are threatened by human activities and climate change [1, 2]. One significant anthropogenic pressure impacting these ecosystems is light pollution, which is observed by the adverse effects of artificial lighting on natural habitats [3, 4].

It has been estimated that 22.2% of the world's coastlines are exposed to anthropogenic light at night (ALAN) [5], while its spatial range is globally increasing by around 6% per year [6]. At the same time, skyglow extends the spatial effect of anthropogenic night light through the reflection of light from cloud cover [7]. ALAN with respect to light intensity and differences in light quality (color spectrum) may have serious physiological and ecological consequences for all taxa [6] as species have evolved under natural conditions of moonlight, sunlight and starlight [5]. The extent of these consequences may be crucial given that a great proportion of the global biodiversity is active during the night with more than 60% of all invertebrates described as nocturnal [8].

Most species have developed endogenous timekeeping mechanisms which are also called biological rhythms as an adaptation to day–night cycles [9]. Endocrine systems may be among the first to be affected by ALAN, as hormones are sensitive to environmental changes, leading to behavioral and fitness variations [10, 11]. Melatonin (*N*-acetyl-5-methoxytryptamine), also known as the "hormone of darkness", can be particularly affected by ALAN. It is present in all taxa, from bacteria and fungi to plants and animals, and its primary biological function is as a free radical scavenger and an antioxidant [12, 13]. The role of this hormone expanded through evolution to encompass a range of additional activities, including its function as a key regulator of circadian rhythm [14, 15]. Given that melatonin synthesis is influenced by photoperiod and triggered by the light–dark cycle, endogenous circadian rhythms may be disrupted by ALAN through the suppression of nocturnal melatonin production [16]. Changes in the melatonin secretion pattern may also affect the reproductive rhythm as shown by its effect on gonadotropins' gene expression in European perch [17]. Furthermore, animal stress response may be impacted as it has been documented that melatonin may reduce stress effect in teleost fish ([18] and references therein). The antioxidant activity

of melatonin may also be affected, resulting in reduction of biological fitness [13, 16].

Melatonin was first isolated from the bovine pineal gland by Lerner et al. [19] and has since been found in several vertebrate tissues (e.g., retina, gastrointestinal track, salivary glands etc.) (comprehensive review in [20]). In invertebrates, melatonin has been identified in various organs and tissues, including the cerebral ganglion, eyes and reproductive tissues (e.g., [21–23]). The melatonin biosynthetic pathway (Fig. 1) was first described in the 1960s in bovine pineal gland and rat liver [24, 25]. This pathway in invertebrates is deemed to be similar to that in vertebrates although further research is needed [26, 27]. Specifically, some precursors such as serotonin, as well as enzymes that catalyze several steps in the melatonin biosynthetic pathway, are known to be present in various invertebrates [27]. In general, two main strategies for serotonin synthesis exist across different taxa, both leading to melatonin production [26]. The first strategy is common in microorganisms and plants and involves the decarboxylation of tryptophan to tryptamine to form serotonin by the action of tryptamine 5-hydroxylase [26]. The second strategy, which is known in vertebrates, is through the decarboxylation of tryptophan to 5-hydroxytryptophan and then into serotonin by the action of tryptophan hydroxylase and an aromatic amino acid decarboxylase, respectively [26]. However, tryptamine has also been mentioned as a precursor of serotonin in mammals [28]. Subsequently, serotonin is converted to *N*-acetylserotonin (NAS), through *N*-acetyltransferase (NAT) activity, while *N*-acetylserotonin *O*-methyltransferase (ASMT) catalyzes NAS conversion into melatonin [26, 27].

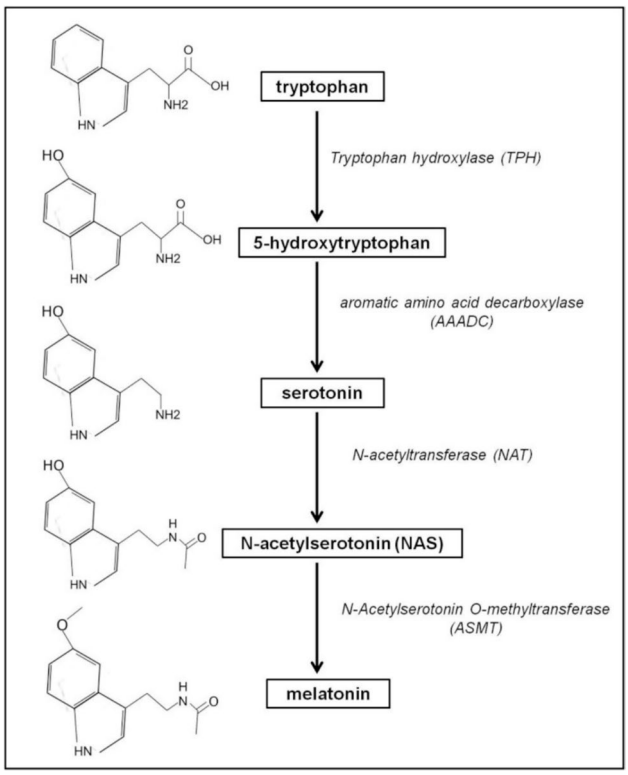


Fig. 1 Melatonin biosynthetic pathway in vertebrate's pineal gland (modified figure from [16]). Enzymes catalyzing the different synthesis steps are shown in italics

Although melatonin has been found in several invertebrates (e.g., [21, 29, 30]), few studies have investigated the effects of light on melatonin concentrations and potential physiological and behavioral changes (e.g., [16, 31–33]). In annelids, melatonin may be involved in the reproduction cycle by suppressing the levels of "juvenile" hormone, which controls growth, regeneration and reproduction [34]. Furthermore, melatonin plays an important role in circadian ciliary swimming regulation [35], which may be evolutionary connected to sleep regulation in vertebrates [36]. However, to our knowledge, no studies have examined the effects of light on melatonin concentrations in annelid species.

To investigate the potential effects of ALAN on annelid's melatonin, we chose the bearded fireworm *Hermodice carunculata* (Pallas, 1766), a large-sized amphinomid annelid, which mostly lives in the hard substrates of the infralittoral zone [37]. The distribution of this species is across the Atlantic, the Red Sea, and the Mediterranean coasts [38, 39]. *H. carunculata* is a predator with high foraging activity during night-time [40]. Specifically, this annelid hides in crevices and under rocks during the day and emerges from dusk to dawn to feed [39, 41]. This nocturnal activity has been observed even under laboratory conditions [40]. Therefore, the nocturnal activity of this fireworm along with its size and high abundances made this a candidate species for the investigation of potential effects of ALAN in melatonin secretion of annelids. In this study, we test whether continuous exposure to ALAN alters the diurnal concentration patterns of melatonin and its related hormones, serotonin and tryptamine, in the annelid *H. carunculata*.

2 Materials and methods

2.1 Animal collection and laboratory conditions

Specimens of *H. carunculata* were collected in February 2023 from the hard substrate of the Alykes area (Crete, Eastern Mediterranean, 35°24′52″N 24°59′18″E) by scuba divers at a depth of 10 m. 150 animals (Stocking density 0.21 individuals L^{-1}) were transferred in a closed re-circulating system in the Marine Ecology and Biodiversity laboratories of the Hellenic Centre for Marine Research (HCMR) to the constant laboratory conditions with a 12 h light/12 h dark photoperiod (L:D 12 h:12 h; light onset at 8:00 am) and a mean temperature of 20.8 °C ± 0.2 °C

(monitoring every 30 min by a temperature logger). The tanks (350 L per tank) were equipped with two fluorescent T5 lamps (Arcadia, one marine white at 10,000 K and one marine blue 420 nm) and light emission measurements were taken using a light meter (LI-250A, LI-COR) from the seawater surface (light intensity of 200 lx; irradiance of 1.88 ± 0.05 W/m^2; Photosynthetically Active Radiation (PAR) of 17.82 ± 0.2 µmol/m^2/s) and the bottom (light intensity of 95 lx; irradiance of 0.86 ± 0.05 W/m^2; PAR 8.66 ± 0.2 µmol/m^2/s). There were no hideouts available in the tanks to ensure that the animals were fully exposed to the respective light conditions and to not cause potential biases. Furthermore, the tanks were covered with black plastic bags in order to avoid external light source effects. Throughout the experimentation period, the animals were fed with frozen sardines every second day ad libitum.

2.2 Experimental setup

In order to investigate the potential effects of ALAN on melatonin concentration of *H. carunculata*, two experimental treatments were performed. Specifically, one experimental tank (control group) was set at a photoperiod of L:D 12 h:12 h (light onset at 8:00 am), while the other one was set at constant light (Group L:L). The photoperiod in the Group L:L was gradually increased by one hour/day before the sampling to acclimate the animals to constant light conditions. In both treatments, the total acclimatization period was 12 days, as indicated by Tilden et al. [31].

Eight individuals (three to five for melatonin and three for serotonin/tryptamine analysis, Table 1) per experimental group were collected every three hours for a 24 h time period. The entire organism was immediately frozen using liquid nitrogen. All samples were stored at − 80 °C until further analysis. During the dark phase, samples were collected under dim red light.

2.3 Melatonin analysis

For all analyses, the annelids' heads (prostomium and peristomium until the first segment) were cut off as they include the multicellular cerebral eyes with photoreceptor cells and the brain [40, 42]. The selection of this body part was also based on the fact that melatonin production has been reported to take place in brain photoreceptors of other annelid species [35]. The heads were homogenized in PBS at ratios of 1:20, 1:30 and 1:40 according to tissue weight and then centrifuged at 1600 rpm at 4 °C for 10 min. The supernatant was used for the quantification of melatonin concentration using of a commercial enzyme immunoassay kit (DRG Melatonin ELISA, DRG Instruments GmbH, Germany) according to the manufacturer's instructions. The kit results were evaluated and showed < 10% of intra-assay and inter-assay coefficient of variation.

2.4 Serotonin and tryptamine analysis

Serotonin, a precursor of melatonin, and tryptamine, a serotonin-related indolamine [43], were measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Similarly with melatonin analysis, annelid heads were cut off and analyzed.

The head tissue from each annelid was weighed and placed in 2-mL Safe-Lock tubes (Eppendorf; Hamburg, Germany) along with two 5 mm stainless steel beads (Qiagen; Hilden, Germany) and 1,000 µL of acetonitrile containing 0.1% formic acid and 0.1% butylated hydroxytoluene, as an antioxidant. Tissue disruption was performed in a TissueLyser II (Retsch, Qiagen; Hilden, Germany) set at 28 Hz for 10 min. The samples were then incubated at -20 °C for 30 min and centrifuged at 14,000 g for 10 min at 4 °C. The supernatants were collected in amber glass vials and spiked with 10 µL of an internal standard solution (*N*,*N*-dimethyl-L-phenylalanine, 0.2 ng µL^{-1}). After brief vortexing, the samples were evaporated to dryness at 30 °C using an EZ-2 centrifugal evaporator (Genevac Ltd.; Ipswich, the United

Table 1 Time points and replicate numbers in each experimental group selected for melatonin, serotonin and tryptamine analysis

	Melatonin		Serotonin		Tryptamine	
Time points	L:D	L:L	L:D	L:L	L:D	L:L
8:00	4	5	3	3	3	3
11:00	4	5	3	3	3	3
14:00	4	5	3	3	3	3
17:00	4	4	3	3	3	3
20:00	4	4	3	3	3	3
23:00	4	4	3	3	3	3
2:00	4	4	3	3	3	3
5:00	3	4	3	3	3	3
8:00	3	4	3	3	3	3

Kingdom) and reconstituted in 200 µL of 20% methanol. Serotonin and tryptamine were analyzed by LC–MS/MS using the chromatographic conditions and the optimized operating parameters for the electrospray ionization source as detailed in Papadaki et al. [44]. The system operated in the positive ion scan mode using dynamic multiple reaction monitoring (d-MRM) for enhanced selectivity and specificity. Two MRM transitions (one quantitative and one confirmatory) were acquired for each analyte and the instrumental parameters of those transitions were thoroughly optimized (Table 2).

2.5 Statistical analysis

The differences in melatonin, serotonin and tryptamine concentrations of annelids between the two treatments (L:D and L:L group) and across the nine different time points of the experiment were assessed using a two-way ANOVA. Shapiro–Wilk and Kolmogorov–Smirnov tests were used to test for the normality of residuals assumption, while Levene's test was used to test for the homogeneity of variances assumption; in cases where the assumptions of ANOVA were violated (i.e., where residuals were not normally distributed), the log transformation ($\log_2(x)$) was used and data met the ANOVA's assumptions. Tukey's HSD test was chosen for the post-hoc comparisons. Box plots displaying the concentration of melatonin, serotonin and tryptamine over time, their median values and the sampling points that were statistically different were also created. The aforementioned analyses were conducted using the packages tidyverse v.2.0.0 [45], ggpubr v.0.6 [46] and ggforce v.0.4.2 [47] in R version 4.4.1 [48].

3 Results

Melatonin was detected in the annelid heads at all time points and for both treatments (Fig. 2). Two-way ANOVA of the 24 h cycle data showed that there is not a statistically significant interaction between treatment and time and that only time had a significant effect on the melatonin concentrations (Table 3). Due to high variability between the specimens, as well as the high number of time points per treatment, the data were tested, separately in two 12 h periods (daytime period 8:00–20:00 and night-time period 20:00–8:00). Two-way ANOVA within the daytime 12 h period (8:00–20:00) revealed that treatment, time and the interaction of time and treatment had a significant effect on the melatonin concentrations (Table 3). Significant differences were observed between the different time points within the L:D treatment (Fig. 2a, Table S1), showing that melatonin levels in the annelid heads had a statistically significant diurnal variation reaching maxima values at 11:00 (mean ± SD: 1.17 ± 0.79 pg· mg^{-1} tissue; $P < 0.01$) (Fig. 2a). In the treatment with constant light conditions, this peak was shifted at 14:00 (Fig. 2b; $P < 0.05$), with the maximum melatonin concentration for the L:L group being 1.20 ± 0.34 pg· mg^{-1} tissue. Furthermore, when comparing the two different treatments within different time points, significant differences were only observed at 8:00, with the average melatonin concentration in the L:L group being significantly higher than in the one of the L:D group ($P < 0.05$). Regarding the night-time 12 h period (20:00–8:00), neither treatment, time, nor their interaction had a significant effect on melatonin concentrations.

Serotonin (Fig. 2c and d) and tryptamine (Fig. 2e and f) were also detected in the annelid heads at all time points and for both treatments. However, in contrast to the melatonin results, in most cases, serotonin and tryptamine concentrations were not significantly affected by treatment, time, or their interaction in any time period (Table 3). The only exception was tryptamine in the daytime12 h period (8:00–20:00), where treatment was found to have a significant effect in the concentrations ($P < 0.05$).

4 Discussion

In this study, melatonin in *H. carunculata* heads was observed to peak during the light period. Similar daytime peaks have also been observed in some crustacean species (e.g., [23, 32]) although the nocturnal secretion in

Table 2 Optimized instrumental parameters for the MRM transitions that were used for the LC–MS/MS analysis of serotonin and tryptamine

Compound	Retention Time (min)	Precursor Ion (m/z)	Fragmentor Voltage (V)	Product ion m/z	Collision energy (eV)	Cell accelerator voltage (V)
Serotonin	1.93	176.9	135	159.8[a]	9	2
				114.9[b]	30	2
Tryptamine	3.50	144.0	158	116.9[a]	20	4
				126.9[b]	20	5

[a]Quantifier ion

[b]Qualifier ion

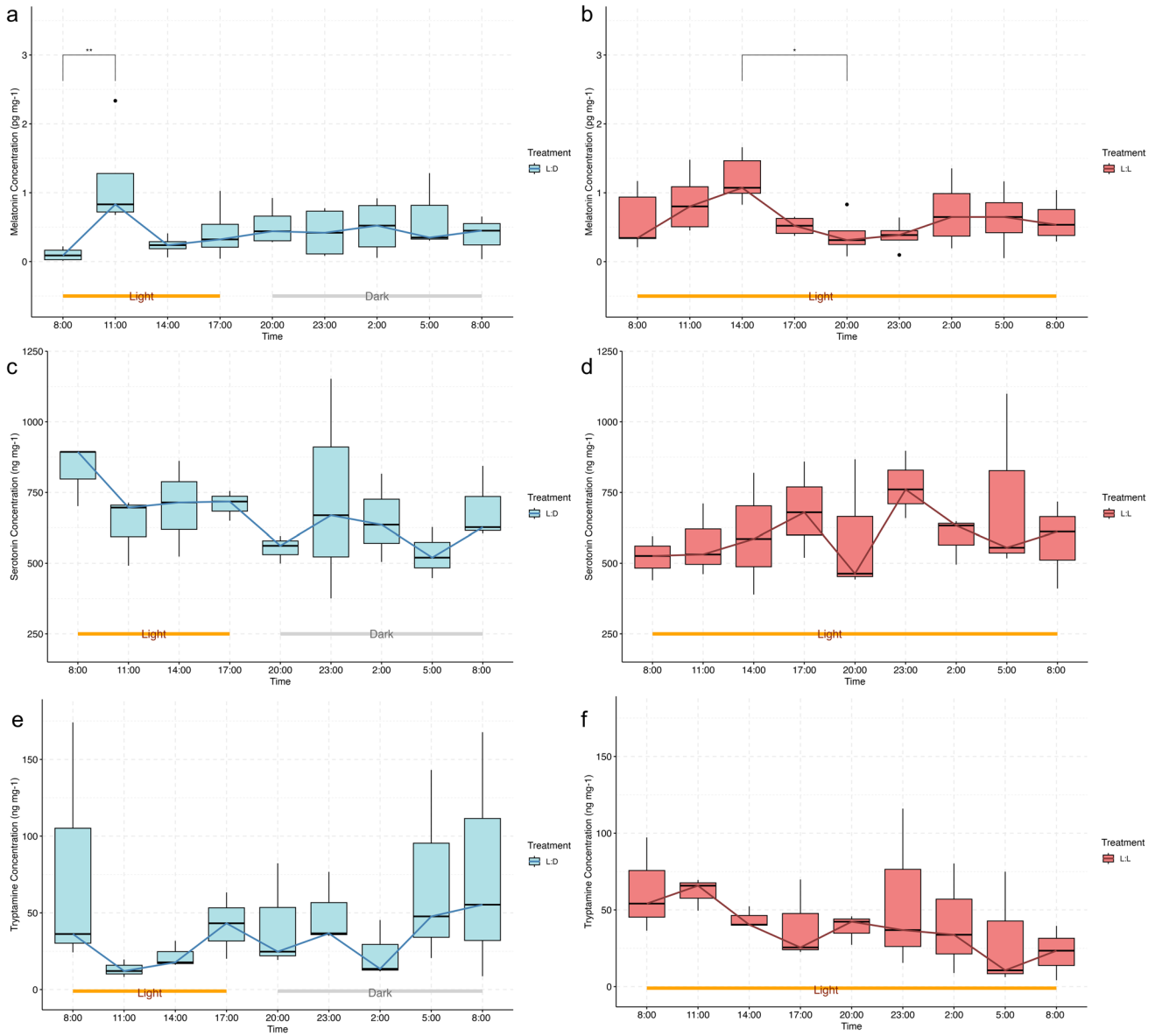


Fig. 2 Box plots of melatonin, serotonin and tryptamine concentrations over time. **a**, **b** Melatonin concentration (pg· mg^{-1} tissue), **c**, **d** Serotonin concentration (ng·g^{-1} tissue) and **e**, **f** Tryptamine concentration (ng/g tissue) in *H. carunculata* heads under a L:D 12 h:12 h photoperiod (blue line) and constant light (red line) over 24 h cycle. Data are presented as median ± SEM ($n = 3$–5 per group for melatonin analysis, $n = 3$ per group for serotonin and tryptamine). Outliers are indicated as dots. Significant differences between the time points are indicated by asterisks (*$P < 0.05$, **$P < 0.01$)

invertebrates is more common (e.g., [21, 30, 35]). In most cases, melatonin signals the onset of night to activate the night-time behavior of organisms [21]. For example, in *Platynereis dumerilii*, a model species of zooplankton, nocturnal melatonin modulates the length and the frequency of ciliary arrests, a behavior associated with different phases of diel vertical migration [35]. Hence, the melatonin secretion of *H. carunculata* during the light period may be related with its nocturnal activity. In the 1960s, Fitzsimons [40] proposed that the diurnal activity rhythm of *H. carunculata* may be regulated by cerebral neurosecretory cells releasing their products into the blood vessels of the prostomial cavity above the brain. Subsequent work by Tosches et al. [35] demonstrated melatonin production in brain photoreceptors of *Platynereis dumerilii.* Therefore, melatonin may have been among the neurosecretory products Fitzsimons hypothesized though it was not specifically identified.

The current study provides evidence regarding the ALAN influence on melatonin secretion in the annelid *H. carunculata.* Gradually increasing light conditions until a constant 24 h light exposure appears to disturb the circadian rhythm by shifting the melatonin secretion pattern (Fig. 2).

Table 3 Results of two-way ANOVA for the melatonin, serotonin and tryptamine concentrations of annelids under different light cycles

			F	*d.f*	*P* value
Melatonin	24 h cycle	Treatment	3.874	1	0.0541
		Time	2.198	8	0.0416*
		Treatment x Time	1.987	8	0.0653
	12 h cycle (8:00–20:00)[#]	Treatment	10.542	1	0.00268**
		Time	4.347	4	0.00621**
		Treatment x Time	4.569	4	0.00478**
	12 h cycle (20:00–8:00)	Treatment	0.141	1	0.710
		Time	0.503	4	0.734
		Treatment x Time	0.326	4	0.858
Serotonin	24 h cycle	Treatment	0.837	1	0.366
		Time	0.549	8	0.812
		Treatment x Time	0.880	8	0.543
	12 h cycle (8:00–20:00)	Treatment	2.925	1	0.103
		Time	0.743	4	0.574
		Treatment x Time	1.211	4	0.337
	12 h cycle (20:00–8:00)	Treatment	0.070	1	0.794
		Time	0.641	4	0.639
		Treatment x Time	0.490	4	0.743
Tryptamine	24 h cycle[#]	Treatment	0.067	1	0.797
		Time	0.627	8	0.750
		Treatment x Time	1.495	8	0.193
	12 h cycle (8:00–20:00)[#]	Treatment	5.511	1	0.0293*
		Time	1.391	4	0.2729
		Treatment x Time	2.328	4	0.0913
	12 h cycle (20:00–8:00)	Treatment	0.875	1	0.361
		Time	0.236	4	0.915
		Treatment x Time	0.762	4	0.562

Significant results are indicated by asterisks

[#]Log-transformed values

*$P < 0.05$

**$P < 0.01$

However, the circadian rhythm still remains under constant light conditions which, according to Gao et al. [33], may indicate that other factors (e.g., biological rhythm genes) also influence melatonin production. Potential shifts in the melatonin secretion pattern may affect the night-time behavior of the *H. carunculata* annelid as melatonin acts as signal for activity–inactivity behavior and therefore in establishing its nocturnal state [21, 35]. Furthermore, melatonin production patterns have been associated with photoperiod signaling, which drives the physiological processes of reproductive cycles. For example, melatonin can act as a seasonal marker for reproduction in the colonial anthozoan *Renilla koellikeri* [49]. It has also been mentioned that melatonin may inhibit GnRH expression, thereby affecting gonadal development in bivalves [50]. Thus, *H. carunculata* reproduction, in terms of synchronization and sexual development, may be affected by nocturnal illumination even at low light intensities [51, 52].

Serotonin and tryptamine were also observed in *H. carunculata* heads. Under normal photoperiod conditions, no diurnal variations were observed in serotonin and tryptamine levels. Regarding serotonin, the absence of a diurnal variation has been also observed in other invertebrate taxa, such as the annelid *Perinereis aibuhitensis* [53] and the mollusk *Octopus vulgaris* [22]. However, in the lobster *Homarus americanus*, diurnal fluctuations have been demonstrated, regulated by a light-entrainable endogenous rhythm [54]. Likewise, serotonin levels in the planarian *Dugesia japonica* exhibited diurnal variation indicating the presence of a circadian timekeeping mechanism [55]. Thus, these results suggest that the presence or the absence of a circadian rhythm in serotonin may be phylum-specific. Regarding tryptamine, to our knowledge, no studies have investigated the secretion pattern of this indolamine in marine invertebrates.

The investigation of the melatonin biosynthetic pathway serves as a valuable approach for assessing the impact of

ALAN on marine animals [56]. According to the results, tryptamine was only affected by constant light but it did not show any diurnal variation. In contrast, serotonin levels remained unaffected under constant light conditions, suggesting that ALAN may not disturb its secretion in the annelid *H. carunculata*. The observed changes in tryptamine levels in response to light exposure may not be related to its role in the serotonin biosynthesis but rather to its specific functions. Specifically, Vanderlinden and Mallefet [57] proposed that tryptamine may act as a neurotransmitter involved in ophiuroid luminescence. Since *H. carunculata* has been reported as a luminescent organism, it is reasonable to assume that the changes of tryptamine levels observed in this study may be associated with its bioluminescent function [58]. However, since serotonin, a direct precursor of melatonin, was not affected by constant light conditions, these findings suggest that the observed diurnal variations in melatonin secretion were not related to the availability of its related indolamines [22].

In summary, the data obtained from this study suggest that ALAN adversely affects melatonin by changing its secretion pattern in the annelid *H. carunculata*. However, serotonin and tryptamine, which are some of the related indolamines of melatonin were not affected, indicating that ALAN may have different impacts on the various physiological aspects. Zapata et al. [59] have proposed that quantifying and understanding linkages across different levels of biological organization (from individual to ecosystem) are crucial for the development of an ALAN research and conservation framework. Therefore, as differences in physiological responses among species have been documented, further research is needed to assess the impact of ALAN on various marine invertebrates, as well as the ecological implications of these hormonal variation patterns.

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Data availability All relevant data will be shared on reasonable request to the corresponding author. The code for the analysis of the results is available on https://github.com/cpavloud/ALAN_melatonin

Declarations

Conflict of interest The authors declare no competing or financial interests.

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