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Antioxidant defense system rhythms in crustaceans and possible roles for melatonin

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1. ABSTRACT

Animals in their habitats are subject to many cyclical patterns for different environmental parameters, resulting in selective pressure to develop biological rhythms for metabolism. To avoid oxidative stress, a rhythmic variation in the antioxidant defense system (ADS) should be associated with aerobic metabolic rhythms. In this review, we summarize and discuss the latest findings on rhythmic variations of the ADS in different tissues of crustaceans, as well as possible mechanisms for their regulation. In vertebrates, melatonin has been shown to be an important molecule in the regulation of the ADS and to be a high-capacity scavenger of reactive oxygen species. Given that this indoleamine has been identified in crustaceans, we also discuss the possible implications of this molecule in crustacean ADS regulation.

2. INTRODUCTION

The radical transformation of the primitive atmosphere, from an extremely reducing to an oxidizing environment, is considered to be a landmark in the evolution of different types of organisms. The simple act of fixing CO_2 in the presence of light to produce glucose and O_2 allowed the emergence of new organisms capable of oxidizing organic compounds to produce energy for their development and evolution. Although these aerobic organisms benefited from such an efficient method for the acquisition of energy from oxidized organic compounds, they were (and are) also affected by molecules generated during these oxidative processes that are extremely reactive and have the potential to cause damage (1). In fact, these so-called reactive oxygen species (ROS), e.g., hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hydroxyl

radical (HO·), can react with protein, lipids and DNA and/or disrupt the redox state of the cell (1-3). To avoid this drawback of oxidative metabolism, aerobic organisms must have a specialized antioxidant defense system (ADS) that counteracts the activities of ROS. The ADS is composed of enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-*S*-transferase (GST), as well as non-enzymatic molecules, such as α-tocopherol, ascorbate, β-carotenes and the tripeptide glutathione (GSH) (4, 5). GSH is particularly important for the redox state of the cell (3-5). Therefore, any disruption of redox signaling and/or any oxidative damage to biomolecules will cause oxidative stress, potentially affecting the survival of the animal in its habitat (1, 3, 4).

In association with the development of ADS, aerobic organisms developed mechanisms to anticipate changes in environmental stressors over time (hours, days, seasons) to avoid oxidative stress. Biological rhythms were developed due to the predictability of natural and cyclical changes in environmental factors such as photoperiod, temperature and salinity. These biological rhythms occur in almost all groups of animals and, in many cases, are linked with metabolic adjustment, involving signaling molecules, key enzymes and hormones (6-10).

Melatonin is an important molecule that is virtually ubiquitous in living animals and is involved in the modulation of many biological rhythms in vertebrates (11). This indoleamine was first isolated in the 1950s by Lerner and colleagues from thousands of bovine pineal glands (12). At least in vertebrates, melatonin production from the pineal gland peaks during the dark phase and is modulated by the key enzyme arylalkylamine N-acetyltransferase (aaNAT). Although melatonin synthesis also occurs in other tissues, such as the gonads, the harderian gland and the intestines, melatonin from these tissues may not have the same temporal profile. In these cases, melatonin may have a paracrine function instead of being released into the bloodstream to control the biological rhythms of different tissues (13-15). Along its evolutionary history, melatonin has become a multifunctional molecule, with actions varying from pigment migration (the first verified effect, hence its name) to serving as an internal signal for photoperiod length (12, 13). In the last decades, melatonin has been studied not only due to its ROS scavenging capacity but also due to its ability to modulate some components of the ADS (6, 8, 16). Given that melatonin has antioxidant capabilities and modulates a variety of biological rhythms, it may have an important role in the modulation of ADS rhythms in animals.

The Subphylum Crustacea comprises more than 50,000 described species, with an unknown number of species yet to be discovered (17). All crustaceans must inhabit water during the early stages of their development, but can subsequently inhabit every possible habitat (terrestrial, semi-terrestrial and fresh and marine aquatic environments). Studies are performed on these animals because they have multiple important roles: as significant members within the food chain (e.g., krill and copepods), as good indicators of contaminated and polluted environments (e.g., barnacles), as part of commercial fishing and aquaculture (e.g., lobster, shrimp and crabs), and as good models for evolution and adaptation studies (all kinds of crustaceans) (17-20).

In this review, we analyze the latest findings on ADS rhythms in the different tissues of crustaceans and discuss how this system is regulated. In addition, the variation of melatonin content in crustaceans and its effects will be correlated with findings in vertebrate animal models and discussed.

3. RHYTHMIC VARIATIONS OF THE ANTIOXIDANT DEFENSE SYSTEM (ADS) IN DIFFERENT TISSUES OF CRUSTACEANS

The ADS system of any organism has a close relationship with aerobic metabolism. Any variation in aerobic metabolism function should generate a corresponding variation in ROS production, which should, in turn, induce an alteration of the ADS system to avoid cell disturbance. Due to the natural photoperiod, animals generally possess locomotor activity fluctuation along the day/night cycle, which also affects aerobic metabolism. Locomotor activity rhythms and oxygen consumption (VO_2) have been studied in crustaceans for a long time (21-29). In the portunid crabs *Callinectes similis* and *Portunus spinicarpus* and in the shrimp *Farfantepenaeus aztecus*, for example, higher VO_2 was shown to occur in the same period as higher locomotor activity (26). In contrast, few studies have addressed the daily variations in the content or activity levels of components of the ADS system and their relationships with aerobic metabolism. Prieto-Sagredo (30) observed that different photoperiods and light intensity affect the hemolymph redox state in the crayfish *Procambarus clarkii* and *Procambarus digueti*. When both species were exposed to a 20:4 photoperiod, the level of GSSG (oxidized glutathione, a pro-oxidant molecule) increased, indicating photo-oxidative stress due to the decrease in the GSH/GSSG ratio. In fact, the 20:4 light-darkness (LD) cycle produced a small, but noticeable, increase in the rate of crayfish mortality. One year later, Durán-Lizarraga (31) reported the first known rhythmic variation of the crustacean ADS, in *P. clarkii*. When the crayfish was submitted to 12:12 and constant darkness (DD) photoperiods, the GSH/GSSG ratio and GR activity in the midgut and the GSH/GSSG ratio in hemolymph showed characteristics of a circadian rhythm due to their free running in DD. A particularly interesting finding of this study was the correlation of GSH status (GSH and GSSG content and GSH/GSSG ratio) argetwith scotophase, given that *P. clarkii* is a nocturnal species (32) and its aerobic metabolism may be high during this period. Along a similar vein, Fanjul-Moles and colleagues (33) studied the same components of the ADS in the midgut gland and hemolymph of *P. digueti*, in addition to measuring GPx activity in the midgut gland of both *P. digueti* and *P. clarkii*. Some differences between the ADS of the two both species were observed in this study. As previously observed, the 20:4 LD cycle and increased light irradiance altered the GSH system in *P. digueti*, increasing GSH in the

midgut and GSSG in hemolymph. The activities of GR and GPx in *P. digueti* and of GPx in *P. clarkii* were higher at scotophase, which again fits with the period of maximal locomotor activity for these species. Given that the activities of GPx in *P. digueti* and GR in *P. clarkii* varied in a circadian fashion, the midgut gland could potentially be coupled to the circadian clock, regardless of clock location (including within its own digestive system), whereas GSH status in the hemolymph may be affected by the oxidative balance of many tissues. Recently, the same group (34) published new data about the ADS in *P. clarkii*, focusing on the nervous tissue, complex optic lobe-brain (OL-B) and retina, which are putative pacemaker sites for circadian rhythms in this species (35). Again, some components of the ADS from both tissues displayed characteristics of circadian rhythms under the DD condition but not as clearly under the 12:12 LD condition. In fact, an increase in the GSH/GSSG ratio was reported under LD 12:12 photoperiod treatment, indicating that the ADS was up-regulated to avoid photo-oxidative stress. This study also examined daily variations in the levels of lipoperoxidation (LPO), which is a measure of damage index. Under the 12:12 LD cycle, the maximum values in the OL-B were found at scotophase, whereas the maximum values in the retina were found at photophase. These data are consistent with the expected results due to the effect of light. In contrast, a paradoxical result was observed in the retina under the DD condition, in which the LPO was higher in the total absence of light than in the 12:12 LD. A possible explanation is that the electrical activity of these sensorial cells is augmented to favor ROS generation and thus results in increased damage. With respect to the ADS, an interesting observation that emerged from these studies is that the midgut gland and the complex optic lob-brain seem to be coupled by two self-sustained oscillators, whereas the retina appears to be a passive oscillator coupled with the zeitgeber light/dark cycle.

Crustacean ADS rhythms have also been investigated in our laboratory in the estuarine crab *Neohelice granulata* (previously known as *Chasmagnathus granulata/granulatus* – see ref. 36), which is a brachyuran species. This species is a semi-terrestrial crab with nocturnal locomotor activity (37). Under a 12:12 LD condition, Maciel and colleagues (29) verified two peaks in oxygen consumption in the gills and hepatopancreas of these animals that were separated by 12-h intervals. This observation leads to an interesting question: does the ADS in the gills and hepatopancreas follow a circadian rhythm, as locomotor activity does, or a tidal rhythm, as tissue VO_2 does? Given that the observed phenomenon depends on which specific antioxidant defense and which specific tissue is considered, this question will not be easy to answer. In fact, Maciel and colleagues (29) found differences in enzymatic activities (e.g., CAT and GST) and non-enzymatic content (e.g., non-proteic sulfhydryl groups, -NP-SH), as well as LPO levels, between these two tissues. Different CAT activity patterns were observed between the two tissues, with high but stable levels during the entire light/dark cycle in the gills and an activity peak at night in the hepatopancreas. The opposite situation was observed for NP-SH levels, with peak activity levels occurring at night in the gills and no variations in observed in the hepatopancreas. LPO levels were higher at photophase in the gills and at scotophase in the hepatopancreas, an observation that reinforces the tissue specificity of different antioxidant components. In a recent publication, Maciel and colleagues (10) verified that H_2O_2 induces an *in vitro* increase in NP-SH levels in the gills of *N. granulata* to the same level previously observed during the night period (29), suggesting that H_2O_2 may be the main signaling compound for activation of the GSH system. Furthermore, the same group showed a daily bimodal profile for the total peroxyl radical scavenging capacity, which serves as a general measurement of the activity of the entire ADS against peroxyl radicals generated *in vitro*, in the gills of intact and eyestalkless crabs, suggesting that gills are driven by a biological clock not located in the eyestalks. This bimodal variability may be due to antioxidants that were not studied, including enzymes, such as SOD and GPx, and non-enzymes. These observations suggest that the gill ADS follows a tidal rhythm. These studies in crayfish and crabs can be used to construct a putative flux model of the influence of photoperiod on the GSH status of crustaceans (Figure 1), in which cell damage can be initiated directly by the photoperiod or indirectly through an information flux that is initialized in the photoreceptors. This flux is then transmitted to the biological clock that adjusts metabolic and locomotor activities, in turn leading to rhythmic variation in the levels of GSH and LPO in peripheral tissues.

Over the last ten years, other studies have focused on seasonal variations in the levels of LPO and enzymatic antioxidants in crustaceans. In addition to photoperiod, other environmental aspects, including temperature, salinity and rainfall level, and others can be extremely variable throughout the year in different ecosystems. This variability is apparent in South Asia, where monsoons significantly alter many physical and chemical aspects of the environment, leading to seasonal metabolic adjustment in the local organisms. In the digestive gland of the barnacle *Balanus balanoides*, Niyoge and colleagues (38) observed higher activity of CAT and SOD during the pre-monsoon (March-June) period with a gradual decrease during the monsoon (July-October) and post-monsoon (November-February) periods, whereas an opposite profile was seen for LPO. Temperature decreases and a possible reduction in food availability during the post-monsoon (winter) period may lead to classical oxidative stress in *B. balonoides* due to the injured metabolism. Two studies showed seasonal LPO variations in males and females of three amphipod species, i.e., *Hyalella pleoacuta*, *H. castroi* and *H. curvispina* (39, 40). In females of *H. pleacuta* and H. *castroi*, higher LPO levels were found in autumn, whereas in females of *H. curvispina*, higher LPO levels were observed in summer. These periods are correlated with reproduction in each species, when females carry juveniles in the marsupium after the eggs have hatched. In addition, the highest LPO levels for males occurred in autumn and winter for *H. pleoculata*, in summer and winter for *H. curvispina* and in autumn for *H. castroi*. Again, these variations are probably linked to

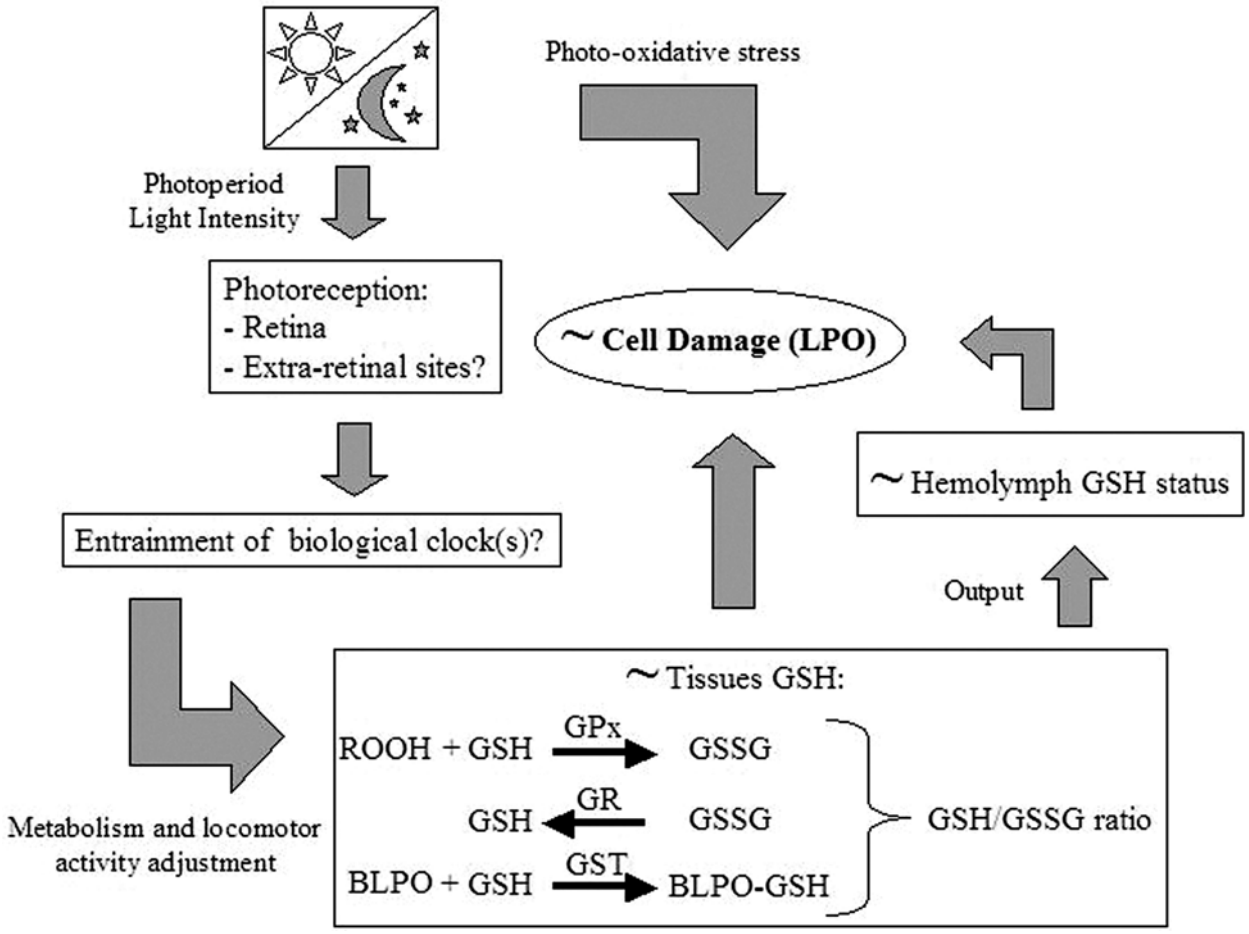

Figure 1. Putative flux model of photoperiod influence on the GSH status of crustaceans. Light can injure cells directly through its photo-oxidative capability or indirectly through photoreception. Photoreception occurs in the retina and perhaps in extra-retinal sites, with the photoperiod/light intensity information transmitted to the putative biological clock, which may facilitate the entrainment of metabolic and locomotor activities. This transmission may subsequently cause tissue-specific rhythmic variations in the GSH/GSSG ratio as the levels of GSH change. Given that variations in LPO and GSH output have been seen in the hemolymph, cellular damage to these tissues may rhythmically vary. GSH: reduced glutathione; GSSG: oxidized glutathione; GSH/GSSG ratio: reduced glutathione and oxidized glutathione ratio; ROOH: hydroperoxide; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-*S*-transferase; BLPO: byproducts of lipid peroxidation; ~: rhythmic variation.

copulation activity, which occurs in autumn, and to an increase in light irradiance during the summer, which may be an additional environmental stressor for these species. Another factor to consider is the variability of food antioxidant properties from season to season, which might affect the overall antioxidant capacity of these animals. Kong and colleagues (41) verified seasonal variations in CAT, SOD and GPx activities as well as LPO levels in the gills of the mud crab *Scylla serrata*. Although activities of all these enzymes were higher in summer, none were sufficient to prevent a peak of LPO during the same season. A second peak of GPx activity was observed in winter, but LPO levels also peaked during this period due to low activity of CAT and SOD. Summer appears to be the most stressful season, despite the higher activity of enzymatic antioxidants, probably due to food availability and type as well as other factors not considered in this study, such as light irradiance and/or temperature.

4. POSSIBLE MECHANISMS OF REGULATION FOR ADS

As described above, ROS are routinely generated during aerobic metabolism. These molecules cannot just be regarded as harmful by-products of aerobic metabolism because they also serve fundamentally important roles in intracellular signaling pathways. However, all current knowledge regarding the role of ROS signaling in ADS regulation is based upon mammalian studies, implying that no studies on this topic have been performed in invertebrates, including crustaceans. Given that some signaling pathway cascades are phylogenetically preserved and that ROS have been present since the formation of the aerobic atmosphere (1, 3, 4), ADS regulation by ROS signaling can plausibly be assumed to be similar in mammals and crustaceans despite their phylogenetic distance. Therefore, a very brief overview of ADS

regulation in mammals will be presented here for comparison with putative mechanisms in crustaceans.

Many reports have indicated that the GSH/GSSG ratio is an important "sensor" for ADS regulation (42). Increased GSSG levels, resulting in a decreased GSH/GSSG ratio, occur in parallel to incremental increases in ROS generation (mainly for H_2O_2). These changes promote the oxidation of protein cysteinyl thiols, which activate or deactivate specific enzymes in the signaling cascades (42-44). In fact, this oxidant-dependent mechanism can stimulate the gene expression of antioxidant enzymes through the regulation of nuclear factor (NF) κB, mitogen-activated protein kinase (MAPK), activating protein-1 (AP-1), the phosphoinositide 3-kinase (PI3K)/Akt pathway, p53 activation and the heat shock response (45). The transcription factors Nrf2 (erythroid-derived 2) and class O of forkhead box (FoxO) are also involved in the ADS signaling pathway (46, 47). It is interesting that so many signaling pathways are involved in ADS stimulation, although the likely explanation involves the role of ADS as a general response to a variety of stressors. H_2O_2 is one of the most important messengers in these signaling pathways because of its constant generation within mitochondria, its relative stability and its ability to cross a variety of membrane barriers due to its small size (48). As previously mentioned, H_2O_2 can induce an increase in the NP-SH levels in the gills of *N. granulata* (10), a similar signaling action reported in mammals (42-44).

In addition to its well-known variation following the circadian rhythm, melatonin also acts as scavenger of ROS or enzyme activity, or as an expression modulator (for review see ref. 8 and 15) in the ADS. Several studies have demonstrated the effects of melatonin on antioxidant enzymes in mammals as well as other groups of animals. Melatonin can also regulate the gene expression of antioxidant enzymes, such as CuZnSOD, MnSOD and GPx, but the specific signaling pathways involved have not yet been fully described (16). A few reports have suggested that the regulation of antioxidant enzymes by melatonin occurs through its activation of secondary messengers (e.g., cAMP, phospholipase C or intracellular calcium) via specific membrane receptors (e.g., MT1 and MT2). As a result, several transcription factors, including antioxidant enzymes, could be activated through the stimulation of MAP kinase cascades by melatonin-bound membrane receptors (16, 49). Although there are no published reports about melatonin signaling pathways in crustaceans, the effects of melatonin on the ADS in crustaceans may be similar to those reported for mammals.

5. EFFECTS OF MELATONIN ON VERTEBRATE ADS

In the early 1990s, several studies reported that, in addition to its function as a messenger that conveys information about the length of the internal photoperiod, melatonin showed higher and more direct scavenging of ROS than other, more traditional antioxidants, such as GSH (50-52). At the same time, Barlow-Walden and colleagues (53) and Pablos and colleagues (54) showed that melatonin indirectly affected ROS, through the increased activity of GPx in rat brain and several chicken tissues. Liu and Ng (55) and Ozturk and colleagues (56) also showed enhanced SOD activity in livers of rats treated with melatonin. Additional evidence supporting the regulation of ADS by melatonin was provided by Pablos and colleagues (57) and Albarran and colleagues (58), who showed that inhibition of melatonin production by light inhibits the increased activities of SOD and GPx in several chicken tissues and in rodents (59, 60). As mentioned above, the pineal gland is the main site of melatonin production and secretion in vertebrates. Accordingly, circulating melatonin levels are dramatically decreased when this gland is extirpated. This removal, in turn, may affect the whole ADS of the animal. In fact, Baydas and colleagues (61) observed decreased GPx activity in some tissues of pinealectomized rats. As the aging process is characterized by increased ROS generation and a decline in many physiological functions, melatonin might prevent or decrease the damages associated with senescent processes (62). Along those lines, Mauriz and colleagues (8) observed a decrease in LPO levels in the livers of aged rats treated with melatonin as compared to aged control rats. Antioxidant enzymes, such as cytosolic CuZnSOD as well as mitochondrial GPx and CAT, presented higher activities in melatonin-treated rats than in aged controls, reinforcing the importance of melatonin in ADS regulation. The antioxidant activity of melatonin may not be as clear in fish as in mammals and birds, although only a few reports have been published to date. López-Olmeda and colleagues (63) observed a slight decrease in muscle LPO levels in goldfish submitted to hypoxia/re-oxygenation and treated with melatonin ($3mg.kg^{-1}$) during re-oxygenation, but no effect was seen in liver. In the same study, melatonin was not capable of suppressing LPO production in the muscle and liver of fishes exposed to H_2O_2 for 1 h. Sreejith and colleagues (64) verified the influence of this indoleamine on the ADS *in vitro* in the teleost *Anabas testudineus*. Cell culture revealed higher LPO levels in the livers of fish kept under constant light (LL) compared to livers of control fish held under the standard 12:12 LD cycle. In contrast, cells treated with melatonin (15-min incubation) had decreased levels of LPO. In addition, melatonin increased GPx and GR activities as well as GSH levels, suggesting its involvement in fish glutathione metabolism.

As previously mentioned, melatonin is capable of stimulating not only the activity of antioxidant enzymes but also their gene expression both *in vivo* and *in vitro*. Antolin and colleagues (65) observed an increase in the mRNA levels of CuZnSOD and MnSOD in the Harderian gland of Syrian hamsters after melatonin treatment. Similar results were verified in the brain cortex of rats injected with melatonin (66). Moreover, Mayo and colleagues (16) also verified increased mRNA levels of antioxidant enzymes *in vitro* when melatonin was added to the growth medium of PC12 cells and human neuroblastoma SK-N-SH cells. Melatonin may or may not be an effective enhancer of antioxidant gene expression, however, depending on the tissue and the experimental conditions. Mauriz and colleagues (8) studied the effect of aging on antioxidant

gene expression in rats and showed that melatonin did not stimulate the activities of MnSOD and CAT in the livers of aged animals, whereas CuZnSOD and both cytosolic and mitochondrial GPx activities were higher in the livers of aged animals treated with melatonin. Recently, Jimenez-Ortega and co-workers (67) reported no daily variations in the gene expression levels of CuZnSOD, MnSOD and CAT in the rat medial basal hypothalamus. However, increased gene expression of these enzymes was seen at certain time intervals, indicating that melatonin can regulate antioxidant gene expression, at least in mammals.

The mitochondrion is the organelle responsible for energy production, but ROS generation is inevitable due to leakage in the electron transport chain. Under basal metabolic conditions, cellular ADS can counteract ROS action despite the occurrence of basal levels of damage. However, the ADS may be adjusted to avoid cell disturbance under stress conditions or daily and seasonal variations in aerobic metabolism. Thus, melatonin can be inferred to specifically regulate mitochondria for maintenance of cell homeostasis through its roles as an ROS scavenger or an ADS modulator. In fact, melatonin is a highly lipophilic molecule capable of crossing cell membranes, including those of mitochondria (68, 69), and of binding to specific receptors on these membranes (70, 71). Several reports have shown that melatonin stabilizes mitochondrial inner membranes (72) and increases the activities of respiratory complexes I and IV (73, 74), both preventing inner calcium overload and helping maintain the membrane potential for ATP generation (75). All these functions may be general features of melatonin in aerobic organisms. Additional studies on this topic should be performed in both vertebrates and invertebrates, including crustaceans, to link every possible divergence of the evolution of melatonin as a signaling molecule.

6. MELATONIN VARIATIONS IN CRUSTACEANS

Melatonin has been identified in a wide variety of vertebrate species since its first discovery by Lerner and colleagues (12) in bovine pineal glands. By contrast, the number of studies on melatonin in invertebrate is much lower, although the increasing efforts on it during the last years. With respect to crustaceans in particular, even less is known about this indoleamine. Only ten crustacean species (including crabs, prawns, isopods, lobster, crayfish and krill – see Table 1) were examined for the presence of melatonin. Some of these species showed rhythmic variations in the regulation of melatonin similar to those seen in vertebrates. Variability among species makes it difficult to establish a general pattern for the rhythmic regulation of melatonin.

Vivien-Roels and Pévet (76) published the first known report on the presence of melatonin in crustaceans, which was identified using the radioimmunoassay (RIA) method in the eye and eyestalk of the crab *Carcinus maenas* in a concentration ranging between 2,700 and 3,650 ρg.eye^{-1}. Interestingly, although no daily variation was observed, different levels of melatonin were reported during different times of the year, suggesting the existence of a putative seasonal control over its production. In the optic lobes of the freshwater prawn *Macrobrachium rosenbergii*, Withyachumnarnkul and co-workers (77) observed an increase in melatonin content at 1500 h (5.5 ρg.μg of protein^{-1}) compared to the lowest value at 2400 h (0.5 ρg.μg of protein^{-1}), which is a temporal pattern opposite to those generally reported for vertebrates. In another study, Withyachumnarnkul and colleagues (78) showed that melatonin levels in the optic lobes of sub-adult males and females of *Penaeus monodon* collected at 0900 h did not differ (30-35 ρg.optic lobes^{-1}).

Agapito and colleagues (79) showed, for the first time, melatonin content in the circulating fluid of *Procambarus clarkii* kept in a 14:10 LD cycle. Melatonin levels in the circulating fluid peaked at photophase (1900 h; 172 ρg.ml^{-1}), with increasing levels in the eye plus eyestalk at the same time point (656 ρg.eye^{-1}). Animals kept in an artificial 8:16 LD cycle also had a peak in melatonin levels in the eye at photophase, at 1600 h (749 ρg.eye^{-1}). In contrast, Balzer and co-workers (80) observed a melatonin peak in the eyestalk of *P. clarkii* at scotophase (0300 h; 1,813 ρg.eyestalk^{-1}). In this study, the amplitude of melatonin was higher (the lowest value was 30 ρg.eyestalk^{-1} at 1500 h) than in the previous work. Differences in the temporal regulation and concentration of melatonin could be related to the geographical localization of decapod species, with the crayfish in the former and latter studies from Spain and Mexico, respectively. Chemical interference or differing sources for the melatonin antibody used in the RIA method cannot be ruled out as additional possible explanations.

Tilden and colleagues (81, 82) observed that the pattern of daily melatonin content varied in the eyestalks of *Uca pugilator* fiddler crabs kept under LD and DD. Under LD 12:12, a single melatonin peak occurred at the photophase (230 ρg.eyestalk^{-1} at 1300 h), whereas two peaks that were 12 hours apart occurred in the DD condition (196 ρg.eyestalk^{-1} at 1600 h and 111 ρg.eyestalk^{-1} at 0400 h). The second DD peak, which was not present in the 12:12 LD condition, seemed to be influenced by light and thus displayed an exogenously controlled component drift. Interestingly, melatonin levels in the LL condition were higher at almost all time points, with the maximum value at 1300 h (431 ρg.eyestalk^{-1} at 1300 h), compared to treatments with other photoperiods. This response is the opposite of the well-known pattern in mammals, in which light suppresses melatonin production. Meyer-Rochow (83) showed an absence of daily variation of melatonin content in the eye of the freshwater crayfish *Astacus fluviatilis*, in the head of the isopod *Saduria entomon* and in the eyestalk of *Carcinus maenas*. Instead, a seasonal regulation may be involved in these cases, as seems likely for *C. maenas*. In the optic lobes of the crab *Neohelice granulata*, variations in melatonin content were observed (84) at levels similar to those reported in *Uca pugilator* (81, 82). Under the 12:12 LD and DD conditions, two peaks separated by 12-h intervals occurred, and all melatonin variation was abolished in the LL condition. This pattern was similar to that reported for vertebrates but different from that reported for *U. pugilator*. Despite the similarity in the 12:12 LD and

Table 1. Day-night variations and range concentrations of melatonin in different tissues of crustacean species, as measured by distinct techniques

Species	Tissues	Day-Night variations	Range Concentrations	Technique	Reference
C. maenas	Eye plus eyestalk	No variation	2,700 – 3,650 ρg.eye^{-1}	RIA	76
M. rosenbergii	Optic lobes	Photophase	0.5 - 5.5 ρg.μg of protein^{-1}	RIA	77
P. monodon	Optic lobes	Not studied	30 – 35 ρg.optic lobes^{-1}	RIA	78
P. clarkii	Eyestalk and hemolymph	Photophase	50 – 656 ρg.eye^{-1} and 20 – 172 ρg.ml^{-1}	RIA	79
P. clarkii	Eyestalk	Scotophase	30 – 1,813 ρg. eyestalk^{-1}	RIA	80
U. pugilator	Eyestalk	Photophase	110 – 230 ρg. eyestalk^{-1}	RIA	81, 82
A. fluviatilis	Eye	No variation	Not informed	RIA	83
S. entomon	Head	No variation	Not informed	RIA	83
N. granulata	Optic lobes, hemolymph and muscle	Photophase and scotophase	7 – 20 ρg.optic lobes^{-1}; 102.8 ρg.ml^{-1} and 1.26 ng.mg^{-1} of muscle	RIA; ESI-LC-MS/MS	9, 10, 84
E. superba	Eyestalk and hemolymph	No variation	6.3 ρg.eyestalk^{-1} and 2.9 ρg.ml^{-1}	HPLC/ELISA	85
N. norvegicus	Hemolymph	No variation	40 ρg.ml^{-1}	LC-MS/MS	86

DD melatonin profiles, the concentration range in *N. granulata* (7–20 ρg.optic lobes^{-1}) was more similar to that reported for *P. monodon* (78). Recently, Maciel and co-workers (10) verified that melatonin levels (measured only in the morning interval from 0900 to 1100 h) in the hemolymph of *N. granulata* kept under the DD condition were similar to those observed in *P. clarkii* (81.8-123.8 ρg.ml^{-1}).

Pape and colleagues (85) reported the absence of a daily variation in melatonin levels in the eyestalks and hemolymph of the Antarctic krill *Euphausia superba*. Variations in melation levels were not seen in animals collected in summer (6.1 ρg.eyestalk^{-1} and 2.4 ρg.ml^{-1}) or in winter (6.3 ρg.eyestalk^{-1} and 2.9 ρg.ml^{-1}). These values are also lower than those for the different species mentioned above. Of note, this study employed a different methodology for melatonin measurement, consisting of an ethanol–chloroform extraction followed by HPLC purification and ELISA, rather than the RIA typically used in previous reports. The Norway lobster *Nephrops norvegicus* also did not display any differences in the concentration of melatonin between day and night (86). In this study, which utilized liquid chromatography/tandem mass spectrometry (LC–MS/MS), animals kept under an 12:12 LD condition with a light intensity of 10 lx, melatonin levels in hemolymph ranged between 2 to 8 ng.ml^{-1}, whereas animals exposed to the same photoperiod regime with a light intensity of 0.1 lx had even lower melatonin levels (0.03 – 0.04 ng.ml^{-1}). Geihs and colleagues (9) detected melatonin in non-neuronal tissues for the first time in crustaceans. High levels of melatonin (1,258.6 ρg.mg^{-1}), measured in the subjective morning by electrospray interface (ESI) LC–MS/MS, were seen in the locomotor muscle of *N. granulata* kept under a DD condition. This concentration is similar to that found in the eyestalk of *P. clarkii* (80). In a pioneering study in crustaceans, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), the main metabolite of melatonin in mammals, was detected in the optic lobes (53.7 ρg.mg^{-1}) and supraesophageal ganglion (85.7 ρg.mg^{-1}) but not in muscle.

Thus, different crustacean species possess different melatonin profiles and concentrations in the tissues that have been analyzed. Although the number of studies has increased, a general consensus regarding melatonin variation in crustaceans still remains difficult to establish.

7. EFFECTS OF MELATONIN IN CRUSTACEAN ADS

Some studies have shown that melatonin can be involved in a variety of crustacean physiological parameters, such as electroretinograms, pigment migration, limb regeneration, hemolymph glucose and lactate rhythms and synaptic transmission (80, 81, 87-90). Studies on ADS regulation by melatonin, however, are scarce. Although some reports have shown that photoperiod-influenced variations and light intensity alter antioxidant status as an indirect and putative effect of melatonin action, only two reports (9, 10) have actually demonstrated the regulatory role of this molecule on some components of the ADS in crustaceans.

Maciel and colleagues (10) studied daily variations in the total peroxyl radical scavenging capacity (TOSC) in the gills of *N. granulata* and showed a biphasic profile similar to that of oxygen consumption in the same tissue (29). One peak occurred at photophase and the other at scotophase, separated by a 12-h lag. Interestingly, eyestalk ablation delayed the peaks by 3 hours but did not affect the overall profile. In contrast, treatment of eyestalkless crabs with 2x10^{-12} mol.animal^{-1} of melatonin abolished this TOSC profile, suggesting a metabolic suppression that decreases ROS production and, consequently, decreases the general antioxidant defense against peroxyl radicals, especially when taking into account the melatonin-induced decrease in *in vitro* gill VO_2 (10). Within this framework, Geihs and co-workers (9) observed that the locomotor muscle of eyestalkless crabs kept in DD and pre-treated (short-term, 30 min) with low doses of melatonin (0.002 and 0.02 ρmol.crab^{-1}) showed increased VO_2, γ-GCL activity (the rate-limiting enzyme for GSH synthesis) and GSH content without changes in ROS concentration, antioxidant capacity or LPO levels. The effects of melatonin were reduced at higher dosages (2 and 20 ρmol.crab^{-1}), probably due to the desensitization of specific membrane receptors, which suggests an increase in aerobic metabolism (opposite to what is seen in the gills), but only under non-stressful conditions. In animals pre-treated over the long-term (570 min), low dosages of

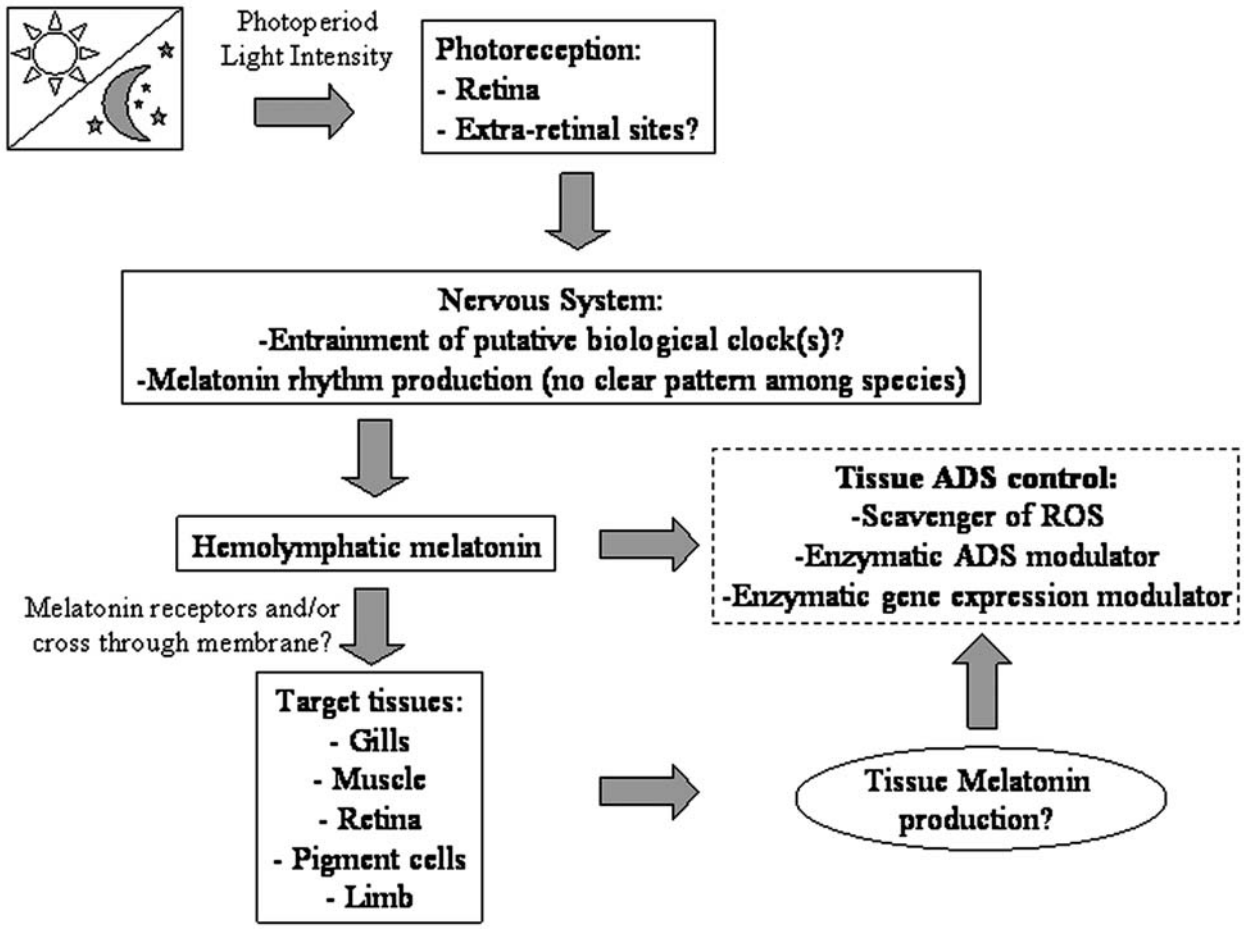

Figure 2. Putative flux model of the effects of melatonin on crustacean ADS. Photoreception occurs in the retina and perhaps in extra-retinal sites, with the photoperiod/light intensity information transmitted to the nervous system, the putative biological clock site. This biological clock may facilitate the entrainment of melatonin synthesis, which may be a species-specific pattern, as no clear overall pattern has been seen to date in crustaceans. Melatonin secreted in the hemolymph may act in target tissues via either specific membrane receptors or transport across the cell membrane. These target tissues may also produce melatonin that may or may not act in a corresponding fashion with circulating melatonin to induce tissue-specific ADS control by scavenging ROS and modulating the gene expression of ADS enzymes. ADS: antioxidant defense system; ROS: reactive oxygen species.

melatonin decreased antioxidant capacity and CAT activity, whereas higher dosages reduced VO_2 and increased antioxidant capacity. The variability in the response of crab muscle to melatonin suggests that melatonin is capable of affecting antioxidant status in a time- and dosage-dependent manner.

8. CONCLUSIONS AND PERSPECTIVES

The ability of animals to detect natural signals from the environment and, thus, follow or even anticipate these cyclical changes by adjusting their metabolism and antioxidant defenses is fascinating. Rhythmic variations in ADS and melatonin content are well described in mammals, and knowledge about their regulation is advanced and constantly progressing. In crustaceans, however, only a dozen reports on ADS and melatonin rhythms have been presented, providing a basic understanding of their regulation (Figure 2). Photoperiod information is transmitted from the photoreceptor site to the nervous system, where the entrainment of the putative biological clock and melatonin rhythm production occurs. This variably produced melatonin may affect the control of tissue ADS and/or paracrine melatonin production through hemolymphatic transport. Some results in crustaceans are similar to those seen in mammals, but others possess high intra- and inter-specific variabilities. Therefore, increased experimental efforts are needed for the following issues:

1. Identification of intracellular messengers for pre-existing ADS regulation;
2. Characterization of the signaling pathway for enzymatic ADS gene expression;
3. Measurement of melatonin concentration rhythms in different tissues from different species at different times of the day and on different days in different seasons to establish a generalized profile;

4. Identification of the signaling pathway and the key enzymes for melatonin synthesis;
5. Assessment of the capability of melatonin to modulate the gene expression of ADS enzymes.

Given that crustacean species are clustered in a large subphylum, the development of a generalized rhythmic consensus pattern for ADS regulation and the role of melatonin may be difficult. With increasing numbers of reports, however, a future review on this subject may provide updates on some or all of the above issues.

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