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Serotonin expression in the optic lobes of cavernicolous crustaceans during the light–dark transition phase: Role of the lamina ganglionaris

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The expression pattern of serotonin neurotransmitter (5-HT) was investigated in the optic lobes of the marine cave mysid *Hemimysis margalefi* (Crustacea, Mysidacea) during the dark to light and light to dark transitions by fluorescence immunohistochemistry. The intensity of the expression was evaluated according to the number of immunoreactive elements. Experiments were carried out under laboratory controlled conditions of illumination. The results show that some structures as the X-organ and medulla terminalis are always immunoreactive during light or dark periods. Other ones as the rhabdom of the reticular cells (rh), the distal pigment cells (dpc), the internal medulla (im), the external medulla (em), and the sinus gland (sg) are immunoreactive during light exposure. In these latter structures, immunoreactivity is quickly detected after illumination (30 min) while, back to darkness, it takes longer to disappear (1 h for rh and dpc, 2 h for im and more for em and sg). The 5-HT expression pattern in the lamina ganglionaris is different. During the dark to light transition, immunoreactive cells are already detected after 30 min of light exposure; after 1 h of exposure, the lamina ganglionaris expresses many immunoreactive cells, then, they are less numerous after 2 h; finally, the detection limit is reached after 3 h of exposure. During the light to dark transition, 5-HT is expressed in the lamina ganglionaris only after 1 h of darkness. The number of 5-HT neurones was increased after 2 h of darkness but no reactivity is detected after one night. To go further, same experiments were carried out in an other mysid species, *Leptomysis lingvura* that lives at same depth but in a shallow lagoon. In contrast to *H. margalefi*, *L. lingvura* is not subject to nycthemeral migrations. A different 5-HT expression pattern in the lamina ganglionaris has been observed: immunolabelling was detected as soon as 30 min, whatever the considered transition phase, dark to light or light to dark. These results are discussed considering the behaviour of each species. We suggest that the expression pattern of 5-HT in the lamina ganglionaris can play the role of a photoreception signal integrator during the light–dark transition phases and that cavernicolous mysid crustaceans can be considered as a good model to study the mechanisms of determination and regulation of circadian migrations of zooplankton.

Keywords: Lamina ganglionaris; Light–dark transitions; Mysidacea; Serotonin; Zooplankton

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1. Introduction

Mysid crustaceans are important elements of the marine food webs. They live in various habitats and have been recorded from shallow to deep waters in the world oceans and at all latitudes and longitudes; moreover, some live in marine caves where they can account for a significant part of the community biomass. In the open sea, many planktonic species accomplish vertical and circadian migrations that are homologous with the nycthemeral migrations of some cave mysids (Passelaigue and Bourdillon, 1986). The distribution and the circadian migrations of cavernicolous *Hemimysis* species have been previously studied (Chevaldonné and Lejeusne, 2003; Coma et al., 1997; Macquart-Moulin and Passelaigue, 1982; Passelaigue and Bourdillon, 1986). However, the physiological mechanisms involved in the control of these migrations are unknown.

Crustaceans display a variety of circadian rhythms that are controlled by periodic functions of the nervous system (Fanjul-Moles and Prieto-Sagredo, 2003). In the Decapoda crustaceans, although some of these rhythms are well documented, the regulation processes are not completely understood. It has been suggested that some tissues of the optic lobe, such as the lamina ganglionaris (Aréchiga and Rodríguez-Sosa, 1998), the retina (Aréchiga and Rodríguez-Sosa, 1997; Escamilla-Chimal et al., 1998), and the X-organ-sinus gland complex (Sáenz et al., 1997) may play a role in this regulation.

In decapods, serotonin (5-hydroxytryptamine, 5-HT) is involved in the regulation of several behaviours and biological functions by acting either as a classical neurotransmitter, a neuromodulator or a neurohormone (Beltz, 1999). Indeed, 5-HT plays a key role in feeding and reproductive behaviours and also aggressive, postural and social behaviours (Edwards et al., 1999; Harris-Warrick and Kravitz, 1984; Kravitz, 1988; Livingstone et al., 1980; Weiger, 1997; Yeh et al., 1997). In the decapod optic lobes, 5-HT has been located in all neuropiles. Although 5-HT has been proposed to modulate the release of some hormones from neurosecretory cells (Sáenz et al., 1997) such as the crustacean hyperglycaemic hormone (CHH), the red pigment dispersing hormone (RPDH) and the neurodepressing hormone (NDH) (Crow and Bridge, 1985; García and Aréchiga, 1998; Kravitz, 1988), 5-HT plays a major role in the response to light or obscurity. Indeed, the 5-HT content in the entire eyestalk is higher at night (Fingerman and Fingerman, 1977) and 5-HT has been shown to enhance the

sensitivity to light in crayfish retinal photoreceptors (Aréchiga et al., 1990). However, little is known about the role of this biogenic amine in Crustacea other than Decapoda. In Mysidacea, 5-HT neurons have been evidenced and mapped in the brain and optic lobes only recently (Moreau et al., 2002).

As light intensity is thought to be the major factor influencing the migrations of crustaceans, the purpose of this study was to investigate the effects of various conditions of illumination, especially during the light–dark and dark–light transition phases, on the expression pattern of 5-HT in the optic lobes of two mysid species easily kept alive in laboratory conditions. These investigations were conducted on a Mediterranean endemic marine cave species that exhibit nycthemeral migrations, *Hemimysis margalefi* Alcaraz, Riera and Gili, 1986, and on *Leptomysis lingvura* G.O. Sars, 1866, a shallow lagoonal species living at comparable depth and not subject to circadian migrations.

2. Materials and methods

2.1. Experimental designs

During winter 2003–2004, biological material was collected off the French Mediterranean coast: the specimens of *H. margalefi* were obtained from the Figuier cave (15 m depth) near Marseille and *L. lingvura* from the Brusca lagoon near Toulon. Experiments were carried out under artificial lighting (cool-white fluorescent light at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$). Adult specimens of *H. margalefi* were randomly sampled under low red light intensity.

For both species, eight groups of 10 randomly chosen specimens were maintained one night (12 h) in the dark at 21 °C in natural seawater. For each species, one group was fixed in the dark after the 12 h in darkness. Four groups were illuminated with cool-white fluorescent light and respectively fixed after 30 min, 1 h, 2 h or 3 h of illumination. Three groups were illuminated with cool-white fluorescent light for 3 h and then, placed back in the dark and, respectively, fixed in darkness after 30 min, 1 h or 2 h. The period of light exposure was not longer than 3 h in order to preserve the functional integrity of the visual system of the cave mysids.

For each condition, the specimens were fixed in saline phosphate buffer (PBS, 0.1 M pH 7.2) containing 4% paraformaldehyde (PFA) and used for immunoassays. Fixed samples were stored at 4 °C until immunohistochemical assay.

2.2. Immunohistochemistry

The detailed protocol was previously described by Moreau et al. (2002). Briefly, specimens were fixed in PBS-PFA and then rinsed in PBS containing 0.6% Triton X-100 for 2 days. Entire animals were embedded in Cryomount® (Histolab, Göteborg, Sweden) and frozen in liquid nitrogen. To saturate non-specific sites, the frontal sections of the optic lobes (18 µm) were pre-incubated 20 min at room temperature in 0.1 M PBS (pH 7.2) containing 0.6% Triton X-100, 10% goat serum, 2% bovine serum albumin (PBS-T buffer) and 10 µM pargyline (Sigma, Saint Quentin-Fallavier, France). Slides were incubated overnight at 4 °C in a dilution (1:200) of an anti-5-HT antibody, raised in rabbit (Sigma, Saint Quentin-Fallavier, France), in PBS-T containing 10 µM pargyline. Sections were rinsed 30 min three times in PBS-T then incubated 1 h at room temperature with a goat anti-rabbit IgG secondary antibody (1:200) labelled with AlexaFluor 488 (Interchim, Montluçon, France). Sections were then mounted and observed under an epifluorescence microscope (Leica M1560). Some sections were also observed under a confocal laser microscope (Olympus IX70, Fluoview Program). We have estimated the number of 5-HT immunoreactive elements (cell bodies and fibres) at each experimental condition and in each structure, then we have assigned the sign “++” when we have detected the presence of numerous 5-HT immunoreactive elements, the sign “+” when 5-HT immunoreactive elements were less numerous than previously, the sign “+/-” at the detection limit and, finally, the sign “-” in absence of 5-HT immunoreactivity.

The specificity of the 5-HT antiserum was determined by preincubating the diluted antiserum with 1.5 mM 5-HT (Sigma, Saint Quentin-Fallavier, France) overnight at 4 °C before normal treatment of the nervous system. Other controls were done by incubating the slides without the primary antibody. Immunostaining was almost totally abolished by preincubation with 5-HT and totally abolished in absence of the primary antibody.

3. Results

The nomenclature of the histological structures of the optic lobes is that previously detailed in Moreau et al. (2002).

3.1. *H. margalefi*

All the results are summarized in Table 1. First of all, whatever the experimental conditions, i.e. during

Table 1

Serotonin-like immunoreactive areas in the optic lobes of the cave dwelling mysid *Hemimysis margalefi* kept in the dark for one night and then exposed to light and placed back in darkness

Duration of light or dark periods	XO	Medullae			sg	lg	rh	dpc
		mt	im	em				
1 night darkness	+	+	-	-	-	-	-	-
30 min light	+	+	+	+	+	++	+	++
1 h light	+	+	+	+	+	++	++	+
2 h light	+	+	+	+	+	+	++	+
3 h light	+	+	++	+	+	+/-	+	++
30 min darkness	+	+	+	+/-	+	-	+	+
1 h darkness	+	+	+	+	+	+	-	-
2 h darkness	+	+	-	+	+	++	-	-

dpc: distal pigment cells; lg: lamina ganglionaris; em: external medulla; im: internal medulla; mt: medulla terminalis; rh: rhabdom of the reticular cells; sg: sinus gland; XO: X-organ; ++: presence of numerous serotonin-like immunoreactive elements; +: presence of some serotonin-like immunoreactive elements; +/-: detection limit; -: absence of serotonin-like immunoreactivity.

light or dark periods, a 5-HT-like immunolabelling in the medulla terminalis and in the X-organ is observed (Fig. 1A–C).

The rhabdom of the reticular cells, the distal pigment cells, the internal medulla, the external medulla, and the sinus gland (Fig. 1D) are immunoreactive during light exposure. In these structures, 5-HT-like-immunoreactivity is quickly detected after illumination (30 min). Back to darkness, it takes longer to disappear: 1 h for rh and dpc, and more for the others. Some 5-HT somata and fibres are observed after 1 h in the external and internal medullae (Fig. 1E) and in the sinus gland. The 5-HT-like-immunoreactivity persists up to 2 h in the external medulla and the sinus gland (Fig. 1D).

After one night in darkness, no immunoreactive elements are detected in the lamina ganglionaris (Fig. 2A). The 5-HT-like immunoreactivity appears quickly, i.e. after 30 min of exposure to light (Fig. 2B) and many 5-HT elements are present at 1 h of light exposure (Fig. 2C). Then, their number decreases progressively to reach the detection limit at 3 h of exposure to light (Fig. 2D). Back to darkness, it needs 1 h to observe a 5-HT-like-immunolabelling represented only by portions of 5-HT fibres (Fig. 2E). After 2 h of darkness, the number of 5-HT-like-immunoreactive elements increases since more 5-HT somata and axons are detected (Fig. 2F,G).

The rhabdom of the reticular cells and the distal pigment cells are always immunoreactive when exposed to light. When the mysids are placed back to darkness, after an illumination period of 3 h, the number of 5-HT-like-immunolabelled structures decreases progressively:

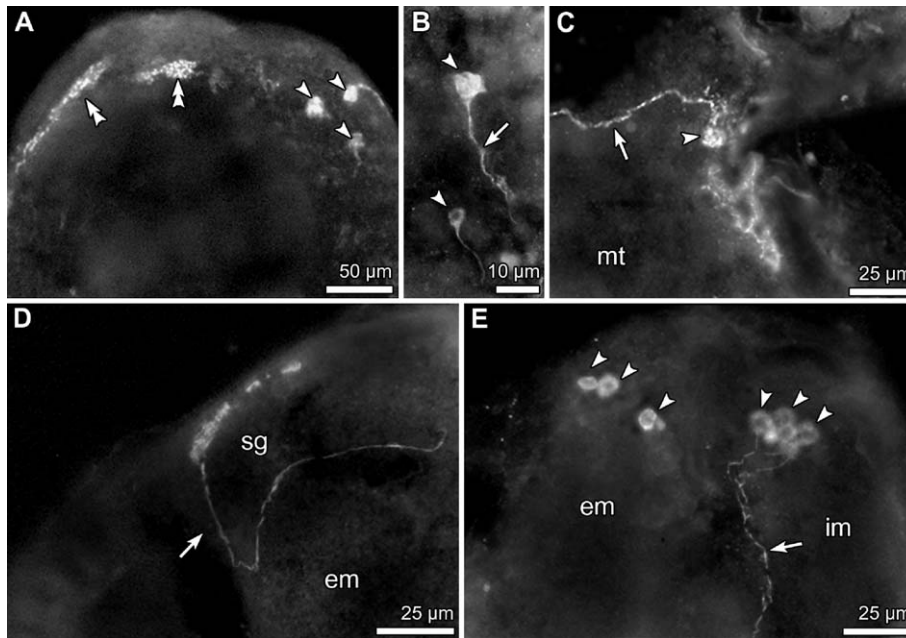


Fig. 1. Serotonin-like immunoreactivity in the optic lobe of the cave-dwelling mysid *Hemimysis margalefi* under artificial conditions of illumination. (A, B) Medulla terminalis. Terminal network after 2 h of illumination (A) and cell bodies after 1 h of darkness (B) of 5-HT neurons. (C) X-organ after 3 h of illumination. (D) Long 5-HT axon that ends in the sinus gland (sg) after 2 h of illumination. (E) External and internal medulla after 1 h of darkness. Arrowhead, 5-HT cell body; double arrowhead, 5-HT terminals; arrow, 5-HT fibres; em, external medulla; im, internal medulla; mt, medulla terminalis.

5-HT is still expressed after 30 min of darkness (Fig. 2H) but not any longer after 1 h.

3.2. *L. lingvura*

To go further, the 5-HT expression pattern in the lamina ganglionaris of *L. lingvura* was investigated. The results are summarized in Table 2. An absence of 5-HT-like immunolabelling after one night of darkness is followed by a response of the lamina ganglionaris: many 5-HT neurons are observed after 30 min (Fig. 3A) and 1 h of light exposure (Fig. 3B). Then, after 2 h of light exposure, their number decreases considerably (Fig. 3C). No 5-HT-like immunoreactivity is detected in the lamina ganglionaris when *L. lingvura* are exposed 3 h to light. When *L. lingvura* are placed in the dark, i.e. after the light–dark transition, 5-HT-like immunolabelling is observed in the lamina ganglionaris as soon as 30 min (Fig. 3D). When darkness is longer, the 5-HT-like immunoreactivity is no more detected.

4. Discussion

The first evidence for the presence of 5-HT in mysids (Crustacea, Mysidacea) has been reported by Moreau et al. (2002). In the present study, the X-organ and the

medulla terminalis are always 5-HT immunoreactive during light or dark periods. The rhabdom of the reticular cells, the distal pigment cells, the internal medulla, the external medulla, and the sinus gland are 5-HT immunoreactive during light exposure. In these latter structures, immunoreactivity is quickly detected after illumination (30 min) while, back to darkness, it takes longer to disappear. The 5-HT expression pattern in the lamina ganglionaris is very different and is discussed regarding to its possible implication during the light–dark transition phases.

In *H. margalefi* and *L. lingvura*, at least six 5-HT immunoreactive cell bodies are found in the lamina ganglionaris with many 5-HT-like fibres that project towards the reticular cells (Moreau et al., 2002). Similar organization has been found in decapod species (Beltz, 1999; Sandeman et al., 1988). Although the structure of the lamina ganglionaris and some of its expressed neurotransmitters are well known in various arthropods, the functional implication of this first neuropile involved in the treatment of light information arising from reticular cells is still poorly understood. In crayfish, Aréchiga and Rodríguez-Sosa (1998) have suggested an implication of the lamina ganglionaris in the generation of circadian rhythmicity. However, despite the numerous works conducted on the circadian

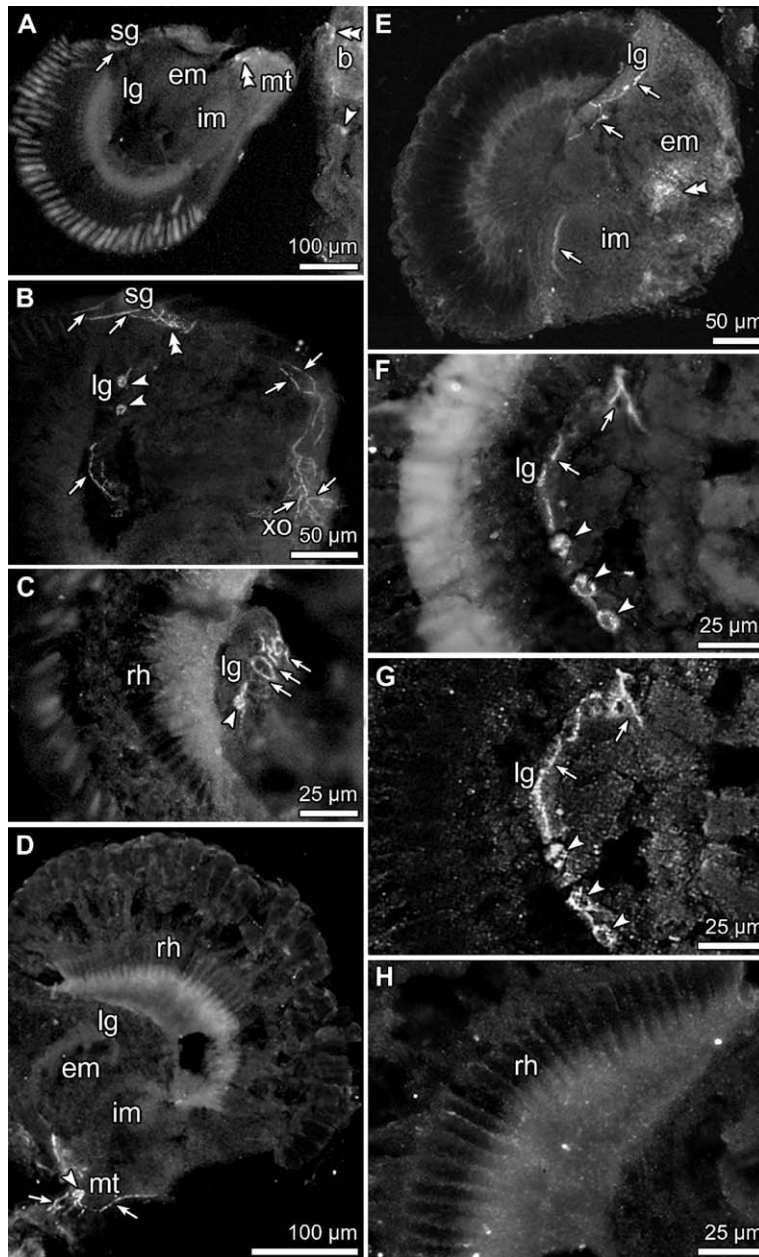


Fig. 2. Serotonin-like immunoreactivity in the optic lobe of the cave-dwelling mysid *Hemimysis margalefi* under various periods of illumination. (A) Absence of 5-HT-like labelling in the lamina ganglionaris after one night in darkness. (B, C, D) 5-HT-like immunoreactivity after 30 min, 1 h and 3 h of exposure to light, respectively. (E) 5-HT-like-immunoreactivity of the lamina ganglionaris 1 h after darkness. (F, G) 5-HT-like-immunoreactivity of the lamina ganglionaris 2 h after darkness: comparison between observation with an epifluorescence (F) or a confocal (G) microscope. (H) 5-HT-like-immunolabelling in the rhabdom of reticular cells after 30 min of darkness. b, brain; lg, lamina ganglionaris; rh, rhabdom; XO, X-organ. For other abbreviations see legends to Fig. 1.

clock function in the eyestalks of decapod crustaceans, little is known about the role of lamina ganglionaris. Most of the studies have focused on the ommatidial changes. For example, Escamilla-Chimal et al. (1998) have reported daily changes in 5-HT-like immunoreactivity in the retina, and a circadian rhythm in the retinal

electrical activity has been described in isolated retinas (Aréchiga and Rodríguez-Sosa, 1997). Morphological evidence of changes in the rhabdom shape suggests a possible sequence of events involving production, utilization and degradation of the photoreceptor membranes during changes from light to dark and dark to

Table 2

5-HT-like immunoreactivity of the lamina ganglionaris (lg) of the shallow lagoonal mysid *Leptomysis lingvura* kept in the dark for one night and then exposed to light and placed back in darkness

Duration of light or dark periods	lg
1 night darkness	-
30 min light	++
1 h light	++
2 h light	+
3 h light	-
30 min darkness	+
1 h darkness	-
2 h darkness	-

++: presence of numerous serotonin-like immunoreactive elements; +: presence of some serotonin-like immunoreactive elements; -: absence of serotonin-like immunoreactivity.

light (Itaya, 1976). During light adaptation, the eye of the nocturnal crayfish *Cherax destructor* reduces the ommatidial acceptance angle by pigment migrations (Bryceson and McIntyre, 1983). Balzer et al. (1997) have proposed that, in crayfish, melatonin, which is synthesized from 5-HT, acts on the gain of photoreceptors and on the movements of accessory pigments toward the “dark-adaptation position” and that melatonin is involved in the expression of circadian rhythms. In insects, it has also been proposed that 5-HT neurons located in the medullae are involved in circadian rhythms (Leitinger et al., 1999).

The present study underlines a fundamental role of 5-HT neurons of the lamina ganglionaris during the light–dark transition phase in mysid crustaceans, and especially in *H. margalefi*. From the present results, it can be ascertained that the expression pattern of 5-HT in the lamina ganglionaris is linked to the daily migrations of this cave species. Indeed, *H. margalefi* swarms have been noticed to have different positions in the cave according to the outside light intensity (Chevaldonné P and Lejeusne C, personal observations). By the past, nycthemeral movements of *Hemimysis* swarms have been well documented (Macquart-Moulin, 1979; Macquart-Moulin and Passelaigue, 1982; Passelaigue and Bourdillon, 1986). It is established that the swarms display circadian migrations in and out of the caves: during daytime, the cave shelters an important population of *Hemimysis* mysids; each night, these mysids undergo horizontal migrations, leaving the cave at sunset and turning back into the cave at daybreak. These migrations are homologous to vertical circadian migrations of the plankton and have been related to fluctuations in solar and lunar light intensities (Passelaigue and Bourdillon, 1986). The cave mysid species for which the nycthemeral migra-

tion pattern has been well established is *H. spelunca* Ledoyer, 1963. Since June 1999, due to a regional warming of the NW Mediterranean, *H. spelunca*, which was the unique cave mysid species of the Figuié cave, has totally disappeared from this marine cave and has been replaced by the more thermotolerant *H. margalefi* (Chevaldonné and Lejeusne, 2003; Lejeusne and Chevaldonné, 2005). As both *Hemimysis* species share very similar biology and ecology (Chevaldonné

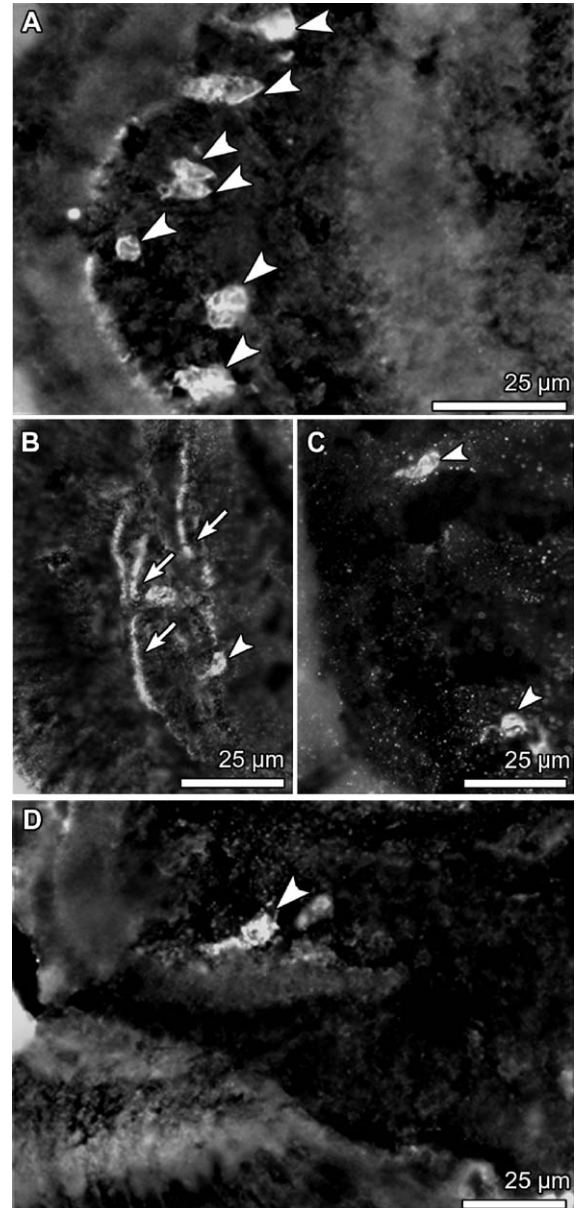


Fig. 3. (A–D) Serotonin-like immunoreactivity in the lamina ganglionaris (lg) of the shallow lagoonal mysid *Leptomysis lingvura* after 30 min (A), 1 h (B), 2 h (C) of light exposure and 30 min of darkness (D). For other abbreviations see legends to Fig. 1.

and Lejeusne, 2003; Lejeusne and Chevaldonné, 2005), it is interesting to confront data obtained for the migration pattern of *H. spelunca* and data of expression of 5-HT obtained for *H. margalefi* in present study. During daytime, *Hemimysis* mysids are confined to the darkest parts of the caves. A progressive migration of these animals from those parts of the cave has been evidenced showing that the swarm progresses slowly towards the outside (Passelaigue and Bourdillon, 1986). As long as the solar illumination is higher than the lunar one, the swarm stays at the entrance of the cave. When the solar illumination becomes lower than the lunar one, the number of mysids at the entrance decreases slowly and the exit is general only after moonset. At the end of the night, mysids go back into the cave half an hour before daybreak. The rapidity of the return migration contrasts with the progressive movement toward the exit. Therefore, it seems that the sensitivity to light of these animals is lower at the beginning of the night than at the end (Passelaigue and Bourdillon, 1986). In the same way, the present study shows that the 5-HT-like immunoreactivity in the lamina ganglionaris appears quickly, i.e. at 30 min, after the beginning of the light exposure, while when animals are brought back to darkness immunolabelling is detected only after 1 h. When *H. margalefi* is exposed to artificial light, a strong 5-HT-like immunolabelling occurs in the lamina ganglionaris after 1 h. In their natural habitat, the return migration of the whole population of *H. spelunca* back into the cave, takes only 1 h (Passelaigue and Bourdillon, 1986). The 5-HT-like immunoreactivity increases when mysids are placed in obscurity for 2 h. The exit of the cave is progressive and requires up to 5 h for the entire population. For both *H. margalefi* and *L. lingvura*, the 5-HT-like immunoreactivity of the lamina ganglionaris is transient: it disappears after 3 h of illumination and after one night in darkness. However, the comparison between the 5-HT-like immunoreactivity in the lamina ganglionaris of *H. margalefi* and *L. lingvura* reveals a different pattern of 5-HT expression. In contrast to *H. margalefi*, 5-HT-like immunoreactivity is rapidly detected in *L. lingvura*, i.e. as soon as 30 min, and this, whether the transition is from light to dark or dark to light. It is noteworthy that specimens of *L. lingvura* form swarms in shallow water in places where they are submitted to light variations. Moreover, *L. lingvura* does not undergo nycthemeral migrations. Therefore, in *H. margalefi*, it seems that the 5-HT expression in the lamina ganglionaris acts as the departure signal for the migration during the dark-to-light and light-to-dark transitional phases.

In the present work, the observed transient 5-HT-like immunoreactivity in the lamina ganglionaris may underline a possible function of the lamina ganglionaris as a photoreception signal integrator and, especially, for species showing circadian migrations. In the decapods, a circadian rhythmic function has been suggested for the lamina ganglionaris (Aréchiga and Rodríguez-Sosa, 1997). In these animals, environmental factors such as light intensities are known to modulate tonic patterns of behaviour. In crayfish, the product of the *Drosophila per*-gene (period gene), involved in the genesis of rhythms in insects and vertebrates, has been detected in cell bodies of the lamina ganglionaris (Aréchiga and Rodríguez-Sosa, 1998). It would be interesting to investigate the relationships between 5-HT and *per*-gene in the genesis of these rhythms.

In the decapods, the biological mechanisms that regulate circadian rhythms have been investigated for several decades. In fact, more than one circadian pacemaker may operate in the control of some circadian rhythms (Aréchiga et al., 1993). Two main organs have been identified as essential for the maintenance of circadian rhythms in decapod crustaceans: the optic neuropile in the eyestalk and the supraoesophageal ganglion (Aréchiga et al., 1993; Escamilla-Chimal et al., 2001; Fanjul-Moles and Prieto-Sagredo, 2003). In the eyestalk, it is likely that at least two sources of circadian rhythmicity exist, one at the level of the retina and the lamina ganglionaris, generating the photoreceptor rhythmicity, and one near or in the medulla terminalis, responsible for neurosecretory rhythms (Aréchiga and Rodríguez-Sosa, 1997). Pacemaker neurons undergo circadian rhythms of electrical and of secretory activity. Besides their endogenous rhythmicity, they are also influenced by neurotransmitters and modulators mediating the effects of light and darkness (Aréchiga, 2003). Furthermore, 5-HT has been proposed as a local modulator of the retinal activity (Aréchiga et al., 1990). Horizontal migrations of *Hemimysis* species, which consist in leaving and turning back into the cave, are under control of an endogenous circadian rhythm that depends on light and darkness. To our knowledge, the present study is the first report suggesting that a neurobiological phenomenon is synchronized with horizontal migration, an important crustacean behaviour. It is likely that 5-HT expression in the lamina ganglionaris is one of the key elements to explain this complex behaviour. Therefore, the circadian behaviour of the *Hemimysis* cave mysids may correspond to a complex physiological mechanism involving neurotransmitters, neurohormones and several pacemakers.

With regard to light intensity, it has been suggested that the horizontal migrations of cavernicolous *Hemimysis* species are similar to the vertical migrations of many zooplankton organisms. Indeed, it has been demonstrated that the illumination in the blind end of the caves, i.e. the diurnal habitat of these mysids, corresponds to that prevailing at depths about 400 to 500 m in the open sea (Passelaigue and Bourdillon, 1986). Therefore, for further investigations, we agree with these authors to consider cavernicolous mysids as a very good model for the study of the mechanisms determining and regulating the circadian migrations of zooplankton.

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