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Influence of Melatonin on Gonad Maturation in *Lineus lacteus* (Heteronemertini, Lineidae)

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ABSTRACT The annual reproductive cycle of *Lineus lacteus* is regulated by photoperiod and thermoperiod. Gonad maturation depends on a gonad-inhibiting hormone (GIH) secreted by the cerebral ganglia, in which melatonin was also observed. Results in the worm showed that melatonin concentrations seemed to vary at the two most appropriate times of the year (spawning and sexual rest), in an inverse manner to the state of gonad maturation. Exogenous melatonin did not significantly inhibit but delayed the advanced maturation of gonads triggered by decapitation of the worm. We also showed that the GIH is not melatonin. We therefore assume that the gonad-inhibiting action of melatonin is indirect, and acts via the GIH.

In *Lineus lacteus,* the following facts are known: 1) Photoreceptor cells occur at the eyes and cerebral ganglia (Vernet, '74); 2) sexual maturation is controlled by a cerebral gonad-inhibiting hormone (GIH) (Vernet and Bierne, '88); 3) the annual reproductive cycle is regulated by photoperiod and thermoperiod (Vernet, '90); and 4) melatonin is present in the eyes and brain. Its concentration varies according to a nyctohemeral rhythm (Arnoult et al., '94).

Given that variations in circulating melatonin levels throughout the year condition sexual maturation in vertebrates (Chaudhuri and Maiti, '89; Haldar and Ghosh, '90; Malpaux et al., '93), the question was whether melatonin was the gonad-inhibiting hormone in *Lineus lacteus* (Vernet and Arnoult, '92).

MATERIALS AND METHODS

The worms used were collected close to the Arago laboratory at Banyuls-sur-mer, France, from gravel at the air/seawater interface. The rearing conditions at Reims, France, followed strictly those recommended by Gontcharoff ('51), i.e., absolute darkness, and constant temperature of 12°C.

Gonadogenesis in the female of Lineus lacteus *(Fig. 2)*

In *Lineus lacteus,* like in most nemertean worms, there are males and females. The gonads are simple pouches limited by a flat epithelium with elongated nuclei. They seem to form de novo at each period of reproduction. Maturation of sexual products requires several months. Gonads are numerous and are located between the intestinal cæca. They communicate with the outside by a short gonoduct. During the period of sexual rest and shortly after, males and females are undistinguishable.

In the female, gonadogenesis occurs in the following way. Cells of the germen colonize areas between intestinal diverticles. The number of these cells is low. Some of them grow with reserve material, other cells evolve into gonad walls and gonoduct. During their differentiation, ovocytes go through four successive stages: premeiosis, previtellogenesis, vitellogenesis, and maturity (spawning).

During the premeiotic stage, ovogonies have a big nucleus. During the previtellogenesis, ovocytes I are incorporated in a conjunctive layer forming a young ovary. They grow slowly from August to December to attain a diameter of 60 μm. During vitellogenesis, ovocytes grow rapidly and increase from 60 to 250 μm in 12 weeks (from December to March). Ovocytes are then mature; they do not grow any more, but their membranes are modified. Spawning occurs from March to May; ovocytes are discharged in the seawater where fecondation rapidly takes place.

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Preparation of animals for melatonin assays

The animals were subjected to a temperature of 12°C and an 8:16 LD photoperiod; the light was on from 9 a.m. to 5 p.m. Since the conditions under which the melatonin assays were carried out were the same throughout the year, any observed variations in hormone level would therefore be seasonal.

The assays were performed on brains removed at the same time each day (9 p.m.), according to a protocol previously described (Arnoult et al., '94). The two assays were performed in April 1992 (on a batch of 40 animals) and June 1992 (on a batch of 75). We decided to use two times a year since they correspond to periods most likely to generate significant results. April is in the middle of the reproductive period, and June is in a period of sexual quiescence.

Since this sort of experiment requires numerous animals (several dozen each time), to date, we have limited ourselves to making two indicative measurements only.

Test of melatonin toxicity

To determine a possible toxic effect of melatonin on *L. lacteus,* groups of ten animals were placed in solutions of differing hormone concentrations, and survival rates were monitored over 2 months. Given the results obtained by other workers on planarians (Yoshizawa et al., '91), a phylum related to nemerteans, six concentrations were tested (25, 50, 75, 100, 150, and 200 mg/l). Melatonin-free seawater acted as control medium. In each case, three batches of ten animals were used. Batches of ten animals were placed in small dishes, each one containing 50 ml of seawater with or without melatonin, and kept in incubators at 12°C in continuous darkness.

Experimentally advanced gonad maturation—the role of melatonin

If decapitated, *L. lacteus* will develop gonads within 30 days (Gontcharoff and Lechenault, '58). The phenomenon is clearly more remarkable outside the period of reproductive activity, from December to May. We therefore used this characteristic in our study of melatonin in sexual reproduction.

Groups of ten decapitated *L. lacteus* were placed in either seawater alone or seawater containing melatonin at a range of concentrations. On each occasion, the number of days required for mature gonads to appear was recorded.

RESULTS

Melatonin levels at the most appropriate time of the annual reproductive cycle of Lineus lacteus

The assayed values for melatonin in the samples, prepared as described in the first paragraph of Materials and Methods, were as follows (Fig. 1): The lowest value (2.3 pg) was obtained in April, in the middle of the worms' period of sexual reproduction (from mid-March to mid-May); the highest value (7.5 pg) was obtained in June, just after the animals, having released their gametes into the natural environment, begin their annual period of sexual quiescence.

These preliminary results suggest that the melatonin concentration in *L. lacteus* varies at the two periods of the year at which measurements have been performed (Fig. 2).

It was not our intention to trace changes in melatonin levels over an annual seasonal cycle, but melatonin measurements have been realized at the most appropriate time of year (spawning and sexual rest) in order to obtain the maximal difference between melatonin levels. Each value represents a pool of 40 and 75 animals, respectively, for April and June so that no mean ± SEM is indicated. A previous study showed that melatonin levels vary ±0.5 pg during the nyctohemeral cycle (Arnoult et al., '94). Thus, the April data (spawning) are significantly different from those obtained in June (sexual rest). It was not possible to perform more than two measurements because of limitations in the number of worms.

Action of melatonin on advanced maturation of gonads in L. lacteus

Since our team had already shown that amputating the animal's brain outside the reproductive period triggered the advanced maturation of gonads, we were thus able to study the degree of melatonin influence. Clearly, before doing so, its possible toxicity on the animals had to be studied first.

Toxicity test

The animals placed in "normal" seawater (i.e., without melatonin) remained very healthy. This was as expected since the conditions were effectively standard rearing conditions. As concerns the melatonin solutions, the higher the concentration, the faster the worms died (Fig. 3). At concentrations of 150 and 200 mg/liter, all subjects died within 12 days. At 100 mg/liter, the time doubled; at 75 mg/liter, 60 days were required to reach this

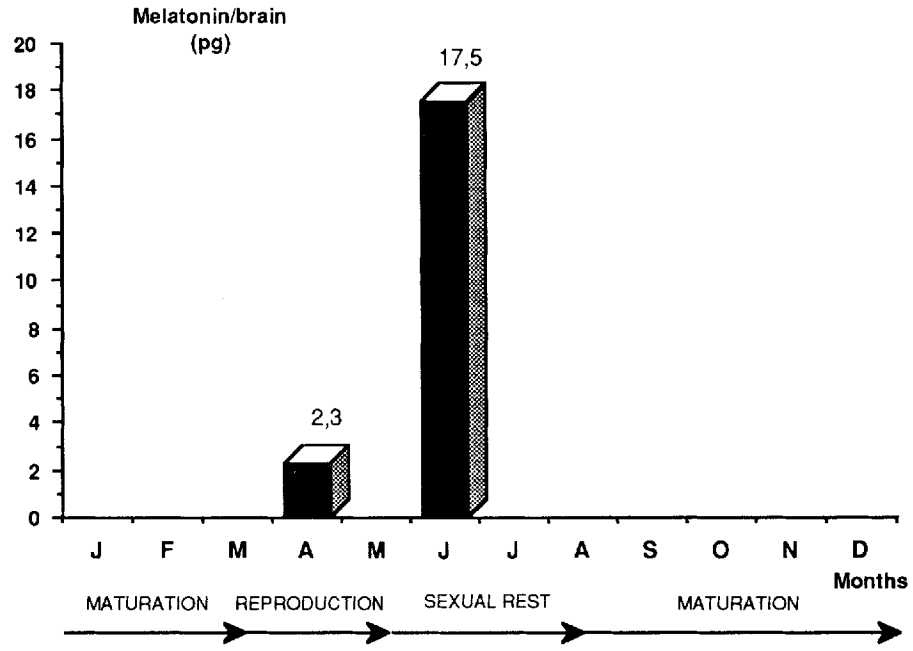

Fig. 1. Melatonin measurement in *Lineus lacteus* during the reproductive period and the sexual rest. Values correspond to melatonin amounts in the brain at 9 p.m. Experimental conditions: 8:16 LD photoperiod, constant temperature of 12°C, lights on from 9 a.m. to 5 p.m. Measurements are realized on batches of worms, so that there is no mean ± SEM.

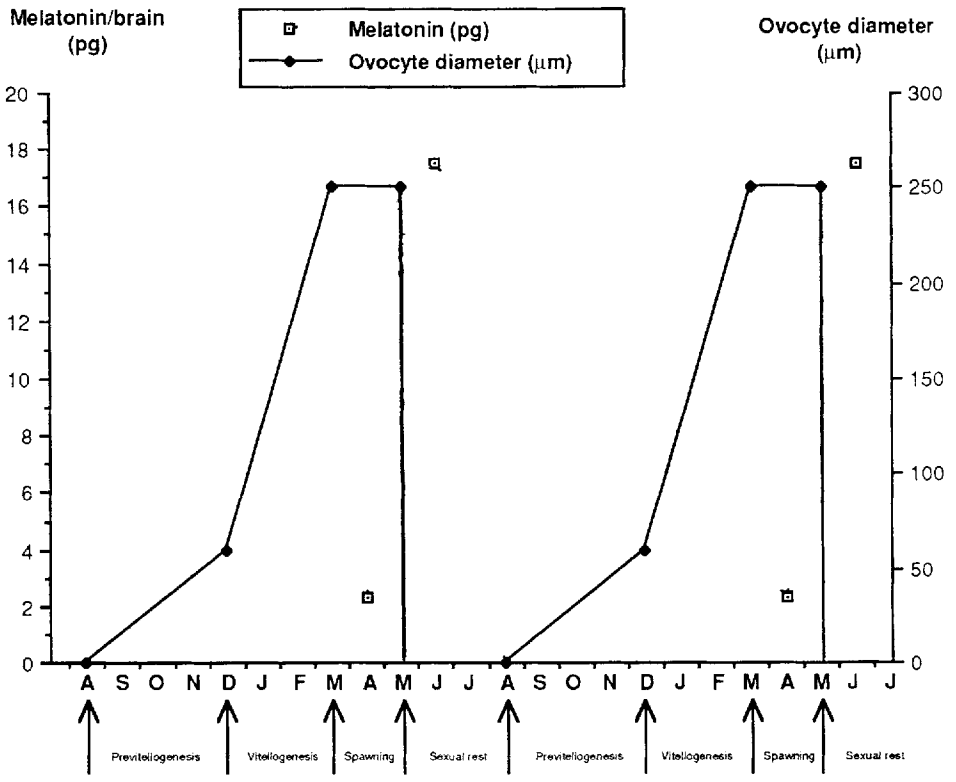

Fig. 2. Evolution of ovocyte diameter during the seasonal reproductive cycle of *Lineus lacteus*. Melatonin levels at two appropriate times of this cycle.

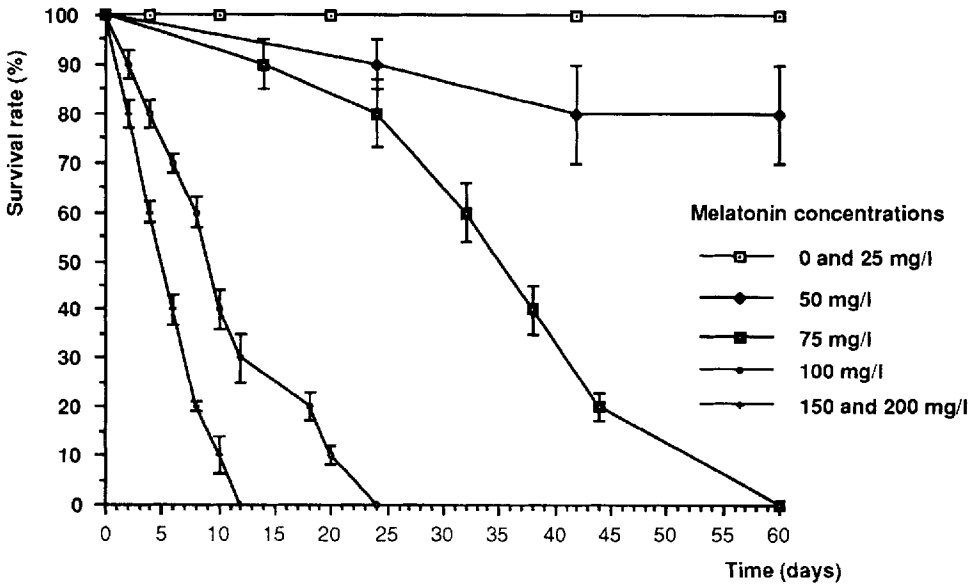

Fig. 3. Evolution of the number of *Lineus lacteus* in different concentrations of melatonin solutions (0, 25, 50, 75, 100, 150, and 200 mg/liter). Each point represents a mean value from three separate assays. Bars indicate SEM.

result. At 50 mg/liter, the survival rate at the end of 60 days was 80%. At 25 mg/liter, the survival rate was always 100%.

Given these results, it was decided that the maximum concentration to be used in future experiments would be 50 mg/liter. The seawater solutions actually used contained 0, 25, and 50 mg/liter of melatonin.

As yet, we have no hypothesis to explain how melatonin kills the animals in the toxicity tests. It may be a direct or indirect toxicity, especially when concentrations are high. In our experiments, we used subacute concentrations. To propose a hypothesis, complementary studies are still necessary. We have now started some investigations in this direction to more precisely assess the effects of melatonin and its toxicity in relation to histological analysis.

Melatonin and advanced development of gonads

Under standard conditions (seawater only, permanent darkness, and constant temperature of 12°C), the gonads of all decapitated animals matured within 30 days (Fig. 4).

When the animals were placed in a 25 mg/liter melatonin solution, the time required for gonad maturation increased: For the three trials carried out, the durations were 40, 45, and 60 days. In two cases, one individual out of ten did not present gonads. Since a sexual maturation rate of 90% is significant, the experiment could nevertheless be stopped. It should be noted that in the animals reared in the melatonin solutions (25 and 50 mg/liter), the gonads which develop were quantitatively and qualitatively comparable to those of controls.

In the 50 mg/liter solution, the time required for maturation reached 75 and 80 days. Beyond this, the proportion of mature animals reached 50–60% according to case. One animal per batch of ten died. This was in agreement with the toxicity tests carried out earlier. Nevertheless, after 3 months in the 50 mg/liter medium, the animals' state of health had deteriorated. Their movements in the dish were more sluggish, and their integument became fragile.

DISCUSSION

Melatonin is a chemical messenger transmitting photoperiod information and allowing the animal to adjust its reproductive period to favorable environmental conditions (Poeggeler, '93). In mammals, the seasonal gonad-inhibiting action of melatonin (Thibault and Levasseur, '91) requires the intermediary of gonadotropic hormones of the hypothalamo–hypophyseal axis (Fraschini et al., '68; Martin and Klein, '76; Martin et al., '80; Hastings et al., '87; Maywood et al., '89; Lincoln, '92; Malpaux et al., '93) and/or acts on the gonads directly (Ellis, '72; Fiske et al., '84; Webley and Luck, '86; Ayre and Pang, '94).

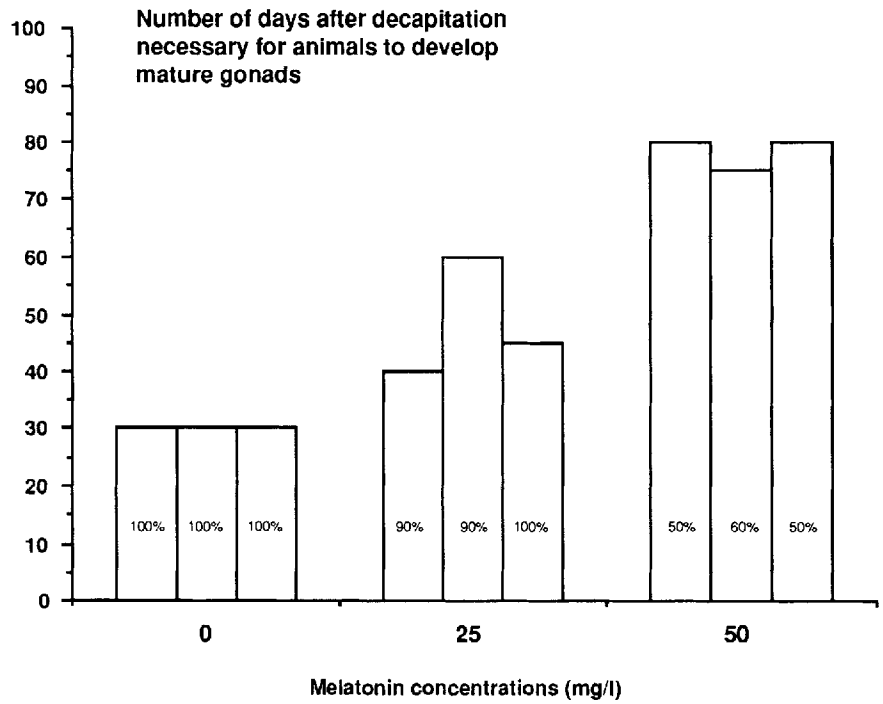

Fig. 4. Effect of melatonin on advanced maturation of gonads in *Lineus lacteus*. For each melatonin concentration, three groups of ten worms were used. Percentages indicate the quantity of sexually mature animals for each group of ten.

Melatonin has also been observed to have a circannual gonad-inhibiting effect in birds. There is an inverse relationship between the activity of the pineal gland (melatonin synthesis) and the development of gonads during the year (Chaudhuri and Maiti, '89; Haldar and Ghosh, '90; Chakraborty, '93).

In one species of tortoise (*Lissemys punctata punctata*), an "obvious" variation of pineal gland activity through the year has been observed (Mahata-Mahapatra and Mahata, '92). Outside reproduction, the nuclei of pineal cells are large; similarly, the gonads are lightest during this period. During reproduction, the opposite occurs, both in terms of gonad weight and functioning of the pineal cells.

The results of our own experiments presented here indicate variations in melatonin concentrations at two periods of the year (spawning and sexual rest), which probably condition gonad maturation in *Lineus lacteus*.

The question may therefore be whether melatonin acts on the gonads directly, or indirectly via a gonadotropic hormone, in the present case GIH.

To answer this, we should re-examine the results obtained during our attempts to inhibit gonad maturation with melatonin. For inhibition to occur, the animals had to be treated with such quantities of product (50 mg/liter) that their mobility and external integument deteriorated. Hence, we cannot conclude that melatonin has a specific action on gonadal development, but instead promotes a disease process. At lower melatonin concentrations (25 mg/liter), the animal still devoted a substantial proportion of its resources to maintaining its physiological state, hence the delay in gonad maturation observed.

In other words, it is reasonable to think that, in *L. lacteus*, melatonin does not have a direct action on the gonads but a deleterious effect on the animal overall. The following chain of events may therefore be put forward to explain the phenomena arising between perception of environmental light stimuli and sexual reproduction in *L. lacteus*.

The light from the natural milieu would be perceived by the organism's photosensitive type cells located both in eyes and cerebral ganglia (Vernet, '74). Perception of light conditions melatonin production (Arnoult et al., '94), known to be directly dependent on photoperiod. Thus, the hormone could be hypothesized to control the synthesis of gonad-inhibiting hormone in the cerebral ganglia,

the hormone itself being responsible for controlling gonad maturation (Vernet and Bierne, '88). A similar regulatory pathway has already been proposed for mammals (Pévet, '92).

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