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IN VITRO MELATONIN RHYTHM REVEALS A CLOCKED PINEAL IN THE EUROPEAN SEA BASS, *DICENTRARCHUS LABRAX*

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Abstract

The melatonin-generating system of the European sea bass, *Dicentrarchus labrax*, was studied *in vitro* using a perfusion tissue culture methodology. The examined properties included photic entrainment in the pineal gland, and circadian rhythmicity in alternating dark and light, in continuous darkness, and in continuous light. Pineal glands were removed from adult fish and placed in a perfusion tissue culture at a constant temperature of 24°C. Melatonin discharge into the culture medium was measured using radioimmunoassay. Melatonin concentrations during a photoperiod of 12h light:12h dark were low during the light periods and high during the dark. The melatonin discharge was inhibited by continuous light but strong and rhythmic in continuous dark with a circadian period (Tau) of about 24 h (n = 10). The results demonstrate that the European sea bass pineal gland is photosensitive, with ability to be entrained, and contains an internal circadian oscillator that regulates melatonin production.

Introduction

The European sea bass *(Dicentrarchus labrax*, L.) is an important commercial marine species. Females grow 20-50% faster than males and, therefore, are in greater demand for mariculture. Much effort has been spent on developing female monosex populations (Zanuy et al., 2001). In *D. labrax*, as in many gonochoristic fish species, phenotypic gender is the result of both environmental and genetic factors (Devlin and Nagahama, 2002; Gorshkov et al., 2003). Adjusted photoperiods have been used to manipulate reproduction by postponing puberty and spawning (Carrillo et al., 1995; Zanuy et al., 2001). Since photoperiod influences repro-

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duction, it was natural to study the pineal gland. The pineal gland receives light information directly from photoreceptor cells that release a hormone, i.e., melatonin, according to a daily and seasonal rhythm (reviewed by Falcon, 1999). A high concentration of melatonin during dark periods and a low concentration during light periods were reported in *D. labrax* by Sanchez-Vazquez et al. (1997), Garcia-Allegue et al. (2001), and Bayarri et al. (2002; 2004), similar to many other vertebrate species (reviewed by Falcon, 1999).

During vertebrate evolution, two types of pineal glands developed in fish. In the pineal glands of most species so far studied, intrapineal oscillators (also called a "clock") drive the rhythmic production of melatonin (Falcon, 1999; Okimoto and Stetson, 1999; Ron and Okimoto, 1999). In other fish species, the rhythmic melatonin production of the pineal gland is directly controlled by the light:dark cycle (Okimoto and Stetson, 1997; Falcon, 1999).

In this study, I present data on the photic regulation of the melatonin-generating system in the European sea bass, *D. labrax*. The commercially important *D. labrax* is a native of the Mediterranean Sea and an excellent species to serve as a gonochronistic example since it readily breeds in captivity and can easily be maintained in high densities. The objectives of this study were to (a) investigate the properties of the circadian oscillator *in vitro* in *D. labrax* and (b) determine whether there is a pineal gland clock in *D. labrax*.

Materials and Methods

Animals. Adult European sea bass, *D. labrax*, were maintained at ambient water temperature (22-24°C), under the natural photoperiod, at the National Center for Mariculture in Eilat, Israel. The fish were kept in 5 m^3 fiberglass tanks in a flow-through, seawater system (41 ppt) and fed a commercial feed (Koppens, Holland) according to their weight (Lupatsch et al., 2001).

Pineal gland removal. Fish were anesthetized with clove oil prior to decapitation and removal of the pineal gland (Bressler and Ron, 2004). The animals were decapitated during the late afternoon on the days of the trials. The pineals were removed under a surgical microscope. A coronal cut through the frontal bone was made to obtain the skullcaps. Brain tissue was gently removed from the skullcap, leaving behind the appended pineal gland organ. The pineal gland was carefully separated from the skullcap and preincubated in a sterile culture medium.

Tissue culture of the pineal gland. Dissected pineal glands were placed in a perfusion tissue culture in tissue chambers. Ten tissue chambers, each containing one pineal gland and perfusion tissue culture, were housed in a flow-through light-impermeable box. The perfusion tissue culture was Eagle's Minimal Essential Medium (MEM, M4144), supplemented with sodium bicarbonate (S5761; 2.2 g/l), L-glutamine (G5763; 0.292 g/l), penicillin-G (P3032; 1000 units/l), streptomycin sulfate (S-9137; 0.1 g/l), and amphotericin-B (A2942; 2.5 µg/l). The culture medium and supplements were obtained from Sigma Chemicals, Rehovot, Israel. A peristaltic pump was used to perfuse the glands with the culture medium at a flow rate of 1.0 ml/h. The medium in the reservoir was bubbled with a light flow of 95%O_2/5%CO_2. The culture was kept at a constant temperature of 24°C. An 18 W fluorescent bulb was mounted on the upper inner surface of the box to provide light according to the desired light:dark cycle. The lighting was regulated by a programmed electronic timer. Light intensity at the level of the tissues was 1100 lux. Perfusion samples were collected hourly with a fraction collector and frozen at -30°C until melatonin determination.

Exposure to light. The photic entrainment of the pineal glands was investigated using culture methodology (Ron and Okimoto, 1999) with a photoperiod of 12h light:12h dark or 14h light:10h dark (lights on at 08:30) for 5 days at a constant temperature of 24ºC. To test for the existence of a pineal gland clock, the glands were exposed to 12h light:12h dark (lights on 08:30) for one day, then constant dark or light for 3-4 days, also at 24ºC. Melatonin concentrations in the culture medium were measured by radioimmunoassay.

Melatonin radioimmunoassy. Melatonin was assayed directly from the culture medium using a two-phase counting system, as described by Nagy et al. (1996) but with modifications. In brief, standards (0.4-200 pg/tube) were made by serial two-fold dilutions from a 10 ng/ml stock solution of melatonin (M5250; Sigma) and 400 µl of each standard was used in the melatonin RIA. Melatonin antiserum (S704-8483; Stockgrand Ltd., Guildford, UK) was used at a final test tube dilution of 1/22,000 (100 µl/test tube). Tritiated melatonin tracer (^{3}H-*N*–acetyl–5-methoxytryptamine; specific activity 84 Ci/ mmol; Amersham Pharmacia Biotech, Sweden) was diluted in a tricine buffer (pH 5.5) to 4000 cpm for every 50 µl and this volume (50 µl/tube) was added to test tubes. The test tubes were vortexed and incubated at 4°C for 18 h. Except for three 'total count' test tubes, 500 µl dextran-coated, activated charcoal was added to each tube, vortexed, and incubated on crushed ice at 4°C for 15 min. The test tubes were centrifuged for 15 min at a speed of 3000 rpm with a Universal 32 R centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany). The supernatant (1050 µl) was decanted into miniature 6-ml polyethylene vials for scintillation counting (Packard BioScience, Groningen, Netherlands) and 4 ml of scintillate was added to each vial (Ultima Gold, Packard BioScience, Groningen, Netherlands). Each vial was counted for 5 min in a TRI-CARB 2100TR liquid scintillation analyzer (Packard Instrument Company, Meriden, CT, USA). Standards were assayed in triplicate and samples run individually. For assay validation, competitive binding curves between serial dilutions of the melatonin standard (0.4-200 pg/tube) and a collection of scotophase medium (pool) containing *D. labrax* pineal glands were examined for parallelism. The scotophase pool was parallel to the standard curve over a range of 25-400 µl (data not shown). The recovery of added cold melatonin standards to a pool of scotophase medium was linear and parallel to that of the unspiked melatonin standards over the range tested (data not shown).The intra- and inter-assay coefficients of variation were 8.7% and 13.5%, respectively.

Statistics. Data were analyzed using the GraphPad Prism Version 4.00 software package (GraphPad Software, San Diego, CA, USA). In some cases, hormone release was normalized by expressing the amount of melatonin released by an individual pineal gland as a percentage of the highest discharged melatonin value (100%) under dark conditions during each sampling interval to account for the high variation between glands. Normalized data were arc sine transformed before being analyzed by two-way ANOVA for the effects of photoperiod and time. *Post hoc* comparisons were made with the LSMEANS test. Results were considered significant at $p<0.05$. A periodogram technique (Fourier Transform) was used to calculate the period (Tau) of the melatonin rhythm in four days of darkness.

Results

Melatonin was released rhythmically, with a higher release during the dark. *In vitro*, the *D. labrax* pineal gland seems to be directly photosensitive and responsive to changes in the light:dark cycle (Figs. 1a,b). An endogenous rhythm reflecting a free-running oscillator was seen during constant dark (Fig. 1c), indicating the existence of a pineal gland clock in *D. labrax*. When placed under continuous light, the cultured glands were unable to produce melatonin (Fig. 1d). Periodogram analysis (Fourier transform, S-Plus) of the melatonin rhythm during dark revealed that the European sea bass pineal gland clock is circadian in nature (Tau = 22.1-26.4 h).

Conclusions

The rhythmic melatonin release in response to the light:dark cycle indicates that the pineal gland of *D. labrax* is photosensitive (e.g., contains photoreceptors) and that photic entrainment is present in *D. labrax*. The rhythmic melatonin release in continuous dark suggests the presence of a functional pineal gland clock. Further characterization of the melatonin-generating systems of the European sea bass is in progress.

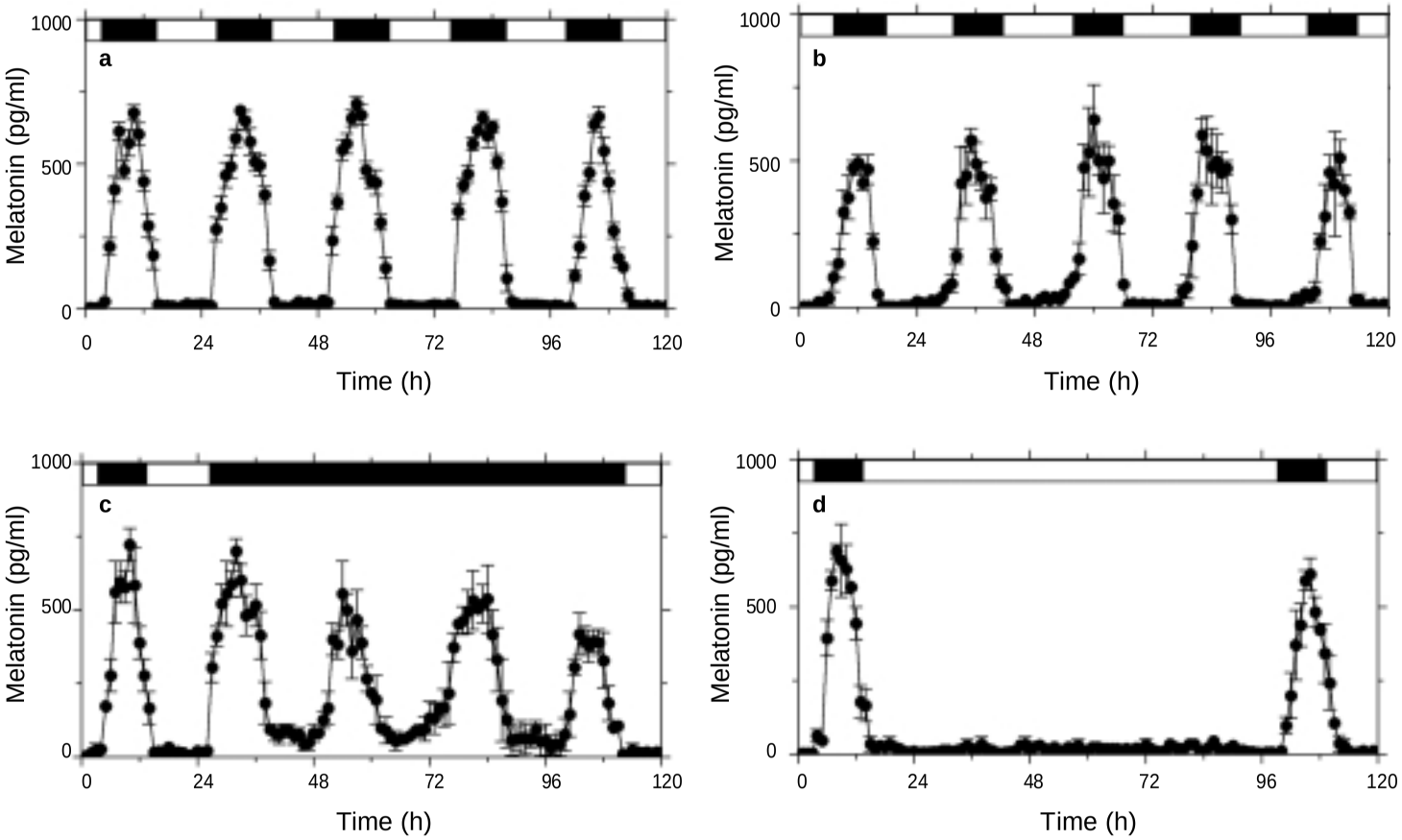

Fig. 1. Melatonin release from pineal glands of adult European sea bass (*Dicentrarchus labrax*) in a perfusion tissue culture system exposed to (a) 12h light:12h dark for five days, (b) 14h light:10h dark for five days, (c) 12h light:12h dark for one day followed by continuous darkness for 3.5 days, then 6h light on the fifth day, and (d) 12h light:12h dark for one day followed by continuous light for 3 days and 12h dark on the fifth day. Temperature was maintained at 24°C. Black boxes along the x-axis indicate dark periods; white boxes represent periods of 1100 lux light. Value are means ± SEM (n = 10).

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