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Rhythmic melatonin secretion in different teleost species: an *in vitro* study

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Abstract The rhythmic production of melatonin is governed by intrapineal oscillators in all fish species so far investigated except the rainbow trout. To determine whether the latter represents an exception among fish, we measured in vitro melatonin secretion in pineal organs of nine wild freshwater and six marine teleost species cultured at constant temperature and under different photic conditions. The results demonstrate that pineal organs of all species maintain a rhythmic secretion of melatonin under light:dark cycles and complete darkness, and strongly suggest that most fish possess endogenous intrapineal oscillators driving the rhythm of melatonin production, with the exception of the rainbow trout.

Key words Melatonin · Pineal organ · Photoperiod · Rhythm · Teleost fish

Abbreviations *LD* light:dark · *DD* dark:dark · *NAT* *N*-acetyltransferase · *RIA* radioimmunoassay

Introduction

Melatonin is one of the signals elaborated by the pineal organ of vertebrates. This hormone is involved, as an internal "Zeitgeber", in the control of various circadian and seasonal rhythms. In all vertebrates investigated so far, the LD cycle is the principal environmental factor controlling melatonin secretion. Melatonin biosynthesis is low during daytime and high during nighttime.

In fish pineals, biosynthesis takes place in photoreceptor cells (Falcón et al. 1994a) which are structurally and functionally analogous to retinal photoreceptors. During the course of evolution they have been gradually replaced by modified photoreceptors, mainly found in lizards and birds, and then by pinealocytes *sensu stricto*, mainly found in snakes, birds and mammals (Collin et al. 1986, 1989).

The rhythmic production of melatonin in fish, lizard and chicken is governed by intrapineal circadian oscillators probably localized in each photoreceptor cell (Menaker and Wissner 1983; Kezuka et al. 1989; Takahashi et al. 1989; Falcón et al. 1992; Pickard and Tang 1993, 1994; Bolliet et al. 1994). In mammals, the locus of the circadian oscillators is the suprachiasmatic nuclei.

In vitro studies of rhythmic production of melatonin in fish are particularly scarce. Intrapineal oscillators have previously been localized in only three teleost species: the pike, *Esox lucius*, the goldfish, *Carassius auratus*, and the white sucker, *Catostomus commersoni* (Falcón et al. 1989; Kezuka et al. 1989; Iigo et al. 1991; Zachmann et al. 1992b). In contrast, no intrapineal oscillator was identified in the rainbow trout, *Oncorhynchus mykiss* (Gern and Greenhouse 1988; Randall et al. 1991; Gern et al. 1992; Max and Menaker 1992). In this species, pineal organs cultured in complete darkness display continuously high melatonin release. Whether this species represents an exception among fish remains to be elucidated.

The present study was undertaken to investigate endogenous production of pineal melatonin in a variety of fish species. For this purpose, we measured in vitro melatonin secretion in pineal organs of nine wild freshwater and six marine teleost species, cultured at constant temperature and under LD and DD conditions.

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Materials and methods

Animals

Nine freshwater and six marine teleost species were used in this study (Table 1). Pike (*Esox lucius*), catfish (*Ictalurus punctatus*), yellow sunfish (*Lepomis gibbosus*), largemouth bass (*Micropterus salmoides*), golden shiner (*Notemigonus chrysoleucas*) and yellow perch (*Perca flavescens*) were caught in May in the "rivière des Prairies", Québec, Canada. Creek chub (*Semotilus atromaculatus*) were obtained from a commercial source in Montreal in the last week of June. Animals were kept for one night in aerated water at 15 °C before sacrifice. Carp (*Cyprinus carpio*) and eel (*Anguilla anguilla*) originating from ponds of the Poitou-Charentes, France, were obtained in March from a commercial hatchery and immediately sacrificed. Alewife (*Alosa pseudoharengus*), cod (*Gadus morhua*), sea raven (*Hemitripturus americanus*), winter flounder (*Pseudopleuronectes americanus*), mackerel (*Scomber scombrus*) and white hake (*Urophysis tenuis*) were caught in August in St. Andrews, New Brunswick, Canada. Fish were kept in outdoor tanks for no more than a few hours before sacrifice.

The water temperature in the natural habitat of all species was about 13–15 °C. Natural photoperiods are given in Table 1. All animals were anesthetized with MS 222 and killed by decapitation during daytime. After removing the skullcap, the pineal organ was quickly detached from the meninges and immersed in 1 ml sterile culture medium (RPMI 1640) supplemented with Penicillin/Streptomycin (100 IU·ml^{-1}) for 10 min.

Culture

A static culture was used for pineals of all species investigated except the creek chub (superfusion system). The culture medium used in all experiments was RPMI 1640 supplemented with bicarbonate (2 g·l^{-1}), glucose (2 g), fungizone (5 µg·ml^{-1}), penicillin (100 IU·ml^{-1}) and streptomycin (100 µg·ml^{-1}) (all from Gibco, Brl Canada).

Static organ culture

Four pineals per species were transferred into 24-well culture plates (Falcon 3047, Becton Dickinson Labware), each well containing 500 µl of culture medium and one organ. All pineals were covered with a piece of sterile gauze to facilitate medium change. Culture media were renewed every 3 h and samples were kept at −20 °C until analysis.

Flow-through organ culture (superfusion)

Four pineal organs were cultured with a perifusion system previously described in Falcon et al. (1989). Each pineal was placed in an incubation chamber delimited by two Teflon plugs (final volume 500 µl). The culture medium was supplemented by means of a peristaltic pump at a flow rate of 1 ml·h^{-1}. Fractions were collected automatically every 2 h and stored at −20 °C until analysis.

Experimental conditions

Since cultures were run in different laboratories, the availability of the incubation chambers did not allow us to use conditions of photoperiod and temperature similar to natural ones. Experimental conditions used for each species are summarized in Table 1.

Melatonin radioimmunoassay (RIA)

Culture medium concentrations of melatonin were measured by means of a direct RIA previously described and validated by Zachmann et al. (1991).

Data analysis

The amounts of melatonin released in the media were measured every 3 h for six to seven consecutive 24 h cycles for each pineal. The resulting data series were analyzed to detect a period and its length. Because periods cannot be detected when a trend is present, a linear equation was fitted to the data series. The series were detrended using the variate-difference method (Legendre and Legendre 1983), only when the slope of the fitted equation was significantly different from zero ($P < 0.05$). This resulted in stationary series which were subsequently smoothed using the moving-average method for a

Table 1 Experimental conditions. (*LD* light/dark schedule, *DD* continuous darkness)

Species	Experimental photoperiod and temperature of culture	Natural photoperiod	Schedule of culture
Freshwater species			
Anguilla anguilla	18 °C; 12L$_{(0800\ 2000h)}$/12D	12L$_{(0700\ 1900h)}$/12D	2LD/4DD/1LD
Cyprinus carpio	18 °C; 12L$_{(0800-2000h)}$/12D	12L$_{(0700-1900h)}$/12D	2LD/4DD/1LD
Esox lucius	15 °C; 15L$_{(0600\ 2100h)}$/9D	15L$_{(0600\ 2100h)}$/9D	LD/4DD/1LD
Ictalurus punctatus	15 °C; 15L$_{(0600-2100h)}$/9D	15L$_{(0600-2100h)}$/9D	LD/4DD/1LD
Lepomis gibbosus	15 °C; 15L$_{(0600-2100h)}$/9D	15L$_{(0600-2100h)}$/9D	LD/4DD/1LD
Micropterus salmoides	15 °C; 15L$_{(0600-2100h)}$/9D	15L$_{(0600-2100h)}$/9D	LD/4DD/1LD
Notemigonus chrysoleucas	15 °C; 15L$_{(0600\ 2100h)}$/9D	15L$_{(0600\ 2100h)}$/9D	LD/4DD/1LD
Perca flavescens	15 °C; 15L$_{(0600\ 2100h)}$/9D	15L$_{(0600-2100h)}$/9D	LD/4DD/1LD
Semotilus atromaculatus	18 °C; 16L$_{(0500-2100h)}$/8D	16L$_{(0500\ 2100h)}$/8D	2LD/4DD/1LD
Marine species			
Alosa pseudoharengus	20–22 °C; 12L$_{(0800-2000h)}$/12D	14L$_{(0630-2030h)}$/10D	LD/4DD/1LD
Gadus morhua	20–22 °C; 12L$_{(0800\ 2000h)}$/12D	14L$_{(0630\ 2030h)}$/10D	LD/4DD/1LD
Hemitripturus americanus	20–22 °C; 12L$_{(0800\ 2000h)}$/12D	14L$_{(0630-2030h)}$/10D	LD/4DD/1LD
Pseudopleuronectes americanus	20–22 °C; 12L$_{(0800-2000h)}$/12D	14L$_{(0630-2030h)}$/10D	LD/4DD/1LD
Scomber scombrus	20–22 °C; 12L$_{(0800\ 2000h)}$/12D	14L$_{(0630\ 2030h)}$/10D	LD/4DD/1LD
Urophysis tenuis	20–22 °C; 12L$_{(0800\ 2000h)}$/12D	14L$_{(0630\ 2030h)}$/10D	LD/4DD/1LD

window of three observations (Legendre and Legendre 1983). Series that did not show any significant trend ($P > 0.05$) were directly smoothed with the moving-average method. Data for each species were finally converted to a contingency periodogram (Legendre et al. 1981) in order to detect significant rhythms in melatonin release. Period lengths of the melatonin secretion were determined for data series representing four 24 h cycles starting after the first nocturnal surge.

Results

When submitted to a LD cycle, pineal organs of all species investigated maintained a rhythmic production of melatonin, with high amounts being released during the dark phase and low amounts released during the light phase.

Under DD conditions melatonin secretion from some pineal organs was either progressively increasing or decreasing throughout the dark period, resulting in significant trends in the data series (Fig. 1A). In this case, data were first detrended using the variate-difference method (Fig. 1B) prior to smoothing with a moving average of three observations (Fig. 1C). With this procedure, two data points were lost at the beginning of the series and one at the end.

Of the four pineals tested for each species, one to three of these maintained a significant rhythm of melatonin secretion for four consecutive 24 h dark cycles (Table 2). Depending on the pineal and the species, the analysis revealed periods ranging from 20/22 h to 27/30 h (Figs 2, 3; Table 2). Melatonin concentrations in the media were high during the subjective night and low during the subjective day. In both freshwater and marine species, the amplitude of the rhythm was dramatically reduced when compared to that observed under LD cycles (Figs. 1–6). Depending on the pineal, different rhythmic patterns of melatonin secretion were observed. The amplitude of the oscillations was either well sustained throughout the dark period (Fig. 3) or tended to dampen after the first one or two 24 h cycles (Fig. 4).

In most pineals of marine species and in pineals of the pike and the yellow perch, the maximum levels of melatonin observed under DD conditions were similar to or higher than those observed under LD conditions (Figs. 3, 5). In these species the oscillation patterns remained relatively constant throughout the dark period. In the sea raven melatonin secretion increased continuously throughout the culture duration (Fig. 1A). In contrast, most pineal organs of freshwater species showed a marked drop in peaks of melatonin secretion under complete darkness (Figs. 4, 6). In these species the secretory activity generally decreased throughout the dark period and the rhythmic activity tended to dampen.

When no significant circadian rhythm was maintained for four consecutive 24 h cycles, the melatonin secretion was (i) markedly suppressed after a few days, probably due to damage at removal; (ii) rhythmic only at the beginning of the dark period; or (iii) rhythmic at the end of the DD period, after one or two transient cycles (data not shown).

Application of a 24 h LD cycle at the end of the DD period generally reestablished a rhythmicity of high amplitude (Figs. 1, 3, 5). However, in pineals that released decreasing levels of melatonin throughout the dark period, the final LD cycle did not restore nocturnal levels of melatonin secretion as high as before continuous darkness (Figs. 4, 6).

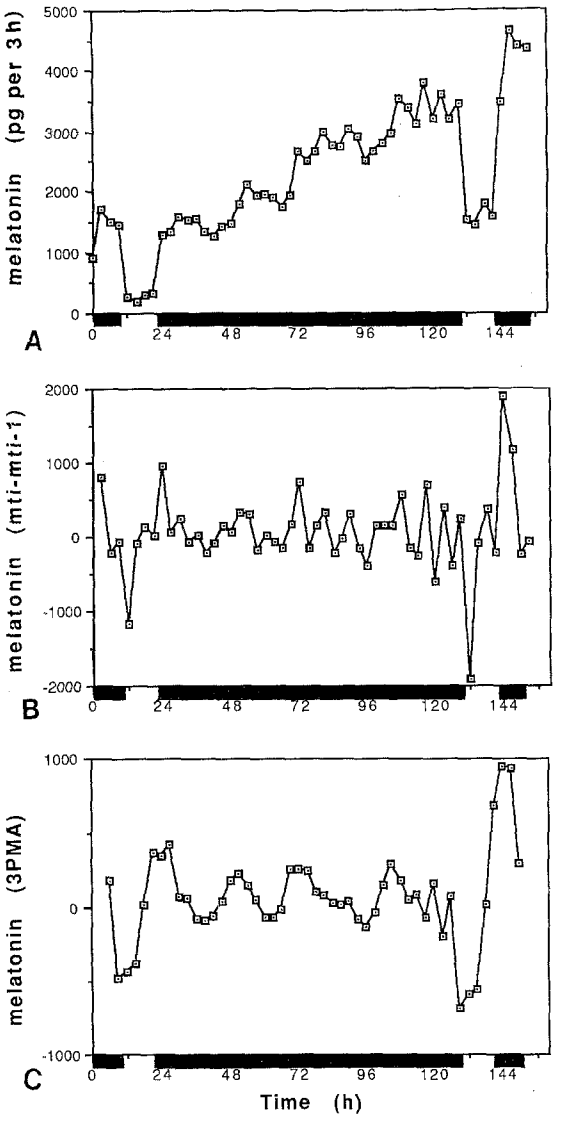

Fig. 1A–C Melatonin release from a pineal of *Hemitripturus americanus* cultured at 20–22 °C: **A** raw data series. The release of melatonin increases progressively throughout the culture period, resulting in a significant trend. Each point is plotted at the start of the collection interval: **B** detrended data series; **C** smoothed stationary series. Each point represents the mean of three points plotted at the start of the collection interval. Lighting conditions are indicated along the abscissa (*filled box = dark*). At time 0 (2100 hours), the pineal was submitted to one $12L_{(0800-2000)}/12D$ cycle and one night. Complete darkness was then applied for four 24 h cycles. The experiment was ended by a 24-h LD cycle.

Table 2 Trends and periods of the rhythmic secretion of melatonin from pineals of different teleost species cultured under complete darkness. Trends were detected by fitting a linear equation to the data series. Period length of melatonin release was detected for four 24-h dark cycles using the contingency periodogram. *n*: number of pineal per species showing a significant rhythm of melatonin secretion for four consecutive 24-h dark cycles; y: significant trend in the data series: n: no significant trend in the data series

Species	*n*	Period		Trend
Freshwater species				
Anguilla anguilla	2	27 h	$P = 0.02$	y
		27 h	$P = 0.01$	y
Cyprinus carpio	2	24 h	$P = 0.004$	n
		24/27 h	$P = 0.01$	y
Esox lucius	3	24 h	$P = 0.001$	y
		24/27 h	$P = 0.01$	n
		27 h	$P = 0.01$	n
Ictalurus punctatus	1	24 h	$P = 0.009$	y
Lepomis gibbosus	2	24/27 h	$P = 0.008$	y
		27/30 h	$P = 0.02$	y
Micropterus salmoides	1	24 h	$P = 0.03$	y
Notemigonus chrysoleucas	3	24 h	$P = 0.02$	n
		24 h/27 h	$P = 0.03$	y
		24 h/27 h	$P = 0.01$	y
Perca flavescens	2	24/27 h	$P = 0.02$	n
		27 h	$P = 0.03$	n
Semotilus atromaculatus	3	20/22 h	$P = 0.007$	y
		22 h	$P = 0.0005$	y
		22 h	$P = 0.0003$	n
Marine species				
Alosa pseudoharengus	2	27 h	$P = 0.02$	n
		27 h	$P = 0.01$	n
Gadus morhua	3	27 h	$P = 0.02$	y
		27 h	$P = 0.01$	n
		24/27 h	$P = 0.01$	n
Hemitripturus americanus	2	24/27 h	$P = 0.01$	y
		27 h	$P = 0.01$	y
Pseudopleuronectes americanus	3	27 *h*	$P = 0.01$	n
		27 h	$P = 0.001$	n
		27 h	$P = 0.04$	n
Scomber scombrus	3	24/27 h	$P = 0.007$	n
		27 h	$P = 0.005$	n
		27/30 h	$P = 0.02$	n
Urophysis tenuis	2	24/27 h	$P = 0.02$	n
		27 h	$P = 0.03$	n

Discussion

When submitted to a LD cycle isolated pineals of nine freshwater and six marine teleost species maintained a rhythmic secretion of melatonin, with high amounts being released during nighttime and low amounts released during daytime. These findings are in good agreement with in vivo and in vitro results previously obtained for the pike, white sucker, goldfish, brook trout, rainbow trout, and lamprey, *Petromyzon marinus* (Falcón et al. 1987, 1989; Gern and Greenhouse 1988; Kezuka et al. 1989; Randall et al. 1991; Zachmann et al.

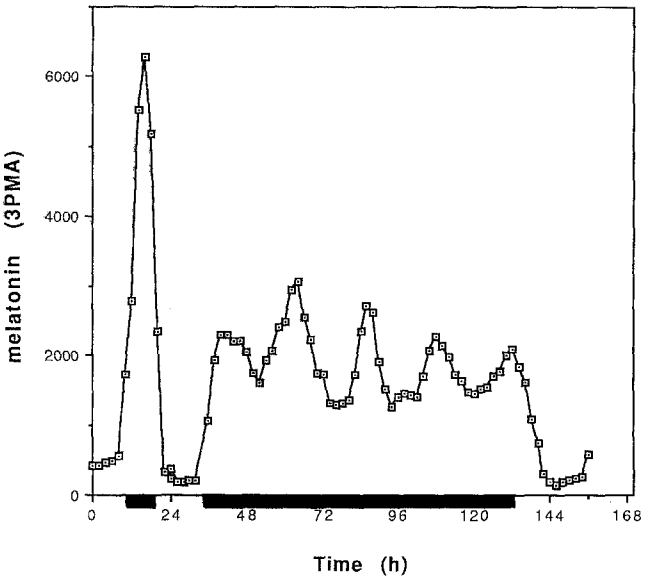

Fig. 2 melatonin release from a pineal organ of *Semotilus atromaculatus* cultured at 18 °C with a perfusion system. The initial raw data series was smoothed using a moving-average method with a window of three points. Lighting conditions are indicated along the abscissa (*filled box = dark*). At time 0 (0800 hours) the pineal was submitted to two $16L_{(0500-2100)}/8D$ cycles. Complete darkness was then applied for four 24-h cycles. The experiment was ended by a 24-h LD cycle. Each point represents the mean of three points plotted at the start of the collection interval. Under complete darkness, melatonin was released in a rhythmic manner with a period of 22 h

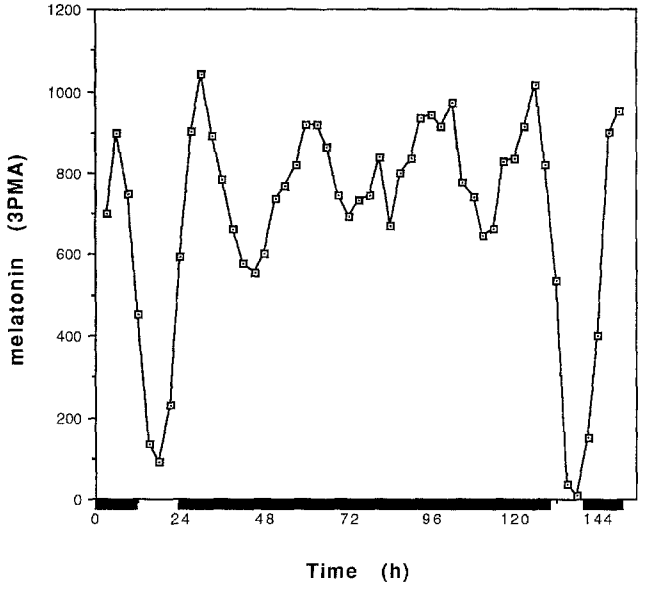

Fig. 3 Melatonin release from a pineal organ of *Scomber scombrus* cultured at 20–22 °C. The initial raw data series was smoothed using a moving-average method with a window of three points. Lighting conditions are indicated along the abscissa (*filled box = dark*). Same experimental conditions as in Fig. 1. The period revealed by the periodogram analysis for the cycles in DD is of 27/30 h

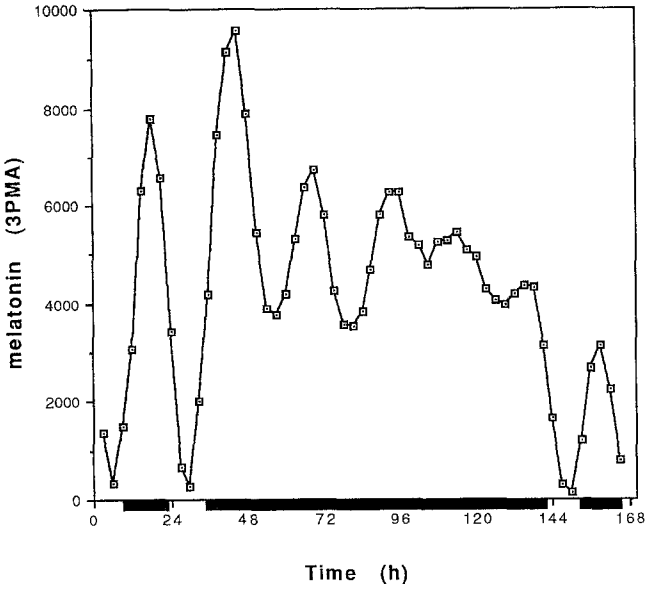

Fig. 4 Melatonin release from a pineal organ of *Cyprinus carpio* cultured at 20 °C. The initial raw data series was smoothed using a moving-average method with a window of three points. Lighting conditions are indicated along the abscissa (*filled box = dark*). At time 0 (0800 hours) the pineal was submitted to two $12L_{(0800-2000)}/12D$ cycles. Complete darkness was then applied for four 24-h cycles. The experiment was ended by a 24-h LD cycle. Each point represents the mean of three values plotted at the start of the collection interval

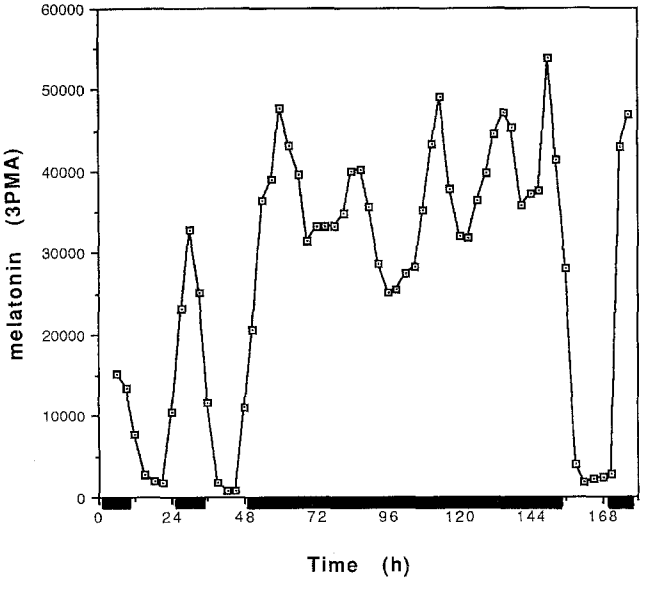

Fig. 5 Melatonin release from a pineal organ of *Esox lucius* cultured at 15 °C. The initial raw data series was smoothed using a moving-average method with a window of three points. Lighting conditions are indicated along the abscissa (*filled box = dark*). At time 0 (2000 hours), pineal was submitted to two $15L_{(0600-2100)}/9D$ cycles and one night. Complete darkness was then applied for four 24-h cycles. The experiment was ended by a 24-h LD cycle. Each point represents the mean of three values plotted at the start of the collection interval

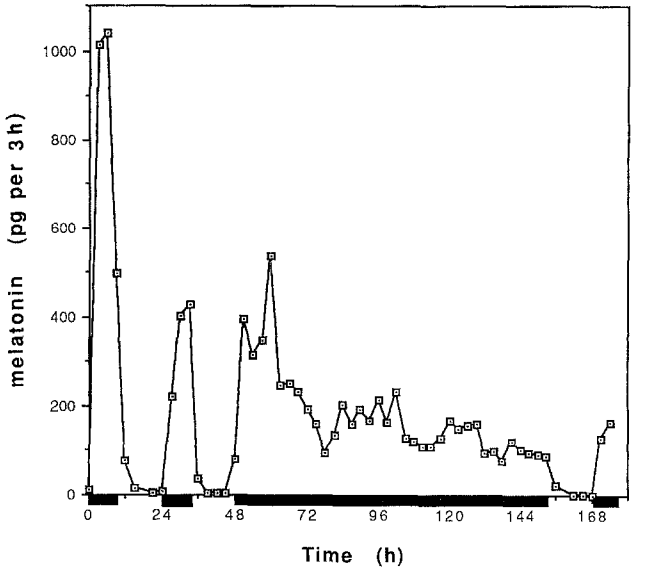

Fig. 6 Raw data of melatonin release from a pineal organ of *Notemigonus chrysoleucas* cultured 15 °C. Lighting conditions are indicated along the abscissa (*filled box = dark*). Same experimental conditions as in Fig. 5. Each point is plotted at the start of the collection interval

1992a, b; Bolliet et al. 1993). As demonstrated in vitro and in vivo in the pineal of the pike and the rainbow trout, the nycthemeral fluctuation of melatonin secretion depends on daily changes in NAT activity, a key enzyme in the melatonin biosynthesis pathway (Falcon 1987, 1989; Morton and Forbes 1988).

The rhythm of melatonin production is governed by endogenous oscillators in all vertebrate species so far investigated (see Introduction), except in the rainbow trout and the lizard, *Dipsausorus dorsalis* (Gern and Greenhouse 1988; Underwood 1989; Randall et al. 1991; Max and Menaker 1992). In these species the pineal organ functions as a simple photometer in term of melatonin production. Without the control of oscillators, the secretion of melatonin remains low in the light and high in darkness independently of the duration of each phase.

As the rhythmicity of melatonin secretion had been previously investigated in only four teleost species (see Introduction), we undertook this study in order to extend our knowledge to a larger number of species. It is shown here that a significant rhythm of melatonin secretion was maintained for four 24-h dark cycles in one to three isolated pineals of 15 wild teleost species. These results support the idea that most fish species possess intrapineal oscillators controlling melatonin production, and distinguish the rainbow trout as an exception. While the lack of endogenous rhythmicity remains unexplained in the lizard *Dipsausorus*, it was suggested that in the rainbow trout it might be the result of genetic selection for desirable rearing qualities in this strain of trout (Gern and Greenhouse 1988; Gern et al. 1992). Further experiments on wild

salmonids are now required in order to strengthen this hypothesis.

The lack of sustained circadian rhythm of melatonin secretion in some pineals used in the present study needs an explanation. On the one hand, it is possible that pineals were damaged at removal, especially in species such as the largemouth bass or the catfish which possess a very rigid skullcap. On the other hand, it has been previously stated that the profile of the rhythms may result from the experimental conditions [Bolliet et al. (1994) and references therein]. A large number of pineals can be studied at the same time in static culture, but the successive renewal of the medium might affect the organ. Finally, it has been demonstrated in chick pineal cells that medium osmolarity could affect the expression of the oscillators (Zatz and Wang 1991). Although the RPMI 1640 osmolarity was relatively low for marine species (300 mosmol·l^{-1}), it is unlikely that it could affect their melatonin production. Indeed, isolated pineals of cod cultured with this medium and with RPMI adjusted to 450 mosmol·l^{-1} showed a similar pattern of melatonin secretion under DD conditions (unpublished data).

The oscillations of melatonin release from most pineals of marine species cultured at 20–22 °C remained stable or increased slightly throughout the dark period. In contrast, the release of melatonin from most pineals of freshwater species cultured at 15 °C progressively decreased under complete darkness. These results are consistent with those reported in pineals of the white sucker showing an increase of melatonin release at 20 °C and a decrease at 10 °C (Zachmann et al. 1992b). In this species, it was suggested that temperatures of both acclimatization and culture were important in determining the profile of the rhythm (above reference).

In poïkilothermic vertebrates, it is believed that the pineal acts as a transducer of both photic and thermic informations (Underwood 1989; Zachmann et al. 1991; Falcon et al. 1994b). Low temperatures reduce the amplitude of the melatonin rhythm and can even abolish the circadian oscillations of melatonin secretion (Vivien-Roels et al. 1988; Delgado and Viviens-Roels 1989; Zachmann et al. 1992b; Bolliet et al. 1994). Taken together these results suggest that the different patterns of melatonin secretion are not species dependent but might result, at least in part, from the different temperatures used for the culture. On the other hand, it has been demonstrated in the pike that the center of the pineal organ began to degenerate after 1 or 2 days of culture (C. Thibault, personal communication). This suggests that the progressive decrease in melatonin release observed in some freshwater species might also reflect a degeneration of the organ. This hypothesis is supported by the fact that the final LD cycle did not restore high nocturnal melatonin levels in these species.

It is noteworthy that an endogenous rhythmicity of melatonin secretion was observed in pineals of pike cultured at 15 °C in the present study but not in pineals of pike originating from France and cultured at the same temperature (Bolliet et al. 1994). In pineals of the French pike cultured in the dark, it was demonstrated that the NAT activity was maximal at 18–25 °C (Thibault et al. 1993). Similar experiments need to be undertaken with Canadian pike which live for extended periods in lakes covered by ice. This would determine whether ecological habitat can affect the thermal sensitivity of the enzyme and thus, whether the difference observed between the French and Canadian pike can be the result of different selective processes.

The 3-h sampling interval used in the present study (except for the creek chub) did not allow us to determine precisely the length of the free-running period. In most species investigated the periodogram analysis revealed a significant rhythm of melatonin secretion with a period longer than 24 h (24/27 h). Since the moving-average method can slightly lengthen the period of a series it is difficult to compare our results with those previously reported in the literature. However, it is noteworthy that, despite the use of this averaging method, pineals of creek chub (the only species sampled every 2 h and cultured with a perfusion system) released melatonin rhythmically with a period of less than 24 h (20/22 h). It is unlikely that these variations depend on the sampling interval or the type of culture. Indeed, pineals of pike cultured with a superfusion system and sampled every 1, 2 or 3 h under DD conditions secrete melatonin in a rhythmic manner with a circadian period of 26/28–24/27 h (Bolliet et al. 1994; unpublished data). In addition, pineals of the white sucker cultured in static or in superfusion and sampled every 3 h, released melatonin with a circadian period of 21/24 h (Zachmann et al. 1992b; unpublished data). The functional significance of the length of the period remains unknown. The periods of circadian rhythms are generally less than 24 h, in day-active animals and greater than 24 h in night-active animals [Hastings et al. (1991) and references therein]. The results obtained in the present study do not support this hypothesis in fishes. On the other hand, it has been reported in birds that the photoperiod used prior to the dark period could affect the period of the circadian rhythm. As the creek chub was the only species acclimated to a long photoperiod, one cannot exclude a similar phenomenon in fishes. Further experiments with fishes need to be undertaken in order to determine whether or not there are species differences in the length of the circadian period and whether these differences may have a functional significance.

In conclusion, the present study provides the first evidence that isolated pineals of a large number of wild freshwater and marine teleost species maintain a rhythmic secretion of melatonin for four 24-h cycles in complete darkness. Our results strongly suggest that most fish possess endogenous oscillators driving the rhythm of melatonin production with the exception of the rainbow trout. Comparative studies between the pineal of

the trout and that of any other of the species investigated to date might prove useful in elucidating the mechanism of the clock.

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