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PHOTIC ENTRAINMENT OF CIRCADIAN ACTIVITY PATTERNS IN THE TROPICAL LABRID FISH *HALICHOERES CHRYSUS*

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ABSTRACT

Yellow wrasses (*Halichoeres chrysus*) show clear daily activity patterns. The fish hide in the substrate at (subjective) night, during the distinct rest phase. Initial entrainment in a 12h:12h light-dark (12:12 LD) cycle (mean period 24.02h, SD 0.27h, $n = 16$) was followed by a free run (mean period 24.42h, SD 1.33h) after transition into constant dim light conditions. Light pulses of a comparable intensity as used in the light part of the LD cycles did not result in significant phase shifts of the free-running rhythm in constant darkness. Application of much brighter 3h light pulses resulted in a phase-response curve (PRC) for a fish species, with pronounced phase advances during late subjective night. The PRCs differed from those mainly obtained in other vertebrate taxa by the absence of significant phase delays in the early subjective night. At that circadian phase, significant tonic effects of the light pulses caused a shortening of the circadian period length. Entrainment to skeleton photoperiods of 1:11 LD was observed in five of six wrasses exposed, also after a 3h phase advance of this LD cycle. Subsequently, a 1:11.25 LD cycle resulted in entrainment in four of the six fish. It is suggested that the expression of the circadian system in fish can be interpreted as a functional response to a weak natural zeitgeber, as present in the marine environment. This response allows photic entrainment as described here in the yellow wrasse. (*Chronobiology International, 17*(*5*), *613–622, 2000*)

Key Words: Circadian rhythm—Daily—Entrainment—Fish—Phase-response curve.

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INTRODUCTION

Light is the main environmental factor that entrains circadian rhythms (Aschoff 1981). In vertebrates, formal properties of entrainment have been documented extensively in mammals and birds (Johnson 1997). In fish, however, the evidence for entrainment by light, studied under light-dark (LD) cycles and in continuous light conditions, is scarce.

Tonic effects of light on rhythms in fish have been reported in the pioneering studies of Schwassmann (1971). He confirmed Aschoff's rules of impact of light intensity on the period of activity patterns in constant conditions in electric fish. Similar observations have been collected in many other fish species (Tabata et al. 1989). Arguments for phasic effects of light can be drawn indirectly from studies of annual effects on timing of activity. As shown by Müller (1978) and Eriksson (1978), several species change phase of activity (day, night, or crepuscular) according to the season. However, as shown by several authors (Schwassmann 1971; Kavaliers 1978; Gerkema 1992), the majority of fish species kept in constant darkness lose circadian activity patterns within some weeks.

Discrepancy between rhythmic patterns in nature and the lack of rhythmicity in laboratory conditions have led to the generalized statement that, in fish, "the rhythm finds full expression only under suitable environmental conditions" (Lissmann and Schwassmann 1965, p. 168). It is thus understandable that, so far, the effect of light pulses on the phase of circadian rhythms has not been summarized in fish in the form of a phase-response curve (PRC).

In this study, we report on activity patterns in *Halichoeres grisonimus*. This species, like other labrid fish species, shows discrete rest/activity cycles by hiding under sand cover during inactivity (Videler 1986). From preliminary observations, we noticed that this species shows free-running circadian activity rhythms in constant darkness (DD) characterized by a remarkable intraindividual stability of the circadian period length τ. We constructed a PRC and a τ response curve by measuring the phasic and tonic effects of light pulses on activity rhythms. In addition, we studied synchronization in LD cycles and entrainment in skeleton photoperiods of two light pulses per day in DD conditions (Pittendrigh 1981). By the latter, we avoided the problems of masking induced by the application of LD cycles (Aschoff et al. 1983), which has been reported especially in fish (Ali 1992).

EXPERIMENTAL

Animals and Housing

The 16 yellow wrasses (*Halichoeres chrysus*) used in this study came from the Philippines, in the middle of the west-central Pacific Ocean. The wide distribution area of this species ranges from 30° N, 120° W, to 35° S, 155° E. Yellow wrasses stay at depths of 7–60 m, although rarely below 20 m (Randall and Allen 1980). In the experiments, the fish, about 80 mm long, were kept individually in $60 \times 180 \times 85$ cm aquaria, supplied with a sand layer of several centimeters. In nature, this bright yellow labrid species inhabits sandy territories around coral areas. Like many other wrasse species, *Halichoeres chrysus* buries in the sand at night as well as during parts of the day (Randall and Allen 1980; Dakin 1992; Nelson 1994). A seawater filter system replenished the aquaria with a constant 500 L of artificial seawater (HW Meersalz®, Wiegandt, Urefeld,

BRD) with a salinity of 30%. Aquaria were contained in a climate room held at a constant temperature of 18°C. To obtain visual isolation of the aquaria, wooden boxes were placed around each. Fish were fed brine shrimp (Artemia Brine Shrimp®, Sera, Meinsberg, BRD) and fish food flakes (Tetramin®, Melle, BRD) at irregular times (Boujard and Leatherland 1992) between 07:00 and 17:00 three times a week.

Light Conditions

Apart from the light conditions specified below, a low-intensity red light was used as background light. During an initial 10 days, fish were kept in an LD cycle of 12h light and 12h dark (12:12 LD). Light was supplied by a bulb (75 W) during the light phase. Thereafter, constant low-light conditions (dim LL) were achieved by a halogen bulb (15 W). Subsequently, 3h light pulses, applied by a 38 W fluorescent lamp (Joel Flexlight®, Wiegandt) interrupted the dim LL conditions once every 10 days. Spectral composition and irradiation of the light sources used were determined by a Licor Incorporated® spectrophotometer (LI185B, Wateringen, The Netherlands). The strip light irradiance spectrum was found to have peaks in the orange (600 nm) and green (530 nm) part of the spectrum, besides some lower peaks in the blue part. For each light condition, intensities were measured at the bottom and near the water surface in the aquaria (Table 1).

Activity Recording

The recordings were taken from a computerized locomotor monitoring setup with two photoelectric light-emitting diodes (LEDs; Omron Inc.® E3JK-R2M2, Keip, Groningen, The Netherlands) placed lengthways in each aquarium 10 cm above the substrate. The number of interruptions of LED emissions were counted and accumulated in 2-minute bins continuously by an event recording system. The accuracy of these recordings was verified by infrared video observations during 5 days in 12:12 LD conditions.

Procedure

After synchronization to a 12:12 LD cycle for at least 10 days, 3h light pulses ($N = 31$) were applied interrupted by 10 days of constant light conditions. After a resynchronization for 14 days to a 12:12 LD cycle, fish were exposed to a skeleton photoperiod of 1h light and 11h dim light for 20 days. An immediate 3h advance shift of this skeleton photoperiod was followed by a continuation of this photoperiod for 18 days. Consecutively, a second skeleton photoperiod of 1h light and 11.25h dim light was ap-

Table 1. Irradiance Levels (Micro E) Integrated Between 400 and 700 nm of Different Light Sources Measured at Two Levels in the Aquaria

Level	LD (bulb)	Dim L (halogen)	Light pulse (strip)
Bottom (depth 0.6 m)	16.51	0.0021	40.25
Water surface	36.58	0.0051	69.90

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plied for 17 days. To measure stability and period length of the individual free-running rhythms, activity recordings in dim LL for 3 weeks finalized the experimental procedure.

Data Analysis

Activity recordings were analyzed to calculate the length of the activity phases and of the period length. Significance of periodicity was determined using chi-square periodogram analysis (Sokolove and Bushell 1978). Stability of the rhythm was estimated by measuring the difference between rhythmicity index *Qp* and the corresponding significance level of the chi-square distribution at the $p < .05$ level for the highest peak of the periodogram (delta *Qp*; Gerkema et al. 1994). In addition, coefficients of variation of the time between two subsequent onsets of activity for 10 successive days were calculated (Pittendrigh and Daan 1976). Phase shifts were calculated by regression analyses of onsets of activity. The initial phase position was determined on the basis of onsets of activity during 7 days preceding the pulse. Immediate shifts of onset of activity at the day after the light pulse were distinguished from steady-state phase shifts by backward regression of onsets during days 3–10 after application of the pulse. Changes in period length τ (delta τ) were calculated as the difference between τ after the pulse minus τ before the pulse. Video observations were analyzed, differentiating activity as less or more than one change in spatial position per 2 minutes.

RESULTS

All 16 *Halichoeres chrysus* synchronized to a 12:12 LD cycle (mean period length = 24.02h; SD = 0.27h). Activity onset preceded onsets of light significantly (phase angle difference = 0.26h [SD = 0.19]; $p < .0005$). The length of the activity bout was 11.17h (SD = 0.94), and fish disappeared in the sand substrate before lights went off. Comparison of video observations and automated locomotor recordings revealed a full temporal match. Onsets of activity showed a free run after release into continuous light conditions (mean period length = 24.42h, SD = 1.33h). These free runs in LL started from the original phase position, indicating entrainment during the initial LD cycle in all fish. Whereas the circadian period length increased in free-running conditions, the activity bout decreased significantly to 9.99h (SD = 0.94; paired *t* test $p < .05$) when compared with the entrainment to 12:12 LD. Release into constant light conditions did lower the precision of the activity rhythms, although not significantly (paired *t* test, $p > .05$) with respect to the delta *Qp* values obtained in the periodogram analysis. The coefficient of variation of intervals between consecutive onsets (calculated as the quotient of SD of the offset intervals and the average value of τ), however, increased significantly, from 0.012 in LD conditions to 0.049 in constant light conditions (paired *t* test, $p < .005$).

During constant light conditions, 3h light pulses were applied to nine fish. Length and intensity of the light pulses were based on the results of pilot experiments in which pulses of 1 h (60 W bulb: 20·E) did not result in phase shifts.

In Fig. 1, six actograms are shown in which the impact of light pulses at different circadian times (CTs) is demonstrated. During subjective day (CT0–12), only small phase shifts (delta φ) were generated (Figs. 1A, 1D; delta $\varphi = -0.11$h and -0.21h, respectively). Phase delays and advances up to about 20 minutes were generated in the early subjective night (Figs. 1B, 1E; delta $\varphi = -0.44$h and 0.20h, respectively). Substantial phase ad-

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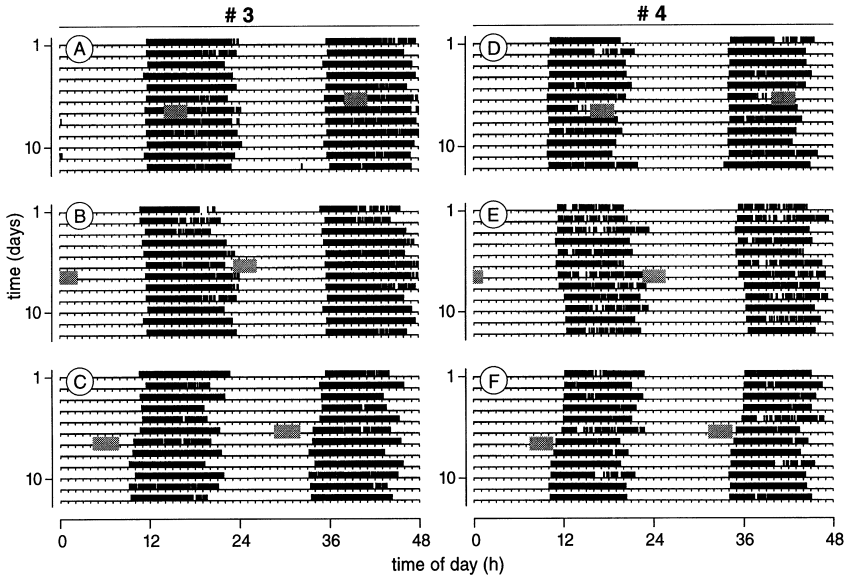

FIGURE 1. Double-plotted actograms of two yellow wrasses (fish 3: A–C; fish 4: D–F) during constant darkness. The fish were subjected to 3h light pulses during subjective day (A, D), resulting in small delay phase shifts; during early subjective night (B, E), leading to both small advances and delays; and during late subjective night (C, F), followed by relatively large phase advances.

vances of more than 1h occurred in response to a light pulse in the late subjective night (Figs. 1C, 1F; delta K = 1.17h and 1.42h, respectively).

The phase shifts are summarized in a PRC (Fig. 2), differentiated for the activity state of fish exposed to the light pulses. As indicated in Fig. 2, fish stayed inactive in response to a light pulse regularly ($n = 9$). The resulting phase shifts were never larger than 1h and did not significantly differ from zero (paired t test, $p > .05$). Although the resulting PRC is not much affected (Fig. 2), only those phase shifts were analyzed that were obtained in fish showing activity during the light pulse. Averaged in 2h bins, these phase shifts revealed significant differences for CT (analysis of variance [ANOVA], $p < .05$). In contrast to the prominent phase advances during the late subjective night (paired t test, $p < .001$), no significant phase delays occurred during the subjective day or early subjective night. At those CTs, phase shifts were relatively small, with only two exceptions of 1–2h delays in the early night. Besides effects on phase, light pulses also caused tonic effects on the circadian system by changes of the circadian period length τ (Fig. 3). Although overall changes of τ with CT just failed to be significant (ANOVA, $p = .07$), a significant shortening of the period length of the free-running circadian rhythm was observed during early subjective night (τ change from CT12 to CT16; paired t test, $p < .05$, two tailed). Lengthening of τ of more than 0.4h occurred at the transitions of subjective night and day only.

In preparation for the application of subsequent skeleton photoperiods, behavioral records were obtained during initial 12:12 LD cycles in six fish. All animals were rather well synchronized (Fig. 4; period length = 23.93h, SD = 0.06). In five fish, entrainment during the first episode of a skeleton photoperiod (1:11 LD cycle) was established (period

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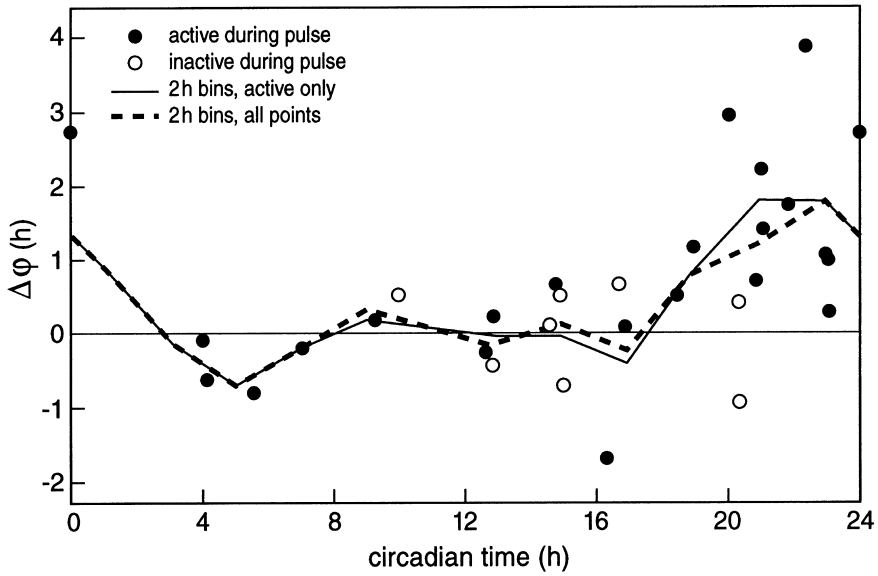

FIGURE 2. Phase-response curve (PRC) for light pulses. Phase shifts in the onset of activity are plotted against circadian phase. Closed dots indicate exposure of fishes in active state; open dots indicate inactive state during the light pulse. The middle of the 3h light pulse was used as the reference phase. The dotted line represents the PRC based on mean values of all phase shifts per 2h bin. The PRC based on mean values of phase shifts for fish active during the pulse per 2h bin is given by the straight line.

length = 23.96h, SD = 0.04). The sixth animal combined masked activity during the "dawn" light pulse with activity restricted to the subjective day. This last activity showed a free run with a period of 23.87h. Later, this fish lost most of its activity for several days regularly. The remaining activity deviated entirely in phase and period from the applied skeleton photoperiods. All five other animals reentrained after a 3h phase advance in this LD cycle. The exposure to a skeleton photoperiod of 24.5h (1:11.25 LD cycle) failed to entrain a second fish. The two fish discussed so far showed a free-running activity pattern during the subsequent recording in dim LL (period length = 23.78h, SD = 0.12). The 1:11.25 LD skeleton photoperiod resulted in entrainment in four fish, as confirmed by periodogram analysis (period length = 24.5h, SD = 0.03, significantly deviating from the preceding period length, $p < .005$, paired t test). These four fish also showed free-running rhythmicity in constant light conditions (period length = 23.85h, SD = 0.18). The initial phase position of activity onset indicated entrainment in the previous 1:11.25 LD cycle.

DISCUSSION

The yellow wrasse *Halichoeres chrysus* showed clear activity patterns in 12:12 LD cycles. Activity onsets often preceded the light phase by several minutes, indicating entrainment of an internal circadian activity pattern. The fish anticipated the night also

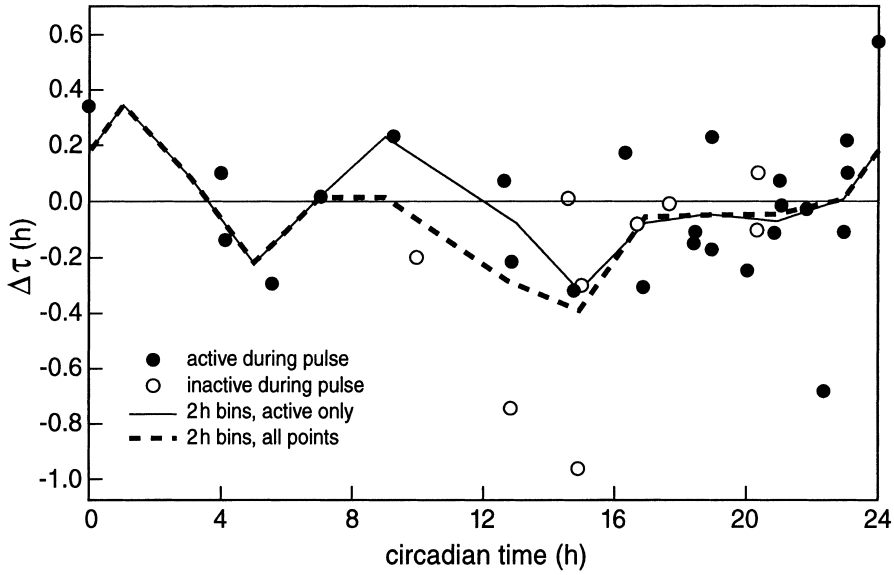

FIGURE 3. Change in circadian period length following the application of a 3h light pulse plotted against circadian phase of the pulse. Symbols distinguish between fish active and inactive during the light pulse. The middle of the 3h light pulse was used as the reference phase. The dotted line is the response curve based on mean values of all phase shifts per 2h bin. The straight line gives the response curve based on mean values of phase shifts for fish active during the pulse per 2h bin.

at the transition to darkness by hiding below the sand surface. In constant light conditions, yellow wrasses demonstrated the circadian nature of their locomotor activity. Precision of free-running patterns, expressed as the coefficient of variation in timing of activity onsets, was lower than that of rodents, but comparable with that of humans (Gerkema and Daan 1985). Synchronization occurred to changes in zeitgeber cycles. It is only in the specific situation of skeleton photoperiods that some individuals are no longer synchronized to the photic zeitgeber and show free-running rhythmicity. In other reports on circadian rhythmicity in fish (for reviews, see Thorpe 1978; Ali 1992) patterns are characterized as labile, and in many instances, phase jumps at certain phases of LD cycles have been observed. In comparison, *Halichoeres chrysus* shows a precise entrainment to LD conditions that is similar to photic entrainment in birds and mammals.

Interestingly, the light intensity applied during the LD cycles, which were effective zeitgebers to which the fish entrained, failed to cause phase shifts when applied when 1h light pulses interrupted constant darkness. Phase shifts occurred only when the light intensity was approximately doubled and light pulses were extended to 3h, a finding that is reminiscent of similar light-doses dependent results obtained for rodents (Meijer et al. 1992). Responsiveness to the bright light pulses resulted in phase advances during late subjective night. Phase delays larger than 30 minutes rarely occurred. The stability of the period length before and after application of the light pulses allowed the establishment of a PRC, to our knowledge the first one published for fish. With the exception of the beginning of the subjective night, the resulting PRC is comparable with those obtained

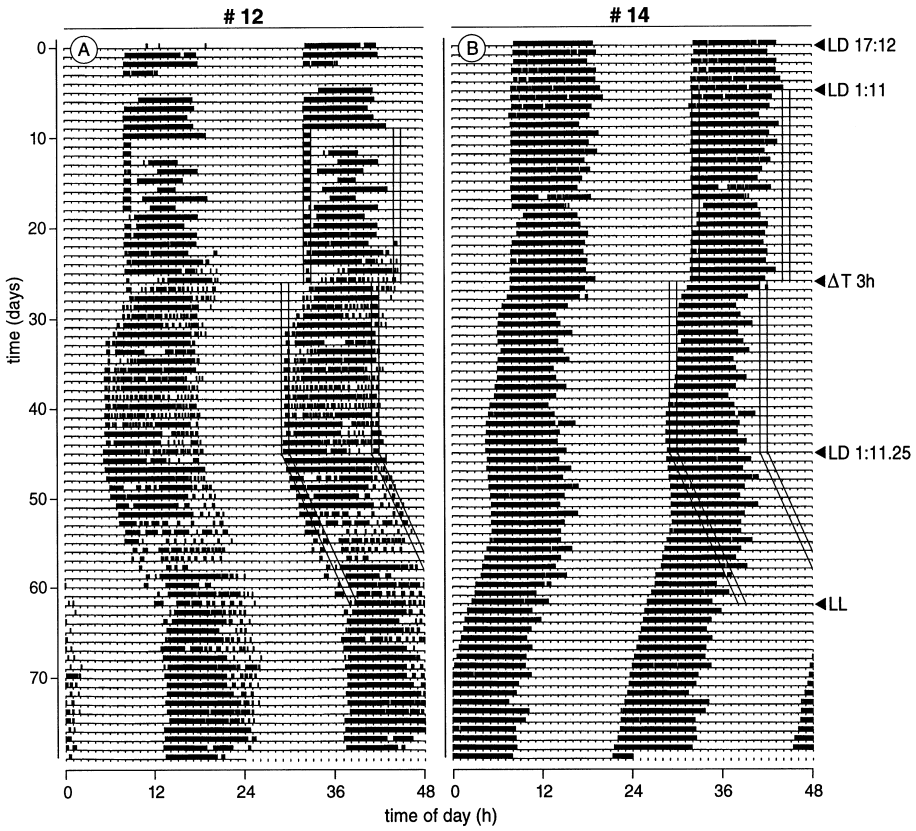

FIGURE 4. Double-plotted actograms of fish during skeleton photoperiods. Initial entrainment to a 12:12 LD cycle (days 1–10), followed by a 1:11 LD cycle (days 11–46). Phase advance of 3h in zeitgeber cycle at day 28. Subsequent 1:11.25 LD cycle (days 47–62), followed by release in constant low light conditions (LL; days 63–79). Note the complete entrainment in all LD cycles in fish 12, while fish 14 starts to free run in 1:11.25 LD.

in birds and mammals (Johnson 1997). The extended advance portion and the low number of delays can be explained only partly by the higher occurrence of inactivity during the first part of the subjective night. Light pulses also had long-term implications for the circadian system: The shortening of the circadian period caused by pulses in the beginning of the subjective night could contribute to the mechanism by which light entrains the circadian system.

Simultaneous effects of light pulses on phase and period length have been described before (for instance, Gerkema et al. 1993). The implications of this finding for our understanding of entrainment have been discussed recently by Beersma et al. (1999). However, both tonic and phasic effects of the light pulses advanced the activity patterns of the fish. Together with the observation that the free-running period length of the wrasses did not exceed 24h consistently, we cannot fully explain the stable entrainment to LD cycles with a zeitgeber period equal to or larger than 24h by the pulse experiments. Based on anatomical findings (Holmqvist et al. 1992), one can expect that photic entrain-

ment mechanisms are present in all vertebrate taxa, but it can be expected also that properties of the oscillator mechanisms are adapted to the natural environment. Daily light changes will differ according to specific environmental conditions, such as attenuation of day-night differences with water depth (Lythgoe 1979). The relatively weak expression of the circadian system in fish can be interpreted as a response to relatively small differences in irradiance levels between light and dark in a marine environment. In birds, it has been shown that experimental lowering of amplitude of the circadian system leads to faster reentrainment in LD cycles with a low amplitude (Hau and Gwinner 1994). This functional response could also apply to fish. A reduction of the self-sustaining capacity of the pacemaker may be correlated with a higher sensitivity of the circadian system to a "weak" zeitgeber and thus forms the basis of the photic entrainment described in this study.

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