Exogenous and Endogenous Control of Swimming Activity in *Astyanax mexicanus* (Characidae, Pisces) by Direct Light Response and by a Circadian Oscillator

I. Analyses of the Time-Control Systems of an Epigean River Population

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*Astyanax*, Epigean Ancestor, Circadian, Swimming Activity, Light Response

1. The swimming activity of 6 specimens of an *Astyanax mexicanus* river population was tested with regard to its time control under various light-dark(LD)cycles and under constant conditions.

2. Activity is classified into three different forms according to the special experimental arrangement: surface activity, bottom activity and the sum of both (total activity).

3. All applied LD-cycles act as so-called forcing signals (Zeitgeber) and entrain the activity.

4. The maximum values of surface activity correspond to the dark phases of a LD-cycle, those of bottom activity to the light phases. This inversion causes a less strong entrainment of the total activity up to a loss of a significant oscillation in extreme cases.

5. This inverse pattern is kept the more stronger the more the period length of a LD deviates from 24 h. In the range of resonance about 24 h there is a greater flexibility with regard to the phase relation of the maximum values.

6. Activity reacts very sensitive to the differential parameter of the forcing signal. Therefore, no phaseangle difference occurs between forcing and forced signal. Moreover, the system needs no swing-in time to become entrained when starting a LD.

7. After transition from LD to DD (= constant darkness) the forced signal does not die away immediately, but damps out within one or a few cycles with decreasing amplitude and unchanged frequency.

8. In nearly all applied LDs a non-synchronized circadian rhythm can be observed in addition to the entrained frequency, which is dominant.

9. Also, in DD a freerunning circadian rhythm is detectable.

10. We suggest the model that first a passive system with a nearly unlimited range of entrainment controls activity. In contrary to extremely passive systems, it is able to oscillate. Moreover, it acts like a linear system with respect to frequency transfer. In the tested cases, output and input frequency are equal. In addition, activity is under control of an endogenous circadian oscillator. Its effects are overlapped under forcing conditions, but they become obvious under constant conditions. Furthermore, nonstationary processes are features of this circadian rhythm. The properties of a passive and a circadian system alone do not explain the flexible patterns in the range of resonance. Therefore, a time-dependent controller is demanded to control the phase relation of the maxima.

Introduction

*Astyanax mexicanus* is a common characin of Mexico. The widespread epigean river fish is the recent ancestor of several hypogean cave forms. All forms belong to the same species because fertile crosses are possible (Sadoglu [1]). Therefore, *A. mexicanus* is a suitable object for studies in evolutionary processes including genetically fixed time-

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The tests were carried out at a temperature of 25 °C ± 1 °C in a temperature controlled room, which could be illuminated with fluorescent tubes of the type “white”. The light-dark-cycles (LDs) were applied as rectangular signals with 1 lux on an average in the light phases. During the dark phases and constant darkness (DD) illumination was switched off. The LD-programs were continued for 7 to 14 days. Each LD was followed by DD for about 14 days. Swimming activity was measured by interruptions of IR-light barriers (AEG E52 with IR-filter). Two photocells were used for each aquarium, one near the bottom and one near the surface. It was impossible even for the larger fishes to cause impulses at both photocells at the same time. Because of this special arrangement the activity of each fish was recorded in the form of two separate time series, called bottom and surface activity. By total activity, we denote the simple addition of both time series. In the beginning of the experiments a third IR-light barrier in the middle between the two photocells was used. It gave no further information but a mixture of the two features of surface and bottom activity (Fig. 1). So it was used no longer. The swimming activity was tested in various LDs, which can be classified in two types: first, LDs with period lengths of 24 h (LD 12:12 h, 16:8 h) or a harmonic of 24 h (LD 6:6 h, 4:4 h, 2:2 h), and second, LDs with a period length differing from 24 h and its harmonics (LD 16:16 h, 9:9 h, 8:8 h, 3:7 h). Constant conditions were applied mainly as DD and in one case as constant illumination (LL) at 1 lux. DD was chosen because the fishes continued to swim at both levels whereas they moved down to the bottom in most cases in LL and lowered their activity.

The impulses of each photocell have been registered, and the activity has been defined as the number of impulses during half an hour. Thus, the sequence of activity measures is a time series with a sampling interval of Δt = 0.5 h. The time series have been analysed by using the interactive computer system TIMESDIA (Martin et al. [12]). The calculations have been performed on an IBM 370/168 at the RHRZ of the University of Bonn. The general analysis of biological time series by using TIMESDIA is well documented in literature, but for clarity, we briefly describe the most important features we have used in this analysis.

Mathematically, an oscillator produces a periodic signal coming from minus infinity and going to in-
Fig. 1. Activity of a specimen during 4 days in a LD 12:12 h. Abscissa: time scale (clocktime: MEZ); ordinate: amount of swimming activity per hour; black bars indicate the positions of dark phases. The maximum values of surface activity (left) correspond to the dark phases, those of bottom activity (right) to the light phases of the LD. The activity, recorded by a photocell in the middle between surface and bottom (middle), is a mixture of both features.

finity. The shape of such a periodic signal is arbitrary, but for reasons of mathematical ease of tractability, the periodic signal will be composed into a Fourier series, i.e. an infinite sum of sine functions with distinct amplitudes and phases. The frequency of the first term of the sum (fundamental frequency) is equal to the frequency of the underlying periodic signal, and the frequencies of the higher terms of the sum (harmonics) are integer multiples of the fundamental frequency. Clearly, such a model is not applicable in any practical situation. But, a slight modification gives a reasonable model: in the strictly periodic model, the fundamental harmonic is given by $A \sin (\omega t + \theta)$. Now, we allow the amplitude $A$ and the phase $\Theta$ to be time-dependent functions, i.e. our model takes on the form $A(t) \sin (\omega t + \Theta(t))$. Thus, we have modelled changing amplitudes and frequencies. Estimates of these instantaneous amplitudes $A(t)$ and instantaneous phases $\Theta(t)$ are obtained by complex demodulation. The Figures (4, 5, 10) show estimated instantaneous amplitudes and phases against time. By the re-modulated signal we denote the estimates of the time-varying model. Our model remains valid even if we add noise or we consider the data as a realization of a nonstationary process (Martin [10]). Thus, if $\Theta(t)$ is a linear function (a straight line in the figures) the frequency of the signal remains unchanged, i.e. stable. Only in this case, periodogram analyses and the calculation of average signals are valid. In a periodogram analysis, we perform an estimation of the energy of a supposed periodic signal. Energy of a signal is equivalent to probabilistic variance. Significance of a frequency is tested in comparing the signal's energy to the total energy. In the figures, total energy is normed to one, and a 95% confidence limit for each frequency is obtained by assuming the noise to be Gaussian white noise.

One specimen of the river fish was previously observed by IR-videotechniques in a LD 3:3 h under comparable conditions as described above. During this test activity was not recorded by photocells. These observations were made in order to get an idea of the typical behaviour during the test situations. There was no intention to get a complete ethogram. Under this point of view, the behaviour of the river fish was classified into 4 main components:

1. Swimming near the surface
   The fish is calmly swimming near the surface, i.e. in the upper part of the aquarium without stopping for a while.

2. Swimming near the bottom
   The fish is swimming near the bottom, i.e. in the lower part of the aquarium without stopping for while.

3. Resting near the bottom
   The fish is resting near the bottom. It only moves slightly up and down keeping the same spot by beating its fins synchronously. These movements would be recorded by photocells as impulses. (The corresponding component: Resting near the surface could be observed, but it occurred so seldom that it was neglected.)

4. Swimming up and down the pane at the small side of the aquarium.
Results

IR-video-observations

The results of the IR-video-observations are shown in Fig. 2. Each column in the histograms represents the percentual amount of each behavioural component with regard to the total time of observation. Thus, their frequencies of appearance are involved in the analysis, but the duration of each component is of more importance. In Fig. 2a the components 1 to 4 are presented. The component 1 appears predominantly during the dark phase of the LD, whereas the course of the components 2 and 3 is exactly inverse. These two forms reach their maximum values during the light phase. Therefore, the “activity” near the bottom is composed of two different contributions: real moving around and moving on the spot. In the dark phase, the component 2 is reduced and the component 3 disappears. The component 4 seems to be a typical behaviour of irritation. It mainly appears in the ranges of illumination changes.

The time series, recorded by photocells, only allow to discriminate between surface and bottom activity. For this reason, the 4 components are summarized to the 2 components SURFACE (component 1 + 1/2 component 4) and BOTTOM (component 2 + component 3 + 1/2 component 4). Obviously, two clear-cut oscillations result with a phaseangle difference to one another of 180° (Fig. 2b). The oscillation SURFACE builds up its maximum in the dark phase, the oscillation BOTTOM builds up its maximum in the light phase of the LD.

Analyses of times series

General remarks

To start with, some general remarks about the main features of the actograms should be pointed

![Fig. 2. Distribution of behavioural components of a specimen in a LD 3:3 h. Abscissa: number of observations (1–8); ordinate: percentual amount of the components in comparison to the total observation time (100%). Below the histograms the LD (1–0 lux) is marked as a rectangular signal. Black bars indicate positions and lengths of the 8 observations. a) Distribution of each single component: 1. Swimming near surface; 2. Swimming near bottom; 3. Resting near bottom; 4. Swimming up and down the pane. b) These 4 components are reduced to: 1. SURFACE (comp. 1 + 0.5 comp. 4) and 2. BOTTOM (comp. 2 + comp. 3 + 0.5 comp. 4).]
out because they are obvious without using sophisticated mathematical methods. The above described distribution of activity under LD-regime is well represented in the time series. But a detailed analysis shows more complicated patterns. In general, the maximum values of the surface activity correspond to the dark phases of a LD (Fig. 1). Their amount is not as high as in the bottom activity. The pattern shows clearcut maxima. There is no or only very few activity in the minima, i.e. during the light phases. The actograms generally give the impression of activity and rest. The course of the bottom activity is in general inverse to the surface activity. The maximum values mainly correspond to the light phases and they reach a higher amount (Fig. 1). Normally, the maxima are clearcut comparable to the surface activity. But in contrast to the latter, there is always activity even in the minima, i.e. during the dark phases. Therefore, it cannot be distinguished between activity and rest but only between two levels of permanent activity. The difference between the two levels is important.

It must be emphasized that the described patterns of both activity forms are not strictly kept in LDs with period lengths of 24 h. In these particular cases both forms are able to react flexible with regard to the phase in which the maxima are build up. What does “flexible” mean? In some cases the surface activity builds up its maxima in the light phases of the LD so that both activity forms are in phase. Sometimes, the surface activity does not show a coherent maximum but a bimodal pattern with peaks in the ranges of light changes (Fig. 3). These peaks are not exclusively due to direct responses to a change of illumination, since the activity can increase or decrease hours before or after a light change. This flexibility is especially a feature of the bottom activity. During the course of a LD-experiment, the maximum values may first correspond to the light...
Fig. 5. Complex demodulation of time series of surface- (Fig. 5a), bottom- (Fig. 5b) and total activity (Fig. 5c) in a LD 12:12 h and following DD. A: estimated instantaneous amplitude; B: estimated instantaneous phase; C: observed data and remodulated signal; demodulation period: 24 h; right passband edge: 22.00 h⁻¹; stopband starts at 20.75 h⁻¹. Under LD-conditions surface- and bottom activity are entrained and oscillate with a phaseangle difference of 180° to one another. During 4 days after beginning of DD a section of instability in which phaseshifts take place occurs in surface activity. Thereafter the oscillation stabilizes. Bottom activity becomes arrhythmic in DD. Total activity exhibits under LD-conditions a weak entrainment because of the inversity of both single oscillations.
phases, then they may change to the dark phases and later on back to the light phases again. In extreme cases, the pattern looks unentrained (Fig. 4). It must be pointed out again that this flexibility of the patterns — bottom as well as surface — is restricted to LDs with period lengths in the range of resonance about 24 h. The more a period lengths removes from this range the stronger the inverse pattern is kept.

Under DD-conditions some remarkable changes occur in the actograms. 1. The clearcut patterns of maxima and minima disappear. The oscillations display their maxima against an increasing background noise (Fig. 5a). It becomes more obvious that maxima and minima do not mean “activity” and “rest”, but are expressions of the instantaneous amount of permanent activity. 2. Surface and bottom activity do not show any longer a phaseangle difference of 180°. Their maxima become more or less congruent. Normally, it is the surface activity in which the necessary phase shift takes place when the light remains switched off. The phase shift can occur in one step (Fig. 6) or in a softer way within some periods (Fig. 5a). In general, the amount of the bottom activity is lowered, probably, because the behavioural component “resting near the bottom” is no longer kept. The amount of the surface activity does not change in this systematic way.

Data analysis

All applied LDs are so-called forcing signals (Zeitgeber) and entrain the activity in the described way (Fig. 7). Features of the entrained rhythms are slight modulations of the amplitudes (see amplitude

Table I. Shifts of period length (in h) of surface activity (step width: 6 h). Left: in a LD 12:12 h, measured from \( t = 58.0 \) h after beginning of LD until \( t = 298 \) h; right: in DD after LD 12:12 h, measured from \( t = 12 \) h after beginning of DD until \( t = 219 \) h.

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Fig. 6. Surface activity of a specimen in a LD 16:8 h and in following DD. The histogram is doubled in order to give a better impression of the oscillation in DD. Left: \( n^{th} \) day; right \((n+1)^{th}\) day. The oscillation immediately shifts its phase about 180° as soon as light remains switched off (arrows).
diagram in Fig. 5) and slight shifts of the phases (see phase diagram in Fig. 5). The shifts of the corresponding instantaneous periodicities are in the range of one hour (Table I). The signal energies of the average signals vary under different LD-conditions but not in a systematical way (Table II). In LDs with period lengths differing from 24 h and its harmonics they are not lower than in the other cases. Interindividual differences are as important as different applied LDs. Furthermore, there is no difference between surface and bottom activity. The values reach at maximum 58% of the total energy amount of the process and fall at minimum to 17%. These results correspond to all applied LDs. Thus, it does not matter whether the applied LD is a harmonic of 24 h or not.

A completely changed pattern will result if surface and bottom activity are added up to the total activity (Fig. 5c). The clearly entrained rhythms of both forms are no longer detectable because of their inverse course. The resulting rhythm is more or less weak and in some cases no longer significant. The phase of this oscillation is determined by the phase of the bottom activity, which normally shows the higher amplitude.

The activity reacts quickly upon the forcing light-dark signal. In general, its reactions become obvious within the first measuring interval of 1/2 h after environmental conditions have changed. So, there is no measurable phaseangle difference between forcing and forced signal. As expected from these results, the forced oscillation needs no swing-in time to become

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entrained by a LD. When LD-regime is finished and constant conditions (DD) begin, the forced oscillation does not always disappear at once. It damps out within at least one or more periods with a decreasing amplitude and an unchanged frequency (Fig. 8).

The question arises whether the entrained rhythms in LDs with period lengths differing from 24 h are the only ones detectable in the data. Of great interest are oscillations in the circadian range. Regarding the frequency analyses of the residuals (after subtraction of the average signal of the entrained period length) two facts become obvious: 1. An additional circadian rhythm determines the activity in nearly all cases at least in one activity form (Fig. 9). Two exceptions are the LDs 6:6 h and 16:16 h. In LD 6:6 h only the harmonic can be observed but not the basic oscillation (this is in accordance with the theory of self-sustained oscillations (Hayashi [13]; Meyer and Guicking [14])). 2. If the period length of a LD is a harmonic of 24 h the additional circadian rhythm will be relatively strong and stable. If the period length is no harmonic the additional circadian rhythm will be less strong and stable but still detectable. But in general, the entrained rhythms are very dominant, and the detection of an additional circadian component was only possible by using our data procedures.

The persistence of a circadian rhythm under constant conditions was tested mainly in DD. In DD after release of all forcing conditions a freerunning cir-
A circadian rhythm can be detected at least in one activity form (Fig. 10). It appears mostly in surface activity while the bottom activity often becomes arrhythmic (Fig. 5 b). There are several features of the freerunning rhythm: 1. It is in general not as strong as under synchronizing conditions, i.e. the energy of the average signal of freerunning circadian rhythm is considerably lower than that of the entrained rhythm (Fig. 5 a). 2. Spontaneous changes of the freerunning period are not unusual (Fig. 5 a). Therefore, the estimated instantaneous periodicities show shifts in the range of several hours (Table I). 3. The lowered amplitude remains modulated (Fig. 5 a). 4. The oscillation of the total activity is in most cases stronger than under LD-regime because the phaseangle difference between the two activity forms is no longer kept (Fig. 5 c).

It should be pointed out again that under LL-conditions a freerunning circadian rhythm also appears (Fig. 11). As already mentioned, these time series were not mathematically analysed. But the existence of a significant rhythm is obvious even in the histogram. The modulation of the amplitude and the relatively unstable phase can be recognized. This is in good agreement with the results of DD besides the relatively strong signal being present in LL.
Discussion

The results clearly underline the importance of proper techniques in collecting and analysing data. A method, which allows to record the total swimming activity, would not be suitable in these cases since it gives no information about the movements in vertical levels of space.

Forms of activity

Under LD-conditions the river form of *A. mexicanus* is exhibiting two different types of activity, called surface and bottom activity. Both can be strongly entrained, in general with a phaseangle difference of 180° to one another. The maximum values of the surface activity correspond mainly to the dark phases, those of the bottom activity to the light phases of a LD. This invercity causes a completely changed pattern when both are added up to the total activity. The clearcut rhythm of each single oscillation disappears. The result is a more or less unstable rhythm, which is even no longer significant in some extreme cases. Therefore, a strong entrainment could be overlooked very easily if the data give no informations about the activities at different space levels. The reason why the river form displays these particular activity patterns is not yet clear. Little is known about its behaviour in the natural habitat. Therefore, it is impossible to decide of what ecological relevance this behaviour could be. One could object that this form of activity answer to light stimuli is an artificial product of unnatural conditions in the laboratory. The isolated keeping over months is very unusual for this species, which normally lives in schools. This may give reason for strange behaviour. On the other hand, all tested fishes behaved in a similar way from the very beginning of the experiments to the end. This is a reason to believe that the particular activity patterns are components in the natural behaviour of this species. Perhaps, the form of actogram depends on the form of the forcing signal. Thìnès et al. [7] kept the river form under natural LD, and they describe it as mainly active at dawn and dusk without differentiation between different activity forms. This is in contrast to the activity patterns, presented here, which were observed in LDs being applied as rectangular signals with a very low amplitude of light intensity. Bimodal patterns, comparable to those described by Thìnès actually occurred in the surface activity. But they appear too seldom to be considered as a general feature. The answer to these questions must be subjected to further research.

The inverse course of surface and bottom activity becomes the more obvious the more the period length of a LD deviates from 24 h. In the range of resonance about 24 h the patterns can exhibit a great flexibility. Its extent differs from individual to individual. This form of answer to light stimuli cannot be explained by the physical properties of a self-sustained or a passive system. As a preliminary and merely qualitative model one could imagine a kind of controller for each activity form, which determines their relation to the light – or dark phases of a LD. This controller should be inserted between the control system and the triggered function. In the range of resonance being in accordance with the natural conditions it is easy to switch.

The results do not allow to speak of a preadaptation of the epigean river form to a future living in caves with regard to its light behaviour. The higher amount of activity near the bottom during illumination could be explained as a form of “photonegative” reaction. But this is not enough reason to call it preadaptation.

The actograms show a permanent activity though at least under LD-conditions the “activity” near the bottom is not only “moving around”. But video-observations suggest that in DD the permanent activity is a real moving for the most part. This result could be closely related to the applied methods. On the other hand, Hahn [15] and Vandel [16] also report that blinded river fishes and those which were kept under constant darkness show permanent activity. Perhaps, the animals try to compensate in this way the loss of visual information by a more frequent exposition of their lateral organ to external stimuli (Hahn [15]).

The passive control-system

The activity of the river fish is entrainable by all applied LDs (here entrainment is not used in the sense of Aschoff et al. [17], but in the sense of an entrainment of a forced oscillation without assuming the forced system as selfsustained or externally sustained). That leads to the conclusion that a passive system controls the activity. Furthermore, the system acts like a linear system because in the tested range the output frequency (activity oscillation) is always
equal to the input frequency (LD). There are two types of passive systems (Röhler [18]): 1. an extremely passive one being unable to oscillate; 2. a passive one being able to oscillate. Under forcing conditions both systems show similar reactions, but differentiation is possible just after a transition from LD to constant conditions. In this case, the forced signal of an extremely passive system dies away immediately in an exponential form while the forced oscillation of a passive system exhibits a damping out oscillation. The data analyses of the river fish clearly show that after a transition from LD to DD the entrained oscillations damp out within one or a few periods with an unchanged frequency. That means, the system is passive but not extremely passive because it is able to oscillate. Another feature of passive systems is the speed they need to reach a new steady state under forcing conditions. The system is "weakly damped" if it takes a long time to reach the new steady state, and it is "strongly damped" if it takes a short time to become adapted. Normally, the activity of the river fish reacts very quickly upon the light stimuli, i.e. within the first measuring interval of 1/2 h after environmental conditions have changed. Therefore, no phaseangle difference occurs between forcing and forced oscillation. That characterizes the passive system as strongly damped in comparison to a circadian time scale. This form of answer to external light stimuli especially occurs in the case of their rectangular application whereas in the case of sinusoidal forcing signals the forced signal appears more smoothed (Lamprecht and Weber [19]). But that does not mean: in the first case the external stimulus has pushed on a passive system and in the second case it has not. If the passive system is sensitive to the differential parameter of a forcing signal the answer to a rectangular stimulus with a high differential value must be stronger than the answer to a sinusoidal stimulus with a lower differential value. An entrainment out of the empirical range of an endogenous circadian system is not very unusual at all and is described in literature as "masking" (Aschoff [20]; Hoffmann [21]). This definition is typical of the point of view under which this phenomenon is seen up to now. It is an unwanted form of tuning an external stimulus into an internal function. It overlaps the true reaction of the interesting endogenous oscillator. The underlying system itself has been disregarded more or less. Recently, Robinson et al. [22] and Robinson and Scott [23] were successful in separating an endogenous oscillator from a passive system and in describing the properties of the latter in Trifolium repens leaves. They were able to determine the spontaneous frequency of the passive system and they figure out a model of the leaf oscillator. The passive system of Astyanax cannot be described so detailed by the applied methods. It would be necessary to test the swimming activity in sinusoidal forcing signals in order to ease the evaluation of the results.

Features of the entrained rhythms in Astyanax are modulations of the instantaneous amplitude and phase. The extent of the modulations can hardly be evaluated because of the lack of comparable data in other species. Moreover, relatively high values of signal energies and low levels of background noise are characteristics.

The nonlinear selfsustained circadian system

The question arises whether a selfsustained circadian oscillator is involved in the activity control in addition to the passive system. To prove the existence of such a system it is necessary to detect persistent oscillations within the circadian frequency range while a forcing signal is absent. The data analyses of constant conditions make obvious that freerunning rhythms do exist. It must be emphasized that the freerunning rhythm is less strong than the rhythm in the case of synchronisation if one uses the energy of the average signal as a measure. Furthermore, freerunning rhythms occur mainly in the surface activity but not in the bottom activity as well. This is also in contrast to LD-conditions. Another feature are spontaneous changes of the frequency so that a frequency analysis including the whole time series often gives the impression of a weak or insignificant rhythm. This nonstationary processes seem to be characteristics of the circadian activity rhythm in the river form of A. mexicanus. Using suitable analysing techniques, they can be detected and they do not call in question the existence of a nonlinear, selfsustained, circadian oscillator.

The data do not allow a determination of the range of entrainment, but, nevertheless, they give indications for further research. The existence of a circadian rhythm in addition to the entrained 18 h-rhythm in a LD 9:9 h shows that the lower limit of
this range is already overstepped. Theoretical considerations (Wever [24]) demand an asymmetrical range of entrainment in the sense of a larger extension to longer periods. A lot of examples are known to prove this demand (review: Bruce [25]). The lack of an additional circadian rhythm in the LD 16:16 h could be a hint that this period length is still within the range of entrainment. But this must be subjected to further research.

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