NAT. CROAT. | VOL. 10 | No 3 | 141–152 | ZAGREB | September 30, 2001

ISSN 1330-0520
UDK 579.9:591.521

original scientific paper / izvorni znanstveni rad

CIRCADIAN RHYTHMICITY, RESPIRATION AND BEHAVIOR IN HYPOGEAN AND EPIGEAN SALAMANDERS

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Hervant, F., Mathieu, J. & Durand, J. P.: Circadian rhythmicity, respiration and behavior in hypogean and epigean salamanders. Nat. Croat., Vol. 10, No 3, 141–152, 2001, Zagreb.

Comparisons of circadian rhythmicity, behavior, and metabolism between surface and cave-dwelling salamanders enable elucidation of evolutionary trends in these processes. The obligate cave-dwelling proteid *Proteus anguin*us did not show any apparent daily rhythm of activity or resting metabolic rate. In contrast, the surface-dwelling salamandrid *Euproctus asper* had a circadian SMR and activity cycle. These circadian rhythms possessed an endogenous component. The lives of both salamanders studied were characterized by long periods of inactivity punctuated by bouts of foraging or exploratory/predatory behavior. *Proteus anguinus* had reduced resting metabolic and spontaneous activity rates (considerably lower than most surface-dwelling amphibians), and therefore appears to be a good example of a vertebrate as a low-energy system. The low metabolic and activity rates of *P. anguinus* are interpreted as adaptations to a subterranean existence where poor and discontinuous food supplies and/or intermittent hypoxia may be present for long periods.

Key words: amphibian, metabolism, activity, cave, surface, Proteidae

Hervant, F., Mathieu, J. & Durand, J. P.: Cirkadijalni ritam, disanje i ponašanje hipogejskih i epigejskih repaša. Nat. Croat., Vol. 10, No 3, 141–152, 2001, Zagreb.

Usporedbe cirkadijalnog ritma, ponašanja i metabolizma kod površinskih i špiljskih proteida omogućuju rasvjetljavanje evolucijskih trendova u tim procesima. Obligatorni stanovnik špilja, proteid *Proteus anguinus*, nije pokazivao nikakav vidljiv dnevni ritam aktivnosti ili posebni metabolizam prilikom odmora. Naprotiv, salamandrid *Euproctus asper* koji boravi na površini, imao je cirkadijalni SMR i ciklus aktivnosti. Ti cirkadijalni ritmovi imali su endogenu komponentu. Životi obaju proučavanih životinja bili su karakterizirani dugim periodima neaktivnosti, prekidanih hranjenjem ili izviđačkim/predatorskim ponašanjem. *Proteus anguinus* imala je smanjen metabolizam tijekom odmaranja i tijekom spontane aktivnosti (znatno niži od većine površinskih vodozemaca), pa se čini dobrim primjerom kralježnjaka kao niskoenergetskog sustava. Niske vrijednosti metabolizma i aktivnosti *P. anguinus* interpretiraju se kao prilagodbe na život u podzemlju, gdje je u duljim periodima prisutna slaba i nekontinuirana opskrba hranom i/ili hipoksija.

Ključne riječi: vodozemci, metabolizam, aktivnost, špilja, površina, Proteidae

INTRODUCTION

Aquatic subterranean biotopes, including karstic aquifers, are relatively stable with respect to abiotic factors such as darkness, high moisture, temperature, and water chemistry, as well as to biotic factors such as low predation pressure and food limitation. In addition to depigmentation and reduction (or loss) of eye structures, hypogean species possess characteristics that allow them successfully to exploit the subterranean environment. These generally include the elongation of the body and/or appendages, the development of extraoptic sensory structures, and a reduced metabolic rate compared with closely related epigean species (reviews in HÜPPOP, 1985; HERVANT *et al.*, 1998).

Physiological circadian clocks are synchronized by a few daily environmental cycles. In lower vertebrates, the main external »zeitgebers« of these clocks are the rhythmic changes of light and darkness and/or temperature. The absence of light (and therefore photoperiod) and significant temperature variations in subterranean biotopes means that there is no exogenous synchronization of daily rhythms such as respiratory metabolism or activity. Several studies showed the loss (or reduction) of the circadian rhythm of activity in hypogean beetles, crustaceans and fishes (reviews in: LAMPRECHT & WEBER, 1982; TRAJANO & MENNA-BARRETO, 1995). Nevertheless, we know very little about the circadian rhythmicity of the reduced metabolism typical of cave species (MATHIEU, 1973).

The proteid amphibian *Proteus anguinus* (the »grottenolm«) is the only European vertebrate that lives exclusively in caves. Its range is restricted to the caves of the Adriatic karst (BRIEGLEB, 1962; PARZEFALL *et al.*, 1999). *P. anguinus* has its main habitat in a widely distributed system of small karst fissures, inaccessible by humans (BRIEGLEB, 1962). This species is now considered an excellent model for organisms that have colonized this extreme biotope, as it has to cope frequently with limited food supply and/or low oxygen tensions and permanently with darkness (ISTENIC, 1971; PARZEFALL *et al.*, 1999; UIBLEIN *et al.*, 1992). Therefore, *P. anguinus* exhibits marked troglobitic traits, including a slow development rate (they reach the adult stage between 14 and 18 years, and live more than 60 years), elongated body and thin limbs, depigmentation, ocular degeneration, and retention of larval traits such as external gills and caudal fins.

As a permanent cave dweller, *P. anguinus* is morphologically well adapted to aquatic subterranean habitats (i.e., with poorly developed eyes and almost unpigmented skin: DURAND, 1971). Therefore, it is reasonable to expect that it might also exhibit physiological and/or metabolic adaptations (e.g., a reduced resting metabolic rate).

The salamandrid *Euproctus asper* is found in the French and Spanish Pyrenees, in springs, small rivers, and lakes, as well as in some caves (CLERGUE-GAZEAU & MARTINEZ-RICA, 1978). *E. asper* is morphologically similar to *P. anguinus* (although it does not show troglomorphic traits), but not taxonomically closely related, as most subterranean species are either phylogenetic or distributional relicts.

Cave-dwelling species are excellent models for chronobiological studies focusing on the functions of circadian rhythmicity because (i) they enable tests of hypotheses

on external versus internal selection (TRAJANO & MENNA-BARRETO, 1995), especially in comparison with facultative cave-dwelling species, and (ii) they may be favorable subjects for studying the biological significance of the circadian clock system (LAMPRECHT & WEBER, 1985). Therefore, we aimed to compare the degree of adaptation (metabolic rate and circadian rhythm) to cave biotopes exhibited by both epigean and hypogean aquatic salamanders. Two questions are addressed in this paper:

1) does the cave-dwelling species *P. anguinus* possess reduced metabolic and activity rates?

2) is there still a circadian rhythm of activity and metabolism in *P. anguinus*?

MATERIALS AND METHODS

Animals

Individuals of the obligate cave dweller *Proteus anguinus* (Proteidae) and the facultative cave dweller *Euproctus asper* (Salamandridae) originated from a stock established (in natural caves for *P. anguinus*; in aquaria for *E. asper*) in the CNRS cave laboratory at Moulis (France) (JUBERTHIE *et al*., 1996). Specimens of *P. anguinus* aged three (fresh mass = 2.6 ± 0.1 g, juveniles, n = 8), seven (4.7 ± 0.3 g, juveniles, n = 11), and nineteen (18.3 ± 0.6 g, adults, n = 16) years old, and of *E. asper* aged three (1.8 ± 0.1 g, juveniles, n = 8), and eight (8.9 ± 0.3 g, adults, n = 16) years old were used during the experiments. For the study of circadian rhythms of oxygen consumption or activity, only adults were used.

Salamanders were transferred to the HBES laboratory (University Lyon I, France) and raised under semi-natural conditions, in aquaria containing stones and recirculating groundwater (pumped from the underground aquifer below the University of Lyon I). They were fed chironomid larvae (»blood worms«) once a week. Aquaria were kept in darkness in a controlled temperature facility (T = 13 ± 0.2 °C). Individuals of both species were acclimated to laboratory conditions during one month before experimentation.

Measurement of oxygen consumption

For both species, minimum resting rates of oxygen consumption under standardized conditions (standard metabolic rate: SMR) were measured in constant darkness at the same period of the day to cancel the effects of a possible circadian rhythm of respiration. SMRs were measured for 2 hours with a closed respirometer placed in a constant temperature chamber at 13 ± 0.2 °C and supplied with aerated freshwater (PO_2 = 8.1 ± 0.2 mg O_2 l^{-1}). Immediately before the experiments began, salamanders of both species were transferred individually into an 800-ml Plexiglas metabolic chamber, which was part of the respirometer. This chamber was too short to allow animal displacements, and therefore oxygen consumption values did not include an activity component. Each individual was starved for two days before experiments to ensure that digestive metabolism did not modify the results. A con-

stant low rate of water flow (25 ml.min^{-1}) was maintained through the respirometric system during each experiment, using a peristaltic pump, to prevent localized oxygen depletion around the electrode. Oxygen depletions inside the system were monitored with a MOCA 3600 oxygen meter/recorder (Orbisphere Laboratory, sensivity: 0.01 mg O_2 l^{-1}). One hour before the experiments began (adaptation phase), individuals were transferred into the respirometric chamber.

For the specific study of the circadian rhythm of resting oxygen consumption, measurements were performed with the same respirometric system, but during 6 successive 2-hour periods, at different periods of the day between 01h00 and 22h00.

Measurement of spontaneous activity

Two hours before the experiments began (adaptation phase), salamanders of both species were transferred individually into a large glass aquarium containing stones and supplied with aerated freshwater (13 ± 0.2 °C), in constant darkness. Percentage of activity time/hour (including displacements, head and/or tail movements) was registered for 2 hours using an infrared camera (Aaton 30, Newicon) equipped with a 70 mm focal length macro-objective, associated with a VHS video recorder. The illumination was an IR light source. For the study of the daily pattern of activity, measurements were performed at different periods of the day between 01h00 and 22h00.

General remarks and statistical analyses

In some cases, a slight overshoot in oxygen consumption and a slight hyperactivity, probably due to laboratory stress, were observed just after the transfer of individuals into a metabolic chamber or an aquarium at the start of the experimentation. Consequently, to minimize this confusing effect, the first hour(s) of measurements (adaptation phase) were not taken into account for all further metabolic or activity rate calculations.

Values are presented as means ± SE. StatView version 5 from SAS Institute Inc. (Montclair, CA, USA) was used for statistical tests. For analyses between means, a *t*-test was used, whereas tests among means were conducted with a one-way ANOVA. To differentiate the measurement time periods in the cycles, a multiple range comparison test (Tukey test) was used as appropriate.

RESULTS

Oxygen consumption

The data on SMR in *P. anguinus* and *E. asper* are summarized in Fig. 1. Juveniles (3 and 7 years old) of the blind salamander and adults (8 years old) of the epigean newt *E. asper* displayed extremely low metabolic rates in comparison with numerous amphibians (HUTCHISON, 1971; LICHT & LOWCOCK, 1991; GATTEN *et al.*, 1992). Moreover, adults (19 years old) of the cave-dwelling neotenous *P. anguinus* showed a very low SMR, with a value half that of adults *E. asper* (at 13 °C). Juveniles (3

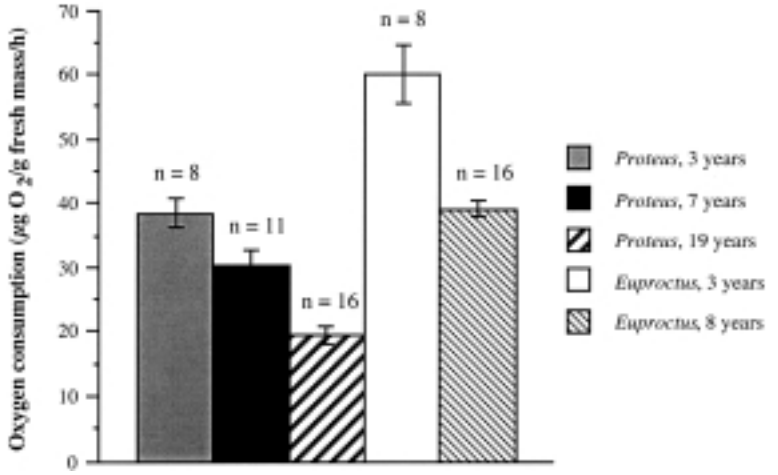

Fig. 1. Oxygen consumption (SMR, mg O_2/g fresh mass/h) in *Proteus anguinus* and *Euproctus asper* of various ages, at 13 °C, under conditions of constant darkness. Values are means ± SE, for n = 8–16 individuals. All five values were significantly different ($p < 0.05$).

years old) of *E. asper* showed »normal« SMR, included in the range of values summarized in HUTCHISON (1971), LICHT & LOWCOCK (1991) and in GATTEN *et al*. (1992). All five values were significantly different ($p < 0.05$).

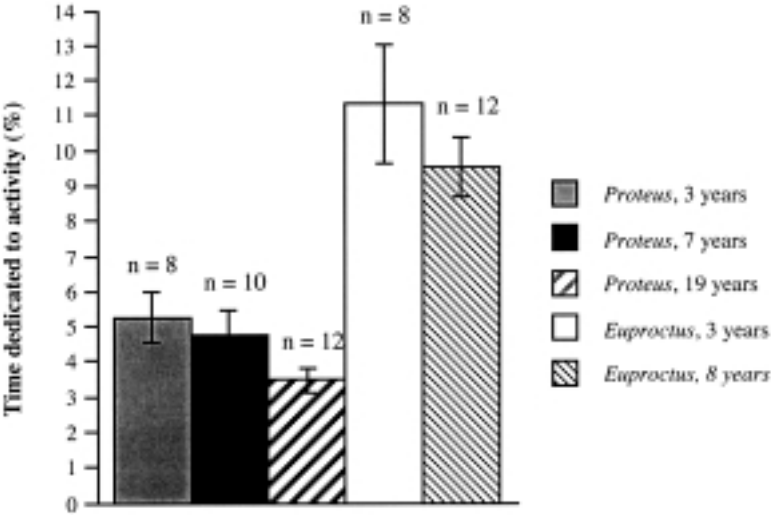

Fig. 2. Spontaneous activity (percentage activity per hour) in *Proteus anguinus* and *Euproctus asper* of various ages, at 13 °C, under conditions of constant darkness. Values are means ± SE, for n = 8–12 individuals.

Activity

Figure 2 shows that the subterranean *P. anguinus* displayed a very low spontaneous activity rate, approximately half that of the facultative cave-dwelling *E. asper*. Juveniles of both species showed a higher locomotory activity than adults (Fig. 2). Locomotory activity decreased with age (Fig. 2). Both species exhibited periods of high activity interspersed with periods of inactivity or low activity (not shown).

Circadian rhythm of oxygen consumption and locomotion

The obligate cave-dweller *P. anguinus* lacked any apparent daily rhythm of activity or SMR. In contrast, *E. asper* presented circadian components of SMR (Fig. 3) and activity (Fig. 4). *E. asper* appears to be a nocturnally active species, showing maxima of SMR and activity at night, between 20h00 and 03h00. In this organism, daily patterns of SMR were stable during constant darkness, and the oscillation of oxygen consumption seemed to be unrelated to light-dark cycles.

DISCUSSION

Circadian rhythmicity

Many animals show rhythmic sequences of behavioral, physiological, and metabolic events, controlled by endogenous and/or exogenous time-keeping mechanisms

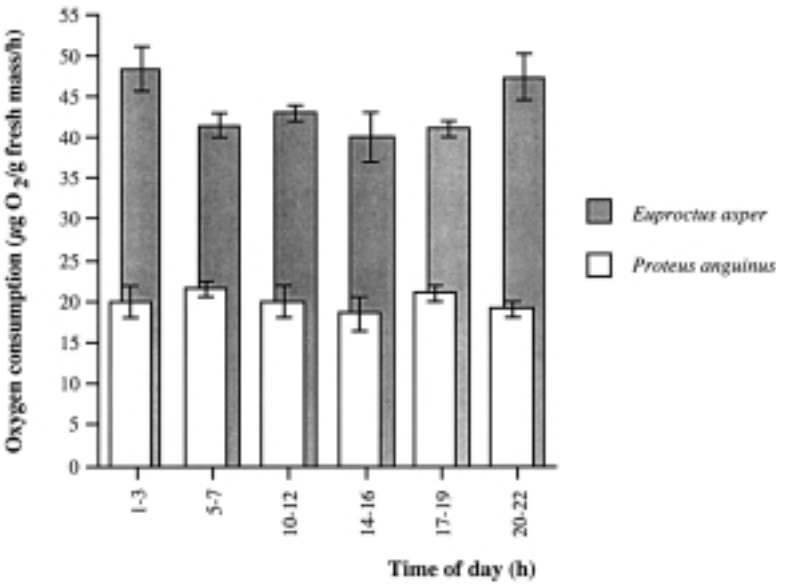

Fig. 3. Circadian trends in oxygen consumption (SMR, mg O_2/g fresh mass/h) in *Proteus anguinus* and *Euproctus asper*, at 13 °C, under conditions of constant darkness. Values are means ± SE, for n = 12 individuals.

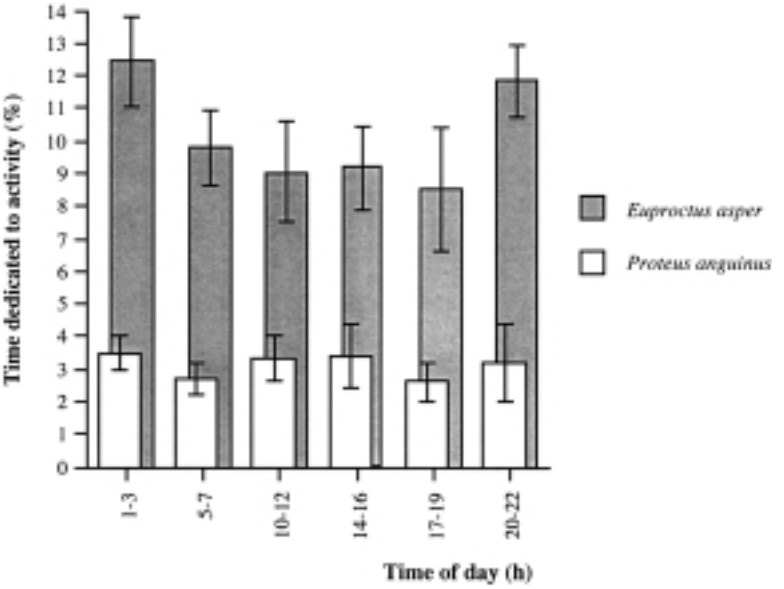

Fig. 4. Circadian patterns of spontaneous activity (% of the total observation time dedicated to activity) in *Proteus anguinus* and *Euproctus asper*, at 13 °C, under conditions of constant darkness. Values are means ± SE, for n = 12 individuals.

(among them circadian clocks) (LAMPRECHT & WEBER, 1985). Usually, circadian events are controlled by daily environmental changes. Daily cycles of spontaneous activity and/or resting metabolism occur in amphibians when they are exposed to a light-dark cycle (MILLER & HUTCHISON, 1979).

Our work showed that, in darkness, a daily rhythm of oxygen consumption and activity were evident in the facultative cave-dwelling species (*E. asper*, not permanently exposed to a light-dark cycle in his natural ecosystem), whereas no rhythmicity occurred in the permanently hypogean *P. anguinus* (living continuously in darkness). In the absence of daily external »zeitgebers« (e.g. light, food, temperature), this circadian rhythm clearly has an endogenous component in the epigean *E. asper*.

In the absence of periodic environmental changes (i.e., one of the main characteristics of cave habitats), *P. anguinus* lacked circadian periodicities of activity and metabolism. A persistent daily locomotory rhythm (under constant darkness) was reported for this species by BRIEGLEB & SCHATZ (1974), but repetition of this experiment could not confirm that result (SCHATZ *et al.*, 1977). Although the circadian activity of a cave-dwelling population of the fish *Astyanax fasciatus* has been connected to light-dark cycles, this rhythmicity disappeared immediately in constant darkness (ERCKENS & MARTIN, 1982). Several authors (reviewed by POULSON & JEGLA, 1969; LAMPRECHT & WEBER, 1985; TRAJANO & MENNA-BARRETO, 1995) have also observed the loss or reduction of circadian rhythms in cave-restricted species as diverse as insects, crustaceans, and fishes. Therefore, LAMPRECHT & WEBER (1985)

concluded that during the course of regressive evolution under cave conditions the circadian system degenerates without any residue.

Any evolutionary process requires time, and species living permanently or temporarily in subterranean habitats may present specific adaptive traits in any stage of regression. In »less specialized« hypogean species (such as the blind catfish *Pimelodella kronei*: TRAJANO & MENNA-BARRETO, 1995) and in facultative cave dwellers (such as *E. asper*), circadian rhythmicity is sometimes persistent, corroborating the hypothesis that exposure to circadian cycles is a major selective pressure. Non-photophobic troglobite species living near cave entrances (where food availability is higher), as in the case with part of some *P. kronei* populations (TRAJANO & MENNA-BARRETO, 1995), or animals that periodically leave subterranean habitats (for the same nutritional reason, as the case with cave-dwelling bats: MARIMUTHU, 1984), are exposed to light-dark and temperature cycles (circadian »zeitgebers«). Exposure to light (sometimes of extremely low intensity: stars and/or moon light) may contribute to the daily resetting of the circadian clock system. TRAJANO & MENNA-BARRETO (1995) concluded that, for both kinds of species, the maintenance of an »internal temporal order« represented an important adaptive trait, enabling such animals to adopt the optimal time to start outside activities. In a few hypogean organisms, ecological advantages have prevented or limited the regression of the circadian (or annual) clock system (LAMPRECHT & WEBER, 1985).

Reduced metabolic and activity rates

The minimum resting rates of oxygen consumption under standardized conditions (SMR) is generally regarded as a valuable standard of comparison in the analysis of variables of biological relevance, indicating the effects of many ecological, physiological, pharmacological, and/or evolutive (between closely related species) factors.

Subterranean organisms tend to have lower activity and metabolic rates than closely related epigean species (for review, see HÜPPOP, 1985; HERVANT *et al.*, 1997; 1998). *Proteus anguinus* is no exception, with oxygen consumption up to one-half that of facultative cave dwellers *Eurycea multiplicata griseogaster* (the gray-bellied salamander: BROWN & FITZPATRICK, 1981) and *E. asper*, and considerably lower (up to one-eighth) than that of most surface-dwelling amphibians (reviews in HUTCHISON, 1971; LICHT & LOWCOCK, 1991; GATTEN *et al.*, 1992: values and references therein). Only two epigean giant salamanders, the closely related proteid *Necturus maculosus* and the amphiumid *Amphiuma means* (LICHT & LOWCOCK, 1991), shared similar SMR values with the subterranean *P. anguinus*, but their mass specific oxygen consumption does not make them truly comparable, since their fresh masses are too dissimilar.

Amphibians are typically quiescent (POUGH *et al.*, 1992). Unsurprisingly, the lives of both studied salamanders were characterized by long periods of inactivity punctuated by bouts of foraging or exploratory/predatory behavior. Amphibians generally display low SMR compared to other ectotherms, and possess low capacities for sustained locomotor activity (review in POUGH *et al.*, 1992, and references therein).

Thus, *P. anguinus* is a good example of a vertebrate representing a low-energy system.

The presence of reduced metabolic and activity rates in subterranean species as diverse as crustaceans, fishes (HÜPPOP, 1985; HERVANT *et al*., 1998), and (in the present study) amphibians implies that this is one of the most important adaptations to subterranean environments (HERVANT *et al*., 1998). Low and discontinuous food supplies and/or alternately hypoxic and normoxic waters (encountered by numerous subterranean species, among them *P. anguinus*: ISTENIC, 1971; UIBLEIN *et al*., 1992) were certainly the most important factors controlling adaptations of metabolic and activity rates in aquatic hypogean organisms (HERVANT *et al*., 1997; 1998).

HERVANT *et al*., (1998) suggested that a reduced SMR (i.e., a lower metabolic cost) shown by hypogean animals i) improves survival in the harsh, stressful, subterranean environments, and ii) reflects a lower capacity for locomotion, due to reduced visual predator-prey interaction. This latter hypothesis was corroborated by the results summarized in UIBLEIN *et al*., (1992), showing that *P. anguinus* locates prey using a non-visual, mechanically and chemically guided approach instead of a widely foraging mode (as the case for *E. asper*).

ACKNOWLEDGEMENTS

This research was supported by the University Claude Bernard-Lyon I and the French National Centre of Scientific Research (CNRS).

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SAŽETAK

Cirkadijalni ritam, disanje i ponašanje hipogejskih i epigejskih repaša

F. Hervant, J. Mathieu & J.-P. Durand

Usporedbe cirkadijalnog ritma, ponašanja i metabolizma kod površinskih i špiljskih proteida omogućuju rasvjetljavanje evolucijskih trendova u tim procesima. To je bio i poticaj za istraživanje metabolizma i ponašanja dvaju vodozemaca – akvatičkih repaša, špiljske (*Proteus anguinus*) i površinske (*Euproctus asper*) vrste. Osim toga, željeli smo usporediti stupanj prilagodbe (potrošnju kisika i cirkadijalni ritam) na špiljska staništa, a koju su pokazale i epigejska i hipogejska vrsta.

Proteidni vodozemac *Proteus anguinus* (čovječja ribica, »grottenolm«) je jedini Europski kralježnjak koji živi isključivo u špiljama. Njen areal je ograničen na špilje Jadranskog krša, široko rasprostranjen sustav malih pukotina u kršu (nedostupan ljudima). *P. anguinus* pokazuje izrazite troglobitske osobine, uključujući spori razvoj (dostižu odrasli stadij sa 14–18 godina, i žive više od 60 godina), izduženo tijelo i tanke udove, depigmentaciju, degeneraciju očiju i zadržavanje ličinačkih osobina kao što su vanjske škrge i repne peraje. Salamandrid *Euproctus asper* živi u Francuskim i Španjolskim Pirenejima, u izvorima, rječicama i nekim jezerima (od 400 do 2500 m), kao i u nekim špiljama (od 250 m). *E. asper* je morfološki sličan *P. anguinus*, iako ne pokazuje troglomorfne osobine.

Obligatorni špiljski proteid *Proteus anguinus* koji nikad nije bio podrvrgnut fotoperiodičkim podražajima nije pokazivao nikakav očit dnevni ritam aktivnosti ili potrošnje kisika (*resting metabolic rate*). Zato možemo postaviti hipotezu da tijekom regresivne evolucije u špiljskim uvjetima cirkadijalni sustav degenerira bez ostataka. Nasuprot tome, površinski salamandrid *Euproctus asper* posjeduje cirkadijalni ritam potrošnje kisika i aktivnosti. Ti cirkadijalni ritmovi imaju endogenu komponentu. Život obiju proučavanih vrsta karakteriziraju dugi periodi neaktivnosti prekidani periodima hranjenja i istraživačkog/predatorskog ponašanja. *Proteus anguinus* imala je smanjen metabolizam odmaranja i spontane aktivnosti (znatno niže nego kod mnogih površinskih vodozemaca). Niski metabolizam i aktivnost (tj. *jeftiniji* metabolizam) kod *P. anguinus* tumače se kao prilagodbe na život u podzemlju, gdje je hrana slabo i nekontinuirano prisutna i/ili hipoksija može biti prisutna dulje vrijeme. Ova podzemna vrsta se smatra izvanrednim modelom organizama koji su naselili ekstremno zahtjevna/teška staništa, i čini se dobrim primjerom kralježnjaka kao niskoenergetskog sustava.

SUMMARY

Circadian rhythmicity, respiration and behavior in hypogean and epigean salamanders

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Comparisons of circadian rhythmicity, behavior, and metabolism between surface and cave-dwelling salamanders enable elucidation of evolutionary trends in these processes. Therefore, a comparative study of metabolism and behavior has been realized in two aquatic urodele amphibians, a cave-dwelling (*Proteus anguinus*) and a surface-dwelling (*Euproctus asper*) species. In addition, we aimed to compare the degree of adaptation (oxygen consumption and circadian rhythm) to cave biotopes exhibited by both epigean and hypogean salamanders.

The proteid amphibian *Proteus anguinus* (the »grottenolm«) is the only European vertebrate that lives exclusively in caves. Its range is restricted to the caves of the Adriatic karst, in a widely distributed system of small karst fissures (inaccessible by humans). *P. anguinus* exhibits marked troglobitic traits, including a slow development rate (they reach the adult stage between 14 and 18 years, and live more than 60 years), elongated body and thin limbs, depigmentation, ocular degeneration, and retention of larval traits such as external gills and caudal fins. The salamandrid *Euproctus asper* is found in the French and Spanish Pyrenees, in springs, small rivers, and some lakes (from 400 to 2500 m), as well as in some caves (from 250 m). *E. asper* is morphologically similar to *P. anguinus*, although it does not show troglomorphic traits.

The obligate cave-dwelling proteid *Proteus anguin*us, never submitted to a photoperiodic stimulus, did not show any apparent daily rhythm of activity or oxygen consumption (resting metabolic rate). Therefore, it could be hypothesized that during the course of regressive evolution under cave conditions the circadian system degenerates without any residue. In contrast, the surface-dwelling salamandrid *Euproctus asper* had a circadian oxygen consumption and activity cycle. These circadian rhythms possessed an endogenous component. The lives of both salamanders studied were characterized by long periods of inactivity punctuated by bouts of foraging or exploratory/predatory behavior. *Proteus anguinus* had reduced resting metabolic and spontaneous activity rates (considerably lower than most surface-dwelling amphibians). The low metabolic and activity rates (i.e., a lower metabolic cost) of *P. anguinus* are interpreted as adaptations to a subterranean existence where poor and discontinuous food supplies and/or intermittent hypoxia may be present for long periods. This subterranean species is now considered as an excellent model for organisms that have colonized an extremely challenging/harsh biotope, and appears to be a good example of a vertebrate as a low-energy system.