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BEHAVIOURAL, PHYSIOLOGICAL AND METABOLIC RESPONSES TO LONG-TERM STARVATION AND REFEEDING IN A BLIND CAVE-DWELLING (*PROTEUS ANGUINUS*) AND A SURFACE-DWELLING (*EUPROCTUS ASPER*) SALAMANDER

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Summary

The effects of long-term starvation and subsequent refeeding on haematological variables, behaviour, rates of oxygen consumption and intermediary and energy metabolism were studied in morphologically similar surface- and cave-dwelling salamanders. To provide a hypothetical general model representing the responses of amphibians to food stress, a sequential energy strategy has been proposed, suggesting that four successive phases (termed stress, transition, adaptation and recovery) can be distinguished. The metabolic response to prolonged food deprivation was monophasic in the epigean *Euproctus asper* (Salamandridae), showing an immediate, linear and large decrease in all the energy reserves. In contrast, the hypogean *Proteus anguinus* (Proteidae) displayed successive periods of glucidic, lipidic and finally lipido-proteic-dominant catabolism during the course of food deprivation. The remarkable resistance to long-term fasting and the very quick recovery from nutritional stress of this cave organism may be explained partly by its ability to remain in an extremely prolonged state of protein sparing and temporary torpor. *Proteus anguinus* had reduced metabolic and activity rates (considerably lower than those of most surface-dwelling amphibians). These results are interpreted as adaptations to a subterranean existence in which poor and discontinuous food supplies and/or intermittent hypoxia may occur for long periods. Therefore, *P. anguinus* appears to be a good example of a low-energy-system vertebrate.

Key words: starvation, refeeding, cave, surface, amphibian, intermediary metabolism, energetic metabolism, activity, blood variables, oxygen consumption, *Euproctus asper*, *Proteus anguinus*.

Introduction

Numerous groundwater ecosystems, including caves and karstic aquifers, are characterized by severely limited food supplies during most of the year because of the lack of autotrophic production and sporadic, unpredictable, allochthonous input. Because of the temporal and spatial patchiness of food availability in most cave biotopes, periods of prolonged starvation are common events in the life of subterranean (i.e. hypogean) organisms (Poulson, 1964; Hüppop, 1985; Hervant et al., 1997; Hervant et al., 1999).

Several hypogean species are able to survive for long periods without food – nearly 1 year in invertebrates, and up to several years in cave fishes and salamanders (Poulson, 1964; Mathieu and Gibert, 1980; Hervant et al., 1997; Hervant et al., 1999). Periods of nutritional stress may influence both the geographic and temporal distribution of a species (Hervant et al., 1997; Hervant et al., 1999). Knowledge of changes in the behaviour and in the physiology of hypogean species under severe food limitation and refeeding could improve our understanding of their competitive abilities. In addition, a considerable number of studies on the behavioural, morphological, physiological and/or metabolic effects of starvation in fishes, birds and mammals have been made, but this type of stress has been very little studied in amphibians.

Periods of starvation are encountered by most epigean and hypogean animals, but they can adjust their metabolism to the lack of food by utilizing metabolites stored during times when food is abundant. Much information about the influence of starvation in vertebrates concerns qualitative and quantitative changes in body composition (Newsholme and Stuart, 1973; Le Maho, 1981; Merkle and Hanke, 1988a; Koubi, 1993). The relative importance of these metabolic reserves and their order of utilization varies with species. Moreover, periods of drastically reduced food intake are regular seasonal events for many epigean amphibians (Merkle and Hanke, 1988b). It is therefore not surprising that numerous species have the ability to survive periods of prolonged starvation such as hibernation, aestivation and/or the spawning season. Among them, the water frog *Rana esculenta* showed a survival time during starvation of 12 months (Grably and Piery, 1981) and the South

African clawed toad *Xenopus laevis* of 18 months (Merkle and Hanke, 1988a).

The hypogean salamander *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). Briegleb (1962) states that *P. anguinus* has its main habitat in a widely distributed system of small karst fissures that are inaccessible to humans (Briegleb, 1962). This species is now considered as an excellent model for organisms that have colonized this 'extreme biotope' because it has to cope frequently or permanently with darkness, limited food supply and/or low oxygen tensions (Istenic, 1971; Uiblein et al., 1992; Malard and Hervant, 1999; Parzefall et al., 1999; Hervant et al., 2000). In addition, it has been reported (Michaehelles, 1831; Schreiber, 1875; Nusbaum, 1907; Gadeau de Kerville, 1926) that *P. anguinus* could survive food deprivation for exceptional periods, ranging between 18 and 96 months, with no signs of illness.

In the French and Spanish Pyrenees, the epigean salamander *Euproctus asper* is found in surface fresh water (Clergue-Gazeau and Martinez-Rica, 1978). *E. asper* is morphologically close to *P. anguinus* (although this surface-dwelling species does not show troglomorphic traits, i.e. poorly developed eyes and almost unpigmented skin; Durand, 1971; Durand, 1983), but is not taxonomically closely related because most subterranean species are either phylogenetic or distributional relicts.

P. anguinus represents a good model animal for research on physiological and behavioral adaptations to extreme environments (Hervant et al., 2000). Therefore, we aimed at comparing the degree of adaptation to cave biotopes exhibited by epigean and hypogean aquatic salamanders. Thus, experiments were conducted on these urodeles during long-term starvation and subsequent recovery with the following objectives: (i) to determine the metabolic changes, the use and resynthesis of energy reserves, the behavioural and haematological adaptations to starvation/renutrition and, hence, the ecological importance of these processes, and (ii) to understand better the ecological problems concerning the survival of aquatic subterranean amphibians in their food-limited habitats. These aims were met by recording behavioural, physiological and metabolic variables during a 240-day starvation period and a subsequent 15-day renutrition phase in the cave-dweller *P. anguinus* and during a 90-day starvation period and a subsequent 15-day refeeding phase in the surface-dweller *E. asper*.

Materials and methods

Animals

Individuals of the cave-dweller *Proteus anguinus* Laurenti (Proteidae) and the surface-dweller *Euproctus asper* Duges (Salamandridae) originated from a stock established in the CNRS cave laboratory at Moulis, France. Adult specimens of *P. anguinus* aged 19 years (17.5±0.9 g, N=16, mean ± S.E.M.) and of *E. asper* aged 8 years (8.7±0.3 g, N=17, mean ± S.E.M.) were used during the experiments.

Most of the animals had been freshly caught in the field, where numerous environmental factors are superimposed on feeding conditions. These problems should be minimized in animals bred under artificial, controlled conditions. Therefore, salamanders of both species were transferred to the HBES laboratory (University Lyon 1, France) and raised, under semi-natural conditions, in aquaria containing stones and recirculating ground water (pumped from the underground aquifer below the University). They were fed with chironomid larvae ('blood worms') once a week. The aquaria were kept in the darkness in a controlled-temperature facility (12.8±0.15 °C).

Individuals of both species were acclimated to laboratory conditions for 3 months before experimentation, then separated into control (N=8) and treatment (N=8–9) groups. The control group was fed as described above. The treatment group was deprived of food. To investigate responses to food stress, individuals were maintained under conditions of starvation and removed at intervals of 7, 15, 30, 60, 90, 120, 150, 180, 210 and 240 days for *P. anguinus*, or 7, 15, 30, 60 and 90 days for *E. asper*, to measure their rates of oxygen consumption and activity and to sample blood and muscle.

To investigate responses to recovery from long-term lack of food, individuals were starved for either 240 days (*P. anguinus*) or 90 days (*E. asper*) before subsequent refeeding (individuals were fed every 4 days over 15 days). Individuals of both species were then removed at intervals of 7 and 15 days to measure their rates of oxygen consumption and spontaneous activity and to sample blood and muscle. No deaths occurred during the experiments.

Measurement of oxygen consumption

For both species, rates of oxygen consumption was measured under standardized conditions, at the same time of the day to counter the effects of a possible circadian rhythm of respiration. Oxygen consumption was measured for 2 h (in darkness) in a closed respirometer placed in a constant-temperature chamber at 12.8±0.15 °C and supplied with aerated fresh water (P_{O_2}=15.7±0.4 kPa). One hour before the experiments began, fed and starved salamanders of both species were transferred individually into an 800 ml Plexiglas metabolic chamber which was part of the respirometer. Fed individuals were starved for 2 days before experiments to ensure that digestive metabolism did not affect the results. A constant low rate of water flow (25 ml min^{-1}) was maintained through the respirometric system during each experiment, using a peristaltic pump, to prevent local oxygen depletion around the electrode. Oxygen depletion inside the system was monitored with a MOCA 3600 oxygen meter/recorder (Orbisphere Laboratory) accurate to ±0.1 kPa.

Measurement of spontaneous activity

Three hours before the experiments began, individuals of both species were transferred individually into a large glass

aquarium containing stones and supplied with aerated fresh water (12.8±0.15 °C). The percentage of activity time per hour (percentage of the total observation time dedicated to activity, including locomotory activity, head and/or tail movements) and displacement speed (i.e. swimming/walking speed) were then recorded for 2 h (in darkness) using an infrared camera (Aaton 30, Newicon) equipped with a 70 mm focal length macro-objective and a VHS video recorder. The illumination was an infrared light source. Video tapes were digitized (six points per animal), and activity variables were quantified/analysed using special trajectometric software (Coulon et al., 1983).

Blood and muscle sampling, metabolite assays

To investigate changes in levels of key metabolites and in body composition, control, starved and refed individuals were removed from the aquaria, immediately weighed, and then anaesthetized by placing the animals for 5 min into a 0.5 g l^{-1} tricaine methane sulphonate solution (Sandoz MS-222). Blood (0.4 ml) was then obtained from caudal vessels using a heparinized syringe. Whole blood was used immediately for the determination of haematocrit, haemoglobin and glucose concentrations, as described previously (Johansson-Sjöbeck et al., 1975). The rest of the blood was centrifuged (3000 ***g*** for 10 min at 4 °C). The blood plasma was kept at −30 °C until analyzed for levels of serine, glycerol, urea and non-esterified fatty acids (as described by Fynn-Aikins et al., 1992). A percutaneous biopsy (50 mg) of muscle (twitch fibres, this tissue representing the bulk of the musculature in urodeles; Chanoine et al., 1989) was sampled under MS-222 anaesthesia from the caudal musculature (3 cm behind the level of the cloaca) using a Bergström needle (Depuy, Phoenix, AZ, USA). The muscle biopsy was weighed (fresh mass) then immediately deep-frozen in liquid nitrogen before being lyophilized (Virtis lyophilizator, Trivac D4B). The lyophilized muscular tissues were weighed (dry mass), homogenized (as described by Hervant et al., 1996) and stored in capped vials at −75 °C until analyzed for glycogen, protein and triglyceride contents.

The following metabolite concentrations were determined, as described previously (Hervant et al., 1995; Hervant et al., 1996), using standard enzymatic methods (Bergmeyer, 1985): glucose, glycerol, serine and glycogen. Total proteins and triglycerides were extracted from muscle, and urea from blood (according to Elendt, 1989), and their concentrations were then determined using specific test combinations (Bœhringer-Mannheim). All assays were performed in a recording spectrophotometer (Beckman DU-6) at 25 °C. Enzymes, coenzymes and substrates used for enzymatic assays were purchased from Bœhringer (Mannheim, Germany) and Sigma Co. (St Louis, MI, USA). The accuracy of each assay was measured by testing samples with and without an added internal standard. The sensitivity of the assays used was approximately 1 μmol g^{-1} dry mass for all metabolites.

General remarks and statistical analyses

Control (fed) organisms showed no changes in behavioural, physiological or biochemical variables during the experimental period (not shown). In some cases, a slight increase in the rate of oxygen consumption and increased activity, probably due to stress, were observed just after the transfer of individuals into a metabolic chamber or an aquarium at the start of an experiment. Consequently, to minimize this effect, the first 30 min of measurements was not taken into account for all metabolic or activity rate calculations. Control organisms showed no immediate and/or long-term changes in haematological and/or biochemical variables induced by the MS-222 anaesthesia.

Values are presented as means ± S.E.M. For comparison between means (at the $P<0.05$ level) and after verification of normality of the values, a Tukey test was used. Comparisons among means were conducted with a one-way analysis of variance (ANOVA) using a Bonferroni test for multiple comparisons as appropriate.

Results

Activity and displacement speed

Figs 1A, 2A show that fed (t=0) subterranean *Proteus anguinus* displayed a very low spontaneous rate of activity, 3.3 times lower than that of the surface-dweller *Euproctus asper*. Both (fed) species exhibited periods of high activity (foraging or exploratory/predatory behavior) interspersed with periods of inactivity or low activity. In *P. anguinus*, starvation resulted in a period of hyperactivity (4.7-fold increase) at 30 days of food deprivation, followed by a decrease in locomotory activity, reaching 40 % of the initial value (fed animals, t=0) on day 120, after which it remained constant (Fig. 1A). In contrast, starved *E. asper* showed a significant hyperactivity (4.3-fold increase at 15 days; 1.7-fold at 90 days) for the duration of the experiments (Fig. 2A). During refeeding, the activity of both species increased rapidly (on day 7, a 2.3-fold increase in *P. anguinus* and a 3.9-fold increase in *E. asper*), then returned to the fed (t=0) value in *P. anguinus* at day 15 (Figs 1A, 2A).

In both salamanders, starvation induced a slight increase in displacement speed immediately after the onset of nutritional stress (Figs 1A, 2A), followed from day 60 in both species by a decrease in speed. During refeeding, displacement speed was no longer different from the fed level (t=0 value) by day 15 in both species (Figs 1A, 2A).

Oxygen consumption

The cave-dwelling *P. anguinus* displayed extremely low metabolic rates, with a value half that of *E. asper* (at 12.8 °C; Figs 1B, 2B). In *P. anguinus*, starvation initially resulted in a slight increase in oxygen consumption (+26 %) at 30 days of food deprivation, after which metabolic rates decreased, reaching 37 % of the initial value on day 180, when they levelled off (Fig. 1B). In *E. asper*, starvation resulted in an immediate increase in the rate of oxygen consumption (+40 % at day 30, a larger increase than in *P. anguinus*), after which

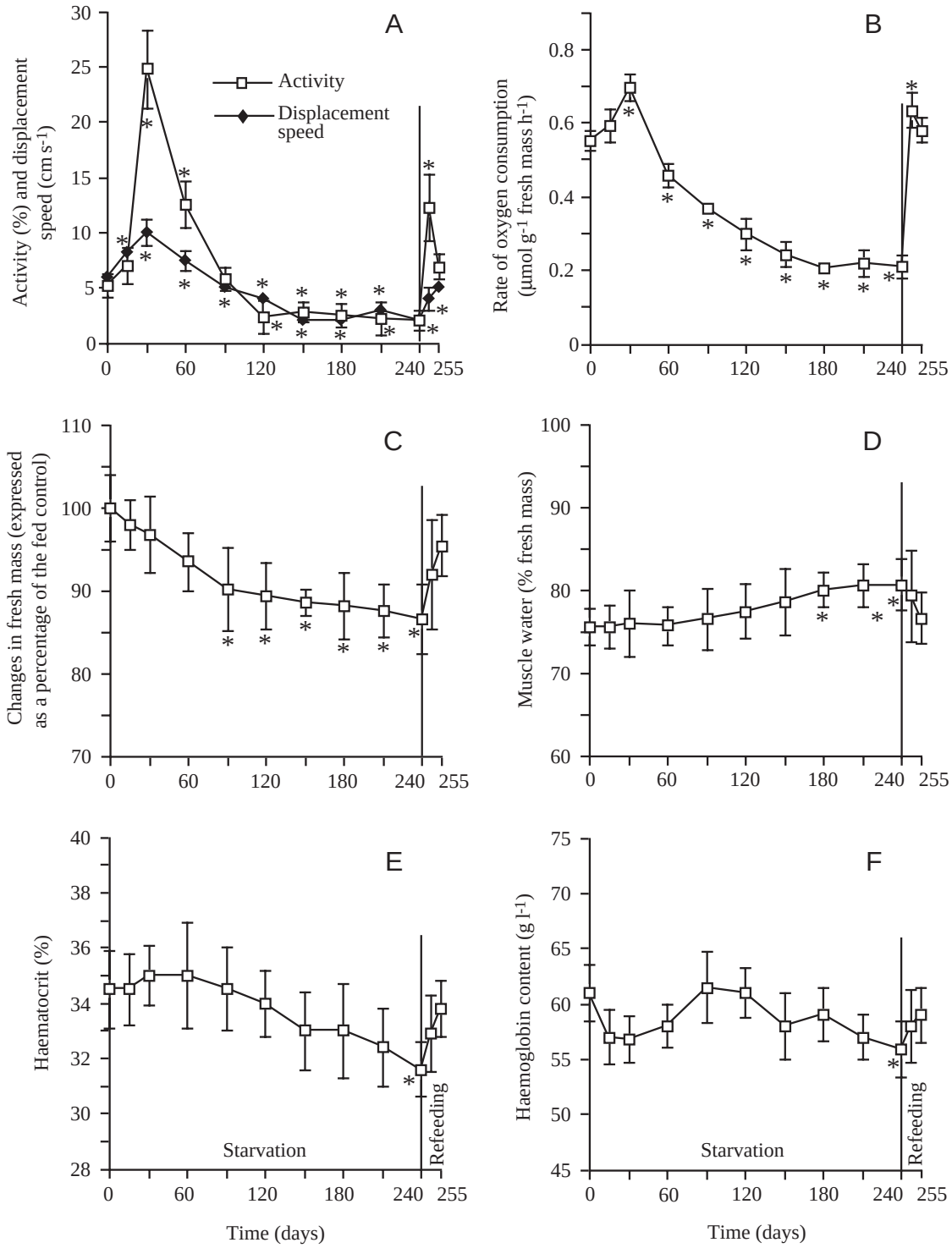

Fig. 1. Changes in (A) spontaneous activity (percentage activity per hour) and displacement speed, (B) rate of oxygen consumption, (C) fresh mass, (D) percentage muscle water content, and (E,F) haematological variables during long-term starvation (240 days) and subsequent refeeding (15 days) of the subterranean amphibian *Proteus anguinus* at 12.8 °C in darkness. Values are means ± S.E.M. for N=8 animals. An asterisk indicates a value that is significantly different from the fed control (t=0) (P<0.05).

oxygen consumption decreased to reach the fed value by day 90 (Fig. 2B).

Both species showed a large increase in rates of oxygen consumption (Figs 1B, 2B) immediately after the onset of refeeding, with values significantly higher than those found in fed animals. After day 7, metabolic rate gradually declined; the rate of oxygen consumption was no longer different from the fed level after 15 days of refeeding.

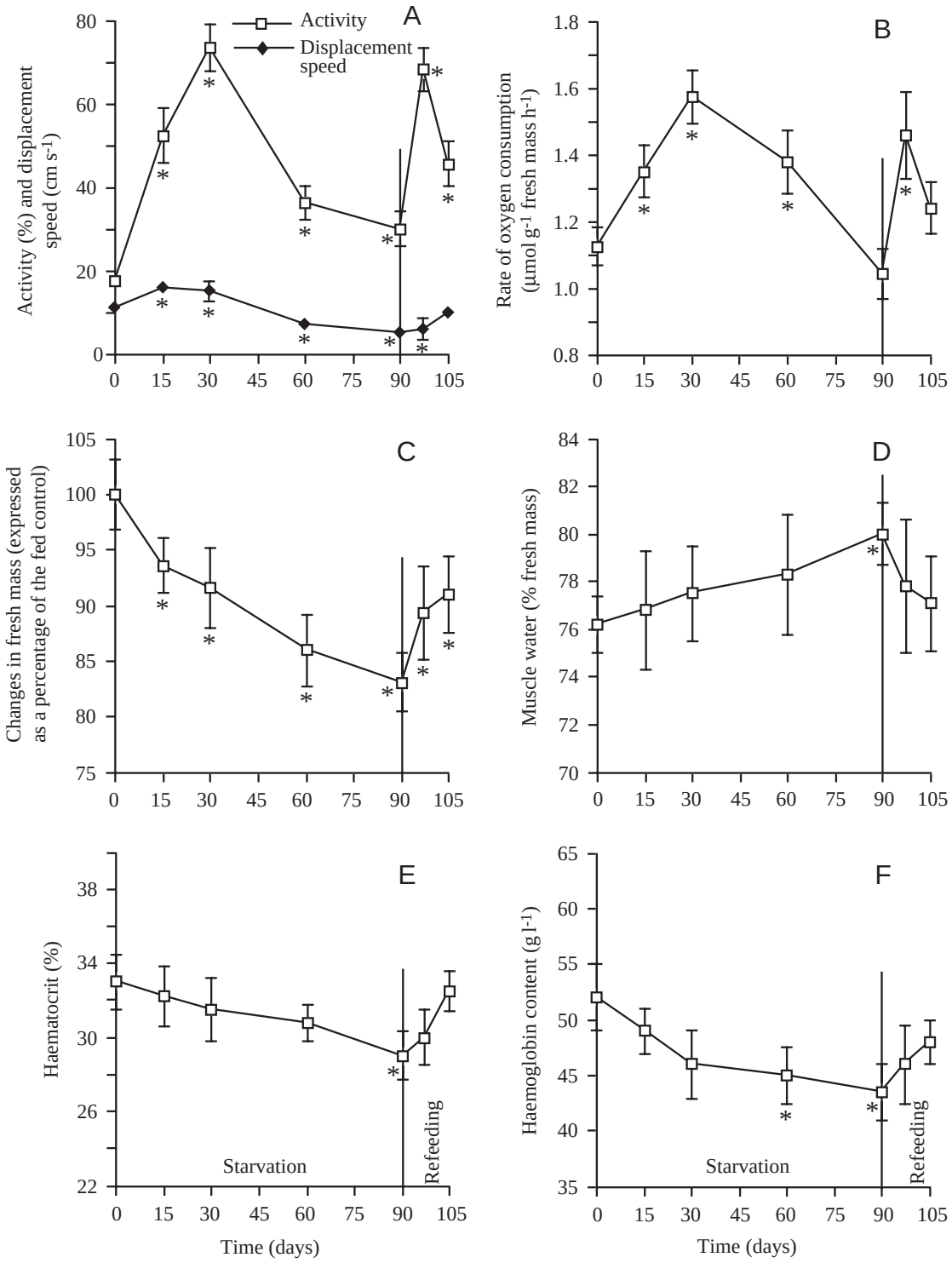

Fig. 2. Changes in (A) spontaneous activity (percentage activity per hour) and displacement speed, (B) rate of oxygen consumption, (C) fresh mass, (D) percentage muscle water content, and (E,F) haematological variables during long-term starvation (90 days) and subsequent refeeding (15 days) of the surface-dwelling amphibian *Euproctus asper* at 12.8 °C in darkness. Values are means ± S.E.M. for N=9 animals. An asterisk indicates a value that is significantly different from the fed control (t=0) (P<0.05).

Body mass and water content

Starved animals of both species showed a significant decrease (from day 90 in *P. anguinus* and from day 15 in *E. asper*) in mean percentage fresh mass (−13.4 % after 240 days in *P. anguinus*, −15.2 % after 90 days in *E. asper*; Figs 1C, 2C). Starvation resulted in a small increase (Figs 1D, 2D) in mean percentage muscle water content (+5.1 % after 240 days in *P. anguinus*, +5.0 % after 90 days in *E. asper*) in both

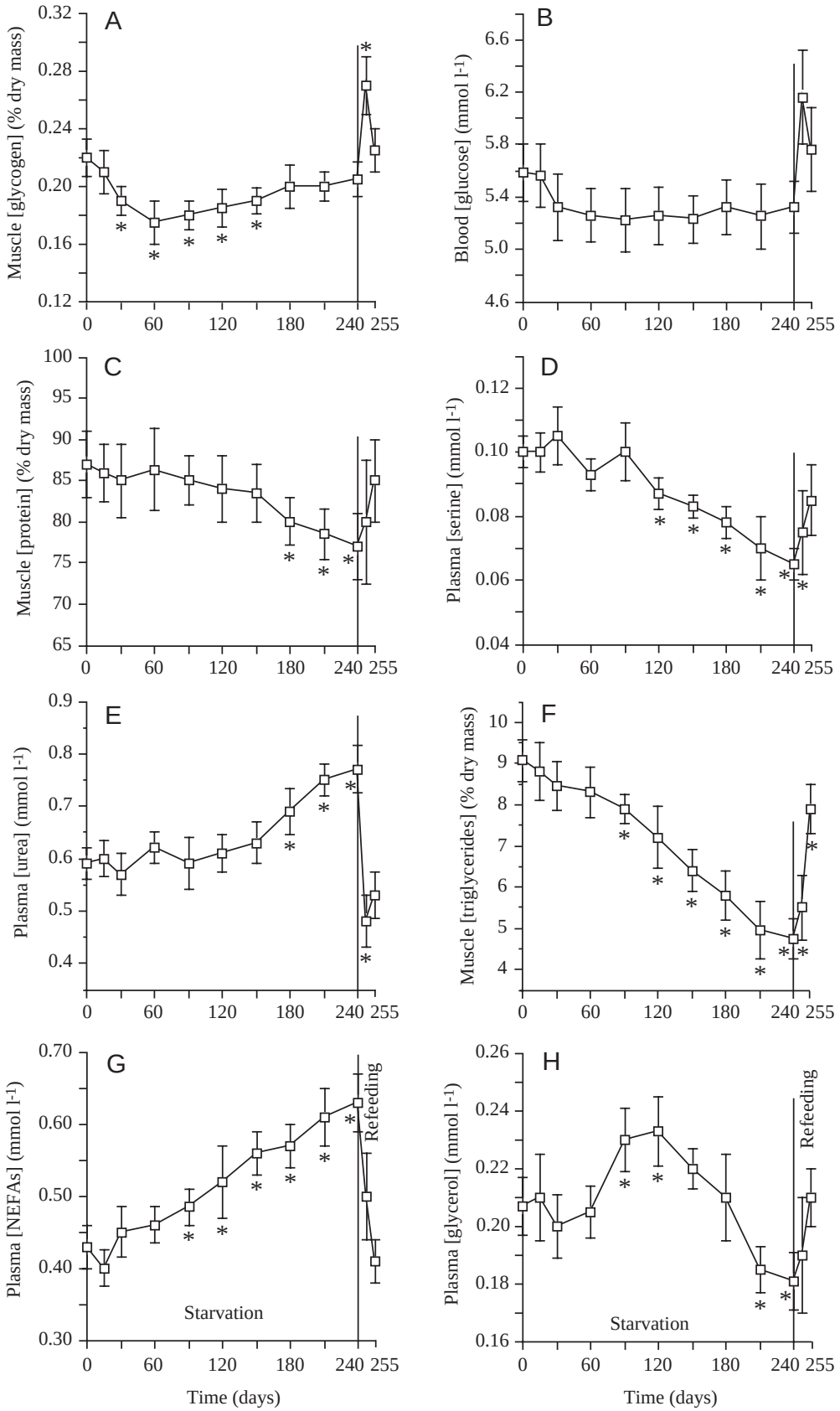

Fig. 3. Changes in levels of muscle and blood metabolites during long-term starvation (240 days) and subsequent refeeding (15 days) of the subterranean amphibian *Proteus anguinus* at 12.8 °C in darkness. Values are means ± S.E.M. for N=8 animals. An asterisk indicates a value that is significantly different from the fed control (t=0) (P<0.05). NEFAs, non-esterified fatty acids.

species, although this increase was not significant until day 180 in the subterranean species and until day 90 in the epigean species. During refeeding, muscle water content returned to pre-starvation levels (Figs 1D, 2D); fresh mass increased slightly (but significantly, $P<0.05$) in both species (at day 15, +8.7 % in *P. anguinus* and +6.2 % in *E. asper*) (Figs 1C, 2C).

Blood variables

When starved, both species showed a slight decrease (at day 240 in *P. anguinus* and at day 90 in *E. asper*) in haematocrit (–8.4 % in *P. anguinus*, –12.0 % in *E. asper*; Figs 1E, 2E). In addition, both urodeles showed a significant decrease (at 240 days in *P. anguinus* and at 60 days in *E. asper*) in haemoglobin concentration (–8.4 % after 240 days in *P. anguinus*, –16.4 % after 90 days in *E. asper*; Figs 1F, 2F). During refeeding, haemoglobin content and haematocrit immediately returned to fed levels.

Effects of starvation and subsequent refeeding on muscle and blood metabolite concentrations in the subterranean Proteus anguinus

Muscle glycogen content decreased from 30 days of food deprivation in the cave-dwelling *P. anguinus*, reaching 80 % of its initial concentration on day 90 (Fig. 3A) and returning to the pre-starvation level after 180 days. Glycogen content showed a dramatic increase during the first week of recovery from starvation (reaching 123 % of the fed value), before returning to the pre-starvation level (Fig. 3A). No significant changes in the blood concentration of glucose were observed during food deprivation and subsequent renutrition (Fig. 3B).

During starvation, muscle proteins were catabolized only after 180 days, muscle protein content reaching 89 % of the fed level by day 240 (Fig. 3C), which corresponded to a utilization of $0.10\,g\,g^{-1}$ dry mass. During refeeding, protein content returned to the pre-starvation level by day 15 (Fig. 3C). During food deprivation, plasma serine concentration was significantly decreased by day 120, reaching 65 % of the fed level by day 240 (Fig. 3D). During subsequent renutrition, plasma serine content returned to the pre-starvation level after 15 days (Fig. 3D). Plasma urea concentration followed a biphasic pattern: no significant changes were observed until 180 days of nutritional stress, followed by a large increase (+30.5 % on day 240; Fig. 3E). During refeeding, urea content immediately decreased, reaching 81 % of the fed level by day 7, then returning to the pre-starvation level (Fig. 3E).

During starvation, muscle triglycerides were metabolized after 90 days, reaching 52.3 % of the initial value on day 240 (Fig. 3F), equating to a utilization of $0.043\,g\,g^{-1}$ dry mass. During renutrition, triglyceride stores increased immediately and reached 87 % of the control level by day 15 (Fig. 3F). During starvation, non-esterified fatty acid (NEFA) concentrations changed in a reciprocal pattern to that of triglyceride concentration (Fig. 3G). During recovery from nutritional stress, plasma NEFA content immediately returned to the control level (Fig. 3G). Plasma glycerol concentration followed a triphasic pattern during food deprivation. No significant changes were observed for 60 days, followed by a slight increase up to day 120, then a decrease (Fig. 3H). During refeeding, glycerol content returned to control levels by day 15 (Fig. 3H).

Effects of starvation and subsequent refeeding on muscle and blood metabolite concentrations in the epigean Euproctus asper

During starvation, muscle glycogen content decreased immediately and drastically to reach 27 % of its initial concentration after 90 days (Fig. 4A) in the surface-dwelling *E. asper*, which corresponded to a utilization of $0.012\,g\,g^{-1}$ dry mass. Glycogen content showed a significant increase during the first week of recovery from nutritional stress (reaching 118 % of the fed value), before it returned to the pre-starvation level (Fig. 4A). During starvation, blood glucose content decreased immediately, reaching 82 % of its initial concentration by day 90 (Fig. 4B). Glucose concentration rapidly returned to fed value during refeeding (Fig. 4B).

During starvation, levels of muscle proteins decreased from day 30, reaching 78.6 % of the fed level after a 90-day period (Fig. 4C), equating to a utilization of $0.18\,g\,g^{-1}$ dry mass. During refeeding, protein content increased slowly, reaching 89 % of the fed level after 15 days (Fig. 4C). During food deprivation, plasma serine concentration increased slightly by day 30, reaching 111 % of the fed level by day 60 and returning to the fed level after 90 days (Fig. 4D). During subsequent renutrition, plasma serine content remained at pre-starvation levels (Fig. 4D). Plasma urea content had increased by 30 days of starvation and was 118 % of the fed level on day 90 (Fig. 4E). During refeeding, urea concentration decreased immediately, reaching 87 % of the initial level after 15 days of recovery (Fig. 4E).

During starvation, muscle triglyceride content had decreased by day 30, reaching 41 % of the initial value on day 90 (Fig. 4F), equating to a utilization of $0.047\,g\,g^{-1}$ dry mass. During recovery, triglyceride stores increased slowly, reaching 82 % of the fed value after 15 days (Fig. 4F). Plasma NEFA concentration had increased significantly by 30 days of food deprivation and was 134 % of the fed level on day 90 (Fig. 4G). During recovery from nutritional stress, NEFA content returned immediately to the pre-starvation level (Fig. 4G). During starvation, plasma glycerol concentration was significantly elevated between day 30 and day 90 (Fig. 4H). During refeeding, plasma glycerol content decreased to 87 % of the fed value after 7 days, then returned to the control level (Fig. 4H).

Discussion

The ability to withstand and recover from periods of inadequate/poor nutrition is a very important adaptation for the survival and reproductive potential of any species that has to cope sporadically with prolonged food deprivation. This

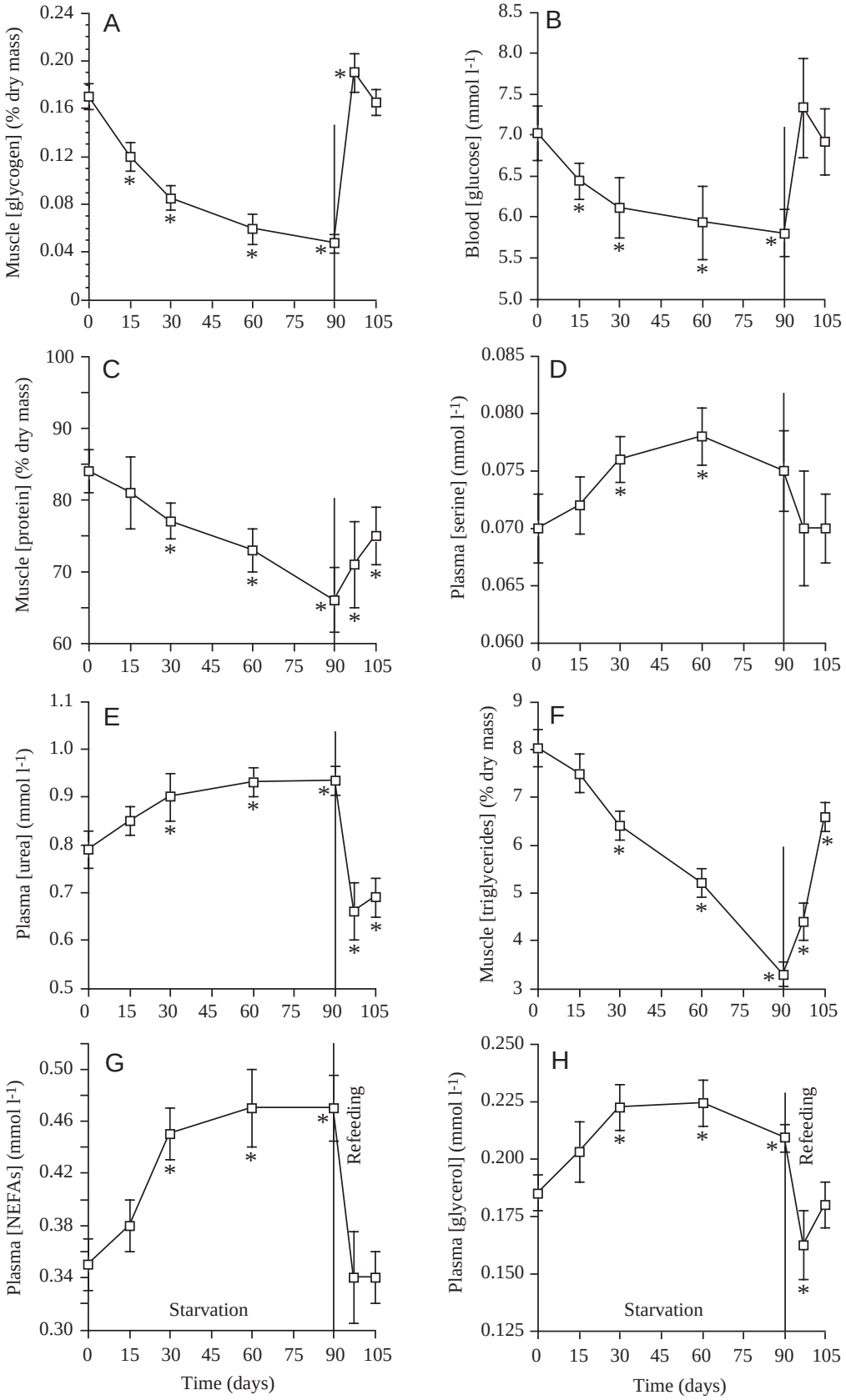

Fig. 4. Changes in levels of muscle and blood metabolites during long-term starvation (90 days) and subsequent refeeding (15 days) of the surface-dwelling amphibian *Euproctus asper* at 12.8 °C in darkness. Values are means ± S.E.M. for N=9 animals. An asterisk indicates a value that is significantly different from the fed control (t=0) (P<0.05). NEFAs, non-esterified fatty acids.

capability depends on the presence of appropriate nutrient stores, together with the necessary adaptive responses (i.e. adjustments in behavior, physiology and/or energy and intermediate metabolism) for ensuring that these stored metabolites are utilized and/or maintained optimally, i.e. in a manner that improves energetic efficiency.

In a review of numerous studies on fishes, Mendez and Wieser (1993) pointed out that selection might have favoured a sequential energy strategy in response to long-term starvation and subsequent refeeding, such that four successive phases (termed stress, transition, adaptation and recovery) can be distinguished (on the basis of changes in oxygen consumption and spontaneous activity) (Mendez and Wieser, 1993). To provide a hypothetical general model (i.e. a sequence of events) representing the responses of amphibians to food stress, and because clear evidence for the presence of these phases has been found in the cave-dwelling *P. anguinus* and the surface-dwelling *E. asper*, this nomenclature was employed in the present study. Long-term fasting birds and mammals generally show a similar energy strategy, but including a critical phase after the adaptation phase (Le Maho, 1984). This fifth phase is characterized by a drastic increase in both body mass loss and protein utilization rate; the animals then rapidly die if the fast continues. A starvation period of 240 days in *P. anguinus* and 90 days in *E. asper* was not sufficient to reach this dangerous, occasionally lethal, phase. In addition, Merkle and Hanke (1988a) also demonstrated that metabolic responses to long-term starvation appear to proceed in two distinct phases (equivalent to the transition and adaptation periods) in the clawed toad *Xenopus laevis*, but they ignored the initial 15-day 'stress' phase (Merkle and Hanke, 1988a).

Stress

At the start of the starvation period (0–60 days for both salamanders), both species increased locomotor activity and displacement speed (and therefore rates of oxygen consumption) at first, presumably to increase the chance of finding prey. Phases of hyperactivity reflecting an increase in food-searching behaviour have been found in food-deprived fishes (for a review, see Mendez and Wieser, 1993) and subterranean crustaceans (Danielopol et al., 1994; Hervant et al., 1997).

The only physiological/metabolic change observed in *P. anguinus* during this first period was a slight decrease in muscle glycogen stores, whereas *E. asper* showed a large utilization of triglyceride (with a corresponding increase in plasma glycerol and NEFA levels), glycogen and protein (with a corresponding increase in plasma urea level) reserves in muscles, associated with a slight decrease in fresh mass and haemoglobin content.

Transition

After the phase of hyperactivity, both salamanders responded to continued food deprivation with a reduction in the rate of oxygen consumption, in displacement speed and in spontaneous activity. These reductions were more drastic in the subterranean species than in *E. asper*. In addition, the surface-dwelling species showed a significant decrease in both haematocrit and haemoglobin content. This transition phase was completed by day 90 in the epigean *E. asper* and by day 150 in the hypogean *P. anguinus*.

It was observed that, during this second phase, energy metabolism in the swimming muscles of *P. anguinus* shifted from a carbohydrate-dominated to a lipid-dominated form, whereas no specific adaptation was observed in *E. asper* (i.e. throughout the nutritional stress, this epigean organism used all three stored metabolites rapidly and linearly). In addition, the absence of a change in urea plasma concentration in *P. anguinus* up to this point demonstrated that protein stores did not contribute to energy metabolism during this period (i.e. this species displayed efficient protein sparing). Therefore, this subterranean urodele could rapidly begin searching for prey once food became available again.

Adaptation

No significant adaptation phase was observed in *E. asper*; this species seemed to enter the critical phase directly (as defined by Le Maho, 1984). For *P. anguinus*, this third period was characterized by stable metabolic and activity rates and a speed of displacement that remained at the reduced levels reached at the end of the transition phase. This subterranean species is the first organism in which a constant minimal rate of oxygen consumption has been recorded for so long. At the end of this phase, *P. anguinus* showed a slight decrease in both haematocrit and haemoglobin content.

Prey rarity and darkness require longer search times in cave biotopes. In addition, the prey is invisible and often dead (carried in by occasional floods) and/or aggregated in patches. It has been demonstrated (Uiblein et al., 1992) that hypogean predators such as *P. anguinus* locate prey using a non-visual, mechanically and chemically guided approach instead of the widely foraging mode used by *E. asper*. The present study confirms that, during long-term starvation, *P. anguinus* uses an efficient food-search strategy, drastically reducing its locomotory activity, slowly and methodically exploring each part of the aquarium (using 'long-distance' mechano- and chemosensory prey detection as defined by Uiblein et al., 1992). Therefore, this hypogean amphibian is likely to have a reduced cost of exploratory/predatory activity (i.e. it maximizes the net energy gain, for example by capturing more prey per unit energy expended in searching). In contrast, the epigean *E. asper* attemps to localizes prey using fast and 'hazardous' displacements, without any marked decrease in locomotory rate and, therefore, suffers high energetic costs.

During the adaptation phase (from 150 days of starvation), energy metabolism in the swimming muscles of *P. anguinus* shifted progressively from a lipid-dominated to a lipid/protein-dominated form, whereas no specific adaptation was observed in *E. asper*. Contrary to the situation in subterranean crustaceans (Hervant et al., 1999), the increase in plasma urea

concentration observed during the adaptation phase suggested that the cave-dwelling urodele was not capable of prolonging total protein sparing after a 150-day starvation period. In addition, this hypogean species rapidly resynthesized its muscle glycogen stores during this period. This *de novo* synthesis may have been the result of an increased conversion/utilization of some amino acids (originating from proteolysis) or of glycerol (from lipolysis) to glycogen by glyconeogenesis. This hypothesis is supported by the decrease in plasma serine and glycerol contents (two major gluconeogenic compounds) at this time in *P. anguinus*. This ability rapidly to restore the reserves of metabolites used during the initial phase of food deficiency allows subterranean organisms successfully to withstand a prolonged hypoxic period subsequent to (or associated with) the nutritional stress (Istenic, 1971; Malard and Hervant, 1999) and will, therefore, increase their competitive ability.

Recovery

During refeeding, both species showed marked hyperactivity (and therefore a significant increase in oxygen consumption) corresponding to active food-searching behavior. Because of its higher muscular protein content and its higher sensitivity to the presence of potential food (Uiblein et al., 1992), prey detection was economical, more efficient and more rapid (a few seconds) in the starved hypogean salamander than in *E. asper* (approximately 30 s). This suggests that there is not only protein sparing during fasting in the hypogean species but also a selective utilization of muscle proteins, thus sparing those that are involved in essential functions such as locomotion. After prey detection, both amphibians started feeding immediately.

Immediately after the onset of renutrition, both species showed a large increase in rates of oxygen consumption, probably partly as a result of digestive metabolism. After a few days, respiration rate gradually declined and fed levels were achieved. The 'feeding efficiency' (i.e. the gain in body mass per gram of food eaten) during refeeding was 1.4 times higher in *P. anguinus* than in *E. asper*. Hypogean and epigean crustaceans showed similar results (Hervant et al., 1997; Hervant et al., 1999). There will obviously be a selective advantage for an animal in such a harsh environment in using the available food energy optimally.

It is ecologically very important for organisms to recover quickly and completely from nutritional stress when food becomes available. The hypogean *P. anguinus* resynthesized muscle glycogen, proteins and triglycerides very rapidly: 1.2–3 times faster than the epigean *E. asper*. This ability to maintain and rapidly restore high levels of metabolic stores for use during food deficiency (and/or lack of oxygen; Malard and Hervant, 1999) allows subterranean organisms successfully to fuel an ensuing starvation (and/or hypoxic) period and, therefore, increases their competitive ability (Hervant et al., 1999).

In both amphibian species, muscle glycogen content displayed a large but transitory increase during refeeding, its concentration exceeding the control level during the first week of refeeding. This metabolic response may represent an adaptation for the rapid storage of food energy to be later mobilized for the synthesis of body materials such as triglycerides and proteins. The hypogean crustaceans *Niphargus virei* and *N. rhenorhodanensis* show a similar energy strategy (Hervant et al., 1999).

Metabolic responses to prolonged starvation

The features referred to above allow the subterranean amphibian *P. anguinus* to tolerate a reduction in food availability by maximizing the period for which metabolism can be fuelled by a given food ration and/or a given energy reserve. This supports the suggestion (Hoffmann and Parson, 1991) that difficulties in obtaining food in stressful environments may select for conservative energy use.

Subterranean organisms tend to have lower activity levels and metabolic rates than closely related epigean species (for reviews, see Hüppop, 1985; Hervant et al., 1997; Hervant et al., 1998). *P. anguinus* is no exception, with a rate of oxygen consumption approximately half that of *E. asper* and considerably lower (up to one-eighth) than that of most surface-dwelling amphibians (for reviews, see Hutchison, 1971; Licht and Lowcock, 1991; Gatten et al., 1992: values and references therein). In addition, *P. anguinus* showed a considerably lower activity level than most other amphibians studied to date (Gatten et al., 1992; Pough et al., 1992). The presence of reduced metabolic and activity rates in (fed) subterranean species as diverse as crustaceans, fishes (Hüppop, 1985; Hervant et al., 1998) and amphibians (present study; Hervant et al., 2000) implies that this is one of the most important adaptations to subterranean environments (Hervant et al., 1998). The low and discontinuous food supplies and/or alternately hypoxic and normoxic waters encountered by numerous subterranean species, including *P. anguinus* (Istenic, 1971; Uiblein et al., 1992; Malard and Hervant, 1999), are likely to be the most important factors controlling adaptations of metabolic and activity rates in aquatic hypogean organisms (Hervant et al., 1997; Hervant et al., 1998; Hervant et al., 1999; Hervant et al., 2000).

It has been suggested (Hervant et al., 1998; Hervant et al., 2000) that the reduced metabolic rate (i.e. a lower metabolic cost) of hypogean animals (i) improves survival in harsh, stressful, subterranean environments, and (ii) reflects a lower capacity for locomotion due to reduced visual predator–prey interactions.

The metabolic response to prolonged food deprivation was monophasic in *E. asper*, which showed an immediate, linear and large decrease in all its energy reserves. In contrast, starvation developed in three successive phases in *P. anguinus*, with an 'immediate' but lower rate of depletion of glycogen stores (from day 30 to day 150), followed by a rapid utilization of triglycerides (from day 90 to day 240), then finally proteins (from day 180). The subterranean species displayed successive periods of glucidic, lipidic and finally lipido-proteic-dominant catabolism during the course of the 240-day experiment.

If the amounts of glycogen, proteins and lipids utilized are completely oxidized to CO_2 and H_2O, then the energy provided by each metabolite can be calculated (according to Elendt, 1989). Because of the rarity of the species studied, this analysis was unfortunately restricted to blood and muscle, but it is assumed here that these tissues are representative of the energy metabolism of the whole animal. In *P. anguinus*, proteins (56 % of the energy consumed in the muscle during the 240-day starvation period) and lipids (44.4 %) were the most metabolized substrates in terms of total energy, whereas glycogen did not contribute to the energy metabolism (0.6 %). *E. asper* displayed a different energy strategy: proteins (67.2 % of the energy losses during the 90-day starvation period) and total lipids (29.5 %) were the most metabolized stores in the muscle, whereas glycogen reserves seemed not to be preferentially used (3.3 %). During food deprivation, this surface-living species did not appear to use the glyconeogenesis pathway from amino acids and/or glycerol.

In the muscle, the total energy content was reduced by only $17\,J\,g^{-1}$ dry mass day^{-1} in *P. anguinus*, whereas *E. asper* utilized $70\,J\,g^{-1}$ dry mass day^{-1}. Our data are in agreement with previous results (Hervant et al., 1999) for starved epigean ($169\,J\,g^{-1}$ dry mass day^{-1}, for the whole body) and hypogean ($29–38\,J\,g^{-1}$ dry mass day^{-1}) crustaceans. In addition, the relative metabolic rate of the cave-dwelling organism during starvation (i.e. the metabolic rate during starvation divided by that before starvation) was considerably lower than that of the epigean species. In this way, approximately 60 % of the metabolic energy dissipated by the well-fed subterranean species was saved by the starving one, while no energy was saved by *E. asper*. Similar responses have been obtained for epigean and hypogean crustaceans (Hervant et al., 1997; Hervant et al., 1999). Indeed, subterranean organisms can survive long periods of food deprivation at a low energetic cost. This reduction in energy demand (associated with a very low standard metabolic rate) will sustain the metabolic reserves for as long as possible, therefore increasing survival time during starvation.

During food deprivation, *P. anguinus* metabolized muscle glycogen, proteins and triglycerides at relatively low rates, 3–13 times lower than in the epigean *E. asper*. In addition, fed *P. anguinus* had larger muscle glycogen and triglyceride reserves, respectively 29 % and 13 % greater than in fed *E. asper*, making the fuelling of energy metabolism possible for longer during starvation. Both urodele species displayed a significant decrease in body mass during food deprivation, but the subterranean species showed the lower responses to long-term starvation, with a rate of decrease in percentage fresh mass three times slower than that of the surface-dwelling species. For comparison, numerous epigean mammals of a similar size suffer the same rate of mass loss as *E. asper* during starvation (Larsson and Lewander, 1973; Merkle and Hanke, 1988a, and references therein; Hung et al., 1997). Epigean and hypogean crustaceans displayed similar metabolic responses (Hervant et al., 1999). The possession of larger metabolic stores and the lower stored metabolite utilization rates ought to ensure prolonged survival during periods of food deprivation.

No effect of fasting on glucose level was observed in *P. anguinus*, demonstrating that this organism can maintain glucose homeostasis during prolonged nutritional stress, whereas *E. asper* showed substantial hypoglycaemia. Surprisingly, a similar decrease in blood glucose levels as an effect of fasting has also been observed in some starvation-tolerant fishes (Larsson and Lewander, 1973, and references therein; Hung et al., 1997). Gluconeogenesis (from glycerol and/or gluconeogenic amino acids) probably contributed significantly to glucose production in *P. anguinus*, maintaining, in particular, the glucose supply to the nervous system.

Conflicting results exist in the literature concerning the effects of starvation on haematological variables, including plasma glucose level, in aquatic amphibians (Merkle and Hanke, 1988a; Boutilier et al., 1992). In both urodele species, prolonged fasting led to a significant decrease in both haematocrit and haemoglobin content. This suggests a limitation of both erythropoiesis and the production of plasma proteins by the liver. The decrease in both haematological variables was 4–5 times lower in the cave-dwelling than in the surface-dwelling species, demonstrating that oxygen transport ability was better maintained in the hypoxia-tolerant *P. anguinus*, which would ensure prolonged survival during hypoxic periods (e.g. by supplying oxygen to the tissues more effectively during the decrease in oxygen tension).

Adaptive strategy for a hypogean amphibian during starvation

An energy strategy has been proposed (Hervant et al., 1997; Hervant et al., 1999) for a hypogean invertebrate that involves the ability to withstand long-term starvation and the efficient use of consumed food. Resistance to starvation involved a ‘sit-and-wait’ behaviour, a period of depressed locomotion, ventilation and oxygen consumption, during which the subterranean crustaceans subsisted on a high-energy reserve, mainly lipid stores.

The present study confirms the existence of this same strategy in hypogean vertebrates and demonstrates that cave-dwelling urodeles have lower energetic requirements and larger metabolic stores and, therefore, are better adapted to long-term food deprivation than surface-dwelling ones. The remarkable resistance to long-term fasting and the very rapid recovery from nutritional stress of this cave-dwelling organism may be explained partly by its ability to remain in extremely prolonged states of protein sparing and temporary torpor, as observed in some small birds and hibernating mammals (Le Maho, 1981). These adaptive responses might be considered for *P. anguinus* (and probably numerous other subterranean organisms) as an efficient energy-saving strategy in an environment where starvation periods of variable duration alternate with sporadic feeding events. Therefore, *P. anguinus* appears to be a good example of a ‘low-energy’ vertebrate (Hervant et al., 2000).

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