Comparative Biochemistry and Physiology, Part A 155 (2010) 77–83

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Selection of physiological and metabolic adaptations to food deprivation in the Pyrenean newt *Calotriton asper* during cave colonisation

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ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 1 October 2009
Accepted 2 October 2009
Available online 8 October 2009

Keywords:
Amphibian
Blood variables
Cave
Energetic metabolism
Fasting
Hypogean/epigean
Oxygen consumption
Refeeding

ABSTRACT

Food restriction is one of the major and most common constraints that subterranean animals face in their biotope. Cave-dwelling organisms thus have to cope with fasting periods that can extend from a month to a year. However, adaptive fasting resistance previously found in subterranean fauna has only been highlighted by direct comparisons with phylogenetically distant epigean organisms, which could severely impact conclusions. Here we report physiological and metabolic responses to 42 days of fasting followed by 10 days of refeeding in two populations (one subterranean and one epigean) of *Calotriton asper*. In the fed state (control), the hypogean population exhibited a hypometabolism together with higher glycogen (+25% in liver and muscles) and triglyceride stores (+50% in muscles). During the fasting period, cave individuals exhibited a 20% decrease in VO_2 whereas epigean individuals experienced no significant change. In addition, the energetic reserves always remained higher in the hypogean population. According to phylogenic and biogeographic data, cave colonization by this species dates back to less than 10,000 years, suggesting a rapid selection of adaptive traits related to fasting. This study strongly suggests that cave colonization induces a decrease in metabolism together with a higher capacity to accumulate energy reserves and therefore to withstand unpredictable fasting periods.

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1. Introduction

Groundwater biotopes (caves, karstic and porous aquifers) are generally characterised by unpredictable and severe variations in trophic resources. As primary production is absent from these biotopes, food is only available during a few periods along the year, after important rainfalls that drain nutrients across ground layers or an increase in the infiltrating stream flow. Following this allochtonous input, the nutrients are quickly consumed by bacteria and the subterranean fauna (Gibert et al., 1994). Furthermore, dissolved organic matter is particularly heterogeneously dispatched (Malard and Hervant, 1999). Thus, apart from some exceptions e.g. nutrient-rich tropical caves, cave-dwelling organisms have to cope with frequent and long fasting periods that can extend from a month to a year (Hervant et al., 1997a,b). In order to face such extreme constraints, hypogean (i.e. subterranean) organisms have evolved efficient behavioural, physiological and metabolic adaptations which extend their survival during food deprivation. For instance, several hypogean species are able to tolerate long periods without food — nearly 1 year in invertebrates, and up to several years in cave fishes and salamanders (Poulson, 1963; Mathieu, 1982; Hervant et al., 1997a,b, 1999, 2001).

Based on these studies, Hervant and Renault (2002) proposed a general adaptive strategy for groundwater organisms that includes a reduced activity, a lower metabolic rate and higher amounts of fuel stored (glycogen, triglycerides, proteins) compared to epigean species, and lower utilization rates of stored metabolites, making the fuelling of food deprivation possible for a longer time. Moreover, during refeeding, hypogean organisms generally exhibit a faster reconstruction of energy reserves than epigean ones, underlying an opportunistic strategy when food is *de novo* available.

From an evolutionary point of view, such adaptations should have been selected in the epigean ancestor during the colonisation of the subterranean biotope (Notemboom, 1991). Among the different selective pressures peculiar to the hypogean biotope, several authors have hypothesized that food scarcity may be one of the strongest pressures encountered by colonising organisms and thus should favour the selection of fasting adaptations (Malard and Hervant, 1999). Although this assumption seems obvious, it remains relatively difficult to demonstrate in practice due to the relative phylogenetic isolation of subterranean species (Lefebure et al., 2006a,b, 2007). These elements explain why adaptive fasting resistance, like hypoxia or cold resistance exhibited by subterranean fauna, has only been highlighted by direct comparisons with phylogenetically distant epigean organisms (generally from the same order or suborder, Hervant et al., 1997a,b, 1999, 2001; Issartel et al., 2005a,b, 2006, 2009). As a result, the evolutionary conclusions of such comparative studies could

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doi:10.1016/j.cbpa.2009.10.002

be impaired by multiple confounding effects due to phylogenetic distance (for a review see Garland et al., 2005).

Calotriton asper is an endemic urodel from the Pyrenean range (France) that lives in oxygenated streams at altitude ranging from 700 to 2500 m. Surprisingly, *C. asper* specimens were recently discovered deep inside a few Pyrenean caves (Miaud and Guillaume, 2005). It has been demonstrated that these populations realize their entire lifecycle inside caves. Furthermore, primary phylogenetic analyse have demonstrated that these cave-dwelling populations have been strongly isolated (no between-populations gene transfer; Mila et al. submitted article) for approximately 10.000 years (Miaud and Guillaume, 2005). In spite of this cave isolation, no troglomorphic traits (i.e. eye regression, skin depigmentation) have been observed in these populations. This "recent" colonisation thus provides a rare case study that could bring reliable (non biased) evolutionary information regarding selective pressures endured by organisms colonising the subterranean biotope.

Here we thus report experiments designed to elucidate whether a recent colonisation of cave environment induces physiological and metabolic adaptations to fasting in an amphibian, i.e. an increased capacity to depress the metabolism, a lower utilization rate and higher storage of metabolites. We thus compared the fasting responses of cave- and surface-dwelling populations of the salamander *C. asper*. The standard metabolic rate together with the use and reconstruction of energy reserves during a 42-day fasting period, followed by a subsequent 10-day refeeding phase were assessed.

2. Materials and methods

The present investigation was carried out according to the ethical principles of the French (Ministère de l'Agriculture) and European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Council of Europe, no. 123, Strasbourg, 1985) at the Station of Experimental Ecology of Moulis, France (Veterinary Services : No. A09583).

2.1. Animals

Adult individuals of a surface-dwelling population of *C. asper* Duges (6.57 ± 0.21 g, $N = 40$) were sampled in a food abundant stream (mean annual temperature $= 11.4 \pm 2.8$ °C; Guillaume, personal communication), the rau de Cass-Ratz (42.876078°N, 2.315065°E, St-Just-et-le-Bézu, Aude, France). Adult specimens of a cave-dwelling (6.45 ± 0.19 g, $N = 40$) population were sampled deep inside a cave (42.999328°N, 1.534564°E, cave Bernard; St-Martin-de-Caralp, Ariège, France) alimented by a stream (mean annual temperature $= 11.5 \pm 2$ °C; Guillaume, personal communication). Hypogean newts were collected in the subterranean stream in which temperatures range from 9.4 to 13.5 °C along the year (Guillaume, personal communication). Then, newts of both populations were transferred to the Station of Experimental Ecology (Moulis, France) and raised, under semi-natural conditions, in aquaria containing stones and controlled water. Before the experiment, they were fed with chironomid larvae ('blood worms') twice a week during 3 months in order to avoid many environmental factors that could interfere with feeding conditions. The aquaria were kept in the darkness in a controlled-temperature facility (12 °C).

After acclimation to laboratory conditions, individuals were separated into control ($N = 8$) and treatment groups ($N = 8$–9 for each treatment conditions). The control group was fed as described above. The treatment group was deprived of food and individuals were weighted once a week. To investigate responses to food stress, individuals were maintained under fasting conditions and removed at intervals of 21 and 42 days to measure oxygen consumption and to sample blood, muscle and liver.

To investigate responses to recovery from long-term lack of food, individuals were starved for 42 days before subsequent refeeding. Oxygen consumption was assessed at intervals of 1 day and 10 days after refeeding. Blood, liver and muscle were sampled 10 days after refeeding. No deaths occurred during the experiments.

2.2. Measurement of oxygen consumption

There was no difference in body mass among treatment groups or between populations. Hence body mass effect on metabolic rate was considered to be negligible and oxygen consumption rates (VO_2) are expressed as $\mu molO_2/g/h$.

Oxygen consumption was assessed as described in Hervant et al. (2001). For both populations, rates of oxygen consumption were measured under standardized conditions, at the same time of 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 °C and supplied with previously aerated fresh water. Before metabolism measurement, individuals were submitted to 4 days of fasting to ensure that they were postabsorptive. One hour before the experiments began, the salamanders were transferred individually into an 800 mL Plexiglas chamber. A constant low rate of water flow (25 mL min^{-1}) was maintained in 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 an electrode (TriOx EO 200) coupled to an O_2 meter (WTW Oxi 2000).

2.3. Blood, liver, muscle sampling, and metabolic 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 in a 0.5 g L^{-1} tricaine methane sulphonate solution (Sandoz MS-222). Blood (1 mL) was then obtained from the heart using a heparinized syringe. The whole blood was used immediately for the determination of haematocrit and glucose concentrations, as described previously (Hervant et al., 2001). The rest of the blood was centrifuged (3000*g* for 10 min at 4 °C). The

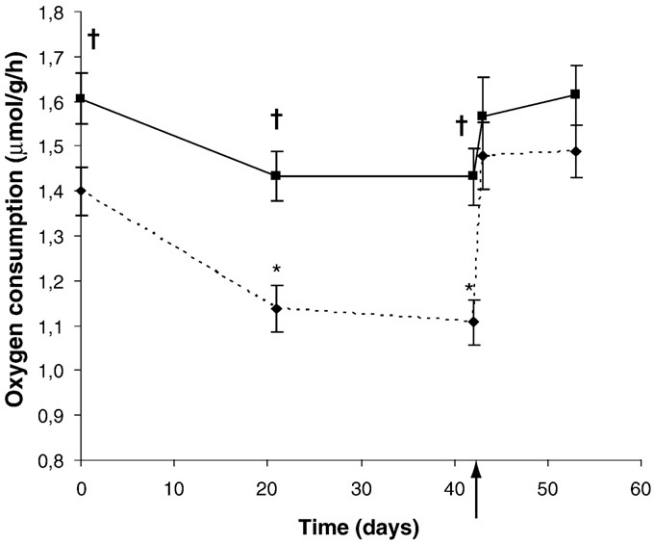

Fig. 1. Measurement of oxygen consumption during starvation (42 days) and subsequent recovery (10 days) in cave- and surface dwelling *Calotriton asper* (dotted and continue lines, respectively). The arrow represents the refeeding day. Values are means ± SEM ($N = 8$). * = significantly different form control ($P < 0.05$). † = significant difference between populations ($P < 0.05$).

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