Long-Term Starvation in Cave Salamander Effects on Liver Ultrastructure and Energy Reserve Mobilization

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ABSTRACT The morphological alterations of hepatocytes of cave-dwelling salamander Proteus anguinus anguinus after food deprivation periods of one and 18 months were investigated and the concentrations of glycogen, lipids, and proteins in the liver were determined. Quantitative analyses of the hepatocyte size, the lipid droplets, the number of mitochondria, and volume densities of M and P in the hepatocytes were completed. After one month of food deprivation, the cytological changes in the hepatocytes are mainly related to the distribution and amount of glycogen, which was dispersed in the cytoplasm and failed to form clumps typical of normal liver tissue. After 18 months of food deprivation hepatocytes were reduced in size, lipid droplets were less numerous, peroxisomes formed clusters with small, spherical mitochondria, and specific mitochondria increased in size and lost cristae. Lysosomes, autophagic vacuoles, and clear vacuoles were numerous. The liver integrity was apparently maintained, no significant loss of cytoplasmic constituents have been observed. Biochemical analysis revealed the utilization of stored metabolic reserves in the liver during food deprivation. Glycogen is rapidly utilized at the beginning of the starvation period, whereas lipids and proteins are utilized subsequently, during prolonged food deprivation. In the Proteus liver carbohydrates are maintained in appreciable amounts and this constitutes a very important energy depot, invaluable in the subterranean environment. J. Morphol. 00:000–000, 2013.

KEY WORDS: liver; morphology; ultrastructure; starvation; energy reserves; cave salamander

INTRODUCTION

The cave-dwelling salamander Proteus anguinus anguinus, like most other cave animals, is subject to poor and discontinuous food supply and/or intermittent hypoxia that may occur for long periods in subterranean environments. It is important, therefore, that the organisms be able to adjust their metabolism and utilization of metabolic reserves. Many vertebrates are subject to a natural starvation period during some part of the year and have, therefore, developed an ability to survive long periods of starvation. Several subterranean species are able to live for long periods without food, nearly one year in invertebrates, and up to several years in cave fishes and salamanders (Vandel, 1965; Hüpopp, 1985). Species of the genus Proteus can survive periods of food deprivation for between 18 and 96 months or more, with no signs of illness (Vandel, 1965; Bulog et al., 2000; cited in Hervant et al., 2001). This remarkable resistance to long-term fasting and also the quick recovery from nutrition stress exhibited by this cave salamander has been explained in terms of its ability to remain in an extremely prolonged state of protein sparing and temporary torpor (Hervant et al., 2001). As a consequence, Proteus has lower energy requirements and larger metabolic stores than surface-dwelling species. An adaptive strategy of Proteus during starvation has been described that involves “sit-and-wait” behavior, a period of depressed locomotion, ventilation and oxygen consumption and subsistence on a high-energy reserve, mainly lipids. In addition, Proteus is the most anoxia-tolerant amphibian and one of the most anoxia-tolerant vertebrates (Issartel et al., 2009).

The large amount of energy reserves in the liver of Proteus (Bulog et al., 2000; Bizjak Mali et al., 2001), its remarkable resistance to long-term fasting, and its low-metabolic rate which is considerably lower than those of the most surface dwelling amphibians (Vandel, 1965; Bulog et al., 2000; Hervant et al., 2000, 2001) triggered our interest to study effect of food deprivation on liver ultrastructure, with special reference to liver energy reserve mobilization. The liver is the central organ for metabolic activity in vertebrates and also an important storage depot (Hadley, 1985) for energy storage. Therefore, we studied morphological and biochemical changes of liver tissue of Proteus...
during food deprivation periods. With a combination of qualitative morphological and quantitative biochemical data, we aim to evaluate the adaptive capability of the *Proteus* liver and the importance of the liver as an energy storing organ.

**MATERIALS AND METHODS**

*Proteus anguinus anguinus* Laurenti, 1768 (Amphibia, Caudata) is a neotenic cave salamander living only in the underground waters of Dinaric Karst region from the Isonzo-Soca River in Italy in the northwest to the river Trebićnjica in Herzegovina in the southeast. Altogether almost 250 locations are known, most in Slovenia (Sket, 1997). It is the only vertebrate in Europe permanently living in the caves.

Adult specimens were collected in the Planina Cave (Plana, Slovenia) with the permission of the Ministry of the Environment and Spatial Planning of the Republic of Slovenia which administers rigorous national conservation laws aimed at protection of *P. anguinus anguinus*. The number of captured animals available for research is strictly limited and for this study, we used six animals with body lengths from 226 to 270 mm. All of them were sexually mature females with different oocytes stages in the ovarium. Vitellogenic stage IV oocytes were present in almost all females; only one female had previtellogenic stage II oocytes as the most mature oocyte stage. The animals were acclimated to laboratory conditions for three months before experimentation. Animals were kept in a speleological laboratory in darkness in experimental tanks containing aerated water at temperature of 10°C and were fed with isopods once a week. After three months, two animals were sacrificed and served as controls. The other four animals were food-deprived for four weeks (N = 2), and 18 months (N = 2).

**Specimen Preparation**

All sampling was accomplished at midmorning. The animals were anaesthetized in 0.3 g/L tricaine methane sulphonate solution (Sandoz MS-222) before being sacrificed. Standard length and body mass were determined. The whole liver was removed in saline (Sandoz MS-222) before being sacrificed. Standard length and body mass were determined using the anthrone reagent (Seifter et al., 1950), total proteins were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The concentrations of glycogen, total lipids, and proteins were expressed as mg/g of wet tissue.

For light microscopy, three pieces of the anterior, median, and posterior part of the liver were fixed overnight by immersion in 10% neutral buffered formalin, dehydrated through graded alcohols, cleared in xylene and embedded in paraplast. Serial sections 5 μm in thickness were used for the estimation of the number of mitochondria per CSA of the hepatocytes and for volume densities estimation of lipid droplets in hepatocytes of fed and food-deprived animals. Three randomly selected liver tissue blocks were used from each animal. From each block, three different semithin sections were made and from each section three fields were selected and photographed at 400× magnification. All selected cells were sectioned mediially and had a visible nucleus. *In toto*, 20 liver cells per animal were analyzed. CSA of the hepatocytes was measured on images of semithin sections using freehand tool of Image J, a public domain Java image processing program, available online at http://rsb.info.nih.gov/ij/.

Images for quantitative estimation of lipid droplets in hepatocytes were processed using AdobePhotoshop 5.5 software. Processing was carried out as follows: at first margins were drawn around the hepatocytes, and then the background was erased with the “eraser” tool. Using the “color transforming” tool, we colored the lipid droplets black and the remainder of the cytoplasm white, whereas the nucleus and background were colored grey.

With these changes, we obtained black and white pictures in which we determined black and white contrasts from a histogram using an Excel programme. The percentage of lipid droplets in the cytoplasm of liver cells was calculated as:

\[
\text{percentage of lipid droplets} = \frac{\text{Surface of lipid droplets}}{\text{Surface of the cell-surface of the nucleus}}
\]

The number of mitochondria per CSA of the hepatocytes and the volume densities (Vv), or fractional volumes, of the mitochondria and peroxisomes in the cytoplasm of the hepatocytes were determined on electron micrographs. For each animal, two ultrathin sections from three different regions (anterior, median, and posterior) of the liver were randomly selected and six electron micrographs were recorded from each section.

The micrographs of the whole hepatocytes were recorded at 2400 magnification and used for counting mitochondria and measuring the CSA of hepatocytes using freehand tool of Image J. The number of mitochondria was expressed per mm² of hepatocyte CSA.

For volume densities estimation of the mitochondria and peroxisomes, the micrographs were recorded at magnification of 7200. Volume densities (Vv) were estimated by placing a lattice with 100 test points (PT) on micrographs using Image J and determining the fraction (Pv/PT) of these points enclosed within profiles of the structure investigated (Pv) (Weibel et al., 1969). The cytoplasm of hepatocytes was selected as reference.
space and test points falling on extracellular space or the hepa-
tocyte nucleus were subtracted from the total number of test
points.

The data were statistically evaluated by Kruskal–Wallis test
and Dunn's multiple comparison test. Values are given as
medians with interquartile ranges.

RESULTS

Morphology of the Hepatocytes of Normal Liver Tissue

The liver parenchyma of Proteus anguinus anguinus consisted of large hepatocytes arranged
in laminae two or more cells thick, separating adja-
cent sinusoids (Fig. 1a). There was no lobulation
of the liver tissue. The hepatocytes were polyhe-
dric with large spherical and euchromatic nuclei,
usually with one prominent nucleoli (Figs. 1a, 2a).
Numerous pigment cells were present among he-
patocytes which were grouped in large clusters
(Fig. 1a). The pigment cells contained mainly hae-
mosiderin, positive to Perl's reaction in histological
sections (Fig. 1b) and a smaller amount of melanin
(Fig. 1c). The ultrastructure and histochemistry of
pigment cells in Proteus liver has already been
described in detail separately (Prelovšek et al.,
2008).

The lipid droplets were major cytoplasmic inclu-
sions (Figs. 1a, d, 2a) and differed greatly in size
and shape. The size of single lipid droplet may
exceed that of the nucleus. Either spherical or
irregular lipid droplets were observed (Fig. 2a–c)
in the cytoplasm of individual hepatocytes in the
same liver tissue. Irregular lipid droplets were
predominant and usually surrounded by the
intense electron-dense deposits that are positive to
periodic acid-Schiff's reaction in semithin sections
(Figs. 1d, 2a, c). Glycogen was also abundant and
randomly distributed in the cytoplasm in mainly
large clusters of alpha glycogen or rosettes (Fig.
2c, d). The PAS histochemical reaction on semithin
sections was strong (Fig. 1d).

Mitochondria were most frequent cytoplasmic
organelles scattered throughout the cytoplasm and
often close to the lipid droplets (Fig. 3a, b). They

Fig. 1. The liver of the Proteus anguinus anguinus. Light micrographs. (a) The laminae of hepatocytes separating adjacent sinu-
soids (S) and large clusters of pigment cells (PC). N, nucleus of the hepatocyte. Semithin section stained with Azur II–methylene
blue. (b) Perl's method for iron identification in the pigment cells. (c) Masson–Fontana stains for melanin in the pigment cells. (d)
Semithin section of the liver stained with histochemical PAS method for glycogen. N, nucleus; S, sinusoid.

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had moderately electron dense matrix with sparse cristae and few electron dense granules. Mitochondria with short and sparse cristae are also common features in amphibians and reptiles (Ferrer et al., 1987; Storch et al., 1989; Osman et al., 1991). A rough endoplasmic reticulum was moderately developed and mainly arranged in parallel stacks around the nucleus, at the lateral border of the cell and close to the bile canaliculi (Fig. 3a–c). The smooth endoplasmic reticulum was vesiculated and closely related to glycogen particles (Fig. 3c). Circular peroxisomes consisting of a single membrane without a crystalline core and with a finely granular matrix were also scattered in the cytoplasm always near the mitochondria or in intimate association with lipid droplets (Fig. 3b, c). Lysosomes were scarce and had variable size and shape (Fig. 3a). The Golgi complex was not very prominent, and consisted of four to five closely arranged cisternae (Fig. 3d). The terminal portion of the cisternal profiles of the Golgi apparatus (GA) were dilated and contained dense granular

Fig. 2. The hepatocyte ultrastructure of the control animals of Proteus anguinus anguinus. (a) The hepatocyte with numerous lipid droplets (L) and glycogen. N, spherical and euchromatic nucleus; n, nucleolus. (b) The large spherical lipid droplet (L) with the coalescent smaller droplets. (c) Irregular lipid droplets (L) with dense fuzzy coat of glycogen (arrow). Gly, glycogen. (d) Glycogen deposit (Gly) consists mainly of α-glycogen particles. M, mitochondrion; SER, smooth endoplasmic reticulum.
Fig. 3. The hepatocyte ultrastructure of the control animals of *Proteus anguinus anguinus*. (a) A part of the apical cytoplasm facing the bile canaliculus (bc). Arrow, the junctional complex; GA, Golgi apparatus; Gly, glycogen; Ly, lysosome; M, mitochondrion; P, peroxisome. (b) Supranuclear part of the cytoplasm with mitochondria (M) in close apposition to the lipid droplet (L) and rough endoplasmic reticulum (GER) around the nucleus (N). P, peroxisome. (c) Lateral part of the cytoplasm. GER, rough endoplasmic reticulum; Gly, glycogen; L, lipid droplet; M, mitochondrion; P, peroxisome; SER, smooth endoplasmic reticulum. (d) The Golgi complex (GA) at the higher magnification. M, mitochondrion. (e) The junctional complex with tight junction (arrow) and numerous desmosomes (arrowhead). bc, bile canaliculus.
material. The GA typically lied in apical cell regions, often near bile canaliculi, and some cells had two or more. Bile canaliculi were surrounded by 2–4 hepatocytes and had short, stout microvilli (Fig. 3a). The lumina of biliary canaliculi were sealed with junctional complexes and included a tight junction (zonulae occludentes) and numerous desmosomes (Fig. 3e). In all cells, a perinuclear cytoplasmic layer was present, practically free of particulate material such as vesicles or glycogen, and had a homogeneously granular appearance of moderate density.

**Morphology of the Hepatocytes after Food Deprivation**

The hepatocyte ultrastructure after the first month of food deprivation was almost the same as

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that in control animals (Fig. 4a). The most striking differences were in distribution, appearance, and amount of glycogen. The large alpha rosette clusters typical for normal liver tissue had disappeared and only small β-glycogen particles were scattered throughout the cytoplasm (Fig. 4c). Glycogen particles were condensed primarily around lipid droplets (Fig. 4a, c). The PAS histochemical reaction on semithin section remained positive, but less intense than in control animals. Numerous lipid droplets filled the cytoplasm (Fig. 4a) and were in an intimate spatial relationship with the mitochondria and peroxisomes (Fig. 4b, c), as also with the heavy electron dense cisternae of the rough endoplasmic reticulum (Fig. 4b). The smooth endoplasmic reticulum was abundant. The Golgi complex (GA) was prominent and synthetic very active (Fig. 4d). The cisternae on the forming face of GA, the terminal part of the GA and vesicles were heavily stained (Fig. 4d). The ultrastructural arrangement of the hepatocytes after one month of food deprivation indicated metabolically very active cells.

After 18 months of food deprivation further ultrastructural changes were found in the hepatocytes. At this point, glycogen was mostly in the monoparticulate form and tends again to form clusters (Fig. 5a, d). The PAS positive reaction was also intense (Fig. 5b). Lipid droplets were still present, but they were less numerous (Fig. 5a) and numerous peroxisomes formed clusters with small and round mitochondria (Fig. 5a, c). Two or three mitochondria in each cell increased in size and lost cristae (Fig. 5a, d). Lysosomes, autophagic vacuoles with different content and clear vacuoles were also numerous (Fig. 6a–c). The intercellular space was expanded and included electron dense granular material (Fig. 7a). The concentration of electron dense particles within intercellular space could be prominent and visible also on light micrograph of semithin section (Fig. 7b). Numerous membrane vesicles were present along the lateral and basal plasma membrane. No degenerative cells were evident, and only traces of cellular debris were found in the bile canaliculi and sinusoids.

The Liver–Somatic Index

In comparison with control animals, liver weight reduction was observed to have occurred during food deprivation (Table 1). After one month starvation, the liver somatic index decreased by 27–50% of the control level and after 18 months starvation by 18–25% of the control.

Biochemical Analyses

The results of biochemical analyses are summarized in Table 1. After the first month of food deprivation the glycogen concentration in the liver significantly decreased from 61.5 and 66.9 mg/g wet tissue in control animals to 21.5 and 23.9 mg/g wet tissue. After 18 months of food deprivation, the glycogen concentration was 32.5 and 37.3 mg/g wet tissue, slightly higher than in the one-month-starved animals.

Lipids and proteins were utilized later during prolonged starvation. The total lipid concentration was clearly lower after 18 months of food deprivation (171.7 and 199.7 mg/g wet tissue) than in one-month-starved animals (259.2 and 267.2 mg/g wet tissue), and control animals (258.2 and 260.4 mg/g wet tissue).

Similarly, the protein concentration in one-month-starved animals (94.3 and 103.0 mg/g wet tissue) was almost the same as in controls (93.9 and 120.2 mg/g wet tissue), showing a decrease only after 18 months of food deprivation (69.7 mg/g wet tissue).

Quantitative analysis

For the estimation of the size reduction of the hepatocytes during starvation, CSAs of the hepatocytes on the semithin sections were compared. The size of the hepatocytes was reduced during starvation (Fig. 8). The CSA of the cells in 18-month-starved animals was significantly lower ($P < 0.0001$) in comparison to one-month-starved individuals and to controls. No significant differences were found between hepatocytes of control and one-month-starved animals.

Quantitative analysis of the lipid droplets in hepatocytes confirmed the utilization of lipids during prolonged food deprivation. In animals starved for 18 months, the amount of lipid droplets in the cells was markedly diminished ($P < 0.0001$) in comparison to controls and to one-month-starved individuals (Fig. 9). The proportion of lipid droplets in hepatocytes of control animals was between 10 and 60%. The median value was 40%—almost the same as in one-month-starved animals (44%), and significantly higher than in 18-month-starved ones (12%). Lipid droplets in hepatocytes after animals were starved for 18 months decreased significantly to between 0 and 30%, and most of the cells had 10% of lipid droplets. No sampled cells had more than 30% of lipid droplets.

The morphometric results of the mitochondrial number per cross sectional area of the hepatocytes and the volume densities of mitochondria, as well as peroxisomes confirmed the abundance of these structures in hepatocytes of 18-month-starved animals observed by TEM (Figs. 10, 11a, b). The mitochondrial number per cross sectional area and volume density were significantly increased in 18-month-starved animals ($P < 0.0001$ for mitochondrial number and $P < 0.005$ for mitochondrial volume density). For the volume density of the peroxisomes, the significant difference was only found between hepatocytes of one-month-starved and 18-month-starved animals ($P < 0.01$).
DISCUSSION

The liver parenchymal organization of *Proteus anguinus anguinus* resembles that described in other amphibian species and the hepatocytes show almost the same fine structural organization in other amphibians (Spornitz, 1975; Haar and Hightower, 1976; Miscalencu et al., 1978; Osman et al., 1991). The liver is constituted of plates, two or more cells thick, separated by sinusoids. No hepatic lobulation is observed. The main ultrastructural characteristics of the hepatocytes of *P. anguinus anguinus* are numerous lipid inclusions and also abundant glycogen particles, many small mitochondria, a relatively well-developed rough endoplasmic reticulum, abundance of vesicles of smooth endoplasmic reticulum, few lysosomes, and relatively common peroxisomes, which are not very prominent. The nucleus with one or two

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nucleoli is spherical and euchromatic. However, the liver of *Proteus* also shows some conspicuous features. It contains numerous pigment cells that have been described in detail by Prelovšek et al. (2008), and abundant amounts of lipid droplets with an intense fuzzy coat as well as abundant glycogen deposits. The hepatocytes in vertebrates store glycogen and lipids, and the ratio between them can differ greatly among different groups of animals. The liver of certain teleost fish contains lipid-rich hepatocytes; in other species, glycogen predominates (Welsch and Storch, 1973; Chapman, 1981; Robertson and Bradley, 1992). Large amounts of glycogen in hepatocytes is a typical amphibian characteristic (Spornitz, 1975; Hadley, 1985; Fenoglio et al., 1992) and some species, especially urodele amphibians, also have larger amount of lipids. Such species include the newt *Ambystoma mexicanum* (Godula, 1970), *Notophthalmus viridescens* (Haar and Hightower, 1976), and Japanese newt *Cynops*

*Fig. 6. The hepatocyte ultrastructure of the 18-months food-deprived animals of Proteus anguinus anguinus.* (a): Lysosomes (Ly) in the apical part of the cytoplasm facing the bile canaliculus (bc). Gly; glycogen; M, mitochondrion; VA, clear vacuole. (b) Autophagic vacuoles (arrows) with glycogen (Gly), rough endoplasmic reticulum (GER) and lipid (l). L, lipid droplet; M, mitochondrion. (c) Clear vacuoles (VA). M, mitochondrion.
pyrrhogaster (Osman et al., 1991). In general, the amphibian’s liver plays a more important lipid storage role than in other vertebrates, on account of the poorly developed adipose tissue (Hadley, 1985). Lipids are an important energy rich storage material in the hepatocytes of all ectothermic animals, including fishes, amphibians, and reptilian sauropsids (Welsch and Storch, 1973; Storch et al., 1989; Osman et al., 1991). Hepatic fat is important in vitellogenesis (Peute et al., 1978); it serves as energy reserve during hibernation and in many vertebrates during starvation (Yamamoto, 1964; Hightower and Haar, 1978; Storch et al., 1989) and it is an ideal storage material because it liberates twice as much energy as carbohydrates (Allen, 1976).

During hibernation and starvation, ultrastructural changes appear in hepatocytes of vertebrates, which could be minor or could lead to complete degradation of the cells (Cardell, 1971; Duveau and Piery, 1973; Barbeau and Cardell, 1975; Hightower and Haar, 1978). Some authors (Pfeifer, 1973, Segner and Braunbeck, 1988) have reported that most hepatocytes atrophy during hibernation and starvation as a consequence of reduced synthetic activity, and degradation of the cell content, including glycogen, is evident. The present study shows that during starvation, hepatocytes of liver in Proteus undergo size reduction and ultrastructural changes which are mostly related to synthesis, storage, and release of reserve material. However, our results revealed that no severe changes or degenerative cells are observed, and P. anguinus anguinus is evidently able to maintain liver integrity during prolonged starvation.

The main cytological changes in hepatocytes of P. anguinus anguinus after short-term starvation

### Table 1. Liver weight, liver–somatic index (JSI) and concentrations of glycogen, lipids and proteins in mg/g wet tissue of the liver of Proteus anguinus anguinus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Animal length (mm)</th>
<th>Animal weight (g)</th>
<th>Liver weight (g)</th>
<th>JSI</th>
<th>Glycogen (mg/g)</th>
<th>Lipids (mg/g)</th>
<th>Proteins (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>262</td>
<td>22.0</td>
<td>1.35</td>
<td>6.1</td>
<td>66.9</td>
<td>260.4</td>
<td>93.9</td>
</tr>
<tr>
<td>C2</td>
<td>268</td>
<td>23.5</td>
<td>1.42</td>
<td>6.0</td>
<td>61.5</td>
<td>258.2</td>
<td>120.2</td>
</tr>
<tr>
<td>S1m_1</td>
<td>260</td>
<td>18.8</td>
<td>0.75</td>
<td>4.5</td>
<td>37.3</td>
<td>199.7</td>
<td>69.7</td>
</tr>
<tr>
<td>S1m_2</td>
<td>226</td>
<td>17.0</td>
<td>0.75</td>
<td>4.4</td>
<td>21.5</td>
<td>259.2</td>
<td>103.0</td>
</tr>
<tr>
<td>S18m_1</td>
<td>250</td>
<td>11.4</td>
<td>0.56</td>
<td>4.9</td>
<td>32.5</td>
<td>171.7</td>
<td>/</td>
</tr>
<tr>
<td>S18m_2</td>
<td></td>
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C, control animals \((N = 2)\); S1m, one-month-starved animals \((N = 2)\); S18m, 18-month-starved animals \((N = 2)\).
are related to the distribution, appearance, and amount of glycogen. The glycogen was more dispersed in the hepatocytes and did not form clumps typical of normal liver tissue. Our morphological and biochemical data show clearly that stored glycogen is greatly depleted after the first month of food deprivation, whereas the lipid content remains unchanged. A marked decrease in liver glycogen has been reported in eel *Anguilla anguila* between 95 and 145 days of starvation (Larsson and Lewander, 1973), in the newt *Notophthalmus viridescens* starved 2–16 weeks (Hightower and Haar, 1978), in frog *Rana papiens* starved 59 days (Baic et al., 1979) and in the frog *Rana esculenta* after hibernation (Fenoglio et al., 1992). Duveau and Piery (1973) found glycogen only in isolated areas of frog livers after eight months of starvation. In contrast, no significant variability in glycogen content in hepatocytes was found in *Xenopus laevis* starved for between 1 and 5 months (Sporntz, 1975). Reorganization of glycogen from α- to β-particles was observed in *P. anguinus anguinus* hepatocytes after starvation. This reorganization of glycogen has also been described in the liver of amphibians after hibernation and after starvation (Spiegel and Spiegel, 1970; Baic et al., 1979, Fenoglio et al., 1992; Barni et al., 1999), in fasted teleosts (Segner and Braunbeck, 1990), and also in fasted mammals (Cardell, 1971). According to Baic et al. (1979), reorganization from the α- to the β-configuration is consistent with a different molecular organization and thus a different stability. The arrangement of other cellular organelles in *Proteus* hepatocytes after first month of food deprivation, especially rough endoplasmic reticulum with

![Fig. 8. The cross-sectional area (CSA) of the hepatocytes in μm² of control and food-deprived animals of *Proteus anguinus anguinus*. *P* < 0.0001 for hepatocytes of S18m animals vs. control and S1m, respectively. S1m, one-month-starved animals; S18m, 18-month-starved animals.](image1)

![Fig. 9. The proportion of lipid droplets in hepatocytes of control and food-deprived animals of *Proteus anguinus anguinus*. *P* < 0.0001 for hepatocytes of S18m animals vs. control and S1m, respectively. S1m, one-month-starved animals; S18m, 18-month-starved animals.](image2)

![Fig. 10. Mitochondrial number per cross sectional area (mm⁻²) of the hepatocytes of control and food-deprived animals of *Proteus anguinus anguinus*. *P* < 0.0001 for mitochondrial number of S18m animals vs. controls. M, mitochondria; S1m, one-month-starved animals; S18m, 18-month-starved animals.](image3)

![Fig. 11. Mitochondrial (a) and peroxisomal (b) volume density in cytoplasm of control and food-deprived animals of *Proteus anguinus anguinus*. *P* < 0.0001 for mitochondrial volume density of S18m animals vs. control. *P* < 0.01 for peroxisomal volume density of 18m vs. S1m. M, mitochondria; P, peroxisomes; S1m, one-month-starved animals; S18m, 18-month-starved animals.](image4)
heavy electron dense cisternae close to the lipid droplet as well as GA with heavy staining cisternae on the forming face, suggests the presence of metabolically active cells.

After prolonged starvation, hepatocytes size reduced in size, as also more evident morphological changes in hepatocytes of *Proteus* were observed. These changes are mainly concerned with the number, distribution, and shape of the cellular organelles, especially the mitochondria and peroxisomes, and also with the distribution and amount of the lipid droplets and glycogen. The most obvious changes in the majority of the hepatocytes in *Proteus* liver are the numerous peroxisomes which tend to form clusters with numerous small and round mitochondria with sparse cristae. The number of mitochondria per cross sectional area of the hepatocytes, as also the volume density of the mitochondria is significantly higher in 18-month-starved animals than in controls and one-month-starved animals and confirmed the results observed by TEM. For peroxisomes, the only significant difference was observed between one-month-starved and 18-month-starved animals. An increased number of peroxisomes arranged in clusters and in close associations with mitochondria and lipid droplets were reported for hepatocytes of lizard during starvation and also at low temperatures (de Brito-Gitirana and Storch, 1998, 2002) and in teleosts and mammals after exposure to cold (Horowitz, 1976; Braunbeck et al., 1987). Peroxisomes are highly versatile organelles; they contain enzymes that are essential to various catalytic and anabolic pathways (van den Bosch et al., 1992), and they have a role in cholesterol metabolism, oxidation of fatty acids, and in glyconeogenesis (Ghadially, 1997). It is well known that peroxisomes are abundant in tissue active in lipid metabolism (van den Bosch et al., 1992; Ghadially, 1997) and proliferation of peroxisomes and clustering with numerous small mitochondria observed in *Proteus* hepatocytes could be related to increased β-oxidation of fatty acids during starvation because the lipid droplets, which were numerous in controls and one-month-starved animals, were diminished after 18 months of fasting. The biochemical analysis of lipids as well as the quantitative analysis of lipid droplets in semithin sections confirmed utilization of lipid reserves in *Proteus*’s liver. The total lipid concentration in the liver decreased by 25% from the control value, also a proportion of lipid droplets in hepatocytes decreased almost fourfold relative to the control. Most of the hepatocytes contained between 0 and 10% of lipid droplets in the cytoplasm.

Enlarged mitochondria with almost no cristae were also evident in *Proteus* hepatocytes after 18 months of food deprivation, as were numerous electron dense bodies, autophagic vacuoles, and clear vesicles. Enlarged mitochondria or giant mitochondria with altered internal structures have been observed in liver and a variety of tissue of mammals under diverse situations, including protein, vitamin and related nutrition deficiencies, hormonal imbalances, toxic factors, and carcinomas (Ghadially, 1997). The phase of enlargement was said to be followed by a phase of degeneration and disintegration of internal architecture and autolysosome formation. In the present case, giant mitochondria could signify a phase prior to disintegration of organelles and clear vesicles a phase after lysis. Altered mitochondria transformed into electron dense masses or clear vesicles have been described for *Salamandra salamandra* liver following hibernation (Miscalencu et al., 1978). Increases in size of mitochondria until the largest were lysed have been described in the newt *Notophthalmus viridescens* during starvation (Hightower and Haar, 1978). In mammalian liver, no changes in the mitochondrial structure were observed after starvation, only clustering of the mitochondria and changes into round shapes was reported (Cardell, 1971). An increased number of autophagic vacuoles in the hepatocytes of food-deprived specimens of *Proteus* is expected and the autophagy in the liver is important for the balance of energy and nutrients for basic cell functions, and the turnover of major subcellular organelles under both normal and pathophysiological conditions (Yin et al. 2008).

Another visible characteristic in *Proteus* hepatocytes after prolonged starvation is the presence of dilated intercellular spaces with electron dense granular material and membrane vesicles with the same material along the lateral and basal plasma membrane indicating intense intercellular transport and transport towards or from the sinusoid. A similar arrangement has been reported in hepatocytes in starved fishes (Yamamoto, 1964) and amphibians during starvation (Miscalencu et al., 1978). The dilation of the intercellular space could also be induced by the reduction of the size of the hepatocytes. Further studies to clarify the significance and functional role of granular material inside the intercellular spaces and transports will be necessary. Nevertheless that our specimens were females with vitellogenic stage IV oocytes in the ovaries cannot be overlooked and this could be due to the process of vitellogenesis. The presence of vitellogenic stage oocytes in the ovaries of starved animals indicates that oocytic maturation is not affected by starvation.

During starvation animals meet their energy demand by depleting tissues and stored reserve substances. The importance of metabolic reserves and their order of utilization varies within species. Our biochemical results show that the liver glycogen was rapidly utilized at the beginning of food deprivation, whereas the lipid and proteins were utilized later, during prolonged starvation. After 18 months of starvation, the liver glycogen was decreased by...
50%, proteins by 35%, and lipids by 25%. Also, the liver weight reduction was mainly a consequence of metabolized glycogen and loss of water. The liver somatic index decreased by 27–50% of the control level after one-month starvation and by 18–25% of the control after 18-month starvation. These results suggest the same metabolic response to prolonged food deprivation as has been described in *P. anguinus anguinus* by Hervant et al. (2001). They displayed successive periods of glucidic, lipidic, and finally lipid-proteic-dominant catabolism in tail muscles during the course of a 240-day food deprivation.

While in many other vertebrates, fasting causes depletion of liver carbohydrates, these reserves are maintained in quite large amounts in the liver of *P. anguinus anguinus*. Glycogen was decreased by approximately 50% after 18 months of starvation and this decrease is significantly lower than in amphibians which are known to be tolerant to long-term fasting under laboratory conditions, for example, *Rana esculenta*, which is known to tolerate fasting for more than 18 months at 18–20°C and in which the liver glycogen concentration decreased by 95% (Grably and Piery, 1981), and in *Xenopus laevis* which is able to withstand 12 months of starvation in good condition at 20°C in aerated water, although its glycogen levels were reduced by 80–90% (Merkle and Hanke, 1988a, b).

This liver glycogen level preservation during starvation in *P. anguinus anguinus* could be a very important energy depot to satisfy the energy needs during hypoxic periods characteristic of subterranean habitats (Istenicˇ, 1979, 1986; Bulog et al., 2002). Namely, glycogen in the liver could also ensure energy in anoxic condition, whereas catabolism of lipids requires oxygen. The glycogen pool in liver could contribute to the high anoxia tolerance of *P. anguinus anguinus* described by Issartel et al. (2009), and also to the glucose homeostasis during prolonged nutritional stress as well as to the rapid recovery after starvation that has been described by Hervant et al. (2001).

In conclusion, our research reveals morphological and functional flexibility of hepatocytes during food deprivation as well as the important role of the liver as depot organ for energy-rich reserves that are used to meet metabolic needs during food deprivation. Remarkable accumulation of lipid and glycogen deposits in the liver, and glycogen level maintenance during food deprivation, contribute to the superior adaptation of *P. anguinus anguinus* to stress situations in subterranean environments, such as sporadic and discontinuous food supplies and/or hypoxia.

**LITERATURE CITED**


