Isoforms of enzymes of intermediated metabolism during the hibernation of the water frog *Rana ridibunda*

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Low temperatures and the lack of food during the winter lead the water frog to hibernate in order to survive. In this situation animals reduce their metabolic rate and use the stored glycogen and fat to produce energy. Metabolic depression occurs either by changes in the properties of mitochondria, or by changes in the concentration or efficiency of the enzymes, or in the concentration of substrates. The activation of anaerobic mechanism is an adaptation ensuring the continuous energy fuelling during hibernation. In the present work the various isoforms of enzymes of intermediated metabolism were studied during the hibernation of the water frog *Rana ridibunda*. The electrophoretic patterns of the enzymes a-GPD, MDH and LDH in heart and in skeletal (gastrocnemius) muscle were studied. According to our results a tissue-specification for the enzymes LDH and a-GPD was detected, probably due to the type of metabolic pathway of the tissues. The major pathway in heart is the aerobic metabolism whereas in skeletal muscle the anaerobic. For MDH, the electrophoretic pattern in heart showed only one isoform. The electrophoretic pattern was the same for all three enzymes in all the phases of hibernation. Therefore, we believe that the production of different isozymes in frog tissues is associated with the functional nature of particular tissues.

Key words: *Rana ridibunda*, hibernation, glycolytic enzymes, isoforms LDH, MDH, aGPD.

INTRODUCTION

Amphibians are ectotherms and for that reason almost every aspect of their physiology and behavior is affected by temperature. In temperate zones they face problems related to low ambient temperatures during winter. In order to survive they show a variety of both cellular and behavioral adaptations to low temperatures, including hibernation and alterations of varying degrees in metabolic rate and activity (Duellman & Trueb, 1994; Wells, 2007). With the decrease or increase of temperature this effect is modi-

fied by various mechanisms that can be adaptive, thus extending the range of thermal resistance of species. During long-term adaptation to low temperature, these mechanisms include regulation of activity of enzymes through changes in the a) concentration or effectiveness of enzymes, b) concentration of particular substrate, c) energy levels required for the accomplishment of the catalyzed reaction, and d) intracellular environment (Hochachka & Somero, 2002). In short term, the efficiency of enzymes is largely influenced by alterations in the intracellular environment in which they act. Changes in the concentration of ions (and particularly K^+) as in the pH, alter the enzyme activity. Responding to temperature decrease, pH increases (becomes more alkaline) and leads

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to significant stabilisation of the protein structure, resulting in reduced temperature-dependence of the enzymes (Storey, 1997).

Long term acclimation or acclimatization to low temperature affects the concentration of enzymes and more specifically influences the synthesis and the structure of proteins (Somero, 2004). This concerns usually certain enzymes controlling reactions of complicated activity or limiting the metabolic rate. Still, because most enzymes exist as a "variety" of isozymes with different mobilities, a possible solution for maintaining the catalytic properties of enzymes is to change the present "set" of isozymes. In frogs, and also in fishes, low temperatures favour the synthesis of isozymes LDH-B (typically existing in heart) in skeletal muscles and thus the oxidation of pyruvate at the expense of its reduction to lactate by the more normal type of LDH-A of skeletal muscle (Hochachka & Somero, 2002).

Isozymes are enzyme variants that catalyze the same reaction but are structurally different and are coded by separate gene loci. Isozymes can vary greatly in their properties to support the different functional needs for a particular reaction in different organs or different compartments of the cell. They can be found in different tissues of the same organism, e.g. the two forms of alkaline phosphatase in liver and placenta, as well as in different parts of a cell, as the malate dehydrogenase in mitochondria and soluble cytoplasm.

Many studies have been devoted to hibernation of frogs and to their cellular responses (Storey & Storey, 1986, 2004; Tattersall & Ultsch, 2008). Metabolic responses during hibernation have been studied on European frogs as well, mainly to the common frog (*Rana temporaria*) (Pasanen & Koskela, 1974; Pasanen & Sorjonen, 1994), the water frogs *Rana ridibunda*, *Rana lessonae* and their hybridogenetic species *Rana esculenta* (Holenweg & Reyer, 2000; Voituron *et al*., 2003, 2005). The above ranid species are widely distributed in Europe (Gasc *et al*., 2004) and particularly *R. temporaria* occurs above the Arctic Circle. Consequently, the duration of the hibernating season may fluctuate from three to nine months depending on latitude and altitude.

In Greece adult marsh frogs of *R. ridibunda* (recently named as *Pelophylax ridibundus*) usually enter hibernation in the second half of November when the temperature in ponds drops below 9°C and the hibernating period lasts until the middle or end of February, depending on the weather conditions, while subadults hibernate later and may be active on sunny

and warm days of December or February (Kyriakopoulou-Sklavounou, 1983). Hibernation of *R. ridibunda* in Greece is typically aquatic and hibernation sites include mud at the bottom of the ponds or holes in river banks (Kyriakopoulou-Sklavounou & Kattoulas, 1990; Loumbourdis & Kyriakopoulou-Sklavounou, 1996). At a previous paper we studied the glycolytic adjustments in tissues of *R. ridibunda* during hibernation (Michaelidis *et al*., 2008). At this period animals decrease their metabolic rate to survive under unfavourable conditions. It has been suggested that differential expression of protein isoforms provides another important mechanism for regulating enzyme function following temperature acclimation or hibernation (Hochachka & Somero, 2002). This study focus on the various isoforms of certain enzymes of intermediated metabolism in the Greek populations of the frog *R. ridibunda* during hibernation in order to assess the adaptations of this species to these unfavourable conditions and provide further data for an integrative study in global level.

MATERIALS AND METHODS

Animals and experimental design

Adults of *R. ridibunda* used in this study were collected from Lake Vistonis (41°25′ N, 25°77′ E, Thrace, NE Greece) in October 2006. They were transported to the laboratory within the same day and were put into plastic tanks measuring 50 cm height, 80 cm width and 1.50 m length. The plastic tanks contained soil and water, in order to simulate the ponds where the frogs were collected from and were placed outdoors so that frogs were exposed to natural conditions of light and temperature. Covering of the tanks protected them from floating by rain. Frogs were fed with mealworms (*Tenebrio molitor*) every day until the first days of December when temperature dropped below 10°C. Feeding was stopped as frogs started to be less active and remained longer in the water. At different periods starting from the first day of November (2006) until the middle of March (2007) heart tissue and skeletal (gastrocnemius) muscle were dissected from four randomly selected individuals, freeze-clamped between aluminum tongs cooled in liquid nitrogen and ground under liquid nitrogen. Tissues were stored at –25°C.

Electrophoresis

Tissues samples of 0.2-0.3 g were homogenized in a double quantity of homogenization solution (50 mM

imidazole, 10 mM EDTA, 10 mM EGTA, 100 mM Naf and 30 mM mercaptoethanol). Homogenates were centrifuged in Eppendorf tubes for 5 min at 7000 g. The supernatant was transferred immediately to an eppendorf tube and stored at –25˚C. Horizontal starch electrophoresis was carried out in 10% starch gels. Two different buffer systems were used; (i) Tris-citrate buffer pH = 8.2 (Ridgway *et al*., 1970) for the enzymes lactate dehydrogenase (LDH E.C. 1.1.1.27) and a-glycerophosphate dehydrogenase (aGPD E.C. 1.1.1.8) and (ii) N-(3-aminopropyl) morpholine-citrate buffer pH = 6.1 (Clayton & Tretiak, 1972) for the enzyme malate dehydrogenase (MDH E.C.1.1.1.37). Staining techniques were from Allendorf *et al*. (1977) for the enzyme MDH and from Pasteur *et al*. (1987) for the enzymes LDH and aGPD. Calculation of the frequency of an allele was done by applying the equation: $f = 2Ho + He / 2N$, where *Ho* is the number of homozygotes, *He* the number of heterozygotes, and *N* the sample size. Diagrams of electrophoretic patterns were done in Photoshop program.

RESULTS

The nomenclature of isozymes which have been adopted is illustrated in Figures 1-5. The isozymes of a given system are denoted in terms of the order of their anodal mobility. The results for the five allozymic loci are summarized in Table 1.

Lactate dehydrogenase

The electrophoretic patterns of LDH isozymes in heart and gastrocnemius muscle in relation to temperature

TABLE 1. Loci, alleles and allele frequencies found in the examined samples of *Rana ridibunda*

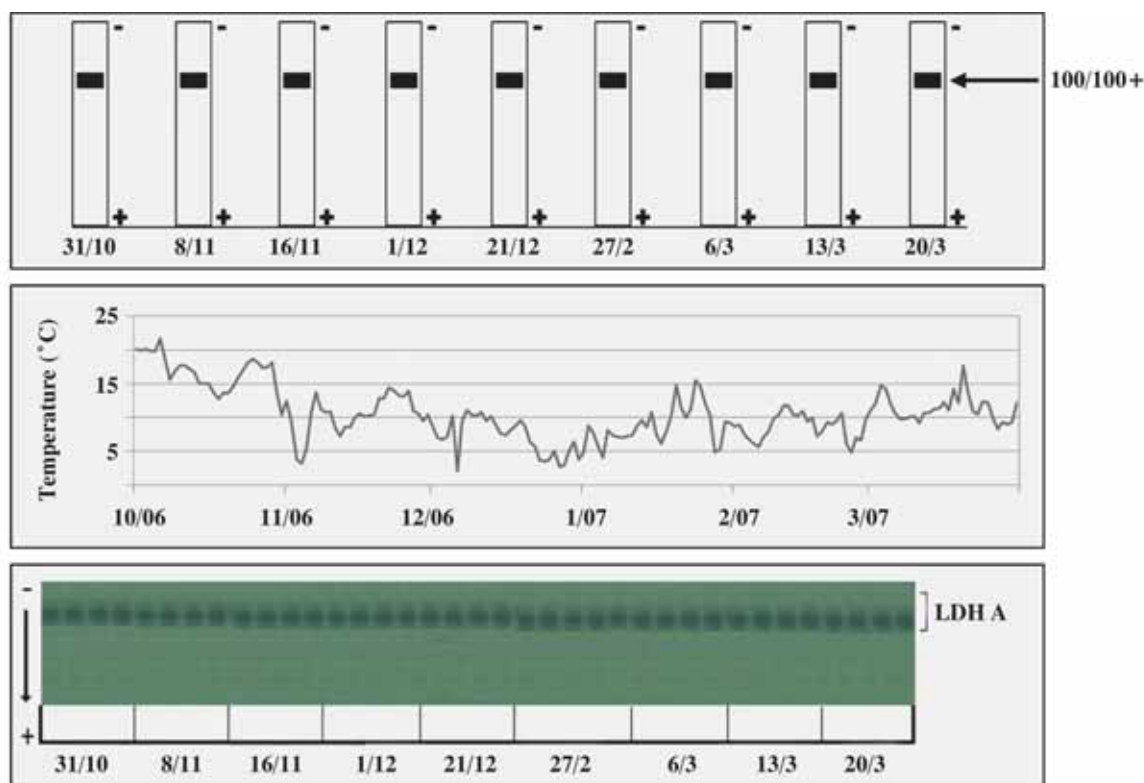| Loci	Alleles	Allele frequencies
LDH-A	100	1.00
LDH-B	100	0.95
	80	0.05
a-GPD-A	100	1.00
a-GPD-B	100	1.00
MDH-A	100	1.00

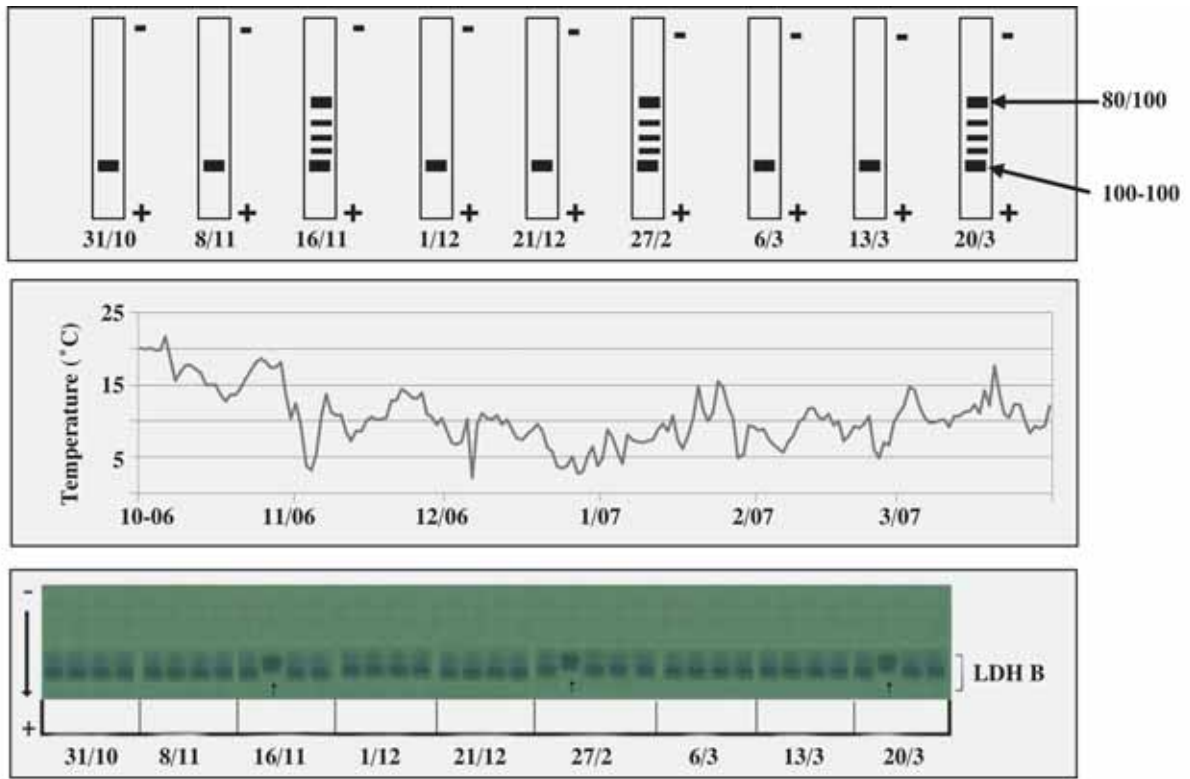FIG. 1. Electrophoretic patterns of LDH-A in gastrocnemius muscle related to seasonal temperature.

FIG. 2. Electrophoretic patterns of LDH-B in heart related to seasonal temperature.
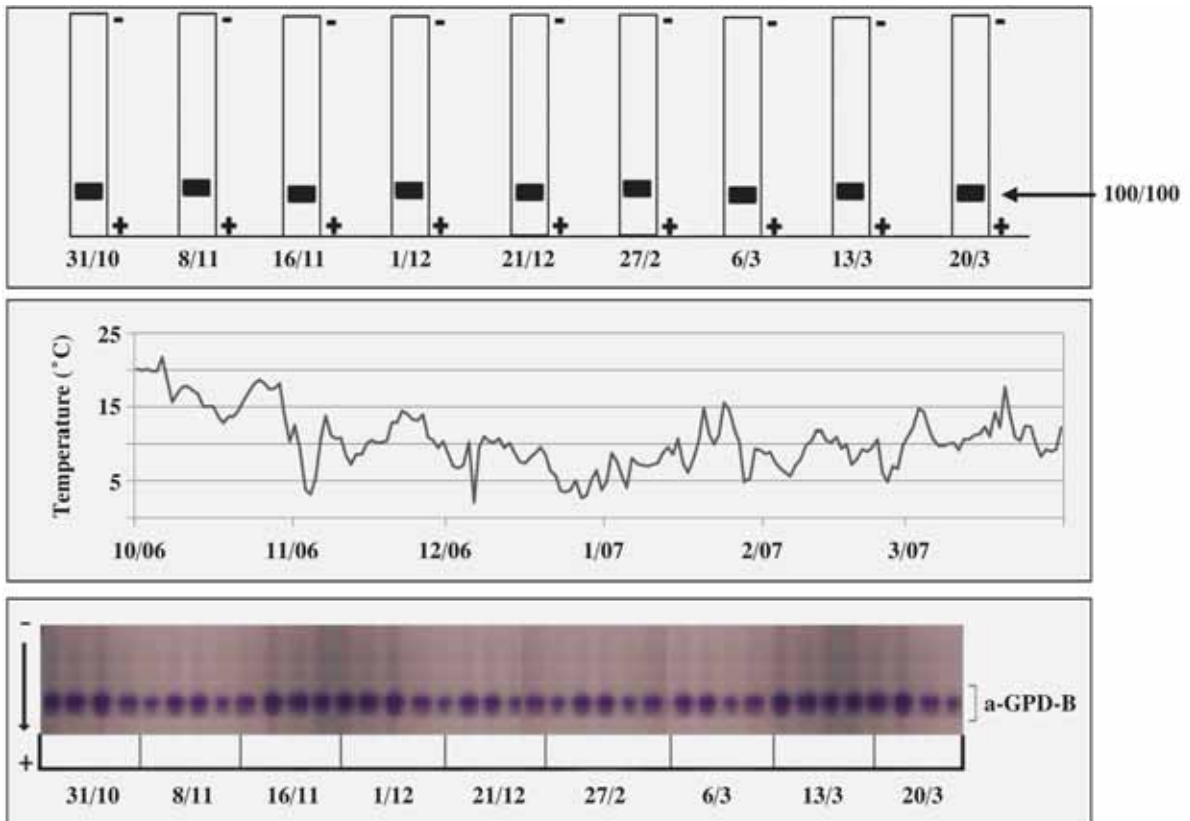

FIG. 3. Electrophoretic patterns of aGPD-B in heart related to seasonal temperature.
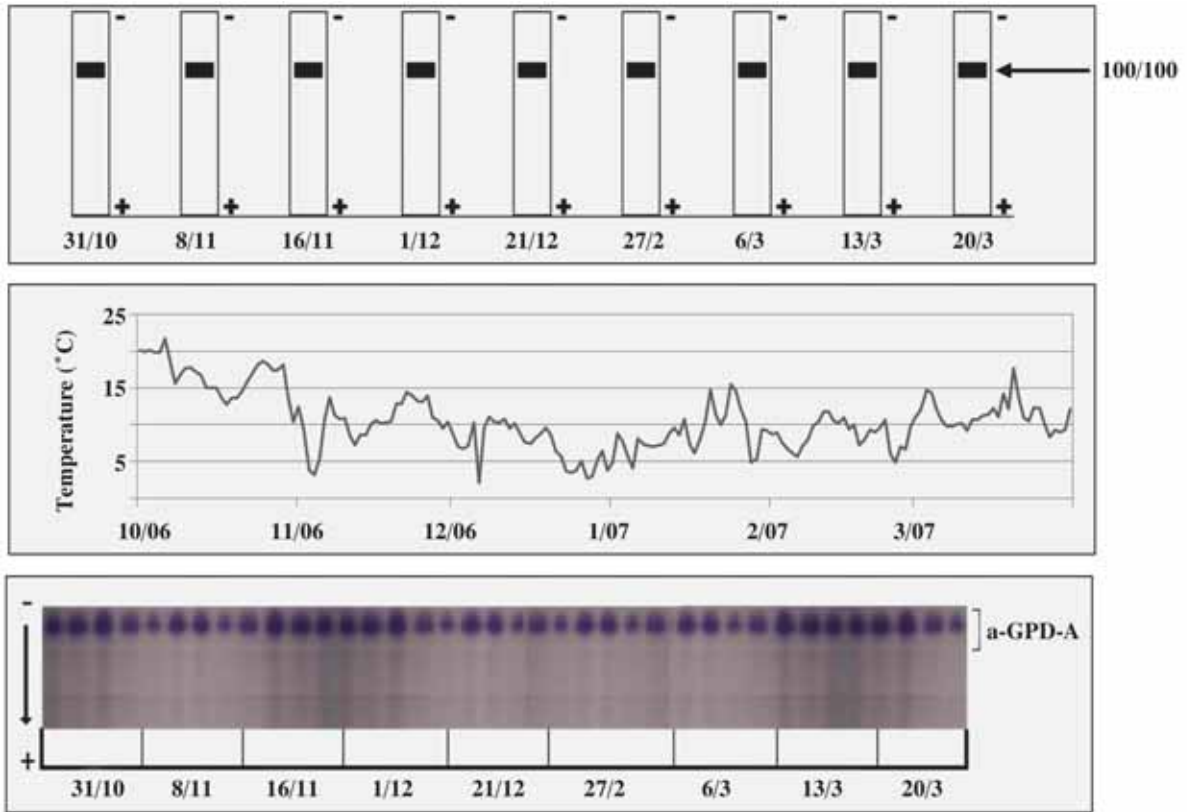
FIG. 4. Electrophoretic patterns of aGPD-A in gastrocnemius muscle related to seasonal temperature.
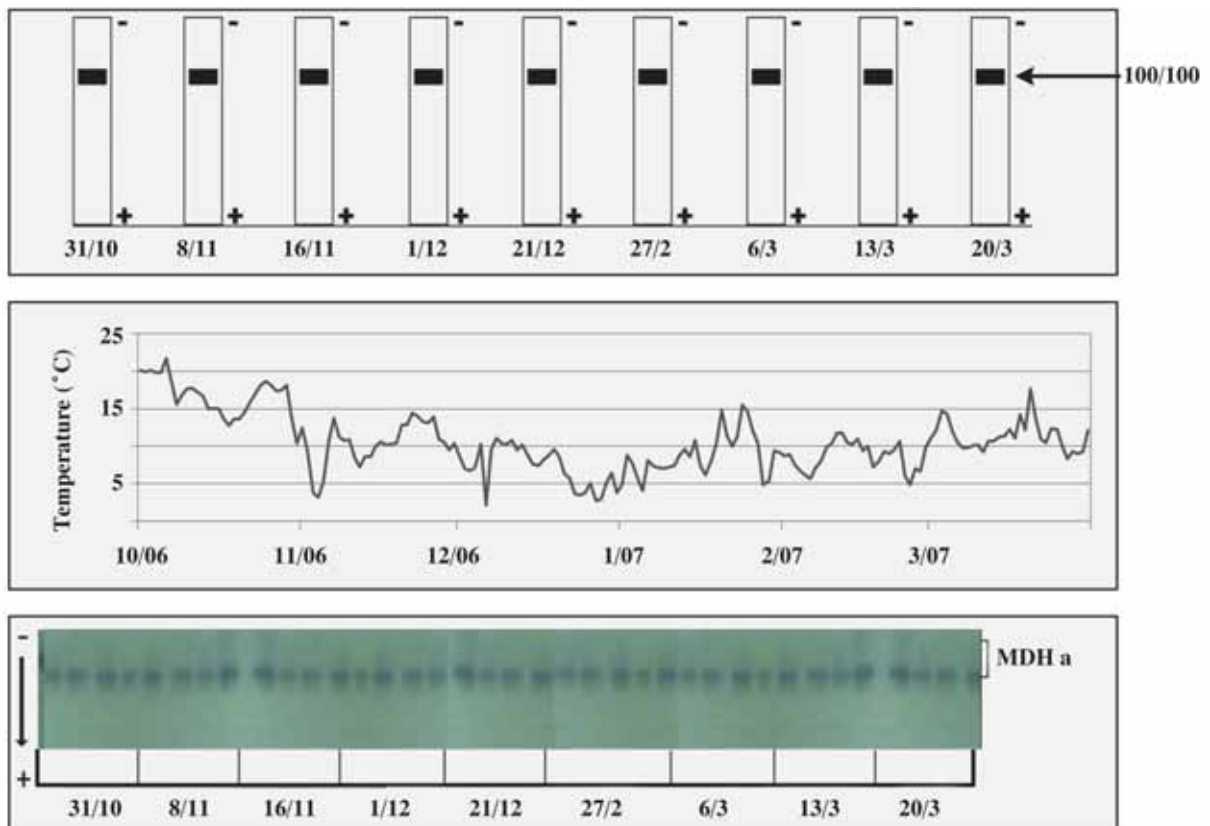

FIG. 5. Electrophoretic pattern of MDH in heart related to seasonal temperature.

during hibernation are shown in Figures 1-2. Two loci were found, LDH-A and LDH-B. In all samples the monomorphic locus LDH-A was found only in gastrocnemius muscle while LDH-B locus was detected exclusively in heart. Three heterozygotes (80/100) were found in 16/11, 27/2 and 20/3 while the rest individuals were homozygotes (100/100).

a-Glycerophosphate dehydrogenase

Two loci were found in a-glycerophosphate dehydrogenase (a-GPD) which are expressed in different tissues. The most anodal a-GPD–B was revealed in heart as one isoform (Fig. 3) and a-GPD–A was revealed in gastrocnemius also as one isoform (Fig. 4). Both gene loci were monomorphic in all samples analyzed.

Malate dehydrogenase

The enzyme malate dehydrogenase (MDH) was examined in heart. Only one gene locus MDH-A was found that was also monomorphic (100/100) during the whole hibernation period (Fig. 5).

DISCUSSION

Many studies have focused on the mechanisms of metabolic depression in hibernating ectotherms. Based on the published data the main mechanisms inducing metabolic depression are: a) the reverse phosphorylation of key-enzymes of glycolytic pathway, b) changes in the way of connection of enzyme and cellular structural elements, c) change in the levels of the 2, 6 diphosphoric fructose, d) modification of the allosteric control of enzymes, and e) changes in the levels of enzymes (Brooks & Storey, 1997; Storey, 1997). Furthermore expression or activation of different enzymatic isoforms seems to play a crucial role in maintaining a basal metabolic rate in the tissues of hibernating animals (Hochacka & Somero, 2002).

The electrophoretic patterns of the enzymes studied here have revealed only one isomorph of MDH in the heart of *R. ridibunda* frogs, maintained during hibernation (Fig. 5). Grainger & Kunz (1966) also reported one form of MDH at all developmental stages of the frog *Rana temporaria*. MDH appears with one anodic, one cathodic and an intermediate isoform that can be polymorphic in skeletal muscle of *R. catesbeiana* (or *Lithobates catesbeiana*) adults. Several differences between the tadpoles and adults were observed in other tissues like liver and the intermediate

zones appeared only in tadpoles (Manwell, 1966).

Two forms of the a-glycerophosphate dehydrogenase (a-GPD) were present in hibernating *R. ridibunda*, a cathodic in the gastrocnemius muscle and an anodic in the heart (Figs 4 and 3 respectively). This is a dimeric enzyme that supplies the glycolytic path with dihydroxyacetone, which is an intermediate product of glycolysis. Agrell & Kjellberg (1965), in an electrophoretic study of adult individuals of the frog *Rana temporaria*, found three anodic zones of isoforms of the enzyme a-GPD in heart.

The enzyme lactate dehydrogenase (LDH) determines the rate of pyruvate oxidation to lactate under low levels or lack of oxygen. The isozyme forms of LDH present in different tissues are well studied, and thus it is possible to correlate their kinetic properties to tissue energy demand and metabolic profile under several conditions. It has been reported that LDH is a tetrameric enzyme which consists of the combination of the two subunits A and/or B that are coded by two different genes (Pasteur *et al.*, 1987). In mammals and in certain species of birds, a third locus has also been found. The electrophoretic pattern of LDH revealed two gene loci, the LDH-A in gastrocnemius muscle and the LDH-B in the heart of *R. ridibunda* (Figs 1, 2). Hornby *et al.* (1989) found five and four isozyme isoforms of LDH in frogs *Xenopus laevis* and *R. temporaria*, respectively. The LDH-B locus was observed in the skeletal muscle of the species *X. laevis*. Probably this muscle contains elements from white and red muscle that remained during the growth of species (Kunz, 1973). Goldberg & Wuntch (1967) found at least three isoforms of LDH in the heart of *R. pipiens* (or *Lithobates pipiens*) and one isoform in gastrocnemius muscle. The European pool frog *R. lessonae* is largely polymorphic for two common alleles *e* and *b* at LDH-B locus. The individuals that are homozygotes for allele *e* reached metamorphosis earlier and were heavier than homozygotes for allele *b*. The two alleles showed the same performance ranking when combined in hybrids with *R. ridibunda* allele *a*. The hybrid species *R. esculenta* seems to receive mostly the allele *e* than *b* (Hotz & Semlitsch, 2000).

The lack of observable differences in the isozyme patterns of hibernating *R. ridibunda* indicates that frogs do not alter the isozyme production as a mean of temperature compensation. Even if this strategy of a "variety" of isozymes with different optimal temperature is not common between the species, there are many animals that express different isozymes in

different tissues (liver, heart and muscle) but also in the same tissue. The ability to alter gene expressions and thus switch between protein components in response to seasonal (or even diurnal) change is a key factor in the adaptive repertoire of some animals with particularly wide temperature tolerances (Hochacka & Somero, 2002). However the transient appearance of a second anodic band for LDH-B in the heart of hibernating *R. ridibunda* needs further discussion. It remains unknown whether it is another isozymatic form and what its physiological role is during hibernation, though it seems to be an allele of this locus.

The leopard frog, *R. pipiens*, appears to synthesize the usual LDH-M and LDH-H monomers, as evidenced by the isozyme distribution patterns. However, the predominance of LDH-M4 and LDH-M3H in both the pure heart LDH and the crude extract is seen at pH 8.4, a pH at which most LDH isozymes are routinely separated. Altman & Robin (1969) found apparently similar patterns at pH 8.8 for the pond turtle, *Pseudemys elegans*, heart and muscle LDH, and suggested that the predominance of LDH-M4 in these tissues is an important mechanism enabling the animal to survive long anaerobic periods using glycolysis as a principal energy source. This proposal was supported by their finding of almost identical pyruvate inhibition curves for pond turtle heart and muscle LDH, in each case reflecting anaerobic metabolism. Blix & From (1971) also reported identical LDH patterns for the common eider (*Somateria mollissima*), a diving bird.

It is known that the LDH-A locus functions better in the gastrocnemius muscle (in anaerobic metabolism) and LDH-B is present in the heart where the aerobic mechanism remains. During the anaerobic glycolysis the transformation of pyruvic acid to lactic is important for continuous production of ATP. The LDH can change the pyruvate in lactate but also reversely (in the liver with glyconeogenesis). The gene locus A that controls the LDH-A is related mainly with the anaerobic metabolism and usually it participates in the transformation of pyruvate to lactate while the LDH-B participates in the transformation of lactate to pyruvate. In the frog *R. pipiens* the LDH-B locus expressed in the heart, is inhibited by high concentrations of pyruvic acid and is sensitive to heat. The form LDH-A of the skeletal muscle is not suspended by high concentrations of pyruvic acid neither is influenced by heat (Wright & Moyer, 1973). The rate of glycogen reduction is the same in the heart under hypoxic and normal conditions, indicating that

anaerobic mechanism does not contribute to ATP turnover (Donohoe & Boutilier, 1998). Frogs that find shelter in water adopt dermal breathing and thus enriching the skin blood vessels. However, sensitive in hypoxia internal body organs do not take the required oxygen to function. Since about the 35% of body mass of frogs is constituted from skeletal muscles we can conclude that the reduction of blood flow and, at the same time, the reduction of oxygen that passes to them contribute to the total reduction of metabolic rate of the entire individual. This mobilizes the anaerobic mechanism with decreased production of ATP which is constant during hibernation. Nonetheless in the heart, no reduction of ATP has been observed, a fact suggesting that aerobic mechanism is still in use. This is happening because heart should function regularly in order to receive oxygenated blood by the dermal respiration and to channel it in the brain.

In conclusion, we suggest that the production of different isozymes in frog tissues is due to the functional nature of each particular tissue. The LDH-A is expressed better in the gastrocnemius muscle which is mainly anaerobic, while the LDH-B is expressed better in the heart which continues to metabolize in an aerobic way. The same pattern is also followed by a-GPD. In order to understand the adaptive significance of any alterations in isozyme expression it is also necessary to consider structural and kinetic data.

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