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Enzymatic Urea Adaptation: Lactate and Malate Dehydrogenase in Elasmobranchs

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Summary

Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) electrophoretic tissue patterns of two different orders of Elasmobranchii: Carchariniformes (*Galeus melanostomus* and *Prionace glauca*) and Squaliformes (*Etmopterus spinax* and *Scymnorinus licha*) were studied. The number of loci expressed for these enzymes was the same of other elasmobranch species. Differences in tissue distribution were noted in LDH from *G. melanostomus* due to the presence of an additional heterotetramer in the eye tissue. There were also differences in MDH. In fact, all the tissues of *E. spinax* and *G. melanostomus* showed two mitochondrial bands. Major differences were noted in the number of isozymes detected in the four compared elasmobranchs. The highest polymorphism was observed in *E. spinax* and *G. melanostomus*, two species that live in changeable environmental conditions. The resistance of isozymes after urea treatment was examined; the resulting patterns showed a quite good resistance of the enzymes, higher for LDH than MDH, also at urea concentration much greater than physiological one. These results indicated that the total isozyme resistance can be considered higher in urea accumulators (such as elasmobranchs) than in the non-accumulators (such as teleosts).

Key words
Elasmobranchs • Environmental adaptation • Lactate dehydrogenase • Malate dehydrogenase • Urea denaturation

Introduction

Elasmobranchs are an ancient group, evolved independently from bony fishes for more than 400 million years (Schaeffer and Williams 1977). The last common ancestor of the sharks and teleosts must have lived in the Silurian Period (Carrol 1988), and the two groups differ considerably in many aspects of their morphology, physiology and biochemistry. Two major subgroups of modern chondrichthyans are currently recognized: the Holocephali, or elephant fish, and the Elasmobranchii, or sharks, skates and rays. The trees resulting from several studies suggest that the Elasmobranchii consists of two major clades: the Galea (or Galeomorphii) and the Squalea. This group of fishes currently occupy environments ranging from polar oceans

to tropical seas and from surface waters to the abyss. It is of scientific interest for three main reasons: their evolutionary position, their osmotic strategy and their low incidence of neoplasia. They are one of the oldest extant groups of jawed vertebrates and have a simple anatomical organization. This simplicity extends to other aspects of their biology and considerable insight into the design of metabolism can be gained by studying elasmobranchs (Browing 1978). Structural and functional differences between isozymes, as well as their regulatory and distribution patterns, have been largely used to suggest phylogenetic relationships among fish species (Buth 1983, 1984, Kettler and Whitt 1986, Whitt 1987).

The expression of single specific genes or groups of genes takes places on a strict space-temporal developmental schedule and determines the ultimate different fates of the cells involved. The specific genes are activated by some specific proteins, which are coded by other genes which, in turn, are activated by still further specific factors (Markert and Ursprung 1971, Raff and Kaufman 1983, Davidson 1986, Kettler *et al.* 1986, Beloussov 1988). Lactate dehydrogenase (LDH; EC 1.1.1.27) and malate dehydrogenase (MDH; EC 1.1.1.37) are two of the most extensively studied isozyme systems. In fact, they are very suitable systems for studying several metabolic, genetic, ecological and evolutionary features, and they are very useful in systematic studies (Almeida-Val *et al.* 1990, 1991, 1992, Coppes 1990). Many characteristics of elasmobranch metabolism show differences from that of higher vertebrates. Estimation of enzyme activities support the view that elasmobranchs have a low aerobic capacity, so that the study of LDH and MDH assumes a special importance. In fact, low levels of citrate synthase in tissues of elasmobranchs (Alp *et al.* 1976, Dickson *et al.* 1988, Moon and Mommsen 1987) imply low aerobic capacity of their tissues, unlike with more active fish species. Elasmobranch fishes (sharks, skates and rays) are often incorrectly perceived as primitive animals, which have remained relatively unchanged for many millions of years. In truth, members of this group of animals have adapted to exploit many different environments, spanning extremes in temperature, pressure and salinity. A broad range of reproductive, osmoregulatory and metabolic strategies can be found within elasmobranch fishes, making them excellent comparative models for the study of physiological processes. In general, fishes have proven to be indispensable as experimental models in many scientific fields because of their strategic evolutionary and ecological position (Powers 1989, Stock and Powers 1995, 1998).

The primary solute of elasmobranchs, urea, is a chaotropic agent that disrupts hydrophobic interactions (Yancey and Somero 1979). Urea has disruptive effects on protein structure because hydrophobic interactions are important in maintaining the three-dimensional structure of proteins and in subunit association of multimeric enzymes. Such effects on structure can disrupt essential protein functions, such as catalysis and substrate binding of enzymes. Elasmobranchs appear to have two responses to this problem. One of them is a modification of the proteins to maintain the function in the presence of urea; the other is to use the other major organic solute they contain, the methylamines, to counteract the disruptive effects of urea.

The present paper describes the electrophoretic patterns of LDH and MDH from some elasmobranch species as well as the isozymes distribution in five different tissues. Moreover, the inhibitory effects of urea have also been studied. The different isozymes distribution of tissues (tissue-specific enzymatic pattern) represents a direct proof of their functional efficiency and it is certainly indicative of their metabolic peculiarity and demands of various tissues, as well as of the characteristics of the various species studied here. The uninterrupted presence of the elasmobranchs on the earth for more than 400 million years attests to their viability. The simplicity of their model, not only as concerns the anatomy, but also the metabolic organization, may have contributed to their success, demonstrated among other things by their adaptability to environmental conditions.

Methods

Animals

Specimens were taken from South Tyrrhenian Sea. The fish caught belonged to two different orders of elasmobranchs: Carchariniformes (*Galeus melanostomus* and *Prionace glauca*) and Squaliformes (*Etmopterus spinax* and *Scymnorhinus licha*). They have different anatomical and morphological features.

Preparation of tissue extract

The tissues were dissected immediately after capture and kept in a freezer (−20 °C) until analysis. The desired tissues, white muscle, heart, brain, liver and eye, were cut into small pieces and homogenized (2:1 v/w) in a 0.1 M Tris-HCl buffer at pH 7.5 containing phenyl-

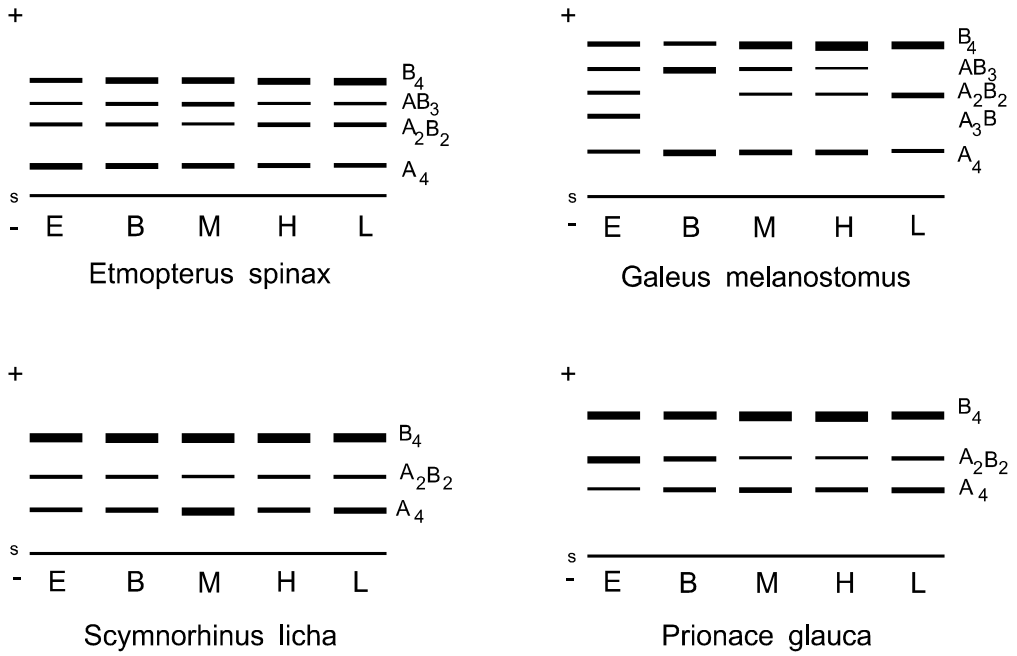

Fig. 1. Diagrammatic representation of LDH isozyme patterns in in *Etmopterus spinax*, *Galeus melanostomus*, *Scymnorhinus licha* and *Prionace glauca* tissues. Eye (E), brain (B), muscle (M), heart (H), liver (L). Note that in this and subsequent figures A_4, A_2B_2, AB_3, B_4 indicate subunit composition of LDH isozymes. s indicates sample origin; +, anode; -, cathode.

methyl sulfonyl fluoride (PMSF), using a Potter-Elvehjem tissue grinder, at 0 °C and then centrifuged at 36,300 x *g* for 30 min at 4 °C. The resulting supernatants were used for electrophoretic analysis.

Electrophoretic analysis and enzyme staining

Vertical electrophoresis was performed on 7.5 % polyacrylamide gel, applying a constant voltage of 2.5 mV per tube, with a Tube Cell Model 175 (in a cold room kept at 4 °C). The LDH and MDH isozymes were characterized with the 0.3 M Tris-HCl gel buffer (pH 8.9) and 25 mM Tris, 192 mM glycine tank buffer (pH 8.5). Running time was 3 h. Gel preparation, voltage, current and running time were carefully controlled in order to provide virtually identical electrophoretic conditions. LDH and MDH staining solutions consisted of 60 mM lithium lactate (LDH) or malate (MDH), 0.336 mM nicotinamide adenine dinucleotide (NAD^+), 0.168 mM Nitro-blue tetrazolium (NBT), 0.056 mM phenazine methosulfate (PMS) in 0.1 M phosphate buffer (pH 7.0). After applying the stain, the gels were incubated in the dark for 2 h at 37 °C. To check the specificity of the staining reaction control gels were stained without lactate or malate for nonspecific dehydrogenases. After incubation, the gels were washed and kept in a conserving solution (7 % acetic acid).

Urea inactivation

Urea was dissolved in 0.5 M Tris-HCl buffer to a final concentration of 8.0 M and with a final pH of 9.0. Different amounts of this stock urea solution were mixed with 0.5 M Tris-HCl (pH 9.0) buffer to generate different urea concentrations. After electrophoresis, some gels were placed in the appropriate urea-plus-buffer mixture for 35 min before the staining solutions were added. The concentrations of urea were 8 M, 4 M, 2 M and as control one sample without urea. The gels were washed and stained for LDH and MDH activity as described above.

Results

The electrophoretic profiles of lactate and malate dehydrogenase from five tissues of the four elasmobranchs analyzed showed multiple forms with different mobility. Moreover, the treatment of tissues extract with several denaturant concentrations of urea evidenced a different inhibitory effect against the isoforms.

Lactate dehydrogenase

The electrophoretic patterns of LDH isozymes of each of the four species studied are shown in Figure 1 (with the molecular interpretation of each component). As in all elasmobranchs, the A and B genes for LDH

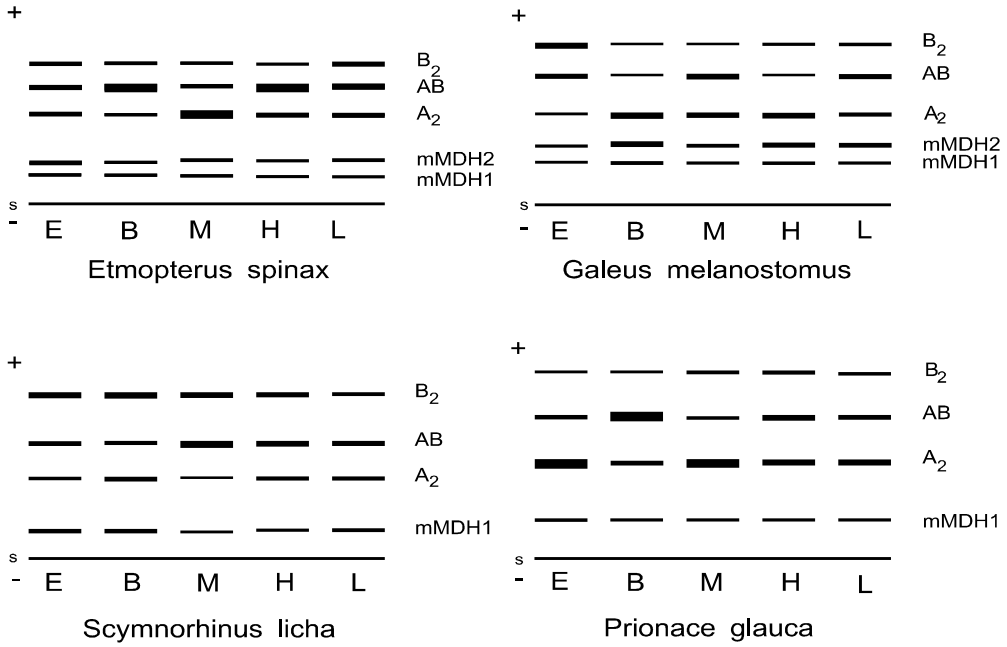

Fig. 2. Diagrammatic representation of MDH isozyme patterns in *Etmopterus spinax*, *Galeus melanostomus*, *Scymnorhinus licha* and *Prionace glauca* tissues. Eye (E), brain (B), muscle (M), heart (H), liver (L). Note that in this and subsequent figures A_2, AB, B_2 indicate subunit composition of MDH isozymes, mMDH1 and mMDH2 the mitochondrial enzymes. s indicates sample origin; +, anode; -, cathode.

were detected, resulting in two homotetramers (A_4 and B_4) and one or more heterotetramers. All the tissues of *P. glauca* and *S. licha* showed three bands with little intensity differences among them. LDH-B_4 was the most represented isoform, except for muscle of *Scymnorhinus* and eye tissues of *Prionace*, in which its concentration was almost the same as LDH-A_4 and LDH-A_2B_2, respectively. In *E. spinax* two heterotetramers were detected in all the analyzed tissues, with little differences in their relative concentration. LDH-B_4 was abundant in the liver, heart and muscle, whereas in brain it was similar to the concentration of LDH-A_4 and in the eye tissue it was found at even lower concentration levels.

The enzyme expression in *G. melanostomus* showed a tissue-specific pattern with different relative proportions of the isozymes. The homotetramer LDH-B_4 was predominant in the heart and muscle, whereas in the eye tissue an additional heterotetramer (A_3B) was observed. Moreover, the heterotetramers A_2B_2 and AB_3 could not be observed in brain and liver tissues, respectively

Malate dehydrogenase

The MDH isozyme patterns of the four analyzed elasmobranchs are shown in Figure 2. In all the tissues the expression of three separate gene loci (Mdh-M, Mdh-A and Mdh-B) were found. The presence of MDH-AB heterodimers in all tissues indicates the simultaneous synthesis of both MDH-A and B subunits in many differentiated cells. The four species considered exhibited essentially similar, although species-specific patterns of gene expression in five tissues examined. The differences observed concern essentially the electrophoretic mobility and relative intensity of the detected isozymes.

In *P. glauca* the MDH-A_2 homodimer predominated in eye and muscle; in heart and liver it was almost the same as AB, that was higher in the brain tissue. Eye and brain tissues of *S. licha* showed the predominance of MDH-B_2; its intensity was similar to the heterodimer in the heart, whereas the homodimer A_2 was the highest in muscle.

E. spinax tissues were characterized by an additional mitochondrial band (m-MDH2), but cytosolic MDH (s-MDH) was always higher than m-MDH. In this case the MDH-A_2 was predominant in muscle and the heterodimer AB in brain, heart and partly in liver tissues; s-MDH of eye was almost equally distributed between isoforms.

G. melanostomus also showed two mitochondrial isoforms, indicated as m-MDH1 and m-MDH2 and the latter was predominant in most cases. The liver isozymes intensities were similar each other, whereas the

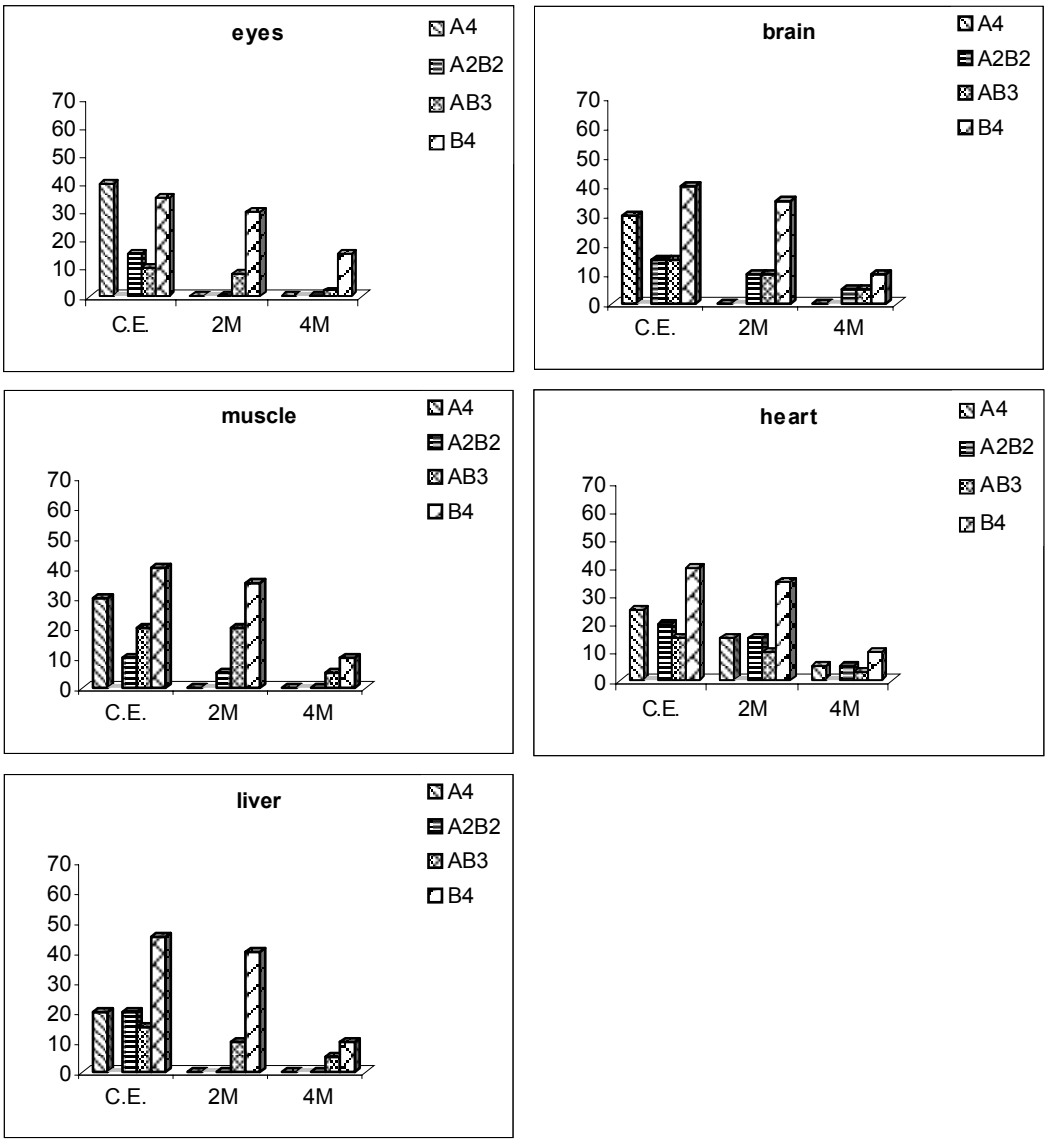

Fig. 3. Percent LDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Etmopterus spinax* tissues.

remaining tissues showed generally the predominance of two isoforms: both cytosolic or one cytosolic and one mitochondrial.

Urea inactivation

In general, for increasing urea concentration, an increased disruptive effect on the three-dimensional structure of proteins was observed. In this work we analyzed the effect of 2 M, 4 M and 8 M urea concentrations on MDH and LDH isozymes, but the results obtained for the highest concentration of urea were not reported because in all cases its effect was the total inactivation of both enzymes.

LDH isozyme patterns after urea inactivation are shown in Figures 3-6. Generally, urea treatment resulted in a differential sensibility of the isozymes. In the studied fishes, the LDH-B_4 homotetramer was present in all tissues treated with 2 M urea and in some of them, one or two heterotetramers were also detected. In contrast, treatment with 4 M urea resulted in a total inactivation of most of the LDH isozymes. In fact, in almost all the fish tissues treated with 2 M urea, the most cathodic bands and, sometimes, those at medium mobility disappeared. The inhibitory effect was higher with 4 M urea; at this concentration, in the most of tissues, only the anodic isozyme was detected.

In *E. spinax* tissues the treatment with 2 M urea resulted in the inactivation of homotetramer LDH-A4 and the reduction of the other isozymes, particularly due to loss of LDH-A subunits. Generally, the increase of urea

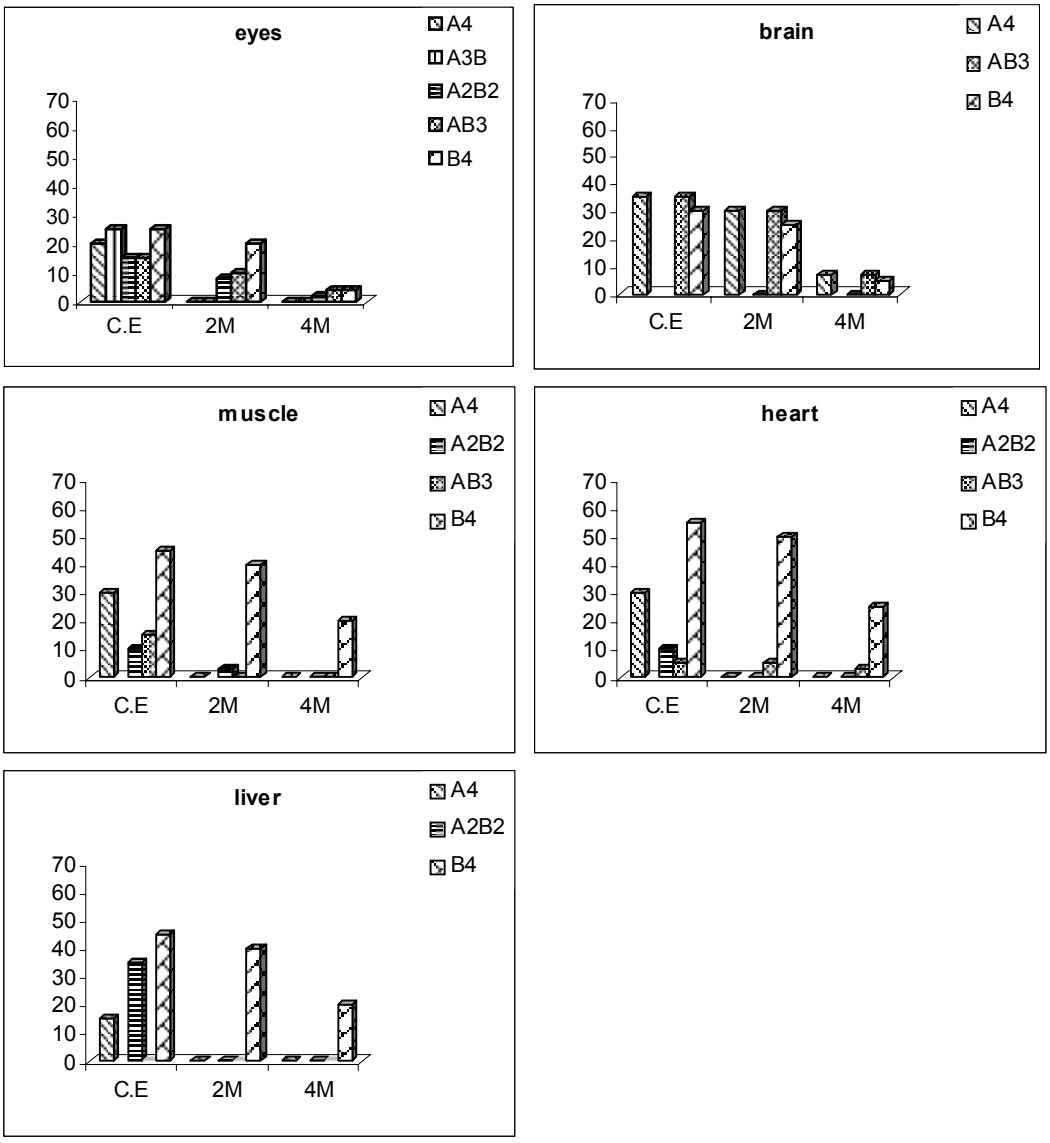

Fig. 4. Percent LDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Galeus melanostomus* tissues.

concentration caused a loss of isozymes activity. An exception to this behavior was given by heart tissue, in which all isoforms were detected in both the electroforetic pattern obtained after 2 M and 4 M urea treatment, even if the activities were lower.

Urea effects on *G. melanostomus* tissues were almost the same as for *Etmopterus*, except for the brain tissue, in which the behavior was the same observed for *Etmopterus* heart.

The treatment with 2 M urea was just sufficient for a total inactivation of LDH-A subunits in all *S. licha* tissues. Only LDH-B_4 was detected, even after treatment with 4 M urea, in all examined tissues, whereas the heterotetramer LDH-A_2B_2 was resistant to urea treatment only for the eye and brain tissues. These effects were more pronounced for the higher urea concentration (4 M).

In brain, heart and liver tissues of *P. glauca*, only LDH-B_4 was detected after 2 M urea treatment. An exception was represented by eye and muscle tissues. In fact, for the eye tissue examined even at 4 M urea concentration, the heterotetramer LDH-A_2B_2 showed a quite good resistance in comparison to the other tissues, whereas the A_4 homotetramer was detectable in the muscle extract treated even with 4 M urea.

MDH isozymes showed generally a quite good resistance after 2 M urea treatment in all tissue extracts obtained from the four analyzed fishes (Figs 7-10). The increase of urea concentration caused a further decrease of all isoforms activity, the drop being more evident for the isozymes with lower mobility. The most important

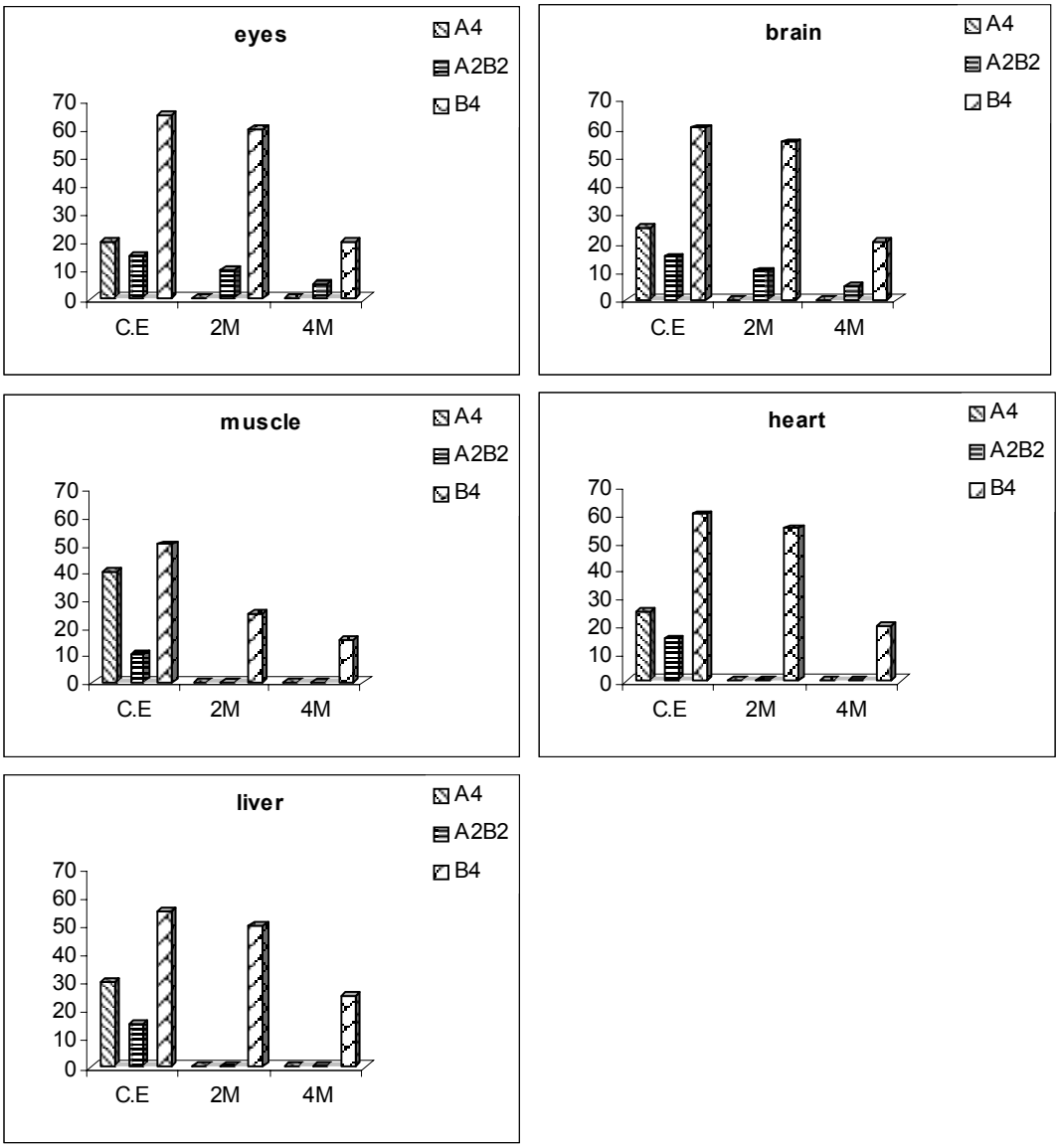

Fig. 5. Percent LDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Scimnorhinus licha* tissues.

differences observed among the fishes tissues are summarized as follows.

The electrophoretic pattern of MDH obtained from *E. spinax* tissues showed that all the isoforms of this enzyme were quite urea resistant. In fact, urea treatment caused a reduction, but not a total inactivation of both soluble and mitochondrial isozymes in all the tissues, with the exception of liver, in which only MDH-B subunits could be detected at higher urea concentration.

In *G. melanostomus* tissues, the treatment with 2 M urea reduced MDH activities in general, with 4 M urea treatment some isoforms disappeared completely, probably due to their very low initial concentration.

In *S. licha*, all the isozymes of eye, muscle, heart and liver tissues were detected to have reduced activity, after 2 M urea treatment, except for mMDH of brain tissue. In 4 M urea, the heart tissue showed all s-MDH, whereas in the other tissues only MDH-B subunit was detected.

In *P. glauca* both s-MDH and m-MDH were quite resistant also at 4 M urea concentration. In fact, only eye and heart tissues did not show m-MDH, probably due to its very little concentration.

Discussion

The tissue distribution is quite different between distinct fish groups. One of the main evolutionary patterns can be seen in isozyme systems since it involves regulatory genes and represents tissue metabolic

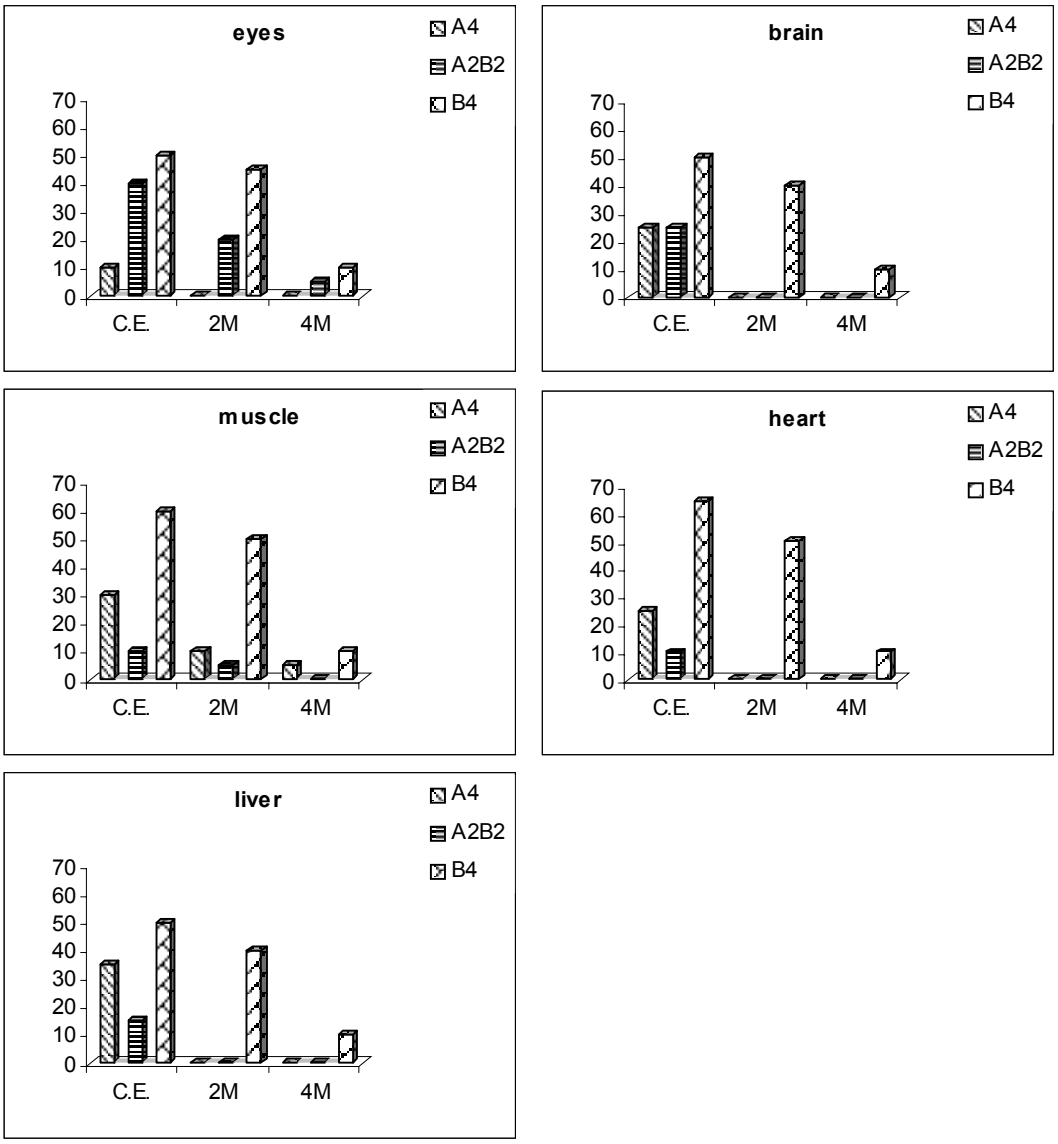

Fig. 6. Percent LDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Prionace glauca* tissues.

preferences, which may reflect adaptive properties of the species. According to Whitt (1983) the evolution of molecular and cell mechanisms contributes to the differential expression of isozymes in tissues and may constitute the regulatory evolution of structural genes. Thus, less specialized enzymes would be expressed in a great number of tissues, and the most specialized ones would be characteristic for a few tissues (Ferris and Whitt 1977, 1979). Some aspects of the metabolism of elasmobranch fishes are unique when compared with those of other vertebrates.

Although many features of their metabolism can be attributed to their primitive evolutionary position (e.g. fewer isoforms of enzymes and other proteins), some unique features include the specific synthesis and use of urea as an osmoregulatory agent (Smith 1936). The four species of elasmobranchs studied in the present paper showed two gene loci for LDH, Ldh-A and Ldh-B. A and B subunits of LDH are different polypeptides but are homologous. Evidence for such a homology is in the ability to interact among them (Markert *et al*. 1975).

According to Goldberg and Wuntch (1967), the presence of a three-band pattern for LDH A and B detected in most tissues of the species analyzed here would be a combination of the subunits in homodimers and consequently in homotetramers, thus yielding the isozymes A_4 and A_2B_2 and B_4. In contrast, all the tissues of *E. spinax* and some tissues of *G. melanostomus* showed a higher number of LDH heterotetramers. Perhaps the reduced number of the isoforms is in

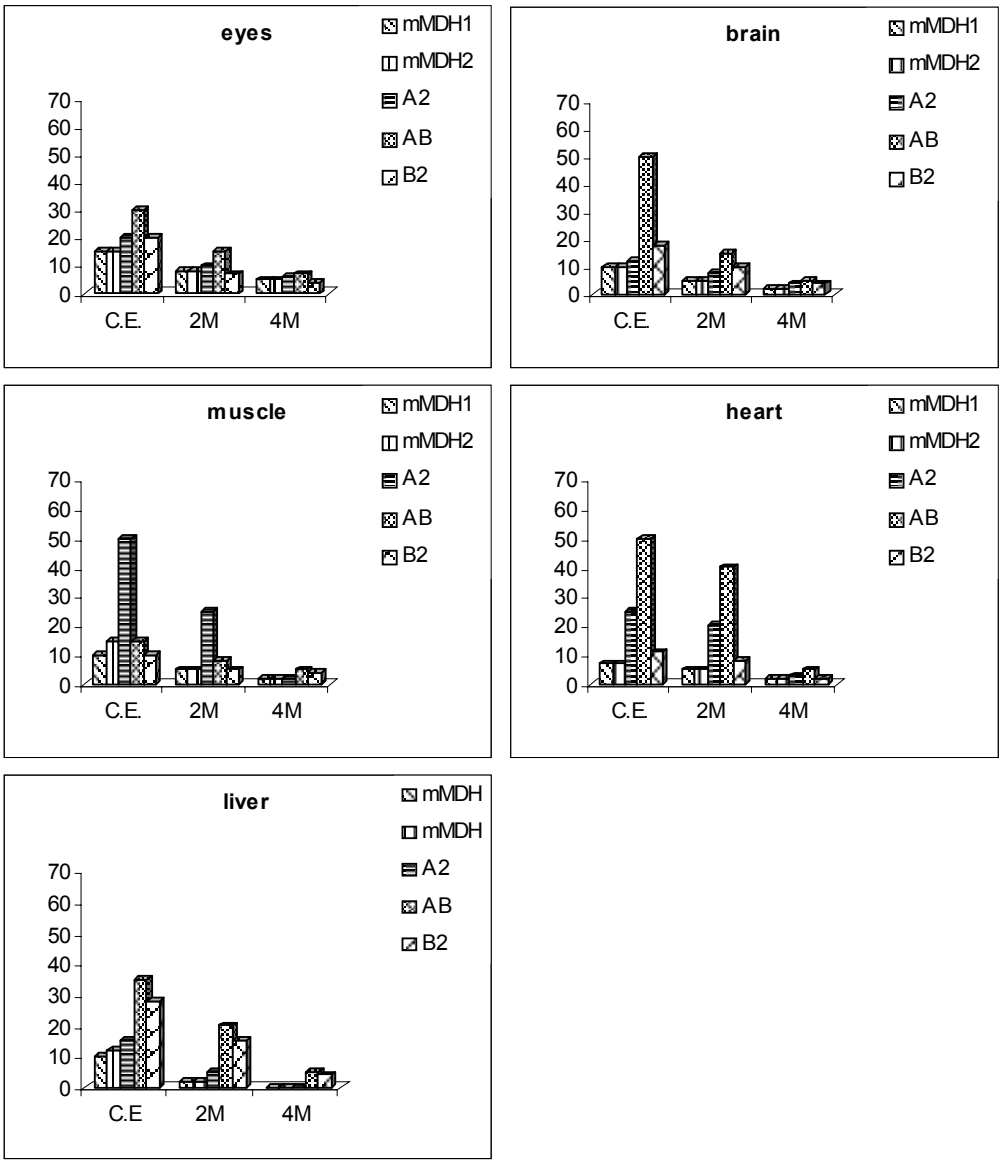

Fig. 7. Percent MDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Etmopterus spinax* tissues. In this case, the electrophoretic pattern revealed two mMDH isoforms, indicated as $mMDH_1$ and $mMDH_2$.

accordance with the relative simplicity of metabolism of this group, which are due to simple food availability and somewhat stable environmental conditions. The greater polymorphism detected in *E. spinax* and still more in *Galeus*, in which a different distribution with a greater number of isoforms in the eyes appears, is possibly related to the depth variation to which they are exposed. We have to consider that the number of species belonging to these two genera is small; so they have a different feeding pattern even if they are essentially carnivorous, with respect to other species, for which they are the quarries. So they put into action some complex mechanisms, such as mimicry: *Galeus* has spots according to the depth where it lives and *Etmopterus* masks itself with mud.

As found for teleosts fishes by other authors (Fisher *et al*. 1980, Basaglia 1989), the MDH was expressed with its mitochondrial and cytosolic enzymes also in the four elasmobranch species analyzed in this work.

The MDH isozyme patterns showed the presence of MDH-AB heterodimers in all tissues which indicates the simultaneous synthesis of both MDH-A and MDH-B subunits in many differentiated cells. In terms of electrophoretic mobility and band intensity the observed tissue patterns were quite different in these four species. Moreover, also in these cases, the tissues of *G. melanostomus* and *E. spinax* showed a higher number

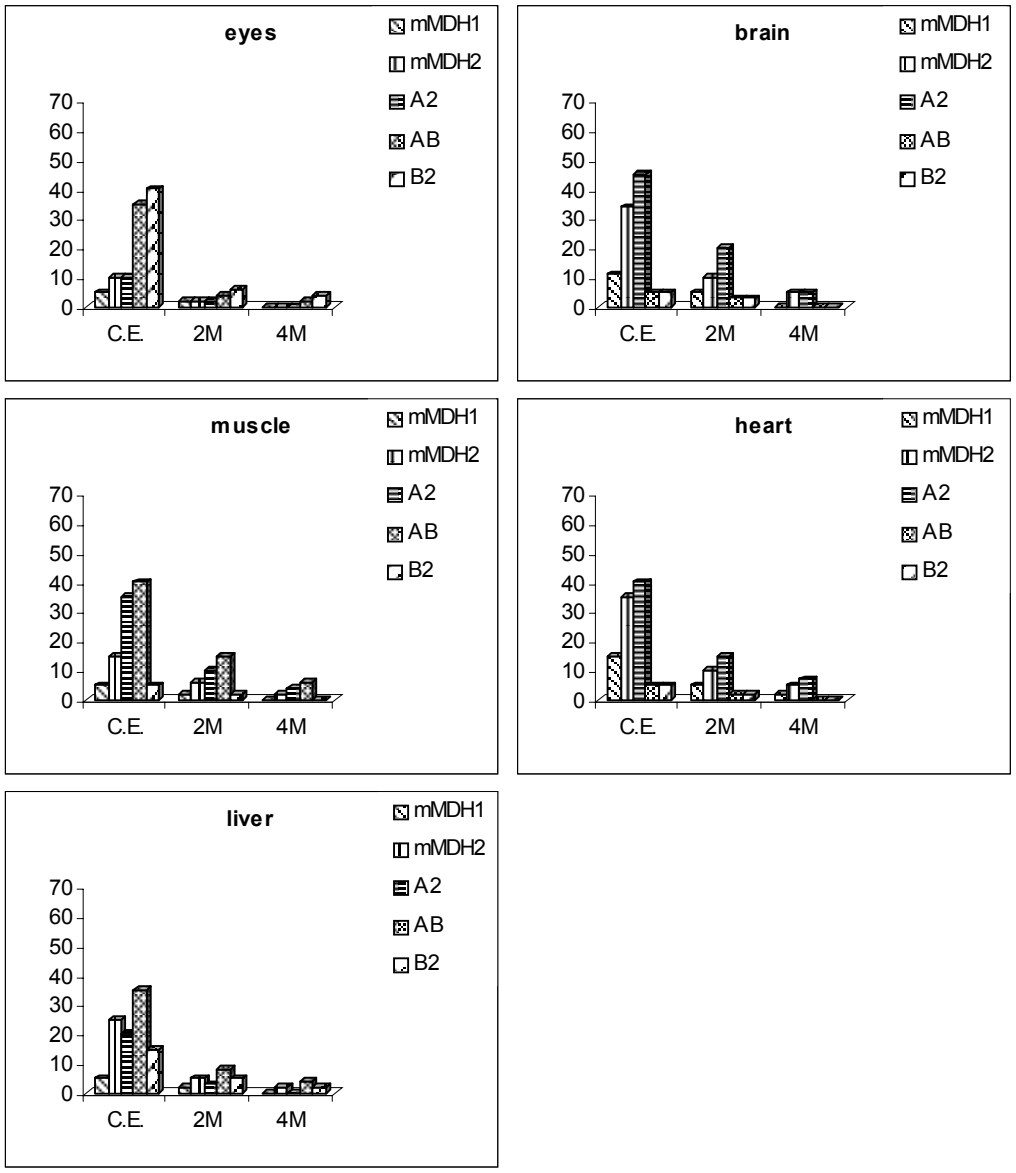

Fig. 8. Percent MDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Galeus melanostomus* tissues. In this case, the electrophoretic pattern revealed two mMDH isoforms, indicated as $mMDH_1$ and $mMDH_2$.

of bands. The higher polymorphism and the different tissue patterns observed in *E. spinax* and *G. melanostomus* for these isozyme systems is probably due to the different depth range, which they inhabit. Moreover, their size is relatively small and they frequently serve as food for other large elasmobranchs. The presence of a low number of isoforms of glycolytic enzymes is proof of a low evolutionary position with respect to the complexity increase of the metabolic model of vertebrates. On the other hand, the adaptation to different environmental conditions causes at a molecular level a higher enzymatic polymorphism which is evidenced in two of the species studied here, *Etmopterus* and *Galeus*, in which there are five isoforms of MDH in all the considered tissues; but they have different relative percentages in the function of specific tissue metabolism. Elasmobranchs are osmoconformers, using a combination of solutes to maintain the extracellular and intracellular osmolarity close to that of the environment. It is unusual that about 40 % of the osmolarity is due to urea with concentrations reaching 680 mM (Browing 1978). Such levels pose potential threats to the structure and function of proteins and other cellular components.

Generally, the isozymes of LDH and MDH differ in their sensitivity to urea with the anodic isozyme being more resistant than the cathodic one. MDH

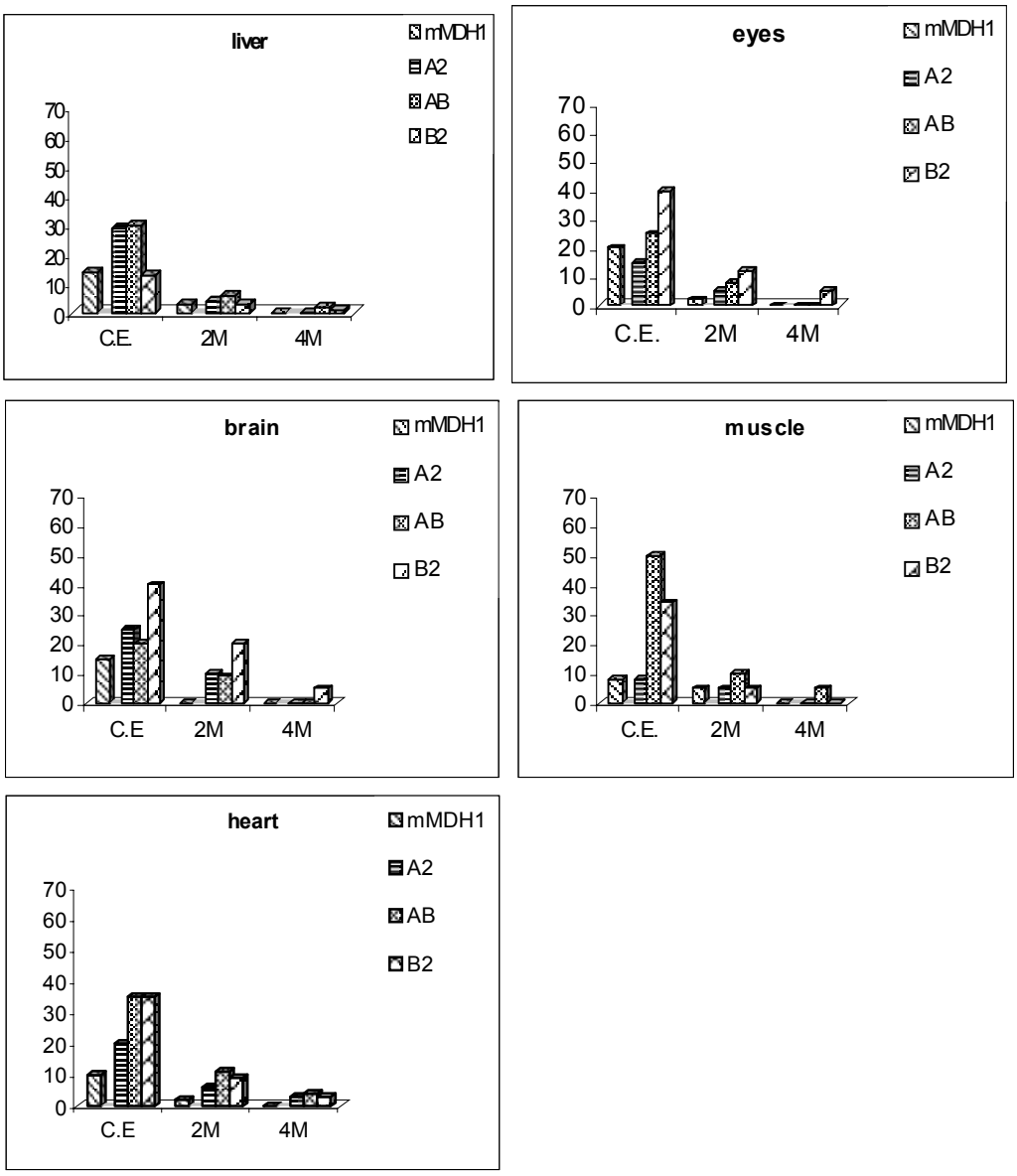

Fig. 9. Percent MDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Scimnorhinus licha* tissues. In this case, the electrophoretic pattern revealed one mMDH isoform, indicated as $mMDH_1$.

responses to the inhibitory effects of urea in elasmobranchs are certainly related to the synthesis, storage and different tissue distribution of this osmoregulator (Browing 1978). Tissue urea concentrations from 300 to 400 mM are normally found in tissues of elasmobranchs and this requires an enzymatic adaptation to high and variable urea levels which is reflected in the different qualitative and quantitative isozyme distribution – an effective answer to the different metabolic demands of different tissues (Anderson 1986). Interaction between catalysis and urea in elasmobranchs is of particular importance for the enzymes, because they must be not only resistant to high urea concentrations but also their catalytic efficiency depends on high concentrations. The effect of urea on the physiological levels of LDH isozymes in the species of elasmobranchs assayed here was lower than the effect in most animals that do not accumulate urea (Fuery *et al.* 1997). At these concentrations the sensitivity of MDH isozymes was even lower. This suggests that the enzymes produced by the urea accumulators are kinetically different from those of the non-accumulators.

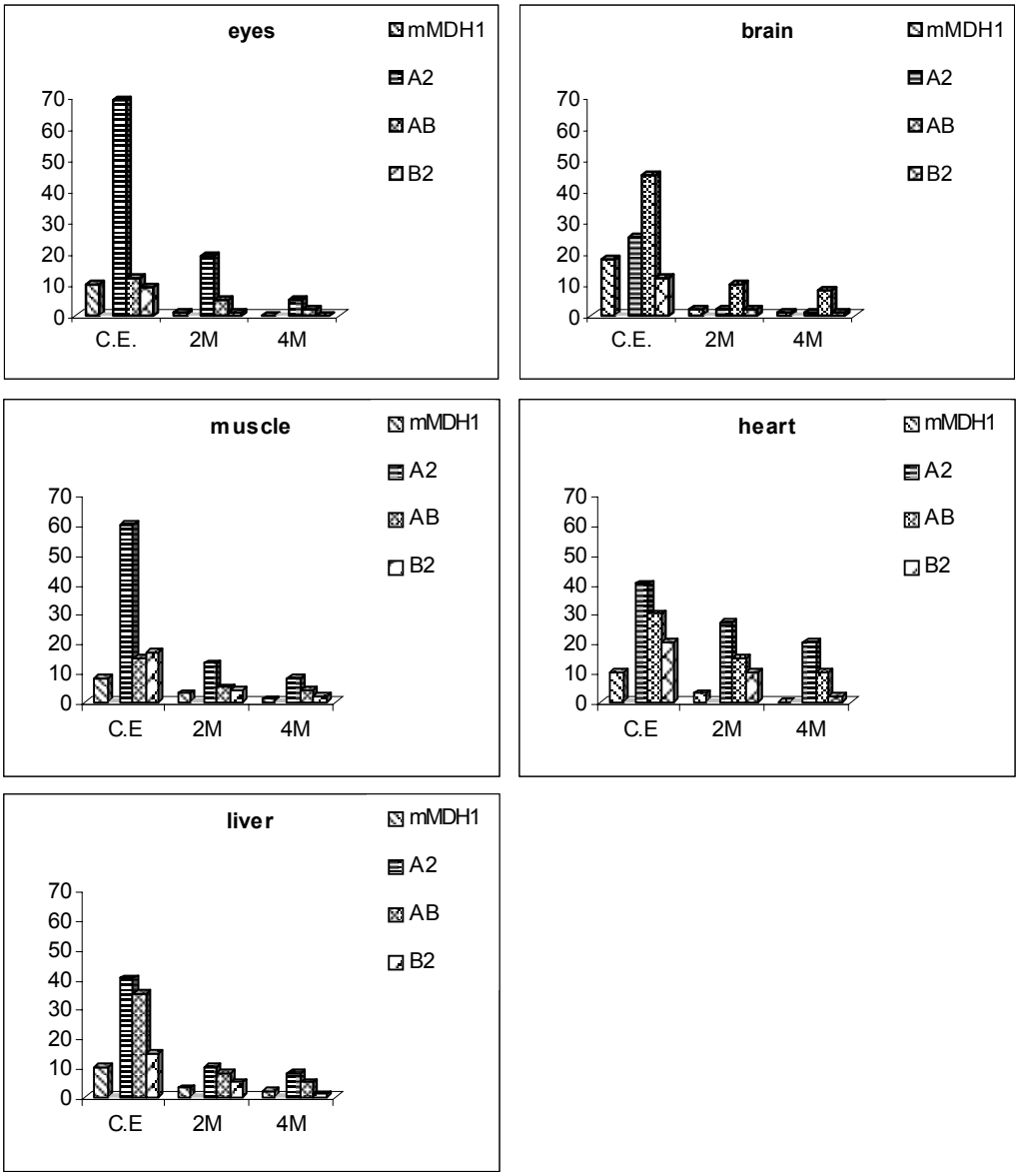

Fig. 10. Percent MDH isozyme activities in untreated crude extract (C.E.) and after urea treatment (2M and 4M represent urea molarity) in *Prionace glauca* tissues. In this case, the electrophoretic pattern revealed one mMDH isoform, indicated as $mMDH_1$.

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