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ANTARCTIC FISH METABOLIC RESPONSES AS POTENTIAL BIOMARKERS OF ENVIRONMENTAL IMPACT

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ABSTRACT

Most of the biochemical and physiological studies of Antarctic fish were directed to understand the molecular adaptation of these ectotherms to low temperatures and limits of their metabolic plasticity associated with thermal stress. The principal objective of this review is to discuss the energy metabolism, of L-arginine, antioxidant defense and the xenobiotics of Antarctic fishes as possible markers of environmental change. With the establishment of the circumpolar current and the physical isolation of the region, Antarctic fishes evolved under stable low temperatures. The elevated endemism, low diversity, and the probable stenothermality motivated a number of studies on Antarctic fishes. The presence of enzymes with physical and chemical properties adapted to low temperatures was evident in various studies and fortified the cold adaptation concept of these organisms. By ratifying the Antarctica Environmental Protection Treaty (Madrid protocol), the member countries committed themselves to monitoring the environment of the region. Though, it is one of the most pristine regions of the planet, increasing human activity in Antarctica has given rise to concerns about pollution from combustion oils, heavy metals and sewage. As the energy sustainability of the tissues requires that the velocity of the ATP generator systems should be in step with the energy demand, changes in the environment would lead to elevation in the energy demand of the organisms, which in turn demanded an adjustment of the ATP synthesis velocity. In these cases the enzymatic upregulation and downregulation have been used as biomarkers of the energy metabolism. Traditionally, the antioxidant defense enzymes and the xenobiotic metabolism of fishes have been used as biomarkers to access the natural or anthropic environmental change. However, these studies must correlate the levels of enzymes with the principal risk factors in the environmental pollution. L-arginine metabolism enzymes have been used as biomarkers of the physiological response in mammals, but there are few studies on Antarctic fishes. The central role of the amino acid L-arginine in the synthesis of nitric oxide, polyamines and as a signal in various physiological processes has drawn the attention of scientists. The tissue distribution and the isoforms distribution, along with its control via upregulation and downregulation of the arginase enzymes, nitric oxide synthase and ornithine decarboxylase in Antarctic fishes are potential biomarkers for environmental change.

Keywords: Antarctica; fish; biomarkers; metabolism; environment.

RESUMO

RESPOSTAS METABÓLICAS DE PEIXES ANTÁRTICOS COMO MARCADORES DE IMPACTO AMBIENTAL. Boa parte dos estudos bioquímicos e fisiológicos com peixes Antárticos foram conduzidos objetivando entender a adaptação molecular desses organismos ectotérmicos às baixas temperaturas, bem como os limites da sua plasticidade metabólica associada com o estresse termal. O principal objetivo desta revisão

é discutir os metabolismos energético, da L-arginina, de xenobióticos e da defesa antioxidante dos peixes antárticos. Com o estabelecimento da Corrente Circumpolar Antártica e o isolamento geográfico da região, os peixes antárticos evoluíram sob temperaturas baixas e estáveis. O número elevado de espécies endêmicas, a baixa diversidade e a provável estenotermia motivaram diversos estudos com peixes antárticos. A presença de enzimas com propriedades físico-químicas adaptadas a baixa temperatura ficou evidente em vários estudos e reforçou o conceito de adaptação ao frio. Ao ratificar o Protocolo de Proteção Ambiental para o Tratado da Antártica (protocolo de Madri), os países membros se comprometeram com o monitoramento ambiental da região. Embora seja considerada a região mais preservada do planeta, a presença humana crescente na Antártica tem motivado preocupações em relação à poluição por óleo combustível, metais pesados e esgoto. A sustentabilidade energética dos tecidos requer que a velocidade de síntese de ATP pelos seus sistemas geradores esteja em consonância com a demanda energética. Mudanças ambientais podem elevar a demanda energética dos organismos e, por sua vez, demandar ajustes na velocidade de síntese de ATP. Nesse caso, os aumentos ou reduções dos níveis enzimáticos tem sido utilizados como biomarcadores do metabolismo energético. Tradicionalmente, enzimas da defesa antioxidante e do metabolismo de xenobióticos de peixes tem sido utilizadas como biomarcadoras para indicar alterações ambientais naturais ou antrópicas. Estes estudos devem correlacionar os níveis de atividade enzimática com a presença do principal fator de risco da poluição ambiental. As enzimas do metabolismo da L-arginina tem sido utilizadas como biomarcadoras de respostas fisiológicas em mamíferos, mas há poucos estudos sobre peixes antárticos. A função do aminoácido L-arginina na síntese de óxido nítrico, poliaminas e como sinalizador em vários processos fisiológicos tem chamado a atenção de pesquisadores. A distribuição tecidual e a distribuição de isoformas, bem como o controle via *upregulation* e *downregulation* das enzimas arginase, óxido nítrico sintase e ornitina descarboxilase de peixes antárticos representam potenciais biomarcadores de alterações ambientais.

Palavras-chave: Antártica; peixe; biomarcador; metabolismo; ambiente.

RESUMEN

RESPUESTAS METABÓLICAS DE PECES ANTÁRTICOS COMO POTENCIALES MARCADORES DE IMPACTO AMBIENTAL. La mayoría de los estudios bioquímicos y fisiológicos de los peces antárticos fueron realizados para entender las adaptaciones moleculares de estos organismos ectotérmicos a las bajas temperaturas, así como también los límites de su plasticidad metabólica asociado al estrés térmico. El principal objetivo de esta revisión es discutir como posibles marcadores del cambio ambiental a los metabolismos energéticos de la L-arginina, de los xenobioticos y de las defensas antioxidantes de los peces antárticos. Con el establecimiento de la Corriente Circumpolar Antártica y el aislamiento geográfico de la región, los peces antárticos evolucionaron en condiciones estables de baja temperatura. El elevado endemismo, la baja diversidad y la probable estenotermia motivaron numerosos estudios en peces antárticos. La presencia de enzimas con propiedades físico-químicas adaptadas a las bajas temperaturas fue evidente en varios estudios y reforzó el concepto de adaptación al frío de estos organismos. En la ratificación del Protocolo al Tratado Antártico sobre Protección del Medio Ambiente (Protocolo de Madrid), los países miembros se comprometieron al monitoreo ambiental de la región. Si bien es una de las regiones más prístinas del planeta, el aumento de la actividad humana en Antártida ha generado preocupación en relación con la polución generada por los aceites de combustión, los metales pesados y las aguas de desecho. La sustentabilidad energética de los tejidos requiere que la velocidad de síntesis de ATP, a partir de sus sistemas generadores, deba estar sincronizada con la demanda de energía. Cambios en el ambiente conllevarán un aumento en las demandas energéticas de los organismos, que a su turno demandarán un ajuste de la velocidad de síntesis del ATP. En estos casos, los aumentos o disminuciones de los niveles enzimáticos han sido utilizados como biomarcadores del metabolismo energético. Tradicionalmente, las enzimas de defensa antioxidante y del metabolismo de xenobioticos de peces han sido utilizadas como biomarcadores para indicar alteraciones ambientales naturales o de origen antrópico. Estos estudios deben correlacionar los niveles de actividad enzimática con los principales factores de riesgo de la polución ambiental. Las enzimas del metabolismo de la L-arginina han sido utilizadas como biomarcadores

de las respuestas fisiológicas en mamíferos, pero existen pocos trabajos en peces antárticos. El rol central del aminoácido L-arginina en la síntesis de óxido nítrico, poliaminas y como señal en varios procesos fisiológicos ha llamado la atención de los investigadores. La distribución de tejidos e isoformas, así como también su control, vía la regulación de las enzimas arginasas, síntesis de óxido nítrico y la ornitina descarboxilasa en peces antárticos son potenciales biomarcadores de cambios ambientales.

Palabras clave: Antártida; peces; biomarcadores; metabolismo; ambiente.

INTRODUCTION

The present Antarctic fish fauna evolved under the selective pressure of low temperatures and seasonality imposed by the long austral winters. After the breakup of the super continent, Gondwana and the movement of Antarctica towards the South Pole (\approx 40 million years ago), the temperatures of the austral ocean decreased gradually from 15°C to the near freezing point. This cooling was accelerated in the last 2 million years, when the temperature declined (0.25°C/100,000 years) from about 5°C to the present values, but still considered enough for the adaptive evolution of Antarctica fishes (Eastman 1993).

The south Antarctic Polar Frontier compiled data of ichthyofauna show the existence of 322 species distributed in 50 families and dominated by benthic fishes (70%) of the Notothenioidei family (Figure 1) (Eastman 2005). Even though the marine fishes are represented by approximately 16,764 species, the ichthyofauna of Antarctica is only 1.9% of these species (Eschmeyer *et al.* 2010). Every year, about 100-150 new species of marine fish are described in a total of 5,000 not reported (Eschmeyer *et al.* 2010). In the Antarctic marine environment, the unknown ichthyofauna was estimated to be about 64 species by Eastman (2005). The most important molecular change in the adaptive evolution of Antarctic fish was the antifreeze glycoproteins (AFGP) in biological fluids about 5 to 14 million years ago. These class of proteins probably derived from mutation in the trypsinogen gene, is responsible for the fluidity of the biological fluids in notothenioids in subzero temperatures (Chen *et al.* 1997).

Studies of the physicochemical properties of enzymatic proteins and non-enzymatic proteins of Antarctic fishes and their distinction from their homologues encountered in temperate water fishes are corroborated to understand the physiological systems of fishes in subzero temperatures (Somero 2004). The

elevated values of enthalpy and the polymerization entropy of isolated tubulin in Antarctic fishes *Notothenia coriiceps*, *Notothenia gibberifrons* and the icefish *Chaenocephalus aceratus*, compared to those of temperate water fishes and homeothermic animals, are compatibles with the polymerization of tubulin at 0°C (Detrich III 1991). Considering the central role played by ATP generation process and the energetic demand of the physiological systems, biochemists and physiologists have concentrated their studies on the energy metabolism. Enzymes with low values of K_m and $K_{0.5}$, along with the elevated values of K_{cat} , have sustained the evolutionary metabolic compensation of the Antarctic fishes at low temperatures (Clarke 2003, Somero 2004). The enzyme phosphofructokinase (PFK) in the Antarctic fish *Trematomus bernacchii*, considered as the principal regulator enzyme in the glycolytic pathway, has $K_{0.5}$ value for the substrate fructose-6-phosphate 20 fold less at 5°C than at 20°C (Rodrigues *et al.* 1994).

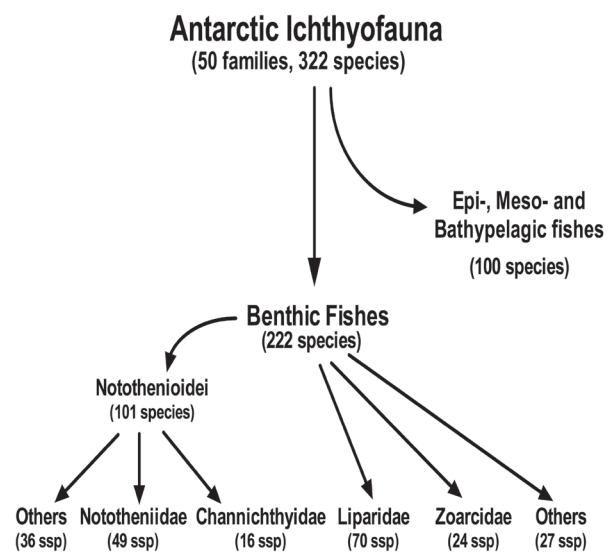


Figure 1. Diversity of the Antarctic fish fauna in the Southern Ocean. Notothenioidei, Liparidae and Zoarcidae taxa represent the largest number of benthic Antarctic fish species (60.6%). Data compiled by Eastman (2005)

Even though the Antarctic is one of the planets well preserved environment, scientists are concerned about increasing human activity in the region, particularly the localized pollution near scientific stations and near the anchorage of research and tourist ships. The sinking of Paradise Bay in 1989, near the Anvers Island in the Antarctic Peninsula spilled 600,000L of diesel oil and contaminated Antarctic fishes and invertebrate in an area of 100km² (Kennicutt II 1995, Janiot *et al.* 2003).

Discharge of sewage, burning of fossil fuels in the scientific stations, along with the leakage of small quantities of combustion oil when transferring it from ships to coastal stations are the principal sources Antarctic coastal regions pollution along with heavy metal and petroleum derivatives hydrocarbon (Duquesne & Riddle 2002, Ahn *et al.* 2003).

Following the ratification of the Protocol on Environmental to the Antarctic Treaty (Madrid Protocol) in 1991, the members of the treaty have committed themselves to environmental monitoring and have made efforts to reduce the anthropic impact of their scientific activities in the region. The Specially Managed Antarctic Area (ASMA) in the Admiralty Bay (362km²), located on the King George island (62°02'S and 58°21'W), South Shetland Archipelago, has scientific stations from 5 nations (Brazil, Ecuador, Peru, Poland and USA) and is of Site of Special Scientific Interest (SSSI N° 8) (Braun *et al.* 2001, Arigony-Neto *et al.* 2004). The human impact in the ASMA in Admiralty Bay and the environmental risks are higher during austral summer, as there is much movement of scientific and tourists ships, as well as the corresponding increase in the personnel and logistics, particularly those involving transfer of combustible from ships to scientific stations.

The monitoring of Admiralty Bay ASMA integrates goals of the National Institute of Antarctic Science, Technology and Environmental Research (INCT-APA) of the Brazilian Antarctic Program. Using a long time series of the physical, chemical and biological processes, INCT-APA is interested in understanding the natural and anthropic impacts in the region. The incorporation of biological information in the abiotic data amplifies the monitoring possibilities, predicting its effects on the biota and evaluating the environmental risk in case of contamination. All the biological responses arising from the changes in the

physical and chemical environment have their origin at the molecular level, and reflected in the upper levels of the hierarchy such as biochemical, physiological, cellular and tissues (van der Oost *et al.* 2003).

The term biomarker is used to express measurable changes in the biological tissues and fluids at the molecular, biochemical, cellular and physiological levels. The changes arise at the sub individual level and are induced by the changes in the physical and chemical environments (van der Oost *et al.* 2003, Lam 2009). The advantages of using biochemical biomarkers in environmental monitoring as compared to the biological response at higher levels (populations, communities and ecosystems) is principally on the low cost of analysis, specificity and early changes to the biological answers (Lam 2009).

The use of fishes as bioindicators in environmental monitoring programs, rests on the fact that the species are easy to identify, are distributed in distinct trophic levels and have long life. Different from the sessile organisms, fishes express the stress factors within an area and offer ample information about the environment (Whitfield & Elliott 2002, Shahidul Islam & Tanaka 2004).

Biochemical biomarkers of fishes have been used to evaluate the pollutants impact on the environment and in the recovery of contaminated environments (Regoli *et al.* 2002, Linde-Arias *et al.* 2008). The selection of the sentinel organisms and biomarkers should be used with a lot of criteria, based on the scientific studies of the desired specificity and sensitivity to the detection of the toxic stress arising from changes in the environment.

The increasing anthropic activity in the region has given rise to concerns about the pollution of environment around the scientific stations and places where ships anchor. This article summarizes the enzymatic information on the L-arginine, xenobiotic, antioxidant defense and energy metabolism in Antarctic fish and their potential to study the environmental impacts.

ENERGETIC METABOLISM

Physiologists and biochemists have studied the energy metabolism of Antarctic organisms as the ATP generation process plays a central role in the functioning of other physiological systems (Clarke 2003). In

vertebrates, three main metabolic pathways are used to maintain the tissue ATP level: a) generation of ATP from creatine phosphate; b) anaerobic catabolism of substrate (fermentation); and c) aerobic catabolism of the substrate (respiration) (Hochachka & Somero 2002a). To sustain the tissues energy requirements, the velocity of the ATP generation systems must be in synchronism with the energy demand. The metabolic rate in Antarctic fishes depends partly on the expression of the enzymatic proteins with thermal kinetic properties that retain the catalytic efficiency at subzero temperatures. The presence of enzymes with more flexible structures, with elevated K_{cat} values and low K_m have been interpreted as the homologous of those found in fishes in temperate waters, and have the adaptive advantages at low temperatures (Somero 2004). The quantity of the expressed proteins in the tissues is also an important modulator of the energy metabolism and is related to the metabolic rate in subzero temperatures (Hochachka & Somero 2002a). This strategy permits the increase in the concentration of key energy metabolic enzymes (upregulation), compensating the negative effect of low temperature on the velocity of enzymatic reactions. Haschemeyer (1983) investigating the protein synthesis in the tissues of the Antarctic fish *Notothenia coriiceps*, *Notothenia gibberifrons* and *Chaenocephalus aceratus*, found that the velocity of the process is tissue specific and is expressively less in the icefish compared to the nototheniids. The protein synthesis was related to the alimentary state as well as the adaptation mechanisms to low temperatures.

Working with the isolated hepatocytes of the high Antarctic notothenioids (*Trematomus eulepidotus*, *Trematomus pennellii*, *Trematomus lepidorhinus*, *Trematomus bernacchii* and *Artedidraco orianae*) and the zoarcid *Pachycara brachycephalum*, as well as with the sub-Antarctic *Lepidonotothen larseni*, Mark *et al.* (2005) found that the energy allocated to the RNA synthesis, protein synthesis, Na,K-ATPase activity and mitochondrial ATP synthases, calculated from the inhibition effect of the oxygen consumption, is little influenced by the temperature in the range of 0°C to 15°C. Assuming that this allocation of energy in the cell is maintained in the thermal range, the authors believed that the thermal limits could be related to the availability of O₂ and also the energetic limitations at the higher organismal levels. The Antarctic fishes

are a species with major stenothermality arising from the geographic isolation of the region and its subsequent chronic cooling (Coppes Petricorena & Somero 2007). A knowledge of the thermal metabolic plasticity limits and the degree of stenothermality of Antarctic fishes are important for understanding the impact of raising temperatures on marine ecosystems (Drinkwater *et al.* 2010, Pörtner 2002). Temperature is considered as the principal stress agent that directly effects the velocity of chemical reactions, the fluidity of biological membranes, and indirectly all the physiological systems (Hochachka & Somero 2002c). Thermal alterations in the aquatic environments affect the solubility of the dissolved oxygen (Wu 2002) and hence the respiratory system in fishes. In Antarctic fishes *Pagothenia borchgrevinki* and *Trematomus bernacchii*, an increase in temperature (between 0 e 6°C) is reflected in an increase in the velocity of oxygen consumption (MO₂) and a reduction in the osmolality and plasma glucose (Lowe & Davison 2005). On the other hand, the post-exercise MO₂ in *P. borchgrevinki* declined when the acclimatization temperature was raised from 3°C to 6°C, indicating that the availability of oxygen limits the aerobic behavior at elevated temperatures (Lowe & Davison 2006, Morrison *et al.* 2006). As the aerobic energy metabolism is the principal segment responsible for the ATP generation in mitochondria rich tissues and is limited by its utilization in the electron transfer chain (Hochachka & Somero 2002b), the relation between oxygen availability and thermal changes could prevent important physiological activities in Antarctic fishes, such as locomotion.

The MO₂ converted into energy units has been used to calculate the energy budget and the allocation of energy in the cell. The energy budget allocated for routine metabolic activities in the Antarctic fish *Pachycara brachycephalum*, does not have any significant alterations when acclimatization to temperatures between 0°C and 6°C. However, changes in the metabolic parameters suggest that the total energy demand could be exceeding the total energy obtained from the feeding (Brodte *et al.* 2006a). An increase in the mitochondrial respiration in Antarctic fishes arising from an increasing temperature, could be exceeding the ATP demand or the availability of oxygen and, consequently accelerate the formation of reactive oxygen species in the respiratory chain.

In this case, the increase in concentration of the uncoupling proteins (UCP) in the mitochondria of the Antarctic fish *P. brachycephalum*, acclimatized to 5°C, has been considered as an important factor in the protection, accelerating the elimination of protons across the mitochondrial internal membranes and minimizing the formation of reactive oxygen species (Mark *et al.* 2006).

The ability to use oxygen as the final oxidant in the metabolic processes has been associated with the tissue level of enzymes of the energy metabolism. Working with fishes from different habitats, Torres & Somero (1988a, 1988b) found a narrow relation between the velocity of oxygen consumption and the levels of the enzyme citrate synthase (CS) and lactate dehydrogenase (LDH). The enzymatic activities and the MO_2 of fishes was conducted at 10°C, using a Q_{10} of 2.0, the Antarctic fishes with elevated stenothermality (*T. bernacchii* and *Pagothenia borchgrevinkii*) showed larger values in

the CS activity as part of the cold adaptation (Torres & Somero 1988b).

The specific activity of the citric acid cycle enzymes [isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH) and citrate synthase (CS)], the glycolytic pathway [hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK) and LDH], along with other energy segments of the metabolism [pyruvate dehydrogenase (PDH), creatine kinase (CK), Na,K-ATPase, glycogen phosphorylase (GPase) and cytochrome c oxidase] in fishes have been investigated as possible biomarkers for thermal (Altringham & Johnston 1985, Brauer *et al.* 2005, Hardewig *et al.* 2004, Miller *et al.* 2009), osmotic stress (Morrison *et al.* 2006, Nakano *et al.* 1998, Sangiao-Alvarellos *et al.* 2005) and for hypoxia (Feller *et al.* 1991). The principal interrelations between the enzyme levels of the energy metabolism and the environmental stress agents is shown in Figure 2.

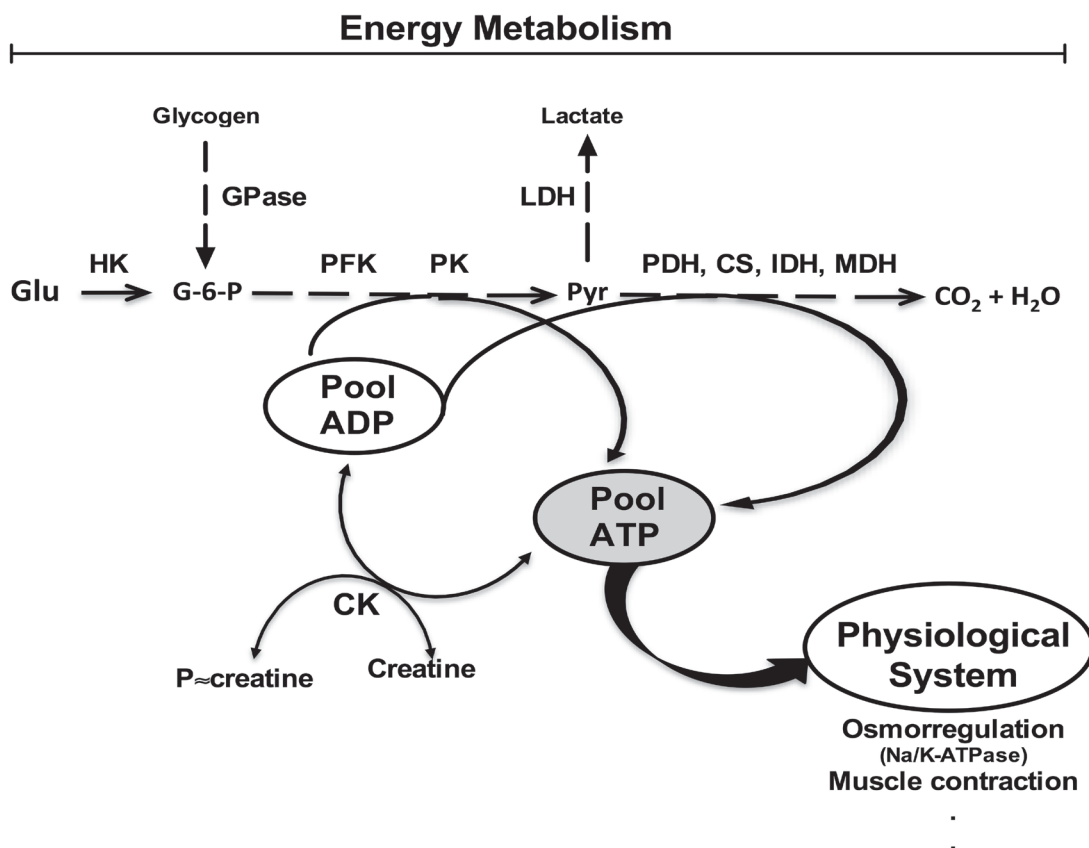


Figure 2. Interrelation between the energy metabolism, and energy demand, of the physiological systems. The glucose (Glu) oxidation in pyruvate (Pyr) and, in sequence to $CO_2 + H_2O$ is one of the main pathways of energy obtainment. The enzymes HK, PFK, PK, GPase, LDH, PDH, CS, IDH, MDH and CK has been used as biomarkers of the energy metabolism and Na/K-ATPase as biomarker of the energy demand to osmoregulation.

As enzymatic specificity permits the determination of the enzymatic activity in extracts of tissues and biological fluids, in the presence of hundreds of other proteins, most used are spectrophotometric methods, which is simple, rapid, specific and has low cost. However, the biological responses to the stress agents, are tissue and organism dependent, and require additional studies to identify the best biomarkers for a given change in the environment. Some enzymes are highly specific markers of environmental change. An example is δ -aminolevulinic acid dehydratase (ALAD) that is inhibited by lead. However, most of the enzymatic markers respond to a wide range of stress agents in the environment (Lam 2009).

Changes in the composition of non-enzymatic components related to energy metabolism in the tissue and biological fluids have also been used as biomarkers for environmental change. After acclimatization of the Antarctic fish *P. borchgrevinki* to 4°C for 5-6 weeks was accompanied by a reduction in the osmolarity and an increase in the plasma glucose from $4.6 \pm 0.7 \text{ mmol L}^{-1}$ to $5.8 \pm 1.1 \text{ mmol L}^{-1}$ (Lowe & Davison 2005). Metabolite changes in tissues and biological fluids are often accompanied by changes in the blood hormone levels, which are also used as stress markers (Fiess *et al.* 2007).

Biomarker studies must also take into consideration the energy demands of the tissues. The energy demand in some tissues, increase as a function of specific physiological activities. Unlike the locomotor system, the cerebral levels of the enzymes CS and LDH, in a variety of fishes, are not affected by the energy demand for specific activities nor the nutritional state (Kawall *et al.* 2002). However, according to Soengas & Adalgunde (2002), the cerebral metabolism of fishes is similar to those of other vertebrates and shows a elevated dependence on glucose as combustible energy. Depriving the alimentation alters the metabolic routine of this tissue, which then uses the ketone bodies derived from the hepatic metabolism of lipids. The enzyme levels of GPase, LDH and β -hydroxybutyrate dehydrogenase (β -HBDH) increase, the levels of HK, PFK and glycogen synthase (GSase) are reduced. The cerebral energy metabolism of fishes is also affected by pesticides, which reduce the tissue levels of LDH, HK and GSase, and elevate the levels of glycogen phosphorylase (GPase).

On the other hand, in teleost fishes, the muscle represents a major part (more than 60%) of the body mass. The activity of this tissue is of vital importance in the escape from the predators and prey capture (Fernández & Calvo 2009). The aerobic energetic muscular routine in these Antarctic fishes had been associated to the presence of large quantity of lipids, high mitochondrial density, oxygen consumption and the presence of mitochondrial fat droplets (Lin *et al.* 1974, Kamler *et al.* 2001, Johnston 2003). The dominant lipids present in the notothenioids tissues are triacylglycerols composed of monounsaturated and polyunsaturated fatty acids (Hagen *et al.* 2000, Kamler *et al.* 2001). High lipid content in the tissues has been associated with the maintenance of neutral buoyance of these fish (Clarke *et al.* 1984) and easy oxygen diffusion into cells as well as to the subcellular compartments (Egginton & Sidell 1989). The triacylglycerol composition of unsaturated fatty acids is strictly related with the metabolic preference in using these fatty acids as energetic fuel. The mitochondrial oxidative capacity is high for unsaturated fatty acid and the activity of the enzyme carnitine palmitoyl transferase is 2.4 times more with palmitoleoyl~SCoA than with palmitoyl~SCoA (Crockett & Sidell 1993). The enzymes fatty acyl~SCoA synthetase (Grove & Sidell 2004) and hormone-sensitive lipase (Hazel & Sidell 2004) also has preference to unsaturated fatty acids and unsaturated triacylglycerol.

Antarctic fishes have varied diet based on proteins and lipids, coming from capture of diverse Antarctic organisms, such as euphausiids, salps, small fishes, bivalves and others (Barrera-Oro 2003). Studies with Antarctic fishes of Admiralty Bay revealed that the fatty acids composition of *Notothenia coriiceps* is typical of principal Antarctic benthic species (Amphipoda, *Himantothallus grandifolius* and *Curdiea racovitzae*) that serve as food to this nothothenioid (Kamler *et al.* 2001). The levels of vitamin C and E in the blood of the Antarctic fishes *Pagothenia borchgrevinki* and *T. bernacchii* are five to six times those found in the blood of the temperate water species (Gieseg *et al.* 2000), and could be related to the important physiological role of the natural antioxidants of the monounsaturated and polyunsaturated fatty acids

The gills of Antarctic fishes, in contrast to the muscles, have a low oxidative capacity for fatty acids and use a variety of circulating energetic fuels,

especially glucose and the amino acid glutamate, to meet their energy requirements (Crockett *et al.* 1999). In this case, the oxidative deamination of the glutamate, catalyzed by the mitochondrial enzyme glutamate dehydrogenase, introduces its carbon chain into the citrate cycle and provides the aminic nitrogen in the form of ammonia for excretion. Amino acids such as aspartate and alanine, could have their aminic nitrogen channelized to α -ketoglutarate, via transamination, originating glutamate. This process permits that the nitrogen of these amino acids can be excreted in the form of ammonia through the oxidative deamination of glutamate. As urea cycle is physiologically inactive in most of the fish, the use of glutamate as ammonia acceptor in the reaction catalyzed by glutamine synthetase leads to the glutamine formation, which function as “ammonia nitrogen buffer” (Hochachka & Somero 2002a). Before the central role of glutamate in the fish metabolism, some studies with the enzyme glutamate dehydrogenase of fish revealed its potential as biochemical biomarker in relation to the presence of pesticides and heavy metals (Mary Chandravathy & Reddy 1994, Begum 2008).

Other key amino acid of energetic metabolism is the L-arginine. In fishes this amino acid should be present in diet and represents about 6% of the muscle amino acids (Ball *et al.* 2007). Of the various end products of the L-arginine metabolism, the transfer of its amid group to the amino acid glycine initiates the formation of creatine, an important “energy buffer” in fishes, marked by the reversible transfer of the high energy phosphate of ATP to creatine, catalyzed by the enzyme creatine kinase. This high energy phosphate reservoir meets the energetic requirements of the fish muscles for about 30 seconds, independent of the oxygen availability (Gibb & Dickson 2002). Studies of the effect of fungicides (Li *et al.*), genotoxic and cytotoxic pollutants (Napierska *et al.* 2009) and heavy metals (Almeida *et al.* 2002) on the fish metabolism indicate that the enzyme creatine kinase is a potential biomarker of environmental pollution.

On the other hand, the hydrolysis of L-arginine, catalyzed by the enzyme arginase, introduces the non protein amino acid L-ornithine in the intermediate metabolism and permits the synthesis of polyamines, proline and glutamate (Jenkinson *et al.* 1996). In addition to participating in the intercellular transfer of amino groups, establishing the equilibrium and

composition of the cellular amino acids, along with the synthesis of glutamine in the ammonia buffer (Hochachka & Somero 2002a), glutamate in the blood is a potential energy substrate in the gills of Antarctic fishes (Crockett *et al.* 1999).

L-ARGININE METABOLISM

For fishes, dietary amino acid L-arginine is essential for various metabolic activities among which growth, immunity improve, larval metamorphosis, reproduction, resistance to stress factors and infections (Li *et al.* 2009). The principal tissue demands of L-arginine are for protein synthesis, nitric oxide synthesis (NO), phosphocreatine and L-ornithine (Hird 1986, Wu & Morris 1998).

The recognition of food and predators by fish, as well as their defense response and pheromonal communications are activated by detection of odors (Hashiguchi *et al.* 2008). The capacity of the olfactory system to discriminate odorant amino acids including L-arginine (Lipschitz & Michel 1999) can be related with the selection of food rich in essential amino acids. With few exceptions, the requirement of fishes for L-arginine is 1.2 to 1.5g/100g of food (Ball *et al.* 2007). In the young turbot (*Psetta maxima*) the L-arginine during the growth phase is used principally for protein synthesis (Fournier *et al.* 2002, Fournier *et al.* 2003, Gouillou-Coustans *et al.* 2002). Considering that urea cycle is non-operating in these fishes, the quantity of urea excreted is related with dietary levels of L-arginine and, the hydrolysis of L-arginine has been accepted as the principal ureogenic pathway in turbot. The strong relation between the dietary levels of L-arginine and the arginase expression in the liver indicate that this is the principal enzyme responsible for ureogenic activity in turbot. Though this may not be the metabolic behavior common to all fishes, Raymond & DeVries (1998), have registered a possible relation between the diet rich L-arginine and the ureogenic activity of Antarctic fishes.

The control of L-arginine in biological fluids has been related with the tissue argininolytic activities and arginase subcellular localization. In ureotelic animals, the hepatic arginase levels have been related to the urea cycle activity and the detoxification of ammonia (Jenkinson *et al.* 1996). Considering that hepatic tissues and the renal function as metabolic

filters for the entry and exit of various compounds, respectively, the presence of elevated levels of arginase in the distal renal segment and the liver of

Antarctic fish *Notothenia neglecta* (Figure 3) could be related to the systemic control of L-arginine (Rodrigues *et al.* 2006).

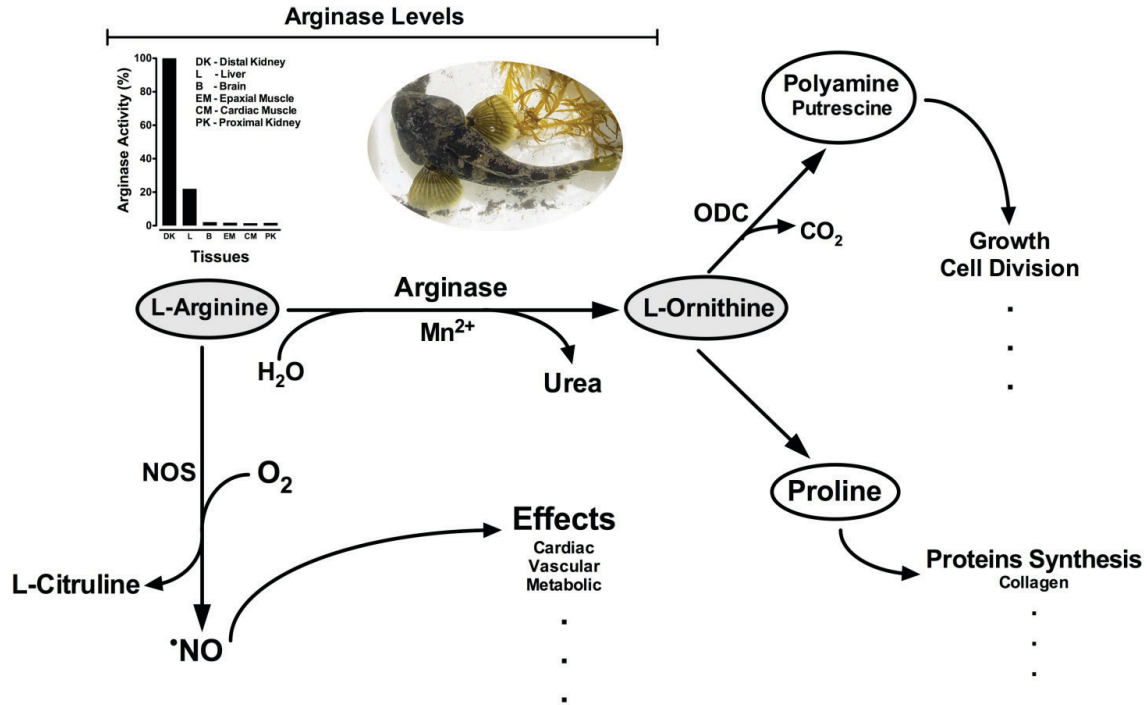


Figure 3. L-arginine metabolism. Virtually in all tissues Arginase and nitric oxide synthetase (NOS) compete for the amino acid L-arginine. The nitric oxide (*NO) synthesized from L-arginine has many physiological effects. Non-protein amino acid L-ornithine is essential to polyamine and proline. Antarctic fish *Notothenia coriiceps neglecta* has high levels of the arginase in the kidney and liver, data from (Rodrigues *et al.* 2006).

The hydrolysis of L-arginine also provides the non-protein amino acid L-ornithine for metabolism of the polyamines and proline. The decarboxylation of ornithine to putrescine, catalyzed by the enzyme ornithine decarboxylase (ODC) which is considered as the limiting step in the synthesis of polyamines. Found in all vertebrate cells, the polyamines participate in control of cell division and modulate the genes activity involved in cell growth (Moinard *et al.* 2005), hence the growth and tissue regeneration in fishes has been associated with the synthesis of these compounds (Koza *et al.* 1992, Berge *et al.* 2002). The elevation of cerebral polyamine levels in goldfish *Carassius auratus*, during it acclimatization to 5°C for 2 months, indicated that these polyamines have possible protection effect in the cold adaptation of goldfish. Within the observed biochemical responses, the increase in the levels of ODC and L-ornithine during the first 10 days of acclimatization has drawn

attention (Notari *et al.* 2004). Proline synthesis is not yet totally understood, however the synthesis of proline is capable to sustain the collagen synthesis (21% proline + hydroxyproline) for fish growth, considering that proline is not essential amino acid (Li *et al.* 2009).

After Ignarro *et al.* (1987) and Palmer *et al.* (1987) independently confirmed the chemical nature of the “endothelium-derived relaxing factor”, originally described by Furchgott & Zawadzki (1980) was the nitric oxide (*NO), later Palmer *et al.* (Palmer *et al.* 1988) demonstrated that L-arginine is the substrate used in the *NO synthesis. The knowledge about the synthesis and the *NO physiological effects were amplified in the last 20 years. The L-arginine oxidation reaction forming L-citrulline and *NO is catalyzed by enzyme nitric oxide synthases (NOS) in almost all tissues (Jobgen *et al.* 2006, Mori & Gotoh 2000). Three isoforms of NOS have been identified

and denominated as neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3). The control of NO synthesis is regulated at transcriptional, post transcriptional (mRNA stabilization), post-translational (phosphorylation, glycosylation) and at level of substrates and cofactors (L-arginine) (Balligand *et al.* 2009). NO has a wide tissue specific effect on physiological systems. In addition to its effects on the vascular endothelium and the cardiac muscle, NO also affect the metabolism of carbohydrates, lipids, proteins and the interorgan metabolism of the energy substrates (Jobgen *et al.* 2006). As both arginase and NOS compete for the L-arginine substrate, the tissue argininolytic activity has been postulated as an important modulator of NOS activity in the endothelial inflammatory processes (Bachetti *et al.* 2004), as well as the myocardial contraction mediated by NO (Jung *et al.* 2006).

Nitric oxide is one of the oldest molecular signal in animal tissues for inter and intracellular communication. Even though most of the physiological and pathological studies of the NOS/ NO system were in mammals, studies in fishes reveal some particulars that make these organisms interesting biological models of the diversity of NO action at the molecular level (Agnisola & Pellegrino 2007). In goldfish, Wang & Mangel (1997) showed that endogenous NO modulates the horizontal cone cells in the retina and regulates the circadian rhythm. The iNOS expression in the liver macrophages and the heterophylic granulocytes in the kidneys of the rainbow trout has been interpreted as a defense mechanism of this fish (Barroso *et al.* 2000) and as in mammals, NOS activity modulates the cardiac function in the fish *Anguilla anguilla* (Imbrogno *et al.* 2001).

The central role of NO as a signal in various physiological activities is modulated by NO dioxygenation of hemoglobin, myoglobin and flavohemoglobins (Figure 4), which protect against the toxic effect of nitrogen (Gardner 2005). The absence of hemoglobin in the fish family Channichthyidae and of myoglobin in some species of the family is unique among vertebrates (Kock 2005, Sidell & O'Brien 2006). Investigating the NO metabolism in transgenic rats without myoglobin and with the over expression of iNOS in the heart, Gödecke *et al.*

(2003), found that this condition leads to hypertrophy and the excess of NO to cardiac failure. However, this loss of myoglobin expression in the cardiac muscle is not an obstacle for the adaptation of some species of fish of the Channichthyidae family. The absence of respiratory pigments in the blood and tissues of icefish reduces the protection against stress from nitrosatives compounds as compared to red blooded Antarctic fish. Comparing fishes in the suborder Notothenioidei, Morlá *et al.* (2003) showed that the expression of eNOS and nNOS in the skeletal muscle is more in white blooded fish than in red blooded fish, reflecting a greater potential to generate NO .

Understanding how fishes of the Channichthyidae family, transport, supply and utilize O_2 as a final oxidizing agent in the respiratory processes, without the traditional respiratory pigments, has been an important question (Bacila *et al.* 1989, Di Prisco 2000). Compared to the red blooded Antarctic fishes, icefishes have a more dense microvasculature and capillaries with wider luminal diameter (Fitch *et al.* 1984, Eastman & Lannoo 2004), elevated number and mitochondrial density (Sidell 1998, O'Brien & Sidell 2000), and elevated cardiac debit (Sidell & O'Brien 2006). As NO has a remodeling effect on the capillary network growth (Conway *et al.* 2001), and acts as an activation messenger in the mitochondrial biogenesis process (Nisoli *et al.* 2004), it should be expected that NO_x (nitrate + nitrite) in the blood of icefish should be higher than those in the red-blooded Antarctic fish. However, Beers *et al.* (2010) found that the levels of NO_x , which indicate the levels of NO , in the blood as well as in tissues of icefishes were much less than those found in red-blooded fishes. It is possible that NOS is being down regulated in tissues, which could be responsible for the low expression. The real NO tissue concentrations, that defines the physiological effects, depending on the equilibrium between the synthesis and degradation of nitric oxide, hence the absence of the respiratory pigments in the icefishes reduces the degradation of NO as compared to the red blooded fishes.

Hence, a number of open questions remain to be solved regarding the metabolism of NO in Antarctic fishes. One of them is the control of the NOS enzyme activity by arginase. Various studies in mammals, particularly humans, indicate that the control of

L-arginine levels by arginase is an important control point of NOS (Palm *et al.* 2008, Jung *et al.* 2010).

ANTIOXIDANT DEFENSE

Polar waters have high oxygen concentration (0.18-0.36mmol/L) due to increased oxygen solubility at lower temperatures (Hardy & Gunther 1935). Since the formation of reactive oxygen species (ROS) usually increases in biological systems with increased oxygen concentration, formation of free radicals could be easier in polar marine organisms (Ansaldo *et al.* 2000). However, little is known about antioxidant defense system in polar fishes [reviewed by Abele & Puntarulo (2004)]. Antioxidants prevent cellular damage caused by metabolically and environmentally produced ROS such as hydrogen peroxide (H₂O₂), superoxide anion radical ([•]O₂⁻) and hydroxyl radical (Halliwell & Gutteridge 1999). Antioxidant include enzymes such as superoxide dismutase (SOD), which catalyzes the dismutation of [•]O₂⁻ to water and oxygen; catalase, which reduces H₂O₂ to water and oxygen; and glutathione reductase, which regenerates reduced glutathione used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme glutathione peroxidase. Vitamins E and C are non-enzymatic antioxidants (Halliwell & Gutteridge 1999). The formation of ROS and the antioxidant defense are summarized in the Figure 4.

Comparing SOD and catalase activity in the tissue of Nototheniidae (red-blooded) and Channichthyidae (white-blooded) fishes families, Witas *et al.* (1984) and Cassini *et al.* (1993) found differences in SOD activities between these fish groups. Ansaldo *et al.* (2000) investigated the activity of SOD, catalase and glutathione peroxidase in the liver, gills, muscle and heart and found that, Channichthyidae fish often have low catalase activity in these tissues and high SOD activity in gills compared to Nototheniidae. The latter have high levels of SOD in blood and glutathione peroxidase in the gills compared to white-blooded fish. The erythrocytes usually contain a significant SOD fraction. However, it is important to emphasize that white-blooded fish have very few erythrocytes without hemoglobin and consequently low SOD levels in the blood. The same authors found that vitamin E content is exceptionally high in the liver of *Notothenia rossii* in relation to same tissue of other

Antarctic fishes investigated and it is clearly high in the heart and muscles of Cannichthyidae compared to Nototheniidae. Investigating antioxidant and cytochrome c oxidase enzymes in the liver of Arctic and temperate teleosts, Speers-Roesch & Ballantyne (2005), found that cold-adapted fish have reduced antioxidant enzyme when compared to temperate species, although cytochrome c oxidase activity was higher in polar than temperate zone fish. Analyzing the data of Ansaldo *et al.* (2000), Fujisawa *et al.* (2010) concluded that great aerobic demand is not directly compensated with higher antioxidant enzyme activity. Thus, cold-adapted teleosts may have greater dependence on other antioxidant molecules such as vitamin E for defense against ROS damage.

Several processes such as transportation across the membrane, molecular signaling and electron transport chains of mitochondria are reliant on the physicochemical structure of cellular membranes. Ectothermic organisms regulate their membrane properties in response to temperature by altering the lipid composition of their membranes. Cold adaptation and low temperature acclimation are achieved by increasing content of low molecular weight, unsaturated and branched-chain fatty acids in their cell membranes. However, this modification renders cold-adapted ectotherms more vulnerable to oxidative damage since membrane unsaturated lipids, particularly poly-unsaturated fatty acids, are a primary target for ROS in the initiation of radical-chain lipid peroxidation (Fujisawa *et al.* 2010). Lipid hydroperoxides are unstable and their decomposition products, such as malondialdehyde, can damage cellular components, including DNA (Marnett 1999, Fujisawa *et al.* 2010). A specific constituent of vitamin E designated “marine-derived tocopherol” (MDT) occurs exclusively in marine organisms, being particularly abundant in cold-water fish, was also found in Antarctic fish (Dunlap *et al.* 2002). These authors investigated two Channichthyidae species and *Gobionotothen gibberifrons* (Nototheniidae). They found that Channichthyidae species had a lower total amount of vitamin E in tissues such as liver and spleen than *G. gibberifrons*, but a higher percentage of MDT in these and other tissues (gonads, the pectoral and myotomal muscles) than the Nototheniidae specie. Moreover, this Nototheniidae showed a high amount of α -tocopherol in the liver (Dunlap *et al.*

2002). Comparing antioxidant properties of MDT in relation to α -tocopherol in fish oil at 0°C revealed that MDT has greatest reactivity to peroxy radicals when measured at low rates of radical flux (Yamamoto *et al.* 2001). MDT is likely to have greater diffusion than α -tocopherol in cold viscous lipids providing evidence that it reduces lipid peroxidation in marine ectotherms inhabiting a cold-water environment (Yamamoto *et al.* 2001, Dunlap *et al.* 2002).

Nitric oxide and superoxide anion form peroxy nitrite, a strong biological oxidant and nitrating compound that triggers cytotoxic effects such as lipid peroxidation, protein nitration and oxidation, DNA oxidative damage, activation of matrix metalloproteinases, and inactivation of several enzymes (Liaudet *et al.* 2009). To keep appropriate $\cdot\text{NO}$ level in the cell, heme protein $\cdot\text{NO}$ dioxygenases (NODs) catalyze the reaction of O_2 and $\cdot\text{NO}$ to yield the biologically benign nitrate anion (NO_3^-). Hemoglobin and myoglobin exhibit NOD activity and they also have antioxidant role in mammals (Schopfer *et al.* 2009). However, red-blooded Antarctic notothenioids have fewer erythrocytes and reduced hemoglobin concentration than temperate and tropical species. Moreover, 16 Icefish species of Channichthyidae lacked hemoglobin (Russo *et al.* 2010). Myoglobin is also lacking in skeletal locomotory muscle in most of notothenioid and in cardiac muscles in some species of the group (Sidell *et al.* 1997). In anemia induced *Notothenia coriiceps* (red-blooded notothenioid), significantly higher concentration of nitric oxide metabolites in their plasma was due to decrease of $\cdot\text{NO}$ degradation (Borley *et al.* 2010). In this case, $\cdot\text{NO}$ mediated induction of genes related to angiogenesis to compensate lower oxygen carrying capacity in the blood of anemic fishes (Borley *et al.* 2010). However, excessive production of $\cdot\text{NO}$ in the absence of NOD activity provided by hemoglobin and myoglobin could be deleterious due to potential formation of peroxy nitrite. Recently, gene for neuroglobin, a monomeric heme-containing globin, which is able to bind oxygen and $\cdot\text{NO}$ (Brunori *et al.* 2005) was found in the brain of red-blooded notothenioids and most species of icefishes (Cheng *et al.* 2009). Although the physiological function of neuroglobin is still unknown, ROS scavenging role in an extreme oxygen-rich environment is an attractive hypothesis.

The most studied oxidative stress biomarkers are glutathione-S-transferase (GST), catalase, SOD and glutathione peroxidase activities. Additionally, DNA strand breaks caused by the generation of toxic metabolites or excessive ROS production can be detected with the comet assay – a biomarker of genotoxicity (Nahrgang *et al.* 2010). Indices of oxidative stress have been investigated in fishes and include determinations of: a) lipid peroxides and end products of lipid peroxidation, such as malondialdehyde, as a marker of oxidative damage in the lipid fraction; b) protein carbonyls as indicator of oxidative damage to proteins; and c) total thiols content, because free thiols groups are considered part of cell protection against ROS and related with many redox-sensitive processes (Lushchak & Bagnyukova 2006). The glutathione is a low molecular weight thiol, which status represents the most important indicator and determinant of the cellular redox environment (Schafer & Buettner 2001). Nevertheless, indices of oxidative stress in Antarctic fishes have been few reported. Heise *et al.* (2007) determined thiobarbituric reactive substances (TBARS) as a marker of lipid peroxidation, and also protein carbonyls, reduced and oxidized glutathione, iron content (catalyst for Fenton reactions that enhances formation of hydroxyl radicals and leads lipid radical formation), lipid radicals, α -tocopherol, antioxidant enzymes (SOD and glutathione peroxidase) and tert-butyl hydroperoxide-initiated chemiluminescence rate (indicates imbalance between pro and antioxidant processes resulting from depletion of antioxidant compounds like glutathione, vitamin E and vitamin C in *Pachycara brachycephalum*. This species is an endemic Antarctic eelpout species and non-notothenioid teleost (Brodte *et al.* 2006b). Heise *et al.* (2007) comparing *P. brachycephalum* acclimated at 0°C and 5°C for 11 weeks, verified that only TBARS level and glutathione peroxidase activity was increased in acclimated fishes at 5°C whereas other parameters were not significantly different between both groups. These authors also compared antioxidant defense between *P. brachycephalum* and *Zoarces viviparus* (temperate eelpout) and concluded that the Antarctic eelpout has higher antioxidant capacity, including high degree of glutathione oxidation, than the temperate eelpout.

Nahrgang *et al.* (2010) investigated mRNA expressions of genes for xenobiotic metabolism and antioxidant defense in Arctic fish (*Boreogadus saida*) after exposure to crude oil. Expression of genes for catalase, cytosolic superoxide dismutase,

phospholipid hydroperoxide glutathione peroxidase, and catalase activity was investigated. However, they were poor for oil monitoring purposes because the lack of clear pollutant concentration-dependent responses.

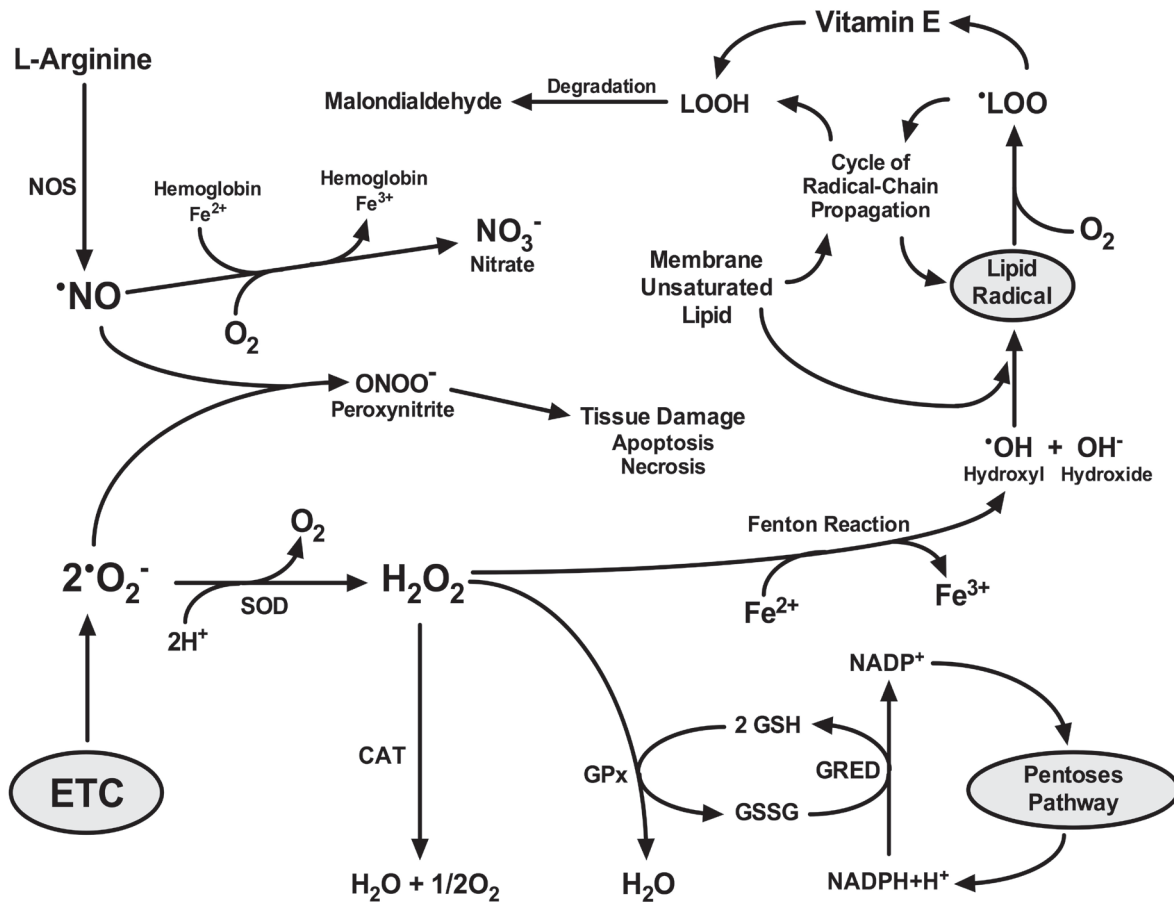


Figure 4. Formation of reactive oxygen species and antioxidant defense. CAT (catalase), GPx (glutathione peroxidase), GRED (glutathione reductase), NOS (nitric oxide synthase), SOD (superoxide dismutase), GSH (reduced glutathione), GSSG (oxidized glutathione), •OH (hydroxyl radical), •LOO (lipid peroxy radical), LOOH (lipid hydroperoxide), •NO (nitric oxide). Vitamin E is an efficient terminator of free radical propagation in membrane lipids.

Chen *et al.* (2008) carried out transcriptome analyses between *Dissostichus mawsoni* (Nototheniidae) and non-notothenioid warm-water teleost fishes to identify cold-specific expressed genes. The results revealed upregulation of several genes related to antioxidant defense, which was assembled in 3 groups: a) gene related to ROS scavenging such as for phospholipid hydroperoxide glutathione peroxidase, peroxiredoxin-5, superoxide dismutase and haptoglobin, that alleviates hemoglobin-driven oxidative stress in blood cells; b) antiapoptotic

genes for Bax Inhibitor 1 (BI-1) and Translationally Controlled Tumor Protein (TCTP) with known roles in mitigating ROS-mediated apoptosis and cellular oxidative stress; c) genes involved in iron transport and storage: for ferritin and serotransferrin that bind free iron and the small peptide hormone hepcidin, that reduces iron absorption. The expression of these genes would be consistent with the need for controlling free Fe²⁺-catalyzed ROS generation (Chen *et al.* 2008). These results open a new range of targets for oxidative stress biomarkers in Antarctic fishes.

XENOBIOTICS IN ANTARCTIC ENVIRONMENT

Organisms are constantly exposed to compounds such as synthetic drugs, poisons and antibiotics which have no metabolic function and would be harmful if accumulated in the cells. These potentially damaging compounds are often referred as xenobiotics. Some xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), are resistant to degradation and these compounds are listed as persistent organic pollutants (Testa & Krämer 2006, Corsolini 2009).

PAHs are present in petroleum, coal, and are by products of fuel combustion. Some of them are carcinogenic, mutagenic and teratogenic. They are lipophilic and the larger compounds are present in the soil and sediments as they are less soluble in water, have low volatility and hence are less prone to evaporation. PAHs are the most widely spread organic pollutants (Hodgson 2004, Fetzer 2007). PCBs are synthetic organic chemicals with low water solubility, the breakdown is inversely related to the degree of chlorination where as lipophilicity is directly related. Lighter PCBs are more likely to evaporate into the atmosphere and heavy ones settle into sediments. Fish and marine mammals such as seals and whales accumulate PCBs, and may reach levels thousand times higher than in water (Hodgson 2004).

Anthropogenic influence is closely related to the presence of PAHs in the marine environment (Kennicutt II 1995, Soclo *et al.* 2000, Ferguson *et al.* 2003). Even though Antarctica remains a relatively pristine location, with ever expanding scientific activity, fisheries and growing tourism it is vulnerable to pollution. Accidents of tourist ships MS Nordkapp and MS Explorer in 2007 are clear examples (Curtosi *et al.* 2007). Pollution is responsible for presence of measurable amounts of PAHs and PCBs. About 90 million liters of fuel used by the scientific research activities ever year, of which 75% is diesel fuel, this leads to the release of hydrocarbons directly into the environment (Cripps 1992, Cripps & Shears 1997).

The first report of contamination in the Antarctic ecosystems was in 1966 (George 1966, Risebrough *et al.* 1968). Over the last fifteen years many studies of hydrocarbon contaminants associated with research stations were conducted. Kennicutt II (1995) analyzed

sediment, bivalves, limpets and fish collected from Winter Quarters Bay (WQB) at McMurdo Station, Palmer Station and remote sites of McMurdo. Widely occurring PAH and unusually high concentrations of PCB were observed in the sediment of WQB other than accumulation of PCBs in the organisms of this region. This demonstrates that PCBs have accumulated in the Antarctic food web at these locations. Sediment and organisms from most control and remote sites showed little or no PCBs. This indicates that long distance atmospheric transport plays a minor role in the dispersal of contaminants and the most likely source of contamination is human activity. This study also shows that intertidal zones are particularly vulnerable to exposure due to the nature of contaminant release and is most probable site of long term chronic exposure. Intertidal limpets collected at Palmer Station had higher concentrations of PCBs than subtidal limpets, this also suggests runoff source.

Weber & Goerke (1996) analyzed various Antarctic fish species for ten non-polar xenobiotics compounds and found hexachlorobenzene (HCB) concentrations expressed on the basis of extractable organic matter did not differ from those of North Sea fish. It is possible that PCB are distributed globally by the atmospheric processes and condense in the Polar Regions. Martins *et al.* (2004) reported the concentrations of PAHs range from 9.45 to 270.5ng g⁻¹ in surface sediments near the Brazilian station in Admiralty Bay. The highest values occurred at sites 20 meters from the out fall and lower concentrations from sites farther away than 50 meters. Martins *et al.* (2004) in his work showed that the aliphatic and PAHs concentrations were, in general, similar in other Antarctic areas.

Jubany station in Potter Cove in King George Island, South Shetland Islands has been operating for more than 50 years, and other than visits of large ships, is also involved in tourist activities. Potter Cove is therefore, exposed to both chronic and acute events of hydrocarbon spillages and contamination (Vodopivec *et al.* 2008). Curtosi *et al.* (2009) studied levels of 25 PAHs at Potter Cove in suspended particulate matter (SPM), surface sediment and marine organisms as fish (*Notothenia coriiceps*) of pelagic fauna, bivalve (*Laternula elliptica*) a filter feeder and intertidal limpet (*Nacella concinna*) a grazer of benthic community.

In sediments, PAHs were reported to be similar to those of low level pollution sites, 14-20ng g⁻¹ dw in Signy Island (Cripps 1992) and 14-280 ng g⁻¹ dw at Admiralty Bay (Martins *et al.* 2004). Whereas sites studies near shore had levels closer to 21-1572ng g⁻¹ dw at Arthur Harbour (Kennicutt II *et al.* 1992) or 1100-2100ng g⁻¹ dw near McMurdo station (Negri *et al.* 2006). The total levels of PAHs in SPM were similar to sediment samples agreeing with those found in coastal soils (Curtosi *et al.* 2007). This data support the hypothesis that fine soil particles absorb PAHs and transported from contaminated land sites to cove by summer melt water. The PAHs concentrations (below 200ng g⁻¹) exhibited in *N. corriceps* tissues were low compared to the environment levels and higher levels of PAH were in the liver tissue when compared with other tissues.

Persistent organic pollutants (POPs) transport in Antarctica can be explained by global POPs models (Wania & Mackay 1995, Wania & Su 2004). The low temperatures and winter darkness slows down the degradation of POPs and traps them in ice. POPs are released into the environment with ice melt and enter into the trophic webs. Polar organisms do not have a well defined system to deal with foreign

substances and their enzyme system for detoxification is not yet fully understood (Corsolini 2009). Hence, the accumulation of foreign substances, and their potential toxicity could be good indicators of the biological impact of pollutants.

Monitoring the contaminants (PCBs, PAHs and heavy metals) occurring naturally or from human activity is very difficult. Examining the changes in the biochemical response, particularly the impairment of the physiological processes in organisms exposed to contaminants could be used to monitor the changes in the environment. One promising approach could be to examine the impairment of the physiological processes in organisms exposed to contaminants. Biotransformation and antioxidant enzymes involved in detoxification of xenobiotics and their metabolites have been investigated as potential biomarkers.

XENOBIOTICS METABOLISM

The toxicity of the xenobiotic compounds to an organism can be affected by metabolism, where beneficial detoxification or harmful bioactivation can occur. Possible sequences of biotransformation of xenobiotics are show in the Figure 5.

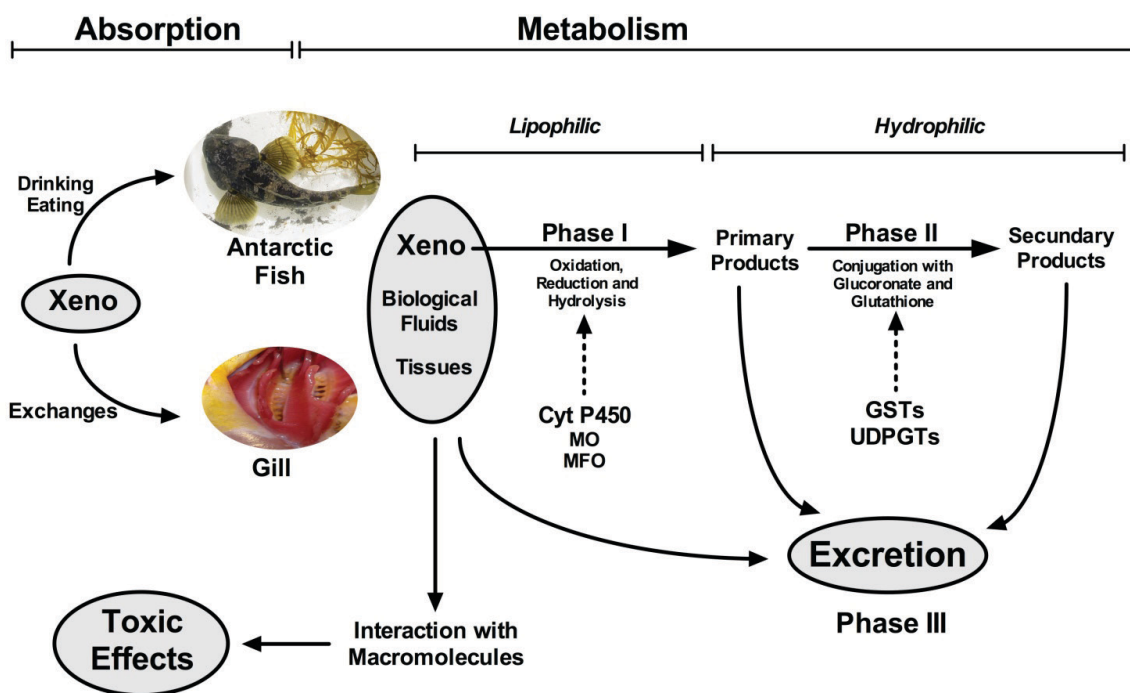


Figure 5. Metabolic pathways of xenobiotics (Xeno) in Antarctic fish. The virtually main access of xenobiotics to the tissues and biological fluids in fish is through gills and digestive tract which are the principal routes. The oxidation, reduction and hydrolyses of xenobiotics (phase I) form more soluble primary products which can be more, less or equally toxic. The conjugation with glucuronate and glutathione (phase II) origin more hydrophilic secondary products and can be excreted preferably through bile (phase III).

In fish, liver is most commonly involved in the detoxication of foreign compounds, which makes it as target organ. The xenobiotic metabolism is divided into three phases (Figure 5), phase I involves cytochrome P450 oxidase (CYP450) which introduces reactive or polar compounds, phase II the modified compounds are then conjugated to polar compounds. These reactions are catalyzed by glutathione S-transferase (GTS) and UDP-glucuronyl transferase (UDPGT). The conjugated hydrophilic compounds of phase II are pumped out or may be further metabolized before being excreted in phase III (Jakoby & Ziegler 1990, Commandeur *et al.* 1995, van der Oost *et al.* 2003).

The xenobiotic compounds in phase I are catalyzed by microsomal monooxygenase (MO) enzymes, also known as the mixed function oxidase (MFO) system, which involved mainly the cytochrome P450 (CYP450), cytochrome b5 (cyt b5) and NADPH cytochrome P450 reductase (P450 RED). CYP450 enzymes are a widely spread super family of heme proteins; some of these enzymes are especially inducible by their substrates (Nelson *et al.* 1993, Bucheli & Fent 1995). A subfamily, CYP1A is recognized as an important biomarker to exposure to PAHs, PCBs and halogenated dibenzo-p-dioxins (Goksøyr 1995). In most fishes CYP1A expressed is very low and are effectively induced by many planar hydrocarbons through their binding to cytosolic arylhydrocarbon receptor (AhR) and this receptor binds with nuclear translocator and acts as transcriptional activator in the promoter of CYP1A1 and other responsive genes (Handschin & Meyer 2003). The study of *T. bernacchii* exposed to laboratory β -naphthoflavone (β -NF) indicated that hepatic CYP1A showed higher sensitivity after β -NF exposure and higher methoxyresorufin O-deethylase (MEROD) activity than ethoxyresorufin O-deethylase (EROD), phase II enzymes were not affected by the exposure, indicating that their modulation is not mediated by Ah receptor (Di Bello *et al.* 2007).

T. bernacchii is an indicator species of CYP1A induction to assess the level of pollutants impact on local biota. Results of liver tissues from fish collected from WQB and Backdoor Bay (BDB) which is remote from human activities showed fluorescent aromatic compounds (FACs) two fold higher and 37 fold higher CYP1A expression in WQB, the CYP1A

induction correlated positively with biliary FACs, even though McMurdo station cleaning program was begun in 1988 the results of present study, show that a significant contamination still exists. Since hydrocarbon breakdown is slow in Antarctic due to cold temperatures, the contamination effect remains for a considerable period of time. In fish CYP1A is catalytically expressed principally through the EROD activity (Miller *et al.* 1999).

Lurman *et al.* (2010) also evaluated the impact of environmental pollution on fish in McMurdo, using as biomarkers EROD, pentosylresorufin O-deethylase and biliary naphthalene and phenanthrene with samples of *T. bernacchii* and *Trematomus hansonii* from a control site BDB and WQB. The differences in EROD activity between polluted and control locations seem to be similar with other previous works. The WQB *T. hansonii* EROD activity was 101 pmol min⁻¹ mg⁻¹ and 14 pmol min⁻¹ mg⁻¹ for BDB *T. hansonii*. The EROD response is not proportional to the level of sediment pollution, where PAH is high. This may be due to differences in species, where *T. bernacchii* may be accounted for by inhibition of EROD activity by PCBs which are also high in WQB, the same was also observed with copper and cadmium also 5 to 10 fold higher in WQB. Many authors have documented inhibition of CYP1A by PCBs, and this reduction of EROD in *T. bernacchii*, this could be a confounding factor. Lurman *et al.* (2010) also observed in his study that *T. bernacchii* collected from BDB and caged in WQB for 2 and 4 weeks, at 2 weeks had highest EROD activity and with 4 weeks caged the fish had greater PCB, copper and cadmium burden, thus suppressing the EROD response. Similar results are obtained in work with *T. bernacchii* from Gerlache Inlet, Terra Nova Bay, Ross Sea. Induction caused by PAH was suppressed by co-exposure with Cd and Cu, these results reveal a complex interaction pathway between different co-exposures (Benedetti *et al.* 2007).

Lepomis species, commonly known as sunfish, which is abundant in North America, was studied by Brammell *et al.* (Brammell *et al.* 2010) as potential biomonitoring organism for halogenated aromatic hydrocarbon and PAH, using induction of CYP1A and phase II enzymes as GST and UDPGT. *Lepomis* hepatic CYP1A exhibited weakly induction for PCB and highly induced for PAH indicating that sunfish could be excellent biomarker for PAH pollution.

Whereas phase II enzymes remained unchanged may be indicating that these enzymes are not sensitive biomarkers for pollutants. Recently *Lipophrys pholis* has been pointed out as PAH pollution monitor. Exposure to PAH showed sufficient induction of EROD activity together with accumulation of FAC in the bile, suggesting use of these fish in pollution monitoring for chronic contamination as well for oil spill accidents of moderate scale. Another point to take into account determination of EROD and FACs is not expensive, rapid and easy to use (Santos *et al.* 2010). Three spined sticklebacks (*Gasterosteus aculeatus*) were exposed to humic substance (organic molecules that constitute the major part of the dissolved/natural organic matter in freshwater) of different origins and EROD activity was measured in liver and gills. EROD induction was observed primarily in gills, this would be significant to utilize EROD activity as biomarker (Andersson *et al.* 2010).

The available literature about xenobiotics metabolism in Antarctic fish is mainly on *T. bernacchii*. The research with other endemic fish of Antarctica would give a more accurate data about utilization of xenobiotic metabolism enzymes as biomarkers. The available data of phase I and phase II enzymes indicates that phase I has more sensitivity on pollutants effects than phase II, so phase I enzymes like EROD are preferred in using as biomarkers in Antarctic fish.

HEAT SHOCK PROTEIN

The protein activity is determined by the sequence of amino acids. The correct folding of the amino acid sequence and the compartmentation of the newly formed protein, depend on the protein assistants called chaperons. This class of proteins also prevent the aggregation of denatured protein structures and help refold them to the native state (Hochachka & Somero 2002a). The term chaperone has its origin in the studies of the heat shock protein expression (Hsp). The expression of Hsps under thermal stress, in various organisms, help stabilize the denatured proteins, refold proteins that are denatured, and give directions those proteins that have suffered irreversible denaturation to the degradation pathways, thus avoiding cellular damage (Glickman & Ciechanover 2002, Kalmar & Greensmith 2009).

Comparing the gene expression of Hsps in ectotherms inhabiting thermally stable aquatic

environments with those inhabiting moderately stable and highly variable thermal aquatic environments, Tormanek (2010) found that ability to induce the thermal protective response were absent or is very low in the organisms in the stable environments. Hofmann *et al.* (2000) investigating the induction of thermal shock responses in *T. bernacchii* exposed to 10 °C for 2 hours, found that this highly stenothermic Antarctic notothenioid does not have the capacity to induce the synthesis of Hsp70. This apparent loss of function was interpreted as a consequence of the evolution to stable subzero temperatures over a period of about a million years. Working with *T. bernacchii*, Buckley *et al.* (2004), found that the cells when exposed to thermal shock of up to 12 °C, failed to induce an increase in the mRNA hsp79 levels, the heat shock factor 1 activity (hsf1), or for that matter any other class of hsp. The inability of *T. bernacchii* to up-regulate the expression of various types of heat shock proteins is quite wide and is not limited to just Hsp70. For instance, the co-chaperone Hsp40 of this notothenioid is weakly induced (1.1 times) under conditions of thermal stress (Buckley & Somero 2009).

The inability of the Antarctic notothenioid, to up-regulate the Hsp, probably occurred after the separation of the Bovichtidae family from the family of Antarctic notothenioid fishes. Through the long evolution, the thornfish *Bovichtus variegatus* and the non-Antarctic notothenioid, which inhabit the marine environments around New Zealand, South America and Australia, retained the capacity to synthesize them RNA of Hsp70, but only *B. variegatus* has a robust synthesis of the Hsp70 (Hofmann *et al.* 2005). The inability of the Antarctic notothenioid to induce the Hsp70 gene under thermal stress conditions, probably reside in the alteration of the gene adaptation to low temperature where it has a constantly higher levels of Hsp70. There are evidences that the adaptation to sub zero temperatures could require naturally elevated levels of chaperones to avoid the protein denaturation induced by the cold or to compensate the negative effects of low temperatures on protein folding (Place & Hofmann 2005).

CONCLUSION

Initial studies of the metabolic enzymes of Antarctic fishes were directed to understanding the mechanisms of adaptation to the cold. However,

human presence in the region has introduced additional new variables into these ecosystems. The levels of the energy metabolism enzymes (CS, MDH, PFK, HK, PK, IDH, CK, Na/K-ATPase, cytochrome oxidase, β -HBDH and GPase), L-arginine metabolism (arginase and NOS), antioxidant defenses (SOD, CAT, GPx and GRED) and xenobiotics (EROD e GST) have been investigated as possible biomarkers of the environmental changes mostly in tropical and temperate zone animals. Though a considerable work has been done on the utility of these enzymes as biomarkers in various organisms, there are very few studies with Antarctic organisms, especially fish. There are a number of open questions regarding the: a) the actual controls involved in the metabolism of nitric oxide involving NOS and arginase and L-arginine, b) the changes in the antioxidant metabolism in Antarctic fish arising from the enhanced production of reactive oxygen species by xenobiotics and their relation to the pollution levels, c) the actual defense mechanisms against xenobiotics, and the metabolic alterations induced by co-exposure to heavy metals whose natural concentrations are high in this region. Therefore, it is very important to have good understanding of the metabolic biomarkers to monitor the changes caused by the anthropogenic activities.

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