ARTICLE

Aerobic and anaerobic enzyme activity in the hake *Merluccius gayi gayi* related to the Oxygen Minimum Zone off central-southern Chile

Actividad enzimática aeróbica y anaeróbica de la merluza común
Merluccius gayi gayi, relacionada con la Zona de
Mínimo Oxígeno de Chile centro-sur

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Resumen.- El Sistema de Corrientes de Humboldt de la costa de Chile posee una Zona de Mínimo Oxígeno (ZMO) entre 30 y 250 m de profundidad, con concentraciones de oxígeno < 0,5 ml L^{-1}. Las mayores densidades de merluza *Merluccius gayi gayi* se asocian a esta zona, sugiriendo que posee adaptaciones metabólicas para permanecer en ella. Se estimó el potencial aeróbico y anaeróbico de esta especie demersal mediante la medición de enzimas metabólicas claves y del sistema de transporte de electrones (ETS) en diferentes tejidos (músculo blanco, corazón, cerebro e hígado). La actividad de la enzima citrato sintasa (CS) y de la ETS fueron mayores en el cerebro y corazón, confirmando la predominancia del metabolismo aeróbico en los órganos vitales diferentes al músculo. La actividad promedio de la enzima anaeróbica lactato deshidrogenasa (LDH) fluctuó entre 55 ± 17,5 and 263 ± 79 UI g^{-1} ww, siendo menor en el corazón y cerebro. Esta baja actividad de LDH y una alta razón MDH/LDH en estos órganos vitales indican que ambos serían capaces de lidiar con periodos de acidificación metabólica producida por hipoxia ambiental. Los niveles de LDH fueron excepcionalmente altos en el hígado, lo que podría relacionarse con factores ambientales como la contaminación ambiental. La baja razón MDH/LDH y la menor actividad de CS y ETS en el músculo, sumado a la alta actividad de LDH en el hígado indicarían un perfil metabólico de *M. gayi gayi* para tolerar condiciones de hipoxia y por lo tanto permanecer en la ZMO, pero disminuyendo su actividad natatoria.

Palabras clave: *Merluccius gayi gayi*, zona de mínimo oxígeno, actividad enzimática, hipoxia

Abstract.- The Humboldt Current System (HCS), off the coast of Chile, has an Oxygen Minimum Zone (OMZ) between 30 and 250 m depth, with oxygen concentrations < 0.5 ml L^{-1}. Densities of *Merluccius gayi gayi* associated with this zone are highest between 100 and 300 m depth, suggesting metabolic adaptations allowing it to remain in the OMZ. The aerobic and anaerobic potential of this demersal fish was estimated through the measurement of key metabolic enzymes and the electron transport system (ETS) of different body tissues (white muscle, heart, brain and liver). The activities of the citrate synthase (CS) enzyme and the ETS were higher in the brain and lower in muscle, confirming the predominance of aerobic metabolism in vital organs, different of muscle. The typical activity of the anaerobic enzyme lactate dehydrogenase (LDH) fluctuated between 55 ± 17.5 and 263 ± 79 UI g^{-1} wet weight, with lower activities in heart and brain. The low LDH activity and high MDH/LDH ratio found in these vital organs indicate that they are able to cope with metabolic acidification during long periods of environmental hypoxia. Exceptionally high LDH activity was found in the liver which may be related to environmental factors such as pollution. Considering the low MDH/LDH ratio of muscle, the decreased activity of CS and ETS in this tissue and the high LDH activity in the liver may indicate metabolic profile of *M. gayi gayi* in order to tolerate hypoxia, and therefore the capability to stay in the OMZ by decreasing its swimming activity.

Key words: *Merluccius gayi gayi*, minimum oxygen zone, enzymatic activity, hypoxia

INTRODUCTION

Oxygen minimum zones are defined as regions where oxygen concentrations are lower than 0.5 ml L^{-1}, and are typically found in the middle of the water column at depths between 10-1300 m (Levin 2003). These zones are usually formed in regions of strong upwelling, which generates high surface productivity that sinks and breaks down, decreasing the oxygen in the water column (Levin 2003).

In the Humboldt Current System (HCS), off the Chilean coast, low-oxygen conditions (< 0.5 ml $O_2 L^{-1}$) can be found at depths as shallow as 30-50 m (northern Chile) and around 250 m (core; central-southern Chile) (Ahumada & Chuecas 1979, Grados 1989), associated with wind-induced upwelling of low-oxygen, nutrient-rich Equatorial Subsurface Waters (ESSW) (Strub *et al.* 1998, Morales *et al.* 1999). This OMZ constitutes one of the largest in the world's oceans (Levin 2003, Quiñones *et al.* 2009) and is considered an important barrier for the vertical distribution of marine organisms (White 1988, Eissler & Quiñones 1999, Escribano & Hidalgo 2000, González & Quiñones 2000, 2002; Ulloa *et al.* 2001, Gallardo *et al.* 2004). Hypoxia in coastal ecosystems has been recognized as an important system-level perturbation affecting both ecological dynamics and fishery sustainability around the globe (Grantham *et al.* 2004, Chan *et al.* 2008, Vaquer-Sunyer & Duarte 2008), which may sometimes generate massive beaching and mortality of fish (Hernandez *et al.* 2010). Moreover, recent studies indicate that hypoxic zones are increasing worldwide, due to global warming and eutrophication (Diaz & Rosenberg 2008, Stramma *et al.* 2010), making it increasingly important to understand the physiological and metabolic adjustments enabling the survival of species living in or near these hypoxic areas.

The biota dwelling permanently or semi-permanently in an OMZ must adapt to the low availability of oxygen (Childress & Siebel 1998), using several strategies such as (i) more effective oxygen incorporation, (ii) reduced metabolic demands, and (iii) use of anaerobic metabolism (Childress & Siebel 1998). It has been suggested that some vertically migrating species can alternate between anaerobic metabolism while in the OMZ and aerobic metabolism while in more oxygenated waters (Childress 1977). These species make an interesting object of study in terms of the biochemical and physiological adaptations that should evolve as a product of this environmental forcing. These adaptations consist largely of efficiently using anaerobic metabolic pathways for obtaining energy under hypoxic conditions, either environmental or physiological (González 2002).

Analyses of enzyme activities are used as an approach to look for greater reliance on anaerobic metabolism in OMZ species (Childress & Siebel 1998). Lactate dehydrogenase (LDH), the terminal dehydrogenase in the production of lactate, is used as an indicator of muscle anaerobic capacity. In contrast, citrate synthase (CS) catalyzes the first step in the Kreb's cycle and is considered an index of muscle aerobic capacity (Hochachka & Somero 1984). The enzyme malate dehydrogenase (MDH) maintains the redox balance during intense anaerobic catabolism (Hochachka & Somero 1984, González 2002). The glycolytic enzymatic activity in white muscle, specifically LDH, is also a good marker of swimming capability and feeding strategies in fish (Sullivan & Somero 1980), with higher activity in burst swimming fish where physiological hypoxia occurs in white muscle.

The common hake *Merluccius gayi gayi* (Guichenot, 1848), an important demersal resource, is found in the HCS off Chile and Peru (Arancibia 1997). It is an important commercial species in Chile (Arancibia & Neira 2008), with an annual catch of 36,900 tons in 2013 (SERNAPESCA 2014), and is currently considered overexploited in Chilean waters (Gatica *et al.* 2015). Adults are distributed across the continental shelf and slope between 50 and 500 m deep, associated with the cold, saline and poorly oxygenated water mass of the ESSW (Avilés *et al.* 1979, Lillo *et al.* 2012, Gatica *et al.* 2015). The species exhibits daily migrations within the water column; with the adults feeding mostly on fish such as anchovy, *Engraulis ringens,* and benthic crustaceans (Gatica *et al.* 2015) from low-oxygen waters at the bottom of the continental shelf. As a result, the highest densities of this species are associated with oxygen concentrations between 0.5 and 1 ml $O_2 L^{-1}$, especially between 29°40'S and 35°10'S (north zone, Fig. 1) (Lillo *et al.* 2002, 2005 and 2012). This suggests that hake should have certain physiological or metabolic features allowing it to remain in the hypoxic zone.

In this study, the aerobic and anaerobic enzymatic activities of different tissues in *M. gayi gayi* have been measured in order to assess the biochemical capacity of this species to tolerate low oxygen conditions.

MATERIALS AND METHODS

COLLECTION AND PRESERVATION OF STUDY ANIMALS

Specimens of *M. gayi gayi* were collected on the continental shelf off central-southern Chile (36°48'S-73°15'W). Samples (n= 30) were taken in April 2005 on board the artisanal vessel 'Princesa III', using gill-net fishing gear located between 100 and 300 m depth.

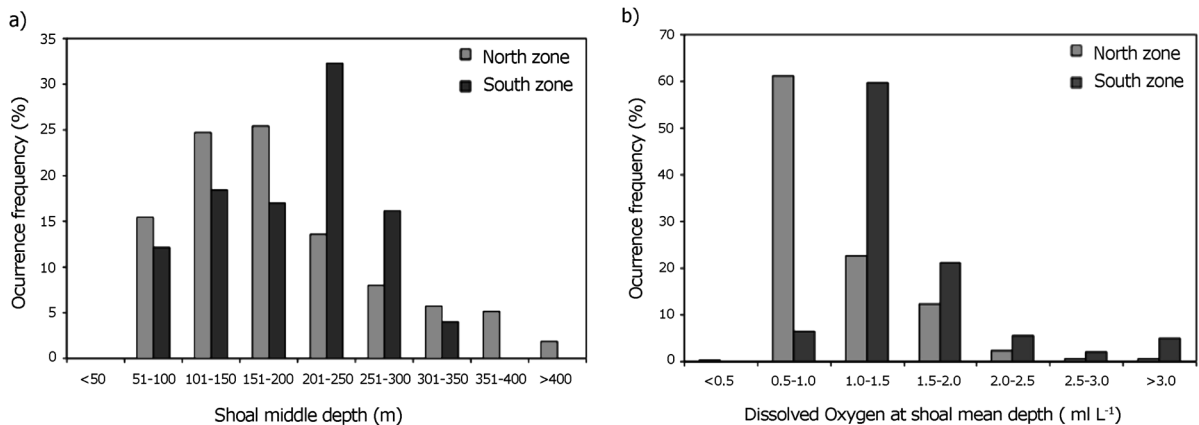Figure 1. a) Mean depth of occurrence of *M. gayi gayi* shoals in northern and southern Chile; b) frequency of occurrence of *M gayi gayi* within different oxygen concentration ranges (modified from Lillo *et al.* 2012) / a) Profundidad promedio de las agregaciones de *M. gayi gayi* en el norte y sur de Chile, b) frecuencia de ocurrencia de *M. gayi gayi* en diferentes rangos de concentración de oxígeno (modificado de Lillo *et al.* 2012)

Once caught, each live hake was sacrificed using a benzocaine overdose (100 mg L⁻¹) (Barker *et al.* 2012) and then measured (fork length, FL). Immediately after, white muscle samples (approx. 1 cm³) were extracted from each individual from the dorsal area, behind the pectoral fin. Samples of liver, heart and brain tissue were also extracted. All samples were immediately stored in liquid nitrogen for later enzymatic analysis. The rest of the fish were kept on ice (less than 5 h) until they were weighed in the laboratory on land.

HOMOGENIZATION

The samples were weighed and homogenized in 200 mM K_2HPO_4 buffer (pH 7.9), 0.3% polyvynilpirrolidone (PVP), 5 mM EDTA, and 0.1% Triton X-100, using an Ultra Turrax homogenizer in an ice bath. The amount of buffer solution added to each sample was calculated using a dilution factor that varied according to the different tissues (1:40 for heart, muscle and brain; 1: 100 for liver). The homogenate was centrifuged for 5 min at 3000 g (4°C), and the supernatant used for the enzyme assays. One part of the supernatant was used for the ETS activity and the rest for measuring enzymatic activity.

DETERMINATION OF ENZYMATIC ACTIVITY RELATED TO ANAEROBIC METABOLISM

Measurements of the various enzyme activities were conducted by spectrophotometry and run in triplicate. Enzyme activities were expressed as µ moles of substrate converted per minute (IU) per gram of wet weight (ww). The average assay temperature was between 15° and 16°C.

Lactate pathway activity (LDH) was analyzed as representative of the anaerobic metabolism. The lactate pathway maintains the metabolic rate under environmental or physiological hypoxic conditions (Livingstone 1983).

The assay mixture was modified from Schiedek (1997) and contained an 80 mM K_2HPO_4 buffer (pH 7.9) and 3.2 mM pyruvate. Before measuring, 0.2 mM NADH was added to the mixture. Finally, an aliquot of supernatant was added and the decay of the NADH absorption at 340 nm was measured. All the enzymatic activities were corrected for nonspecific NADH oxidation.

MDH activity, from oxaloacetate to malate, was measured in all tissues using the procedure described by Childress & Somero (1979) and Vetter *et al.* (1994).

DETERMINATION OF ENZYMATIC ACTIVITY RELATED TO AEROBIC METABOLISM

The activity of the citrate synthase (CS) enzyme, characteristic of aerobic metabolism, was measured using a modified version of the method proposed by Childress & Somero (1979) and Vetter *et al.* (1994). The reaction mixture contained 50 mM Imidazol /HCl pH 8.0 at 20°C, 1.5 mM $MgSO_4$, 0.1 mM acid 5.5 ditiobis (2-nitrobenzoic) (DTNB), 0.06 mM acetyl-CoA. The supernatant was added, and the mixture incubated for 20 min at room temperature. Afterward, 0.2 mM of oxalacetate was added, and absorbance was measured. Absorbance was determined at 412 nm. All the determinations were corrected using a blank containing the supernatant but with the absence of oxalacetate.

The potential activity of the electron transport chain was determined using the ETS technique described by Packard (1971). This is an indirect enzymatic method used to estimate the rate of oxygen consumption as an expression of the maximum potential activity of the electron transporters in the respiratory chain at a mitochondrial level.

In situ ETS activity was calculated with the Arrhenius equation [$S = A \exp (E_a/k \, (1/T_a – 1/T_s))$], where S is the *in situ* ETS, A is the ETS calculated at the assay incubation temperature, E_a is the Arrhenius activation energy, k is the constant of the gases (1.987 cal mol^{-1} deg^{-1}), T_a is the assay temperature (°K), and T_s is the *in situ* temperature (°K). The activation energy used was 16.2 (kcal mol^{-1}) (Arístegui & Montero 1995). The *in situ* temperature was 10°C, which corresponded to the temperature observed at 200 m depth, in the area where common hake are usually found (Lillo *et al.* 2002).

The conversion of ETS activity to oxygen consumption (R) was done using an ETS/R ratio of 2 (Ikeda 1996), based on the assumption that the kinetics of Michaelis-Menten can be applied to respiratory chemistry and that the concentration of the respiratory regulator (*i.e.*, ADP) remains near Km.

All potential activities were expressed as apparent specific activities. The unit used to express LDH and MDH activity is µmol NADH min^{-1} g ww^{-1}. CS activity is expressed as µmol DTNB min^{-1} g ww^{-1} and ETS units as µL O$_2$ h^{-1} g ww^{-1}.

STATISTICAL ANALYSIS

A one-way analysis of variance (ANOVA) was used to evaluate differences in enzymatic activity among tissues, a Tukey post hoc test was performed to see which tissue differed. Regression analysis was conducted to explore the relationship between body weight and enzymatic activity.

RESULTS

Body weight of *M. gayi gayi* specimens ranged from 263 and 861 g. The enzymatic activities of both aerobic and anaerobic metabolisms are shown in Table 1, along with the activity of the electron transport system (ETS).

AEROBIC METABOLISM

CS activities ranged between 0.741 ± 0.19 and 2.302 ± 0.49 µmol DTNB min^{-1} g ww^{-1} (Table 1), with the lowest activity corresponding to the muscle ($F_3 = 30.55$, $P < 0.01$), whereas the other tissues showed more similar activities between them (Fig. 2c). The activity of the electron transport system (ETS) ranged from 135 ± 74 to 872 ± 298 µl O$_2$ h^{-1} g^{-1} ww (Table 1), also with a lower activity in white muscle (Fig. 3d) ($F_3 = 27.46$, $P < 0.01$).

A significant positive and linear relationship ($P < 0.05$) was found between the log transformed body weight and log ETS activity in the liver of *M. gayi gayi* (Fig. 4).

ANAEROBIC METABOLISM

The LDH activity fluctuated between 55.1 ± 17.5 and 263 ± 79 µmol NADH min^{-1} g^{-1} ww (Table 1, Fig. 2a), with significant differences between tissues (one-way ANOVA, $F_3 = 78.17$, $P < 0.01$) and higher activity in the liver, followed by heart, muscle, and finally brain.

Table 1. Enzymes activities, ETS (corrected for *in situ* temperature) activities and oxygen consumption (R) in different tissues from *M. gayi gayi*. LDH, MDH, and CS activities are in IU^{-1} g ww^{-1} (±SD), ETS and R activities are in µl O$_2$ h^{-1} g ww^{-1} (±SD) / Actividades enzimáticas, actividad del ETS (corregido para la temperatura *in situ*) y consumo de oxígeno (R) en diferentes tejidos de *M. gayi gayi*. La unidad de la actividad de LDH, MDH y CS es IU^{-1} g ww^{-1} (±SD). Unidad de las actividades del ETS y R es µl O$_2$ h^{-1} g ww^{-1} (± SD)

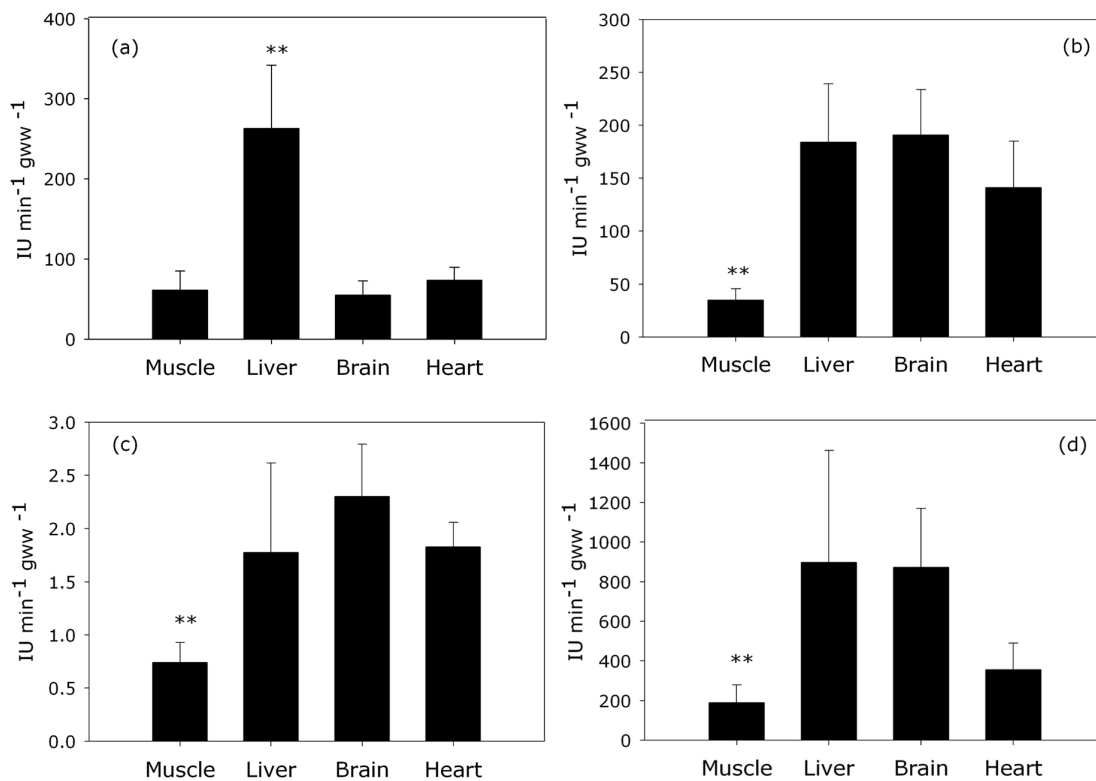|	n	Muscle	n	Liver	n	Brain	n	Heart
LDH	16	61.1 ± 24	13	263 ± 79	14	55.1 ± 17.5	14	73.7 ± 16
MDH	16	35.05 ± 10.6	13	184 ± 55.3	13	190.76 ± 43	13	141.2 ± 44
CS	17	0.741 ± 0.19	9	1.776 ± 0.84	11	2.302 ± 0.49	12	1.829 ± 0.23
ETS	43	135 ± 74	30	807.4 ± 381	10	872 ± 298	11	713.04 ± 269.4
R	43	67.5 ± 37	30	403.7 ± 191	10	436 ± 148	11	356.5 ± 134.7

Figure 2. Mean enzyme and electron transport system activity in different tissues of *M. gayi gayi*. (a) LDH, (b) MDH, (c) CS, (d) ETS. Values are means ± SD. Significant differences between tissues are given by **(*P* < 0.01) / Promedio de la actividad enzimática de enzimas y del sistema de transporte de electrones en los tejidos de *M. gayi gayi*. (a) LDH, (b) MDH, (c) CS, (d) ETS. Valores son medias ± DE. Diferencias significativas entre tejidos se presentan con **(*P* < 0,01)
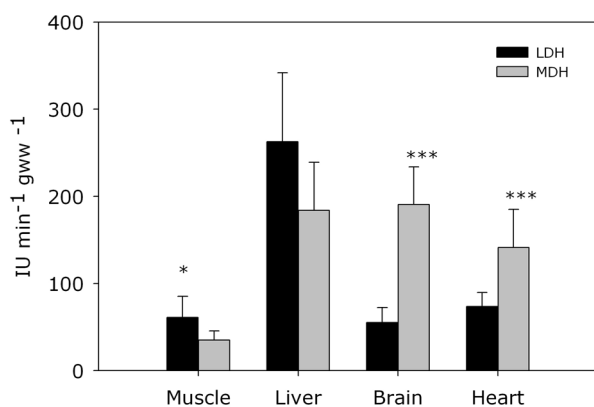

Figure 3. Mean lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activity of different tissues from *M. gayi gayi*. Values are means ± SD. Significant differences between enzymes are given by *(*P* < 0.05), ***P* < 0.0001) / Actividad media de lactato deshidrogenasa (LDH) y malato deshidrogenasa (MDH) en diferentes tejidos de *M. gayi gayi*. Valores son medias ± DE. Diferencias significativas entre tejidos se presentan con *(*P* < 0,05) y ***(*P* < 0,0001)
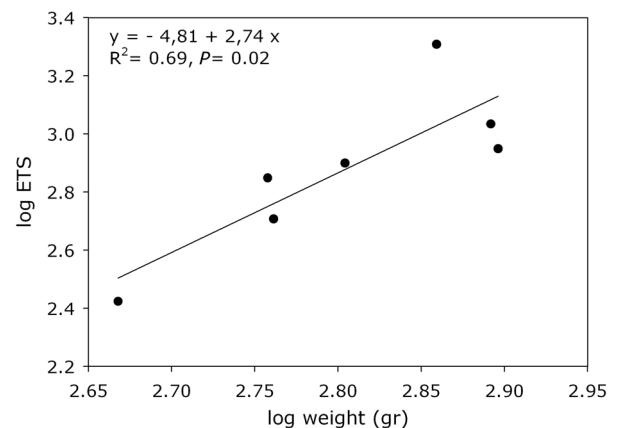
Figure 4. Relationship between body weight of *M. gayi gayi* and ETS (µl O$_2$ h^{-1} g^{-1} wet tissue) in liver (*P* < 0.05) / Relación entre peso corporal de *M. gayi gayi* y ETS (µl O$_2$ h^{-1} g^{-1} tejido húmedo) en hígado (*P* < 0,05)

Table 2. MDH/LDH ratio for different tissues in *M. gayi gayi /* Razón MDH/LDH para diferentes tejidos de *M. gayi gayi*

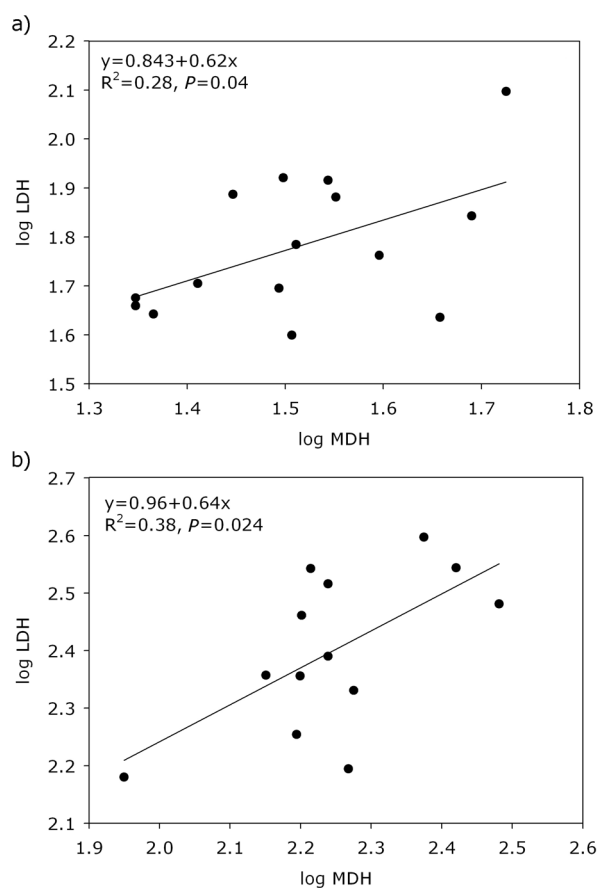|	MDH/ LDH
Muscle	0.666
Liver	0.703
Brain	3.737
Heart	2.024

a)

b)

Figure 5. MDH-LDH relationship in: a) muscle and b) liver of *M. gayi gayi /* Relación entre MDH y LDH en: a) músculo blanco y b) hígado de *M. gayi gayi*

MDH ranged between 35.1 ± 11 and $191 \pm 43\,\mu$mol NADH min^{-1} g^{-1} ww (Table 1, Fig. 2b), and was significantly lower (one-way ANOVA, $F_3 = 47.2$, $P < 0.01$) in white muscle than among the other tissues. The MDH activity pattern in different tissues was fairly similar to that observed for CS and ETS (Figs. 2c,d), and its activity was higher than LDH in heart and brain (Fig. 3) (one-way ANOVA, $F_3 = 49.93$, $P < 0.01$).

The MDH/LDH ratio was calculated in order to indicate which type of metabolism is preponderant in each tissue. This ratio was greater than 1 in the brain and heart, and less than 1 in muscle and liver (Table 2), due to the high levels of LDH in the former two tissues. A slight significant positive relationship ($P < 0.05$) was found between log transformed MDH and log transformed LDH in the muscle and liver of *M. gayi gayi* (Fig. 5).

DISCUSSION

Merluccius gayi gayi is a typical demersal species of central-southern coastal Chile, distributed from surface to 500 m depth (Lillo *et al.* 2005). It feeds primarily on benthic organisms (squat lobster) and euphausiids (Vidal *et al.* 1997, Arancibia 1997, Gatica *et al.* 2015) over the continental shelf. The anaerobic metabolic capacity detected in the present study is consistent with both the vertical distribution and the trophic behaviour of the species, as both characteristics require an adaptive capacity for remaining, for periods of at least a few hours, in waters of the oxygen minimum zone (≤ 0.5 ml O$_2$ L^{-1}).

Lower CS and ETS activities found in the muscle contrast with the high values found in all other tissues. This support the predominance of aerobic metabolism in all the tissues except muscle, agreeing with other studies carried out on fish (Childress & Somero 1979, Yang *et al.* 1992, Panepucci *et al.* 2000, Panepucci *et al.* 2001, Treberg *et al.* 2003) and most vertebrates (Hochachka & Somero 1973).

LDH activity was very high in hake liver, followed by lessening activity in heart, muscle, and, finally, brain. The activities of LDH in white muscle ($61.1 \pm 24\,\mu$mol min^{-1} g^{-1} ww) are similar to those determined in deep-sea fish (Saavedra *et al.* 2016), and lower than in shallow living species which are more continuous swimmers, such as mackerel (Sullivan & Somero 1980). The similarity of this LDH activity with that of other deep living species may also be related to a decreased capacity for carbohydrate metabolism, which in turn has been related to a reduction in growth or swimming activity during chronic hypoxia (Martinez *et al.* 2006). Nevertheless, *M. gayi gayi* has almost exclusively white muscle in its body which powers rapid burst swimming movement for prey capture and predator avoidance, consistent with the low CS and ETS activity in this tissue (Table

1). This burst swimming capacity was described by Queirolo *et al.* (2010) through direct observations of hake behaviour in response to trawling. On the other hand, the higher activities of LDH in liver agree with the increased specific activities found by Martinez *et al.* (2006) in the liver of *Fundulus grandis* acclimatized to hypoxic conditions. They suggest that this increase in carbohydrate metabolism in liver is a result of 'chronic hypoxia', which is consistent with the normal presence of *M. gayi gayi* in the OMZ (Lillo *et al.* 2012).

MDH activities in the liver, brain, and heart were very similar, revealing a potential anaerobic metabolism in these organs under prolonged periods of low-oxygen conditions, preventing lactic acid accumulation (Shapiro & Bobkova 1975, Panepucci *et al.* 2000). In contrast, white muscle shows very low levels of MDH activity, which suggests that *M. gayi gayi* adjusts its metabolism toward efficient anaerobic production of ATP and the use of LDH to maintain cytoplasmic NAD/NADH (Chippari-Gomez *et al.* 2003). This is also observed in the different MDH/LDH ratios between tissues, where white muscle showed the lowest, while brain and heart both had ratios higher than 1, indicating that attenuated pyruvate to lactate flux is produced in these tissues and, as a consequence, carbohydrate metabolism will be largely channelled toward complete oxidation (Almeida-Val & Hochachka 1995 *sensu* Panepucci *et al.* 2000). These results agree with Panepucci *et al.* (2000), who found an extremely high MDH/LDH ratio for the heart of the fish *Rhinelepis strigosa*s, showing the importance of this organ to fish survival in critically hypoxic situations. In brief, the high MDH/LDH ratio found in the heart and brain of *M. gayi gayi*, suggests that hake present metabolic adaptations that allow them to tolerate low-oxygen conditions by protecting their vital organs from prolonged periods of hypoxia. Despite the low MDH/LDH ratio found in muscle and liver, it is possible to see a slight correlation between both enzymes in these tissues (Fig. 5) which suggests that both enzymatic activities could be acting simultaneously during chronic hypoxia, as it has been observed in bivalves during extreme anoxic stress (Grandón *et al.* 2008). Also, the much higher activity of MDH relative to CS in both tissues suggests that, in addition to its function in the citric acid cycle, MDH may play an important role in redox balance in *M. gayi gayi* white muscle tissue.

In relation to the size dependence of enzymatic activities, only liver ETS activity showed an increase with body mass. However, these results may not be conclusive because of the low sample number. This is also the explanation for the absence of size dependence in the other tissues.

From the few enzymatic indicators obtained in the present study, it is possible to affirm that *M. gayi gayi* has a metabolic profile to tolerate hypoxic conditions, and therefore the capability to stay in the OMZ, maintaining slow swimming activity, but with the possibility for burst swimming when necessary.

It is important to highlight the elevated LDH activity found in *M. gayi gayi* liver, which does not agree with the typically low activity of this enzyme in this organ observed in other fish species (*e.g.,* Panepucci *et al.* 2001, Cooper *et al.* 2002), and with the belief that this organ is considered a predominantly aerobic tissue with high blood irrigation (Hinton *et al.* 2009). These high LDH levels could be an indication of metabolic failure of the liver (Orrego *et al.* 2010) due to the exposure of *M. gayi gayi* to pollution sources (industrial waste and domestic sewage) associated with the Biobío river and the coastal zones of central-southern Chile (*e.g.,* Parra *et al.* 1993, Riveros *et al.* 1996, Muñoz 2002), also demonstrated by other studies (Monteiro *et al.* 2007, Orrego *et al.* 2011). Further research is needed to explore this hypothesis.

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