

CO₂-fixing enzymes and phosphoenolpyruvate metabolism in the fish parasite *Hysterothylacium aduncum* (Ascaridoidea, Anisakidae)

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ABSTRACT: CO₂ stimulates the development of many of the intestinal helminths that are able to fix CO₂ by means of phosphoenolpyruvate carboxykinase (PEPCK), such as *Hysterothylacium aduncum*. We determined the activity of CO₂-fixing enzymes such as PEPCK and phosphoenolpyruvate carboxylase (PEPC), although no significant activity was detected for pyruvate carboxylase or carboxylating-malic enzyme. The former act on phosphoenolpyruvate (PEP) to yield oxalacetate. In the helminths studied, PEP has a vital role in glucidic metabolism. Consequently, we determined the activity of other enzymes involved in the crossroad of PEP, such as pyruvate kinase (PK), lactate dehydrogenase and malate dehydrogenase. All enzymes detected showed significant variations in activity during the *in vitro* development of the parasite from the third larval stage to mature adult. Fixing of CO₂ by PEPCK decreased during development (from 228 to 115 nmol min⁻¹ mg⁻¹ protein), while that by PEPC increased (from 19 to 46 nmol min⁻¹ mg⁻¹ protein). This enzyme, which is rare in animals, could play a part in detecting levels of free phosphate, releasing it from PEP when required for processes such as glycogenolysis, glycolysis and adenosine 5'-triphosphate (ATP) synthesis. PK, which showed increasing activity during development up to immature adult (from 56 to 82 nmol min⁻¹ mg⁻¹ protein), could act in combination with PEPC to obtain energy in the cytosol (in the form of ATP) and in the mitochondria (possible destination of the pyruvate formed), compensating for the decrease in activity of PEPCK.

KEY WORDS: Fish parasite · *Hysterothylacium aduncum* · Nematoda · Carbon dioxide fixation · Phosphoenolpyruvate carboxykinase · Phosphoenolpyruvate carboxylase · Phosphoenolpyruvate metabolism

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INTRODUCTION

Parasites in general, and nematodes in particular, require specific stimuli during their life cycle. These trigger the processes that lead to the next developmental stage. Those related to the development of the infective stage for the next suitable host are particularly interesting and may take place within the host or in the environment. In nematodes, these infective stages, often the third larval stage (L3), having attained optimal development, await what is generally the definitive host. Some authors believe that in this stage there is a reduction in metabolism, leaving the larvae in a quiescent state or a state of 'hypometabolic

dormancy' (Petronijevic & Rogers 1987), which can be compared to the 'dauer larva' occurring in free-living nematodes such as *Caenorhabditis elegans*. However, when the parasite reaches the new host, it must recognise the suitability of the latter and restart its own development, as far as the adult stage if the host is definitive, reactivating and adapting its metabolism to the new physiological situation of the habitat it occupies within the new host. These stimuli or triggers for development are not well understood and may be simple or may involve several factors. In nematodes, CO₂ has long been recognised as a trigger for the development of the infective stages, generally the infective L3 (Sommerville 1957, Rogers 1960, Taylor & Whitlock

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1960, Rogers & Sommerville 1963, Petronijevic & Rogers 1987, Hertzberg et al. 2002), although some authors have reported exceptions such as species of *Nematodirus*, in which the acid medium generated by HCl takes the place of CO₂ as a stimulus for moulting to infective larvae (Christie & Charleston 1965, Hertzberg et al. 2002). In the anisakids, we have observed that a combination of factors, such as temperature, partial pressure of CO₂ (pCO₂), acidic pH and pepsin, trigger the development of the infective L3 stage as far as the adult stage, at least *in vitro* (Iglesias et al. 1997, 2001, 2002, Adroher et al. 2004, Dávila et al. 2006), as has been observed in other nematodes (Silverman & Podger 1964). The mechanisms by which parasites recognise these triggers as such are still unknown but undoubtedly involve a change in the expression of the genes of the parasite, which would lead it to enter the next developmental stage adapted to the new host. As we believe that CO₂ affects the metabolism of anisakids, we studied the enzymes involved in CO₂ fixing as an expression of the changes taking place during the development of the parasite within the definitive host, using *Hysterothylacium aduncum* as a model.

Hysterothylacium aduncum is an aquatic parasitic nematode of crustaceans and fish in both freshwater and marine habitats worldwide. Despite being among the most common parasitic nematodes of fish, it has scarcely been studied. Its pathogenicity is under discussion, although Berland (1987) attributed mortality in rainbow trout to their parasitisation by *H. aduncum*. Other workers have shown its damaging effects on the larvae of herring and cod (Balbuena et al. 2000, Karlsbakk et al. 2001, 2003), and even on very young fish, in the case of halibut (Bristow 1990). Carvajal et al. (1995) and González (1998) suggested that the mere presence of the parasite is a cause of stress in fish, aiding the development of other infections and thus affecting the health of the fish. This can be a serious problem in aquaculture by reducing production. Although this does not seem to be a problem in freshwater aquaculture sites, due to the level of control that can be attained, the reverse may be true in a marine setting. Here, the enormous growth of this activity, combined with easy access into the cages for small fish and crustaceans harbouring the parasite, could facilitate its rapid spread through the installation, leading to a reduced yield and serious financial losses (González 1998, Sepúlveda et al. 2004).

In many organisms, the fixing of CO₂ is a source of carbon atoms for a range of cellular metabolic processes. Two enzymes involved in this fixing use phosphoenolpyruvate (PEP) as a substrate, producing oxalacetate (OAA): phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase

(PEPC). Another 2 CO₂-fixing enzymes use either pyruvate rather than PEP as a substrate to obtain OAA (the case of pyruvate carboxylase, PC) or malate (the case of carboxylating-malic enzyme [ME]). Mitochondrial ME is of great importance for metabolism in helminths due to its role in the dismutation of malate, generally functioning in the decarboxylating direction. However, a cytosolic isoform could carboxylate pyruvate to obtain malate in only one step, and this would enter the mitochondrion. All enzymes mentioned have been detected, with a greater or lesser activity, in parasitic helminths (see Barrett 1981 for references, Mallick et al. 1991, Loiseau et al. 1993, Dávila et al. 2006, and others). In previous studies, we have reported the presence of PEPCK and PEPC as CO₂-fixing enzymes in L3 of *Hysterothylacium aduncum* (Iglesias et al. 2005). In the present study, we considered the metabolism of PEP and the fixing of CO₂ throughout the *in vitro* development of this parasite from L3, obtained from paratenic/intermediate host fish, to the adult stage.

MATERIALS AND METHODS

Collection of L3 larvae and cultivation. The L3 of *Hysterothylacium aduncum* were collected from horse mackerel *Trachurus trachurus*. The worms, found free in the host body cavity, were 8 mm or more in length, and were collected with the help of a needle with a blunt tip, placed on a Petri dish with cold 0.9% NaCl solution and washed in it several times. The worms were observed individually under an inverted microscope, and those that showed any kind of internal or external damage were discarded. They were then identified according to morphological features (Yoshinaga et al. 1987, Petter & Maillard 1988, Petter & Cabaret 1995). Next, a number of larvae were frozen (–80°C) for enzyme determination (L3 0 h). The rest of the collected larvae were axenised and individually placed in culture, in a modified RPMI-1640 medium (Iglesias et al. 2002), at 15°C, to determine the exact stage of development of each worm, since this medium enables development of the L3 stage into the mature adult (Iglesias et al. 2002). The development of the cultured *in vitro* parasite occurred as follows: L3 moults to the fourth larval stage (L4) after 5 (range: 4 to 6) d in culture; L4 moults to juvenile adult (L5J) after 23 (range: 18 to 25) d in culture; the L5J to a mature adult stage (L5M) ca. 35 d after starting the culture.

Preparation of the extract and protein determination. The following samples were taken and frozen (–80°C) for enzyme determination: L3 from fish (L3 0 h, 30 larvae per sample), L3 after 48 h of cultivation (L3 48 h, 25 larvae per sample), fourth larval stage after

12 d of cultivation (L4, 12 larvae per sample), juvenile adult after 26 d of cultivation (L5J, 6 worms per sample), and mature adult after 61 d of cultivation (L5M, 3 worms per sample). For enzyme determination, the samples were treated as previously described by Iglesias et al. (2005). Protein determination was carried out according to Bradford (1976).

Enzyme determination. This was followed spectrophotometrically by the disappearance of the reduced form of nicotinamide adenine dinucleotide (NADH) from the assay mixture at 340 nm. The assay temperature was 15°C. The techniques previously described (Iglesias et al. 2005, Dávila et al. 2006) were used or modified to determine CO₂-fixing enzyme activity. In previous assays, the highest initial velocity was obtained using Mn²⁺ instead of Mg²⁺ and inosine 5'-diphosphate (IDP) instead of guanosine 5'-diphosphate (GDP) in the PEPCK assay. Controls were carried out in all cases to rule out other overlapping enzyme activities (e.g. lactate dehydrogenase [LDH] activity when carboxylating-ME or PC activity was being measured). The assay mixture to determine PEPCK activity contained: 32 mM Tris-HCl pH 7.4, 0.96 mM MnCl₂, 0.5 mM β-NADH (disodium salt), 1.5 mM sodium IDP, 1.6 U malate dehydrogenase (MDH, E.C. 1.1.1.37), extemporaneous solution of 16 mM NaCO₃H bubbled with CO₂ and extemporaneous solution of 2 mM sodium PEP (monohydrate) and 25 µg of protein from the extract, in a final volume of 1.0 ml. The reaction was initiated by the addition of IDP. The assay mixture to measure PEPC activity contained: 32 mM Tris-maleate pH 6.0, 3 mM MgCl₂, 0.5 mM β-NADH (disodium salt), 10 U MDH (E.C. 1.1.1.37), extemporaneous solution of 16 mM NaCO₃H bubbled with CO₂ and extemporaneous solution of 12 mM PEP and 25 µg of protein from the extract, in 1.0 ml of mixture. The reaction was initiated by the addition of PEP. The measurement of PC and carboxylating-ME activities was as described by Iglesias et al. (2005). MDH activity was measured using the following assay mixture: 100 mM KH₂PO₄-KOH pH 7.4, 0.25 mM β-NADH, 20 mM sodium OAA and 25 µg of protein from the extract, in 1.0 ml of mixture. The reaction was initiated by the addition of OAA. The assay mixture to measure pyruvate kinase (PK) activity contained (modified after Valentine & Tanaka 1966 and Bueding & Saz 1968): 9 mM triethanolamine-HCl pH 7.5, 0.4 mM MgSO₄, 100 mM KCl, 1 mM sodium adenosine 5'-diphosphate (ADP), 0.2 mM β-NADH, 10 U LDH (E.C. 1.1.1.27), extemporaneous solution of 1 mM PEP and 25 µg of protein from the extract, in 1.0 ml of mixture. The reaction was initiated by the addition of PEP. The LDH activity was measured using the following assay mixture (modified after Bergmeyer & Bernt 1974): 100 mM NaH₂PO₄-NaOH pH 7.5, 0.2 mM β-NADH, 6.9 mM sodium pyruvate and 25 µg of protein from the

extract, in 1.0 ml of mixture. The reaction was initiated by the addition of pyruvate. One unit (U) of enzyme is defined as the quantity of enzyme required to consume 1 µmol of NADH of the reaction mixture in 1 min at the temperature of the assay.

Statistical method. The data were processed using SPSS software (version 14 for Windows). Values were tested for significance by 1-way analysis of variance (ANOVA). When the values were significant, post hoc multiple comparisons by pairs were performed using the Bonferroni test. The significance level was $p = 0.05$.

Chemicals. Culture medium, foetal bovine serum, substrates, enzymes and other reagents were purchased from Sigma Chemical, Boehringer Mannheim and Merck.

RESULTS

Fig. 1 shows the variation in the specific activity of the CO₂-fixing enzymes studied throughout the *in vitro* development of *Hysterothylacium aduncum*. While PEPCK activity decreased significantly (ANOVA, $p = 0.001$) from L3 collected from fish to the juvenile adult stage (L5J) and then remained unchanged up to maturity (L5M), the opposite was true for PEPC activity (ANOVA, $p = 0.010$), although the former was always clearly higher than the latter. Fig. 1 also shows the sum of the CO₂-fixing activities, revealing that this remained more or less stable from the L4 stage to the end of the parasite's development. No significant activ-

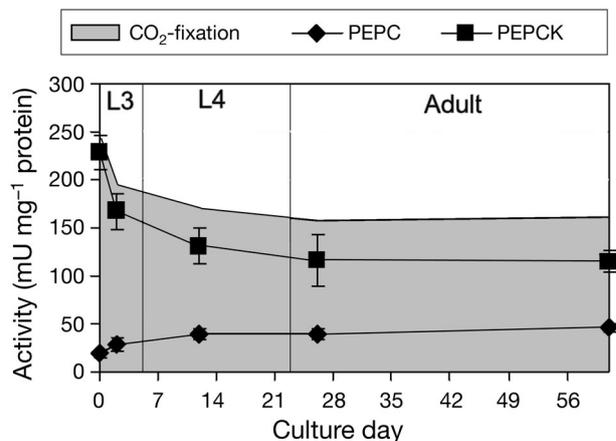


Fig. 1. *Hysterothylacium aduncum*. Specific activity (mU mg⁻¹ protein) of the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) and fixing of CO₂ calculated as the sum of activities of these 2 enzymes, during the *in vitro* development from the third larval stage (L3) to mature adult, measured at 15°C. Each point is the mean ± SE of 3 to 4 experiments in triplicate. The moult of L3 to L4 stage (M3) occurs ca. 5 d post inoculation (p.i.) in culture, and the moult of the L4 to adult stage (M4) occurs ca. 23 d p.i. All juvenile adults (L5J) have matured after 35 d p.i.

ity was detected for PC or carboxylating-ME in any of the developmental stages studied.

Fig. 2 shows the evolution of PK activity during the development of the nematode. This increased from L3 (56 mU mg⁻¹ protein) to juvenile adult (L5J, 82 mU mg⁻¹ protein), decreasing significantly at maturity (L5M, 29 mU mg⁻¹ protein; $p < 0.005$).

Fig. 3 shows the activity of the dehydrogenase enzymes MDH and LDH. In both enzymes, the activity in the L3 recently recovered from the fish was significantly higher ($p < 0.05$) than after 48 h in culture. After moulting, in L4, activity increased slightly and then remained at around 260 mU mg⁻¹ protein until the end of development of the nematode in the case of MDH, whereas in LDH, activity continued to increase slightly as far as L5J, maintaining this level until maturity (124 mU mg⁻¹ protein).

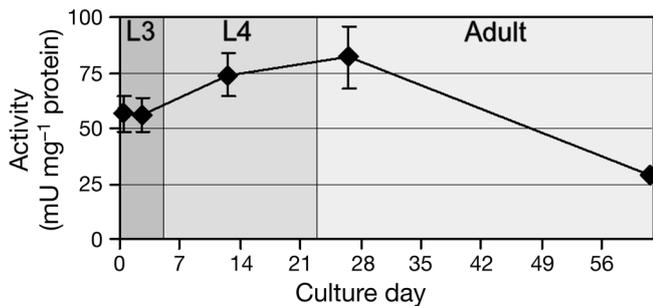


Fig. 2. *Hysterothylacium aduncum*. Specific activity (mU mg⁻¹ protein) of the enzyme pyruvate kinase (PK) during the *in vitro* development from L3 to mature adult, measured at 15°C. Each point is the mean \pm SE of 3 to 4 experiments in triplicate. The M3 occurs ca. 5 d p.i. in culture, and the M4 occurs ca. 23 d p.i. All L5J have matured after 35 d p.i. Abbreviations as in Fig. 1

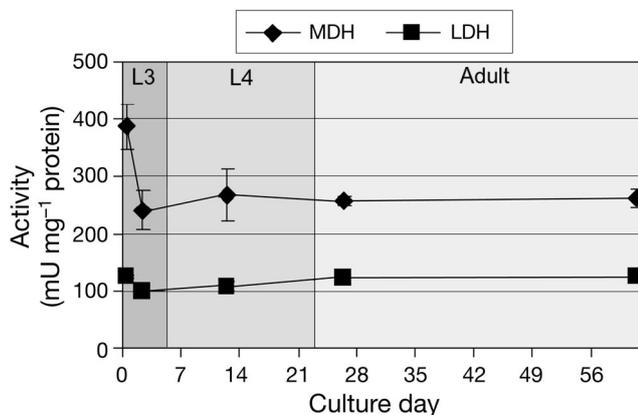


Fig. 3. *Hysterothylacium aduncum*. Specific activity (mU mg⁻¹ protein) of the enzymes malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) during the *in vitro* development from L3 to mature adult, measured at 15°C. Each point is the mean \pm SE of 3 to 4 experiments in triplicate. The M3 occurs ca. 5 d p.i. in culture, and the M4 occurs ca. 23 d p.i. All L5J have matured after 35 d p.i. Abbreviations as in Fig. 1

DISCUSSION

The ascaridoid larval stages that develop in the environment or in intermediate hosts generally exhibit aerobic metabolism with a complete Krebs cycle in their mitochondria. When the larvae reach the definitive host, their metabolism undergoes modification in order to adapt it to the microaerobic/anaerobic conditions of the digestive tract of the host, where they develop, transforming the mitochondria into organelles that specialise in obtaining energy in anaerobic conditions. Thus, malate, produced following the fixation of CO₂ by PEP resulting from the glycolytic metabolism in the cytosol and the reduction of the OAA formed, is incorporated into the mitochondrion, where it undergoes dismutation, finally yielding energy and more or less volatile organic acids as final products (Barrett 1981, Komuniecki & Tielens 2003).

Hysterothylacium aduncum is an anisakid that is an intestinal parasite of fish, its definitive hosts, requiring CO₂ for its *in vitro* development (Iglesias et al. 2002). We decided to study the enzymes of the parasite that could be involved in the fixing of CO₂ as a complement or supplement to their diet, via incorporation of exogenous carbon atoms, possibly related to the between-meals period of the host. In a preliminary study, Iglesias et al. (2005) noted the presence of PEPCK and PEPCK activity in L3 of this parasite and reported changes during M3 (the moult from the L3 to the L4 stage), without detecting PC or carboxylating-ME activity. No significant activity of these 2 enzymes was found in the present study during development from L3 to mature adult, although in the laboratory, we had previously detected decarboxylating-ME activity in all of these stages of the worm by isoenzymatic methods (Iglesias 1999), and other authors have also detected this decarboxylating activity in many parasitic helminths (see Barrett 1981 for references, Park et al. 1984).

Little is known of the metabolism of the anisakid nematodes parasitising fish. Zóltowska et al. (2002) studied the metabolism of glycogen in *Hysterothylacium aduncum*, concluding that, while the most abundant sugar in the larvae (possibly stage L3) was trehalose, it was glycogen and its monomer, glucose, in adults. Glycogen is the storage carbohydrate used by intestinal helminths to span the gap between the meals of the host, and its level varies with the feeding cycle of the host (Komuniecki & Tielens 2003). It can be supposed that both trehalose, via trehalase, and glucose are degraded along the glycolytic pathway, producing PEP (Komuniecki & Harris 1995). In anaerobic or microaerobic metabolism of gastrointestinal helminths, PEP (see Fig. 4) may follow a fermentative pathway to give lactate, via PK and LDH, or malate, via PEPCK and MDH, which is introduced into the mitochondrion

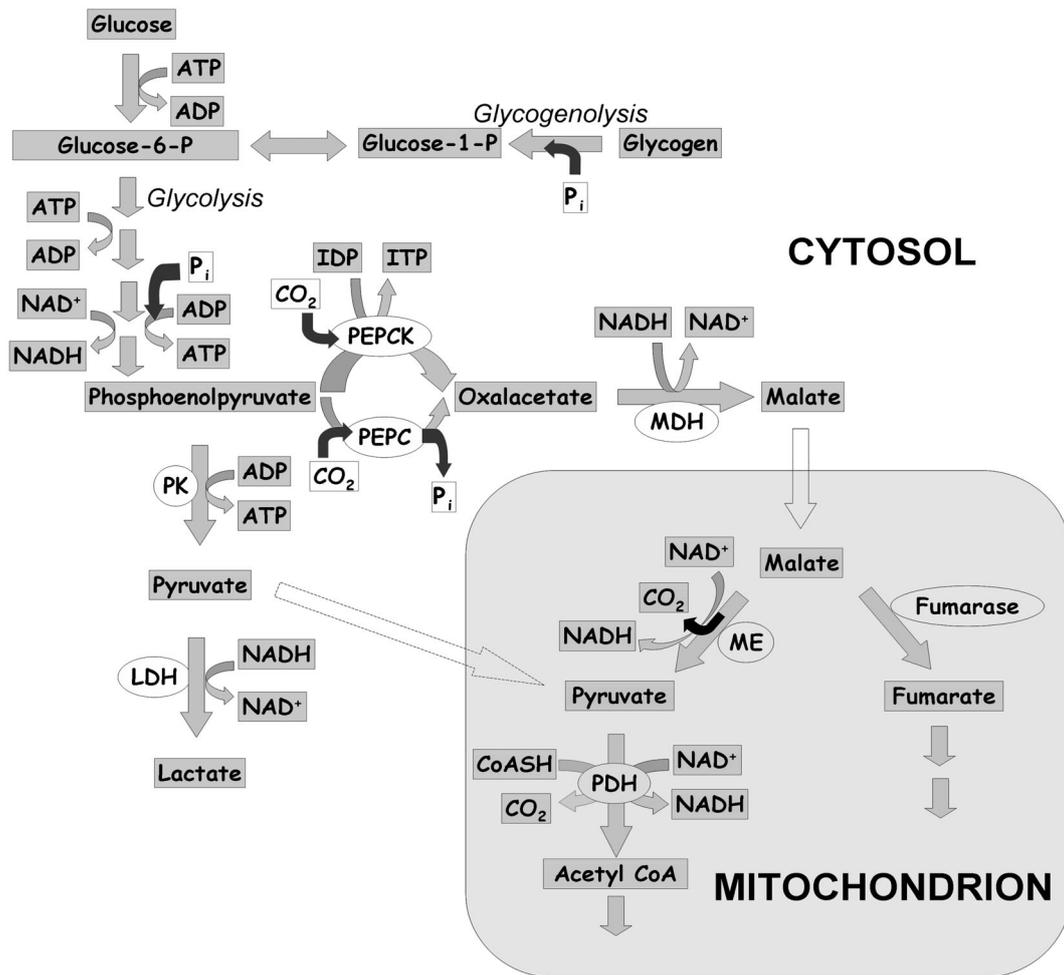


Fig. 4. *Hysterothylacium aduncum*. Proposed scheme for a hypothetical glucidic metabolism after the measured enzymatic activities throughout the third larval stage (L3) to adult *in vitro* development in this work, the data is from Zóltowska et al. (2002), and based on the metabolism from body wall muscle of adult *Ascaris suum* (see Komuniecki & Tielens 2003). Acetyl CoA: acetyl coenzyme A; ADP: adenosine 5'-diphosphate; ATP: adenosine 5'-triphosphate; CoASH: coenzyme A, reduced form; IDP: inosine 5'-diphosphate; ITP: inosine 5'-triphosphate; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; ME: malic enzyme; NAD⁺: nicotinamide adenine dinucleotide, oxidized form; NADH: nicotinamide adenine dinucleotide, reduced form; PDH: pyruvate dehydrogenase complex; PEPC: phosphoenolpyruvate carboxylase; PEPCK: phosphoenolpyruvate carboxykinase; P_i: orthophosphate; PK: pyruvate kinase

to obtain energy, as mentioned previously. While, in vertebrates, acetylCoA plays a key role in carbohydrate catabolism, in gastrointestinal helminths this role is performed by PEP (Lloyd & Barrett 1983). According to Bueding & Saz (1968) and Barrett (1981), the ratio of the activities of the enzymes that act on PEP, i.e. PK/PEPCK, gives an idea of whether the metabolism of a helminth will be preferentially fermentative (with formation of lactate) or CO₂-fixing (with formation of malate, which is incorporated into the mitochondrion to obtain anaerobic energy). In the present study, the ratio was always lower than 1, although in some developmental stages (L4 and L5J) it was higher than 0.5, the limit suggested by Barrett (1981) to define the metabolic tendency of the helminth as CO₂-fixing. In

any case, the greater activity of PEPCK than PK (1.4 to 4.1 times) and of MDH than LDH (2.1 to 3.1 times) –the latter being enzymes that remove the products of the reactions catalysed by PEPCK and PK, respectively– suggest that this helminth, during development from L3 to adult, is mainly CO₂-fixing, as occurs in *Ascaris* (see Barrett 1981 for references). However, Tielens & van den Bergh (1993) questioned the validity of this ratio calculated *in vitro*, since the *in vivo* concentrations of substrates, products, effectors and the enzymes themselves would modify the value of the ratio. If, to this, is then added the presence of other enzymes that can affect the fixing of CO₂, such as PEPC described in this study, or the possible use of pyruvate, formed by PK, as a substrate of the mitochondrial pyruvate dehy-

drogenase complex (PDH; Tielens & van den Bergh 1993), the situation is considerably more complex than that envisaged by Barrett (1981). In fact, there is important PK activity (Fig. 2) that might be explained by the need to recover, in the cytosol, some of the adenosine 5'-triphosphate (ATP) used in glycolysis. The pyruvate formed could be eliminated as lactate or recovered as a substrate of the mitochondrial PDH complex, contributing to the production of anaerobic energy, according to the needs of the parasite.

The high PEPCK activity (Fig. 1) detected may also result in a carbon source, which ensures the survival of L3, which, at least in other anisakids such as *Anisakis simplex*, cannot feed (Sommerville & Davey 1976 and references therein) and in which there is also significant activity of this enzyme (Dávila et al. 2006). In later developmental stages, this activity could be particularly important in the between-meals period of the host, which is when the worm must resort to glycogen and perhaps fixing of CO₂, as a carbon supplement to obtain anaerobic energy.

In addition, there is another enzyme that acts on PEP, viz. PEPC (Fig. 1), which represents an alternative, rare in the animal kingdom, to fixing of CO₂ by PEPCK. Its role is unknown, although it could be involved in the recovery of inorganic phosphate (P_i, used e.g. in glycogenolysis, glycolysis and ATP formation by ATP-synthase) in order to avoid a possible Crabtree effect, like that identified in another ascaroid, *Ascaris suum* (Harpur & Jackson 1976). Loiseau et al. (1993) detected this enzyme in helminths for the first time when studying *Molinema dessetae*, a filaria found in the peritoneal cavity of rodents, and explained its presence as an alternative to the low PEPCK activity in this parasite. This enzyme does not seem to be related to the other PEPC described, since its molecular weight is much lower than the enzyme of bacteria and plants.

Furthermore, PK and PEPC activity could be coordinated to add to or complement the PEPCK activity when P_i is required for cellular processes, but without losing the possibility of obtaining energy through the action of PK or the use, in the mitochondrion, of its product (pyruvate) as a substrate of the PDH (see Fig. 4).

High MDH activity (Fig. 3) was also detected. This removed the OAA resulting from the fixing of CO₂ by PEPCK and PEPC, recovering the oxidised form of nicotinamide adenine dinucleotide (NAD⁺) necessary for glycolysis. In *Ascaris*, this enzyme has mainly been found in the cytosol, reducing OAA. Dubinský & Ryboš (1981) reported that MDH activity in helminths decreases according to the inverse of the partial pressure of oxygen (pO₂), so *in vivo* (into the digestive tract of the host) activity is probably greater than that measured in the present study, since culture was carried out in the presence of oxygen from the air.

LDH activity (Fig. 3) is usual in parasitic nematodes, removing the pyruvate formed by PK, which is not sent to the mitochondrion. It is also believed to take part, together with MDH, in regulating the redox balance in the cytosol of the helminth cells (Barrett 1981).

During the developmental stages studied, the greatest difference in activity of the 2 enzymes was found between L3, recently collected from fish, and L3 48 h culture (Figs. 1 to 3). This may be because after addition to the culture, L3 are reactivated to continue their development towards the next stages of the cycle, while the L3 collected from the fish is still awaiting the arrival of a new paratenic or definitive host. The presence of isoforms depending on the developmental stages may also explain the differences in enzyme activity detected.

Finally, the high carboxylating activity of PEPCK detected in these nematodes, in contrast to its decarboxylating role in vertebrates, the presence of PEPC activity absent from vertebrates and the central role of PEP in helminth metabolism, are all characteristics that may be useful in the development of chemotherapeutic agents to combat this and possibly other helminths of economic and sanitary importance.

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