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Experimental Parasitology 114 (2006) 10–15
Experimental Parasitology
www.elsevier.com/locate/yexpr

Anisakis simplex: CO_2-fixing enzymes and development throughout the in vitro cultivation from third larval stage to adult

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Received 30 September 2005; received in revised form 3 February 2006; accepted 6 February 2006
Available online 5 April 2006

Abstract

We studied the effect of CO_2 on the in vitro cultivation of *Anisakis simplex*, an aquatic parasitic nematode of cetaceans (final hosts) and fish, squid, crustaceans and other invertebrates (intermediate/paratenic hosts), and, occasionally, of man (accidental host). The results showed that a high pCO_2, at a suitable temperature, is vital for the optimum development of these nematodes, at least from the third larval stage (L3) to adult. After 30 days cultivation in air, molting to L4 (fourth larval stage) was reduced to 1/3, while survival was about 1/3 of that when cultivated in air + 5% CO_2. The activity of the CO_2-fixing enzymes, PEPCK and PEPC, was also studied. Throughout the development of the worms studied, PEPCK activity was much higher than that of PEPC (e.g., 305 vs. 6.8 nmol/min·mg protein, respectively, in L3 collected from the host fish). The activity of these enzymes in the worms cultivated in air + 5% CO_2 was highest during M3, and was also generally higher than that of those cultivated in air only, especially during molting from L3 to L4 (e.g., in recently molted L4, PEPCK activity was 3.7 times greater than that of PEPC 2.9 times greater than when cultivated in air).
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Index Descriptors and Abbreviations: Anisakis simplex; Nematode; Anisakiasis; pCO_2; Development; CO_2-fixation; Phosphoenolpyruvate carboxylase; Phosphoenolpyruvate carboxykinase; ATP, adenosine triphosphate; EDTA, ethylene diamine tetraacetic acid; GDP, guanosine diphosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; L3, third larval stage; L4, fourth larval stage; L5, adult stage; M3, molting from L3 to L4; M4, molting from L4 to L5; MDH, malate dehydrogenase; ME, malic enzyme; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogenated; OAA, oxalacetate; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; Tris, tris(hydroxymethyl)aminomethane

1. Introduction

Anisakis simplex is an ascaridoid parasite of the digestive tract of cetaceans although its infective phase (L3) is found in marine fish, squid, and crustaceans in almost all seas in the world. Cetaceans (final host) and man (accidental host) are infected on consuming hosts containing L3s. It is known that, at least in vitro, CO_2 is required for M3 and survival of some nematodes from the digestive tract of mammals (Petronijevic et al., 1985; Rogers, 1960), such as anisakids (Iglesias et al., 1997, 2001, 2002; Sommerville and Davey, 1976).

In many organisms, the fixing of CO_2 has been shown to be a source for carbon atoms for a range of cellular metabolic processes. Two enzymes involved in this fixing use PEP as a substrate, producing OAA: PEPCK and PEPC. Another two CO_2-fixing enzymes use either pyruvate rather than PEP as a substrate to obtain OAA (the case of PC) or malate (the case of carboxylating-ME). Mitochondrial ME is of great importance for the metabolism in helminths due to their 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 the enzymes mentioned have been detected, with a greater or lesser activity, in parasitic helminths (see Barrett, 1981 for references; Loisseau et al., 1993; Mallick et al., 1991; and others). Furthermore, in a previous study, we

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doi:10.1016/j.exppara.2006.02.011

detected PEPCK and PEPC activity in L3 from fish and in vitro just molted L4 of two anisakids, *A. simplex* and *Hysterothylacium aduncum* (Iglesias et al., 2005).

In the current work, we study the activity of these enzymes throughout the development process of this parasite in an atmosphere with or without added CO_2, showing that the activity of the two enzymes is detected throughout all the development of the parasite in vitro (from L3 to adult), with important variations, especially during M3.

2. Materials and methods

2.1. Collection of L3 larvae and cultivation

The L3 of *A. simplex* were obtained from the body cavity of blue whiting (*Micromesistius poutassou*). After washing the larvae with saline solution (NaCl, 0.9%), samples were frozen (−80 °C) for enzyme determination. The rest of the larvae were axenized by immersion in an antibiotic-antifungal solution (Iglesias et al., 1997), and individually placed in culture, in RPMI-1640 medium modified by Iglesias et al. (2001) at pH 4.0 and 36 °C, to determine the exact stage of development of each larva. The culture medium was renewed twice a week. Batches were held in humid atmosphere of air or of air + 5% CO_2.

2.2. Preparation of the samples

The following samples were taken and frozen (−80 °C) for enzyme determination: L3 from fish, L3 after 24 and 48 h of cultivation, L4 just molted (L4-0 h) and L4 after 24 and 48 h of molting, and worms after 10 (L3 in air; L4 with 5% CO_2), 20 (L3 in air; L4 with 5% CO_2), 30 (adults recently molted), 40 (young adults), and 50 (mature adults) days of cultivation. The high mortality of the worms cultured in air without added CO_2 did not allow sampling after 20th culture day. For enzyme determination, the samples were treated as previously described (Iglesias et al., 2005).

2.3. Enzyme determination

This was followed spectrophotometrically by disappearance of NADH from the assay mixture at 340 nm and 37 °C. PEPCK activity was measured by a modification of the assay described by Iglesias et al. (2005). In previous assays, the highest initial velocity was obtained using Mn^{2+} instead of Mg^{2+} and IDP instead of GDP in the PEPCK assay. Controls were carried out in all cases to rule out other overlapping enzyme activities (e.g., lactate dehydrogenase activity when carboxylating-ME or PC activity was being measured). Briefly, the assay mixture to determine PEPCK activity contained: 32 mM Tris–HCl, pH 7.4, 3 mM $MnSO_4$, 0.25 mM β-NADH (disodium salt), 3 mM IDP (sodium salt), 2.5 U MDH (E.C. 1.1.1.37), extemporaneous solution of 16 mM $NaCO_3H$ bubbled with CO_2 and extemporaneous solution of 3 mM PEP (sodium salt, monohydrate) and 40 μg of protein from the extract, in a final volume of 1.555 ml. PEPC, PC, and carboxylating-ME activities were assayed following the techniques previously described (Iglesias et al., 2005). Treatment values were tested for significance by Student's *t* distribution. Substrates, enzymes, and other reagents were from Sigma Chemical Co., Boehringer Mannheim and Merck.

3. Results

3.1. CO_2 and culture

Fig. 1 shows the effect of CO_2 on the development and survival of the larvae. The presence of 5% CO_2 in the culture atmosphere of *A. simplex* was vital for achieving high percentages of molting, both M3 and M4, and a greater survival rate. After 33 days cultivation only 27.6% of the larvae in air survived while, 92.9% of those in air + 5% CO_2 survived. In the culture with CO_2, 100% of the M3 took place between days 3 and 6 while only 35% of the larvae molted, between days 6 and 27, in air. Without added CO_2, M4 was rare (3.3% of L3 or 9.5% of L4), whereas it was frequent when present (71.4% on the 33rd day of culture). This molting mainly took place between weeks 3 and 5 in culture. The distribution of the live larvae in the culture can also be observed for any given moment in Figs. 1(B and D). At the end of the experiment, all the live worms were adults in the presence of 5% CO_2 while, in its absence, almost 50% of the larvae were still L3 and showed very little motility.

A little before molting to L4, the larvae showed little or very little motility. However, immediately after molting motility became high, especially in the presence of CO_2. This was also true for M4, although less obvious. This increase in motility favors the shedding of the larval sheath.

Molting in air + 5% CO_2 takes place in a few hours. However, in air, it may last several days and many larvae degenerate and die without completing the molt. These larvae have difficulty in freeing themselves of the sheath, which often remains attached to the head end of the larvae (Fig. 2A) while, the body frees itself from the rest of the old cuticle. Sometimes, although the entire body is free, the sheath stays stuck to the mouth for several days. If the larva cannot free itself, it degenerates and dies, possibly as a result of it being unable to feed orally.

Although the sheath shed following M3 is quite robust and slightly striated, often intact (Fig. 2B), the sheath shed after M4 seems more fragile with obvious striation (Figs. 2C and D). It breaks into pieces and individual cuticular rings may be observed.

3.2. Enzyme activity

Of the four enzymes assayed, we only detected significant activity in two: PEPCK and PEPC. No significant activity was detected in PC or in carboxylating-ME in any of the development stages studied.

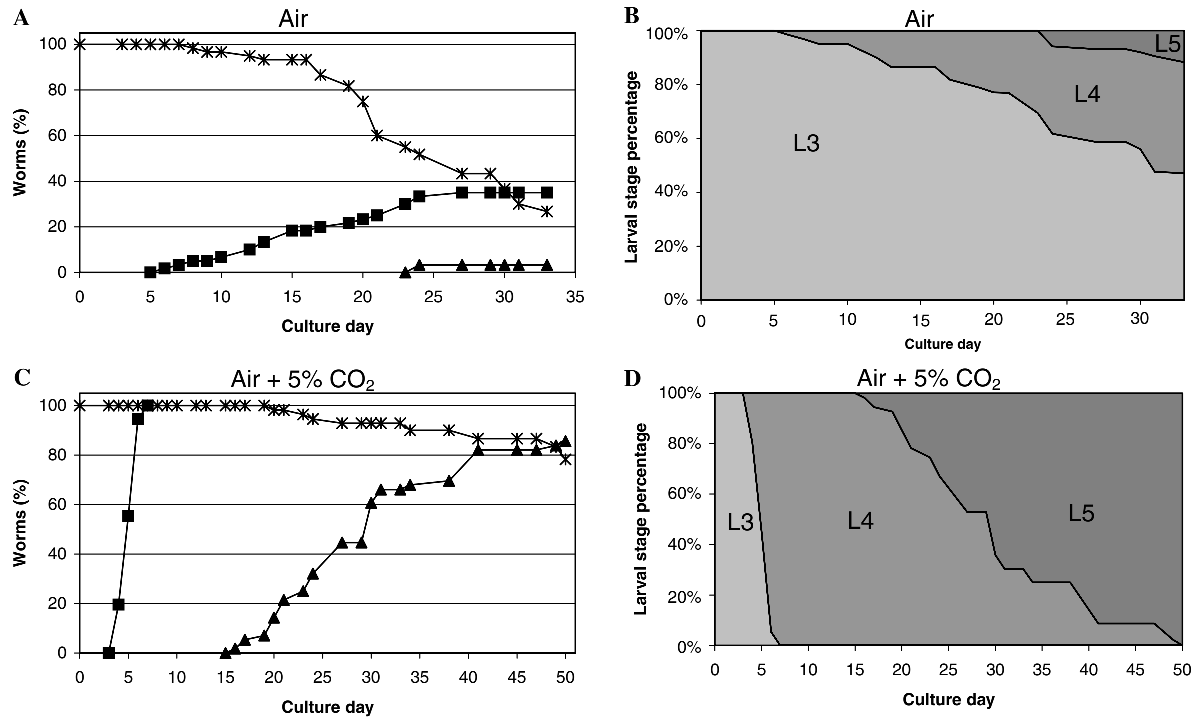

Fig. 1. Effect of CO_2 on the in vitro molting and survival of *Anisakis simplex* larvae in culture. (A and C) Percentage of larvae that completed M3 (■) and M4 (▲) and of worms alive (∗) throughout the culture time. (B and D) Percentage of the third- (L3), fourth-larval (L4), and adult (L5) stage alive throughout the culture time. 100% = all living worms.

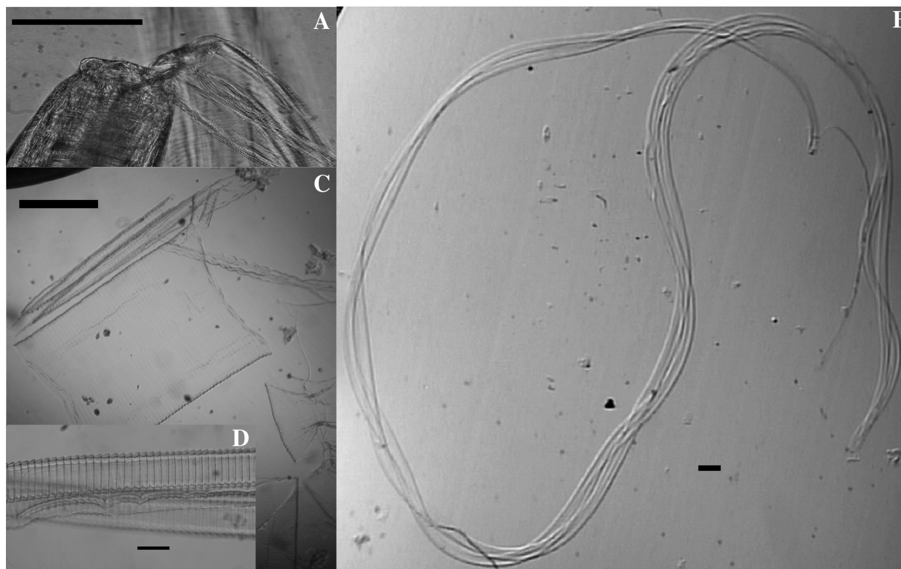

Fig. 2. *Anisakis simplex* molting in culture. (A) The sheath after M3 attached to the head end of the larvae. (B) The sheath following M3 shed in one piece. (C and D) The sheath shed after M4 breaks into pieces; striation can be observed in (D). Bars = 1 mm.

Figs. 3 and 4 show the behavior of the activity of PEPCK and PEPC during the development of the worms cultivated in air with or without 5% CO_2. The M3 period was studied in detail from the moment the L3 was placed in the culture until 48 h after molting to L4. This period is relatively synchronized in air + 5% CO_2, but not in air alone. The activity of the enzymes in the larvae cultivated in air + 5% CO_2 is clearly and significantly higher than in those cultivated in air during all, or almost all, this molting process. The high mortality of the larvae in air did not allow us to continue the study of these enzymes beyond 20 days of culture when three of every four larvae are still L3 (Fig. 1).

Finally, the peak of activity of both enzymes during and immediately after M3 in the presence of 5% CO_2 should be

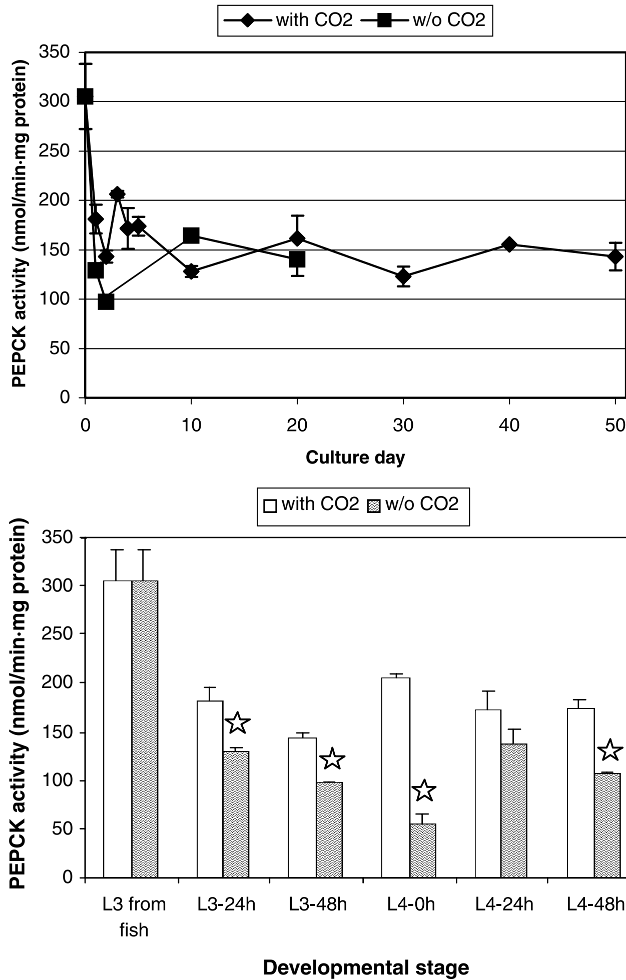

Fig. 3. PEPCK activity throughout in vitro development of *Anisakis simplex* (top) and throughout M3 (bottom). Each point is the mean of two to three experiments in triplicate ±SD. (◆) Worms cultured in air atmosphere + 5% CO_2; (■) Worms cultured in air atmosphere without added CO_2. (Star) Student *t* distribution, $P < 0.05$. L3, third larval stage; L4, fourth larval stage.

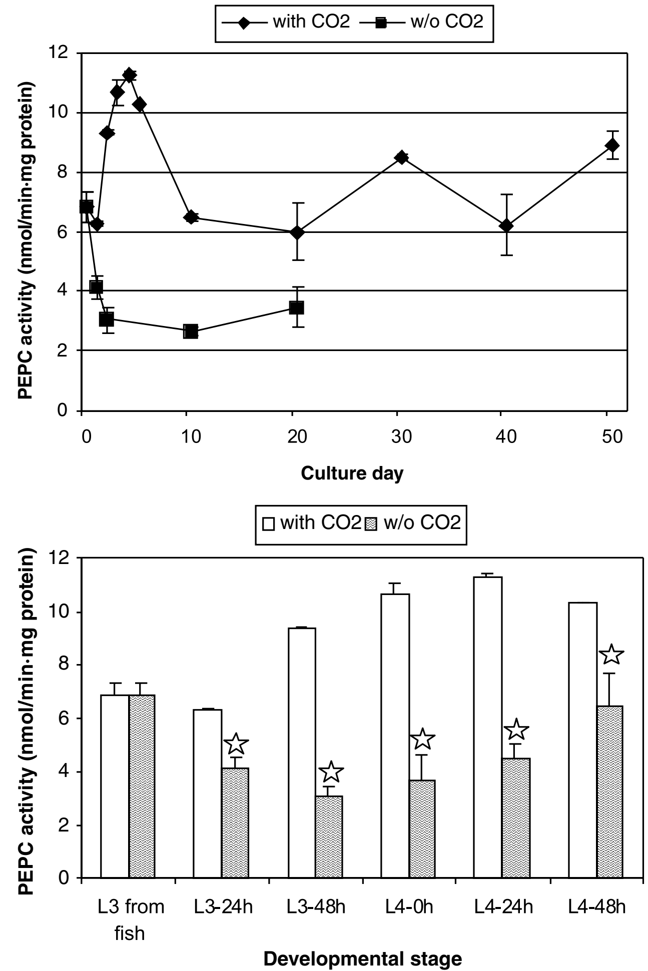

Fig. 4. PEPC activity throughout in vitro development of *Anisakis simplex* (top) and throughout M3 (bottom). Each point is the mean of two to three experiments in triplicate ±SD. (◆) Worms cultured in air atmosphere + 5% CO_2; (■) Worms cultured in air atmosphere without added CO_2. (☆) Student *t* distribution, $P < 0.05$. L3, third larval stage; L4, fourth larval stage.

noted, as should the high CO_2-fixing activity of PEPCK in the L3 freshly collected from the host fish (305 nmol/min · mg protein). After molting, PEPCK activity decreased to around 120 and 160 nmol/min·mg protein.

4. Discussion

Many parasitic helminths require a certain pCO_2, which acts as a stimulus in one or more development stages. In the nematodes, this stimulus generally affects the infective stage and seems to allow it to recognize its surroundings and activate its infectivity. Many gastrointestinal nematodes activate their molting and posterior development in vitro with a high pCO_2, similar to that found in vivo on reaching the digestive tract of the host (Petronijevic et al., 1985; Rogers, 1960), as occurs in aquatic nematodes (Iglesias et al., 1997, 2001, 2002; Sommerville and Davey, 1976). In *A. simplex,* we observed a decrease in survival of the larvae in air, as occurred in other media (Iglesias et al., 1997, 2001), especially from the start of the third week of culture (Fig. 1). We also observed a low percentage of both M3 and M4, with survival being three times lower in air after the first month of cultivation. At the end of the experiment, the L3, which had not molted (50%), were placed in air + 5% CO_2 and it was observed that two of every three reinitiated their development, molted to L4 and finally reached adult stage. This behavior is similar to that of the larvae which were kept in the presence of CO_2 but at suboptimal temperatures (15 °C): their mobility was very low and they did not molt although they retained their capacity for development, since, when the temperature was raised to 36 °C, they reinitiated their development, molting and reaching the adult stage (results not shown). Petronijevic and Rogers (1987) described the infective stages of nematodes as being in a state of "hypometabolic dormancy", remaining in this state until the exposure to suitable stimuli results in molting and posterior development. Our data (this study and Iglesias et al., 1997, 2001) show that a suitable temperature, close to that of the body temperature of

the host, and a CO_2 rich atmosphere, similar to that found in the digestive tract of many mammals, are two stimuli required, as suggested by Sommerville and Davey (1976), and are complimentary for the development of L3 of *A. simplex*, at least in vitro. The inhibition of M3 in L3 cultivated in air also occurs in other parasitic nematodes of the digestive tract of mammals, such as *Ascaris suum* (Vanover-Dettling and Komuniecki, 1989).

The high pCO_2 may be related to the need to incorporate carbon atoms from this gas into the energetic metabolism of the parasite. For this reason, we have studied the CO_2-fixing enzymes. Of the four enzymes studied, we only detected significant activity in two of them throughout the in vitro development of the worm: PEPCK and PEPC. No significant activity was detected for PC or carboxylating-ME in any of the development stages studied. However, in our laboratory, we have, on other occasions, detected decarboxylating-ME activity in all of these stages (Iglesias, 1999), and other authors have also detected activity in many parasitic helminths (see Barrett, 1981 for references; Park et al., 1984). While acetyl-CoA is a key molecule for the catabolism of carbohydrates in vertebrates, in parasitic helminths this function is carried out by PEP (Lloyd and Barrett, 1983). PEP is an important branch-point leading to pyruvate via pyruvate kinase or to OAA and the reverse tricarboxylic acid cycle via PEPCK. In helminths, the formation of OAA from PEP is generally catalyzed by PEPCK, although PEPC may also catalyze this reaction. In both cases, this occurs due to CO_2-fixing. In *A. simplex*, the CO_2-fixing activity, both by PEPCK and by PEPC, is greater in the parasites cultivated in air + 5% CO_2, especially during M3, the difference being greatest in the larvae which have recently molted to L4 ($P < 0.00003$ for PEPCK and $P < 0.005$ for PEPC). Furthermore, PEPCK activity is much greater than that of PEPC (Figs. 3 and 4). In the presence of CO_2, no significant changes ($P > 0.1$) in the activity of the enzymes assayed were detected from L4 from the 20th culture day up to mature adult.

Although, in many vertebrates, PEPCK generally acts as a glyconeogenic enzyme, decarboxylating OAA to PEP, many parasitic helminths of the mammalian digestive tract show greater activity in the opposite direction (see Bryant, 1975 for references). This may be due to a Km for bicarbonate (source of CO_2) several times lower than that of the vertebrates, at least in *A. suum* (Rohrer et al., 1986). Furthermore, the state of hypometabolic dormancy undergone by the infective larvae (Petronijevic and Rogers, 1987) may be applicable to the L3 of *A. simplex* found in the peritoneal cavity of the host fish. The high PEPCK activity detected in them may also result in a carbon source, which ensures the survival of larvae, which, according to some authors, cannot feed (Sommerville and Davey, 1976; Yasuraoka et al., 1967). However, these survive for up to 3 years in their hosts while increasing in size extremely slowly (Smith, 1984; Strømnes and Andersen, 2003). The significant decrease ($P < 0.02$) in PEPCK activity of L3 after being placed in culture, both with and without CO_2, has been related to the activation of the development of the larvae induced by these changes (Iglesias et al., 2005). The posterior significant increase ($P < 0.001$) in activity following M3 in air + 5% CO_2, must be related to a change towards a more anaerobic metabolism in which PEPCK has an important role, similar to that occurring in *A. suum* (Vanover-Dettling and Komuniecki, 1989). Loisseau et al. (1993), working with the filarial *Molinema dessetae*, first showed PEPC activity in parasitic helminths. We have detected this in L3 from fish and in vitro just molted L4 of *H. aduncum* (Iglesias et al., 2005) and, in this work, in L3 from fish and all in vitro development stages of *A. simplex* (L3 up to mature adult). It is not known whether this enzyme is common in other helminths and its role in the metabolism of the parasite is also unknown.

The differences in PEPCK and PEPC activity found throughout the development of the parasite may be due to the regulation of expression or activity of the enzymes or to the expression of different isoforms associated with the development stages of the worm.

Finally, the presence of PEPC in these nematodes (absent in vertebrates), the role of CO_2 in their development, the central role of PEP in the catabolism of carbohydrates and the carboxylating activity of PEPCK in helminths, the opposite of the decarboxylating activity in vertebrates, are all peculiarities which should be exploited as potential therapeutic targets.

Acknowledgments

This work has been funded by the Spanish Grants PB98-1312 from the DGESIC and ACU01-027 from the INIA. D. Malagón is a recipient of a predoctoral fellowship from Consejería de Agricultura y Pesca, Junta de Andalucía (Spain). Our thanks to Manuel Morales and J.A. García Sanjuan for their help with the photographs and to Dr. A. M. Adroher for his help with the Fig. 2. The English translation was by Mr. Robert Abrahams, BSc.

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