

EVIDENCE OF AN ALTERNATIVE OXIDASE IN THE MITOCHONDRIAL RESPIRATION IN THE SCUTICOCILIATE PARASITE *Philasterides dicentrarchi*

Mallo, N.,^a Lamas, J.^b, Leiro, J.^{a,*}

^aDepartamento de Microbiología y Parasitología, Laboratorio de Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, Spain.

^bDepartamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Spain

SHORT TITLE: Alternative oxidase in *Philasterides dicentrarchi*

*Corresponding author

Laboratorio de Parasitología,
Instituto de Investigación y Análisis Alimentarios,
c/ Constantino Candeira s/n,
15782, Santiago de Compostela (La Coruña), Spain
Tel: +34 981 56 31 00
Fax: + 34 881 81 60 27
E-mail address: josemanuel.leiro@usc.

Highlights

- ▶ *Philasterides dicentrarchi* displays a mitochondrial alternative electron pathway.
- ▶ Possesses a cyanide-insensitible AOX inhibitable by salicylhydroxamic acid.
- ▶ AOX is inducible by hypoxia, during infection and in the stationary growth phase.
- ▶ AOX could be considered as potential drug target against scuticociliatosis.

Abstract

Our aim was to detect the presence of an alternative oxidase (AOX) in the mitochondrial respiration of the scuticociliate parasite of turbot, *P. dicentrarchi*. Measurements of O₂ consumption were performed in the presence of 100 μM KCN, an inhibitor of the cytochrome respiratory pathway (CP), and 1 mM salicylhydroxamic acid (SHAM), a specific inhibitor of alternative respiratory pathway (AP). Western blotting using an AOX polyclonal antibody monitored AOX expression. Our results indicate that *P. dicentrarchi* behaves like a microaerophilic microorganisms and possesses a branched mitochondrial electron transport chain, with both cyanide-sensitive and -insensitive oxygen-consumption activities. Mitochondrial respiration was virtually completely inhibited by the combination of cyanide and salicyl hydroxamic acid (SHAM), which is a direct evidence of the existence of an AP in this ciliate. Application of SHAM significantly inhibited the trophozoite growth *in vitro* cultures both under normoxia and hypoxia conditions. In *P. dicentrarchi* AOX is a monomeric protein of approximately 50 kD that is induced by hypoxic conditions, in experimental infections and by CP inhibitors as cyanide and antimycin A or by AP inhibitors as SHAM. The CP is stimulated drastically during the exponential phase of growth and decreases during the stationary phase in which induces the expression of the AOX. In the absence of AOX in the host and since during infection *P. dicentrarchi* breathe primarily via AP coinciding with increased expression of AOX, the possibility of developing non-toxic inhibitors targeting the alternative branches of the respiratory pathway might be exploited as a novel anti-scuticociliate therapy.

Keywords: *Philasterides dicentrarchi*, mitochondrial respiration, hypoxia, alternative oxidase, salicylhydroxamic acid, trophozoite growth.

1. Introduction

The anizoic scuticociliate *Philasterides dicentrarchi* is the aethiological agent of scuticociliatosis, an emergent parasitic disease that cause high mortalities in the farmed turbot *Scophthalmus maximus* (Iglesias et al. 2001; Ramos et al. 2007). Ciliates are an extremely diverse group of protists that can easily adapt to different environmental conditions, such as a large decrease in oxygen concentration (Fenchel and Finlay 2008). Particularly, benthic scuticociliates are microaerophilic taxa of ciliates that are sensitive to high oxygen concentration, and the first appeared at the oxic/anoxic interface and were present throughout the anoxic hipolimnion (Hayward et al., 2003). Many nonparasitic organisms, particularly those that reside in microaerophilic enviroments, have developed a series of modifications in their energy metabolism that facilitates their adaptation to parasitism (Saz, 1981).

Respiration of mitochondria from the scuticociliate *P. dicentrarchi* was comparable to that of mitochondria from other ciliates; however, the respiration cannot be completely blocked by cyanide, an inhibitor of cytochrome c oxidase (mitochondrial complex IV) (Sánchez-Alcazar et al., 1995; Morais et al., 2012), what might be an evidence of the existence of a cyanide-insensitive alternative oxidase (AOX) in this ciliate.

AOX is a respiratory chain protein found in all higher plants, fungi, non-fermentative yeast and protozoans (Moore et al., 2002). Fungi and higher plants mitochondria contain two respiratory electron transport chains: one is the cytochrome pathway (CP) with cyanide-sensitive cytochrome oxidase as the terminal oxidase; another one is the alternative pathway (AP), which branches from CP in the inner mitochondrial membrane at the site of the ubiquinone pool and uses AOX as its terminal oxidase (Moore et al., 2008; Williams et al., 2010; Xu et al., 2012). AOX is a non-haem diiron carboxylate protein that utilize the diiron center to catalyze the oxidation of ubiquinol to the complete reduction of oxygen to water (Affourtit et al., 2002; Berthold and Stenmark, 2003; Moore

et al., 2008). AOX is cyanide-resistant, but sensitive to substituted hydroxamic acids, such as salicylhydroxamic acid (SHAM) (Vanlerberghe, 1997). From a functional viewpoint, in plants and fungi the AOX plays a key role in the release of heat for pollination purposes or for maintaining a warm environment within the flower at low ambient temperatures, photoprotection, to maintaining TCA cycle turnover under high cytosolic phosphorylation potentials, defense against oxidative stress and growth rate and energy charge homeostasis (Finnegan et al., 2004; Zhang et al., 2011; Xu et al., 2012). In lower eukaryotes, AOX was proposed to play a role in the oxidative defense mechanism and represents an important factor for the pathogen survival in macrophages (Magnani et al., 2007). Of particular importance is the presence of AOX in pathogenic protozoa including *Trypanosoma* (Clarkson et al., 1989; Chaudhuri et al., 2006), *Cryptosporidium* (Mogi and Kita, 2010) and, possibly, *Plasmodium* (Torrentino et al., 2010); but are absent from their mammalian host, making AOX a suitable chemotherapeutic target (Roberts et al., 2004).

The principal purpose of this study was investigating the possible existence of a cyanide-insensitive terminal oxidase in the mitochondrial respiration of the asexual scuticociliate *P. dicentrarchi*, perform a molecular characterization and explore its expression level under several physiological conditions.

2. Materials and methods

2.1. Animals

Turbot *Scophthalmus maximus* (L.) of approximately 50 g body weight were obtained from a local fish farm (Galicia, Spain). The fish were held in 250-L tanks, with recirculating, aerated sea water maintained at 14 °C, under a photoperiod of 12L:12K, and they were fed daily with commercial pellets (Skretting, Burgos, Spain). The fish were acclimated to laboratory conditions for 2 weeks before the start of the experiments. All experiments were carried out in accordance with European regulations on animal protection (Directive 86/609), outlined in the Declaration of Helsinki. All experimental protocols were approved by the institutional Animal Care and Use Committee of the University of Santiago de Compostela.

2.2. Ciliate culture and experimental infections

Naturally infected turbot (*Scophthalmus maximus*) showing signs of scuticociliatosis were obtained from a local fish farm (Galicia, Spain). Specimens of *P. dicentrarchi* (isolate I₁; [Budiño et al., 2011](#)) were collected under aseptic conditions from ascitic fluid removed from the intraperitoneal cavity of the fish as previously described by [Iglesias et al., 2001](#). The ciliates were cultured at 21 °C, in complete sterile L-15 medium (Leibovitz, PAA Laboratories GmbH, 10% salinity, pH 7.2) containing 90 mg/L each of adenosine, cytidine and uridine, 150 mg/L of guanosine, 5 g/L of glucose, 400 mg/L of L- α -phosphatidylcholine, 200 mg/L of Tween 80, 10% of heat-inactivated foetal bovine serum (FBS) and 10 mL/L of 100X antibiotic-antimycotic solution (=100 units/mL of penicillin G, 0.1 mg/mL of streptomycin sulfate and 0.25 mg/mL of amphotericin B) as previously described ([Iglesias et al. 2003a](#)). In normoxia experiments, ciliates were cultured in tissue culture flasks with caps fitted with aeration. For hypoxia experiments, argon is bubbled over the complete L-15 medium for 10 min and then sealed culture flasks.

Experimental infections in turbot were performed following the protocol we have previously described (Paramá et al., 2003).

2.3. Determination of anti-ciliate activity by AOX inhibitors

The anti-ciliate activity of SHAM was assayed as previously described (Iglesias et al., 2002), with minor modifications (Leiro et al., 2004). SHAM (Sigma-Aldrich) was prepared in 100 mM stock solutions in dimethyl sulphoxide (DMSO) and maintained at $-20\text{ }^{\circ}\text{C}$ in the dark until use. To investigate anti-ciliate effects, SHAM in 1 ml of complete L-15 medium was added to the wells of 24-well sterile culture plates (Corning) containing 10^5 ciliates per well, to final concentrations of 0.01, 0.1 and 1 mM, and then incubated for three days at $21\text{ }^{\circ}\text{C}$ in . The number of ciliates present on days 1, 2 and 3 after the start of incubation in normoxia and hipoxia (see above) was quantified in a 25 μl aliquot of liquid removed from each well: the ciliates were first inactivated with glutaraldehyde (final concentration 0.25%) for 15 min at room temperature, then counted in a haemocytometer (Iglesias et al., 2002). Five replicates were used in all experiments. To rule out possible effects of the solvent (DMSO), five replicate wells with L-15 medium containing the highest concentration of DMSO used (100 μM) were also included.

2.4. Measurement of mitochondrial oxygen consumption with a Clark electrode

To study the functioning of the mitochondrial respiratory chain, the ciliates were permeabilized with DIG (Sigma) (Cuperus et al. 2010). Ciliates (3×10^6 ciliates / ml)

were centrifuged (700 x g for 5 min), washed twice with L15 incomplete médium (i.e. L-15 medium without nucleosides, glucose, lipids or FBS) and resuspended in 15 mL of incomplete medium L15. PMSF was then added to a final concentration of 1 mM and 100 μ L of 10 mM DIG. The tubes were shaken gently, to avoid breaking the cells, for one minute and re-centrifuged at 700 \times g for 5 min at 4 ° C. The cells were then resuspended in 0.1 mL of incomplete L15 medium. During this time, the oxigraph chamber (Hansatech Instruments, UK) was emptied and filled with 500 μ L of incomplete L15 medium, 50 μ L of 50 mM ADP and 100 μ L of permeabilized cells and thermostated to 25°C. After stabilization of the baseline, 50 μ L each of the 1 M glutamate (G) and 1 M succinate (S) stock solutions in PBS were added with a Hamilton syringe. The final concentrations of inhibitors used were 100 μ M KCN and 1mM of SHAM (Sigma-Aldrich, Spain). All assays were performed in triplicate, and O₂ consumption was analyzed by Oxygraph Plus software (Hansatech, UK). The difference in the rate of O₂ consumption before and after compound addition was calculated to determine the percent inhibition. DMSO used to solubilize the inhibitor SHAM not affected the O₂ consumption at the maximum concentrations used. To analyze the total consumption of O₂ by the ciliates, we used the same protocol as that described above, but without treating ciliates with DIG, and without adding metabolic substrates.

2.5. SDS-PAGE and immunoblotting analysis

Ciliate membrane-associated proteins (MAP) were extracted by phase separation in Triton X-114 solution ([Bordier, 1981](#)) by a previously described method ([Iglesias et al., 2003b](#)). Specifically, 10⁷ cells were resuspended in 1 ml of ice-cold 10 mM Tris-HCl

buffer, pH 7.5, to which 1 ml of ice-cold extraction buffer (300 mM NaCl, 20 mM Tris–HCl, pH 7.5, 2% Triton X-114) was subsequently added. The cytoskeletal elements were eliminated by centrifugation at 16000 x g for 10 min at 4 °C. The supernatant was then transferred to 1.5 ml Eppendorf tubes which were heated for 5 min at 37 °C. At the end of this period the solution clouded as a result of the micellar condensation of the detergent. The sample was then divided among 0.5 ml Eppendorf tubes (200 µl/tube) containing 300 µl of sucrose cushion (6% sucrose, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 0.06% Triton X-114). Subsequently, the detergent and aqueous phases were separated by centrifugation at 300 g for 4 min at room temperature. The resulting supernatants on the sucrose cushion of each tube were carefully extracted and mixed in new 1.5 ml Eppendorf tubes. Subsequently, the extraction process was repeated adding to the aqueous mixture sufficient Triton X-114 to obtain a final concentration of 0.5%. The mixture was then reheated at 37 °C for 5 min and then, after micellar condensation, distributed among the original Eppendorf tubes containing the sucrose cushion and the detergent phase separated in the first extraction. The tubes were then centrifuged again at 300 g for 4 min at room temperature. After centrifugation, the supernatant was discarded and the proteins contained in the detergent phase were precipitated by adding 9 volumes of cold acetone, then resuspended by vortexing and incubated for 30 min on ice. At the end of this period the precipitated membrane proteins were collected by centrifugation at 16000 g for 15 min at 4 °C, and dried in a speed vacuum concentrator (MiVac, GeneVac, UK). Finally, the extracts obtained were resuspended in 10 mM Tris–HCl, pH 7.5, and stored at -80 °C until use.

Total and MAP proteins of *P. dicentrarchi* isolates were separated by SDS-PAGE in 12.5 % linear gels (Piazzon et al., 2008). For polypeptide analysis of total proteins, 3×10^6 ciliates was resuspended in 100 µL of reduction buffer (62 mM Tris–HCl buffer,

pH 6.8, containing 2% SDS, 10% glycerol and 0.02 M dithiothreitol (DTT) and 1 mM PMSF) and were denatured by incubation for 5 min at 100 °C. For non-reduced conditions, integral membrane proteins were prepared in the same sample buffer but without heating and without DTT. The gels were electrophoresed, and then stained with Thermo Scientific GelCode Blue Safe Protein Stain (Thermo Fisher, USA) for qualitatively determining the protein concentration of each sample. Reduced and non-reduced antigens were immunoblotted at 15 V for 35 min to Immobilon-P transfer membranes (0.45 mm; Millipore, USA) in a trans-blot SD transfer cell (Bio-Rad, USA) with the electrode buffer containing 48 mM Tris, 29 mM glycine, 0.037% SDS and 20% methanol, pH 9.2. Membranes were washed with Tris buffer saline (TBS; 50 mM Tris, 0.15 M NaCl, pH 7.4) and stained with Ponceau S to verify transfer, blocked for 2 h at room temperature with TBS containing 0.2% Tween 20 and 5% non-fat dry milk, then washed in TBS and incubated for overnight at 4°C with rabbit anti-AOX1/2 plant alternative oxidase (Agrisera, Sweden) at 1:500 dilution. After washing with TBS, the membranes were then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit Ig (Dakopatts; dilution 1:800) and visualized with enhanced luminol-based chemiluminiscent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, USA) and then photographed with a FlourChem® FC2 imaging system (Alpha Innotech, USA).

In inhibition experiments of the AOX expression, ciliates were incubated for 4 hours with KCN and antimycin A (AMA, Sigma-Aldrich) at a concentration of 100 µM and SHAM a concentration of 1 mM.

2.6. Statistical analysis

The results shown in figures are expressed as means \pm standard error. The statistical significances ($\alpha = 0.05$) of differences were determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons.

3. Results

3.1. *In vitro* growth of *P. dicentrarchi* under normoxia and hipoxia conditions

In a first experiment, we tested whether *P. dicentrarchi* was able to grow *in vitro* cultures under microaerophilic conditions. To this end, ciliates grow in culture conditions in normoxia (available oxygen in the culture medium through the use of flasks with vented caps) and hypoxia culture conditions; in the latter case, these conditions can be achieved by bubbling with argon gas to displace the gases dissolved in the medium, including oxygen, and the sealed flasks were maintained throughout the experiment. The results obtained are shown in Fig. 1, where one can see that the growth kinetics by ciliates cultured in normoxia conditions is very similar to the kinetics of growth by ciliates kept in conditions of hypoxia, there being no statistically significant growth *in vitro* of the ciliates between both.

3.2. Presence of an alternative electron transport pathway in the mitochondrial respiration of *P. dicentrarchi*

To demonstrate the existence of a possible alternative electron transport pathway type, experiments were performed on mitochondrial respiration function of *P. dicentrarchi* by polarographic assays on O₂ consumption in ciliates permeabilized with DIG to which the

respiration was induced by addition of succinate (S), glutamate (G) and ADP. We initially tested the effect exercised only by the SHAM on O₂ consumption by the parasite. As can be seen in Fig. 2A, SHAM adding to the concentration of 1 mM led to a decline in the O₂ consumption of 60% with respect to ciliates incubated in the absence of specific inhibitor of the AOX. Then determined the activity of cyanide and SHAM against parasite respiration. When incubated trophozoites of *P. dicentrarchi* in the presence of 100 μM KCN an mean inhibition of 40% in O₂ consumption was obtained, and these O₂ consumption was completely inhibited upon further addition of 1 mM SHAM (Fig. 2B).

3.3. Growth of *P. dicentrarchi* in the presence of specific AOX inhibitors

This experiment evaluated specifically the inhibitory activity of SHAM at concentrations of 0.01, 0.1 and 1 mM on the *in vitro* growth of ciliates during the exponential phase of culture under normoxic and hypoxic conditions, and results are shown in Fig. 3. SHAM presents from the second day of culturing a significant inhibitory effect on the *in vitro* growth of ciliate at concentrations above 0.1 mM, both normoxia (Fig. 3A) and hypoxia conditions (Fig. 3B).

3.4. Immunochemical identification of the cyanide-insensitive AOX in *P. dicentrarchi*

The identification and characterization of the AOX of *P. dicentrarchi* was performed by Western-blot using a rabbit polyclonal antibody produced after immunization with a synthetic peptide derived from fully conserved C-terminal consensus motif from plant AOX isoforms 1 and 2 conjugated with Keyhole limpet hemocyanin (KLH). Immunoblotting of total reduced proteins of ciliate with polyclonal antibodies against

AOX shows a strong cross-reaction with the ciliate oxidase proteins (Fig. 4). In immunoblots on total protein initially two bands are stained with molecular weight of 70 and 50 kDa (Fig. 4, lanes 1-4). However, when analyzing the recognition patterns of total proteins by anti-AOX from cultures of the parasite in normoxia / hypoxia or from ciliates obtained from experimental infections, it is noted that only the 50 kDa band varies its pattern of recognition. Thus, so in immunoblots of ciliates obtained from hypoxia as ciliates obtained from experimental infections, is an increase of staining of the band of 50 kDa compared to staining of this band in immunoblots from proteins of *in vitro* cultures of ciliates incubated in normoxia conditions. (Fig. 4, lanes 2 and 4). When analyzing the pattern recognition of the 70 kDa band in the different conditions tested, it does not seem to be affected in any of the samples tested.

In immunoblots of MAP only a single band of 50 kDa is observed, disappearing the 70 kDa band which if recognized in the total proteins of the ciliate (Fig. 4, lane 5). Furthermore, the recognition of the 50 kDa band by anti-AOX is not affected by the reducing treatment of MAP with DTT (Fig. 4, lanes 5 and 6).

3.5. Effect of inhibitors of CP and AP pathways on the expression of AOX

This experiment evaluated the effect of CP inhibitors, KCN and AMA to a concentration of 100 μ M, and the inhibitor of PA, SHAM, to the concentration of 1 mM, on the expression of AOX in ciliates from normoxic cultures in their logarithmic growth phase and incubated for 4 h with the inhibitors. To analyze the effect of inhibitors on AOX expression, western-blot was performed using anti-AOX on total proteins of the parasite and the results obtained are shown in Fig. 5.

In all cases, incubation of ciliates in the presence of both CP and AP inhibitors resulted in an increase in the expression of the AOX of *P. dicentrarchi* compared to ciliates control not incubated with these inhibitors. (Fig. 5, lanes 2-4).

3.6. O₂ consumption and level of AOX expression during the *in vitro* growth of *P. dicentrarchi*

Were followed by polarography O₂ consumption by ciliates during the exponential and stationary phase of *P. dicentrarchi* growth under normoxia and, simultaneously, western blots were performed on total proteins from ciliates cultured under such conditions to determine the level of AOX expression during the growth and the results are shown in Fig 6. During the exponential growth phase, appears a strong increase in O₂ consumption that is highest on day 3 of culture and drastically decreases when the ciliate begins to enter the stationary phase of growth from day 4 maintaining during this phase, very low levels of O₂ consumption (Fig. 6A). Moreover, the decrease of the O₂ consumption by ciliates appears to be independent of the level of O₂ present in the culture medium since it is relatively stable throughout the experiment (between 210 and 290 nmol / mL; Fig. 6) . If we analyze the expression levels of AOX in cultures shows that during the exponential phase of growth, the AOX expression is very low or almost nonexistent (Fig. 6B, days 2-3). This decline coincides with the phase of increased consumption of O₂ by the ciliate (Fig. 6A). By contrast, when analyzing the expression of the AOX during the stationary growth phase, which coincides with a marked drop in O₂ consumption by the ciliates (Fig. 6A), the AOX expression begins to markedly increase (Fig. 6B, days 4-7).

4. Discussion

The ciliates are considered to be the dominant protozoan group in marine sediments: more than 800 marine interstitial species, including about 60 scuticociliates have been reported (Wang et al., 2008). In marine biotopes, scuticociliates occur usually abundantly in coastal area, especially in eutrophic mariculture waters (Song, 2000). Usually, scuticociliates accumulating in the microaerophilic zone of oxycline or the monimolimnion (Miracle et al., 1992); and although they can readily grow in either water column or benthos, are typically found in sediments because they contain their preferred (anoxic) niche (Hayward et al., 2003). Our results of *in vitro* growth under hypoxic conditions confirm the microaerophilic nature of *P. dicentrarchi* since this is able to live and reproduce normally both in culture medium at normoxia and hypoxia. The existence of microaerophilic respiratory metabolism between several protozoan parasites may be evidence of their ability to adapt to different environments, in particular the great differences in oxygen tension between the host and the environment (Harris et al., 2000; Müller et al., 2003; Torrentino-Madamet et al., 2010).

In a previous study, we demonstrated that *P. dicentrarchi* displays a mitochondrial respiration that was resistant to cyanide which may indicate the possible existence of an AOX pathway in mitochondrial respiration in this scuticociliate (Morais et al., 2012). AOX had been identified in several species of plants, fungi and some protist including ciliates (Young, 1983; McDonald, 2008) but absent in mammals. KCN, an inhibitor of cytochrome c oxidase (mitochondrial complex IV) (Sánchez-Alcazar et al., 1995), only partially inhibited mitochondrial respiration at 100 μ M in *P. dicentrarchi*. This phenomenon also occurs in other species of ciliates (Pace and Belda, 1944), some of which have been shown to possess AOX, as is the case, for example, of *Tetrahymena pyriformis* (Young, 1983). SHAM and related compounds have routinely been used to document the presence of a cyanide-resistant respiratory pathway in a large number of

organisms (Janes and Wiest, 1982). The cyanide-resistant portion of the *P. dicentrarchi* oxygen consumption was completely inhibited by the AOX inhibitor SHAM as provide the direct evidence for an alternative branched chain respiratory pathway in this ciliate like what happens in other protozoan parasites such as, *Plasmodium* or *Cryptosporidium* (Murphy et al., 1997; Suzuki et al., 2004; Mogi and Kita, 2010), *Acanthamoeba* (Jarmuszkiewicz et al., 2001; Henriquez et al., 2009) and *Trypanosoma* (Chaudhuri et al., 2006)

Though resistant to cyanide, AOX is susceptible to benzohydroxamic acids, such as SHAM (Schonbaum et al., 1971; Stoppani et al., 1980). AOX of protozoan parasites as *Cryptosporidium parvum* and *Trypanosome* are susceptible to inhibition by SHAM (Evans and Brown, 1973; Clarkson et al., 1989; Roberts et al., 2004); however, the inhibition of AOX by SHAM is not sufficient to prevent multiplication of *Acanthamoeba* (Henriquez et al., 2009). In our study, SHAM caused a significant inhibition *in vitro* growth of *P. dicentrarchi* at concentrations above 100 μ M. Other studies have also confirmed the effectiveness of AOX inhibitors to eliminate parasites both *in vivo* and *in vitro* (Grady et al., 1993; Ott et al., 2006; Nakamura et al., 2010) or for potentiate the activity of antiparasitic drugs (Murphy and Lang-Unnasch, 1999).

In this work, the terminal oxidase of *P. dicentrarchi* was described as AOX through the cross-reaction with polyclonal antibodies raised against KLH-conjugated synthetic peptide derived from fully conserved C-terminal consensus motif from plant AOX isoforms 1 and 2 including *Arabidopsis thaliana* AOX1A and AOX2, *Solanum lycopersicum*, *Oryza sativa* (anti-AOX1/2). This antibody also possesses a predicted reactivity with the ciliate *Tetrahymena thermophila*. On reduced total protein of *P. dicentrarchi*, anti-AOX1 / 2 recognized two bands of approximate molecular sizes of 70 and 50kD. The presence of these two bands would suggest the existence of a dimeric form

of the AOX in *P. dicentrarchi*; however, when tested the anti-AOX1/2 vs. membrane proteins of ciliate treated and not treated with DTT, it was revealed that *P. dicentrarchi* AOX functions as a monomer membrane-associated of approximately 50kD and, in contrast to plant AOX, which exists in the mitochondria membrane as a dimer (Umbach and Siedow, 1993; McDonnald, 2009). Two angiosperm-specific domains may be responsible for the non-covalent dimerization of AOX, whereas two indels in the aplastidic AOXs may play a role in their post-translational regulation (McDonnald, 2009). Immunoblotting with monoclonal antibodies raised against the plant AOX revealed three monomeric forms of 32, 35 and 38 kD and a single 65 kD form in the amoebae *Acanthamoeba castellanii*; however, increased amounts of the 35 kD protein are accompanied by an increase in the activity of cyanide-resistant respiration, suggested that in amoebae, AOX occurs as a monomer (Jarmuszkiewicz et al., 1997). All research to date indicates that AOX is always membrane associated and, all eukaryotic AOXs investigated to date are associated with the inner mitochondrial membrane (Nihei et al. 2003, Suzuki et al., 2004; Kirimura et al., 2006). The presence of a higher molecular weight band on the blots containing total protein of ciliate against anti-AOX antibody could also suggest that AOX was synthesized as a cytosolic 70 kD precursor. Thus, in several plants and yeast has been shown that the AOX is synthesized from precursor proteins of higher molecular weight than the mature mitochondrial proteins (Kearns et al., 1992). However, it should be noted that our results do not preclude the possibility that other cytoplasmic proteins are associated with the AOX, which do not react with the anti-AOX antibody.

AOX participates in the antioxidant defence system when the reactive oxygen species (ROS) level increases and when decreasing concentrations of O₂ and its one-electron reductants in the mitochondria (Popov et al., 1997). Several studies indicate that

the AOX expression is regulated by the concentration of O₂ (Szala et al., 2003). Cycles of hypoxia or anoxia followed by reoxygenation occur frequently in both animals and plants produced as a result the generation of ROS which can be destroyed by activation of both peroxidases and AOX (Amora et al., 2000). On the other hand, endoparasites that colonize the host have to face varying O₂ concentrations within different organs and, additionally inflammation itself promotes O₂ consumption within affect tissues and creates a low O₂ environment, as a consequence, parasites have to adapt to rapid changes in O₂ availability (Dietz et al., 2012). In our study, we observed a clear increase in the expression of AOX in both ciliates cultured *in vitro* under hypoxic conditions, as in the parasites obtained from experimental infection in turbot. In certain endoparasites as *Trypanosoma brucei*, the bloodstream forms lack cytochromes and respire exclusively via AOX (Walker et al., 2005).

In several microorganisms has been shown that in normal conditions do not express the AOX, or its expression level is very low (Minagawa and Yoshimoto, 1987; Yukioka et al., 1998); however, chemical inhibition of the CP by respiratory inhibitors, such as AMA or KCN, can induce the synthesis of AOX (Goyal and Tolbert, 1989; Sakajo et al., 1991; Kirimura et al., 2000; Fang and Beattie, 2003; Brown and Tuffery, 2010). We have previously shown that the addition of AMA to *in vitro* cultures generated highly significant increases of ROS production in *P. dicentrarchi* (Morais et al., 2012). In the present study we have also found that not only the addition of CP inhibitors induces the AOX expression but also the presences of the selective inhibitor of AP SHAM have the same effect on the AOX expression in *P. dicentrarchi*.

Under normoxic conditions with dissolved oxygen concentrations in the culture medium constant, *P. dicentrarchi* actively breathes division during the active phase (logarithmic growth phase) indicating that, at this stage activates the CP. However, when

the ciliated stop dividing and enters in the stationary phase of growth, ceases to consume O₂ suggesting that the ciliated enters in a period of AP activation. The phase high O₂ consumption is correlated with low expression of the AOX, while conversely; lower consumption phase correlates with an increased expression of AOX. This phenomenon has also been described in other organisms: cells of the fungi *Candida albicans* in the exponential growth phase, which are actively dividing, have the lowest proportion of cyanide-insensitive activity and cells in the stationary phase have the highest relative activity of the AOX (Brown and Tuffery, 2010).

In conclusion, this work confirms that the scuticociliate *P. dicentrarchi* has a number of physiological characteristics at the level of mitochondrial metabolism that allow a wide adaptation to different environments with large variations in oxygen tension. This scuticociliate possess, in addition to the conventional cytochrome c oxidase, a second terminal oxidase allowing you to get the energy in low oxygen availability and that could also serve to protect from reactive oxygen species produced during the active phase of the ciliate reproduction, or of the ROS produced by the cells of the host immune system during the infection. Because there is no AOX in the vertebrate host, and susceptibility of *P. dicentrarchi* to inhibitors of AOX, could be considered this enzyme as a possible therapeutic target for developing future specific inhibitors they can be used as drugs against fish scuticociliatosis.

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FIGURES LEGENDS

Figure 1. - Graphic representation of the *in vitro* growth kinetics of the ciliates cultured under normoxia and hypoxia. Each symbol represents the mean \pm standard error of ciliate growth (number of trophozoites / mL; n = 5).

Figure 2.- Polarographic analysis, using a Clark electrode, representing the consumption of oxygen (expressed in nmol / min) by trophozoites of *P. dicentrarchi* (c) and permeabilized with digitonin in which the respiration was induced by addition of succinate (S), glutamate (G) and ADP, and incubated with: A) salicylhydroxamic acid (SHAM), a specific inhibitor of the alternative oxidase pathway (AP), at a concentration

of 1 mM and, B) KCN, an inhibitor of cytochrome pathway (CP), at the concentration of 100 μ M and SHAM to the concentration of 1 mM. The curves shown are representative of five independent experiments for each inhibitor and the numbers on the curves indicate the mean values of O₂ consumption obtained from three separate experiments, and the numbers between parentheses indicate the mean inhibition values obtained upon addition of the inhibitors.

Figure 3.- Growth curves for *P. dicentrarchi* obtained after *in vitro* culture for 3 days in the presence of salicylhydroxamic acid (SHAM) at concentrations of 0.01, 0.1 and 1 mM under conditions of normoxia (A) and hipoxia (B). Values presented in the figure are the means \pm standard error (n = 5). Asterisks indicate significant differences relative to controls (**P* < 0.01).

Figure 4. - Western-blot analysis of AOX levels using a polyclonal anti-AOX in: 30 μ g of total cellular extracts of *P. dicentrarchi* obtained from: normoxic cultures (lanes 1 and 3), hypoxic cultures (lane 2), experimental infections (lane 4); and 30 μ g of membrane-associated proteins (MAP) from ciliates grown in hypoxia and treated under reducing (+ DTT, lane 5) or non-reducing conditions (-DTT, lane 6). The arrow and the box indicate the band of recognition that its expression level varies during the experiment.

Figure 5. - Western-blot analysis of 30 μ g of total proteins of *P. dicentrarchi* treated under reducing conditions using a polyclonal anti-AOX, from cultures grown in normoxia conditions (lane 1) and incubated in the presence of 100 μ M of CNK (lane 2) and

antimycin A (lane 3), and 1 mM salicylhydroxamic acid (lane 4). The box and arrow indicates the specific band corresponding to the AOX.

Figure 6.- Effect of growth on O₂ consumption by intact trophozoites of *P. dicentrarchi* cultured in normoxia conditions and determined by polarography using a Clark electrode (A), and on the kinetics of the AOX expression determined by Western blot on total extracts obtained ciliated different days of cultivation (B). In the upper curve shows the evolution of the dissolved oxygen concentration (in nmol/mL) in the culture medium throughout the experiment.

Figure 1

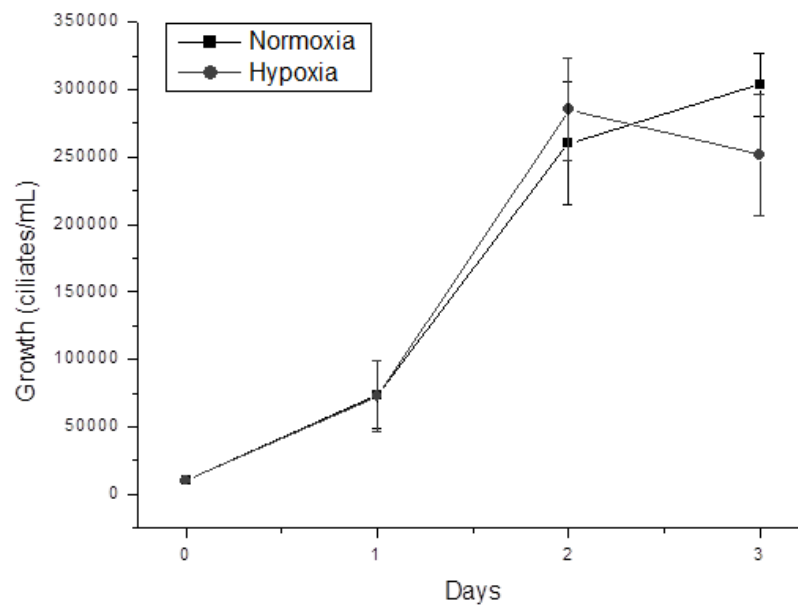


Figure 2

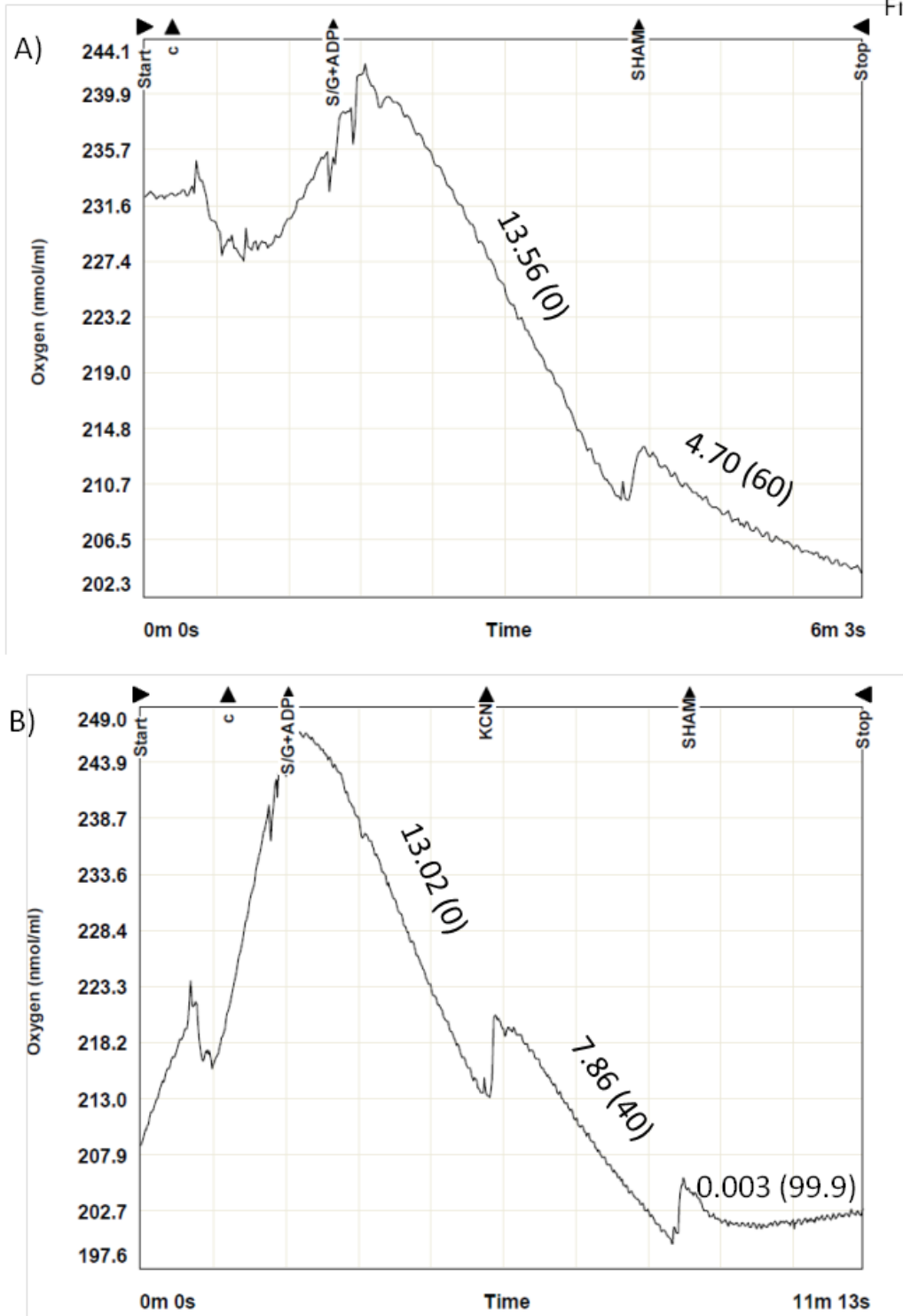


Figure 3

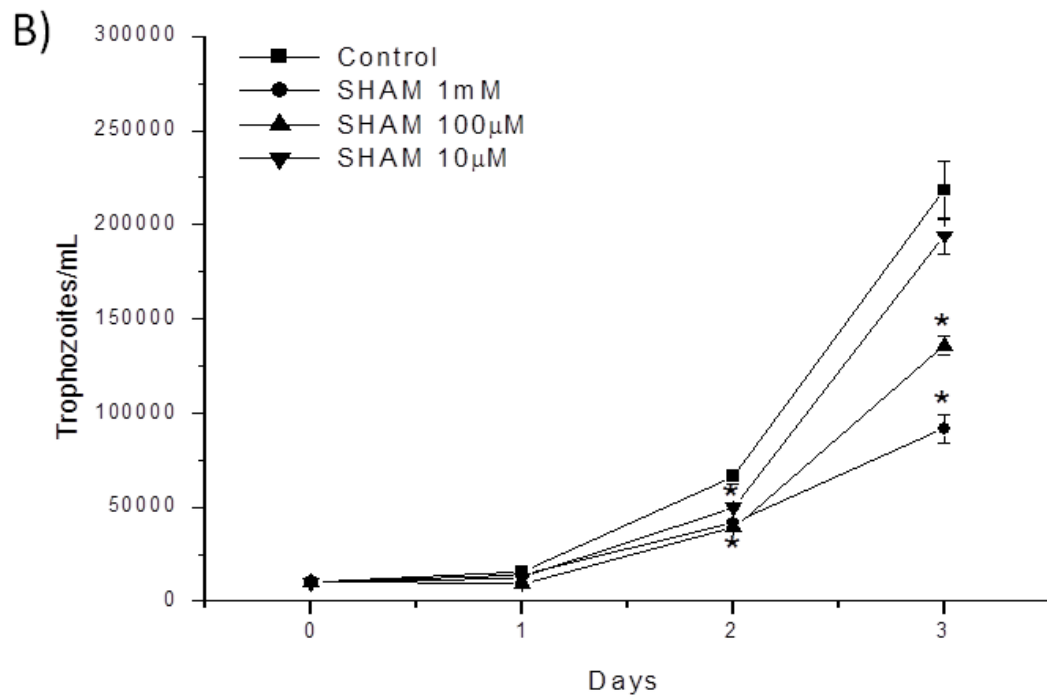
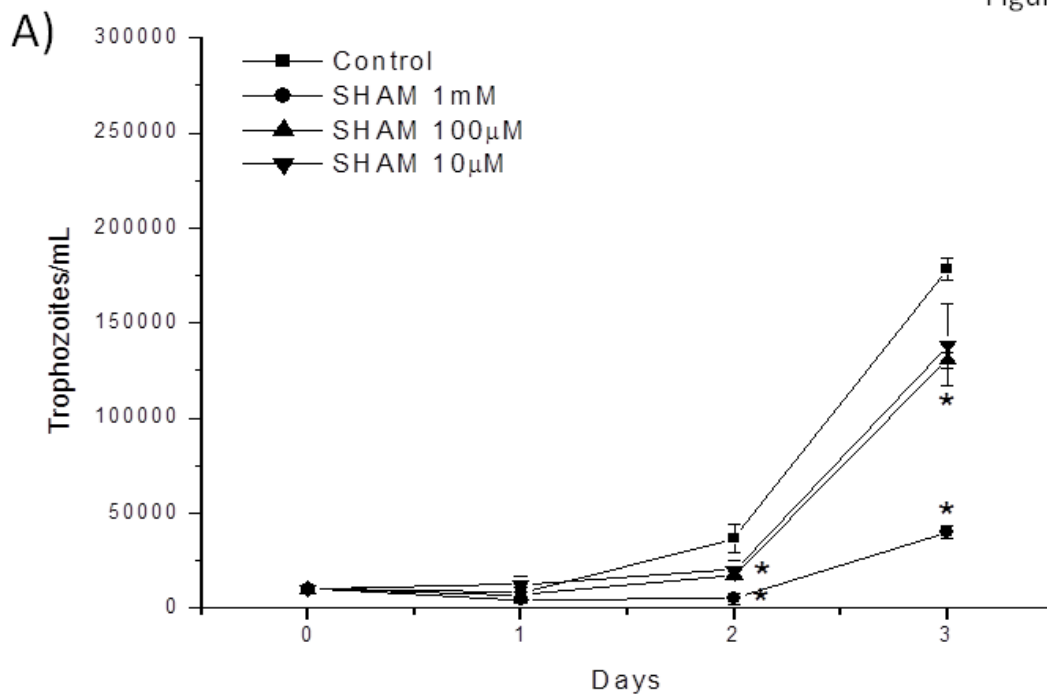


Figure 4

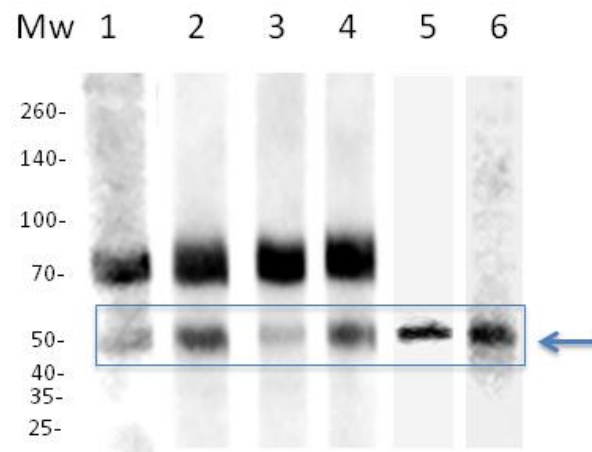


Figure 5

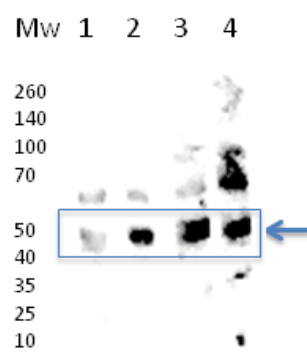


Figure 6

