Effect of resveratrol on oxygen consumption by *Philasterides dicentrarchi,* a scuticociliate parasite of turbot

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Short Title: Resveratrol induces mitochondrial dysfunctions

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Abstract

The phytoalexin resveratrol (RESV) displays antiparasitic activity against *Philasterides dicentrarchi*, a scuticociliate pathogen of turbot, and causes oxidative stress, inhibition of antioxidant enzyme activity and morphological alterations in the parasite mitochondria. In this study, we analysed the mitochondrial biology of *P. dicentrarchi* and assessed the effect of RESV on mitochondrial metabolism. We found that RESV caused dose-dependent inhibition of mitochondrial electron transport and O_2 consumption in ciliates permeabilized with digitonin. Although the RESV molecule has a high capacity for antiradical and antioxidant activity, it induced a high level of pro-oxidant activity against the ciliate, thus causing a significant increase in intracellular ROS production. The increased ROS production was accompanied by mitochondrial collapse and dysfunction of mitochondrial membrane potential ($\Delta\Psi m$) and by a significant increase in intracellular Ca^{+2} levels. RESV inhibited parasite growth in a similar way to antimycin A, an inhibitor of mitochondrial electron transport and ROS generator. The findings confirm the mitochondria as a target in the potential development of effective antiparasitic treatments.

Key words: *Philasterides dicentrarchi*; resveratrol; mitochondrial metabolism; pro-oxidant; reactive oxygen species; oxygen consumption; antiparasitic.

Introduction

The free-living scuticociliate *Philasterides dicentrarchi* is the causative agent of scuticociliatosis in the farmed turbot *Scophthalmus maximus* (Iglesias et al. 2001; Ramos et al. 2007). This ciliate species is capable of becoming a highly pathogenic histiophagous parasite and causing high mortalities in the fish host (Iglesias et al. 2001; Puig et al. 2007). The high virulence of this parasite, together with the legal restrictions on drug use in aquaculture and the fact that some of the compounds that are effective against the parasite are less effective in seawater, restricts the effective control of the disease (Iglesias et al. 2002). Parasites have developed a wide variety of physiological functions to help them survive within the specialized environments of the host, including adaptations to energy metabolism. Several features of mitochondrial physiology provide validated or potential targets for antiparasitic drugs (Kita et al. 2003; Sen and Majumder 2008). Ciliates are an extremely diverse group of protists (Lynn 2003) that can easily adapt to different environmental conditions, such as a large decrease in oxygen concentration (Fenchel and Finlay 2008). For this reason, some authors have suggested that these protozoa are good models for studying the role of mitochondria in intracellular adaptation (Prikhodko et al. 2009).

Resveratrol (RESV; $C_{14}H_{12}O_3$; 3,4′,5-trihydroxystilebene), a natural polyphenol found in abundance in fruits, vegetables and beverages such as red wine, exerts diverse biochemical and physiological actions, including anti-inflammatory, anti-oxidation, anti-proliferation and chemopreventive effects, and also promotes cell differentiation (Yu et al. 2012). RESV is a phytoalexin that in nature protects plants from injury, ultraviolet (UV) irradiation and fungal attack (Shakibaei et al. 2009). RESV is thought to interfere with mitochondrial functions by binding to F1-ATPase, thus inhibiting mitochondrial ATP synthesis (Gledhill et al. 2007). Moreover, RESV can antagonize anti-apoptotic

proteins that prevent induction of the mitochondrial apoptosis pathway in cancer cells (Fulda and Debatin 2004). We have demonstrated that RESV exerts antiprotozoal activity against *P. dicentrarchi* (Leiro et al. 2004). This inhibitory activity is associated with the induction of oxidative stress and with the inability of the parasite to eliminate reactive oxygen species (ROS) as a result of modified activity of antioxidant enzymes (Lamas et al. 2009). The resulting morphological alterations in the mitochondria induce autophagy, which leads to a reduction in the cell volume, and also drastically reduces the ciliate endocytic activity, thereby generating a state compatible with cryptobiosis (Morais et al. 2009).

Mitochondria are important regulators of autophagy-induced cell death, and damaged mitochondria are often degraded by autophagosomes. Inhibition of the mitochondrial electron transport chain (mETC) induces cell death by generating reactive oxygen species (ROS) (Chen et al. 2007). Detailed studies of mitochondrial functions in *P. dicentrarchi* have been hampered by technical difficulties in obtaining workable quantities of functional mitochondria. To circumvent these problems, we explored the possibility of studying mitochondrial functioning by permeabilizing the ciliates with digitonin (DIG), at a concentration that does not affect the mitochondrial membranes (Duan et al. 2003). Mitochondrial electron transport involves ADP-dependent O_2 consumption, which directly reflects coupled respiration or oxidative phosphorylation, and which can be measured by the traditional method of mitochondrial polarography, with a Clark electrode (Li and Graham 2012). We also used the dichlorofluorescein (H_2DCFDA) fluorescent probe to measure intracellular ROS production (LeBel et al. 1992), the lipophilic cationic fluorescent probe JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) to study the mitochondrial membrane potential ($\Delta\Psi m$) (Simeonova et al. 2004), and a fluorescent Ca^{+2} indicator (Fluo-4 AM)

104 to measure in-cell calcium signalling (Cui et al. 2008). Using all of these assays, we
105 showed that antiparasitic effect of RESV on *P. dicentrarchi* is exercised through blockage
106 of electron transport in ciliate mitochondria, which generates a highly significant increase
107 in ROS levels, accompanied by an increase in the intracellular concentration of Ca^{+2} and
108 decreased $\Delta\Psi m$.
109
110 **Results**
111
112 Effects of energy transfer inhibitors and RESV on respiration of *P.*
113 *dicentrarchi* mitochondria
114 We initially tested whether the mitochondrial respiratory chain of *P. dicentrarchi*
115 responded to classical electron transport inhibitors. For this purpose, we permeabilized
116 complete ciliates with DIG at a concentration at which the mitochondrial membranes
117 remained functional. We then induced mitochondrial respiration by adding enzyme
118 substrates such as ADP, succinate and glutamate, and the following inhibitors: rotenone
119 (an inhibitor of complex I), antimycin A (AMA, an inhibitor of complex III), potassium
120 cyanide (KCN, an inhibitor of cytochrome c oxidase - mitochondrial complex IV) and
121 oligomycin (an ATP synthase inhibitor). At the concentrations of inhibitors used,
122 mitochondrial O_2 consumption was completely inhibited by AMA and partially inhibited
123 by rotenone, KCN and oligomycin (Fig.1A-C). RESV (100 μ M) also completely blocked
124 O_2 consumption in the DIG- permeabilized ciliates (Fig. 1B-C).
125 We increased the concentration of KCN, as 10 μM KCN caused only partial
126 inhibition of the O_2 consumption by DIG-permeabilized ciliates. Concentrations of KCN

127 higher than 1 mM inhibited O_2 consumption in the permeabilized ciliates by 70% (Fig.
128 2).

129 RESV exerted an inhibitory effect on O_2 consumption in permeabilized ciliates
130 and in non-permeabilized ciliates. At a concentration of 25 μM, RESV caused a
131 significant dose-dependent inhibition of the total O_2 consumption in non permeabilized
132 ciliates, and concentrations between 50 and 100 μM caused almost complete inhibition
133 of respiration in non permeabilized ciliates (Fig. 3). AMA (100 μM) also caused
134 inhibition of O_2 consumption in non-permeabilized ciliates, similar to that produced by
135 25 μM RESV.

136

137 ## Antioxidant activities of RESV and AMA

138 Both RESV and AMA (at a concentration of 100 μM) displayed a high 2,2-diphenyl-1-
139 picrylhydrazyl (DPPH*) scavenging potential (Fig. 4); that of RESV was significantly
140 higher (86%) than that of AMA (51%).

141

142 ## RESV- and AMA-induced mitochondrial ROS overproduction in
143 scuticociliates

144 We used the fluorescent H_2DCFDA probe to determine intracellular ROS production in
145 *P. dicentrarchi* trophozoites treated with RESV. We also analyzed the effect of AMA (a
146 selective inhibitor of mitochondrial complex III) on ROS production in these ciliates. At
147 concentrations of 50 and 100 μM, both RESV and AMA generated highly significant
148 increases in ROS production relative to untreated controls (Figs. 5A and 5B,
149 respectively). However, comparison of the mean increases per min revealed that at

150 concentrations of 50 and 100 μM, AMA generated a greater increase in ROS than
151 generated by the same concentrations RESV (Fig. 5).

152 ## Effects of RESV on the mitochondrial functions of *P. dicentrarchi*
153 ## trophozoites

154 We used the JC-1 fluorescent probe to analyse the effect of RESV on the $\Delta\psi_m$ in treated
155 and untreated ciliates after incubation for 4 h in L15 medium. At a concentration of 100
156 μM, RESV caused a significant decrease in the mitochondria $\Delta\psi_m$ of ciliates after 2 h
157 (Fig. 6A). We also used the Fluoro-4 AM probe to test the effect of RESV on intracellular
158 levels of Ca^{+2} (Ca^{+2}_i) in ciliates during 3 h of incubation. The Ca^{+2}_i levels increased
159 linearly during the first three hours of incubation. However, the increase was significantly
160 higher in the ciliates treated with 100 μM RESV, than in untreated controls, after the first
161 hour of incubation (Fig. 6B).

162

163 ## Effects of RESV and other electron transfer inhibitors on *in vitro* growth of
164 ## scuticociliates

165 We initially analyzed the effect of RESV at concentrations of 25, 50 and 100 μM on the
166 *in vitro* growth of *P. dicentrarchi*. RESV exerted significant inhibition of the *in vitro*
167 growth of ciliates at the minimum concentration assayed (25 μM) on day 1 of culture
168 (Fig. 7A). However, higher concentrations (50-100 μM) were required to maintain the
169 inhibitory activity, and at 100 μM there was a slight cytocidal effect after 4 days culture.

170 Finally, we also assessed the effect of AMA (an inhibitor of the mitochondrial
171 respiratory chain) on the *in vitro* growth of the ciliate. AMA also had an initial inhibitory
172 effect on the growth at the highest concentrations used (50 and 100 □M), but this effect

occurred from the third day of culture (Fig. 7B). After 3 days, AMA (at concentrations of 50-100 μM) behaved as a cytocidal agent and produced a significant decrease in the number of ciliates per ml in culture (Fig. 7B).

Discussion

The importance of screening potential drug candidates for mitochondrial toxicity is being recognized (Dykens and Will 2008). Measurement of the rates of O_2 consumption are extremely valuable in this regard, as electron transport and oxidative phosphorylation reflect the concerted function of both the mitochondrial and nuclear genomes in expressing functional components of oxidative phosphorylation. Intact cell respiration also reflects the influence of multiple hormonal effects, signalling cascades, and regulated transporters and pathways, and is a good measure of the overall health of cells, particularly because of the susceptibility of mitochondria to oxidative injury (Rogers et al. 2011).

To confirm that respiration of mitochondria from the scuticociliate *P. dicentarchi* was comparable to that of mitochondria from other ciliates, we carried out respirometry experiments that assessed the *in situ* capacity of the mitochondria in DIG-permeabilized cells to carry out oxidative phosphorylation, which is indicative of a functional ATP synthase and electron transport chain. Respiration in *P. dicentrarchi* depended on the presence of the mitochondrial substrates succinate and glutamate, and was stimulated by the addition of ADP. Similar results were obtained in early studies of mitochondria obtained from various isolates of the ciliate *Tetrahymena pyriformis* (Kobayashi 1965; Turner et al. 1971, Unitt and Lloyd 1981). The effects of different inhibitors on the respiratory system of the *P. dicentrarchi* mitochondria are similar to the effects on other

197 mitochondrial systems in ciliates. The complex I inhibitor rotenone (Carrière et al. 2003;
198 2004) causes partial inhibition of electron transport in mitochondria of *P. dicentrarchi.*
199 In *T. pyriformis*, rotenone also partially inhibits electron transport in isolated
200 mitochondria (Turner et al. 1971). As in *T. pyriformis*, mitochondrial ATPase in *P.*
201 *dicentrarchi* is only partially inhibited by the F0-ATPase inhibitor oligomycin (Unitt and
202 Lloyd 1981). At a concentration of 4.10^{-5} M, AMA, which is a well-known specific
203 inhibitor of mitochondrial complex III (Turrens 1997), produces total inhibition of
204 oxidative phosphorylation of the *P. dicentarchi* mitochondria. A similar concentration is
205 required to inhibit succinate oxidation in *Tetrahymena*, but a much lower concentration
206 is required to inhibit respiration in mammals (Kobayashi 1965; Slater 1967). KCN, an
207 inhibitor of cytochrome c oxidase (mitochondrial complex IV) (Sánchez-Alcazar et al.
208 1995), partially inhibited mitochondrial respiration at 10 µM, and inhibition increased
209 very significantly by increasing the concentration to 10 mM. Several authors have found
210 that cyanide does not inhibit respiration in several ciliates (Pace and Belda 1944).
211 Respiration of early-exponential phase cultures of *T. pyriformis* is inhibited by increasing
212 concentrations of cyanide in two stages: inhibition of up to 40-50% occurs at low
213 concentrations (<15 µM) and maximal inhibition is induced by 300 µM-cyanide; at the
214 latter concentration, 20% of the respiration was still unaffected (Lloyd et al. 1980). In the
215 present study, RESV caused significant inhibition of respiration in non permeabilized
216 trophozoites and in DIG-permeabilized trophozoites, and the inhibition was clearly dose-
217 dependent. Studies carried out in mammals suggest that RESV inhibits mitochondrial
218 complex III and ATPase activities (Zini et al., 1999). A similar effect may also occur in
219 *P. dicentrarchi*, although the targets of RESV in ciliate mitochondria remain to be
220 determined.

Flavonoids are renowned for their antioxidant and antiradicalar properties (González et al. 2011). It has been demonstrated that RESV suppresses lipid peroxidation more effectively than vitamins E and C, by chelation of copper and by scavenging of the free radicals (Stojanovic et al. 2001). Use of the general free radical model 2, 2-diphenyl-1-picryl-hydrazyl (DPHH) has shown that RESV has an effective radical and ROS scavenging capacity, with an IC_{50} of 39.6 μM; the antioxidant capacity appears to be related to the presence of OH groups in the molecule (Lorenz et al. 2003). Thus, at least one OH group is required for a radical scavenging reaction and it has been speculated that the scavenging capacity increases with the number of hydroxyl groups in the molecule (Stojanovic et al. 2001). This hypothesis appears to be confirmed by the fact that the AMA, which has only one OH group in its molecule, exerts significantly lower antioxidant activity than RESV, which has 3 OH groups in its molecule.

Some polyphenol antioxidants have been found to exhibit pro-oxidant activity under certain conditions (Lee and Lee 2006). Initial investigations focused on the anti-oxidant and cytoprotective mechanism of action of RESV. However, several studies have demonstrated that RESV can behave either as an anti-oxidant or as a pro-oxidant, depending on the selective microenvironment (Muqbill et al 2012). RESV can exhibit pro-oxidant properties, leading to oxidative breakage of cellular DNA in the presence of transition metal ions such as copper (De la Lastra and Villegas 2007). ROS are formed as by-products of mitochondrial respiration or the action of oxidases, including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO) and certain arachidonic acid oxygenases (Han et al. 2009). Agents that produce ROS can trigger permeabilization of the mitochondrial outer membrane and apoptosis, by overwhelming the antioxidant defence mechanisms of mitochondria, hence causing excessive oxidative damage (Fulda 2010). AMA has been used to generate ROS in several

246 biological systems (Han et al. 2009; Park et al. 2007). The present results demonstrate
247 that at concentrations > 50 μM, both RESV and AMA induced a significant increase in
248 intracellular ROS in *P. dicentrarchi*.

249 Inhibition of electron transport not only causes increased ROS production, but
250 also collapse of the proton gradient across the mitochondrial inner membrane, thereby
251 breaking down the $\Delta\psi_m$ (Panduri et al. 2004; Balaban et al. 2005). Detection of the
252 mitochondrial permeability transition event (PT) provides an early indication of the
253 initiation of cellular apoptosis; this process is typically defined as a collapse in the
254 electrochemical gradient across the mitochondrial membrane - as measured by changes
255 in the Δψm (Zhang et al. 2011). There is evidence that either the presence of ROS or the
256 collapse of $\Delta\psi_m$ opens up the mitochondrial permeability transition pore (PTP), which is
257 accompanied by the release of proapoptotic molecules such as cytochrome c into the
258 cytoplasm (Porcelli et al. 2009). In ciliates, RESV produces a significant decrease in Δψm
259 after only 2 h of incubation, indicating that this polyphenol causes a rapid collapse of the
260 Δψm.

261 Treatment of ciliates with RESV caused a significant increase in $Ca^{+}{}_{i}^{2}$ levels
262 relative to those in untreated ciliates. Cells use $Ca2^{+}$ as a second messenger for most
263 biological processes, including proliferation, gene transcription, post-translational
264 modification of proteins and aerobic metabolism (Clapham 2007). When mitochondria
265 are exposed to an excess of Ca^{2+}, this may induce PT, which is an increase in the
266 permeability of the inner mitochondrial membrane (IMM) to ions and solutes (Bernardi
267 1999). Persistent PTP opening irreversibly commits cells to death by the combination of
268 the following series of events: IMM depolarization, which causes cessation of oxidative
269 phosphorylation and ROS production; matrix swelling and cristae unfolding; and the
270 ensuing breaches in the outer mitochondrial membrane (OMM) with release of stored

Ca^{2+} and of apoptogenic proteins (Zorov et al. 2009). Induction of PTP also enhances ROS generation by stopping the electron flow along the respiratory complexes (Rasola and Bernardi 2011). Irreversible PTP opening is associated with a collapse of $\Delta\Psi_m$, release of cytochrome *c*, and possibly generation of more ROS, resulting in a vicious cycle of further amplification of ROS production, mitochondrial Ca^{2+} overload, and increasingly irreversible cell damage (Dedkova and Blatter 2012). Induction of PTP may occur in the whole cell, prompting necrosis, or it may be a regional event, either initiating apoptosis, or causing local subcellular damage that prompts the autophagic disposal of damaged mitochondria (Rasola et al. 2010), a process also described after treatment with RESV in *P. dicentrarchi* (Morais et al. 2009).

In conclusion, the results indicate that RESV altered *P. dicentrarchi* mitochondria (inhibiting electron transport and modifying the membrane potential) and inhibited ciliate growth. Like RESV, antimycin A, an inhibitor of mitochondrial complex III, also had a significant effect on growth of *P. dicentrarchi*, indicating that mitochondrial electron transport inhibitors may be of potential use as therapeutic agents in turbot scuticociliatosis.

Methods

Cell culture: Infected turbot (*Scophthalmus maximus*) showing signs of scuticociliatosis were obtained from a local fish farm (Galicia, Spain). Specimens of *Philasterides dicentrarchi* (isolate I_1) were collected under aseptic conditions from ascitic fluid removed from the intraperitoneal cavity of the fish (Iglesias et al. 2001). The I_1 isolate is maintained in the culture collection at the Laboratory of Parasitology of the Institute of Food Research and Analysis of the University of Santiago de Compostela,

296 and is available on request from the following address: Laboratorio de Parasitología,
297 Instituto de Investigación y Análisis Alimentarios, c/ Constantino Candeira s/n, E15782
298 Santiago de Compostela, Spain. The ciliates were cultured as previously described
299 (Iglesias et al. 2003), at 18 °C, in complete sterile L-15 medium (Leibovitz, PAA
300 Laboratories GmbH, 10% salinity, pH 7.2) containing 90 mg/L each of adenosine,
301 cytidine and uridine, 150 mg/L of guanosine, 5 g/L of glucose, 400 mg/L of L-α-
302 phosphatidylcholine, 200 mg/L of Tween 80, 10% of heat-inactivated foetal bovine
303 serum (FBS) and 10 mL/L of 100 x antibiotic-antimycotic solution (=100 units/mL of
304 penicillin G, 0.1 mg/mL of streptomycin sulfate and 0.25 mg/mL of amphotericin B).
305 Except where stated, all reagents were purchased from Sigma-Aldrich (Madrid, Spain).
306 **Ethical approval:** All the experiments were carried out in accordance with
307 European regulations on animal protection (Directive 86/609), outlined in the Declaration
308 of Helsinki. All experimental protocols were approved by the Institutional Animal Care
309 and Use Committee of the University of Santiago de Compostela (Spain). For all
310 procedure, the fish were anaesthetized with benzocaine (50 mg/ml), and all efforts were
311 made to minimize suffering.
312 **Measurement of mitochondrial oxygen consumption with a Clark electrode:**
313 To study the functioning of the mitochondrial respiratory chain, the ciliates were
314 permeabilized with DIG (Sigma) (Cuperus et al. 2010). Ciliates (5 x 10^6 ciliates / ml)
315 were centrifuged (700 x *g* for 5 min), washed twice with L15 medium and resuspended
316 in 15 mL of medium L15. PMSF was then added to a final concentration of 1 mM and
317 100 µL of 10 mM DIG. The tubes were shaken gently, to avoid breaking the cells, for one
318 minute and re-centrifuged at 700 × g for 5 min at 4 ° C. The cells were then resuspended
319 in 1.5 mL of L15 medium. During this time, the oxigraph chamber (Hansatech
320 Instruments, UK) was emptied and filled with 500 µL of L15 medium, 50 µl of 50 mM

ADP and 100 µL of permeabilized cells. After stabilization of the baseline, 50 µL 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 as follows: 20 µM rotenone; 30 µ M oligomycin, 40 and 100 µ M AMA; 10 µM and 1, 5 and 10 mM KCN, 25, 50 and 100 µ M RESV. All assays were performed in triplicate, and O_2 consumption was analyzed by Oxygraph Plus software (Hansatech, UK). Neither the dimethyl sulfoxide (DMSO) or the ethanol used to solubilize the inhibitors affected the O_2 consumption, at the maximum concentrations used. To analyze the total consumption of O_2 by the ciliates, we used the same protocol as that described above, but without treating ciliates with DIG, and without adding metabolic substrates. Except where stated, all reagents were purchased from Sigma-Aldrich (Madrid, Spain).

Measurement of intracellular production of ROS in *P. dicentrarchi*: Intracellular ROS production was determined by use of the fluorescent H_2DCFDA (Sigma). A stock solution of the probe was prepared in DMSO (10 mg / mL) and stored at -20 ° C until use. Ciliates grown in L15 medium were centrifuged at 700 x *g* for 5 min and resuspended in Hanks's balanced salt solutions (HBSS; PAA Laboratories) to a final concentration of 5 x 10^6 cells / mL. Assays were performed in black solid polystyrene 96 well microplates (Corning, USA), and a final volume of 100 µL of suspension was added to each well. The suspension contained ciliates, 1 µL probe (diluted 1:10 in HBSS from stock solution) and different concentrations of RESV and AMA (50 and 100 µ M, prepared from 100 mM stock solutions dissolved in DMSO). ROS production was determined by a fluorescence kinetic protocol in a microplate fluorescence reader (Bio-Tek Instruments, USA) (excitation wavelength, 490 nm; emission wavelength 525 nm; sensitivity 70 %; readings up to 1 h at intervals of 60 s, and a constant temperature of 25 °C). In all experiments, the fluorescence produced by the spontaneous oxidation of the

probe was subtracted from the experimentally obtained fluorescence values. For statistical analyses, the average fluorescence values were expressed per min (n = 3).

Evaluation of antioxidant activity: The antiradical activities of RESV and AMA were determined by the free radical DPPH* method initially described by (Brnad-Williams 1995) and modified by (Borlomeazzi et al. 2007). The DPPH was dissolved in methanol at a concentration of $6x10^{-5}$ M. RESV and AMA were dissolved in methanol and were included in the reaction at a final concentration of 100 mM in a volume of 100 µL of solution of DPPH in methanol. The slope of the absorbance at 530 nm was monitored for 0 to 30 minutes. The antiradical effectiveness (antioxidant activity) was expressed as the percentage inhibition of absorbance relative to the controls to which only methanol was added. This was calculated from the following equation:

% inhibition= [Abs_{530} (control) - Abs_{530} (sample) / Abs_{530} (control)] X 100

Determination of Δψm: For the determination of Δψm, the JC-1 kit (Molecular Probes) was used. The kit uses a cationic fluorescent probe, iodide 5,5 ', 6,6'-tetrachloro-1, 1 ', 3,3' - tetraetilbenzimidazolil carbocianine to detect the variation in Δψm. In the trial, $9x10^6$ ciliates (control and treated with RESV 100 µM) were incubated with 250 µL of the probe (diluted 1:10 in L-15) in darkness for 30 minutes. The L-15 medium was then removed by centrifugation (700 x *g* for 5 min), and ciliates were washed twice with previously prepared buffer kit. Finally, the ciliates were added to a 96-well microplate, and the concentration was adjusted o $4x10^5$ciliates/well with the respective JC-1 kit buffer, in a final volume of 100 µL. Fluorescence was initially measured at 560/595 nm (excitation / emission) to quantify the healthy cells, and then at 485/535 nm (altered cells) at 0, 2 and 4 hours in a microplate fluorimeter. The fluorescence intensity ratio was used as an indicator of cell viability.

370 **Determination of intracellular Ca^{+2}:** The fluorescent probe Fluo-4 AM
371 (Invitrogen, USA) was used to determine the intracellular Ca^{+2}. For the assay, the ciliates
372 were washed twice at 700 x *g* and the L-15 medium was replaced with assay buffer (1X
373 HBSS, 20 mM HEPES) to a density of 1.25 $x10^6$ ciliates / mL. Ciliates were incubated
374 with RESV (0 and 100 mM) in 96-well microplates at 21 °C for 1 hour. A stock solution
375 of probe Fluo-4 AM was prepared by adding 5 mL assay buffer and 100 µL of a stock of
376 probe, obtained from a 2X stock, which was stirred vigorously on a vortex for 2 minutes.
377 The solution was used on the same day, and kept in the dark at 4 °C. After preincubation
378 of the plates for 1 hr, 100 µL of probe solution (2X) were added to each well, and the
379 plates were then incubated at 21 °C. Fluorescence measurements were made at
380 494/516nm (excitation / emission) every hour for 3 hours, in a fluorimeter. Positive
381 controls (only probe solutions) and negative (only ciliates) were processed and the the
382 fluorescence intensity values obtained were subtracted from the values obtained in the
383 assay

384 ***In vitro* growth assays:** The activity of RESV and the AMA on the *in vitro* growth
385 of *P. dicentrarchi* was determined as previously described [Iglesias et al. 2002), with
386 minor modifications [(Leiro et al. 2004)]. For all experiments, stock solutions of RESV
387 and AMA were prepared in DMSO, to a concentration of 100 mM, and stored away from
388 light at -80 °C. In order to investigate their effects on the ciliates, RESV and AMA were
389 added to wells of sterile 24-well culture plates (Corning) containing 10^5 ciliates / well in
390 1 mL of medium at concentrations of 25, 50 and 100 µM (along with the respective
391 DMSO control with the highest concentration used). The plates were then incubated for
392 5 days at 21 °C. The number of ciliates per well was counted daily in 25 µL aliquots
393 removed from each well. The ciliates were inactivated with 0.25% glutaraldehyde (final

concentration) for 15 minutes at room temperature, and were then counted in a hemocytometer (Iglesias et al. 2002). Each experiment was replicated five times.

Statistical analysis: The results shown are expressed as means ± standard error of the mean (S.E.M.). Statistical significances ($\alpha = 0.05$) were determined by one-way analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons.

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562 ## Figure legends

563

564 **Figure 1.-** O_2 consumption curves for *P. dicentrarchi* trophozoites permeabilized with
565 digitonin (DIG). O_2 consumption was quantified with an oximeter and a Clark
566 electrode. Mitochondrial respiration was induced in cells by addition of the oxidizable
567 substrates succinate (S, 50 mM), glutamate (G, 50 mM) and ADP (2.5 mM) and the
568 O_2 consumption produced by metabolism of these substrates was measured. The
569 following inhibitors were used: rotenone (20 μM), antimycin A (AMA, 40 μM),
570 potassium cyanide (KCN, 10 μM), oligomycin (30 μM) (A), and resveratrol (RESV,
571 100 μM) (B). The curves shown are representative of five independent experiments
572 for each inhibitor. Table shows the O_2 consumption, before and after the addition of
573 inhibitors expressed in nmoles of O_2/mL/min, and the percentages of inhibition
574 obtained (mean ± standard error) (C).

575

576 **Figure 2.-** O_2 consumption curves for *P. dicentrarchi* trophozoites permeabalized with
577 digitonin (DIG). O_2 consumption was quantified with an oximeter and a Clark
578 electrode. After addition of ciliates (c), 50 mM of the oxidizable substrates succinate
579 (S) and glutamate (G), and 2.5 mM ADP, were added. Potassium cyanide (KCN) was
580 also added at concentrations of 0 (control, C), 1, 5 or 10 mM. O_2 consumption values
581 were measured within 30 sec of the addition of substrates and are expressed in nmoles
582 of O_2/mL/min (mean ± standard error). The curves shown are representative of 3
583 assays. Asterisks indicate significant differences relative to controls ($P< 0.05$; $**P<$
584 0.01).

585

Figure 3.- Curves showing total consumption of O_2 by *P. dicentrarchi* trophozoites. O_2 consumption was quantified with an oximeter and a Clark electrode. After addition of ciliates (c), resveratrol (RESV) was added at concentrations of 25, 50 and 100 μM; antimycin A (AMA) was added at a concentration of 100 μM, or no inhibitors were added (control, C), and O_2 consumption was monitored during 15 min. The curves shown are representative of three assays. The O_2 consumption is expressed in nmoles of O_2/mL/min (mean ± standard error). Asterisks indicate significant differences relative to controls (**P*< 0.05; ***P*< 0.01).

Figure 4.- Histogram showing DPPH radical scavenging activity of resveratrol (RESV) and antimycin A (AMA) at 100 μM. Bars are mean ± standard error (n = 3). Asterisks indicate significance differences relative to controls (**P* <0.01).

Figure 5.- Curves showing intracellular ROS production by *P. dicentrarchi* trophozoites incubated with resveratrol (RESV) (A) and antimycin A (AMA) (B) at 50 and 100 μM. ROS production was measured by use of the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA). The increase in fluorescence in relative units (RFU) was monitored for 1 h, and ROS production is expressed as the mean increase in fluorescence per min ± standard error. The curves are representative of 3 individual trials. Asterisks indicates significance differences relative to controls (**P*<0.01).

Figure 6.- Mitochondrial membrane potential ($\Delta\psi_m$) (A) and intracellular Ca^{+2} ($Ca^{+2}{}_i$) levels (B) evoked in untreated ciliates (control) and ciliates treated with 100 μM resveratrol (RESV), and quantified with fluorescent probes JC-1 and Fluo-4 AM NW,

611 respectively. For determination of $\Delta\psi_m$, data are presented as the ratio of red signal in
612 relative fluorescence units (RFU) at 590 nm, and the green signal (in RFU) at 535 nm.
613 For determination of $Ca^{+2}{}_i$ levels, fluorescence (expressed in RFU) was measured at
614 494/516nm (emission/excitation) every hour. The results are the mean values ±
615 standard error for three assays. Asterisks indicate significant differences relative to
616 controls (*P< 0.01).
617
618 **Figure 7.-** Growth curves for *P.* dicentrarchi obtained after *in vitro* culture for 5 days in
619 the presence of resveratrol (RESV) (A) and antimycin A (AMA) (B) at concentrations
620 of 25, 50 and 100 μM. The final concentration of ciliates in the culture plates was 10^4
621 ciliates/ml. Values presented in the figure are the means ± standard error (n = 5).
622 Asterisks indicate significant differences relative to controls (*P< 0.05; **P< 0.01).