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Respiratory analysis as a tool to detect physiological changes in *Anisakis* larvae subjected to stress

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47 Introduction

148 Anisakiasis has been reported to be an underdiagnosed disease (Bao et al. 2017), characterized by
49 gastrointestinal manifestations of epigastralgia, nausea, abdominal pain and diarrhoea (Shimamura
50 et al. 2016), as well as by a characteristic allergic reaction such as IgE-mediated hypersensitivity,
51 angioedema, urticaria or anaphylaxis (Ventura et al. 2013; Moneo et al. 2017). Consumers can
52 become infected with the live *Anisakis* sp. larvae in the third stage (L3) when parasitized fish is
53 consumed raw or when technological processes, storage or cooking practices do not kill the larvae
54 (Audicana and Kennedy 2008; Nieuwenhuizen and Lopata 2014; Moneo et al. 2017). Therefore, the
55 application of procedures to ensure the mortality of any larvae present in fish is a key factor in
56 prevention of the risk of *Anisakis* infection, and various physical and chemical treatments have been
57 proposed (EFSA Panel on Biological Hazards 2010).

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59 In general, motility of larvae, as demonstrated by spontaneous movements following stimulation by
60 bending with forceps and a needle, is used as the method for assessing viability. However, mobile,
61 treated larvae may not always be infective, and on the other hand, some larvae not showing
62 movement may be erroneously considered dead. Therefore, there is a need to establish other
63 methods to assess whether these larvae are capable of infecting humans (EFSA Panel on Biological
64 Hazards 2010).

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66 Survivability of *Anisakis* in humans is considered one of the determinants for their pathogenicity.
67 Therefore, behavioural testing, such as larval penetrative ability into an agar layer, has been
68 proposed (Ruitenbergh 1970; Oishi and Hiraoki 1973), and this method has recently been
69 incorporated for studying the *in vitro* infective potential due to species differences (Suzuki et al.
70 2010; Arizono et al. 2012) or to technological treatments (Sánchez-Alonso et al. 2018). Similarly, *in*
71 *vitro* studies of acid tolerance in an artificial gastric fluid that simulated the physiological

environment of the human stomach have been conducted (Arizono et al. 2012; Sánchez-Alonso et al. 2018).

Mitochondria are essential organelles not only involved in the production of cellular energy but also performing roles in many cellular processes and signalling pathways (Brand et al. 2013).

Alterations in the oxygen consumption rate are informative indicators of mitochondrial dysfunction and have been widely used in isolated mitochondria, in cells (Brand and Nicholls 2011), and also in whole organisms such as the bacteriovorous soil model nematode *Caenorhabditis elegans* (Navas et al. 2007; Luz et al. 2015; Koopman et al. 2016; Fong et al. 2017; Palikaras and Tavernarakis 2017).

We suggest that mitochondria may become dysfunctional in *Anisakis* L3 as a result of various physical (i.e. freezing, heating, high pressure) or chemical (i.e. acid, marinating, antihelmintics, etc.) treatments that are given to *Anisakis* L3 for inactivation, even if the larvae survive these treatments. In this case, the oxygen consumption rate (OCR) could give valuable information about the actual mitochondrial function, which could be used as an additional indicator to check the pathogenicity of the larvae. Measurements of basal (i.e. without metabolic modulators) respiration rates have been applied in larvae that survived freezing, together with penetration into an agar layer and survival in gastric juice (Sánchez-Alonso et al. 2018). In *C. elegans*, the combined study of basal respiration with maximum and residual respiration has given insights into the spare respiratory capacity of the worms, thus providing a more precise picture of the mitochondrial health which was otherwise not observed (Fong et al. 2017).

The objective of this work was to establish whether respiratory analysis of *Anisakis* L3 could be used to identify subtle differences between larvae that were still considered viable in terms of their mobility, but that had been subjected to stress (thermal and/or chemical). For this purpose, OCR of larvae that survived a freezing treatment was compared with untreated larvae. This comparison was

performed just after thawing, and after several days at 4.6 °C. The effects of temperature (37 °C or 4.6 °C) and incubation medium (artificial gastric juice) on OCR were also studied.

Materials and methods

Raw material

Two batches of hake (*Merluccius merluccius* L.) were caught in the Atlantic Northeast fishing area (FAO area 27). The gutted fish, filleted by a local fishmonger, were transported to the laboratory, kept in the fridge at 4.6±0.3 °C, and processed within 1 h. The fish fillets were inspected visually and any larvae detected were removed from the muscle.

Three batches of *Anisakis* L3 from heavily infected ovaries and viscera of hake obtained from the central fish market in Madrid were used. After receiving the larvae, they were immediately separated from the tissue with forceps, cleaned with 0.145M NaCl, placed in groups of 50 in tubes containing 10 mL of 0.145M NaCl, and stored in the fridge (4.6±0.3 °C) until needed. For taxonomic identification, at least 40 clean larvae per batch were kept frozen stored (−22.0±0.3 °C) until use. Larvae were individualized and each specimen was subjected to identification by using the ITS1 region of the nuclear ribosomal DNA (rDNA) following the taxonomic criteria of D'Amelio et al. (2000) and Abollo et al. (2003) in order to establish the percentage of representation of species or their heterozygote genotypes (*sensu* Mattiucci et al. 2016). Identification was performed using the caudal region of individual L3 stage juveniles placed in an Eppendorf tube storing the rest of the individual for the tissue collection of the Laboratory. DNA was extracted and purified using the Speedtools Tissue DNA Extraction Kit (Biotools) following manufacturer's instructions. Molecular identification was carried out for each individual using PCR-RFLP. The forward primer A 5'-GTCGAATTCGTAGGTGAACCTGCGGAAG GATCA-3' and reverse primer B 5'-GCCGGATCC GAATCCTGGTTAGTTTCTTTTCCT-3' (D'Amelio et al., 2000) were used in reactions containing, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 mM each

of dATP, dCTP, dGTP and dTTP and 1 unit of DNA polymerase (Biotools B and M labs, S.A. Madrid, Spain). Initial denaturalization was carried out for a period of 2 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min followed by a final 7 min extension at 72 °C. Amplified DNA fragments were digested with the restriction enzymes HhaI and HinfI (New England Biolabs, Massachusetts, MA, USA) following manufacturer's instructions. Restriction fragments were separated by electrophoresis in Tris- Borate-EDTA (TBE) buffered 2.5% agarose gel, stained with SYBERsafe and visualized with UV illumination.

Viability

Movement of the larvae spontaneously or in response to stimulation with tweezers was observed (Sánchez-Alonso et al. 2018).

Experimental infection of muscle

Fish were artificially parasitized with *Anisakis* L3. For this purpose, fish fillets were cut into three portions, each weighing about 100 g, trying to obtain the greatest uniformity with respect to length and width. Each portion from one of the fillets was parasitized with 35 mobile *Anisakis* L3, and the matching portion of the other fillet from the same fish was placed on top, thus making a sandwich, which was wrapped in polyethylene film and stored in the fridge for 24 hours to allow larvae to penetrate into the muscle.

Freezing of infected fish and recovery of larvae after thawing

For the study of the effect of freezing, two independent experiments were done within a time lapse of 7 days. For this purpose, two batches of hake were used, whereas the batch of *Anisakis* L3 was the same in both experiments. Three sandwiches per experiment were frozen in a home freezer (GS-310, Radiber S.A., Barcelona, Spain) with work temperature set at -20 °C (-20.0±0.7 °C). The freezing finished when each sandwich reached -10 °C. Samples were then allowed to thaw

overnight in the fridge. Freezer and sample temperatures during freezing and thawing were measured in the thermal centre of each sandwich using T-type thermocouples and recorded (scanning time of 10 s) with a DaqPRO model 5300 data logger (Fourier Systems Ltd.).

Larvae were recovered from the sandwiches after visual inspection, and in some cases with the aid of UV light (366 nm) (Vidaček et al. 2010), and were then placed in 0.145M NaCl for viability and OCR measurements.

Incubation of Anisakis L3

Clean larvae (50 L3/10 mL of 0.145M NaCl) were stored in the fridge (4.6 °C) for up to 11 days. Also, larvae that had survived freezing were stored in similar conditions and analysed periodically for up to 7 days. Another group of control larvae (50 L3/10 mL of 0.145M NaCl) was placed in an incubator at 37 °C for 72 h. Finally, control *Anisakis* L3 were placed in artificial gastric juice and incubated (100 L3, 10 L3/10 mL) at 37 °C as described previously (Sánchez-Alonso et al. 2018).

Respiratory control analysis in Anisakis L3

For the assessment of oxygen consumption during different mitochondrial respiration states in *Anisakis* larvae, a single-chamber Clark electrode (model DW1/AD, Hansatech Instruments Ltd., Norfolk, England) was used. To obtain data for each key mitochondrial parameter (basal respiration, maximal respiration and spare capacity), a total of 25 cycles (loops) of OCR measurements was performed. Two metabolic modulators were added: FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, Sigma-Aldrich, Inc.) and sodium azide (Sigma-Aldrich, Inc.). Each loop consisted of a total of 8 min: a mixing time of 3 min, followed by a waiting time of 2 minutes and measuring time of 3 minutes. For each OCR, five larvae were used, unless indicated otherwise. Except for those larvae that had been incubated at 37 °C, they were previously allowed to warm at this temperature. Then they were carefully placed in the chamber containing 1000 µL of

176 M9 buffer (3 g of KH_2PO_4 , 6 g of Na_2HPO_4 and 5 g of NaCl to 1 L of H_2O , and 1 mL of 1 M
 177 MgSO_4) at 37 °C. Typically, the initial respiration was measured for the first 64 min (8 loops).
 178 After addition of 125 μL of 160 μM FCCP (17.8 μM final concentration in 0.4% dimethyl
 179 sulfoxide), OCR was measured for about 11 loops. Then 125 μL of 500 mM azide was added and
 180 OCR was measured for 6 loops. The OCR values are expressed as nmol/min/worm over time. For
 181 the study of linearity as a function of number of worms only initial respiration was recorded.
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 183 The first point in OCR per run was discarded and the rates until addition of FCCP were recorded.
 184 Respiration after addition of FCCP was calculated once the measurements were considered stable,
 185 so that at least five rates before azide addition were recorded. Likewise, residual respiration was
 186 calculated once measurements after azide addition were stable. Basal respiration was calculated by
 187 the difference between initial and azide respiration rates. Maximum respiration was the difference
 188 between FCCP-added and azide respiration rates, and spare respiration was obtained by the
 189 difference between FCCP respiration and initial respiration rates.
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 191 OCR was measured: a) in control larvae, where four separate respiration profiles were run. They
 192 had been stored at 4.6 °C for 3, 4, 7 or 11 days after the arrival and preparation of *Anisakis* L3; b) in
 193 frozen thawed larvae, where surviving larvae were immediately used for OCR analyses whereas
 194 non-surviving larvae were analysed after 24 h; c) in order to see the stability after freezing, some
 195 surviving larvae were stored at 4.6 °C, and OCR analysed after 1, 2, 5, 6 and 7 days; d) in larvae
 196 after 72 h at 37 °C; e) in mobile larvae after incubation for 24, 96, 120 and 144 h at 37 °C in gastric
 197 juice.

199 **Data analysis**

200 Two-way ANOVA was used to test possible differences between the type of respiration (i.e. initial,
 201 after FCCP addition, and azide-insensitive) and the various factors studied. Then one-way ANOVA

was used to test possible differences as a function of a) respiration type, b) storage days in 0.145M NaCl or in gastric juice, or c) larvae (control, mobile after freezing and non-mobile after freezing). The Levene test was used to check the equality of variances. Where variances were equal, the difference between means was analysed by the F test. Where equality of variances could not be assumed, Welch and Brown-Forsythe's robust test for equality of means was used. Once the difference between means had been assumed, multiple paired comparisons were used to determine differences between means. The Bonferroni test was used where variances were presumed to be equal, and the Tamhane T2 test was used where equality of variances could not be assumed. The effect of incubation (4.6 °C vs. 37 °C) was analysed by a t test of independent samples and the effect of the number of larvae on basal respiration was analysed by linear regression. Differences between samples were set at P<0.05 levels. IBM SPSS Statistics software v24 was used for statistical analysis.

UNE-EN ISO 9001 certification

The Instituto de Ciencia y Tecnología de los Alimentos y Nutrición (ICTAN-CSIC) has been certified since 2008 under UNE-EN ISO 9001 for "Management and execution of research projects and contracts in the area of Food Science and Technology and Nutrition" (certificate number ER-0366/2008).

Results

The predominant species in the three batches of *Anisakis* L3 used was *A. simplex* s.s. (92, 95 and 97%), coexisting with the heterozygote genotype from *A. simplex* and *A. pegreffii*. However, it cannot be discarded that a small percentage of *Anisakis* specimens showing a heterozygote genotype at the ITS region can correspond to the species *A. pegreffii* (Mattiucci et al., 2016). During the whole experiment, untreated larvae (i.e. control) displayed the typical mobility found in these species.

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229 ***Respiration analysis in untreated Anisakis L3***

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Respiration rates in the absence of any metabolic modulator as a function of the number of larvae (Fig. 1) displayed a linear trend, thus showing that OCR results could be normalized per larva, and in the following experiments five individuals were analysed per run.

Fig. 2 shows a typical OCR profile of *Anisakis* L3 stored in the fridge at 4.6 °C in the absence (i.e. initial respiration) and presence of metabolic modulators. The profile had an initial part in which OCR was generally constant. Then, after FCCP addition, there was a gradual and significant ($P<0.001$) increase in this parameter until a plateau was reached, and after this point the addition of sodium azide led to an abrupt and significant ($P<0.001$) loss of oxygen consumption. The spare respiratory capacity calculated by the difference between the initial and the maximal OCR was 1.25 ± 0.09 nmol/min/larva.

In order to check the stability of *Anisakis* L3 during storage at chilling temperatures (i.e. 4.6 °C), the initial, FCCP and azide residual respiration were measured periodically in control larvae (Fig. 3). One-way ANOVA showed that no significant differences were found as a function of time in any of the three parameters studied.

247 ***The effect of freezing of Anisakis L3 and subsequent storage of surviving larvae at chilling temperatures***

Two freezing trials were performed with larvae recovered from experimentally infected sandwiches that had been frozen at -10 °C. The total freezing time to reach -10.1 ± 0.1 °C in the sandwiches and the average time to pass the critical temperature of maximal crystal growth (t_c) were, respectively, 347 ± 26 and 267 ± 22 min, and in these conditions the percentage of survival was 34%.

In freeze-surviving larvae FCCP addition led to a significant increase ($P<0.001$) in OCR as compared to their initial respiration (Fig. 4), and there was an abrupt and significant ($P<0.001$) decrease in this parameter after addition of azide, as in untreated larvae. No significant differences were observed in initial OCR of larvae between those that survived freezing and the controls (Fig. 4). However, the spare respiratory capacity (i.e. maximum minus basal OCR; 0.50 ± 0.06 nmol/min/larva) was significantly lower ($P<0.05$) as compared to the untreated controls. OCR in larvae that did not survive freezing (Fig. 4) had significantly lower mitochondrial metabolism than the controls or the surviving larvae ($P<0.001$), and addition of FCCP or azide had no effect on the respiration rate.

The storage at 4.6 °C of surviving larvae after freezing (Fig. 5) showed that the maximum respiratory capacity increased significantly ($P<0.05$) in the first 24 h after thawing and decreased upon storage for up to 7 days. This observation was consistent with the fact that thawed larvae increased their ability to move when given some hours at room temperature to acclimatize. In fact, for OCR we analysed the mobile larvae after thawing, taking the first five worms that showed the first signs of mobility immediately after thawing.

Incubation of Anisakis L3 at 37 °C in NaCl 0.145M or in artificial gastric juice

Fig. 6 shows that *Anisakis* L3 displayed different respiration rates owing to the effect of the incubation temperature. There was a slightly lower but non-significant difference in initial respiration of larvae that had been incubated at 37 °C. However, both the maximum and spare respiration were significantly lower as compared to larvae stored at 4.6 °C.

We studied to what extent the respiration ability was maintained in the larvae able to survive in gastric juice. After 24 h up to 88% of L3 remained mobile, and viability decreased to 49, 17 and 8%

after 96, 120 and 144 h of incubation, respectively. Fig. 7 shows that the initial and maximum respiratory rates of surviving larvae decreased significantly with incubation time.

Discussion

The respiratory activity of *Anisakis* L3 in the absence of metabolic modulators (Figs. 1, 2) is similar to that found previously (Sánchez-Alonso et al. 2018). The measurement of *Anisakis* L3 oxygen consumption prior to the addition of FCCP does not strictly reflect mitochondrial respiration owing to the variety of oxygenases that would also consume oxygen and thus contribute to the OCR. In other systems (i.e. cells), mitochondrial respiration is usually distinguished from non-mitochondrial by inhibiting the former with a combination of modulators such as Antimycin A and rotenone so the basal respiration rate is calculated by the difference between the initial and non mitochondrial OCR. These metabolic modulators completely inhibit mitochondrial respiration, by acting as inhibitors of complexes III and I of the oxidative phosphorylation system, respectively (e.g. Koopman et al. 2016). In whole organisms, such as *C. elegans*, the impermeability of the cuticle to non-water-soluble compounds does not allow uptake of the above modulators (O'Reilly et al. 2014), and therefore sodium azide, a water-soluble compound which inhibits complex IV (cytochrome c oxidase) and ATP synthase (complex V) (Massie et al. 2003), is used instead (Koopman et al. 2016; Fong et al., 2017). We did not find specific papers devoted to metabolic or respiration physiology in *A. simplex sensu lato* complex neither at genus level, but the mitochondrial genome of *A. simplex* and *A. pegreffii* (Kim et al. 2006; Yamada et al. 2017) is substantially the same that *C. elegans* (Lemire 2005). The high inhibition of the respiratory rate in the presence of azide in *Anisakis* L3 (Fig. 2) ($P < 0.001$), can be interpreted as in *C. elegans*, so that the difference between the initial OCR and the rate after azide addition can be used to distinguish mitochondrial from non-mitochondrial respiration (Koopman et al. 2016), although some authors suggest referring to the residual OCR obtained by azide as azide-insensitive respiration, since azide is able to inhibit other oxidases (Fong et al. 2017).

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306 The significant increase ($P < 0.001$) in OCR in *Anisakis* L3 after the addition of FCCP (Fig. 2) was in
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307 the same range as the increase found in *C. elegans* (i.e. 2- to 3-fold), and the maximum effect
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308 attained by FCCP in *Anisakis* L3 was more gradual than in *C. elegans* (Koopman et al. 2016; Fong
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309 et al. 2017). FCCP uncouples mitochondrial respiration since it disrupts ATP synthesis by
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310 transporting hydrogen ions across the inner mitochondrial membrane instead of the proton channel
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311 of the ATP synthase (complex V) (Zubovych et al. 2010), resulting in a rapid increase in
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312 consumption of oxygen without generation of ATP. The spare respiratory capacity, measured as the
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313 difference in OCR between initial and after FCCP addition is interpreted as the ability of the
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314 mitochondria to respond to increased energy demand (Koopman et al. 2016; Fong et al. 2017).
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2316 *Anisakis* L3 are stable at chilling temperatures for several weeks or months (i.e. Lanfranchi and
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2817 Sardella 2010). Data from fig. 3 showing that initial, maximum and residual respiration rates do not
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318 vary for up to 11 days, is consistent with this stability and it also indicates the robustness of the
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319 OCR measurements. Thus, *Anisakis* L3 responds to the mitochondrial respiration modulators FCCP
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320 and azide, so the basal, maximum, spare and residual respiration rates can be calculated.
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322 We chose freezing to check whether analysis of respiration rates could differentiate between control
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323 and thawed larvae when, according to the assay of mobility, thawed larvae were considered viable.
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324 Previous results showed that thawed *Anisakis* L3 initial respiration ability (i.e. in the absence of
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325 metabolic modulators) in conditions of 50% survival, did not present significant differences with
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326 the untreated controls (Sánchez-Alonso et al. 2018). In the present results lower percentage survival
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327 was obtained but no significant differences with the control were observed either (Fig. 4). The
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328 lower response of frozen larvae to FCCP (Figs. 4, 5) just after thawing, which was overcome after
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329 24 hours (Fig. 5), suggests a gradual adaptation of their metabolism to return to the normal state
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330 after freezing. Similarly, a slower adaptation to temperature increase from 20 to 37 was observed in
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thawed larvae as compared to controls when measuring initial OCR (Sánchez-Alonso et al. 2018).

In addition, the fact that maximum OCR decreased significantly upon storage for up to 7 days shows that the mitochondria, although still functional, had impaired metabolic health as compared to the controls. We also aimed to check the OCR of non-mobile, thawed L3 which were assumed to be dead. The OCR profile of those larvae after freezing (Fig. 4) suggests that although mitochondria retain some basal respiratory activity after the death of the specimen, they are probably uncoupled, owing to the lack of increase in OCR upon addition of FCCP. Thus the complete respiration analysis in whole *Anisakis* L3 is able to show subtle differences between untreated and frozen and thawed larvae.

We also aimed to study the case where larvae do not show signs of mortality yet, but they may suffer some stress, as in the case of the incubation temperature at 37 °C in NaCl 0.145M for several days. Thus, in conditions where all larvae were mobile (i.e. 3 days at 37 °C), the observed decrease in OCR in the presence of FCCP (Fig. 6), did reflect subtle differences as compared to the controls. It is known that *Anisakis* L3 do not survive long in humans, and mortality studies in artificial gastric juice have shown that they die gradually, although some of the larvae may remain mobile after several days at 37 °C in this medium (Arizono et al. 2012; Sánchez-Alonso et al. 2018). The OCR profile of larvae incubated in these conditions is also informative of the progressive deterioration of those larvae which survived gastric juice (Fig. 7).

Therefore, after some treatments or conditions where we would expect stability (i.e. storage at 4 °C) respiratory analysis was stable. When some impairment of the mitochondrial function could be expected (i.e. freezing, storage at 4 °C for some days after freezing, incubation at 37 °C either in NaCl or artificial gastric juice), the method could show differences when viability measured by other means could not give clear results. These different situations show that OCR in the presence of various metabolic modulators can help to identify subtle changes that occur in the larva which

could be used to study different *Anisakis* species, or in the design of physical, chemical, or culinary treatments to prevent the risk of exposure to live *Anisakis* L3. This measurement can be used as an additional tool together with other indicators such as mobility, agar penetration capacity or survival analyses in several incubation media, so that a broader picture of *Anisakis* L3 characteristics and thus its infectivity potential is gained. In this way, they could help to define with more precision at which point larvae are no longer infective after a given treatment since too extensive ones may cause economic and quality losses whereas treatments that are too short may lead to infections. In a broader sense the study of the respiration analysis in *Anisakis* L3 by means of OCR could be taken as part of a holistic approach which, according to Mattiucci et al. (2018) could integrate genetic, ecological, immunobiological, and environmental factors of these nematodes.

Ethics statement

Ethics approval is not applicable according to EU Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, since hakes used in this study were caught for commercial aims, more specifically for human consumption, and not with research aims so that the capture of the animals was not part of the experimental activity proposed in this work. The experimental design did not involve the manipulation of the live animals but of tissues (e.g. muscle fillets and viscera parasitized with *Anisakis* L3) extracted from already non-living specimens acquired in a local fish monger and in a Central Fish Market.

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385 **Conflict of interest**

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386 The authors declare that they have no conflict of interest.

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475   **Legends to Figures**

476   **Fig. 1 Oxygen consumption rate (OCR) as a function of the number of *Anisakis* L3 in the**
477   **absence of metabolic modulators.** The line shows the linear regression of averaged data with a
478   minimum of n=3 experiments per point: $y = 1.443x - 0.299$ $R^2_{adj} = 0.973$

480   **Fig. 2 OCR profile of *Anisakis* L3 showing the initial (i.e. without metabolic modulators),**
481   **maximum (i.e. after FCCP addition), and azide-insensitive respiration in larvae stored at 4.6**
482   **°C.** Data represent the average of 4 experiments. The arrows show the point of FCCP (solid) and
483   sodium azide (dotted) additions

484   **Fig. 3 OCR of untreated *Anisakis* L3 stored in 0.145M NaCl at 4.6 °C for up to 11 days.**
485   Symbols (●,■,▲) represent maximum (i.e. after FCCP addition), initial (i.e. without metabolic
486   modulators) and azide-insensitive respiration, respectively. Error bars show the standard deviation
487   of the different rates for each part of the OCR profile (i.e. initial, maximum, azide-insensitive)

Fig. 4 OCR of *Anisakis* L3 in untreated larvae stored at 4.6 °C (grey bars), treated larvae that survived (diagonal) and did not survive (square) freezing at -10 °C. Data represent the average of 4, 2 and 1 experiments for untreated controls, surviving and dead L3, respectively. Error bars show the standard deviation of the different oxygen consumption rates for the initial (i.e. without metabolic modulators), maximum (i.e. after FCCP addition) and azide-insensitive respiration. Different letters: a, b, c indicate significant differences ($P < 0.05$) in the type of larvae (untreated, mobile, or non mobile after freezing) for each respiration phase of the OCR profile; x, y, z, indicate significant differences as a function of respiration phase (i.e. basal, after FCCP addition and azide insensitive) per each type of larvae

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Fig. 5 OCR of surviving *Anisakis* L3 after freezing at -10 °C and storing at 4.6 °C for up to 7 days. Symbols (●, ■, ▲) represent maximum, initial and azide-insensitive respiration, respectively. Error bars show the standard deviation of the different rates for each part of the OCR profile (i.e. initial, maximum, azide-insensitive). The larvae had been stored in refrigeration for 11 days before being subjected to freezing. Different letters indicate significant differences along incubation time ($P < 0.05$) for each respiration phase of the OCR profile

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Fig. 6 OCR of *Anisakis* L3 as affected by incubation at 4.6 °C (light grey) or 37 °C (dark grey) in 0.145M NaCl. Error bars show the standard deviation of the different oxygen consumption rates for the initial (i.e. without metabolic modulators), maximum (i.e. after FCCP addition) and azide-insensitive respiration. Data from two independent experiments. Different letters: a, b, c, indicate significant differences ($P < 0.05$) in incubation temperature for each respiration phase of the OCR profile; x, y, z, indicate significant differences as a function of respiration phase (i.e. basal, after FCCP addition and azide insensitive) per each incubation temperature (i.e. 4.6 and 37 °C)

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Fig. 7 OCR of *Anisakis* L3 as affected by incubation at 37 °C in artificial gastric juice for 24 (horizontal), 96 (vertical), 120 (diagonal) and 144 hours (square). The solid bars represent the control larvae stored at 4.6 °C for 24 h. Error bars show the standard deviation of the different rates for the initial (i.e. without metabolic modulators), maximum (i.e. after FCCP addition) and azide-insensitive respiration. Data from one experiment. Different letters indicate significant differences (P<0.05) along incubation time for each respiration phase of the OCR profile; x, y, z, indicate significant differences as a function of respiration phase (i.e. basal, after FCCP addition and azide insensitive) per each incubation time (i.e. 0, 24, 96, 120, 144 h)

Figure 2

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Figure 3

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Figure 5