



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

Isabel Sánchez-Alonso<sup>1</sup> · Alfonso Navas<sup>2</sup> · Susana C. Arcos<sup>2</sup> · Miguel González-Muñoz<sup>3</sup> · Noelia Carballeda-Sangiao<sup>3</sup> · Mercedes Careche<sup>1</sup>

Received: 30 August 2018 / Accepted: 11 February 2019 / Published online: 21 February 2019  
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

## Abstract

Human infection due to eating fish parasitized by live *Anisakis* larvae in the third stage is considered an important health problem, and the application of treatments to ensure their mortality in the fish products is crucial to prevent the risk of infection. Mobility is used to assess viability, but mobile larvae may not always be infective and immobile larvae may be erroneously considered as non-viable. The objective was to establish whether the analysis of respiratory activity by means of the oxygen consumption rate (OCR) of *Anisakis* could be used to identify subtle differences between larvae that were still considered viable in terms of their mobility but had been subjected to thermal and/or chemical stress. The metabolic modulators FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone] and sodium azide were used and the basal, maximum, spare and residual respiration rates calculated. Results showed that maximum respiratory capacity of larvae subjected to freezing significantly decreased immediately after thawing, but after some acclimatization, they recovered their capacity fully. However, when these larvae were stored at 4.6 °C, their mitochondria became dysfunctional faster than those of untreated larvae. OCR also showed that mitochondria of larvae were affected by incubation at 37 °C in NaCl or gastric juice. To conclude, OCR of *Anisakis* in the presence of metabolic modulators can help to identify subtle changes that occur in the larva. These measurements could be used to characterize larvae subjected to various stresses so that a broader picture of *Anisakis* pathogenic potential can be gained.

**Keywords** *Anisakis* · Respiration analysis · Oxygen consumption · Freezing · Viability · Fish

## Introduction

Anisakiasis has been reported to be an underdiagnosed disease (Bao et al. 2017), characterized by gastrointestinal manifestations of epigastralgia, nausea, abdominal pain, and diarrhea (Shimamura et al. 2016), as well as by a characteristic allergic

reaction such as IgE-mediated hypersensitivity, angioedema, urticaria, or anaphylaxis (Ventura et al. 2013; Moneo et al. 2017). Consumers can become infected with the live *Anisakis* sp. larvae in the third stage (L3) when parasitized fish is consumed raw or when technological processes, storage, or cooking practices do not kill the larvae (Audicana and

Section Editor: Simonetta Mattiucci

✉ Mercedes Careche  
mcareche@ictan.csic.es; m.careche@csic.es

Isabel Sánchez-Alonso  
isabel.sanchez@csic.es

Alfonso Navas  
alfonsons@mncn.csic.es

Susana C. Arcos  
scobacho@mncn.csic.es

Miguel González-Muñoz  
miguel.gonzalez.munoz@salud.madrid.org

Noelia Carballeda-Sangiao  
noeliacarba@hotmail.com

<sup>1</sup> Institute of Food Science, Technology and Nutrition, Consejo Superior de Investigaciones Científicas (ICTAN-CSIC), C/ José Antonio Novais 10, 28040 Madrid, Spain

<sup>2</sup> Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales (MNCN-CSIC), C/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

<sup>3</sup> Department of Immunology, University Hospital La Paz Institute for Health Research (IdiPaz), Paseo de la Castellana 261, 28046 Madrid, Spain

Kennedy 2008; Nieuwenhuizen and Lopata 2014; Moneo et al. 2017). Therefore, the application of procedures to ensure the mortality of any larvae present in fish is a key factor in prevention of the risk of *Anisakis* infection, and various physical and chemical treatments have been proposed (EFSA Panel on Biological Hazards 2010).

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

Survivability of *Anisakis* in humans is considered one of the determinants for their pathogenicity. Therefore, behavioral testing, such as larval penetrative ability into an agar layer, has been proposed (Ruitenbergh 1970; Oishi and Hiraoki 1973), and this method has recently been incorporated for studying the in vitro infective potential due to species differences (Suzuki et al. 2010; Arizono et al. 2012) or to technological treatments (Sánchez-Alonso et al. 2018). Similarly, in 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 signaling 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 bacterivorous 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  $4.6 \pm 0.3$  °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 tweezers, cleaned with 0.145 M NaCl, placed in groups of 50 in tubes containing 10 mL of 0.145 M NaCl, and stored in the fridge ( $4.6 \pm 0.3$  °C) until needed. For taxonomic identification, at least 40 clean larvae per batch were kept frozen stored ( $-22.0 \pm 0.3$  °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 the manufacturer's instructions. Molecular identification was carried out for each individual using PCR-RFLP. The forward primer A 5'-GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA-3' and reverse primer B 5'-GCCGGATCCGAATCCTGGTTAGTTTCTTTCT-3' (D'Amelio et al. 2000) were used in reactions containing, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 200 mM each of dATP, dCTP, dGTP, and dTTP and 1 U of DNA polymerase (Biotools B and M labs, S.A. Madrid, Spain). Initial denaturation 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, and 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 the 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 h 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  $-20\text{ °C}$  ( $-20.0 \pm 0.7\text{ °C}$ ). The freezing finished when each sandwich reached  $-10\text{ °C}$ . Samples were then allowed to thaw overnight in the fridge. Freezer and sample temperatures during freezing and thawing were measured in the thermal center 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 0.145 M NaCl for viability and OCR measurements.

### Incubation of *Anisakis* L3

Clean larvae (50 L3/10 mL of 0.145 M 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 analyzed periodically for up to 7 days. Another group of control larvae (50 L3/10 mL of 0.145 M 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 min, and measuring time of 3 min. 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 M9 buffer (3 g of  $\text{KH}_2\text{PO}_4$ , 6 g of  $\text{Na}_2\text{HPO}_4$ , and 5 g of NaCl to 1 L of  $\text{H}_2\text{O}$ , and 1 mL of 1 M  $\text{MgSO}_4$ ) at 37 °C. Typically, the initial respiration was measured for the first 64 min (8 loops). After addition of 125 µL of 160 µM FCCP (17.8 µM final concentration in 0.4% dimethyl sulfoxide), OCR was measured for about 11 loops. Then, 125 µL of 500 mM azide was added, and OCR was measured for 6 loops. The OCR values are expressed as nanomoles per minute per worm over time. For the study of linearity as a function of number of worms, only initial respiration was recorded.

The first point in OCR per run was discarded, and the rates until addition of FCCP were recorded. Respiration after addition of FCCP was calculated once the measurements were considered stable so that at least five rates before azide addition were recorded. Likewise, residual respiration was calculated once measurements after azide addition were stable. Basal respiration was calculated by the difference between initial and azide respiration rates. Maximum respiration was the difference between FCCP-added and azide respiration rates, and spare respiration was obtained by the difference between FCCP respiration and initial respiration rates.

OCR was measured (a) in control larvae, where four separate respiration profiles were run. They had been stored at 4.6 °C for 3, 4, 7, or 11 days after the arrival and preparation of *Anisakis* L3; (b) in frozen thawed larvae, where surviving larvae were immediately used for OCR analyses, whereas non-surviving larvae were analyzed after 24 h; (c) in order to see the stability after freezing, some surviving larvae were stored at 4.6 °C, and OCR analyzed after 1, 2, 5, 6, and 7 days; (d) in larvae after 72 h at 37 °C; and (e) in mobile larvae after incubation for 24, 96, 120, and 144 h at 37 °C in gastric juice.

## Data analysis

Two-way ANOVA was used to test possible differences between the type of respiration (i.e., initial, 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 0.145 M 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 analyzed 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 analyzed by a  $t$  test of independent samples, and the effect of the number of larvae on basal respiration was analyzed 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

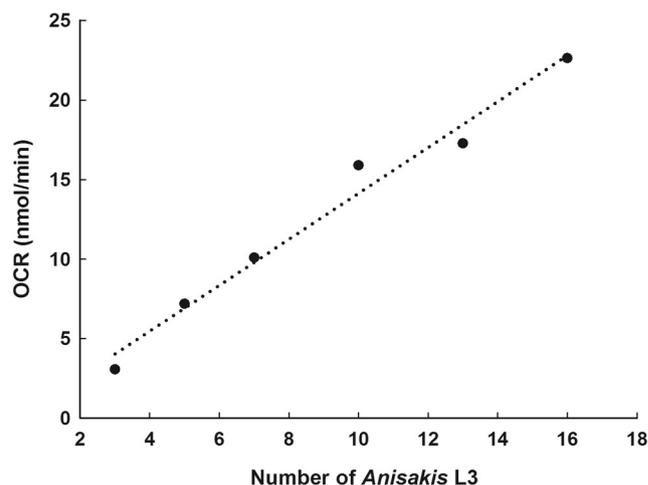
The Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) has been certified since 2008 under UNE-EN ISO 9001 for "Management and execution of research projects and contracts in the area of Food Science and Technology and Nutrition" (certificate number ER-0366/2015).

## 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.

### Respiration analysis in untreated *Anisakis* L3

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 analyzed per run.



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

Figure 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  $1.25 \pm 0.09$  nmol/min/larva.

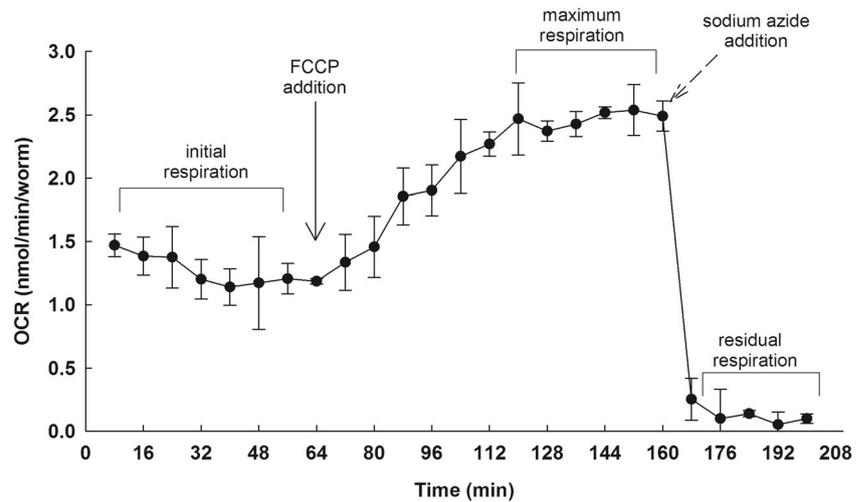
In order to check the stability of *Anisakis* L3 during storage at chilling temperatures (i.e., 4.6 °C), the initial, FCCP, and azide residual respiration were measured periodically in control larvae (Fig. 3). One-way ANOVA showed that no significant differences were found as a function of time in any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

### 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  $-10.1 \pm 0.1$  °C in the sandwiches and the average time to pass the critical temperature of maximal crystal growth ( $t_c$ ) were, respectively,  $347 \pm 26$  and  $267 \pm 22$  min, and in these conditions, the percentage of survival was 34%.

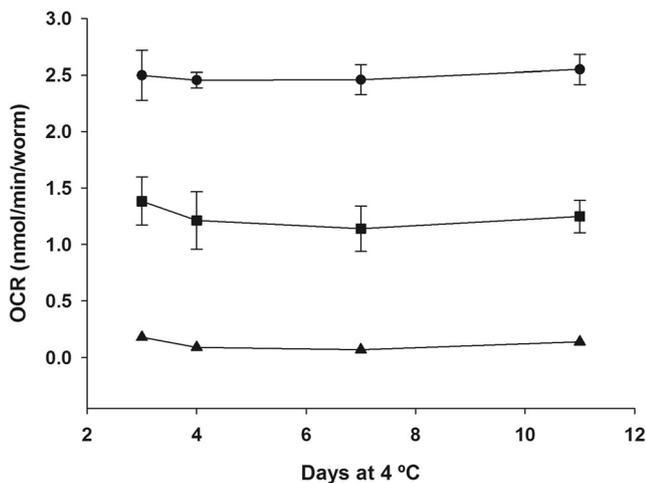
In freeze-surviving larvae, FCCP addition led to a significant increase ( $P < 0.001$ ) in OCR as compared to their initial respiration (Fig. 4), and there was an abrupt and significant ( $P < 0.001$ ) decrease in this parameter after addition of azide, as in untreated larvae. No significant differences were observed in initial OCR of larvae between those that survived

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



freezing and the controls (Fig. 4). However, the spare respiratory capacity (i.e., maximum minus basal OCR;  $0.50 \pm 0.06$  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 analyzed the mobile

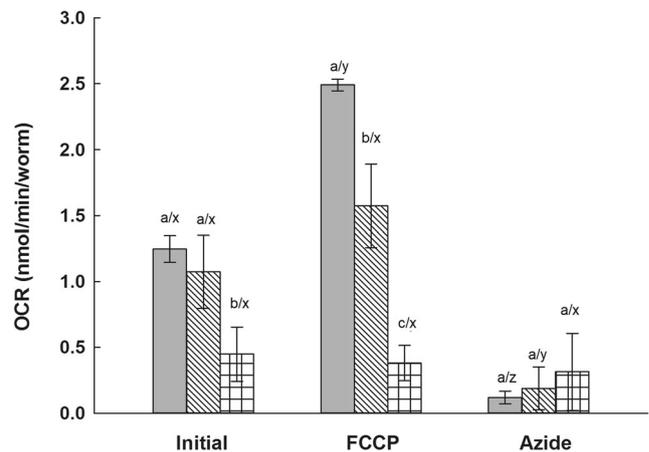


**Fig. 3** OCR of untreated *Anisakis* L3 stored in 0.145 M NaCl at 4.6 °C for up to 11 days. Symbols (●, ■, ▲) represent maximum (i.e., after FCCP addition), initial (i.e., without metabolic modulators), and azide-insensitive respiration, respectively. Error bars show the standard deviation of the different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

larvae after thawing, taking the first five worms that showed the first signs of mobility immediately after thawing.

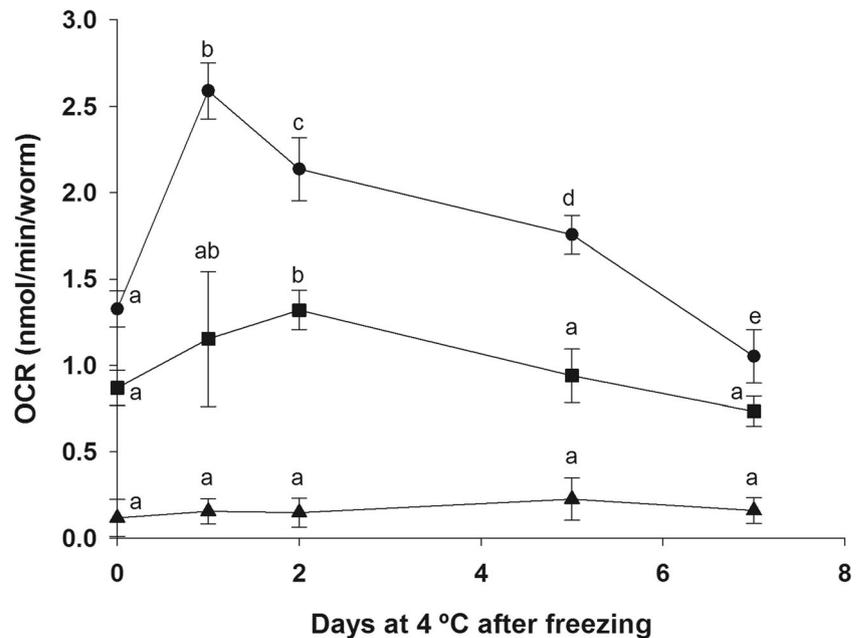
### Incubation of *Anisakis* L3 at 37 °C in NaCl 0.145 M or in artificial gastric juice

Figure 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.

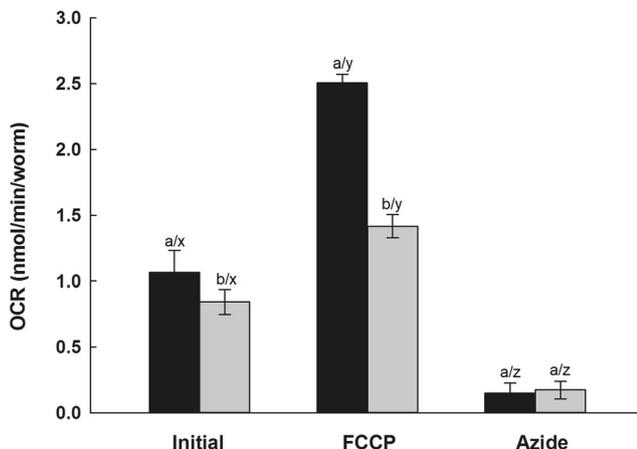


**Fig. 4** OCR of *Anisakis* L3 in untreated larvae stored at 4.6 °C (gray bars), treated larvae that survived (diagonal), and did not survive (square) freezing at -10 °C. Data represent the average of 4, 2, and 1 experiments for untreated controls, surviving, and dead L3, respectively. Error bars show 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, and 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, and z indicate significant differences as a function of respiration phase (i.e., basal, after FCCP addition, and azide-insensitive) per each type of larvae

**Fig. 5** OCR of surviving *Anisakis* L3 after freezing at  $-10\text{ }^{\circ}\text{C}$  and storing at  $4.6\text{ }^{\circ}\text{C}$  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



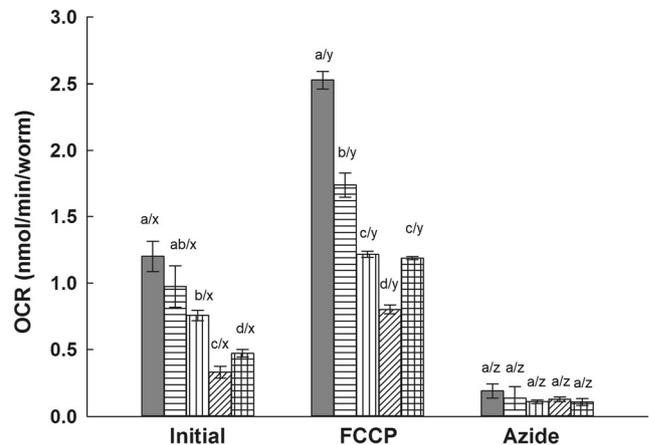
We studied to what extent the respiration ability was maintained in the larvae able to survive in gastric juice. After 24 h up to 88% of L3 remained mobile, viability decreased to 49, 17, and 8% after 96, 120, and 144 h of incubation, respectively. Figure 7 shows that the initial and maximum respiratory rates of surviving larvae decreased significantly with incubation time.



**Fig. 6** OCR of *Anisakis* L3 as affected by incubation at  $4.6\text{ }^{\circ}\text{C}$  (light gray) or  $37\text{ }^{\circ}\text{C}$  (dark gray) in  $0.145\text{ M NaCl}$ . 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*, and *c*—indicate significant differences ( $P < 0.05$ ) in incubation temperature for each respiration phase of the OCR profile; *x*, *y*, and *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  $37\text{ }^{\circ}\text{C}$ )

## Discussion

The respiratory activity of *Anisakis* L3 in the absence of metabolic modulators (Figs. 1 and 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



**Fig. 7** OCR of *Anisakis* L3 as affected by incubation at  $37\text{ }^{\circ}\text{C}$  in artificial gastric juice for 24 (horizontal), 96 (vertical), 120 (diagonal), and 144 h (square). The solid bars represent the control larvae stored at  $4.6\text{ }^{\circ}\text{C}$  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—*a*, *b*, *c*, and *d*—indicate significant differences ( $P < 0.05$ ) along incubation time for each respiration phase of the OCR profile; *x*, *y*, and *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)

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).

The significant increase ( $P < 0.001$ ) in OCR in *Anisakis* L3 after the addition of FCCP (Fig. 2) was in the same range as the increase found in *C. elegans* (i.e., two- to threefold), and the maximum effect attained by FCCP in *Anisakis* L3 was more gradual than in *C. elegans* (Koopman et al. 2016; Fong et al. 2017). FCCP uncouples mitochondrial respiration since it disrupts ATP synthesis by transporting hydrogen ions across the inner mitochondrial membrane instead of the proton channel of the ATP synthase (complex V) (Zubovych et al. 2010), resulting in a rapid increase in consumption of oxygen without generation of ATP. The spare respiratory capacity, measured as the difference in OCR between initial and after FCCP addition, is interpreted as the ability of the mitochondria to respond to increased energy demand (Koopman et al. 2016; Fong et al. 2017).

*Anisakis* L3 are stable at chilling temperatures for several weeks or months (i.e., Lanfranchi and Sardella 2010). Data from Fig. 3 showing that initial, maximum, and residual respiration rates do not vary for up to 11 days is consistent with this stability, and it also indicates the robustness of the OCR measurements. Thus, *Anisakis* L3 responds to the mitochondrial respiration modulators FCCP and azide, so the basal, maximum, spare, and residual respiration rates can be calculated.

We chose freezing to check whether analysis of respiration rates could differentiate between control and thawed larvae when, according to the assay of mobility, thawed larvae were considered viable. Previous results showed that thawed *Anisakis* L3 initial respiration ability (i.e., in the absence of metabolic modulators) in conditions of 50% survival did not present significant differences with the untreated controls (Sánchez-Alonso et al. 2018). In the present results, lower percentage survival was obtained, but no significant differences with the control were observed either (Fig. 4). The lower response of frozen larvae to FCCP (Figs. 4 and 5) just after thawing, which was overcome after 24 h (Fig. 5), suggests a gradual adaptation of their metabolism to return to the normal state after freezing. Similarly, a slower adaptation to temperature increase from 20 to 37 was observed in 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 0.145 M for several days. Thus, in conditions where all larva 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.

**Funding information** This work was supported by the Project ANIRISK (AGL2015-68248-C2), under the Research, Development and Innovation Program oriented to Societal Challenges founded by the Spanish Ministry of Economy and Competitiveness (MINECO) and European Regional Development Fund (FEDER).

## Compliance with ethical standards

**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.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

## References

- Abollo E, Paggi L, Pascual S, D'Amelio S (2003) Occurrence of recombinant genotypes of *Anisakis simplex* s.s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. *Infect Genet Evol* 3(3):175–181
- Arizono N, Yamada M, Tegoshi T, Yoshikawa M (2012) *Anisakis simplex sensu stricto* and *Anisakis pegreffii*: biological characteristics and pathogenetic potential in human anisakiasis. *Foodborne Pathog Dis* 9(6):517–521
- Audicana MT, Kennedy MW (2008) *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev* 21(2):360–379
- Bao M, Pierce GJ, Pascual S, González-Muñoz M, Mattiucci S, Mladineo I, Cipriani P, Bušelić I, Strachan NJC (2017) Assessing the risk of an emerging zoonosis of worldwide concern: Anisakiasis. *Sci Rep* 7(1):–17
- Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochem J* 435:297–312
- Brand MD, Orr AL, Perevoshchikova IV, Quinlan CL (2013) The role of mitochondrial function and cellular bioenergetics in ageing and disease. *Br J Dermatol* 169(Suppl.2):1–8
- D'Amelio S, Mathiopoulou KD, Santos CP, Pugachev ON, Webb SC, Picanco M, Paggi L (2000) Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. *Int J Parasitol* 30(2):223–226
- EFSA Panel on Biological Hazards (BIOHAZ) (2010) Scientific opinion on risk assessment of parasites in fishery products. *EFSA J* 8(4):1543 [1591 pp]
- Fong S, Ng LF, Ng LT, Moore PK, Halliwell B, Gruber J (2017) Identification of a previously undetected metabolic defect in the complex II *Caenorhabditis elegans* mev-1 mutant strain using respiratory control analysis. *Biogerontology* 18(2):189–200
- Kim KH, Eom KS, Park JK (2006) The complete mitochondrial genome of *Anisakis simplex* (Ascaridida: Nematoda) and phylogenetic implications. *Int J Parasitol* 36(3):319–328
- Koopman M, Michels H, Dancy BM, Kamble R, Mouchiroud L, Auwerx J, Nollen EAA, Houtkooper RH (2016) A screening-based platform for the assessment of cellular respiration in *Caenorhabditis elegans*. *Nat Protoc* 11(10):1798–1816
- Lanfranchi AL, Sardella NH (2010) Anisakids survival after microwaving, freezing and salting fish from Argentina. *Food Sci Technol Res* 16(5):499–504
- Lemire B (2005) Mitochondrial genetics. In: Hodgkin J and Anderson P (ed) *The C. elegans* research community. *Wormbook*, pp. 1–10. Doi/10.1895/wormbook.1.25.1. <http://www.wormbook.org>
- Luz AL, Rooney JP, Kubik LL, Gonzalez CP, Song DH, Meyer JN (2015) Mitochondrial morphology and fundamental parameters of the mitochondrial respiratory chain are altered in *Caenorhabditis elegans* strains deficient in mitochondrial dynamics and homeostasis processes. *PLoS One* 10(6):e0130940
- Massie MR, Lapoczka EM, Boggs KD, Stine KE, White GE (2003) Exposure to the metabolic inhibitor sodium azide induces stress protein expression and thermotolerance in the nematode *Caenorhabditis elegans*. *Cell Stress Chaperones* 8(1):1–7
- Mattiucci S, Acerra V, Paoletti M, Cipriani P, Levsen A, Webb SC, Canestrelli D, Nascetti G (2016) No more time to stay 'single' in the detection of *Anisakis pegreffii*, *A-simplex* (s. s.) and hybridization events between them: a multi-marker nuclear genotyping approach. *Parasitology* 143(8):998–1011
- Mattiucci S, Cipriani P, Levsen A, Paoletti M, Nascetti G (2018) Molecular epidemiology of *Anisakis* and anisakiasis: an ecological and evolutionary road map. *Adv Parasitol* 99:93–263
- Moneo I, Carballeda-Sangiao N, González-Muñoz M (2017) New perspectives on the diagnosis of allergy to *Anisakis* spp. *Curr Allergy Asthma Rep* 17(5):27–37
- Navas A, Cobas G, Talavera M, Ayala JA, López JA, Martínez JL (2007) Experimental validation of Haldane's hypothesis on the role of infection as an evolutionary force for metazoans. *Proc Natl Acad Sci U S A* 104(34):13728–13731
- Nieuwenhuizen NE, Lopata AL (2014) Allergic reactions to *Anisakis* found in fish. *Curr Allergy Asthma Rep* 14(8):1–6
- Oishi K, Hiraoki M (1973) Food hygienic studies on *Anisakis* larvae-IV. On the relation between the mortality and the penetration capacity of the larvae into an agar layer. *Nippon Suisan Gakk* 39(12):1345–1348
- O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC (2014) *C. elegans* in high-throughput drug discovery. *Adv Drug Deliv Rev* 69-70:247–253
- Palikaras K, Tavernarakis N (2017) Measuring oxygen consumption rate in *Caenorhabditis elegans*. *Bio Protoc* 6(23):1–10

- Ruitenbergh EJ (1970) Anisakiasis. Pathogenesis, serodiagnosis and prevention. Thesis, Vet Fac Univ of Utrecht, the Netherlands
- Sánchez-Alonso I, Carballada-Sangiao N, González-Muñoz M, Navas A, Arcos SC, Mendizábal A, Tejada M, Careche M (2018) Pathogenic potential of *Anisakis* L3 after freezing in domestic freezers. *Food Control* 84:61–69
- Shimamura Y, Muwanwella N, Chandran S, Kandel G, Marcon N (2016) Common symptoms from an uncommon infection: gastrointestinal Anisakiasis. *Can J Gastroenterol* 2016:1–7
- Suzuki J, Murata R, Hosaka M, Araki J (2010) Risk factors for human Anisakis infection and association between the geographic origins of *Scomber japonicus* and anisakid nematodes. *Int J Food Microbiol* 137(1):88–93
- Ventura MT, Napolitano S, Menga R, Cecere R, Asero R (2013) *Anisakis simplex* hypersensitivity is associated with chronic urticaria in endemic areas. *Int Arch Allergy Immunol* 160(3):297–300
- Vidaček S, De Las Heras C, Solas MT, Mendizábal A, Rodríguez-Mahillo AI, Tejada M (2010) Antigenicity and viability of *anisakis* larvae infesting hake heated at different time-temperature conditions. *J Food Prot* 73(1):62–68
- Yamada A, Ikeda N, Ono H (2017) The complete mitochondrial genome of *Anisakis pegreffii* Campana-Rouget & Biocca, 1955, (Nematoda, Chromadorea, Rhabditida, Anisakidae)-clarification of mitogenome sequences of the *Anisakis simplex* species complex. *Mitochondrial DNA B Resour* 2:240–241
- Zubovych IO, Straud S, Roth MG (2010) Mitochondrial dysfunction confers resistance to multiple drugs in *Caenorhabditis elegans*. *Mol Biol Cell* 21(6):956–969