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Title

Differential cleaving of specific substrates for cathepsin-like activity show cysteine and serine protease activities and a differential profile between *Anisakis simplex s.s.* and *Anisakis pegreffii*, sibling species major etiologic agents of anisakiasis.

Running title

Cathepsins in sibling *Anisakis* spp.

Authors

Verónica Torralbo-Ramírez*, Dolores Molina-Fernández*, David Malagón, Rocío Benítez, and Francisco Javier Adroher[1].

Address

Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, 18071 Granada, Spain

[1] Corresponding author. E-mail: fadroher@ugr.es Orcid ID: http://orcid.org/0000-0002-7969-6658
*These 2 authors contributed equally to this work.

Abstract

Humans can contract anisakiasis by eating fish or squid containing live larvae of the third stage (L3) of the parasitic nematodes of the genus *Anisakis*, majorly from *A. simplex s.s.* and *A. pegreffii*, sibling species of the *A. simplex s.l.* complex. Most cases diagnosed molecularly are due to *A. simplex s.s.*, although *A. pegreffii* has also been identified in human cases. Cathepsins are mostly lysosomal multifunctional cysteine proteases and can participate in the pathogenicity of parasites. Cathepsin B and L activities were investigated in the two sibling species of *Anisakis* mentioned. L3 and L4 of both species were collected during their in vitro development and cathepsin activity was determined in the range of pH 4.0-8.5, using specific fluorogenic substrates. The activity detected with the substrate Z-FR-AMC was identified as cathepsin L (optimum pH = 5.0, range 4.0-6.0, $p<0.001$). Activity was highest in L3 freshly collected from fish, especially in *A. simplex s.s.*, and decreased during development, which could be related to virulence, invasion of host tissues and/or intracellular digestion. Cathepsin B-like activity was not identified with either of the substrates used (Z-RR-AMC and Z-FR-AMC). With Z-RR-AMC, cleaving activity was detected almost exclusively in L4 of *A. simplex s.s.* ($p<0.05$) with optimum pH = 8.0 (range 7.0-8.5). Assays with class-specific protease inhibitors showed this activity was mainly due to serine proteases (up to 90% inhibition with AEBSF), although metalloproteases (up to 40-45% inhibition with 1,10 phenanthroline) and slight cysteine protease activity (<15 % inhibition with E64; putative cathepsin B-like) were also detected. These results show differential serine protease activity between sibling *Anisakis* species, regulated by larval development, at least in *A. simplex s.s.* The higher cathepsin L and serine protease activities detected in this species could be related to its greater pathogenicity, reported in experimental animals, compared to that of *A. pegreffii*.

Key words: nematode; parasite; anisakiasis; sibling species; *Anisakis simplex s.l.*; cathepsins; serine proteases.

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INTRODUCTION

Anisakiasis or anisakidosis is an infection caused by the third stage larvae (L3) of parasitic nematodes of the family Anisakidae. Humans can be infected on consuming fish or squid parasitized by these larvae and which is either raw, undercooked or marinated, smoked, salted, etc. More than 20000 cases have been described worldwide (Chai et al., 2005), perhaps underestimated (Bao et al. 2017), by *Anisakis* (>97% cases), *Pseudoterranova* (2-3%), *Contracaecum* and *Hysterothylacium*. Although more than 90% of cases occur in Japan, anisakiasis also have been often reported in Asian countries such as South Korea (Lim *et al.*, 2015) or European countries such as Spain or Italy (López-Serrano et al., 2000; Pampiglione *et al.,* 2002;). The etiologic agents are usually the larvae of *A. simplex s.s.* or *A. pegreffii*, two species of the complex *A. simplex s.l.* Although cases involving the former appear more frequent in sympatric zones, implying greater pathogenicity, the lack of studies in humans advises caution, although studies on experimental animals seem to confirm this (Rello Yubero, 2003; Suzuki *et al.,* 2010; Quiazon *et al.,* 2011; Romero *et al.,* 2013; Jeon and Kim, 2015).

Cathepsins are peptidases, usually from the papain family and of lysosomic origin, thus acting preferentially in an acidic medium. However, in contrast to the cysteine endopeptidases of vertebrates, the enzymes of helminths exhibit activity within a wide range of pH (pH 4.0-8.0) (Robinson *et al.*, 2013). They are generally cysteine proteases although some aspartyl- or serine-type proteases have also been described. Nematode cathepsins are involved in most of the functions performed by the proteases of parasites, including penetration of host tissues, immune response evasión, virulence, digestion, embryogenesis, moulting, and, particularly, intracellular digestion as a result of their lysosomic origin (Dalton *et al.,* 1996; Hashmi *et al.,* 2002; Guiliano *et al.,* 2004; Robinson *et al.,* 2008; Malagón *et al.,* 2010, 2011, 2013). However, the cathepsins of parasites have not been sufficiently studied despite their being the key to the development of new chemotherapeutic treatments against parasitic nematodes or those

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causing plant diseases, and also useful for diagnosis and development of vaccines (Britton and Murray, 2002; Sajid and McKerrow, 2002; Caffrey *et al.,* 2013).

As cathepsins B- and L-like are those most frequently described in nematodes, the aim of the present study was to detect and partially characterize these two types in the two species of *Anisakis* which are the most common etiologic agents of anisakiasis, while determining differential characteristics associated with each species.

MATERIAL AND METHODS

Sample collection and culture in vitro

Anisakis L3 type I were collected from blue whiting (*Micromesistius poutassou*) from Spanish ports located on the Cantabrian Sea (Ondarroa, Bay of Biscay) and the western Mediterranean Sea (Villajoyosa and Gandía, eastern Spanish coast). The fish were transported and immediately processed to collect the *Anisakis* larvae (see Molina-Fernández *et al.*, 2018). Briefly, the larvae were collected at that time for the L3 sample from fish (L3-0h) and frozen at -20 °C until use. Meanwhile, new L3 batches were prepared and cultured as described by Iglesias *et al.* (1997, 2001). The larvae were removed from the culture at different development times: at 24 h (L3-24h); at 24 h after moulting to L4 (L4-24h) and after 14 days of culture (L4-14d, 10 days after moulting to L4). After harvesting they were frozen at -20 °C until use.

To obtain a sufficient sample of each of the species investigated, the fish were collected from two different geographical areas with predominance of one or other species, according to previous studies (Martín-Sánchez *et al.*, 2005; Mattiucci *et al.*, 2018; Molina-Fernández *et al.*, 2018). Thus, fish from Ondarroa (northern Spain, zone FAO VIIIc) were used for the collection of larvae of *A. simplex s.s.* and those from Villajoyosa and Gandía (eastern Spain, zone FAO 37.1.1) for larvae of *A. pegreffii*.

Genetic identification

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Genetic identification was carried out as described by Molina-Fernández et al. (2015, 2018). After larval DNA extraction, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the rDNA region ITS1-5.8S-ITS2 was performed using the primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (1998). RFLP was performed independently with two restriction enzymes, *TaqI* and *HinfI* Fast Digest (Thermo Scientific). The band pattern generated was visualized by 3% agarose gel electrophoresis and compared to a control for each species to be identified according to D'Amelio et al. (2000) and Abollo et al. (2003). The larvae that showed a mixed banding pattern between *A. simplex s.s.* and *A. pegreffii* with one or other restriction enzyme were considered as L3 type I recombinant genotype larvae.

Preparation of protein extracts

Larval extracts were prepared extemporaneously in tris-HCl buffer at pH 7.8, with 20% glycerin w/v to stabilize the proteins and prevent their degradation. The larvae were homogenized by mechanical means and immersion in liquid nitrogen. Once homogenized, the crude extract was centrifuged at 4 °C and 19,000 x *g* for 20 min (Malagón *et al.*, 2010, 2011). The resulting supernatant was transferred to an Eppendorf and kept on ice until its use as a source of enzymatic activity, its protein concentration being determined (Bradford, 1976).

Cathepsin assays

For the determination of the cathepsin L- and B-like activity of the soluble extract, the procedure of Malagón *et al.* (2010) was followed. The activity was tested in the pH range of 4.0 to 8.5 with increments of 0.5. To achieve a pH-activity profile, it was decided to use a single buffer for the entire pH range. This buffer was tris-maleic 0.2 M, adjusting the pH with HCl or NaOH depending on the case, with 2 mM dithiothreitol (DTT). The maximum ionic strength of the buffer was equivalent to 0.6 M, so NaCl was added to equalize the ionic strength at the different pHs, when necessary.

Fluorogenic substrates Z-FR-AMC (N-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methyl-coumarin), to determine cathepsin B- and L-like activity, and Z-RR-AMC (N-α-

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benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methyl-coumarin) to determine cathepsin B-like activity were used. These substrates show fluorescence when AMC becomes free as a consequence of the hydrolysis of the Arg-AMC bond. The emitted fluorescence was detected with a fluorometer with λ_{ex}=355 nm and λ_{em}=460 nm. Prior to use, the substrates were dissolved in DMSO (dimethylsulfoxide) at 10 mM and frozen at -20 ° C. The final concentration of DMSO in well was 1% in all the assays. The measurements were made in black microplates and each well contained a volume (final concentration) of 100 µl of tris-maleic buffer (100 mM) with DTT (1 mM), 10 µl of extract (10 µg of protein), 30 µl of substrate (15 µM), and bidistilled water to complete a final volume of 200 µl. The reaction was initiated by the addition of the substrate. The measurements were made every 60 seconds for 60 min and, to standardize the process, the most stable zone of the curve was selected for each test, corresponding to 10-30 min. For the inhibition assays, 0.01 mM E64 [L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane], an irreversible inhibitor of cysteine proteases, was added to the reaction mixture. When this did not inhibit 100% activity, pepstatin A (0.02mM) for aspartic proteases, 1,10-phenanthroline (2mM) for metalloproteases and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 2 mM) for serine proteases were also employed as class-specific inhibitors. Enzyme activity was expressed as a variation (Δ) of fluorescence relative units (FRU) x min^{-1} x mg^{-1} protein.

Statistical analysis

The software SPSS 22.0 for Windows was used for the study. As the residuals of the dependent variable did not follow a normal distribution in any case using the Shapiro-Wilk test, the nonparametric Kruskal-Wallis test was performed. The slope of activity for each substrate was used as a dependent quantitative variable, and the variables "larval stage", "species" and "pH" were independent. Since it is a weak method, multiple *post hoc* comparisons were made by pairs when $p<0.1$, using the Mann-Whitney *U* test with the Bonferroni correction. The significance level was set at 0.05.

RESULTS AND DISCUSSION

Genetic identification

A total of 101 larvae of *Anisakis* type I from blue whiting from the port of Ondarroa (northern Spain) were analyzed using the PCR-RFLP technique, identifying 89.1% as *A. simplex s.s.*, 3.0% as *A. pegreffii* and 7.9% as recombinants of the two species. A further 55 larvae of *Anisakis* type I from the Mediterranean ports of Villajoyosa and Gandía (eastern Spain) were identified as 90.9% *A. pegreffii*, 1.8% *A. simplex s.s.* and 7.3% as recombinants. These data coincide with the known distribution of these species, *A. pegreffii* being more prevalent in the western Mediterranean and *A. simplex s.s.* in the northeastern Atlantic, with sympatry to the south and west of the Iberian Peninsula, although these zones were not sampled in this study (Martín-Sánchez *et al.,* 2005; Mattiucci *et al.,* 2018; Molina-Fernández *et al.,* 2018*,* 2019).

Enzymatic activity

The superfamily of papain-like cysteine proteases, to which cathepsins B and L belong, is the best-described group of proteases and are regulated during helminth development (Robinson *et al.,* 2008). Although mainly lysosomic, they have also been detected in the nucleus and cytosol and are secreted into the extracellular medium (Kirschke, 2013).

In the present study it was observed that the pH ranges in which activity of the extracts of *A. simplex s.s.* and *A. pegreffii* was detected did not overlap when a profile of activity-pH was carried out with the two substrates employed. With substrate Z-FR-AMC, which is optimal for cleaving by cathepsins L, although it may also be cleaved by cathepsins B (Robinson *et al.*, 2013), greater activity was observed at acidic pH, with statistically significant differences between pH values ($p<0.001$), with pH 5.0 the most favourable (Fig. 1).

The cleaving of substrate Z-FR-AMC occurred between pHs 4.0-6.0 (maximum at 4.5-5.5) and was almost undetectable at higher pH, always being inhibited by E64, a specific cysteine protease inhibitor. When considered together with the observed lack of fluorescence with substrate Z-RR-AMC (or very low levels not inhibited by E64) at this pH range, it must be supposed that cathepsin B activity is not measured with Z-FR-AMC –as reported by Dalton *et al.* (1996) for *S. manson*i and by Malagón *et al.* (2010) for *H. aduncum*–, suggesting that all the fluorescence detected in this pH range results from cathepsin L-like activity in the species of *Anisakis* studied. The evolution of the activity during development varied according to species. In *A. simplex s.s.* maximum activity was detected in L3-0h, decreasing gradually in each developmental stage ($p<0.006$). In *A. pegreffii*, although showing the same trend, there was an upsurge in activity in L4-24h which then decreased dramatically to a value 14 times lower in L4-14d (Figs. 1 and 2). In spite of this differential behaviour, comparison of the different developmental stages between the two different species only showed significant differences between the most developed stage L4-14d ($p<0.005$), although, overall, *A. simplex* activity was greater than that of *A. pegreffii* ($p=0.06$). The highest activity in infective L3-0h may be related to the greater virulence observed in *A. simplex s.s.* when involved in the processes taking place during host tissue invasion, as occurs in other helminths (Stack *et al.,* 2008; Xue *et al.,* 2019), since, in this stage, the larva is prepared to invade either another paratenic host or its definitive host. However, it may also be related to intracellular digestion since, as several authors have suggested, L3 of *Anisakis*, unlike L4, are not able to ingest food via the digestive system but must obtain nutrients from the extracellular medium through the cuticle (Yasuraoka *et al.,* 1967; Sommerville and Davey, 1976; Iglesias *et al.,* 1997; Dávila *et al.,* 2006). Recently moulted L4 clearly showed greater activity than those which had undergone a longer development time (Fig. 2), which may be related either to the remodelling of the cuticle, as observed in the filarial nematode *Brugia pahangi* (Guiliano *et al.*, 2004) or to adaptation to a new acidic habitat in the stomach chambers of cetaceans (definitive hosts).

Maximum activity for cathepsins L with substrate Z-FR-AMC was at around pH 5 for the two species studied, as in other nematodes from the same superfamily Ascaridoidea such as the

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infective larva of *Toxocara canis* (Loukas *et al.,* 1998) and *H. aduncum* (pH 5-5.5), at least in L3, L4 and adults (Malagón *et al.,* 2010). As these cathepsins are usually of lysosomic origin, their optimal pH is generally 4.5-6.0 (Sajid and McKerrow, 2002; Malagón *et al.*, 2010) although, as mentioned previously, they are also usually active and stable at neutral and even alkaline pH values, in contrast to those of mammals. In this case, their exclusively acidic range of activity would imply their involvement in digestive processes (intracellular and/or intestinal), as reported for *H. aduncum* (Malagón *et al.*, 2010), and perhaps also in processes related to attachment and moulting in an acidic medium associated with the gastric wall of the definitive host. Their relationship with moulting in nematodes has already been established and is considered a conserved function due to the high level of homology within this type of cathepsin (Britton, 2013). In addition, Xue *et al.* (2019) have linked the differential expression of cathepsin L genes with the development and pathogenicity of the nematode *Bursaphelenchus xylophilus*.

With substrate Z-RR-AMC, which is specifically cleaved by cathepsins B-like, activity was detected in both species within the range pH 5.0-8.5, with this activity concentrated within pH 7.0-8.5. Figures 3 and 4 show a good view of the effect of substrate for cathepsin B-like activity results in both species, being very low particularly in *A. pegreffii*. However, note that L4s showed notably higher activity ($p<0.05$) with a maximum at pH 8.0 (Figs. 3 and 4). This appears to coincide with *H. aduncum*, in which a cathepsin B-like has been found with an optimum pH of 7.5 for cleaving Z-RR-AMC (Malagón *et al.,* 2010). However, when the inhibition tests were carried out to determine the activity type of the *Anisakis* extracts, there was only a slight inhibition (<15%) with E64 under our experimental conditions, which shows that only a minimum part of this activity should be of a cysteine protease and, therefore, mostly it is not cathepsin B-like.

Assays with other class-specific inhibitors revealed inhibition of up to 90% with AESBF and 40-45% with 1,10-phenanthroline, showing that the activity detected was mainly due to serine proteases, with some participation by metalloproteases. Serine protease activity has been detected and identified in the excretory-secretory products of L3 of *A. simplex* with optimum pH of 7.5 (Matthews, 1982, 1984; Sakanari and McKerrow, 1990). Later, Morris and Sakanari (1994) isolated, purified and characterized it as a trypsin-like serine protease, 89% homologous with pig trypsin, and able to cleave both Z-RR-AMC and Z-FR-AMC, the former more efficiently, which would explain the detection of activity with the former substrate and not with the latter. These authors reported that $CaCl_2$ was necessary for the enzyme's stability but did not improve its activity. In the present study, the addition of $CaCl_2$ 20 mM, reduced activity by 60-90% (results not shown). Although the cleaving of Z-RR-AMC may be at least partially due to this enzyme, the very low activity of L3 makes this idea questionable. This enzyme may have been preferentially secreted during the L3 stage, possibly to carry out extracorporeal digestion (Buzzell and Sommerville, 1985), which has been observed in nematodes (Feng *et al.*, 2007), and later incorporate the resulting end products of digestion through the cuticle. This could explain the low level of activity detected in the somatic extracts from this stage. However, as L4 are now able to ingest food orally it would not need to secrete the enzyme and it could be accumulated for use in intestinal digestion. Of course, it may be another different serine-protease which appears to express itself differentially in the L4 stage of *A. simplex s.s.* and is almost undetectable under our experimental conditions in *A. pegreffii*. Morris and Sakanari (1994) succeeded in partially characterizing a second serine protease in the somatic extracts of L3 of *A. simplex* which was 85% homologous to a bacterial capable of degrading tissues. It should also be noted that Molina-Fernández *et al.* (2019) found a significantly greater proteolytic activity by serine proteases in all stages of *A. simplex s.s.*, developed at 37 ºC, the same temperature as in the definitive host, than in *A. pegreffii*, although the opposite occurred in L3 collected from the intermediate/paratenic fish poikilotherm host. In addition, Cavallero *et al.* (2018) reported a greater presence of trypsin-like serine protease transcripts in *A. simplex s.s.* than in *A. pegreffii*, albeit with the proviso that the procedure followed may have been more efficient in the former than in the latter. Furthermore, Jasmer *et al.* (2015) reported the low expression of cathepsin B-like cysteine peptidases among the peptidases in the intestine of adult females of *Ascaris suum*, suggesting their possibly scant contribution to nutrient digestion. On the other hand, the lack of activity against the substrate Z-RR-AMC, used for the detection of cathepsins B-like, is not uncommon in nematodes, as in *Dirofilaria immitis* (Richer *et al.*, 1992) or *Ancylostoma caninum* (Dalton *et al.*, 1994). In *C. elegans*, CPR-6, a cathepsin B-like is almost not expressed in the larval stages and overexpressed in adults, showing 70% identity with that of *A. suum* (Britton, 2013). Consequently, this type of activity cannot be discounted in *Anisakis*.

Proteases in general and cathepsins in particular can be regarded as potential therapeutic targets in helminths due to their role in development, survival and pathogenicity for the host (Xue *et al.,* 2019). In fact, some proteases, including cathepsins, are currently being studied with a view to their use in experimental vaccines against trematodes such as *Fasciola hepatica* or *Schistosoma mansoni*, or gastrointestinal nematodes such as *Haemonchus contortus* or *Ostertagia ostertagi* in animals and against the hookworms in humans, with encouraging results so far (Knox, 2012; Hotez *et al.*, 2013; Figueiredo *et al.*, 2015).

CONCLUSIONS

A cathepsin L-like activity has been detected in the two sibling species of the complex *A. simplex s.l.*. The activity of the L3 of *A. simplex s.s.* is higher than *A. pegreffii* L3, which could be related to the higher pathogenicity of the former, and it seems also be involved in the digestion of nutrients. Also, a cathepsin B-like specific substrate is mostly processed by serine protease activity, which has been detected to be significantly higher in *A. simplex s.s.* than in *A. pegreffii*, it could be related to the higher pathogenicity of the former.

Acknowledgements

The authors are grateful to Dr. Manuel Díaz López, from Department of Biology and Geology, University of Almería, for his invaluable advice, to Dr. Miguel Romero Pérez from Department of Pharmacology, Faculty of Pharmacy, University of Granada, for his help with fluorimetry, and to Esperanza Díaz Fernández for her technical assistance. Translation to English was by Robert Abrahams, BSc.

Funding information. This work has been funded by the Agencia Estatal de Investigación (Spanish State Research Agency) and European Regional Development Fund (ERDF), grant number CGL2013-47725-P.

Compliance with ethical standards.

Conflict of interest: none.

REFERENCES

Abollo E, Paggi L, Pascual S, D'Amelio S. Occurrence of recombinant genotypes of *Anisakis simplex s.s.* and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. Infect Genet Evol 2003; 3:175–181. https://doi.org/10.1016/S1567-1348(03)00073-X.

Bao M, Pierce GJ, Pascual S, González-Muñoz M, Mattiucci S, Mladineo I, Cipriani P, Bušelić I, Strachan NJC. Assessing the risk of an emerging zoonosis of worldwide concern: anisakiasis. Sci Rep 2017; 7:43699. https://doi.org/10.1038/srep43699

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Britton C. Proteases of nematodes: From free-living to parasite. In: *Parasitic nematodes. Molecular biology, biochemistry and immunology*, 2nd ed. Kennedy MW, and Harnett W (eds.). Wallingford: CAB International; 2013, pp. 351–374.

Britton C, Murray L. A cathepsin L protease essential for *Caenorhabditis elegans* embryogenesis is functionally conserved in parasitic nematodes. Mol Biochem Parasitol 2002; 122:21–33. PMid:12076767. https://doi.org/10.1016/S0166-6851(02)00066-X.

Buzzell GR, Sommerville RI. The structure of the esophagus in the thirds-stage infective larva of *Anisakis* sp. (Nematoda: Anisakidae). Trans Am Microsc Soc 1985; 104:86–94. https://doi.org/10.2307/3226360.

Caffrey CR, Britton C, McKerrow JH. Helminth cysteine proteases. In: *Handbook of Proteolytic Enzymes*, Volume 2, 3rd ed., Rawlings ND, and Salvesen GS (eds.). Oxford: Academic Press; 2013, pp. 1949–1957. https://doi.org/10.1016/B978-0-12-382219-2.00444-0.

Cavallero S, Lombardo F, Su X, Salvemini M, Cantacessi C, D'Amelio S. Tissue-specific transcriptomes of *Anisakis simplex (sensu stricto)* and *Anisakis pegreffii* reveal potential molecular mechanisms involved in pathogenicity. Parasit Vectors 2018; 11:31. https://doi.org/10.1186/s13071-017-2585-7.

Chai JY, Murrell KD, Lymbery AJ. Fish-borne parasitic zoonoses: Status and issues. Int J Parasitol 2005; 35:1233–1254. https://doi.org/10.1016/j.ijpara.2005.07.013.

D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanço M, Paggi L. 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 2000; 30:223–226. https://doi.org/10.1016/S0020-7519(99)00178-2.

Dalton JP, Clough KA, Jones MK, Brindley PJ. Characterization of the cathepsin-like cysteine proteinases of *Schistosoma mansoni*. Infect Immun 1996; 64:1328–1334.

Dalton JP, Prociv P, Dowd AJ, Brindley PJ, Loukas AC. Secretion of cysteine proteinase activity by the zoonotic hookworm *Ancylostoma caninum*. Am J Trop Med Hyg 1994; 51:341–347. https://doi.org/10.4269/ajtmh.1994.51.341.

Dávila C, Malagón D, Valero A, Benítez R, Adroher FJ. *Anisakis simplex*: CO_2-fixing enzymes and development throughout the in vitro cultivation from third larval stage to adult. Exp Parasitol 2006; 114:10–15. https://doi.org/10.1016/j.exppara.2006.02.011.

Feng J, Zhan B, Liu Y, Liu S, Williamson A, Goud G, Loukas A, Hotez P. Molecular cloning and characterization of AC-MTP-2, an astacin-like metalloprotease released by adult *Ancylostoma caninum*. Mol Biochem Parasitol 2007; 152:132–138. https://doi.org/10.1016/j.molbiopara.2007.01.001.

Figueiredo BCP, Ricci ND, de Assis NRG, de Morais SB, Fonseca CT, Oliveira SC. Kicking in the guts: *Schistosoma mansoni* digestive tract proteins are potential candidates for vaccine development. Front Immunol 2015; 6:21–27. https://doi.org/10.3389/fimmu.2015.00022.

Guiliano DB, Hong X, McKerrow JH, Blaxter ML, Oksov Y, Liu J, Ghedin E, Lustigman S. A gene family of cathepsin L-like proteases of filarial nematodes are associated with larval molting and cuticle and eggshell remodeling. Mol Biochem Parasitol 2004; 136:227–242. https://doi.org/10.1016/j.molbiopara.2004.03.015.

Hashmi S, Britton C, Liu J, Guiliano DB, Oksov Y, Lustigman S. Cathepsin L is essential for embryogenesis and development of *Caenorhabditis elegans*. J Biol Chem 2002; 277:3477–3486. https://doi.org/10.1074/jbc.M106117200.

Hotez PJ, Diemert D, Bacon KM, Beaumier C, Bethony JM, Bottazzi ME, Brooker S, Couto AR, da Silva Freire M, Homma A, Lee BY, Loukas A, Loblack M, Morel CM, Oliveira RC, Russell PK. The human hookworm vaccine. Vaccine 2013; 31:227–232. https://doi.org/10.1016/j.vaccine.2012.11.034.

Iglesias L, Valero A, Adroher FJ. Some factors which influence the in vitro maintenance of *Anisakis simplex* (Nematoda). Folia Parasitol (Praha) 1997; 44:297–301.

Iglesias L, Valero A, Benítez R, Adroher FJ. In vitro cultivation of *Anisakis simplex*: pepsin increases survival and moulting from fourth larval to adult stage. Parasitology 2001; 123:285–291. https://doi.org/10.1017/S0031182001008423.

Jasmer DP, Rosa BA, Mitreva M. Peptidases compartmentalized to the *Ascaris suum* intestinal lumen and apical intestinal membrane. PLoS Negl Trop Dis 2015; 9:e3375. https://doi.org/10.1371/journal.pntd.0003375.

Jeon C-H, Kim J-H. Pathogenic potential of two sibling species, *Anisakis simplex (s.s.)* and *Anisakis pegreffii* (Nematoda: Anisakidae): In vitro and in vivo studies. Biomed Res Int 2015; 2015:ID983656. https://doi.org/10.1155/2015/983656.

Kirschke H. Cathepsin L. In: *Handbook of Proteolytic Enzymes*, Volume 2, 3rd ed., Rawlings ND, and Salvesen GS (eds.). Oxford: Academic Press; 2013, pp. 1808–1817. https://doi.org/10.1016/B978-0-12-382219-2.00410-5.

Knox D. Proteases as vaccines against gastrointestinal nematode parasites of sheep and cattle. In: *Parasitic Helminths: Targets, Screens, Drugs and Vaccines*. Caffrey CR (ed.). Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2012, pp. 399–420. https://doi.org/10.1002/9783527652969.ch24.

Lim H, Jung B-K, Cho J, Yooyen T, Shin E-H, Chai JY. Molecular diagnosis of cause of anisakiasis in humans, South Korea. Emerg Infect Dis 2015; 21:342–344. https://doi.org/10.3201/eid2102.140798.

López-Serrano MC, Alonso Gómez A, Daschner Á., Moreno-Ancillo Á., Suárez de Parga JM, Caballero MT, Barranco P, Cabañas R. Gastroallergic anisakiasis: Findings in 22 patients. J Gastroenterol Hepatol 2000; 15:503–506. https://doi.org/10.1046/j.1440-1746.2000.02153.x

Loukas A, Selzer PM, Maizels RM. Characterisation of Tc-cpl-1, a cathepsin L-like cysteine protease from *Toxocara canis* infective larvae. Mol Biochem Parasitol 1998; 92:275–289. https://doi.org/10.1016/S0166-6851(97)00245-4.

Malagón D, Benítez R, Adroher FJ, Díaz-López M. Proteolytic activity in *Hysterothylacium aduncum* (Nematoda: Anisakidae), a fish gastrointestinal parasite of worldwide distribution. Vet Parasitol 2011; 183:95–102. https://doi.org/10.1016/j.vetpar.2011.07.002.

Malagón D, Benítez R, Kašný M, Adroher FJ. Peptidases in parasitic nematodes: A review. In: *Parasites: Ecology, Diseases and Management*. Erzinger GS (ed.). Hauppage, New York: Nova Science Publishers; 2013, pp. 61–102. Retrieved from: https://www.novapublishers.org/catalog/product_info.php?products_id=40511

Malagón D, Díaz-López M, Benítez R, Adroher FJ. Cathepsin B- and L-like cysteine protease activities during the in vitro development of *Hysterothylacium aduncum* (Nematoda: Anisakidae), a worldwide fish parasite. Parasitol Int 2010; 59:89–92. https://doi.org/10.1016/j.parint.2009.11.001.

Martín-Sánchez J, Artacho-Reinoso ME, Díaz-Gavilán M, Valero López A. Structure of *Anisakis simplex s.l.* populations in a region sympatric for *A. pegreffii* and *A. simplex s.s.*: Absence of reproductive isolation between both species. Mol Biochem Parasitol 2005; 141:155–162. https://doi.org/10.1016/j.molbiopara.2005.02.005.

Matthews BE. Skin penetration by *Necator americanus* larvae. Z. Parasitenkd 1982; 68:81–86. https://doi.org/10.1007/BF00926660.

Matthews BE. The source, release and specificity of proteolytic enzyme activity produced by *Anisakis simplex* larvae (Nematoda: Ascaridida) in vitro. J Helminthol 1984; 58:175–185. https://doi.org/https://doi.org/10.1017/S0022149X00026924.

Mattiucci S, Cipriani P, Levsen A, Paoletti M, Nascetti G. Molecular epidemiology of *Anisakis* and anisakiasis: An ecological and evolutionary road map. Adv Parasitol 2018; 99:93–263. https://doi.org/10.1016/BS.APAR.2017.12.001.

Molina-Fernández D, Benítez R, Adroher FJ, Malagón D. Differential proteolytic activity in *Anisakis simplex s.s.* and *Anisakis pegreffii*, two sibling species from the complex *Anisakis simplex s.l.*, major etiological agents of anisakiasis. Acta Tropica 2019; accepted. https://doi.org/10.1016/j.actatropica.2019.04.003

Molina-Fernández D, Malagón D, Gómez-Mateos M, Benítez R, Martín-Sánchez J, Adroher FJ. Fishing area and fish size as risk factors of *Anisakis* infection in sardines (*Sardina pilchardus*) from Iberian waters, southwestern Europe. Int J Food Microbiol 2015; 203:27–34. https://doi.org/10.1016/j.ijfoodmicro.2015.02.024.

Molina-Fernández D, Rubio-Calvo D, Adroher FJ, Benítez R. Molecular epidemiology of *Anisakis* spp. in blue whiting *Micromesistius poutassou* in eastern waters of Spain, western Mediterranean Sea. Int J Food Microbiol 2018; 282:49–56. https://doi.org/10.1016/j.ijfoodmicro.2018.05.026.

Morris SR, Sakanari JA. Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. J Biol Chem 1994; 269:27650–27656.

Pampiglione S, Rivasi F, Criscuolo M, De Benedittis A, Gentile A, Russo S, Testini M, Villan M. Human anisakiasis in Italy: a report of eleven new cases. Pathol - Res Pract 2002; 198:429–434.

Quiazon KMA, Yoshinaga T, Ogawa K. Experimental challenge of *Anisakis simplex sensu stricto* and *Anisakis pegreffii* (Nematoda: Anisakidae) in rainbow trout and olive flounder. Parasitol Int 2011;

60:126–131. https://doi.org/10.1016/j.parint.2010.11.007.

Rello Yubero FJ. Estudio de los anisákidos parásitos de pescado comercializado en Granada: faneca, sardina y boquerón. 2003. PhD Thesis. Universidad de Granada.

Richer JK, Sakanari JA, Frank GR, Grieve RB. *Dirofilaria immitis*: Proteases produced by third- and fourth-stage larvae. Exp Parasitol 1992; 75:213–222. https://doi.org/10.1016/0014-4894(92)90181-9.

Robinson MW, Brindley PJ, McKerrow JH, Dalton JP. Trematode cysteine endopeptidases. In: *Handbook of Proteolytic Enzymes*, Volume 2, 3rd ed., Rawlings ND, and Salvesen GS (eds.). Oxford: Academic Press; 2013, pp. 1941–1949. https://doi.org/10.1016/B978-0-12-382219-2.00443-9.

Robinson MW, Dalton JP, Donnelly S. Helminth pathogen cathepsin proteases: it's a family affair. Trends Biochem Sci 2008; 33:601–608. https://doi.org/10.1016/j.tibs.2008.09.001.

Romero MC, Valero A, Navarro-Moll MC, Martín-Sánchez J. Experimental comparison of pathogenic potential of two sibling species *Anisakis simplex s.s.* and *Anisakis pegreffii* in Wistar rat. Trop Med Int Heal 2013; 18:979–984. https://doi.org/10.1111/tmi.12131.

Sajid M, McKerrow JH. Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 2002; 120:1–21. https://doi.org/10.1016/S0166-6851(02)00043-9.

Sakanari JA, McKerrow JH. Identification of the secreted neutral proteases from *Anisakis simplex*. J Parasitol 1990; 76:625–630.

Sommerville RI, Davey KG. Stimuli for cuticle formation and ecdysis in vitro of the infective larva of *Anisakis* sp. (Nematoda: Ascaridoidea). Int J Parasitol 1976; 6:433–439. https://doi.org/10.1016/0020-7519(76)90030-8.

Stack CM, Caffrey CR, Donnelly SM, Seshaadri A, Lowther J, Tort JF, Collins PR, Robinson MW, Xu W, McKerrow JH, Craik CS, Geiger SR, Marion R, Brinen LS, Dalton JP. Structural and functional relationships in the virulence-associated cathepsin L proteases of the parasitic liver fluke, *Fasciola hepatica*. J Biol Chem 2008; 283:9896–9908. https://doi.org/10.1074/jbc.M708521200.

Suzuki J, Murata R, Hosaka M, Araki J. Risk factors for human *Anisakis* infection and association between the geographic origins of Scomber japonicus and anisakid nematodes. Int J Food Microbiol 2010; 137:88–93. https://doi.org/10.1016/j.ijfoodmicro.2009.10.001.

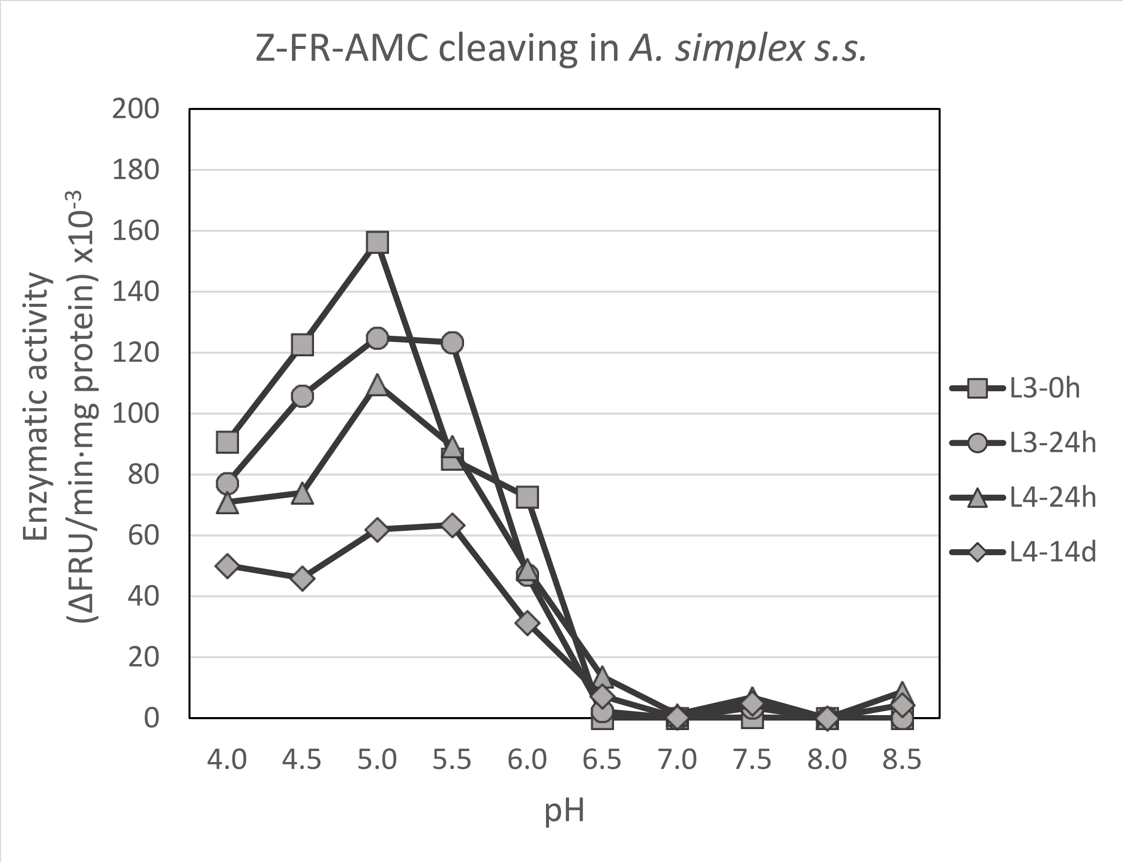
Xue Q, Wu X-Q, Zhang W-J, Deng L-N, Wu M-M. Cathepsin L-like cysteine proteinase genes are associated with the development and pathogenicity of pine wood nematode, *Bursaphelenchus xylophilus*. Int J Mol Sci 2019; 20:215. https://doi.org/10.3390/ijms20010215.

Yasuraoka K, Koyama T, Kato K. Studies on the in vitro axenic development of *Anisakis* larvae (I) [in Japanese]. Japanese J Parasitol 1967; 19:290–291.

Zhu X-Q, Gasser RB, Podolska M, Chilton NB. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. Int J Parasitol 1998; 28:1911–1921. https://doi.org/10.1016/S0020-7519(98)00150-7.

This is the accepted preprint. Article published in *Foodborne Pathogens and Disease* 16 (11): 744-751 (2019).

https://www.liebertpub.com/doi/10.1089/fpd.2019.2633 or https://doi.org/10.1089/fpd.2019.2633.



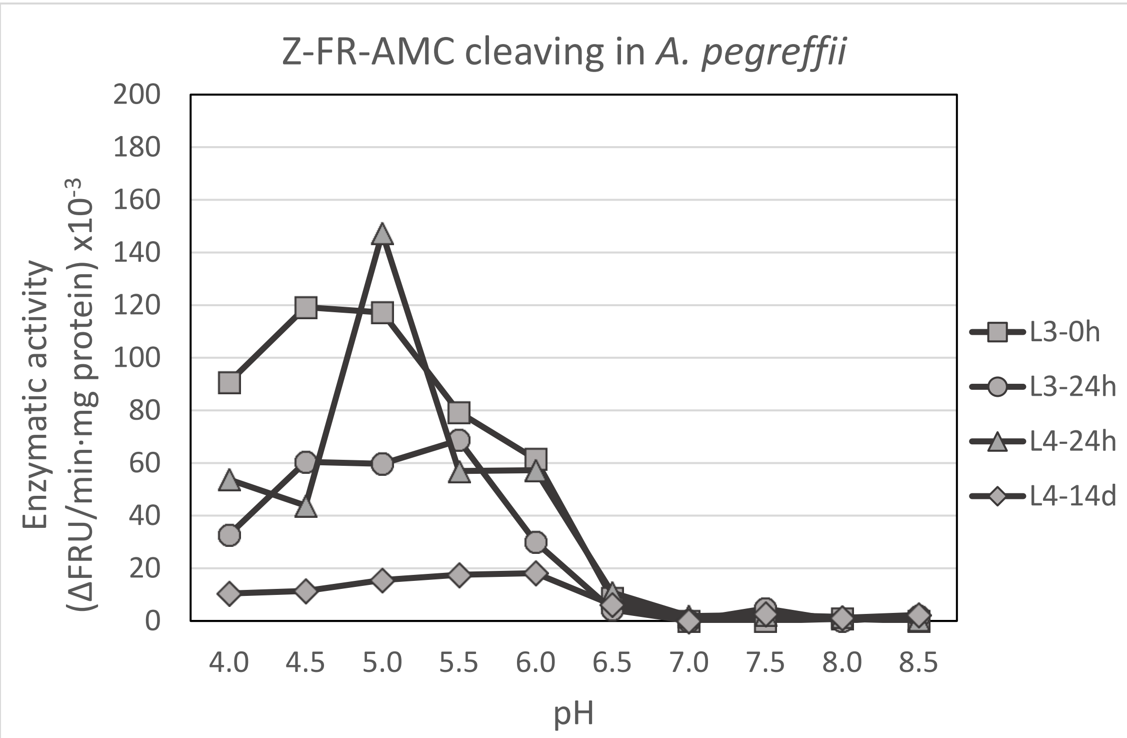


Figure 1.- Profile of cathepsin L-like activity measured by Z-FR-AMC cleavage in *Anisakis* spp. during its development in vitro, depending on pH. Each point is the mean of two or three experiments in triplicate. Upper panel: *A. simplex s.s.* Bottom panel: *A. pegreffii*.

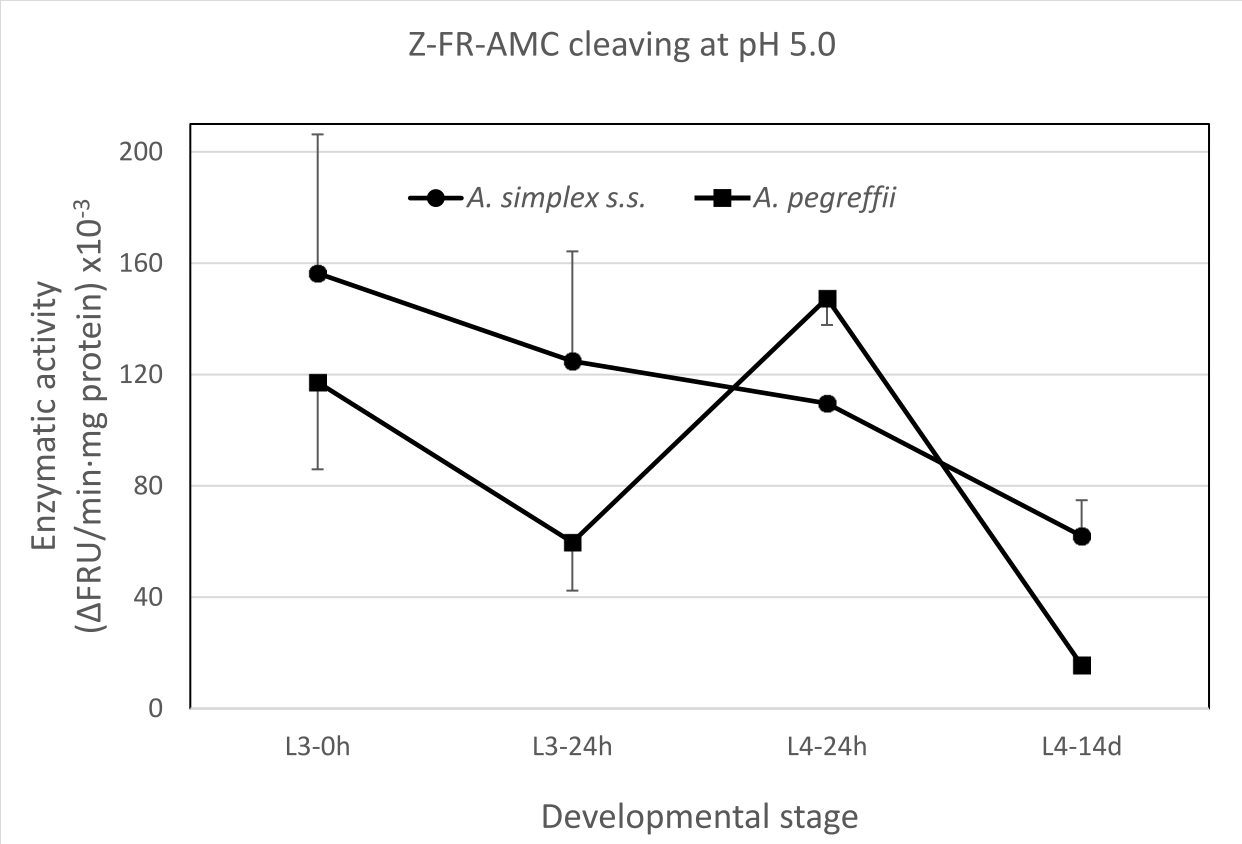


Figure 2.- Profile of cathepsin L-like activity as measured by cleavage of substrate Z-FR-AMC at pH 5.0 in *Anisakis simplex s.s.* (●) and in *A. pegreffii* (■) during its in vitro development. Each point is the mean ± standard deviation of three experiments in triplicate.

466

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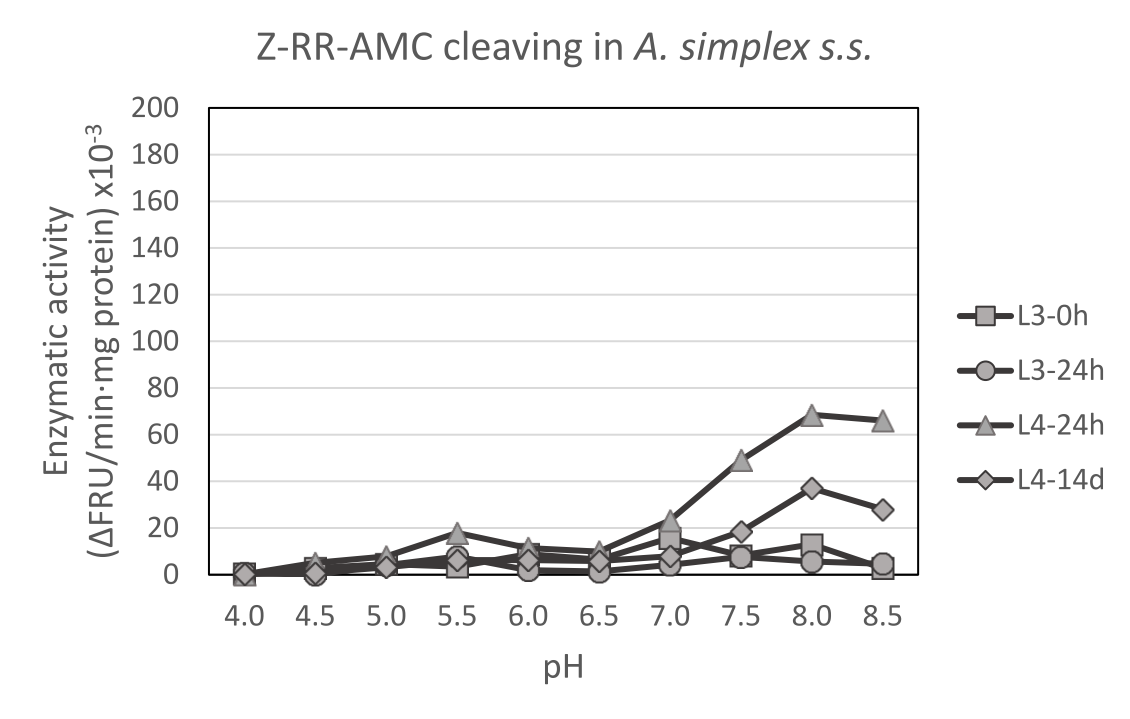

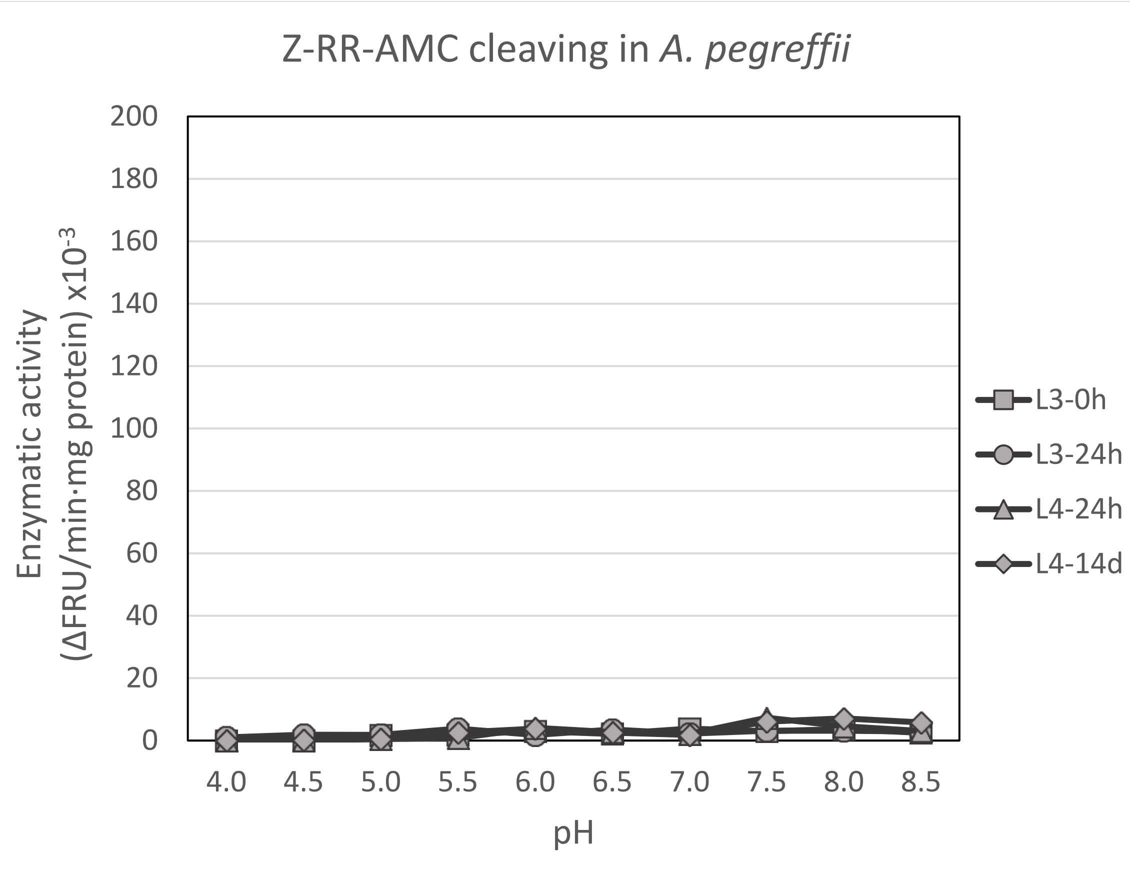

Figure 3.- Profile of enzymatic activity measured by cleavage of Z-RR-AMC in *Anisakis* spp. during its development in vitro, depending on pH. Each point is the mean of two or three experiments in triplicate. Upper panel: *A. simplex s.s.* Bottom panel: *A. pegreffii*.

This is the accepted preprint. Article published in *Foodborne Pathogens and Disease* 16 (11): 744-751 (2019).

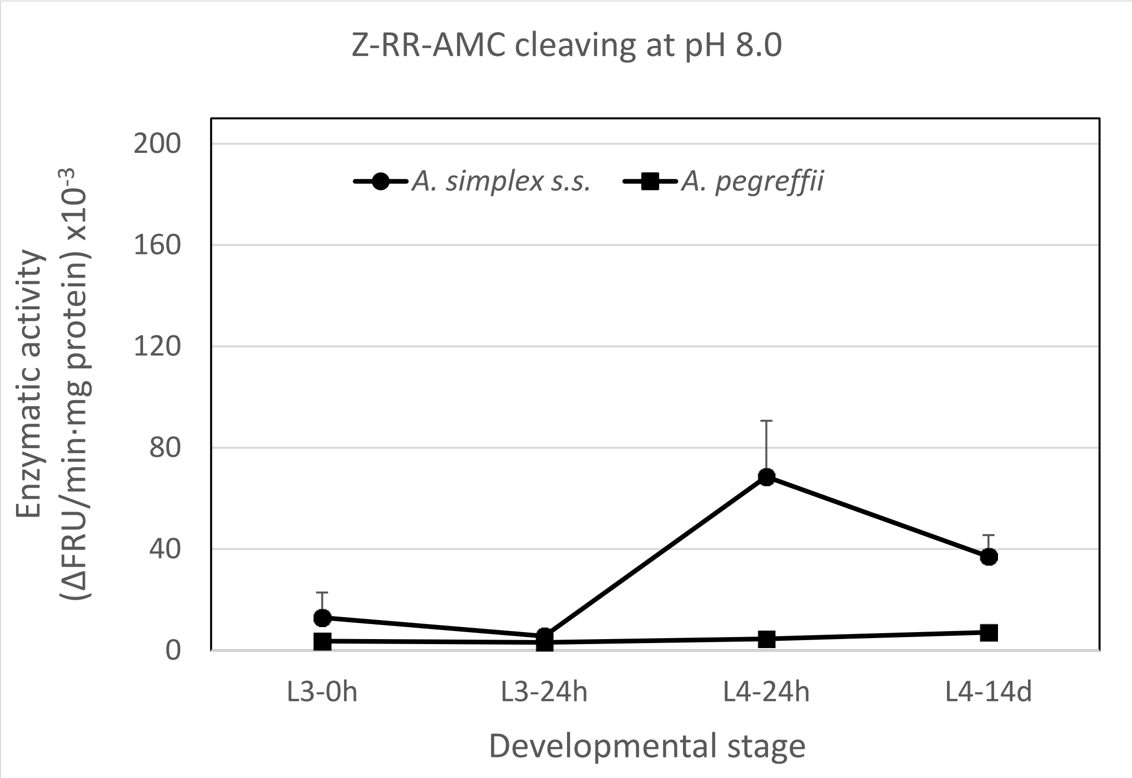

Figure 4.- Profile of enzymatic activity measured by cleavage of substrate Z-RR-AMC at pH 8.0 in *Anisakis simplex s.s.* (●) and in *A. pegreffii* (■) during its in vitro development. Each point is the mean ± standard deviation of three experiments in triplicate.