Epidemiología molecular y desarrollo de anisákidos.

Expresión diferencial de la actividad proteolítica en especies gemelas de *Anisakis simplex s.l.*

**TESIS DOCTORAL**

Programa de Doctorado en Bioquímica y Biología Molecular

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Memoria que presenta la licenciada en Farmacia

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*A mis padres*

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*The influence of a good teacher*

*can never be erased*

# Índice

# Lista de las publicaciones incluidas en la Tesis Doctoral

Los resultados y discusión de esta tesis se presentan como una agrupación de los trabajos de investigación que se han realizado durante el doctorado. Los siguientes artículos se ordenan de acuerdo con su orden de aparición en la memoria:

**Artículo I:**

Molina-Fernández, D.; Malagón, D.; Gómez-Mateos, M.; Benítez, R.; Martín-Sánchez, J.; Adroher, F.J. (2015) Fishing area and fish size as risk factors of *Anisakis* infection in sardines (*Sardina pilchardus*) from Iberian waters, southwestern Europe. *International Journal of Food Microbiology*, **203**: 27-34. doi: 10.1016/j.ijfoodmicro.2015.02.024
Factor de impacto: 3,445 (2015). Nº de citas: 11.
Categoría JCR: Food Science and Technology, posición 14 de 125 (Q1).

**Artículo II:**

Molina-Fernández, D.; Rubio-Calvo, D.; Adroher, F.J.; Benítez, R. (2018) Molecular epidemiology of *Anisakis* spp. in blue whiting *Micromesistius poutassou* in eastern waters of Spain, western Mediterranean Sea. *International Journal of Food Microbiology*, **282**: 49-56. Publicado online 06-06-2018. doi: 10.1016/j.ijfoodmicro.2018.05.026
Factor de impacto: 3,339 (2016). Nº de citas: 1.
Categoría JCR: Food Science and Technology, posición 13 de 130 (Q1; D1).

**Artículo III:**

Valles-Vega, I.*; Molina-Fernández, D.*; Benítez, R.; Hernández-Trujillo, S.; Adroher, F.J. (2017) Early development and life cycle of *Contracaecum multipapillatum* s.l. from a brown pelican, *Pelecanus occidentalis* in Gulf of California, Mexico. *Diseases of Aquatic Organisms*, **125** (3): 167-178. doi: 10.3354/dao03147. Open Access.
Factor de impacto: 1,549 (2016). Nº de citas: 1.
Categoría JCR (2016): Veterinary Sciences, posición 32 de 136 (Q1).

**Artículo IV:**

Molina-Fernández, D.*; Valles-Vega, I.*; Hernández-Trujillo, S.; Adroher, F.J.; Benítez, R. (2017) A scanning electron microscopy study of early development *in vitro* of *Contracaecum multipapillatum* s.l. (Nematoda: Anisakidae) from a brown pelican (*Pelecanus occidentalis*) from the Gulf of California, Mexico. *Parasitology Research*, **116** (10): 2733-2740. doi: 10.1007/s00436-017-5583-y
Factor de impacto: 2,329 (2016). Nº de citas: 2.
Categoría JCR (2016): Parasitology, posición 17 de 36 (Q2).

**Artículo V:**

Molina-Fernández, D.; Adroher, F.J.; Benítez, R. (2018) A scanning electron microscopy study of *Anisakis physeteris* molecularly identified: from third stage larvae from fish to fourth stage larvae obtained *in vitro*. *Parasitology Research*, **117** (7): 2095-2103. doi: 10.1007/s00436-018-5896-5. Open Access.
Factor de impacto: 2,329 (2016). Sin citas aún.
Categoría JCR (2016): Parasitology, posición 17 de 36 (Q2).

**Artículo VI:**

Molina-Fernández, D.; Malagón, D.; Benítez, R.; Adroher, F.J. (2018) Differential proteolytic activity of *Anisakis simplex* s.s. and *Anisakis pegreffii*, two species from the complex *Anisakis simplex* s.l., major etiological agents of anisakiasis. *Acta Tropica*, sometido a evaluación.
Factor de impacto: 2,218 (2016).
Categoría JCR (2016): Tropical Medicine, posición 7 de 19 (Q2).

**Artículo VII:**

Torralbo-Ramírez, V.; Molina-Fernández, D.; Malagón, D: Benítez, R.; Adroher, F.J. (2018) *Anisakis simplex s.s.* and *A. pegreffii*: two sibling species major etiological agents of anisakiasis, with different pathogenicity are showing differential cathepsin-like activity. *Parasitology Research*, enviado.
Factor de impacto: 2,329 (2016).
Categoría JCR (2016): Parasitology, posición 17 de 36 (Q2).

# 1.
# JUSTIFICACIÓN Y OBJETIVOS

Se conoce como anisakidosis la patología producida por larvas de anisákidos, que son nematodos parásitos de la familia Anisakidae. El hombre puede sufrir esta enfermedad cuando consume pescado crudo o poco cocinado que esté parasitado por el tercer estadio larvario (L3) de alguno de los cuatro géneros que se han relacionado con la anisakidosis: *Anisakis*, *Pseudoterranova*, *Contracaecum* o *Hysterothylacium* (Myers 1975; Ishikura et al. 1993; Fernández-Caldas et al. 1998; Takahashi et al. 1998). Esta patología suele cursar con cuadros clínicos digestivos y/o alérgicos, y a pesar de tratarse de una infección subdiagnosticada (Navarro Suárez et al. 2014), se estima que se han producido más de 30.000 casos en todo el mundo, la mayoría de los cuales se han descrito en Japón (Takahashi et al. 1998; Audícana et al. 2003). Además, se calcula que en este país se describen entre 2.000 y 3.000 nuevos casos cada año (Umehara et al. 2007). En España también se han descrito un gran número de casos (Arenal Vera et al. 1991; Repiso Ortega et al. 2003; Del Rey Moreno et al. 2008), siendo el país europeo donde más casos se han diagnosticado, la mayoría de ellos debidos al consumo de boquerones en vinagre (Bao et al. 2017). Las larvas del género *Anisakis* son las más importantes desde el punto de vista sanitario: se estima que alrededor del 97% de todos los casos descritos se deben a este tipo de larvas (Audícana et al. 2003). La patología producida por larvas del género *Anisakis* se conoce específicamente como anisakiasis, y existen varias especies implicadas: *A. simplex sensu stricto*, *A. pegreffii* y *A. physeteris*. Las dos primeras pertenecen a un complejo de especies gemelas indistinguibles morfológicamente, y aunque se ha demostrado mediante estudios tanto *in vivo* en animales de experimentación como *in vitro*, que *A. simplex s.s.* es más patógena que *A. pegreffii*, ambas son capaces de producir anisakiasis (Quiazon et al. 2011; Arizono et al. 2012; Romero et al. 2013).

Una posible forma de abordar esta diferencia de patogenicidad sería mediante el estudio de la actividad proteolítica, dado que algunas proteasas son consideradas como factores de virulencia. Las proteasas son un tipo de proteínas de gran importancia en los nematodos parásitos, donde se han relacionado con su patogenicidad y tienen un papel crucial en la invasión de los tejidos del hospedador (McKerrow et al. 2006), además de participar en diversas funciones biológicas como la nutrición, la muda o el desarrollo embrionario (Malagón et al. 2013). El estudio de la actividad proteolítica de las especies previamente mencionadas podría ayudar a identificar posibles diferencias entre ellas, que justifique esta diferencia en la patogenicidad. Además, los cambios en la actividad proteolítica que ocurran durante el proceso de desarrollo se podrían

relacionar con alguna función biológica. También tienen interés las catepsinas, que son un tipo de proteasas de especial importancia en nematodos parásitos. Este tipo de moléculas se han postulado como posibles dianas terapéuticas además de ser de gran importancia en el desarrollo de vacunas.

Por otra parte, es interesante conocer y analizar la presencia de larvas de anisákidos en peces de interés comercial y culinario, que permita tomar medidas preventivas para evitar la anisakidosis. Para ello nos hemos centrado en dos especies ampliamente consumidas en nuestro país, como son la sardina y la bacaladilla. Se han descrito varias especies de anisákidos que pueden afectar al hombre, por lo que también es relevante conocer qué especies se encuentran parasitando el pescado que consumimos, ya que no todas muestran la misma patogenicidad (Romero et al. 2014; Romero et al. 2013; Arizono et al. 2012).

Igualmente es relevante ampliar el conocimiento de la biología y el desarrollo de estos parásitos, así como identificar las características que nos permitan distinguir entre las distintas especies que pueden afectar al hombre. Además, el ciclo de vida de varias especies de anisákidos no está dilucidado y existen distintas opiniones sobre el desarrollo embrionario que ocurre en el interior del huevo. Conocer a fondo el ciclo de vida de un parásito nos permite diseñar estrategias de control del mismo, lo que es fundamental en cualquier parasitosis. En el caso de los anisákidos, que tienen ciclos de vida complejos y con muchas especies aún no bien diferenciadas implicadas, se necesita aún mucho que investigar.

Por tanto, los objetivos de este estudio son:

-Realizar un estudio epidemiológico molecular de la infección por larvas del género *Anisakis* de bacaladillas y sardinas, peces comerciales muy consumidos en España.

-Ampliar el conocimiento sobre la morfología y la biología del desarrollo de los anisákidos empleando como modelos *Contracaecum multipapillatum* y *Anisakis physeteris*.

-Determinar la actividad proteolítica de dos especies gemelas del complejo *Anisakis simplex s.l.*: *A. simplex sensu stricto* y *A. pegreffii*, nematodos parásitos principales agentes etiológicos de la anisakiasis; su caracterización parcial y sus posibles funciones durante el desarrollo desde la fase L3 procedente del pez hospedador intermediario/paraténico hasta L4 desarrollada *in vitro*.

# 2.
# INTRODUCCIÓN

## 2.1. Anisákidos

Los anisákidos son nematodos pertenecientes a la familia Anisakidae. Los nematodos se caracterizan por tener simetría bilateral y el cuerpo alargado, de sección circular, no segmentado ni dividido. Son unisexuales y presentan dimorfismo sexual en su estado adulto. Tienen una amplia distribución por todo el mundo y son capaces de infectar a una gran variedad de hospedadores vertebrados, como mamíferos, peces, aves, reptiles, e invertebrados (Anderson 2000). Los anisákidos están relacionados con ecosistemas acuáticos, ya que su ciclo de vida incluye peces, mamíferos marinos, y aves piscívoras. Además, la transmisión de estos parásitos normalmente tiene lugar en el agua. Su ciclo de vida es heteroxeno y todos los nematodos pertenecientes a esta familia son parásitos obligados. Varias especies de anisákidos pueden infectar al hombre de manera accidental, provocando una patología conocida como anisakidosis. Dentro de los anisákidos que pueden infectar al hombre encontramos los géneros *Anisakis*, *Pseudoterranova*, *Hysterothylacium* y *Contracaecum*. Estos nematodos suponen un grave problema económico a nivel mundial y se consideran una enfermedad alimentaria emergente.

## 2.2. Clasificación

| | |
|---|---|
| **Phylum** | Nematoda |
| **Clase** | Chromadorea |
| **Orden** | Ascaridida |
| **Superfamilia** | Ascaridoidea |
| **Familia** | Anisakidae |
| **Subfamilia** | Anisakinae |
| **Género** | *Anisakis* |
| **Género** | *Pseudoterranova* |
| **Género** | *Contracaecum* |
| **Subfamilia** | Raphidascaridinae |
| **Género** | *Hysterothylacium* |

El género más estudiado de la familia Anisakidae es *Anisakis*, debido a su alta prevalencia y su gran importancia desde el punto de vista sanitario, ya que las especies de este género son las que han producido más casos de anisakidosis en todo el mundo, así como el género que más frecuentemente podemos encontrar en el pescado, junto con *Hysterothylacium*.

Dentro del género *Anisakis*, tradicionalmente se distinguen 2 tipologías según la morfología de la L3 (Berland 1961). Se distinguen porque en las larvas tipo I la unión del ventrículo con el intestino es oblicua y además presentan un mucrón en el extremo terminal. En las larvas tipo II, la unión ventrículo-intestino es recta y carecen de mucrón.

Las especies que podemos encontrar dentro de cada tipo son las siguientes:

| | |
|---|---|
| ***Anisakis* larva tipo I** | *Anisakis simplex* sensu lato, un complejo de especies gemelas que incluye: |
| | *Anisakis simplex sensu stricto* |
| | *Anisakis pegreffii* |
| | *Anisakis berlandi* |
| | *Anisakis ziphidarum* |
| | *Anisakis typica* |
| | *Anisakis nascetti* |
| ***Anisakis* larva tipo II** | *Anisakis physeteris* |
| | *Anisakis brevispiculata* |
| | *Anisakis paggiae* |

Sin embargo, recientemente se ha sugerido una nueva clasificación del género *Anisakis* en clados, organizados en base a estudios genéticos y evolutivos. Mattiucci et al. (2018) sugieren dividir el género en 4 clados, compuestos por las siguientes especies:

| **Clado I** | *Anisakis simplex sensu stricto* |
| | *Anisakis pegreffii* |
| | *Anisakis berlandi* |

| **Clado II** | *Anisakis ziphidarum* |
| | *Anisakis nascetti* |

| **Clado III** | *Anisakis physeteris* |
| | *Anisakis brevispiculata* |
| | *Anisakis paggiae* |

| **Clado IV** | *Anisakis typica* |

## 2.3. Ciclo biológico

Los anisákidos son parásitos que se localizan en el sistema digestivo de sus hospedadores definitivos, que son mamíferos marinos como los cetáceos (delfines, ballenas, cachalotes, marsopas, calderones, etc.) o los pinnípedos (focas, leones marinos, etc.), de aves acuáticas (pelícanos, cormoranes, …) o de peces. En dicho sistema digestivo se produce la cópula entre macho y hembra, y los huevos generados se expulsan junto con las heces del hospedador, yendo al mar, donde continúa el ciclo biológico (Figura 1). Los huevos eclosionan en el ambiente acuático (o durante la ingestión por un crustáceo), emergiendo larvas de tercer estadio L3 cubiertas por la

cutícula de la fase L2 ( Køie y Fagerholm 1993; Køie et al. 1995; Adroher et al. 2004; Valles-Vega et al. 2017). Estas larvas son ingeridas por pequeños crustáceos, fundamentalmente eufáusidos o copépodos, que forman parte del plancton marino y actúan como hospedadores intermediarios. Los crustáceos infectados son ingeridos sobre todo por peces teleósteos o por calamares (segundos hospedadores intermediarios/paraténicos), donde la larva no cambia de fase, pero sí crece. La localización de los parásitos dentro de estos peces varía, y se pueden encontrar libres en la cavidad visceral o bien encapsulados sobre en las vísceras, sobre todo en el hígado y en las gónadas.

Aunque algunos hospedadores definitivos pueden infectarse directamente a través de los crustáceos del plancton, generalmente las larvas del parásito deben cambiar de hospedador para aumentar de tamaño antes de ser infectivas para su hospedador definitivo.  En los anisákidos, no ocurre ningún cambio de fase en los hospedadores intermediarios ya que en ellos solamente se encuentra el tercer estadio larvario. Por eso podrían considerarse hospedadores de trasporte o paraténicos, no obligatorios en el ciclo biológico. Sin embargo, la L3 recién salida del huevo debe aumentar su talla para volverse infectiva para su hospedador definitivo, para lo que necesita pasar por varios hospedadores. Por esta razón se acepta el uso del término "hospedadores intermediarios/paraténicos" para estos hospedadores ya que no se sabe con certeza el papel que desempeñan en cada caso en el ciclo biológico del parásito.

Finalmente, cuando los peces, crustáceos y calamares infectados son ingeridos por los hospedadores definitivos apropiados, las larvas llegan al sistema digestivo donde se producen dos mudas, de L3 a L4 (M3) y posteriormente a adulto tras la M4. Una vez que los adultos alcanzan la madurez sexual, se produce la fecundación de la hembra por el macho, cerrándose así el ciclo biológico (Figura 1).

El hombre no forma parte de este ciclo biológico, pero puede infectarse al ingerir pescado o calamares que estén insuficientemente cocinados e infectados por las L3 de estos parásitos. Se convierte entonces en un hospedador accidental, y aunque la larva muere a los pocos días/semanas tras la infección, puede producir una patología que se conoce como anisakidosis.
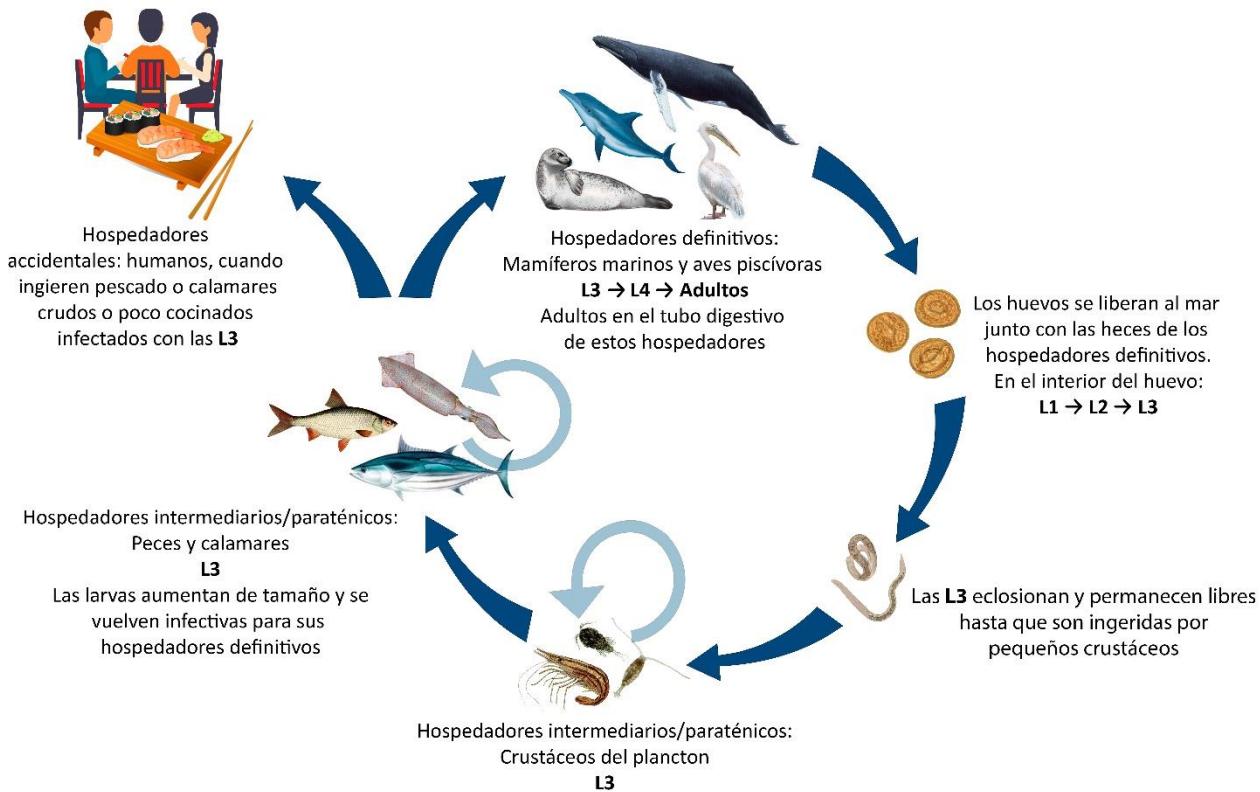
Figura 1. Ciclo de vida de la subfamilia Anisakinae (original de la autora).

Se han descrito cientos de especies de crustáceos que pueden ser hospedadores de anisákidos. También existen cientos de especies de peces y calamares que participan en el ciclo como hospedadores intermediarios/paraténicos, además de los hospedadores definitivos, que también son muy diversos y abarcan a una gran cantidad de especies. Por lo tanto, estamos hablando de un ciclo biológico complejo que involucra a una gran cantidad y diversidad de hospedadores.

Numerosos estudios han relacionado el tamaño de los peces con la prevalencia o la intensidad de la infección por larvas de *Anisakis* (Ruiz-Valero et al. 1992; Adroher et al. 1996; Klimpel et al. 2004; Rello et al. 2008; Bernardi 2009; Rello et al. 2009; Gutiérrez-Galindo et al. 2010). Los peces de menor tamaño suelen tener una menor intensidad, debido a que se alimentan de algunos crustáceos, como los copépodos, donde las larvas de *Anisakis* no son capaces de desarrollarse (Køie 2001). Cuando los peces aumentan de tamaño cambian su alimentación, incluyendo en ella a eufáusidos y otros componentes

del plancton, que sí actúan como hospedadores intermediarios. Además, la intensidad suele ser más alta en peces de mayor tamaño (y por tanto mayor edad) debido posiblemente a la acumulación de parásitos que tiene lugar en el hospedador a lo largo de su vida, ya que, por ejemplo, las larvas de *Anisakis* pueden sobrevivir en estos hospedadores hasta 3 años (Smith 1984).

2.4. Anisakidosis en el mundo

Siguiendo la nomenclatura estandarizada para las enfermedades parasitarias animales (Kassai et al. 1988) se debe utilizar el término "anisakidosis" para describir la parasitosis producida por el tercer estadio larvario de nematodos de la familia Anisakiadae. La mayoría de los casos descritos en el mundo están producidos por el género *Anisakis*, en cuyo caso se denomina "anisakiosis" aunque es más empleado el término "anisakiasis". Sin embargo, hay 4 géneros de anisákidos que son capaces de producir anisakidosis: *Anisakis*, *Pseudoterranova*, *Contracaecum* e *Hysterothylacium*.

Existen dudas sobre si *Hysterothylacium* es capaz de infectar a los humanos o solamente produce reacciones alérgicas. Huang, en 1988, tratando de infectar animales de experimentación, postuló que las larvas de este parásito no son capaces de infectar mamíferos pues son sensibles a las temperaturas superiores a 30 ºC (Huang 1988). Otros autores han llegado a la misma conclusión (Adroher et al. 1991; González 1998). En cualquier caso, se ha descrito un caso de anisakidosis no invasiva por una hembra inmadura de *H. aduncum* (Yagi et al., 1996), quizás porque los adultos pudieran ser más resistentes a la temperatura del cuerpo humano que las larvas. De todos modos, las larvas L3 de *H. aduncum* presentan reactividad cruzada con las larvas L3 de *A. simplex* (Fernández-Caldas et al. 1998) y pueden ser responsables de reacciones alérgicas (Valero et al. 2003).

El primer caso de anisakiasis fue descrito por Van Thiel en 1960 (Van Thiel et al. 1960), en un varón que sufría fuertes dolores abdominales. En un flemón eosinofílico del intestino del paciente se encontró una larva de un nematodo como causante de la lesión, que fue identificada como larva de *Eustoma*, y luego reclasificada como *Anisakis* (Rudolphi, 1809). En 1962 Van Thiel denominó está afección como anisakiasis (Van Thiel 1962).

Japón es el país donde más casos se han descrito, posiblemente debido a sus tradiciones culinarias que incluyen consumo frecuente de pescado crudo como el sushi (Figura 2), además de ser uno de los mayores consumidores de pescado del mundo. Se estima que existe una incidencia de anisakiasis de unos 2.000-3.000 casos al año en este país (Umehara et al. 2007). La mayoría de los casos identificados molecularmente en Japón se deben a *A. simplex s.s.* (Umehara et al. 2007; Arizono et al. 2012), aunque también se han descrito casos provocados por *A. pegreffii* (Umehara et al. 2007), y unos pocos casos de anisakiasis por larvas de *Anisakis* perteneciente al tipo II (Kagei et al. 1978). También se han descrito casos provocados por larvas de *Pseudoterranova* spp. (Mitsuboshi et al. 2017) y el caso por *H. aduncum* ya comentado (Yagi et al. 1996). Ishikura (1990) contabiliza más de 12.000 casos de anisakidosis. Takahashi et al. (1998) indican que hasta ese año ya se habían reportado más de 30.000 casos de anisakidosis, la mayoría de ellos en Japón. Otros estudios posteriores reajustan el número de casos a unos 20.000, habiéndose producido el 90% de los mismos en Japón (Chai et al. 2005; EFSA Panel on Biological Hazards 2010). Cabe destacar un caso clínico donde se extrajeron mediante endoscopia 56 larvas de la curvatura del estómago de una paciente (Noboru y Hiroshi 1992). Hay que tener en cuenta que estamos hablando de una enfermedad subdiagnosticada, por lo que la cantidad de casos reales puede ser mucho mayor.



Figura 2. Sushi, comida tradicional japonesa elaborada a partir de arroz y pescado crudo.

Corea del Sur es otro país donde se describen gran número de casos, unos 200 al año (Lim et al. 2015); por ejemplo, Im et al. (1995) describen 107 casos, la mayoría de ellos provocados por *A. simplex s.l.*. También se han descrito al menos 13 casos de anisakiasis producidos por *P. decipiens* (Na et al. 2013) y 15 de 16 casos identificados molecularmente en dicho país han sido causados por larvas de *A. pegreffii* (Lim et al. 2015).

En Europa se describen unos 500 casos anuales (Lim et al. 2015), siendo España donde más casos se reportan, aunque estudios más recientes indican que esta cifra está muy subestimada (Bao et al. 2017). Aunque por ahora, los casos descritos en nuestro país no han sido identificados molecularmente, sí se han identificado morfológicamente como larvas de *Anisakis* spp. El primer caso encontrado en nuestro país fue en 1991 en un paciente aquejado de dolor abdominal agudo debido a una oclusión de la luz del apéndice, producida por una larva de *Anisakis* (Arenal Vera et al. 1991). La siguiente descripción de anisakiasis en España fue en 1992: un reporte de 3 pacientes que contrajeron la infección mediante el consumo de sardinas crudas (López-Vélez et al. 1992). En dos de los pacientes se encontró una larva de *A. simplex*, y en el tercero, una larva de *P. decipiens*. En 1993 se halló una L4 de *A. physeteris* en una paciente (Clavel et al. 1993), y un caso producido por una L4 de *A. simplex* (Rosales et al. 1999). Existen numerosos artículos en la bibliografía donde se describen múltiples casos de anisakiasis en nuestro país (Olveira et al. 1999; López Peñas et al. 2000; Repiso Ortega et al. 2003; González Quijada et al. 2005; Del Rey Moreno et al. 2008). Aunque la mayoría de los casos solamente involucran una o dos larvas, se ha descrito un caso en Madrid donde se extrajeron más 200 larvas de una paciente mediante endoscopia (Jurado-Palomo et al. 2010). Se han mostrado cientos de casos en nuestro país, a pesar de no ser una enfermedad de Declaración Nacional Obligatoria y a que es una patología infradiagnosticada debido a diversos motivos como la inespecificidad de sus síntomas o a la falta de sospecha por parte de los médicos (Navarro Suárez et al. 2014). Sin embargo, en algunas Comunidades Autónomas sí es de declaración obligatoria, como en Andalucía, donde se declararon 8 casos en 2016 y 9 en 2017 (Consejería de Salud - Junta de Andalucía 2018). La mayoría de los casos en España se producen por el consumo de boquerones en vinagre (Bao et al. 2017) (Figura 3).

Figura 3. Boquerones en vinagre, plato que se elabora con boquerones crudos.

Un estudio reciente predice mediante análisis cuantitativo del riesgo (QRA) teniendo en cuenta solamente el boquerón como medio de adquisición de la anisakiasis, que en España se producen cada año entre 7.700 y 8.320 casos (Bao et al. 2017). Si se tiene en cuenta la migración del parásito que ocurre *post-mortem* desde las vísceras hacia la musculatura del pez, esta cifra ascendería a 91.100 casos anuales en nuestro país (Bao et al. 2017). En cualquier caso, sería una estimación mucho mayor que la se ha reportado previamente para Japón, de unos 2.000-3.000 casos anuales de anisakiasis (Umehara et al. 2007), aunque hay que tener en cuenta que la metodología usada en cada caso ha sido diferente. Además, de esos aproximadamente 8.000 casos estimados, el 42% ocurrirían entre Andalucía y Madrid (Bao et al. 2017). En términos de incidencia, Cantabria sería la Comunidad Autónoma con el valor más alto (35 casos por cada 100.000 habitantes) seguido del País Vasco (31 casos por cada 100.000 habitantes) (Bao et al. 2017).

Italia es otro de los países donde más casos se describen cada año (D'Amelio et al. 1999; Pampiglione et al. 2002; Mattiucci et al. 2013; Mattiucci et al. 2017). D'Amelio y colaboradores describieron en Italia el primer caso que se diagnosticó molecularmente en el mundo mediante PCR-RFLP: fue en 1999 y se produjo por una larva identificada como *A. pegreffii* (D'Amelio et al. 1999). También se han descrito en este país casos por *P. decipiens* (Cavallero et al. 2016). La mayoría de los casos en Italia son producidos por *A. pegreffii* (Mattiucci et al. 2011, 2013, 2017), posiblemente debido a que ésta es la especie predominante en el mar Mediterráneo, especialmente en las costas de Italia. Cabe destacar un estudio que analiza la prevalencia de *Anisakis* en productos listos para consumir elaborados a partir de boquerones y comercializados en supermercados en

Italia (Guardone et al. 2018) en el que se examinaron 107 productos: el 54,2% de éstos tenían al menos una larva de *Anisakis* visible, y se recolectaron en total 1.283 larvas sumando vivas y muertas, siendo el 75,4% *A. pegreffii* y el 24,6% *A. simplex s.s.* Este tipo de productos supone un grave problema económico, al ser rechazados por el consumidor, y sanitario, ya que conlleva riesgo de producir anisakiasis y/o reacciones alérgicas.

Podemos ver que esta patología tiene una distribución cosmopolita, y hasta la fecha se han descrito casos de anisakiasis en países de todo el mundo tales como Australia (Shamsi y Butcher 2011), Estados Unidos (Little y MacPhail 1972; Jackson 1975; Pinkus et al. 1975; Couture et al. 2003), Croacia (Mladineo et al. 2016), Chile (Mercado et al. 1997; Torres et al. 2007), Islandia (Skirnisson 2006), etc.

2.5. Formas clínicas

La anisakidosis o infección por larvas de anisákidos ocurre cuando se ingiere pescado que no ha sido adecuadamente tratado, y que esté infectado por larvas de los géneros *Anisakis*, *Pseudoterranova*, *Contracaecum* o *Hysterothylacium*. La más frecuente es la anisakiasis, producida por larvas del género *Anisakis*. Se estima que el 97% de los casos de anisakidosis en todo el mundo están producidos por larvas del género *Anisakis*, siendo el 3% restante casos de pseudoterranovosis (Audícana et al. 2003; Rello Yubero et al. 2004). Muy pocos casos se han reportado de infección por larvas de *Contracaecum* (Schaum y Müller 1967; Im et al. 1995; Ishikura et al. 1996; Shamsi y Butcher 2011) y sólo uno por *H. aduncum* (Yagi et al. 1996). Se diferencian varias formas clínicas de anisakidosis, que desarrollaremos más adelante:

-Anisakidosis no invasiva: se produce cuando las larvas se expulsan del organismo a través de las heces. Tras la ingesta del parásito aparecen vómitos y sintomatología estrictamente gastrointestinal. Las larvas se eliminan de forma natural sin necesidad de aplicar ningún tratamiento, y una vez eliminadas las larvas remiten los síntomas de manera rápida (Kliks 1983; Hubert et al. 1989; Shamsi y Butcher 2011).

-Anisakidosis invasiva: ocurre cuando la larva invade el organismo, alojándose en alguna región del mismo. Se subdivide en gástrica, intestinal o ectópica, según si la infección ocurre en el estómago (la mayoría de los casos), el intestino o fuera del tracto gastrointestinal.

-Anisakidosis alérgica: ocurre una reacción de hipersensibilidad tras una segunda exposición a los antígenos de las larvas de anisákidos.

-Anisakidosis gastroalérgica: consiste en una reacción de hipersensibilidad generalizada combinada con síntomas gástricos.

La mayoría de los casos de anisakidosis gastrointestinal y gastro-alérgica en nuestro país se producen por el consumo de boquerones en vinagre o crudos. Por ejemplo, en Madrid en 1997, de 96 pacientes diagnosticados con esta patología, 80 confirmaron la ingestión de boquerones en vinagre o crudos (Alonso-Gómez et al. 2004). En el mismo hospital de Madrid se llevó a cabo otro estudio en el año 2000, donde de un total de 22 pacientes diagnosticados con anisakiasis gastroalérgica, 16 afirmaron la ingestión de boquerones; el resto de los casos se dieron por consumo de merluza (4) y sardinas (2) (López-Serrano et al. 2000). En Toledo, el 100% de los pacientes diagnosticados en los dos años que duró el estudio indicaron la ingestión de boquerones antes de la patología (Repiso Ortega et al. 2003). En Antequera, Málaga, de 52 pacientes, 50 confirmaron el consumo de este pescado (Del Rey Moreno et al. 2008; Del Rey Moreno et al. 2013).

2.5.1. Anisakidosis invasiva

**Forma gástrica**: transcurre con unos síntomas típicos de dolor epigástrico, náuseas, vómitos y fiebre ligera, que aparecen entre 1 y 7 horas tras la ingesta de pescado (Oshima 1987; Acha y Szyfres 1989; Kakizoe et al. 1995). Además de los síntomas típicos, la anamnesis es muy importante para el diagnóstico de la anisakidosis, ya que puede ayudar a los facultativos a sospechar sobre esta patología o descartarla (Del Rey Moreno et al. 2008). En algunos casos se puede producir ulceración de la mucosa gástrica, con aparición de hematesis. En estas ocasiones se puede encontrar la larva fuera del aparato digestivo, lo que se conoce como anisakidosis ectópica. El tratamiento de elección es la extracción de la larva mediante endoscopia. Este tratamiento es muy efectivo y tras la retirada de la larva los síntomas remiten de manera rápida (Shimamura et al. 2016). Aun así, conlleva el riesgo de que la extracción no sea completa, por lo que persistirán los síntomas intestinales junto con una inflamación que puede convertirse en crónica. Con respecto al tratamiento farmacológico, hay estudios que evidencian la efectividad del albendazol o de la ivermectina contra las larvas de

*Anisakis* (Dziekońska-Rynko et al. 2002; Pacios et al. 2005). Otros estudios apuntan a remedios de origen natural, como por ejemplo el jengibre o derivados monoterpénicos de plantas, como solución efectiva contra estas larvas (Navarro et al. 2008; Lin et al. 2010), y la citronela y el geraniol para combatir las larvas de *Contracaecum* (Barros et al. 2009).

La mayoría de los casos de anisakiasis cursan de forma gástrica. Por ejemplo, en la recopilación de 12.586 casos de anisakiasis recogidos por Ishikura desde 1968 hasta 1989 (Ishikura 1990) el 92,4% eran gástricos, 4,50 % intestinales y el 2,66 % estaban causados por larvas del género *Pseudoterranova*.

**Forma intestinal**: los síntomas y las manifestaciones no son tan específicas como en el caso anterior. Cursa con dolor abdominal difuso, náuseas, vómitos… Estos síntomas pueden aparecer hasta 7 días después de la ingestión del pescado (Oshima 1987; Acha and Szyfres 1989; Ishikura et al. 1993), por lo que el diagnóstico se dificulta. Además, el paciente no suele referir el consumo de pescado crudo o insuficientemente cocinado por lo que en muchas ocasiones el diagnóstico puede ser erróneo.

Esta forma de anisakiasis puede derivar en complicaciones como obstrucción intestinal, peritonitis o ascitis, o bien derivar en una forma crónica debido a la formación de granulomas eosinofílicos, producidos cuando la larva no se expulsa correctamente. En los casos de patologías más graves, como la obstrucción intestinal, el tratamiento de elección es la cirugía (Takei y Powell 2007). En los casos que no supongan un riesgo grave para el paciente, hay que considerar la terapia conservadora con dieta adecuada, fluidoterapia y tratamiento sintomático (Kawabata et al. 2009; Shrestha et al. 2014). Algunos estudios recomiendan el albendazol como tratamiento farmacológico incluso para obstrucciones intestinales (Pacios et al. 2005).

**Anisakiasis ectópica o extradigestiva**: Ocurre cuando la larva atraviesa la pared intestinal y migra (*larva migrans visceral*) hacia otros órganos o hacia otra zona, como el hígado, la zona esplénica o el pulmón (Yokogawa y Yoshimura 1967; Ito et al. 2007). La sintomatología depende de la zona en la que se encuentre la larva. Es frecuente que se formen granulomas con restos de larvas, que continúan liberando antígenos tiempo después tras la infección. Esto puede ocasionar que el paciente sufra una reacción de hipersensibilidad ante una nueva exposición (Yokogawa y Yoshimura 1967). En los casos de anisakidosis ectópica, lo más común es la extracción quirúrgica del granuloma provocado por la larva mediante cirugía sobre el órgano en el que se encuentra.

Si la anisakiasis digestiva no se trata, los síntomas pueden persistir por un periodo de tiempo desde varias semanas hasta 2 años (Ferre 2001).

### 2.5.2. Anisakidosis alérgica

Las larvas de anisákidos pueden provocar respuestas de hipersensibilidad. La patología transcurre con urticaria, rinitis, broncoconstricción y a veces afectaciones gastrointestinales (Shamsi y Butcher 2011). La reacción de hipersensibilidad es de tipo I y está mediada por inmunoglobulina E. Cuando el alérgeno entra en contacto por segunda vez con el organismo, se activan los anticuerpos y se libera un amplio rango de mediadores inflamatorios como la histamina. Como resultado, aparecen eosinofilia y las manifestaciones clínicas mencionadas (Baird et al. 2014). Algunos de los antígenos son termorresistentes, por lo que pueden provocar este tipo de sintomatología, aunque la larva haya sido destruida mediante altas temperaturas o congelación.

Debido a la dificultad del diagnóstico y a que una parte de la población presenta IgE específica frente a alérgenos de anisákidos, aunque no presenta sintomatología, la anisakidosis alérgica es una enfermedad infradiagnosticada y no se conoce exactamente su prevalencia en la población (Audícana y Kennedy 2008). Existe riesgo de padecer anisakidosis alérgica en individuos sensibilizados tras el consumo de larvas vivas o muertas, incluso en productos listos para consumir si éstos están parasitados. El tratamiento es sintomático, con antihistamínicos o corticoides.

### 2.5.3. Anisakidosis gastro-alérgica

Combina una reacción de hipersensibilidad generalizada, mediada por IgE, junto con síntomas gástricos. Normalmente los síntomas digestivos ocurren primero y aparecen entre 15 minutos y 26 horas tras el consumo de pescado infectado, con una media de 5 horas (López-Serrano et al. 2000). Los síntomas más comunes son la urticaria, hipotensión arterial, angioedema y broncoespasmo (Alonso-Gómez et al. 2004). Hay estudios que indican que solamente se pueden padecer los síntomas alérgicos cuando existe al menos una larva parasitando el tracto digestivo (Alonso-Gómez et al. 2004). Normalmente se tratan los síntomas alérgicos y se procede a la extracción de la larva, tras lo cual los síntomas digestivos suelen desaparecer, aunque a veces pueden durar hasta una semana (López-Serrano et al. 2000). Daschner et al. (2012) sugieren que la alergia a *Anisakis* se desarrolla a partir de una infección previa, con larvas vivas. Una vez que se ha producido la sensibilización, los síntomas alérgicos pueden aparecer aunque la larva ingerida esté muerta (Daschner et al. 2012).

# 3.
# ANTECEDENTES

3.1. Epidemiología y desarrollo de anisákidos

3.1.1. Generalidades

Podemos encontrar larvas de anisákidos en numerosos peces ampliamente consumidos. Se ha descrito la parasitación por larvas de *Anisakis* en más de 200 especies de peces, 25 especies de cefalópodos y adultos en más de 50 especies de cetáceos (McClelland y Ronald 1974b; Smith y Wootten 1978; McClelland et al. 1990; Anderson 2000; Abollo et al. 2001; Klimpel et al. 2004; Cross et al. 2007). Además, algunas de las especies que actúan como hospedadores intermediarios, incluidos numerosos peces de interés gastronómico, presentan una prevalencia que puede llegar al 100% y a veces va acompañada de una alta intensidad (Abollo et al. 2001; Silva y Eiras 2003; Ceballos-Mendiola et al. 2010; Angelucci et al. 2011).

En España, Pereira-Bueno (1992) encontró prevalencias iguales o superiores al 40% en varios peces como la gallineta (*Helicolenus dactylopterus*), brótola de fango (*Phycis blennoides*), bacaladilla (*Micromesistius poutassou*) y abadejo (*Pollacius pollachius*). Sanmartín et al. (1994) también encontraron una prevalencia superior al 40% en varias especies de teleósteos con interés comercial. Los estudios de Abollo y colaboradores en 2001, realizados en Galicia, mostraron una prevalencia del 100% en numerosos peces muy consumidos en España, como en la aguja (*Belone belone*), merluza (*Merluccius merluccius*), abadejo (*Molva dypterygia*), rape común (*Lophius piscatorius*) o gallo (*Lepidorhombus boscii*). También encontró una prevalencia muy alta en la bacaladilla (*Micromesistius poutassou*), con una tasa de infección del 91%, o en el jurel (*Trachurus trachurus*), con el 88% (Abollo et al. 2001).

Numerosos estudios apuntan a que la prevalencia varía según la zona de pesca. Varios estudios demuestran una menor prevalencia en las especies capturadas en el Mediterráneo que en las del Atlántico español (Sanmartín Durán et al. 1989; Ruiz-Valero et al. 1992; Adroher et al. 1996; Valero et al. 2000; Valero et al. 2006). Un ejemplo es la merluza (*Merluccius merluccius*), donde la prevalencia es el doble en las zonas del océano Atlántico que rodean nuestro país que en las costas mediterráneas, con valores de prevalencia del 41 al 87% (Valero et al. 2006).

La zona de captura no solo influye en la prevalencia por anisákidos sino también la especie que se encuentra parasitando el pescado. Se ha observado que *A. simplex s.s.* es la especie más abundante en las zonas del Océano Atlántico cercanas a nuestras costas, mientras que *A. pegreffii* predomina en el Mar Mediterráneo (Ferre 2001;

Martín-Sánchez et al. 2005; Valero et al. 2006; Ceballos-Mendiola et al. 2010; Piras et al. 2014; Costa et al. 2016). Ambas especies pertenecen al clado I. Dentro del clado II del género *Anisakis*, la especie más frecuente en España es *A. physeteris* (Adroher et al. 1996; Valero et al. 2000; Fernández et al. 2005).

En nuestro país, es también bastante frecuente encontrar larvas del género *Hysterothylacium* en diversos peces (Adroher et al. 1996; Valero et al. 2000; Fernández et al. 2005; Rello et al. 2008; Rello et al. 2009; Gutiérrez-Galindo et al. 2010; Madrid et al. 2012) y algunos estudios apuntan que *H. aduncum* es incluso más frecuente que *Anisakis* en algunos como la sardina en el mar Mediterráneo (Rello et al. 2008). Los géneros menos frecuentes en España son *Contracaecum* y *Pseudoterranova*, aunque también están presentes. *Contracaecum* ha sido encontrado tanto en peces como en aves piscívoras (Ferre 2001; Nogueserola et al. 2002; Gutiérrez et al. 2010): se ha descrito la presencia de larvas de este género en congrio (*Conger conger*), platija (*Platichtys flesus*), gobio negro (*Gobius niger*)  o anguila europea (*Anguilla anguilla*) (Sanmartín et al. 2000, 2001; Álvarez et al. 2002; Outeiral et al. 2002). También se ha descrito prevalencia de larvas *Pseudoterranova* en diversos peces capturados en nuestro país (Ferre 2001).

3.1.2. Epidemiología de anisákidos en bacaladillas (*Micromesistius poutassou*)
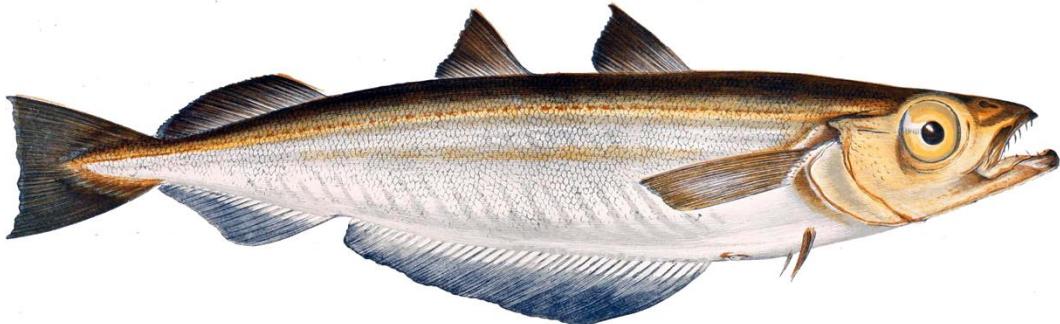


Figura 4. Ilustración de una bacaladilla. Autores: Gervais et Boulart - Les poisons (1877) (Gervais 1877).

La bacaladilla (*Micromesistius poutassou*) (Figura 4) es un pez teleóstero, gadiforme, de cuerpo estrecho que puede llegar hasta los 50 cm de longitud, aunque su media es de unos 22 cm (Cohen et al. 1990). Se trata de una especie batipelágica y oceanódroma, que suele vivir entre los 300 y 400 metros de profundidad, aunque su hábitat puede oscilar entre los 13 y los 3.000 metros (Cohen et al. 1990). Suele residir en aguas templadas de entre los 26º y 79º Norte, y entre los 82º Oeste y 51º Este. Su alimentación está compuesta por pequeños crustáceos decápodos, eufáusidos, pero los individuos más grandes también se alimentan de peces pequeños y cefalópodos. El porcentaje de la dieta que ocupa cada una de estas especies varía según la estación del año y la edad (Macpherson 1977). Pueden vivir hasta 20 años.

El primer estudio epidemiológico llevado a cabo en bacaladillas fue en 1955 por Poljanski, donde se halló una prevalencia del 40% en el mar de Barents, Océano Ártico (Poljanskij 1955). Posteriormente Berland, en 1961, describió una prevalencia del 58,3% y del 100% en dos regiones de Noruega (Berland 1961). En la década de los años 70 se llevaron a cabo numerosos estudios en diversas zonas de Europa, con prevalencias que oscilan entre el 13 y el 100%.

En España, el primer estudio lo realizaron Sanmartín Durán et al. en Galicia en 1989 (Sanmartín Durán et al. 1989) con una prevalencia del 62,3%. Posteriormente Cuéllar et al., en 1991, realizan un estudio epidemiológico en bacaladillas procedentes del Mediterráneo español, encontrando una prevalencia del 30% en este pescado

(Cuéllar et al. 1991). Pereira-Buena et al., en 1992 analizan las bacaladillas del mercado de abastos de Bilbao encontrando un 88,1% de prevalencia (Pereira Bueno 1992).



Figura 5. Bacaladilla procedente de Ondarroa (País Vasco) infectada con L3 de *Anisakis* que se encuentran libres en la cavidad visceral y en la musculatura del pescado (marcadas con flechas).

Con respecto a las especies de anisákidos encontradas en bacaladillas capturadas en España, las más frecuentes son las del complejo *A. simplex s.l.* e *H. aducum*, aunque la proporción de cada especie varía según la zona de captura. Por ejemplo, Martín-Sánchez et al. describen una prevalencia por *A. simplex s.l.* del 81,7% en bacaladillas procedentes del Atlántico, mientras que las del Mediterráneo presentan un 9,1% (Martín-Sánchez et al. 2005). En los últimos años, en la bacaladilla procedente del Océano Atlántico español se han descrito valores de prevalencia por *Anisakis* spp. entre el 78% y el 100% (Chía et al. 2010; Madrid et al. 2012) (Figura 5), mientras en el Mediterráneo esta cifra se sitúa en el 19% según recientes estudios (Madrid et al. 2012).

En bacaladillas procedentes de la costa atlántica de la Península Ibérica se han encontrado las siguientes especies de anisákidos: *A. simplex s.s.*, *A. pegreffii*, individuos recombinantes o híbridos entre ambas especies, *A. physeteris*, *A. paggiae*, *A. brevispiculata*, *A. typica* e *H. aduncum* (Fernández et al. 2005; Martín-Sánchez et al. 2005; Madrid et al. 2012; Romero et al. 2013; Romero et al. 2014; Gómez-Mateos et al. 2016). En bacaladillas procedentes de las costas del Mediterráneo español se han descrito *A. pegreffii*, *A. simplex s.s.*, individuos híbridos entre ambas especies, *A. physeteris*, *A. paggiae* e *H. aduncum* (Valero et al. 2000; Romero et al. 2014).

### 3.1.3. Epidemiología de anisákidos en sardinas (*Sardina pilchardus*)
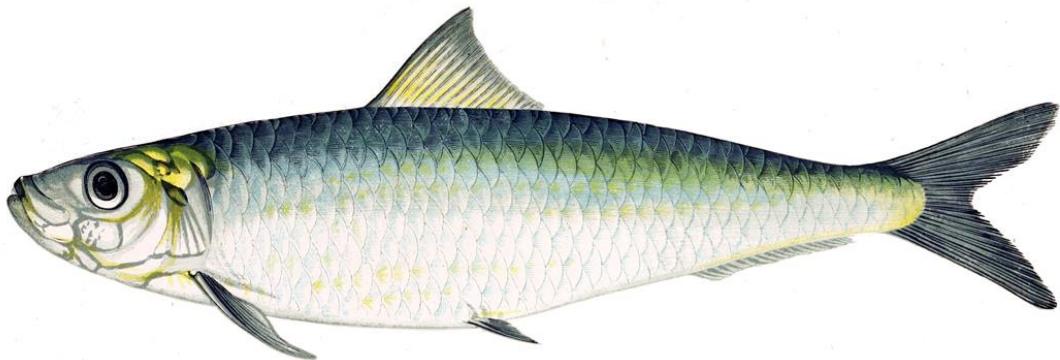


Figura 6. Ilustración de una sardina. Autores:  Gervais et Boulart - Les poisons (1877) (Gervais 1877).

La sardina (*Sardina pilchardus*) (Figura 5) es un pez teleósteo que pertenece a la familia Clupeidae. El género *Sardina* solamente tiene una especie, *Sardina pilchardus*, que se conoce como sardina común o sardina europea. Es una especie pelágica y oceanódroma, que suele vivir entre los 25 y los 100 metros de profundidad, aunque se puede encontrar entre los 10 y los 100 metros. De clima subtropical, se encuentra entre los 14º y los 68º Norte, y entre los 32º Oeste y 43º Este. Su longitud máxima es de 27,5 cm, aunque suele medir unos 20 cm. La edad máxima que se ha reportado en este pez es de 15 años (Fishbase.org). Es una especie litoral, que se alimenta principalmente de crustáceos del plancton, aunque también puede comer organismos de mayor tamaño.

Existen varios casos en los que se relaciona el consumo de sardinas con la anisakiasis. Se ha reportado al menos 4 casos de hipersensibilidad a *Anisakis* ligada al consumo de sardinas enlatadas (Audícana y Kennedy 2008), 2 casos de anisakiasis producidos por el consumo de sardinas marinadas (López- Vélez et al. 1992) y 2 casos de anisakiasis gastroalérgica provocados por el consumo de sardinas (López-Serrano et al. 2000).

La sardina es un pez que suele presentar niveles de prevalencia por *Anisakis* bajos o nulos, dependiendo del área de pesca, y menores que en otros peces capturados en la misma zona. Varios autores no encuentran parasitación por *Anisakis* en sardinas, aunque sí por otros anisákidos como *H. aduncum*, en varios puertos del Mar Mediterráneo como Barcelona, Tarragona, Castellón, Málaga, Almería o Adra. En otros

lugares, como Galicia, o en mercados de Zaragoza, Córdoba o Bilbao tampoco se ha reportado parasitación por *Anisakis* en este pescado (Pereira Bueno 1992; Viu et al. 1996; De la Torre Molina et al. 2000; Abollo et al. 2001). En otras ocasiones sí se ha descrito parasitación por *Anisakis* en sardinas, aunque la prevalencia suele ser baja. Por ejemplo, en mercados de Granada se encontró un 0,9% de prevalencia o un 10% en costas gallegas (Ruiz-Valero et al. 1992; Sanmartín et al. 1994). Los valores de prevalencia más altos se han encontrado en Cerdeña, con el 12%, o Portugal, con el 28,1% (Silva y Eiras 2003; Piras et al. 2014)

3.2. Desarrollo y morfología

3.2.1. Cultivo *in vitro* de anisákidos

El primer intento de cultivo *in vitro* de anisákidos se llevó a cabo en 1921 por Martin, con 2 larvas de *Porrocaecum* (probablemente *Pseudoterranova decipiens*) (Martin 1921), obtenidos de la musculatura del eperlano europeo (*Osmerus eperlanus*). Consiguió la muda a L4 (que llamó "preadultos") empleando un medio de cultivo compuesto por 100 g de una solución de HCl al 0,2% con 30 gotas de un extracto de cuajar pepsina y glicerina, y medio centímetro cúbico de trozos de pescado crudo.

Grainger, en 1959, realizó varias modificaciones del medio descrito por Martin (1921) para cultivar *Porrocaecum* (*Pseudoterranova*) y *Anisakis* obtenidos de la musculatura de bacalao (*Gadus callarias*) (Grainger 1959). Utilizó como base un medio compuesto por 0,4 ml de tampón $Na_2HPO_4$ 0,2 M y 19,6 ml de ácido cítrico 0,1 M, para lograr un pH de 2,2. Estudió la adición de varios componentes como pepsina, tripsina, trozos de pescado, así como varias temperaturas. Durante el cultivo no observó diferencias en el comportamiento entre *Pseudoterranova* y *Anisakis*. Consiguió la muda a L4 e indicó que la adición de trozos de pescado es esencial para que esta muda tenga lugar, además de una alta temperatura (37 ºC).

En 1963, Townsley y colaboradores consiguieron los primeros adultos de anisákidos *in vitro* (Townsley et al. 1963). Cultivaron *Terranova* (=*Pseudoterranova*) *decipiens* obtenidas de la musculatura de bacalao (*Gadus* sp.). Emplearon un medio de cultivo Medium 199 al que añadieron glucosa, extracto de embrión de ternera, extracto de hígado de ternera y antibióticos. De este modo consiguieron el desarrollo hasta adulto de las L3 de *Pseudoterranova*, en un tiempo que, según indican los autores, es aproximadamente es de unos 16 días, muy similar al que el que tardarían en alcanzar este estadio en el tracto digestivo de la foca, su hospedador definitivo (20 días) (Scott 1953).

Van Banning (1971) realizó cultivos *in vitro* con larvas de *Anisakis marina* (=*A. simplex s.l.*). Para el medio de cultivo empleó extracto de hígado a pH 2,0 y sangre de ternera. Consiguió adultos maduros, de hasta 15 cm. También logró la ovoposición de los adultos. Posteriormente, transfirió los huevos a agua de mar a una temperatura de entre 5-6º C, donde el desarrollo tuvo lugar entre 20 y 27 días. Las larvas que obtuvo de los huevos se mantuvieron vivas y móviles durante 6-7 semanas.

Tras estos primeros estudios de cultivo *in vitro* con larvas de *Anisakis*, varios autores comenzaron los cultivos partiendo de huevos obtenidos directamente de los úteros de las hembras. Por ejemplo, McClelland et al. en 1974 estudiaron el desarrollo de los huevos de *Contracaecum osculatum* de hembras obtenidas de focas (McClelland y Ronald 1974b). Estos huevos se incubaron en agua de mar, pero a diferencia de Van Banning (1971), las larvas obtenidas tras la eclosión fueron trasladadas a medio de cultivo compuesto de medio de Eagle (MEM) con 20% de suero bovino fetal, a 15º C. Más de la mitad de las larvas (58%) mudaron a L4 y y sólo cuatro de ellas a adultos (<0,05% de las L4), partiendo de unas 15.000 larvas. Los mismos autores obtuvieron huevos de *P. decipiens* a partir de hembras recogidas de focas y realizaron cultivos hasta el estadio de "preadulto" (nombre con el que denominaban al adulto inmaduro) que era morfológicamente similar a los preadultos obtenidos de las focas (McClelland y Ronald 1974a, 1970). Posteriormente se llevaron a cabo más estudios para dilucidar el ciclo de vida y los factores que afectaban a la muda o la formación de la cutícula (Sommerville y Davey 1976). También se estipuló que el $CO_2$ a una concentración del 5% beneficia el cultivo, permitiendo que más larvas realicen la muda a L4 y una mayor supervivencia de las mismas (Sommerville y Davey 1976). También se sugirió que las L3 no ingieren alimento hasta que no mudan a L4 (Sommerville y Davey 1976).

Grabda en 1976 realizó estudios *in vitro* de larvas de *A. simplex* obtenidas de arenques (Grabda 1976). Para ello empleó una modificación del medio de cultivo descrito por Van Banning (1971), compuesto por hígado fresco de cerdo, que licuaba en una solución de NaCl a los que añadía pepsina y ajustaba a pH 2,0 con HCl, y empleando L3, en 12-14 días tras la muda a L4, consiguió adultos que ovopositaron y de los huevos eclosionaron larvas que sobrevivieron un par de semanas.

En 1982, Threlfall cultivó larvas de *Anisakis* obtenidas de varios peces y calamares, con una modificación del medio de Van Banning (Threlfall, 1982). Este autor no consigue la maduración de las larvas de *Anisakis* de calamares, pero sí de peces, sobre todo de salmón. Encuentra que, a mayor tamaño inicial de las larvas, mejor maduran y mayores son como adultos, lo que a su vez está relacionado con el tamaño del pez hospedador. Hurst en 1984 realizó cultivos con el medio descrito por Grabda (1976). Obtuvo adultos de *Anisakis*, pero no de *Pseudoterranova*, donde debido al escaso número de muestras (146 larvas) solo consiguió que éstas mudaran hasta L4, con una supervivencia máxima de 12 días. En *Anisakis*, donde el número de muestra asciende a 147.000 larvas, la muda a L4 se produjo entre los 5 y 6 días tras el comienzo del cultivo, y a adultos entre los 16 y los 23 días. Likely et al. (1992) llevaron a cabo un cultivo con L3 de *C. osculatum* obtenidas de peces. En este estudio se empleó un sistema de cultivo con dos medios distintos, lo que se conoce como sistema de cultivo en dos pasos.

También empleó una atmósfera con 10% de $CO_2$ y mantuvo el cultivo a 35º C. De este modo, se consiguieron adultos maduros.

Køie y colaboradores, mediante sus observaciones y seguimiento del desarrollo larvario, permitieron analizar y comprender mejor el ciclo de vida de *Anisakis* y *Pseudoterranova* (Køie et al. 1995). Estos autores indican que la fase que sale del huevo es directamente la fase infectiva, es decir, el tercer estadio larvario, cubierta con la vaina de la L2, y no la L2 como se creía anteriormente (McClelland y Ronald 1974b; Measures y Hong 1995). La cutícula del segundo estadio larvario podría ayudar a la flotabilidad de la larva 3 en el caso de *A. simplex*, y en *Pseudoterranova*, la cola de la cutícula de la L2 es adherente y podría ayudar a que la larva se fije a los sustratos, al igual que ocurren con *Contracaecum* (Valles Vega 2014; Valles-Vega et al. 2017).

Estudios posteriores realizados en nuestro laboratorio trataron de desarrollar un medio de cultivo semi-definido, sencillo y eficaz para el cultivo *in vitro* de anisákidos. Se ensayaron diversos medios de cultivo (Iglesias et al. 1997) y finalmente se observó que el que ofrecía mejores resultados era el medio compuesto por RPMI 1640 con 20% de suero bovino fetal inactivado por calor, ajustado a pH 4,0 y con una atmósfera de 5% de $CO_2$. El cultivo se mantenía a 37º C y con renovación del medio 2 veces por semana para *A. simplex*. Posteriormente se descubrió que la pepsina era esencial para éxito del cultivo (Iglesias et al. 2001). Gracias a la adición de pepsina comercial al 1% en el medio descrito por Iglesias et al. (1997), se incrementaba la supervivencia de los parásitos y la cantidad de larvas que completaban la muda de L4 a adulto con éxito, obteniendo un 95,6% de adultos. Además, se trata de un medio de cultivo sencillo y fácilmente reproducible. Este medio de cultivo se utilizó con otro anisákido, *H. aducum*, siendo la primera vez que se conseguían adultos de este parásito *in vitro*. En este caso el cultivo se realizaba a 13º C, también con un 5% de $CO_2$, y con una supervivencia media de 96 días. El pH óptimo para el cultivo de este parásito es pH 4,0. Bajo estas condiciones, todas las larvas mudaban a L4 y más de dos tercios llegaban a adultos, aunque solo el 25-30% de las hembras ponían huevos. Cuando se añadía pepsina al 1%, todas las larvas llegaban a adultos y se producía la ovoposición en el 45% de las hembras. Además, la cantidad de huevos que liberaba cada hembra era 12 veces mayor que en el medio sin pepsina (Iglesias et al. 2002; Adroher et al. 2004).

### 3.2.2. Características morfológicas generales de los anisákidos

Los anisákidos son nematodos y lo por tanto su cuerpo es cilíndrico y delgado, sin segmentar. Son monoicos y presentan simetría bilateral. Todos los anisákidos tienen un sistema digestivo completo, compuesto por boca, esófago, intestino, recto y ano (Moller y Anders 1986). En los peces, el tercer estadio larvario se suele encontrar encapsulado en las vísceras y/o la musculatura, o moviéndose libremente por la cavidad visceral (Audícana et al. 2003)

### 3.2.3. Características morfológicas de las L3 del género *Anisakis*

Las larvas L3 son blanquecinas, alargadas y filiformes, con un diámetro menor a medida que nos acercamos a los extremos, terminando el posterior en punta. La longitud depende de la especie y del grado de desarrollo, pero en las L3 halladas en peces suelen medir entre 20 y 30 mm (Audícana et al. 2003). El rango máximo de medidas de las L3 halladas tanto en peces como en calamares puede oscilar entre 8 y 36 mm de longitud y entre 0,23 y 0,58 de grosor (Koyama 1969; Shiraki 1974; Fagerholm 1982; Smith 1983; Hurst 1984; Chai et al. 1986; Ishii et al. 1989; Pereira Bueno 1992; Iglesias et al. 1997)
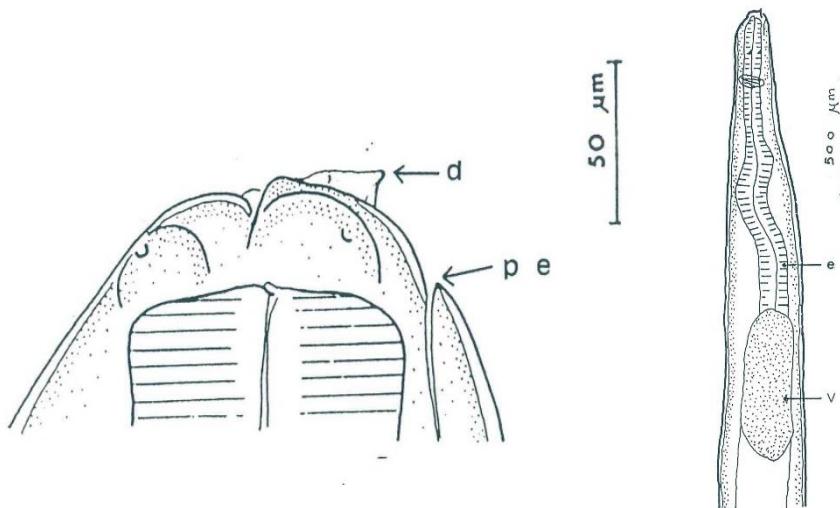


Figura 7. Extremo anterior de una L3 de *Anisakis simplex*. d: diente; p e: poro excretor; e: esófago; v: ventrículo. Tomada de Ruiz Valero (1991).

En el extremo anterior existe un diente triangular (Figura 7), que se emplea para penetrar en la mucosa digestiva del hospedador intermediario/paraténico (Matthews 1982) y una boca triangular rodeada de 3 prominencias labiales: una dorsal bilobulada y dos subventrales monolobuladas. Entre las bases de los labios subventrales encontramos un poro excretor (Grabda 1976) (Figura 7). Los labios presentan papilas alargadas cerca de sus bases: una papila en cada labio suventral y dos en el labio dorsal. Los labios son más prominentes en las L4 y en los adultos que en las L3. El diente solamente se encuentra en la L3 y desaparece cuando las larvas mudan a L4. El anillo nervioso se encuentra cerca del extremo anterior.

Cuando las L3 mudan a L4, aparecen unas crestas dentadas sobre los labios. Se trata de una única fila de estructuras dentadas triangulares, que se extienden a lo largo de la superficie de los labios, dirigidos hacia la apertura de la boca (Weerasooriya et al. 1986).

La longitud del esófago varía entre las distintas especies de *Anisakis*. Está compuesto de tres regiones: región preventricular (de tipo muscular), que es la más larga y está constituida por fibras musculares, zona de transición, y ventrículo, que es de tipo glandular (Buzzell y Sommerville 1985). A lo largo del esófago hay varias glándulas que abren a la luz del mismo. Hay una glándula dorsal en la región preventricular, una glándula subventral en la zona de transición, y dos glándulas, una dorsal y una subventral, a la altura del ventrículo. El ventrículo cambia de forma cuando la larva muda a L4, alargándose y plegándose en una sigmoide. El ventrículo de la L3 es visible a simple vista y se aprecia como una mancha alargada y opaca, cercana al extremo anterior (Figura 7).

El intestino ocupa la mayor parte de la longitud de la larva 3. La luz intestinal es trirradial (Audícana et al. 2003). Las L3 no presentan primordio genital, sí las L4. En las L4 desarrolladas se puede empezar a distinguir la vulva en el caso de las hembras. Los adultos presentan características sexuales más definidas, donde destaca la presencia de vulva en el caso de las hembras, y espícula y papilas caudales en el caso de los machos.
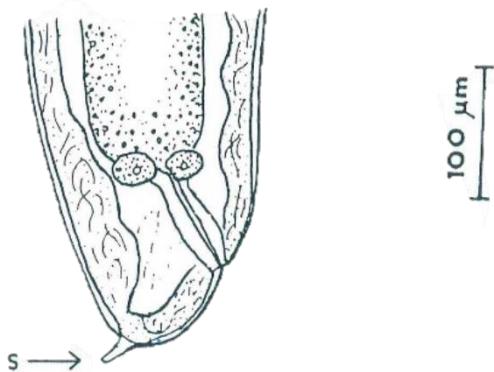
Figura 8. Extremo posterior de una L3 de *Anisakis simplex*. s: espina. Tomada de Ruiz Valero (1991).

El extremo posterior también varía según el tipo de L3 y la especie de *Anisakis*. En esta región se encuentra el recto, que termina en el ano. El ano se encuentra en posición ventral, próximo al extremo posterior, y el recto está rodeado por una glándula ventral y dos glándulas dorsales (Grabda 1976). La cola puede ser redondeada o alargada según la especie y puede presentar o no un mucrón o espina en el extremo de ésta (Berland 1961) (Figuras 8 y 9). El mucrón desaparece cuando la larva muda a L4. Los huevos de *Anisakis* miden unas 40 x 50 µm (Van Banning 1971; Grabda 1976). En los anisákidos de mamíferos marinos, como es *Anisakis*, el poro excretor se encuentra entre los labios subventrales. En los anisákidos de peces, como *Hysterothylacium*, el poro excretor se encuentra cerca del anillo nervioso.
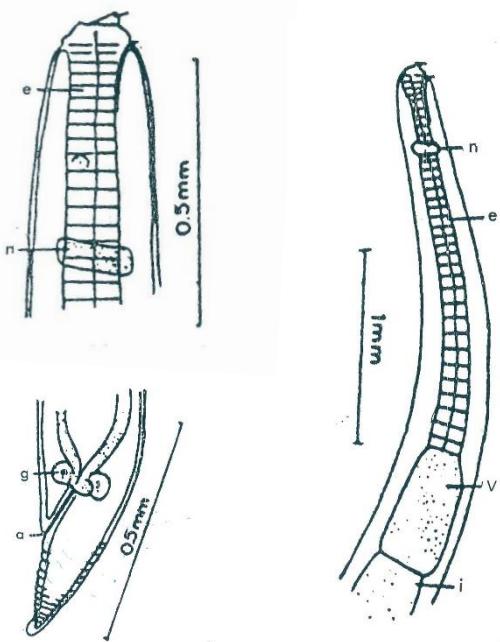
Figura 9. Extremos anterior y posterior de una L3 de *Anisakis physeteris*. e: esófago; n: anillo nervioso; g: glándulas anales; a: ano; n: anillo nervioso; V. ventrículo; i: intestino. Tomada de Ruiz Valero (1991).

Las larvas de *Anisakis* tipo I incluyen las siguientes especies: *A. simplex sensu stricto*, *A. pegreffii*, *A. berlandi*, *A. ziphidarum*, *A. typica* y *A. nascetti*. Las larvas pertenecientes a este tipo se diferencian por tener un ventrículo largo y su unión con el intestino es oblicua. El ventrículo mide entre 0,65 y 1,5 mm. La cola es corta, cónica, y finaliza en un mucrón o espina. Dentro de este tipo I existe un complejo de especies gemelas, *A. simplex s.l.*, compuesto por *A. simplex s.s.*, *A. pegreffii* y *A. berlandi* (Mattiucci et al. 2014). Destacan las dos primeras por su frecuencia y porque ambas son capaces de producir anisakiasis (Quiazon et al. 2011; Jeon y Kim 2015). Aunque estas especies no son distinguibles morfológicamente, un estudio llevado a cabo por Quiazón y colaboradores (Quiazon et al. 2008) indican que podrían diferenciarse por la longitud de su ventrículo, y esto sería válido tanto para las L3 como para las, L4 y adultos. Según se indica en el estudio, la longitud del ventrículo de *A. simplex s.s.* oscila entre 0,90 y 1,50 mm, mientras que el de *A. pegreffii* se encuentra entre los 0,50 y 0,78 mm. Las L4 y los adultos también podrían distinguirse por la relación de medidas entre la longitud del ventrículo y del esófago (Quiazon et al. 2008). Los adultos, además, serían distinguibles por la distribución de las papilas caudales (Quiazon et al. 2008).
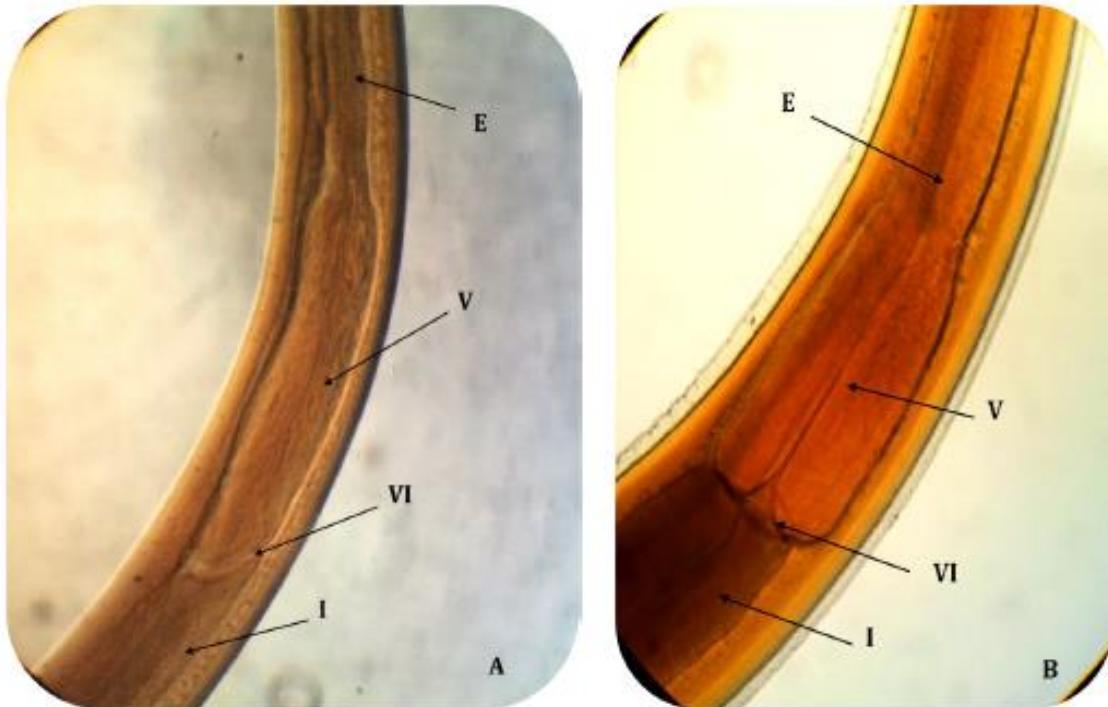
Figura 10. Características distintivas de *Anisakis* tipo I (A) y tipo II (B) en su extremo anterior. E: esófago; V: ventrículo; VI: unión ventrículo-intestino; I: intestino. Tomado de Romero López (2014).
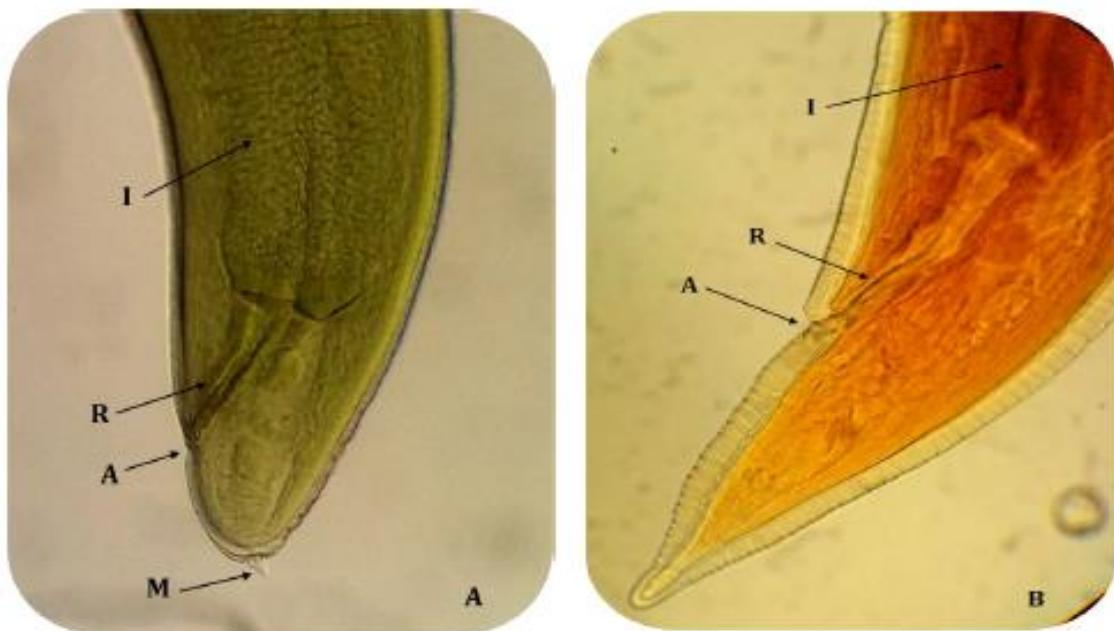


Figura 11. Características distintivas de *Anisakis* tipo I (A) y tipo II (B) en su extremo posterior. I: intestino; R: recto; A: ano; M: mucrón. Tomado de Romero López (2014).

Las L3 pertenecientes- al tipo II incluyen las especies *A. physeteris*, *A. paggiae* y *A. brevispiculata*. Se caracterizan porque el ventrículo tiene una longitud menor que en las larvas tipo I, entre 0,52 mm y 0,75 mm y su unión con el intestino es recta y no oblicua (Berland 1961; Koyama 1969) (Figuras 10 y 11). Las larvas suelen tener una coloración más rojiza en su parte anterior (Figura 10). La cola es larga, cónica y no termina en espina, aunque parece que algunas especies como *A. paggiae* presentan un pequeño lóbulo cónico terminal (Murata et al. 2011).

### 3.2.4. Características morfológicas de las L3 del género *Contracaecum*

La L3 es de color blanquecino, con el extremo posterior cónico. Es alargada y delgada. Las medidas de las L3 halladas en peces oscilan entre los 3,81 y 22,7 mm de longitud, y entre 0,07 y 0,53 mm de grosor (Koyama 1969; Fagerholm 1982; Moravec et al. 1985; Chai et al. 1986; Pereira Bueno 1992). En el extremo anterior presenta una boca triangular rodeada de 3 prominencias labiales y un diente cónico, que ayuda a la penetración de los tejidos del hospedador (Figura 12). Presenta 2 labios subventrales y un labio dorsal. El poro excretor es ventral y abre hacia al diente. Posee un anillo nervioso cerca del extremo anterior (Pereira Bueno 1992) (Figura 12).

El esófago está formado por una parte muscular y el ventrículo, que suele medir entre 0,03 y 0,11 mm (Koyama 1969; Chai et al. 1986), y es pequeño, corto y esférico. Tiene un apéndice ventricular posterior y marcado, cuya longitud se encuentra entre 0,57 y 1,13 mm (Koyama 1969; Chai et al. 1986). Encontramos también un ciego intestinal, que aparece dorsal al esófago, y es más pequeño que el apéndice ventricular, ya que suele medir entre 0,08 y 0,24 mm (Koyama 1969; c et al. 1986) (Figura 12). La presencia o ausencia de ciegos, tanto intestinal como esofágico, es una característica que permite distinguir las larvas de los distintos géneros de la familia *Anisakidae*.
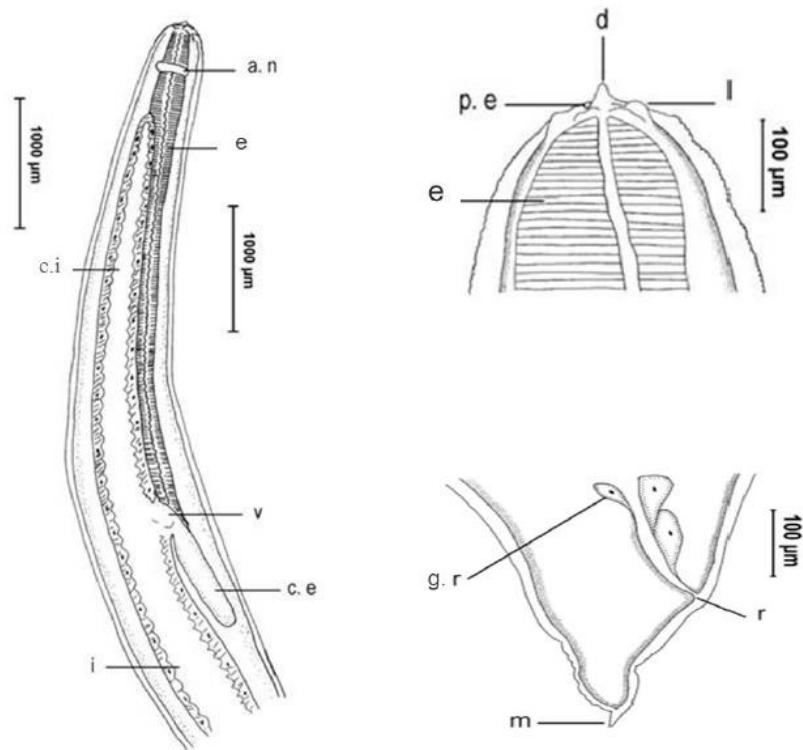
Figura 12. Esquemas de la L3 de *C. multipapillatum s.l.* d: diente cuticular; a.n.: anillo nervioso; l: labios; e: esófago; c.e.: ciego esofágico; c.i.: ciego intestinal; i: intestino; v: ventrículo; m: mucrón o espina; r: recto; g.r.: glándulas rectales. Tomado de Valles Vega (2014).

El extremo posterior de la L3 es cónico, la cola es corta, curvada ventralmente y no presenta mucrón. En el extremo posterior de los adultos, dorsalmente, los machos presentan la cutícula estriada, y ventralmente muestran numerosas papilas, con una distribución característica.

Las larvas del género *Contracaecum* son muy similares a las de *Hystherothylacium*, con la diferencia de que las primeras presentan el poro excretor en la base de los labios subventrales y las segundas cerca del anillo nervioso (Hartwich 1974).

3.3. Actividad proteolítica

3.3.1. Introducción

El término "proteasa" surgió a finales del siglo XIX en Alemania para referirse a enzimas proteolíticas. Se empleó como un término general que englobaba todas las hidrolasas que actuaban sobre proteínas, o degradaban fragmentos de ellas. Grassman y Dykerhoff propusieron el término "proteinasas" a aquellas que actuaban sobre proteínas, y "peptidasas" para las que actuaban sobre oligopéptidos (Grassmann y Dyckerhoff 1928). Más tarde Bergmann y Ross usaron el término "peptidasa" para referirse de una manera más general a hidrolasas que actúan sobre enlaces peptídicos, con la forma extendida de "endopeptidasa" o "exopeptidasa" según si actúan sobre enlaces internos de la cadena de aminoácidos (lejos de los extremos) o bien en los extremos amino o carboxilo terminales (Bergmann y Ross 1936). Esta es la nomenclatura que se recomienda por la IUBMB (Unión Internacional de Bioquímica y Biología Molecular).

El sistema EC (Enzyme Classification) es un esquema internacionalmente reconocido para la clasificación y nomenclatura de todas las enzimas. En este sistema las enzimas se dividen en 6 clases: oxidorreductasas, transferasas, hidrolasas, liasas, isomerasas y ligasas, ordenadas del grupo 1 al 6, con subgrupos. Las peptidasas, que son hidrolasas de enlaces peptídicos, pertenecen al grupo 3.4 según esta clasificación, ya que el grupo 3 engloba a todas las hidrolasas. En la mayoría de la lista EC, las enzimas se definen por el hecho de que catalizan una sola reacción. Esto conlleva diversas implicaciones, como que varias proteínas diferentes se pueden describir como la misma enzima, bajo el mismo código EC ya que catalizan la misma reacción, o a la inversa, una proteína que catalice varias reacciones se tratará como más de una enzima, con más de un código EC. Como ventaja, el nombre de una enzima que catalice una sola reacción se puede formar directamente a partir de la descripción dicha reacción. Sin embargo, las peptidasas no se han clasificado solamente basándose en la reacción que catalizan, como el resto de las enzimas. Esto se debe a que la reacción básica de todas las peptidasas es la misma: la hidrólisis de un enlace peptídico. La especificad de una peptidasa es muy difícil de determinar de una forma rigurosa y es casi imposible usar este dato para la formación de un código EC fiable. Por lo tanto, en 1972 se incluyó el tipo catalítico para un reconocimiento más fácil de las peptidasas (Florkin y Stotz, 1973). Es más útil poder considerar diferentes proteínas como diferentes peptidasas, incluso cuando expresan actividades similares o idénticas.

Dentro del grupo 3.4. las peptidasas se dividen en subclases: desde el 3.4.11 al 3.4.19 para las exopeptidasas, y del 3.4.21 al 3.4.25 junto con 3.4.99 para las endopeptidasas (Tabla 1). Las endopeptidasas se ordenan según su mecanismo catalítico, y las exopeptidasas según si actúan sobre la parte N-terminal o C-terminal de las proteínas. Las treonina peptidasas fueron las últimas en incorporarse a esta clasificación, bajo el código 3.4.25.

Grupo 3.4: Hidrolasas que actúan sobre enlaces peptídicos (peptidasas)

| | |
|---|---|
| **EC 3.4.11** | aminopeptidasas. |
| **EC 3.4.13** | dipeptidasas |
| **EC 3.4.14** | dipeptidil peptidasas y tripeptidil peptidasas |
| **EC 3.4.15** | peptidil dipeptidasas |
| **EC 3.4.16** | carboxipeptidasas tipo serina |
| **EC 3.4.17** | metalocarboxipeptidasas |
| **EC 3.4.18** | carboxipeptidasas tipo cisteína |
| **EC 3.4.19** | peptidasas omega |
| **EC 3.4.21** | serina endopeptidasas |
| **EC 3.4.22** | cisteína endopeptidasas |
| **EC 3.4.23** | aspártico endopeptidasas |
| **EC 3.4.24** | metaloendopeptidasas |
| **EC 3.4.25** | treonina endopeptidasas |
| **EC 3.4.99** | endopeptidasas con mecanismo catalítico desconocido |

Tabla 1. Sistema de clasificación EC. Fuente: Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), Enzyme Nomenclature. (http://www.sbcs.qmul.ac.uk/iubmb/enzyme/EC3/4/)

Este sistema de clasificación tiene las desventajas ya comentadas, y, al tener pocas subclases, agrupa numerosas peptidasas que son muy diferentes entre sí. Y lo más importante: no tiene en cuenta agrupaciones estructurales que podrían dar información sobre relaciones evolutivas entre las peptidasas. En 1992 Rawlings y Barret comenzaron a desarrollar una nueva forma de clasificación de las peptidasas, diseñada para agruparlas de una forma que reflejara sus características estructurales y su relación evolutiva. El sistema se describió en 1992 (Rawlings y Barrett 1993) y en 1996 se publicó bajo el nombre de base de datos MEROPS, nombre de un ave con un comportamiento social que inspiró a los autores para emplear el término "clan". Este sistema clasifica las enzimas proteolíticas en 3 niveles: clan, familia, peptidasa. Cada grupo comienza con una letra que indica el tipo catalítico: A (aspártico), C (cisteín), G (glutámicas), M (metalo), N (asparagina), P (mezcla), S (serín), T (treonin) y U (sin clasificar o desconocidas). El término P se refiere a clanes que contienen peptidasas de más de uno de los siguientes tipos: C, S y T. Un identificador MEROPS es único para cada peptidasa, comienza con el identificador de la familia y se completa con un número decimal (ejemplo: C14.001 es el código para la caspasa-1). Todos los identificadores son estables, solo se cambian bajo condiciones especiales y nunca se reutilizan. Además, la base de datos MEROPS es útil para conocer no solo las enzimas, sino también sus sustratos y sus inhibidores (Rawlings et al. 2012).

### 3.3.2. Clasificación de las proteasas según su mecanismo de acción

a) Aspártico proteasas

Son endopeptidasas; clasificación EC 3.4.23. Este grupo cataliza la escisión del enlace peptídico sin que haya un ataque nucleófilo por parte del grupo de funcional de la enzima. Por lo tanto, no se forma ningún intermediario covalente entre la enzima y un fragmento del sustrato, y la proteolisis ocurre en un solo paso ( Cunningham 1965; Beynon y Bond 2001). El sitio activo tiene dos cadenas laterales aspárticas muy conservadas. El pH óptimo para estas enzimas es ácido ya que el grupo carboxilo está implicado en la reacción. El mecanismo de acción consiste en una reacción ácido-base, con una molécula de agua entre los dos residuos aspárticos de la peptidasa (Cunningham 1965; Beynon y Bond 2001). Se consideran el grupo de peptidasas más conservado (Williamson et al. 2003).

b) Cisteín proteasas

Son endopeptidasas; clasificación EC 3.4.22. Su mecanismo de acción involucra un grupo cisteína que contiene un grupo tiol nucleófilo. En su centro activo poseen el residuo de cisteína que participa en una reacción de tipo covalente (Cunningham 1965). En este caso se forma un complejo intermedio enzima-sustrato a través del ataque del grupo sulfhidrilo (Beynon y Bond 2001). Su pH óptimo es entre 4,0 y 7,0. Dentro de este grupo se encuentra el clan CA (tipo papaína), que está ampliamente distribuido en los parásitos. Dispone de una tríada catalítica en el centro activo compuesta por tres residuos: cisteína, histidina y asparagina (Sajid and McKerrow 2002).

Un tipo de cisteín peptidasas son las catepsinas, que se definen como peptidasas de origen lisosómico que actúan preferentemente en medio ácido (Willstätter y Bamann 1929). La familia de la papaína incluye muchas catepsinas, como las catepsinas B y L. Éstas son las más estudiadas, al ser las más importantes en parásitos y las que más aparecen en nematodos (Sajid y McKerrow 2002).

c) Metalopeptidasas

Dentro de este grupo existen tanto endopeptidasas (EC 3.4.24) como exopeptidasas (EC 3.4.17). Poseen un ion metálico en su centro activo, frecuentemente un catión divalente de Zn, aunque también puede ser Co o Mn, entre otros. El metal está unido a la enzima mediante normalmente tres ligandos, que pueden ser histidina, glutamato, aspartato, lisina o arginina (Cunningham 1965). El catión polariza el enlace peptídico mediante el ataque nucleófilo de un grupo hidroxilo proveniente de una molécula de agua (Beynon y Bond 2001). Al igual que las peptidasas aspárticas, no forman un intermediario covalente. Las metaloproteasas son el grupo más diverso de proteasas, con más de 50 familias hasta la fecha.

d) Serín peptidasas

Endopeptidasas, EC 3.4.21. En su centro activo contienen un tríada formada por tres aminoácidos: serina, aspartato e histidina (Cunningham 1965; Beynon y Bond 2001). En este caso se forma un complejo intermedio entre la enzima y el sustrato de tipo covalente, a través del ataque del grupo hidroxilo de la serina, que es quien lleva a cabo el ataque nucleofílico (Cunningham 1965). La geometría particular de la tríada del centro activo determinará la función específica de cada serín proteasa (Beynon y Bond 2001). Se distinguen dos grandes grupos basados en su estructura: tipo tripsina y tipo subtilisina.

Según hemos visto, las principales peptidasas se pueden dividir en 2 grupos: aspártico y metalo proteasas, que forman complejos enzimáticos covalentes, y cisteín y serín proteasas, que no forman complejos covalentes. Por lo tanto, la estrategia de inhibición de estos dos grupos es completamente diferente. Las enzimas del primer grupo generalmente tienen aminoácidos muy nucleófilos en su centro activo, por lo que los inhibidores para este grupo deben tener grupos electrófilos (Beynon y Bond 2001). En el segundo grupo se cataliza la hidrólisis del enlace peptídico sin el ataque nucleófilo por parte de un grupo funcional de la enzima, por lo que requieren formas más sutiles de inhibición (Beynon y Bond 2001). Es necesario que se formen uniones secundarias a lo largo del centro activo. En el caso de las metaloproteasas, se pueden introducir ciertos grupos como el sulfhidrilo en puntos precisos para conseguir una unión casi irreversible mediante quelación.

### 3.3.3. Funciones de las proteasas en nematodos parásitos

Durante los últimos años, las proteasas de muchos parásitos han sido ampliamente estudiadas, ya que son importantes en la relación parásito-hospedador y se consideran factores de virulencia importantes en algunos parásitos. En muchos casos las proteasas son la principal fuente de antígenos de los parásitos, por lo que ocasionan una gran respuesta inmune en el hospedador. Además, son especialmente inmunogénicas lo que, además de propiciar alergia e hipersensibilidad, las hace muy útiles en el serodiagnóstico y en el desarrollo de vacunas (Todorova y Stoyanov 2000; Sallé et al. 2018).

A grandes rasgos, las proteasas en nematodos:

1. Son esenciales en la digestión de nutrientes de origen proteico. Esta es la función más obvia y generalizada, y permite a los nematodos asimilar y utilizar los nutrientes proteicos o la hemoglobina en el caso de los parásitos hematófagos (Williamson et al. 2003).

2. Intervienen en el desarrollo embrionario del huevo y posteriormente en la remodelación de los tejidos durante el desarrollo larvario (Britton y Murray 2002; Hashmi et al. 2002).

3. Participan en la muda y en la reabsorción de la cutícula de diversos modos: activando prohormonas o proenzimas (Hong et al. 1993), degradando las proteína que fijan la cutícula a la epidermis (McKerrow 1995), facilitando la reabsorción de las proteínas que componen la cutícula o el desprendimiento de la misma (Gamble et al. 1989).

4. Facilitan la penetración en los tejidos del hospedador hasta alcanzar el lugar donde se va a establecer el parásito (Morris y Sakanari 1994). Normalmente este es un proceso digestivo externo donde actúan proteasas excretadas o secretadas.

5. Protegen al parásito frente al sistema inmune del hospedador mediante la degradación de inmunoglobulinas unidas a antígenos de superficie (Hotez y Pritchard 1995).

### 3.3.4. Catepsinas

Las catepsinas son un tipo de proteasas de origen lisosómico, que actúa preferentemente en medio ácido. Son, en su mayoría, cisteín proteasas, aunque también hay algunas catepsinas que son aspártico- o serín- proteasas.

Es importante conocer estas catepsinas ya que están directamente involucradas en la patogenicidad de los parásitos y en invasión de los tejidos. Además, pueden ser clave para desarrollar nuevos tratamientos específicos y efectivos contra las enfermedades producidas por nematodos, y tienen potencial para actuar como posibles dianas en quimioterapia, diagnóstico y vacunas, ya que algunas catepsinas están altamente conservadas en nematodos (Britton y Murray 2002; Hashmi et al. 2002). Las catepsinas intervienen en la penetración de los tejidos del hospedador, evasión de la respuesta inmune, virulencia de los parásitos, y en otros procesos biológicos como la digestión y la muda (Britton y Murray 2002; Hashmi et al. 2002; Guiliano et al. 2004; Robinson et al. 2008; Sojka et al. 2016). A pesar de esto, las catepsinas de los muchos parásitos aún no han sido estudiadas en profundidad.

3.4.5. Funciones de las catepsinas B y L en nematodos parásitos

Las catepsinas B y L son las que más importantes en nematodos, así como las más estudiadas. Pertenecen a la familia de la papaína (EC 3.4.22.1. y EC 3.4.22.15, respectivamente). La principal función de las catepsinas B es la de digerir nutrientes de origen proteico. En nematodos parásitos, se ha encontrado una gran variabilidad interespecífica, en función del pH, la temperatura, la afinidad por el sustrato… Esta gran variabilidad puede estar relacionada con la adaptación que sufre cada parásito en su nicho ecológico (Rehman y Jasmer 1999). Por ejemplo, en *Ancylostoma caninum*, se ha descubierto una catepsina B localizada en el intestino que tiene actividad hemoglobinolítica, y que actúa después de una catepsina D, formando parte ambas de la cascada enzimática que digiere la hemoglobina en este nematodo (Williamson et al. 2004).

También se ha encontrado la misma catepsina B en el tubo digestivo tanto de la larva como del adulto de *C. elegans*, y en el intestino de *Haemonchus contortus* (Pratt et al. 1992; Ray y McKerrow 1992; Britton et al. 1998). Esta catepsina está relacionada con la digestión de nutrientes y se ha demostrado que es un tipo de proteasa muy conservada en nematodos tanto parásitos como de vida libre (Pratt et al. 1992; Ray y McKerrow 1992). También se ha sugerido que las catepsinas B tienen un papel en el desarrollo embrionario y larvario, ya que algunas de estas catepsinas se expresan en la larva infectiva de *A. caninum* cuando aún está en el huevo. En otro parásito, *Angiostrongylus cantonensis*, se ha descrito una catepsina B en los estadios larvarios que causan una disfunción en la barrera hematoencefálica. Por lo tanto, se ha estipulado que puede tener un papel en la invasión del sistema nervioso central y en la inmunorregulación que ocurre en la interacción parásito-hospedador (Han et al. 2011).

Las catepsinas L son menos abundantes que las B en cuanto a su expresión, y parece que están implicadas en procesos más diversos. Entre estos procesos se encuentra la digestión de nutrientes, pero también los procesos de embriogénesis y muda, y otros aspectos relacionados con la patogenicidad como la invasión de los tejidos y la evasión de la respuesta inmune del hospedador (Koiwa et al. 2000; Britton y Murray 2002; Hashmi et al. 2002).

Muchas de estas catepsinas L tienen homólogas en el nematodo de vida libre *C. elegans*, por lo que puede que estén relacionadas con funciones conservadas en nematodos relacionadas con aspectos biológicos. Por ejemplo, se han comparado una serie de catepsinas L de parásitos y de otros nematodos de vida libre, llegando a la conclusión de que éstas estaban relacionadas con el remodelado de la cutícula y de la cubierta del huevo (Lustigman et al. 1996; Hashmi et al. 2002; Guiliano et al. 2004). Se han descrito otras catepsinas L que intervienen en la muda de L3 a L4 (Lustigman et al. 1996), en la embriogénesis (Britton y Murray 2002), o como activadores de otras enzimas y hormonas que están implicadas en la muda (Hashmi et al. 2002). Una cisteín proteasa de tipo catepsina L parece tener un papel muy importante en la muda a L4 (Barker y Rees 1990).

En otro anisákido, *H. aduncum*, se estudió la actividad de ambas catepsinas a lo largo del desarrollo (Malagón et al. 2010). Se observó que las catepsinas L tenían una actividad más alta a pH ácido, y las catepsinas B a pH básico, lo que podría indicar, según los autores, que la primera tiene un papel en los procesos digestivos, mientras que la segunda podría estar involucrada en la renovación de la cutícula y otras funciones.

# 4.
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# 5.
# RESULTADOS

# Fishing area and fish size as risk factors of *Anisakis* infection in sardines (*Sardina pilchardus*) from Iberian waters, southwestern Europe

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## abstract

The sardine (*Sardina pilchardus*) is a fish commonly consumed and appreciated in many countries, although they are more likely to be eaten fresh in western Mediterranean countries such as Spain, Portugal, France or Italy. A molecular epidemiological survey of sardines from 5 fishing areas of the Spanish Mediterranean (Málaga, southern Spain) and Atlantic coasts (southern: Cádiz and Isla Cristina; northern: A Coruña and Ondarroa) was carried out to determine the presence of *Anisakis* spp. larvae. The highest prevalence of these larvae was observed in fish from A Coruña (28.3%), followed by Ondarroa (5%) and Cádiz (2.5%). No *Anisakis* larvae were found in fish from Málaga and Isla Cristina. Three *Anisakis* genotypes were identified: *Anisakis simplex sensu stricto*, *Anisakis pegreffii* and a hybrid genotype between these two species. *A. pegreffii* was the most prevalent species in A Coruña (71% of larvae). Only three *Anisakis* larvae (9% collected larvae) were located in the musculature of sardines: two were identified as *A. pegreffii* while the other was a hybrid genotype. Sardine infection was associated with fishing area and fish length/weight (length and weight were strongly correlated; Pearson's correlation 0.82; $p < 0.001$). Risk factor multivariate analysis showed that the risk of infection increases 1.6 times for every additional cm in the length of the sardines from the same fishing area. Comparison of fish of equal length showed that in sardines from A Coruña the risk of parasitization is 11.5 times higher than in those from other fishing areas. Although the risk of infection by *Anisakis* through consumption of sardines is generally low due to the low epidemiological parameter values (prevalence 10%, mean intensity 1.7 (range 1–5) and mean abundance 0.17), as larger fish are more heavily parasitized, there is an increased risk of infection by *Anisakis* through consumption of large sardines which are raw or have undergone insufficient treatment (undercooked, smoked, marinated, salted, pickled, freezing,…).

## 1. Introduction

The sardine (*Sardina pilchardus*) is a littoral fish which feeds mainly on planktonic crustaceans, appendicularians, diatoms and other organisms (Costalago and Palomera, 2014). This fish is marketed fresh, frozen or canned. It is also consumed dried or salted and smoked or marinated and can be pan-fried, broiled and microwaved. Sardines can harbor parasites such as *Anisakis*, which are transmitted to humans.

*Anisakis* spp. are nematodes which can parasitize a wide range of marine animals. The third larval stage (L3) of this parasite is the etiological agent of human anisakiasis, a disease that causes gastric and intestinal illness and/or allergic reactions. The larvae of *Anisakis*, dead or alive, are also considered to cause food allergy, although this is currently under discussion (Audícana and Kennedy, 2008; Daschner et al., 2012). Reports of cases of anisakiasis are increasing globally. The majority of cases have been reported in Japan, where consumption of raw fish is extremely common.

The life cycle of *Anisakis* is complex and involves a large number of host species. Larvae of *Anisakis* have been reported in numerous invertebrate hosts, mainly crustaceans, which can act as intermediate hosts. L3 of this parasite have been found in a wide range of fish and cephalopods, which are intermediate/paratenic hosts. *Anisakis* parasitization has been reported in more than 200 fish and 25 cephalopod species (Abollo et al., 2001; Klimpel et al., 2004). Cetaceans (final hosts) harbor the adult stage of this nematode. Humans can become accidental hosts, by eating raw, marinated or undercooked fish containing the L3 of these parasites that have not been inactivated during preparatory procedures.

*Anisakis* type I larvae have been categorized into six species: *Anisakis simplex sensu stricto*, *Anisakis pegreffii*, *Anisakis* berlandi, *Anisakis* typica, *Anisakis* ziphidarum and *Anisakis* nascetti (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009, 2014). Although several species have been found to parasitize fish and cephalopods in Japan, human anisakiasis is caused almost exclusively by *A. simplex s.s.* larvae in this country (Arizono et al., 2012; Umehara et al., 2007). Eleven clinical cases attributed to *A. pegreffii* have been reported in Italy, where this species is the dominant in Italian waters whereas none due to *A. simplex s.s.* have been described to date in this area (D'Amelio et al.,1999; Fumarola et al., 2009; Mattiucci et al., 2012). In Spain, since 1991, when Arenal Vera et al. (1991) described the first case of anisakiasis, hundreds of new cases of *Anisakis* infection and *Anisakis* allergy have been described, although no cases have yet been diagnosed molecularly. Several studies conducted in Spain in healthy individuals have revealed high seroprevalence (12–22%) to *Anisakis* related to the high consumption of fish in the Spanish population (Del Rey Moreno et al., 2006; Fernández de Corres et al., 2001; Puente et al., 2008). Among the cases reported, at least 4 of hypersensitivity to *Anisakis* have been linked to the consumption of either fresh or canned sardines (Audícana and Kennedy, 2008), and 3 of human anisakiasis to consumption of marinated sardines (Barros et al., 1992; López-Vélez et al., 1992). Two sibling species, *A. simplex s.s.* and *A. pegreffii*, are sympatric off the Atlantic coasts of the Iberian Peninsula and in the Alborán Sea (Martín-Sánchez et al., 2005; see Mattiucci and Nascetti, 2008 for references). Previous studies showed high parasitization in several species of fish by these two parasites (Abollo et al., 2001) but little or no parasitization in sardines from Spanish coasts and fishmarkets (see Fig. 1 and Table 1) (Abollo et al., 2001; De

la Torre Molina et al., 2000; Gutiérrez-Galindo et al., 2010; Rello et al., 2008; Ruiz-Valero et al., 1992; Viu et al., 1996). However, Silva and Eiras (2003) recorded 28.1% prevalence of *Anisakis* sp. in 57 sardines from the west coast of Portugal, although the mean intensity was low. Romero et al. (2013), working with rats, defined the pathogenic potential of the *Anisakis* larva as its capacity to cause lesions, attach itself to the gastric or intestinal wall, or penetrate them to reach the abdominal cavity. The results obtained by these authors show that *A. simplex s.s.* is more pathogenic than *A. pegreffii*. Due to these differences in the pathogenic potential and the importance of the sardine in the cuisine and economy of the Western Mediterranean, it is useful to carry out a survey to identify the species infecting this host and the risk factors of *Anisakis* infection in sardines from Spanish waters. Fish consumption is high in Spain (Welch et al., 2002) with the fishing fleets of Spain and Portugal landing over 77,000 tons of sardines in 2013 only from the Atlantic Iberian waters, IXa and VIIIc ICES subareas (ICES, 2014).

## 2. Material and methods

2.1. Host and parasites

A total of 190 sardines (*S. pilchardus* Walbaum, 1792; family Clupeidae) from 5 Spanish ports on the Mediterranean (port of Málaga) and Atlantic coasts (Southern Spain: Cádiz and Isla Cristina; Northern Spain: A Coruña and Ondarroa) were surveyed between October 2011 and November 2012 (Fig. 1). After measuring the total length and weight, the fish were dissected to harvest the larvae. The "condition factor" of the fish (CF) was calculated using the formula CF = $100 \times W / L^3$, where W = total weight (g) and L = total length (cm). This CF is considered as an indicator of general fish health. The viscera and the muscle were each subjected to a pepsin digestion (pH 2), as described by Huss and Drewes (1989), at 37 °C for 2 h for the former and 6 h for the later. All larvae morphologically identified (Berland, 1961; Petter and Maillard, 1988) as *Anisakis* L3 type I sensu Berland (1961), were individually preserved in Eppendorf tubes at −20 °C for genetic identification studies.
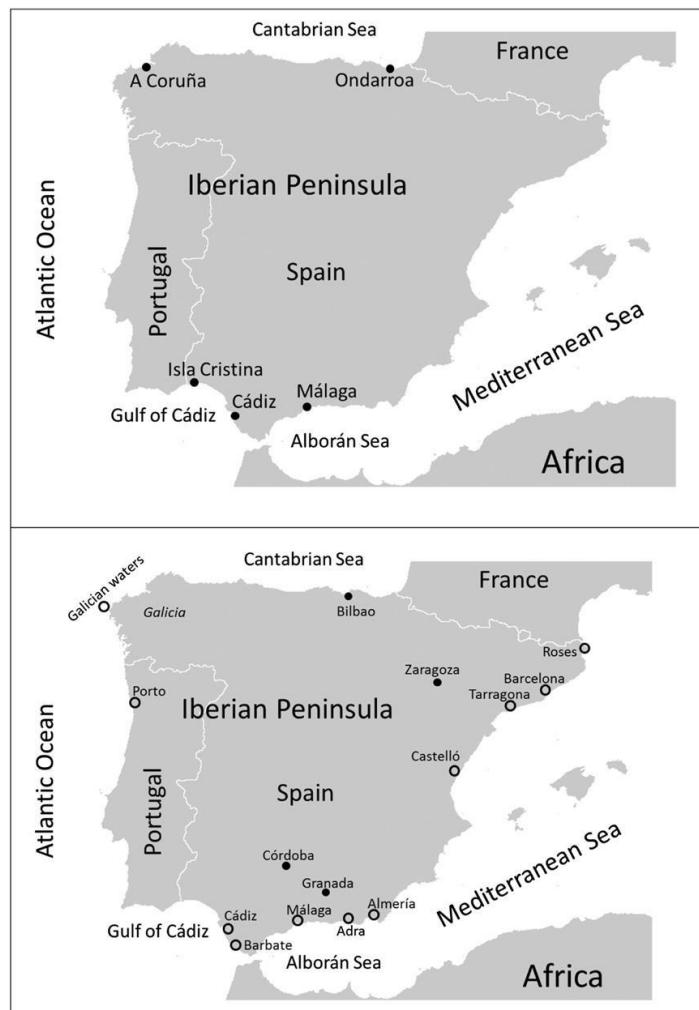
Fig. 1. Maps of Iberian Peninsula. (Top) area of investigation showing sampling ports from south and north coasts. (Bottom) ports (○) and fishmarkets (●) in which the presence of *Anisakis* in sardines has been previously surveyed.

Table 1

*Anisakis* parasitization in sardines *Sardina pilchardus*. Published surveys.

| References | Sardines analyzed | Origin | Sardines parasitized (prevalence of *Anisakis* spp.) | Mean intensity (range) |
|---|---|---|---|---|
| Petter (1969) | 400 | NE Atlantic Ocean: Le Croisic, Region Nantaise,(Western France) | 0 | – |
| Carvalho-Varela and Cunha-Ferreira (1984) | 310 | Portuguese waters | 0 | – |
| Petter and Maillard (1988) | ? | Castiglione, Algeria | Yes | ? |
| Huang (1988) | 22 | Paris — Rungis fishmarket (Northern France) | 1 (4.5%) | 1 (1) |
| Cuéllar et al. (1991) | ? | Castelló waters (Eastern Spain) | 0 | – |
| Ruiz-Valero et al. (1992) | 310 | Granada fishmarket (Southern Spain) | 3 (0.9%) | 1.3 (1–2) |
| Pereira Bueno (1992) | 44 | Bilbao fishmarket (Northern Spain) | 0 | – |
| Sanmartín et al. (1994) | 20 | Galician coasts (NW Spain) | 2 (10%) | 1 (1) |
| Viu et al. (1996) | 204 | Zaragoza fishmarket (NE Spain) | 0 | – |
| De la Torre Molina et al. (2000) | 294 | Northern area of Córdoba province fishmarkets (Southern Spain) | 0 | – |
| Abollo et al. (2001) | 50 | Galician coasts (NW Spain) | 0 | – |
| Silva and Eiras (2003) | 57 | West Portuguese coasts-Porto waters | 16 (28.1%)[a] | |
| Fioravanti et al. (2006) | 1323 | Adriatic Sea | 2 (0.1%) | 1 (1) |
| Karl (2008) | 100 | South of Great Britain | 50 (50%)[a] | 6.3 (?) |
| Rello et al. (2008) | 350 | Mediterranean Spanish coasts: Roses, Barcelona, Tarragona, Castelló, Almería, Adra, Málaga. Atlantic south Spanish coasts: Barbate, Cádiz. | 0 | – |
| Kijewska et al. (2009) | 11 | Northwest African shelf between Morocco and Mauritania | 0 | – |
| Gutiérrez-Galindo et al. (2010) | 160 | Tarragona waters (East Spain) | 0 | – |
| Angelucci et al. (2011) | 5 | Sardinian waters | 1 (20.0%) | 1 (1) |
| Chaligiannis et al. (2012) | 36 | Southern Aegean Sea | 2 (5.5%) | 1 (1) |
| Fioravanti et al. (2012) | 2636: | Mediterranean Sea: | 5 (0.2%) | ~1 (?) |
| | 1591 | NW Adriatic | 3 (0.2%) | |
| | 1045 | Ligurian coasts | 2 (0.2%) | |
| Cavallero and D'Amelio (2012) | 93 | Fishmarkets in central Italy | 1 (1.1%) | 3 (3) |
| Mladineo and Poljak (2014) | 120 | Croatian coast, Adriatic Sea | 4 (3.3%) | 1.25 (1–5) |
| Piras et al. (2014) | 252 | Gulf of Asinara, Northern Sardinia, Mediterranean Sea | 33 (13.1%)[a] | 1.2 (1–3) |
| Serracca et al. (2014) | 750 | Ligurian Sea coast (NW Italia) | 0 | – |
| This report | 190: | Spanish coasts: | 19 (10%) | 1.74 (1–5) |
| | 60 | A Coruña (NW) | 17 (28.3%)[a] | 1.82 (1–5) |
| | 20 | Ondarroa (N) | 1 (5%) | 1 (1) |
| | 40 | Cádiz (S) | 1 (2.5%) | 1 (1) |
| | 30 | Isla Cristina (S) | 0 | – |
| | 40 | Málaga (S) | 0 | – |

[a]Presence of larvae in muscle of sardines is described; prevalence in muscle: 2.8% (Piras et al., 2014); 3.3% A Coruña and total this survey 1.1%; 10.7% (Silva and Eiras, 2003); 0.1% of all larvae in flesh, prevalence ~1% (Karl, 2008).

## 2.2. Genetic identification of the parasites

For the genetic identification study of the larvae, polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) of the ribosomal fragment ITS1-5,8S-ITS2 was carried out. Realpure Kit was employed to extract the genomic DNA of every larva. PCR amplification primers NC5 (forward): 5' GTAGGTGAACCTGCGGAA GGATCATT 3', and NC2 (reverse) 5' TTAGTTTCTTTTCCTCCGCT 3', described by Zhu et al. (1998), were employed. Amplifications were carried out with the following programming: one cycle of 94 °C for 5 min, 60 °C for 60 s, 72 °C for 90 s; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s; and one final cycle of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 5 min, and then cooled and kept at 4 °C until use. The expected size of the amplified fragment was 1000 bp. PCR products were run on gels prior to digestion to verify the success of the amplification process. As controls, we used 2 specimens previously identified by this same technique as *A. pegreffii* and another 2 identified as *A. simplex s.s.* (Martín-Sánchez et al., 2005). RFLP was performed independently with two restriction enzymes, TaqI (5'…T↓CGA…3') and HinfI (5'…G ↓ANT…3') Fast Digest (Thermo Scientific) at 65 °C and 37 °C for 10 min, respectively, using a final enzyme concentration of 0.5 U/µl. The results were visualized through electrophoresis in 3% agarose gel, which permitted the sibling species of *A. simplex* complex to be identified according to the band pattern. In *A. simplex s.s.* controls, digestion with HinfI enzyme produced two fragments of 620 and 250 bp as well as a weaker one of 100 bp; TaqI endonuclease provided three fragments: one of 430 bp, one of 400 bp and a weak one of 100 bp. The *A. pegreffii* controls presented a pattern of three bands of 370, 300 and 250 bp for HinfI enzyme and three of 400, 320 and 150 bp for TaqI enzyme. For hybrid individuals, PCR-RFLP band pattern with the two restriction enzymes, HinfI and TaqI, is the sum of the patterns generated for *A. simplex s.s.* and *A. pegreffii*.

## 2.3. Epidemiological parameters and statistical analysis

The epidemiological parameters such as prevalence, mean abundance and mean intensity of *Anisakis* infection in sardines, were calculated as defined by Bush et al. (1997), i.e., "prevalence is the number of hosts infected with 1 or more individuals of a particular parasite species (or taxonomic group) divided by the number of hosts examined for that parasite species"; "mean intensity is the average intensity of a particular species of parasite among the infected members of a particular host species"; and "mean abundance is the total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined". Differences between prevalence values were evaluated by using the chi-square test or Fisher's exact test, with 95% confidence intervals being determined when possible while a bootstrap 2-sample t-test was used to compare mean intensities and mean abundances. These analyses were performed using free Quantitative Parasitology 3.0 computer software developed by Reiczigel and Rózsa (2005) to address the notoriously left-biased frequency distributions of parasites, based on the theoretical background published by Rózsa et al. (2000).

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2.4. Analysis of risk factors of sardine infection

For analysis of risks, the following variables of the sardines were studied as potential risk factors for infection by *Anisakis*: length, weight, sex, condition factor, Atlantic/Mediterranean origin, fishing area (port where landed) and catch month. A univariate model considering parasitization as the dependent variable, and the above factors as independent variables was developed. Next, a multivariate model selecting variables according to the statistical significance of their association with parasitization was developed. SPSS 20.0 was used for the data analysis.

**3. Results**

3.1. Host

The mean length ± SD (standard deviation) of sardines was 20.9 ± 1.4 cm (n = 190). The mean weight ± SD was 81.0 ± 21.4 g (n = 190) (Table 2). The relationship between weight and length shows a potential line with exponent around 3 (W = 0.0065·L$^{3.094}$; coefficient ± 0.0025 and exponent ±0.128; R$^2$ = 0.7577), thus demonstrating a relationship generally accepted in the literature as cubic (Fig. 2; Fulton, 1904; see Nash et al., 2006). In this sense, total length and weight were strongly correlated (Pearson's correlation 0.82; p < 0.001). The mean CF ± SD per fish was 0.87 ± 0.12 (n = 190) and per fishing areas was 0.86 ± 0.06 (n = 5) (Table 2).

Nineteen of the 190 sardines analyzed were infected (10%) and 33 *Anisakis* larvae found, all of which were identified morphologically as third larval stage (L3) type I sensu Berland (1961). The highest prevalence was found in fish from A Coruña (28.3%), followed by Ondarroa (5%) and Cádiz (2.5%). No parasitization was found in sardines from Málaga and Isla Cristina. Three larvae (9%) were isolated from the muscle of two fish, both from A Coruña. One of these sardines hosted four L3, two in viscera and two in musculature. The remaining larvae were found in the abdominal cavity, free or encapsulated on viscera of the hosts (91% of larvae). A mean intensity of 1.8 (range 1–5) and mean abundance of 0.5 were calculated in fish from A Coruña (Table 2). A relationship between intensity and fish length is depicted in Fig. 3. The mean intensity was 1.7 (range 1–5) and the mean abundance was 0.17 in all the fish examined in this survey.
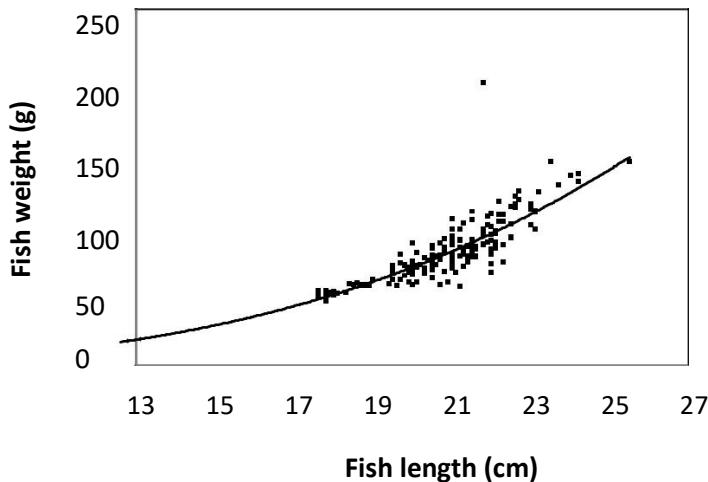
Fig. 2. Relationship between length and weight in sardines surveyed. The potential relationship is $0.0065x^{3.094}$ with $R^2$ = 0.7577.

### 3.2. Genetic identification of *Anisakis* larvae type I by PCR-RFLP

All thirty-three larvae isolated from sardines morphologically identified as *Anisakis* larva type I sensu Berland (1961) were further classified by genetic markers: 21% (7/33) were identified as *A. simplex s.s.*, 70% (23/33) as *A. pegreffii* and 9% (3/33) showed a hybrid PCR-RFLP band pattern with the two restriction enzymes used (TaqI and HinfI). Two of the three *Anisakis* larvae type I isolated from the muscle were identified as *A. pegreffii*; the other was identified as a hybrid genotype. The sole larva collected from Ondarroa was *A. simplex s.s.* and that from Cádiz was *A. pegreffii*.

### 3.3. Molecular epidemiological parameters

The epidemiological parameters, following molecular diagnosis of the L3 of *Anisakis* spp. collected from sardines landed in A Coruña, are shown in Table 3. *A. pegreffii* was 3.5-fold more prevalent than *A. simplex s.s.* (p < 0.02) in an area considered sympatric for these species while the mean abundance of *A. pegreffii* was 3.7-fold that of *A. simplex s.s.* (p = 0.05). However, the mean intensities were similar (p = 0.91). Hybrids of these two species showed similar epidemiological parameters to *A. simplex s.s.* (Table 3).

Table 2: Epidemiological parameters of sardines parasitized by *Anisakis* spp. from the surveyed areas of Iberian waters.

| | West Mediterranean Sea | North-East Atlantic Ocean | | | |
|---|---|---|---|---|---|
| | South Spain | | | NW Spain | North Spain |
| | Málaga | Cádiz | Isla Cristina | A Coruña | Ondarroa |
| No. of fish | 40 | 40 | 30 | 60 | 20 |
| Mean weight ± SD | 68.4 ± 17.7 | 76.1 ± 11.6 | 74.8 ± 7.4 | 99.6 ± 17.7 | 69.9 ± 8.9 |
| (Range) | (45.6–101.3) | (55.8–98.9) | (58.5–92.4) | (63.3–198.3) | (56.1–94.7) |
| Mean length ± SD | 19.7 ± 1.7 | 21.3 ± 0.8 | 20.4 ± 0.8 | 21.9 ± 1.2 | 20.3 ± 0.9 |
| (Range) | (17.6–22.3) | (19.4–23.0) | (19.5–22.0) | (19.8–25.5) | (18.9–22.1) |
| Condition factor ± SD | 0.87 ± 0.07 | 0.78 ± 0.07 | 0.85 ± 0.08 | 0.95 ± 0.15 | 0.83 ± 0.04 |
| (Range) | (0.66–1.04) | (0.59–0.94) | (0.70–1.02) | (0.77–1.91) | (0.76–0.88) |
| Prevalence (%) | 0 | 2.5 | 0 | 28.3 | 5 |
| Mean intensity | – | 1 | – | 1.82 | 1 |
| Mean abundance | – | 0.025 | – | 0.52 | 0.05 |

Weight in g; length in cm. SD = standard deviation. Prevalence = 100·N/F, mean intensity = A/N, mean abundance = A/F; where F is the total number of fish, N is the number of infected fish, and A is the number of larvae.

## 3.4. Analysis of risk factors of sardine infection

Total length, total weight and fishing area have shown their association with parasitization in both univariate and multivariate models. Lack of association has been found with catch month and Atlantic/Mediterranean origin. Sex and CF of the sardine show an association with parasitization in a univariate model but a lack of association in a multivariate model.
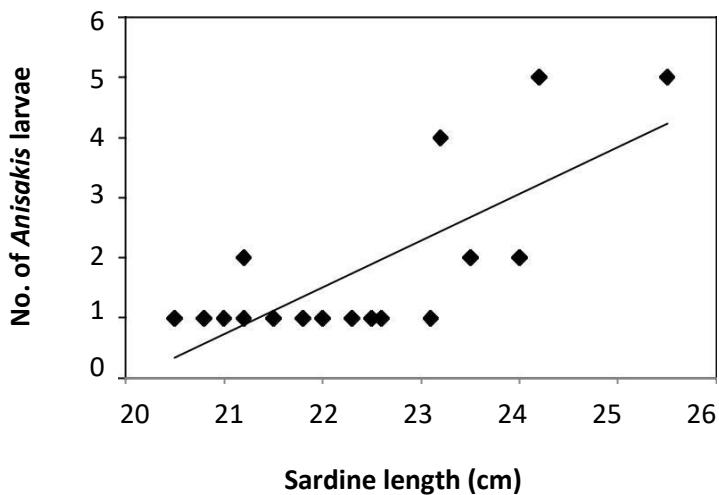
Fig. 3. Intensity of *Anisakis* infection in sardines from A Coruña according their length. The linear relationship is y = 0.7767x − 15.58 with $R^2$ = 0.5602.

### 3.4.1. Univariate models

Using the port of Cádiz as the reference for fishing area, sardines from Málaga, Isla Cristina and Ondarroa have the same risk of parasitization as those from Cádiz (p = 0.62). However, sardines from A Coruña have a risk of infection 15 times higher than those from Cádiz (p < 0.01). Furthermore, statistical association between fish total length/weight and parasitization by *Anisakis* have been demonstrated in this analysis. The risk of infection is multiplied by 2.4 for every additional cm in the length of the sardine. Sardines over 22.5 cm have a risk of parasitization 14 times higher than those under 21.0 cm (p < 0.01). The risk of infection increases 6.6% for every additional gram of fish weight. The risk of infection by *Anisakis* is 13 times higher in sardines over 110 g than in those under 90 g (p < 0.01).

### 3.4.2. Multivariate models

Total length and weight were strongly correlated as indicated above. Consequently, one of them was excluded from the multivariate model, total length being selected to display this model. In fish of equal length, sardines from A Coruña have a risk of parasitization 11.5 times higher than those from Cádiz (OR = 11.5; p = 0.02). Within the same fishing area, the risk of parasitization is multiplied by 1.6 for every additional cm in fish length (OR = 1.6; p ≤ 0.05).

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## 4. Discussion

As sardines are widely caught and consumed in Spain it is useful to know the prevalence of infection by *Anisakis* in this host and to estimate the risk of human anisakiasis due to the consumption of this fish (Table 1). At least three human anisakiasis cases and four *Anisakis* allergy cases associated with consumption of sardines have been described in Spain (Barros et al., 1992; López-Vélez et al., 1992; Audícana and Kennedy, 2008). No parasitization in sardines (*S. pilchardus*) from Spanish Mediterranean coasts has been found, in agreement with other authors who have also reported no parasitization by *Anisakis* in sardines from this area (Cuéllar et al., 1991; Gutiérrez-Galindo et al., 2010; Rello et al., 2008). Several authors have also reported absence of *Anisakis* infection in sardines from Spanish coasts (Abollo et al., 2001; De la Torre Molina et al., 2000; Pereira Bueno, 1992; Viu et al., 1996). Sardines of the Western Mediterranean area, when parasitized, generally show a low prevalence of less than 5% (Ruiz-Valero et al., 1992; see Table 1), except in Sardinian waters (Piras et al., 2014). However, NE Atlantic surveys show a generally higher prevalence, such as occurs in sardines from the coast of NW Portugal with 28.1% prevalence (Silva and Eiras, 2003) and 10.7% larvae found in the muscle. In our study, 28.3% prevalence was found in sardines from A Coruña, and 9.1% of the total number of isolated larvae were found in the muscle, similar data to those from Portuguese waters, probably due to the proximity between the surveyed areas from NW Iberian Peninsula [Porto (Portugal) and A Coruña (Spain), see Fig. 1]. *A. pegreffii* was the dominant species in this survey (70%). In agreement with the higher prevalence of *A. pegreffii* with respect to *A. simplex s.s.*, the only larvae to penetrate the fish muscle were 2 *A. pegreffii* and 1 hybrid of these two species. Differentiation between *A. simplex s.s.* and *A. pegreffii* using ribosomal DNA markers is based exclusively on the existence of two fixed differences (two C/T transitions) at positions 255 and 271 in the ITS-1 sequence, meaning that different restriction patterns are produced with HinfI and TaqI enzymes (Abollo et al., 2003; Ceballos-Mendiola et al., 2010). The restriction enzyme Cfo was not used in this study since it generates the same pattern for the two sibling species. The detection of the mix of genotypes of both species (hybrid genotypes) has been the cause of some controversy in terms of its interpretation. While some authors believe that these mixed genotypes reflect hybridization, others adduce incomplete homogenization in a multiple-copy repeated DNA region (Martín-Sánchez et al., 2005; Hermida et al., 2012).

Table 3

Taxa of *Anisakis* type I larvae identified by genetic markers from sardines from the Atlantic waters of A Coruña.

| | Prevalence (%) 95% CI[a] | Mean intensity (range) 95% CI | Mean abundance 95% CI |
|---|---|---|---|
| *Anisakis* spp. | 28.3 | 1.82 (1–5) | 0.52 |
| | 18.2–40.8 | 1.29–2.65 | 0.30–0.87 |
| *Anisakis pegreffii*[b] | 23.3* | 1.57[ns] (1–5) | 0.37* |
| | 13.9–35.7 | 1.14–2.43 | 0.18–0.63 |
| *Anisakis simplex s.s.* | 6.7 | 1.50 (1–3) | 0.10 |
| | 2.3–16.4 | 1.00–2.00 | 0.02–0.25 |
| *Anisakis* hybrid | 5.0 | 1.00 (1)[uc] | 0.05 |
| | 1.38–13.91 | | 0.00–0.10 |

Prevalence = 100·N/F, mean intensity = A/N, mean abundance = A/F; where F is the total number of fish, N is the number of infected fish, and A is the number of parasites.

[a] CI: confidence interval.

[b] Statistical analysis to compare epidemiological parameters by *A. pegreffii* versus *A. simplex s.s.* and hybrids was statistically significant (*) for prevalence ($p < 0.02$) and mean abundance ($p \leq 0.05$), and not significant ([ns]) for mean intensity. The comparison between *A. simplex s.s.* and hybrids was statistically not significant.

[uc] 95% confidence limits are uncertain.

*A. pegreffii* has a lower capacity to penetrate fish musculature (Suzuki et al., 2010; Quiazon et al., 2011) and rat gastrointestinal wall (Romero et al., 2013) compared to *A. simplex s.s.* Despite these differences, *A. pegreffii* is also capable of penetrating fish muscle and causing lesions in rats and human anisakiasis (Fumarola et al., 2009; Romero et al., 2013). The presence of *Anisakis* larvae in fish muscle poses a greater risk of infection for humans since this is the preferred part of the fish for consumption. These larvae were found in two sardines from A Coruña (1% of all surveyed fish; 3% of fish from A Coruña). Further north in the Atlantic, outside the boundary extension of *A. pegreffii* (Ceballos-Mendiola et al., 2010), Karl (2008) conducted another survey in sardines from southern Great British waters showing 50% prevalence, but only 0.1% larvae in muscle. These data suggest an increase in *Anisakis* parasitism of sardines with increasingly northern latitude in the NE Atlantic Ocean (see Table 2 with data from Isla Cristina and Cádiz). Sardine growth performance is generally lower in the Mediterranean and increases across the northeastern Atlantic from Northern Morocco to the English Channel (Silva et al., 2008). Our results show that the larger sardines were the most parasitized (Fig. 3; see analysis of risk factors in Section 3.4) and that these came from the waters of NW Spain. This association between fish length/weight and *Anisakis* parasitization has long been known in other fish species (Abattouy et al., 2011; Adroher et al., 1996; Grabda, 1974). In addition, the *Anisakis* infection risk in sardines of equal length is 11.5 times higher in fish from A Coruña, i.e., the infection is associated with fishing area, as suggested previously by Rello et al. (2009) for anchovies. The size of the fish has been identified as a risk factor for *Anisakis* infection in other species such as horse mackerel (*Trachurus trachurus*) or mackerel (Scomber japonicus). Similarly to our results, no association was observed in the horse mackerel between *A. pegreffii* infection and Atlantic/Mediterranean catch area (Abattouy et al., 2014). In contrast, the risk of

parasitization was reported to be more than three times higher in mackerel from Atlantic versus Mediterranean waters of the Moroccan coast (Abattouy et al., 2011). We also found no differences in *Anisakis* presence between the sexes of sardines, consistent with results obtained in other species of fish (Abattouy et al., 2011, 2014). Fish CF was not related to parasitization in the multivariate model analysis, suggesting that the higher prevalence of *Anisakis* in sardines from A Coruña (highest CF, Table 2) is related more to the lifespan of the sardines and to the fishing area than to the condition factor, an index of apparent health of fish. In this way, most marine fish ecologists currently consider that the dietary habits of a fish species may depend upon both the availability of prey and the anatomy of the fish (Costalago and Palomera, 2014 for references).

The scarce *Anisakis* parasitization of smaller sardines could be explained by the fact that they feed mainly on copepods and phytoplankton (Palomera et al., 2007). The infection by *Anisakis* is suspected to occur when fish become bigger and euphausiids, known intermediate hosts of *Anisakis*, and other components of the plankton are incorporated into their diet (Cunha et al., 2005; Palomera et al., 2007 for references). Køie (2001) showed that the copepods can be directly infected by ingesting L3 of *Anisakis*, at least experimentally, but these larvae were not developed. The euphausiids were infected by eating these infected copepods, and thus the sardines could not have been infected by *Anisakis* via copepods but rather by eating euphausiids (in the case of large sardines), in which the L3 are developed (Fig. 3). Conversely, Klimpel et al. (2004) showed that, in Norwegian waters, the *Anisakis* lifecycle is sustained using only large carnivorous copepods as first intermediate hosts and planktivorous small fish as second intermediate hosts, without utilizing euphausiids. These authors suggested that *Anisakis* has a great ability to adapt its lifecycle to the autochthonous marine hosts. On the other hand, the higher prevalence and intensity in the largest fish could be also explained by the accumulation of parasites over the life of the fish (Bussmann and Ehrich, 1979; Valero et al., 2000) since these larvae can survive up to 3 years in fish (Smith, 1984). In agreement with Rello et al. (2008), we also suggest that a lower frequency of euphausiids in the Iberian Mediterranean pelagic waters versus Iberian Atlantic waters and the higher presence of cetaceans in the latter, facilitate the maintenance of the *Anisakis* lifecycle in the Atlantic waters of the Iberian Peninsula (Aguilar Vila et al., 1997; Anonimous, 2012; Furnestin, 1968; Papetti et al., 2005; Raga and Pantoja, 2004). The estimated population of cetaceans in Atlantic Iberian Peninsula waters is about 30,000 dolphins and porpoises (Santos et al., 2014), plus the migrating cetaceans. Sardine is the main prey (in terms of reconstructed prey biomass) of the common dolphin in Portugal and second in importance (after blue whiting, *Micromesistius poutassou*) in the dolphins of Galician and Portuguese waters (Santos et al., 2014 for references). The sardine could thus act as an intermediate/paratenic host in the *Anisakis* lifecycle in these Atlantic waters, although blue whiting are frequently parasitized by *Anisakis* and could also act as a source of cetacean infection (Ruiz-Valero et al., 1992).

*A. simplex s.s.* is the prevalent *Anisakis* species in the Atlantic Ocean. This species is widely distributed throughout the eastern Atlantic Ocean, its southern limits being the waters of the Strait of Gibraltar (Mattiucci and Nascetti, 2008). On the other hand, according to Mattiucci and Nascetti (2008), *A. pegreffii* is the dominant species in the Mediterranean Sea

(Romero et al. (2013) observed that the likelihood of finding *A. pegreffii* L3 larvae in blue whiting from Spanish Mediterranean waters is six times higher than in those from Spanish Atlantic waters) although it is also present in Atlantic waters, its northern limits being the North Spanish coast. Therefore, a sympatric area between *A. simplex s.s.* and *A. pegreffii* has been identified along the Spanish and Portuguese Atlantic coasts and in the Alborán Sea (Martín-Sánchez et al., 2005; Mattiucci and Nascetti, 2008 for references). Thus, it is not uncommon to find hybrids of these two species (Abattouy et al., 2011, 2014; Abollo et al., 2003; Ceballos-Mendiola et al., 2010; Hermida et al., 2012; Martín-Sánchez et al., 2005; and others), although Mattiucci et al. (1997) suggested that paratenic hosts of *A. simplex s.s.* were mainly benthic and demersal whereas those of *A. pegreffii* were mainly pelagic. However, as in other fish species, our results show both parasites and their hybrids in a pelagic host albeit with a 3.3-fold greater number of larvae of *A. pegreffii* (Table 3), since 71% of larvae isolated from sardines from A Coruña were identified as *A. pegreffii*.

In summary, we have shown that fishing area is a risk factor of *Anisakis* infection in sardines from Iberian waters, as suggested previously by Rello et al. (2009) for anchovies from the western Mediterranean and the Gulf of Cádiz. Our results also show greater prevalence and intensity with greater sardine size demonstrating that fish size is another risk factor. Although the low number of larvae found in the muscle tissue of the fish represents a lower likelihood of human infection or allergy to *Anisakis* — the meat is the preferred part of the fish for consumption, cases of human anisakiasis through consumption of marinated sardines have been reported in Spain. Clearly, the risk of infection is lower if small sardines or those from fishing areas with low prevalence of *Anisakis* infection are consumed. For example, a sardine of 25.5 cm caught in the waters of A Coruña is 550 times more likely to be parasitized by *Anisakis* than one of 17.6 cm captured in any of the other sample areas. Likewise, a sardine of lower weight also represents a lower infection risk. Thus, one sardine of 112 g presents a risk of infection 18 times higher than two sardines of 56 g. However, the most effective method of preventing human anisakiasis through consumption of sardine or any other fish is to follow public health guidelines; that is, to eat only fish which has undergone a suitable freezing (more than 24 h at −20 °C for whole mass) or cooking process (attaining an internal temperature of more than 60 °C for at least 10 min) (EEC, 1991). However, it is still under discussion whether or not these measures prevent allergy to *Anisakis*, as a food allergy problem (Audícana and Kennedy, 2008; Daschner et al., 2012). Therefore, until this issue is resolved, it may be advisable for people with *Anisakis* allergy to consume smaller fish, which will also reduce the risk of anisakiasis. In addition, knowledge of fishing areas with lower parasite prevalence may be of interest to fishing fleets, which will be able to offer a fish of higher sanitary quality and will also suffer fewer economic losses caused by confiscation of infected fish by the health authorities. The relationship between these studies and the culinary habits of the people of a country or region could enable health authorities to be prepared for the possible incidence of this infection/allergy in the population.

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Compliance with ethical standards

Conflicts of interest: the authors declare that they have no conflict of interest.

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# Molecular epidemiology of *Anisakis* spp. In blue whiting *Micromesistius poutassou* in eastern waters of Spain, western Mediterranean Sea*

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*\*Dedicated to Professor Adela Valero, from our Department, on the occasion of her retirement.*

## a b s t r a c t

The infection of blue whiting *Micromesistius poutassou* from the western Mediterranean Sea, off the eastern coast of Spain, with larvae of *Anisakis spp*. was studied. Between April 2016 and April 2017, 140 fish were analyzed. Total epidemiological data showed that the prevalence of *Anisakis* spp. was 29.3% and the mean intensity 1.8. Of the 74 larvae collected, 61% were type I and the remaining 39%, type II. Of the former, 91% were molecularly identified as *Anisakis pegreffii* (P = 19.3%; MI = 1.4), 2.2% as *Anisakis simplex s.s.* (P = 0.7%; MI = 1.0), while the rest (6.7%) showed a recombinant genotype between the two (P = 2.1%; MI = 1.0). All the type II larvae analyzed were molecularly identified as *Anisakis physeteris* (P = 10.0%; MI = 2.1). Three fish (2.1%) were found to have larvae in the muscle, while two were found with 1 larva of *A. pegreffii* and one with two larvae (1 *A. simplex s.s.* and 1 *A. pegreffii*). Statistical analysis showed that the prevalence of *Anisakis* spp. in blue whiting was higher in spring than in autumn (P < 0.001), probably due to the greater size (and age) of the fish and related to factors as diet shift, accumulation with age and higher food intake. Analysis of the data suggested that blue whiting were first infected with *Anisakis* type I (mean age 2.3 years) and later with *Anisakis* type II (mean age 2.7 years), probably due to the diet changing with age, with the incorporation of the paratenic/intermediate host species of these parasites. In any case, the public health authorities must continue to emphasize the need for suitable thermal treatment (freezing or cooking) of the fish prior to consumption.

## 1. Introduction

The blue whiting *Micromesistius poutassou* is an inexpensive fish which is widely consumed. It is of commercial interest in both the North Atlantic Ocean and the western Mediterranean Sea (WM). The southern blue whiting Micromesistius australis is also appreciated in the countries of the southern hemisphere. In 2014, 1.215.616 tons of the two species were landed, > 96% of which was from the Atlantic Ocean, mainly caught by European fishing fleets (Anonymous, 2016), which shows its commercial importance.

Anisakiasis or anisakiosis is the term for human infection by larval nematodes of the genus *Anisakis*. It is most frequently caused by the third-stage larvae (L3) of *Anisakis* type I (*A. simplex* s.l.) (Arizono et al., 2012; Rello Yubero et al., 2004; Romero et al., 2013), generally ingested via the consumption of raw or insufficiently cooked fish. In addition, a few cases of anisakiasis caused by larvae type II of *Anisakis* have been reported (Asato et al., 1991, and references therein; Clavel et al., 1993; Kagei et al., 1978). Recently, Romero et al. (2014), working with rats, have shown that both *A. physeteris* and *A. paggiae*, both larvae type II, are pathogenic, although less so than *Anisakis* type I. Although the blue whiting is more abundant in the Atlantic Ocean it is also captured in the Mediterranean Sea, for which there have been some studies on infection by *Anisakis* spp. and even fewer where molecular identification of the *Anisakis* larvae has been carried out (Table 1). For this reason, this molecular study of the infection of blue whiting from the western Mediterranean Sea, off the eastern coast of Spain, was carried out, while also analyzing the factors which may affect the infection of these fish with *Anisakis* spp.

## 2. Materials and methods

2.1. Host and parasites

A total of 140 blue whiting *Micromesistius poutassou* Risso were collected through bottom trawl fishing (depth 40–200 ftm; 38.0–38.5° N and 0.0–0.5° W). The trawlers leave the port at dawn and return 10–12 h later. The fish were randomly sampled from those landed, immediately after the arrival of the fishing boats, in the port of Villajoyosa (38.5° N, 0.2° W) in the western WM (Fig. 1), from April 2016 to April 2017 (1 sample in April 2016; 5 in autumn 2016; and 3 in April 2017). The fish were immediately packed in flake ice and transported to the laboratory, where they were promptly measured, weighed and dissected within 15–18 h of being landed. The "condition factor" of the fish (CF) was calculated using the formula CF = 100 × W/L$^3$, where W = total weight (g) and L = total length (cm). This CF is considered as an indicator of general fish health and, according to Monstad (1990), expresses how well-nourished the fish are. Following dissection, the larvae of *Anisakis* spp. visible in the visceral cavity were collected. The viscera and muscle of each fish were individually and separately subjected to pepsin digestion at pH 2 and 37 °C, as described previously (Molina-Fernández et al., 2015), to determine whether larvae were present. Once identified morphologically by optical microscopy as *Anisakis* type I or II (sensu Berland, 1961), the larvae were frozen at −20 °C until their

preparation for molecular identification. In addition, the age of the fish was determined using the formula L = 34.26·(1 - e$^{-0.21 (A+2.58)}$), where L = total length (cm) and A = age (years), after García et al. (1987).

## 2.2. Molecular identification

Each larva was individually prepared for DNA isolation using the RealPure commercial kit according to the maker's instructions. The fragment of rDNA corresponding to the sequence ITS1-5.8-ITS2 was amplified using the primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (1998). The PCR conditions were as previously described (Molina-Fernández et al., 2015). The expected size of the amplified fragment was around 1000 bp. Next, Restriction Fragment Length Polymorphism (RFLP) of the amplicons was performed with the restriction enzymes HinfI and TaqI (Fast Digest, Thermo Scientific), used individually at a final concentration of 0.5 U/μl and temperatures of 65 °C and 37 °C, respectively, for 10 min. Electrophoresis in 3% agarose gel was carried out to visualize the banding patterns of the larvae studied, in order to determine their species according to D'Amelio et al. (2000) and Romero et al. (2014). Some larvae showed a mixed banding pattern between *A. simplex s.s.* and *A. pegreffii* with one or other restriction enzyme and were thus classified, for the purposes of this study, as hybrid type I larvae. The digestion controls with TaqI of the DNA amplicon of *A. simplex s.s.* produced 3 fragments of 430, 400 and 100 bp while those of *A. pegreffii* produced 3 bands of 400, 320 and 150 bp. When digestion was performed with HinfI, the fragments were of 620, 250 and 80 bp for *A. simplex s.s.* and 370, 300 and 250 bp for *A. pegreffii*. In the case of the hybrids of the two species, the banding pattern was the sum of the bands of both species for the enzyme in question. For *A. physeteris*, 3 bands of 300, 280 and 140 were obtained with TaqI and another 3 of 380, 290 and 270 for HinfI.

## 2.3. Epidemiological parameters and statistical analysis

The epidemiological parameters prevalence (P), mean intensity (MI) and mean abundance, defined by Bush et al. (1997), were calculated and compared using the free software Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005) to address the notoriously left-biased frequency distributions of parasites, based on the theoretical work of Rózsa et al. (2000). The differences in prevalence were evaluated using Fisher's exact test. A bootstrap 2-sample t-test (with 20,000 repetitions) was used to compare mean intensities and mean abundances. Student's t-test was used for the statistical comparison of length, weight and condition factor of the fish.

Table 1

*Anisakis* infection in blue whiting, *Micromesistius poutassou*. Published surveys.[a]

| Reference | Hosts analyzed | Origin-FAO fishing zone | Prevalence of L3 *Anisakis* spp. | Mean intensity (range) | Parasites in muscle P; MI[b] (range) | Species |
|---|---|---|---|---|---|---|
| Poljanskij, 1955 | 5 | Barents Sea-27.I | 40% | 1 | | *Anisakis* sp. |
| Berland, 1961 | 12 | Hordaland, W Norway-27.IVa | 58.3% | | | *Anisakis* sp. type I |
| | 3 | Tromsø, N Norway-27.IIa | 100% | | | *Anisakis* sp. type I |
| Raitt, 1968[c] | 20 | Faroe Islands-27.Vb | 100% | 14 | Yes | *Anisakis* sp. |
| | 20 | W Scotland-27.VIa | 25% | 1 | | *Anisakis* sp. |
| Reimer et al., 1971 | 80 | W Ireland-27.Va | 67.8% | | | *A. simplex* |
| | 100 | NW Iceland-27.Va | 70% | | | *A. simplex* |
| Wootten and Smith, 1976 | 610 | W Scotland-27.VIIb | 97.4% | 54.9 | MI=5.2-18.6 | *Anisakis* type I |
| Richards, 1977 | ? | W Scotland-27.VIIb | 13-24% | | | *Anisakis* sp. |
| | ? | Faroe Islands-27.Vb | 27-32% | | | *Anisakis* sp. |
| Bussmann and Ehrich, 1978 | | Faroe Islands-27.Vb | High | | MI=3.5-7.2 | *Anisakis* sp. |
| Grabda, 1978 | 4100 | N Ireland–27.VIa | 98% | (7-679) | P=88%; MI=5.6 (1-20) | *Anisakis* sp. |
| | 50 | W and N Scotland-27.VIa | 92.3% | (1-583) | Yes | *Anisakis* sp. |
| Smith and Wootten, 1978 | 980 | W and N Scotland-27.VIa | 74.3% | | Yes | *Anisakis* sp. |
| MacKenzie, 1979 | 74 | Faroe Islands-27.Vb | High | | P=83.6% | *A. simplex* |
| Højgaard, 1980 | 458 | Faroe Islands-27.Vb | 100% | 60.0 (1-239) | P=63.3%; MI=4.3 (1-19) | *A. simplex* |
| Kusz and Treder, 1980 | 30 | Norwegian Sea-27.IIa | Yes | | Yes | *Anisakis* sp. |
| Schultz et al., 1980 | 829 | Norwegian Sea-27.IIa | Yes | | P=99.4%; MI=5.5 (1-34) | *Anisakis* sp. |
| Zubchenko et al., 1980 | 175 | Celtic Shelf-27.IVa | Yes | | P=27.0% | *Anisakis* spp. |
| Giedz, 1981 | 647 | Faroe Islands-27.Vb | Yes | | P=33.3% | *Anisakis* spp. |
| | 1235 | Norwegian Sea-27.IIa | 99.2% | 43.8 | P=37.9%; MI= 1.5 | *Anisakis* sp. |
| Karasev et al., 1981 | 269 | North Atlantic-27.IIa,IIb, IIIa, Vb, XIVa | Yes | | P=54.1-99.5% | *Anisakis* sp. |
| Dumke, 1988 | 2107 | Italian waters, Mediterranean Sea 37.1.3 and 37.2 | Yes | | | *A. pegreffii*[g] (P=62.1%) *A. physeteris*[g] (P=2.7%) |
| Orecchia et al., 1989 | 487 | W Galicia, NW Spain-27.VIIIc, IXa | 62.3% | 5.8 | | *Anisakis* type I |
| Sanmartín Durán et al., 1989 | 67 | Valencia Gulf, E Spain-37.1.1 | 30.3% | (1-10) | | *Anisakis* type I |
| Cuéllar et al., 1991 | 40 | Bilbao (Spain) fishmarket | 88.1% | 33.5 | P=52.4%; MI=21.9 | *A. simplex* |
| Pereira-Bueno, 1992 | 42 | Granada (Spain) fishmarket | Yes | 13.3 | P=29.1%; MI=7.0 (1-56) | *A. simplex* (P=67.9%) *Anisakis* sp. (P=2.3%) |
| Ruiz-Valero et al., 1992 | 299 | Faroe Islands-27.Vb | 100% | | | *A. simplex* s.l. |
| Køie, 1993 | 10 | Castilla La Mancha (Spain) fishmarkets | 29.3% | | | *Anisakis* spp. |
| López Giménez and Castell Monsalve, 1994 | 82 | W Galicia, NW Spain-27.VIIIc, IXa | 67.0% | 5.9 | P=20.0% | *A. simplex* |
| Sanmartín et al., 1994 | 179 | Zaragoza (Spain) fishmarket | 85.5% | 7.1 (1-61) | P=9.4% | *Anisakis* type I |
| Viu et al., 1996 | 62 | Castilla y León (Spain) fishmarkets | 63.6% | | | *Anisakis* type II *A. simplex* |
| Pereira-Bueno and Ferre-López, 1997 | 11 | | 32.4-65.5% | 1.5-2.3 | | *A. simplex* |
| Manfredi et al., 2000 | 345 | Ligurian Sea-37.1.3 | | 1.2 (1-4) | P=0.3%; MI=1.0 | *A. pegreffii*[g,h] (P=6.7%) *A. physeteris* s.l. (P=2.7%) |
| Valero et al., 2000[d] | 301 | Motril Bay, N Alboran Sea-37.1.1 | 9.0% | | Yes | *A. simplex* |
| Ojeda-Torrejón et al., 2001 | 390 | Gulf of Cadiz, SW Spain-27.IXa | 53.6% | | Yes | Anisakids |
| Osanz-Mur, 2001 | 562 | Tarragona waters, E Spain-37.1.1 | 25.1% | 1.3 (1-3) | Yes | *Anisakis* sp. |
| Silva and Eiras, 2003 | 65 | W Portugal-27.IXa | 93.8% | 14.3 (1-89) | Yes | *A. simplex* s.l. |
| Fernández et al., 2005 | 400 | Rias Baixas, Galicia, NW Spain-27.IXa | 99.3% | 11.1 | Yes | *A. physeteris* (P=0.25%; La=0.04%)[e] |

| Reference | N | Location | Prevalence | Intensity | P; MI | Species |
|---|---|---|---|---|---|---|
| Martín-Sánchez et al., 2005 | 401 | Mediterranean coasts of Spain-37.1.1 | 9.1% | | | *A. pegreffii*[g] (La=59.3%) *A. simplex s.s.*[g] (La=18.5%) Hybrids[f,g] (La=18.5%) New genotype type I[h] (La=3.7%) |
| | | Atlantic coasts of Spain-27.VIIIc, IXa | 81.7% | | | *A. pegreffii*[g] (La=20%) *A. simplex s.s.*[g] (La=66.7%) Hybrids (La=13.3%)[g] |
| Cruz et al., 2007 | 238 | Matosinhos, NW Portugal-27.IXa | 77.7% | 5.8 (1-122) | P=27.7%; MI=2.0 | *Anisakis* sp. |
| Mattiucci and Nascetti, 2007 | ? | Mediterranean Sea-37 | 48.5% | (1-12) | | *A. pegreffii*[g] |
| Chía et al., 2010 | 119 | NW Spain-27.VIIIc, IXa | 100% | 55.9 | P=37.0%; MI=24.2 (1-327) | *Anisakis* type I |
| Angelucci et al., 2011 | 16 | Sardinia-37.1.3 | 87.5% | 10 | P=62.5%; MI=1.4 | *Anisakis* type I (P=81.2%) |
| Meloni et al., 2011 | 17 | Sardinia-37.1.3 | 82.4% | | | *A. pegreffii*[g] (La=90.6%) *A. physeteris*[g] (La=1.3%) Hybrids[g] (La=8.1%) |
| Tedde et al., 2011 | 57 | N Sardinia-37.1.3 | 61.4% | 3.9 (1-50) | P=10.5% | *A. pegreffii*[g,h] *A. physeteris*[g] (P=5.3%) |
| Madrid et al., 2012 | 169 | NE Atlantic-27.VIIIc, IXa | 78% | (1-95) | P=39% | *A. simplex* s.l. |
| | 115 | W Mediterranean Sea-37.1 | 19% | (1-219) | P=7% | *A. simplex* s.l. |
| Romero et al., 2013[d] | ? | Mediterranean coast of Spain-37.1.1 | Only surveyed type I | | | *A. pegreffii*[g] (La=64%) *A. simplex s.s.*[g] (La=19%) Hybrids[g] (La=17%) |
| | ? | Atlantic coasts of Iberian Peninsula-27.VIIIc, IXa | Only surveyed type I | | | *A. pegreffii*[g] (La=28%) *A. simplex s.s.*[g] (La=49%) Hybrids[g] (La=23%) |
| Romero et al., 2014[d] | ? | Mediterranean coast of Spain-37.1.1 | Only surveyed type II | | | *A. physeteris*[g] (La=55%), *A. paggiae*[g] (La=45%) |
| | ? | Atlantic coasts of Iberian Peninsula-27.VIIIc, IXa | Only surveyed type II | | | *A. physeteris*[g] (La=45%), *A. paggiae*[g] (La=50%) *A. brevispiculata*[g] (La=5%) |
| Piras et al., 2014 | 57 | Gulf of Asinara, N Sardinia-37.1.3 | High | (1-50) | P=14%; MI=1 | *A. pegreffii*[g,h] (P= > 66.7%) *A. physeteris*[g] (P=10.5%) |
| Gómez-Mateos et al., 2016 | 100 | Gulf of Cadiz, SW Spain-27.IXa | 82% | 16 (1-328) | P=38% | *A. simplex s.s.*[g,h] (La=50%) *A. pegreffii*[g,h] (La=42.7%) Hybrids[g,h] (La=7%) *A. typica*[g] (La=0.25%) |
| This report | 140 | Villajoyosa port (E Spain)-37.1.1 | 29.3% | 1.8 (1-12) | P=2.1%; MI=1.3 (1-2) | *A. pegreffii*[g,h] (P=19.3%; La=55.4%) *A. simplex s.s.*[g,h] (P=0.7%; La=1.3%) Hybrids[g] (P=2.1%; La=4.1%) *A. physeteris*[g] (P=10.0%; La=39.2%) |

[a] In some surveys the occurrence of other anisakids has been studied: *Hysterothylacium aduncum* L3 has been detected with high prevalence, and less frequently L4 and adults of *H. aduncum*. Also, the presence of L3 of Hysterothylacium sp., *Contracaecum* sp., C. osculatum, and *Pseudoterranova decipiens* has been occasionally reported. See also Karasev (1990).

[b] P, prevalence; MI, mean intensity .

[c] Data from Dr. Kabata

[d] Data calculated from the reference.

[e] La= percentage of *Anisakis* larvae.

[f] Hybrids: recombinant genotype of *A. pegreffii* and *A. simplex s.s.*

[g] Molecular identification.

[h] Also detected in muscle

Fig. 1. Geographical area of the western Mediterranean Sea, port (square) where blue whiting were landed and fishing ground (line in front to landing port).

## 3. Results

### 3.1. Host

The length range of the 140 blue whiting examined was 14.3–30.2 cm, weight range was 24.3–251.1 g and condition factor (CF) range was 0.66–1.06 (Table 2). The relationship between weight (W) and length (L) showed a potential line with an exponent close to 3 (W = 0.0072 × $L^{3.0567}$; coefficient ± 0.0008 and exponent ± 0.0362, $R^2$ = 0.981), showing a relationship generally considered in the literature as cubic (Fulton, 1904; García et al., 1987; Nash et al., 2006). Although length and weight were lower in autumn, CF values were similar throughout the three seasons of sampling (Table 2).

### 3.2. Epidemiological parameters

Total prevalence of L3 of *Anisakis* spp. was 29.3%, with significant variation between spring and autumn (Table 2), being higher in spring, independently of the year. Of the 74 larvae collected from fish, 61% were type I and the remaining 39% type II. Three fish (2.1%) showed larvae in muscle tissue. Table 3 shows the epidemiological parameters according to *Anisakis* L3 morphotype and season (excluding spring 2016) in which the fish were caught. It can be seen that both the total prevalence by morphotype and its seasonal variation within each morphotype was statistically significant, being greater in spring than autumn (P < 0.001).

When the fish were studied according to season of capture and length groups, excluding spring 2016, (Table 4), it was observed that prevalence increased with length, regardless of *Anisakis* type, especially in spring, which is when the largest specimens were captured. The prevalence was still significantly different even when fish of the same length class (17.7–22.3

cm) from autumn 2016 and spring 2017 were compared (Table 4). When extreme lengths (the largest and smallest fish) were removed from this length class so that neither group (autumn 2016 and spring 2017) showed statistical differences in weight, length and CF, significant differences were still observed (P < 0.03) in prevalence and abundance, at least in *Anisakis* type I. Furthermore, comparison of the size of infected fish with uninfected fish revealed that both length and weight were significantly higher in the former, but without affecting the CF of the fish (Table 5). In order to make an approximate estimation of the age at which the blue whiting of the study area first became infected, the average length of fish with a single larva was determined. For *Anisakis* type I, the average length of the blue whiting was 22.0 ± 3.6 cm (n = 28) and for *Anisakis* type II, 23.0 ± 4.5 cm (n = 8), corresponding to a fish age of 2.3 and 2.8 years respectively (García et al., 1987).

### 3.3. Molecular identification of *Anisakis* larvae by PCR-RFLP

Of the 45 *Anisakis* type I larvae collected, 91.1% were molecularly identified as *A. pegreffii*, 2.2% as *A. simplex s.s.*, while the remainder (6.7%) showed a hybrid PCR-RFLP band pattern with one of the restriction enzymes employed (TaqI or HinfI). All the type II larvae analyzed (27 of 29) were molecularly identified as *A. physeteris*. Four larvae (5.4% of all the larvae) were found in muscle after pepsin digestion, all type I: two larvae (1 *A. simplex s.s.* and 1 *A. pegreffii*) in one fish, while two fish each harboured 1 larva of *A. pegreffii* in their muscle.

### 3.4. Molecular epidemiological parameters

The epidemiological parameters for infection by the *Anisakis* species molecularly identified in the fish are shown in Table 6. Prevalence of *A. pegreffii* (19.3%) was almost double that of *A. physeteris* (10.0%) (P < 0.05), while being very low for *A. simplex s.s.* (0.7%). Only 2.1% of the fish were infected with *A. pegreffii*/*A. simplex s.s.* hybrid larvae. The coinfection prevalence was 3.6%: 3 blue whiting harboured larvae of *A. pegreffii* and *A. physeteris*, one fish hosted 1 hybrid larva and 1 *A. physeteris* larva and one blue whiting hosted larvae of *A. pegreffii* (10), *A. simplex s.s.* (1) and *A. physeteris* (1).

## 4. Discussion

Since 1955 (Poljanskij, 1955) numerous studies on anisakid infection of blue whiting from the NE Atlantic Ocean have been carried out. Identification was based on morphological characteristics with different authors reporting L3 of *Anisakis* sp., *Anisakis* type I or *Anisakis simplex* (see Table 1). When the number of fish examined was > 50, prevalence was always very high (> 53%; see Table 1). In addition to *Anisakis*, L3 of *Hysterothylacium aduncum* have often been reported. Other anisakids occasionally found were L3 of *Contracaecum* sp., C. osculatum, Pseudoterranova decipiens, and Hysterothylacium sp. and L4 and adults of *H. aduncum* (references in Table 1, and Karasev, 1990). In the Mediterranean Sea, the first studies were carried out by Orecchia et al. (1989) in Italian waters, and, for the first time, the molecular

identification of *Anisakis* in blue whiting was performed, detecting *A. simplex* A (=*A. pegreffii*, P = 62.1%) and *A. physeteris* (P = 2.7%). Valero et al. (2000) molecularly identified *A. pegreffii* (P = 6.7%) in waters of the northern Alboran Sea. The identification of morphotype II larvae was morphological and reported as *A. physeteris* (P = 2.7%). About 300 km north of the area studied in this work, Osanz-Mur (2001) detected only *Anisakis* type I in blue whiting (P = 22.6%). Here, similar prevalence values (P = 22.9%) were reported for morphotype I (20.7% for *A. pegreffii*, 2.1% hybrids *A. pegreffii/A. simplex s.s.*, and 0.7% *A. simplex s.s.*), but morphotype II (=*A. physeteris*) was also identified (P = 10.0%). Finally, in the coastal waters of Sardinia a P > 60% for *Anisakis* type I (=*A. pegreffii* and a few hybrids *A. pegreffii/A. simplex s.s.*) and P = 5–10% for morphotype II (=*A. physeteris*) larvae were reported (Angelucci et al., 2011; Meloni et al., 2011; Piras et al., 2014; Tedde et al., 2011).

Table 2: Epidemiological parameters of infection by *Anisakis* spp., total and by season, in blue whiting sampled on the Mediterranean coast of eastern Spain.

| Parameter | Total | Spring 2016[#] | Autumn 2016 | Spring 2017[#] |
|---|---|---|---|---|
| N blue whiting | 140 | 13 | 81 | 46 |
| Length ± SD | 20.4 ± 3.6 | 25.5 ± 3.3*** | 18.2 ± 1.8 | 22.9 ± 3.0*** |
| (range) | (14.3–30.2) | (21.1–30.2) | (14.3–22.3) | (17.7–28.4) |
| Weight ± SD | 80.0 ± 45.7 | 155.5 ± 59.4*** | 52.8 ± 17.0 | 106.6 ± 36.2*** |
| (range) | (24.3–251.1) | (84.9–251.1) | (24.3–101.9) | (45.7–173.5) |
| Condition factor ± SD | 0.86 ± 0.06 | 0.90 ± 0.06* | 0.85 ± 0.05 | 0.86 ± 0.07[ns] |
| (range) | (0.66–1.06) | (0.77–0.99) | (0.66–0.98) | (0.71–1.06) |
| Prevalence | 29.3 | 46.2** | 9.9 | 58.7*** |
| CI 95% | (22.1–37.5) | 22.4–74.0 | 4.6–18.4 | 43.4–71.9 |
| Mean intensity (range) | 1.80 (1–12) | 2.67[ns] (1–6) | 1.00 (1) | 1.85[ns] (1–12) |
| CI 95% | 1.39–2.73 | 1.33–4.17 | Uncertain | 1.30–3.22 |
| Mean abundance | 0.53 | 1.23[ns] | 0.10 | 1.09* |
| CI 95% | 0.36–0.83 | 0.38–2.54 | 0.04–0.16 | 0.70–1.98 |

Weight in g; length in cm. SD = standard deviation. Prevalence = 100·F/N, mean intensity = A/F, mean abundance = A/N; where N is the total number of fish, F is the number of infected fish, and A is the number of larvae. CI: confidence interval. Comparison of seasonal prevalence showed $P < 0.001$. Comparison between spring 2016 and spring 2017 not significant for all the epidemiological parameters compared, except length and weight ($P < 0.05$). #Shows the pairwise comparison spring 2016-autumn 2016 and autumn 2016-spring 2017: [ns]not significant; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0001$.

It is accepted that both the fishing area and the size of the fish are risk factors associated with infection by anisakids (Adroher et al., 1996; Molina-Fernández et al., 2015; Rello et al., 2009; and others). In this sense, the area analyzed (western WM) showed a low abundance (Tables 1 and 2) of larvae of *Anisakis* type I in blue whiting with which the prevalence was relatively low compared to other areas surveyed in the Mediterranean Sea (eastern WM) and the NE Atlantic Ocean. There were also notable differences regarding *Anisakis* type II, whose presence in the blue whiting of the surveyed areas of the NE Atlantic Ocean is rare (see Table 1; Adroher and Benítez, in Bay of Biscay, N Spain, unpublished data). In contrast, in the surveyed

areas of the WM, the prevalence of *Anisakis* type II is more or less uniform (P = 5–10%) and identified as *A. physeteris*, although *A. paggiae* has also been described (Romero et al., 2014).

Table 3
Epidemiological parameters of infection by season for the two *Anisakis* L3 morphotypes in blue whiting.

| Parasite | | Total | Autumn 2016 | Spring 2017 |
| --- | --- | --- | --- | --- |
| *Anisakis* type I | Prevalence | 22.9 | 8.6 | 50.0 |
| | CI 95% | 16.3–30.7 | 4.1–17.1 | 35.8–64.2 |
| | Mean intensity (range) | 1.41 (1–11) | 1.00 (1) | 1.57 (1–11) |
| | CI 95% | 1.06–2.63 | Uncertain | 1.09–2.96 |
| | Mean abundance | 0.32 | 0.09 | 0.78 |
| | CI 95% | 0.21–0.61 | 0.02–0.15 | 0.48–1.59 |
| *Anisakis* type II | Prevalence | 10.0** | 1.2$^{\$}$ | 19.6** |
| | CI 95% | 6.0–16.3 | 0.07–6.6 | 10.3–33.6 |
| | Mean intensity (range) | 2.07$^{ns}$ (1–6) | 1.00 (1) | 1.56$^{ns}$ (1–4) |
| | CI 95% | 1.36–3.00 | Uncertain | 1.11–2.33 |
| | Mean abundance | 0.21$^{ns}$ | 0.01* | 0.30$^{ns}$ |
| | CI 95% | 0.10–0.37 | 0.00–0.04 | 0.13–0.59 |

Data of fish caught in spring 2016 not included. CI: confidence interval.
Statistical comparison between morphotypes: $^{\$}$P < 0.07 (Fisher's exact test) but P < 0.04 (Exact unconditional test); *P < 0.05; **P < 0.005. Comparison of the seasonal variation of prevalence within each morphotype shows P < 0.001 in both cases.

Regarding the size (age) of the fish, several authors have shown that the prevalence of anisakid infection increases with the length (age) of the blue whiting (Madrid et al., 2012; Valero et al., 2000; this report). García et al. (1987) reported that the longer, sexually mature blue whiting migrate to greater depths from August to February to spawn, so they are not caught by trawlers in this area of the western WM, being replaced by the blue whiting > 11 cm that climb to the depths of capture. The fact that the larger blue whiting are caught in the spring could explain why a higher prevalence is detected in that season (Gómez-Mateos et al., 2016; Madrid et al., 2012; Valero et al., 2000; this report). However, the comparison of fish of the same length (age), weight and CF between seasons results in a statistically significant difference, at least for *Anisakis* type I, and thus other unknown factors, in addition to age, may have been affecting the seasonal prevalence of infection. The formula to obtain the age of the WM blue whiting, after García et al. (1987), attributes a mean age of 1.4 years to those not infected, of 2.4 years to those infected with *Anisakis* type I (2.3 y for fish hosting one larva) and of 3.8 years to those infected with *Anisakis* type II (2.7 y for fish hosting one larva). These data suggest that blue whiting are not usually infected with *Anisakis* until they are over 1 year old and that infection by type II takes place later than that by type I. This may be related to diet but also to higher food intake in larger fish, since, from the age of 1 year they primarily consume euphausiids and small fish (potential intermediate/paratenic hosts) and there is a significant correlation between

abundance of prey in the diet and in the environment (Macpherson, 1978). However, this author reported that cephalopods, paratenic hosts of *A. physeteris* (Mattiucci and Nascetti, 2008; Orecchia et al., 1989), generally became part of the diet of blue whiting of the WM on the latter attaining a length of 24 cm (~3.2 y), which would, at least partially, explain the results obtained. Angelucci et al. (2011) detected *Anisakis* type II larvae in the squid Todarodes sagittatus (P = 20%) in Sardinian waters, and Picó-Durán et al. (2016) in the squid Illex coindetii (P = 1.6%) in Spanish Mediterranean waters.

Table 4
Variation of the prevalence of *Anisakis* in blue whiting according to length groups and season of capture.[a]

| Blue whiting | Prevalence (F/N)[b] *Anisakis* spp. | | Prevalence (F/N) *Anisakis* type I | | Prevalence (F/N) *Anisakis* type II | |
|---|---|---|---|---|---|---|
| Length class (age)[c] | Autumn 2016 | Spring 2017 | Autumn 2016 | Spring 2017 | Autumn 2016 | Spring 2017 |
| < 17.7 cm (< 1 y) | 9.7 (3/31) | – | 6.5 (2/31) | – | 3.2 (1/31) | – |
| 17.7–22.3 cm[d] (~1–2.5 y) | 10.0 (5/50) | 57.1*** (12/21) | 10.0 (5/50) | 42.9** (9/21) | 0 (0/50) | 14.3* (3/21) |
| > 22.3 cm (> 2.5 y) | – | 60.0 (15/25) | – | 56.0 (14/25) | – | 24.0 (6/25) |

Statistical comparison between seasons: *P < 0.05; **P < 0.005; ***P < 0.001.
[a] Fish caught in spring 2016 not included as cannot be classified into significant groups due to their low number.
[b] Where N is the total number of fish and F is the number of infected fish.
[c] Approximate age class according to García et al. (1987).
[d] This class of length covers the fish included between the minimum size of the spring sample and the maximum size of the autumn one

Furthermore, Valero et al., (2000) observed that the prevalence of *A. physeteris* in blue whiting of > 23 cm from the northern Alborán Sea (WM) was three times that found in smaller specimens. The CF of fish does not seem to be affected by the *Anisakis* infection (Tables 2 and 5). Controversially, some authors have suggested that CF is not only affected when the parasitic intensity is high, but also by factors such as the season or the age (length) of the fish (see Rohde, 1984, and references therein).

Table 5
Epidemiological infection parameters of blue whiting by *Anisakis* morphotype.

| Parameters | | | *Anisakis* spp. | *Anisakis* type I | *Anisakis* type II |
|---|---|---|---|---|---|
| Fish length[a] ± SD | | U | 19.5 ± 2.9 | 19.9 ± 3.4 | 19.9 ± 3.1 |
| | | I | 22. 7± 4.1*** | 22.2 ± 3.7** | 25.3 ± 4.4*** |
| Fish weight ± SD | | U | 68.0 ± 33.2 | 74.6 ± 45.0 | 72.7 ± 36.2 |
| | | I | 109.0 ± 57.9*** | 98.5 ± 44.1* | 146.5 ± 67.1** |
| Fish condition factor ± SD | | U | 0.86 ± 0.06 | 0.86 ± 0.06 | 0.86 ± 0.06 |
| | | I | 0.86 ± 0.07 [ns] | 0.85 ± 0.06 [ns] | 0.84 ± 0.08 [ns] |

Weight in g; length in cm. SD = standard deviation. Abbreviations: U, uninfected fish; I, infected fish.
Statistical analysis to compare morphometrical fish parameters between uninfected and infected fish (Student's t-test): P < 0.05; P < 0.007; ***P < 0.0005; [ns]not significant.
Comparison between morphotypes (Student's t-test): Length (P < 0.04) and weight (P < 0.03) are statistically different in infected fish but the same in uninfected fish (P > 0.7). CF, ns.
[a]According García et al. (1987): mean age of 1.4 years to fish not infected (19.5 cm), of 2.4 years to those infected with *Anisakis* type I (22.2 cm) and of 3.8 years to those infected with *Anisakis* type II (25.3 cm).

Finally, although some *Anisakis* larvae were found in muscle tissue, the risk of human infection seems low as they were few in number in the surveyed zone and the blue whiting is not usually eaten raw. In any case, it should not be forgotten that > 1 million tons of this fish are sold every year, which means that the possibility of cases must be considered by the health authorities. Moreover, although several authors have not detected significant migration of the larvae from the visceral cavity to the muscle in the 72 h after capture (Smith, 1984; Wootten and Smith, 1976; Zubchenko et al., 1980), Madrid et al. (2012) showed a statistical relationship between the number of *Anisakis* larvae in blue whiting muscle and the time since capture (up to 7 days). Furthermore, other authors have described a greater presence of *Anisakis* type I larvae in blue whiting muscle in other fishing zones in both Atlantic and Mediterranean waters (see Table 1) Consequently, suitable thermal treatment is required before consuming any fish product which may be infected with *Anisakis*.

Table 6
Epidemiological infection parameters of blue whiting by species of *Anisakis* genetically identified.

| Parasite | Prevalence CI 95% | Mean intensity (range) CI 95% | Mean abundance CI 95% |
|---|---|---|---|
| *A. physeteris* | 10.0 | 2.07 (1–6) | 0.21 |
| | 6.0–16.3 | 1.36–3.00 | 0.10–0.37 |
| *A. pegreffii*[a] | 19.3* | 1.44 [ns] (1–10) | 0.28 [ns] |
| | 13.5–26.7 | 1.07–2.67 | 0.18–0.54 |
| *A. simplex s.s.* | 0.70 | 1 (1) | 0.01 |
| | 0.04–3.81 | Uncertain | 0.00–0.02 |
| Hybrids[b] | 2.1 | 1 (1) | 0.02 |
| | 0.6–6.3 | Uncertain | 0.00–0.04 |

CI: confidence interval.
[a] Statistical analysis to compare epidemiological parameters *A. physeteris* vs. *A. pegreffii*: P < 0.05; [ns]not significant.
[b] Hybrids: these are the larvae showing a hybrid PCR-RFLP band pattern which was the sum of the bands of *A. simplex s.s.* and *A. pegreffii* for one of the restriction enzymes (i.e. HinfI or TaqI).

Compliance with ethical standards

Conflicts of interest: the authors declare that they have no conflict of interest.

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# Early development and life cycle of *Contracaecum multipapillatum s.l.* from a brown pelican *Pelecanus occidentalis* in the Gulf of California, Mexico

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## abstract

The initial developmental stages of *Contracaecum multipapillatum* (von Drasche, 1882) Lucker, 1941 sensu lato were studied using eggs obtained from the uteri of female nematodes (genetically identified) found in a brown pelican *Pelecanus occidentalis* from Bahía de La Paz (Gulf of California, Mexico). Optical microscopy revealed a smooth or slightly rough surface to the eggs. Egg dimensions were approximately 53 × 43 µm, although after the larvae had developed inside, egg size increased to 66 × 55 µm. Hatching and survival of the larvae were greater at 15°C than at 24°C, and increased salinity resulted in a slight increase in hatching but seemed to reduce survival at 24°C, but not at 15°C. The recently hatched larvae measured 261 × 16 µm within their sheath. When placed in culture medium, the larvae grew within their sheath, and a small percentage (~2%) exsheathed completely (314 × 19 µm). The larvae continued to grow and develop once they had exsheathed, attaining mean dimensions of 333 × 22 µm. Although they did not moult during culture, optical microscopy revealed a morphology typical of third-stage larvae. Finally, the genetic identity between the larval parasites collected from mullet *Mugil curema* and adult female parasites collected from the brown pelican suggests a life cycle of *C. multipapillatum* in which the mullet are involved as intermediate/paratenic hosts and the brown pelicans as final hosts in the geographical area of Bahía de La Paz.

## 1. Introduction

Nematodes of the genus *Contracaecum* (Ascaridoidea: Anisakidae) parasitize fish-eating birds and marine mammals as their definitive hosts, while the larvae use planktonic crustaceans and fish as intermediate/paratenic hosts (Anderson 2000). If accidentally ingested, the third larval stage (L3) found in fish may infect humans, causing anisakidosis (contracaecosis) and possibly allergic reactions. In Germany, Schaum & Müller (1967) described the first human case of contracaecosis in the medical literature. Im et al. (1995) studied 107 cases of gastric anisakiasis in Cheju-do (South Korea), and after examining the collected larvae of the patients, found 1 larva of *Contracaecum* type A (= *C. osculatum* s.l.). Ishikura et al. (1996) later described 2 new cases in Hokkaido (Japan). All cases described to date have been caused by *C. osculatum* s.l. Recently, Shamsi & Butcher (2011) reported the first anisakidosis case in Australia caused by a larva of *Contracaecum* sp. Although no allergic reactions related to the ingestion of larvae of the genus *Contracaecum* in food have been described, these may well occur as they have been reported in other genera of anisakids, such as *Anisakis* (e.g. Daschner et al. 2012) and *Hysterothylacium* (Valero et al. 2003).

*C. multipapillatum* (von Drasche, 1882) Lucker, 1941 (sensu lato) consists of a complex of sibling species which parasitize fish-eating birds, principally of the family Pelicanidae (Mattiucci et al. 2010). The life cycle of these parasites is complex and has yet to be clarified. According to Huizinga (1967), the second larval stage (L2, with the L1 sheath) hatches from the egg into the water and is ingested by a copepod. It then frees itself from the sheath in the intestine of the copepod before passing to the coelom, where it grows without moulting. When the infected copepod is ingested by the first fish host (small planktivorous fish), L2 reaches the abdominal cavity, grows and moults to L3. When this fish is ingested by a larger, piscivorous fish, L3 grows in the abdominal cavity but does not moult. When this fish is ingested by a suitable fish-eating bird host, L3 lodges in the proventriculus where it grows, moulting twice to the adult stage. After fertilization of the female by the male, the eggs are passed in faeces from the final host to the water. The L2 larvae then develop inside the eggs, finally hatching, after which the larvae are free in the water until they are ingested by a new intermediate host.

Deardorff & Overstreet (1980) attempted experimental oral infection of chickens, ducks and rats with L3 of *C. multipapillatum* obtained from fish, without success, although a few larvae placed in the ab dominal cavity of rats developed to the adult stage. At a later date, Vidal-Martínez et al. (1994) again tried to infect animals, this time including cats in the experiment. While the chickens, ducks and rats were not infected orally when using L4 collected from the Mayan cichlid Cichlasoma urophthalmus, the cats were not only infected, but the larvae also developed to the adult stage, at least 3 of these being found firmly attached to the intestine where they caused haemorrhaging similar to that associated with small ulcers. In addition, Barros et al. (2004) infected rabbits experimentally with *C. multipapillatum* L3 from fish, which

caused gastric lesions. All of these authors warned of the potential risks to human health resulting from the accidental ingestion of this parasite.

*C. multipapillatum s.l.* has been found both in commercially important fish of the mullet family Mugilidae (as L3) and in brown pelicans *Pelecanus occidentalis* (as L3, L4 and adults) from Mexico and other regions of America (Deardorff & Overstreet 1980, Iglesias et al. 1998, 2011, Valles-Ríos et al. 2000, Valles-Vega 2011, 2014). Furthermore, in these areas, where mullets form part of the diet of these birds, infections have been found in brown pelicans as young as 2 wk (Humphrey et al. 1978).

The present study describes the early developmental stages of *C. multipapillatum s.l.* and the first attempt at their *in vitro* culture in nutritive media in order to further our knowledge of the biology of this parasite, to determine its life cycle and to describe eggs and larvae using optical microscopy.
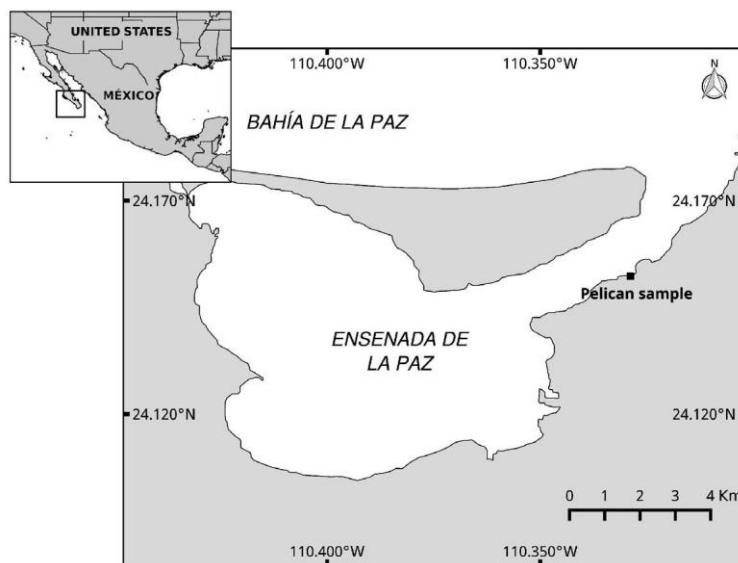


Fig. 1. Geographical zone where mullet *Mugil curema* were collected and a brown pelican *Pelecanus occidentalis* was captured for this study

## 2. Material and methods

### 2.1. Host and parasite collection

A total of 353 adult nematodes were obtained from the digestive tract of male adult brown pelican from Bahía de La Paz, Baja California Sur, Mexico (Fig. 1). Six females of *Contracaecum multipapillatum s.l.*, identified at CICIMAR de La Paz (Mexico) using the morphological characteristics of males that accompanied them (Lucker 1941, Yamaguti 1961), were sent to the Department of Parasitology at the University of Granada (Spain) where they were dissected to extract the eggs. These females were then analysed for molecular identification (see below). The third larval stage (L3) of *C. multipapillatum s.l.* from mullet *Mugil curema* captured in the same geographical area were collected for genetic identification.

### 2.2. Molecular identification

For genetic identification of the L3 larvae of *C. multipapillatum* from mullet and of the adult female *C. multipapillatum* collected from the brown pelican, PCR of the ribosomal fragment ITS1–5.8S–ITS2 of the nuclear genome was carried out. In brief, the procedure described by Ausubel et al. (2002) was employed to extract the genomic DNA of every parasite. The PCR amplification primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (2000) were employed. The expected size of the amplified fragment was ~1000 bp. PCR products were run through electrophoresis in 1% agarose gel and visualized with SYBR Gold dye and a transilluminator. The DNA was purified and sequenced by MacroGen (South Korea).

### 2.3. Analysis of sequences

In brief, the sequences were edited and aligned with Clustal X (version 2.0). A phenetic analysis was carried out. Trees were constructed using neighbourjoining (NJ) analysis, based on Kimura-2-parameter (K2P) distance (Kimura 1980) values, using MEGA 5.05 (Tamura et al. 2011). The reliabilities of the measure of stability of the branches were evaluated using nonparametric bootstrap analysis (Felsenstein 1985) for the NJ tree, with 1000 bootstrap replicates.

### 2.4. Maintenance and cultivation

The eggs were taken from the most external part of the uteri, placed in tubes of tap water, centrifuged at 600 × *g* for 10 min and the supernatant decanted. Next, 9‰ NaCl solution was added, and the tubes were centrifuged at 600 × *g* for 10 min, after which the eggs were placed in an antibiotic-antimycotic solution for 30 min for axenization (Iglesias et al. 1997). The eggs were then washed with sterile 9‰ NaCl solution and centrifuged at 600 × *g*. After resuspension in this saline solution, the solution was divided into 3 aliquots which were then centrifuged, decanted and the eggs resuspended in a volume of sterile NaCl solution at 9, 28

and 35‰, respectively. Aliquots of 1 ml with ~200 eggs were placed in the wells of sterile polystyrene culture plates, in triplicate, which were incubated separately at 15 and 24°C. The development of the eggs was monitored to distinguish the different stages, and we recorded hatching and survival of the larvae in the saline solutions, which were renewed weekly. After 1 mo under these conditions, the saline solution in the wells was changed to a solution of Grace's insect medium supplemented with 2% v/v basal medium Eagle's vitamins (100×) solution, 1 mM L-cysteine, 1 g l$^{-1}$ glucose, 20% v/v heat-inactivated foetal bovine serum and 1% v/v RPMI-1640 amino acid solution (50×), adjusting pH to 7.2, without changing the other experimental conditions and the observation of larval development. The culture medium was renewed weekly. The experiments were deemed to be completed when dead larvae outnumbered live larvae.

## 3. Results

The genetic analyses of the ITS1–5.8S–ITS2 sequences of both adult female *Contracaecum* collected from the brown pelican and L3 larvae collected from mullet in Bahía de La Paz showed 100% identity (Table 1; K2P = 0; Fig. 2).

During the first developmental stages (up to the morula), the eggs from the uteri of female *C. multipapillatum s.l.* placed in different saline solutions measured (mean ± SD) 53.4 ± 3.4 × 43.1 ± 4.1 µm (n = 13). When the larvae developed inside the eggs, these increased in size up to 65.6 ± 5.4 × 54.8 ± 4.5 µm (n = 12). Optical microscopy observations revealed that the eggs had either 2 layers with a smooth covering or 3 layers with a slightly rough covering (Fig. 3).

The eggs exhibited negative buoyancy in all tested saline solutions, depositing on the bottom of the culture wells. Most of the eggs (>85%) left to incubate in the different saline solutions developed as far as forming larvae (Fig. 4A,B) which later hatched (Fig. 4A,C) in large numbers (61−98%). However, the speed of development and the survival of the larvae differed according to incubation conditions. At 15°C (Table 2), salinity did not seem to significantly affect the mean hatch percentage (average range 80−97%) or the survival of the hatched larvae (75−82%, mean percentage of larvae alive at the end of the experiment). Furthermore, a notable increase was observed in the slope of the eclosion curve from Day 11 onwards (not shown).

Table 1: Kimura-2-parameter standard genetic distance in the ITS1 sequence between *Contracaecum multipapillatum* of this study and other *Contracaecum* spp. of birds. *Ascaris suum* was used as an outgroup. GenBank accession numbers are in brackets.

| Contracaecum | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. *C. multipapillatum* (*Mugil curema*) | – | | | | | | | | | | | | | | | | |
| 2. *C. multipapillatum* (*Pelecanus occidentalis*) | 0.00 | – | | | | | | | | | | | | | | | |
| 3. *C. multipapillatum* (AM940057.1) | 0.06 | 0.06 | – | | | | | | | | | | | | | | |
| 4. *C. multipapillatum* B & C (KC437337.1) | 0.06 | 0.06 | 0.00 | – | | | | | | | | | | | | | |
| 5. *C. multipapillatum* (AM940059.1) | 0.06 | 0.06 | 0.00 | 0.00 | – | | | | | | | | | | | | |
| 6. *C. multipapillatum* D (KC4373338.1) | 0.06 | 0.06 | 0.00 | 0.00 | 0.00 | – | | | | | | | | | | | |
| 7. *C. rudolphii* (FM210251.1) | 0.32 | 0.32 | 0.30 | 0.30 | 0.30 | 0.30 | – | | | | | | | | | | |
| 8. *C. rudolphii* F (JF424597.1) | 0.33 | 0.33 | 0.32 | 0.32 | 0.32 | 0.32 | 0.01 | – | | | | | | | | | |
| 9. *C. variegatum* (FM177532.1) | 0.33 | 0.33 | 0.32 | 0.32 | 0.32 | 0.32 | 0.01 | 0.01 | – | | | | | | | | |
| 10. *C. rudolphii* B (AJ634783.1) | 0.33 | 0.33 | 0.32 | 0.32 | 0.32 | 0.32 | 0.01 | 0.01 | 0.01 | – | | | | | | | |
| 11. *C. rudolphii* A (AJ634782.1) | 0.31 | 0.31 | 0.30 | 0.30 | 0.30 | 0.30 | 0.03 | 0.02 | 0.02 | 0.02 | – | | | | | | |
| 12. *C. eudyptulae* (FM177561.1) | 0.32 | 0.32 | 0.30 | 0.30 | 0.30 | 0.30 | 0.02 | 0.02 | 0.02 | 0.01 | 0.02 | – | | | | | |
| 13. *C. bioccai* (JF424598.1) | 0.32 | 0.32 | 0.30 | 0.30 | 0.30 | 0.30 | 0.05 | 0.04 | 0.04 | 0.04 | 0.05 | 0.04 | _ | | | | |
| 14. *Contracaecum* sp. (JF424599.1) | 0.34 | 0.34 | 0.32 | 0.32 | 0.32 | 0.32 | 0.06 | 0.06 | 0.05 | 0.05 | 0.07 | 0.05 | 0.03 | – | | | |
| 15. *C. septentrionale* (AJ634784.1) | 0.34 | 0.34 | 0.33 | 0.33 | 0.33 | 0.33 | 0.05 | 0.05 | 0.04 | 0.04 | 0.05 | 0.04 | 0.04 | 0.06 | – | | |
| 16. *C. microcephalum* (FM177525.1) | 0.34 | 0.34 | 0.32 | 0.32 | 0.32 | 0.32 | 0.09 | 0.05 | 0.08 | 0.09 | 0.10 | 0.06 | 0.09 | 0.09 | 0.09 | – | |
| 17. *C. bancrofti* (EU839572.1) | 0.31 | 0.31 | 0.28 | 0.28 | 0.28 | 0.28 | 0.14 | 0.14 | 0.14 | 0.13 | 0.15 | 0.13 | 0.13 | 0.16 | 0.13 | 0.13 | – |
| 18. *Ascaris suum* (AB571302.1) | 0.49 | 0.49 | 0.47 | 0.47 | 0.47 | 0.47 | 0.38 | 0.37 | 0.36 | 0.36 | 0.37 | 0.36 | 0.38 | 0.39 | 0.38 | 0.34 | 0.34 |

When the same experiment was carried out at 24°C, the notable increase in the slope of the hatching curve occurred towards Day 13 and did not seem to be affected by the salinity of the medium (not shown). However, the hatch percentage was slightly lower than at 15°C (Table 2), while there was a slight increase in mean hatch percentage (71–80%) with increasing salinity. On the other hand, the mean percentage of larvae alive at the end of the experiment was lower at 24°C (30–55%) than at 15°C (75–82%), apparently influenced by the salinity, such that survival decreased as salinity increased (Table 2). In summary, increasing the temperature from 15 to 24°C reduced the percentage of hatching and survival of the larvae; the increase in salinity reduced larval survival at 24°C, with a slight increase in hatching at either temperature.

Recently hatched larvae showed a boring tooth, oesophagus and a developing intestine, which frequently presented 1 or 2 large clear areas in both anterior and posterior zones (Fig. 5A), as well as a striated sheath corresponding to the cuticle of the previous larval stage (Fig. 5). As the larvae developed, the nerve ring, the excretory duct, the ventriculus, the ventricular appendix — with differing degrees of development — and the rectum could also be observed (Fig. 5B). The intestinal caecum could not be observed at this stage, at least not during the period of maintenance.
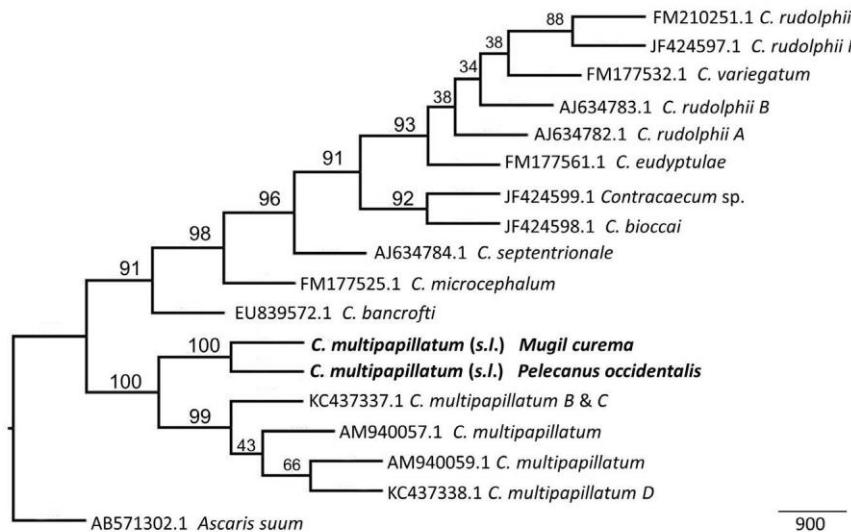


Fig. 2. Neighbour-joining reconstruction between sequences of *Contracaecum multipapillatum* obtained in this study (in **bold**, also showing the host) and sequences of *Contracaecum* species collected from birds, obtained from the NCBI database, with the tree inferred from the ITS-1 data set. The numbers on the tree branches represent the percentage of bootstrap resampling (with 1000 replicates). *Ascaris suum* was used as an outgroup. GenBank accession numbers are in front of species names.

Live larvae collected from the previous experiment at 24°C were placed in wells with 1 ml of Grace's modified medium at the same temperature. These larvae were ensheathed (Fig. 5) and measured $261.0 \pm 38.1 \times 15.8 \pm 2.9$ µm (n = 23), or $297.8 \pm 16.3$ µm (n = 8) including the sheath. The larvae tended to be highly mobile during this stage, exhibiting a flicking motion, but with periods at rest. Some larvae adhered to each other or to fibrous substrates in the culture medium, using the posterior end of the sheath, which finishes in a point (Fig. 5C and see Video S1 in the Supplement at www.int-res.com/ articles/suppl/d125p167_supp/). After 14 d under these conditions, the larvae had grown sufficiently to fill the sheath while some started to free themselves upon reaching a length of around 315 µm ($314.4 \pm 51.1 \times 18.5 \pm 2.0$ µm, n = 7). The larvae freed themselves from the sheath at different times using rapid movements. However, some failed to achieve this under our experimental conditions (Fig. 6A). Over the 25 d of experiments, ~2% of the larvae exsheathed, growing more than 70 µm ($333.4 \pm 39.2 \times 21.9 \pm 5.8$ µm, n = 3) with no new moulting observed. The freed larvae exhibited a more serpentine and less vigorous type of movement than that of the ensheathed larvae and tended not to adhere as much to the substrates of the medium using the posterior end. At the end of the experiment, more than 50% of the larvae were dead. When culture was carried out at 15°C, a similar pattern was observed, although exsheathment did not occur within the timespan of the experiment but was observed later. Exsheathed larvae observed by optical microscopy also revealed an oesophagus, ventriculus, ventricular appendix, incipient intestinal cecum, intestine, rectum and the excretory cell, excretory duct and excretory pore (Fig. 6). From when the larvae began to exsheathe the intestinal contents were seen to actively move, alternating between the ventriculus and the rectum (see Video S2).



Fig. 3. Eggs of *Contracaecum multipapillatum s.l.* under an optical microscope. Egg 'a' has 3 layers, with the outer layer slightly rough. Egg 'b' has 2 smooth layers and contains larvae, and egg 'c' is empty after hatching of the larvae, with the exit hole visible (∗)

## 4. Discussion

The nematodes collected from the brown pelican and mullet were identified as belonging to the complex of species of *Contracaecum multipapillatum s.l.* (K2P < 0.07), clearly separated from other species and complexes of *Contracaecum* that infect birds (K2P > 0.30; Table 1, Fig. 2), as shown by phenetic analysis.

Most of the eggs collected from the uteri of *C. multipapillatum s.l.* females obtained from the brown pelican were already embryonated, probably as a result of the time elapsed between the collection of the nematodes and the removal of the eggs. Huizinga (1967) reported that the eggs collected from the uteri of females were either not embryonated or were in a 2- or 4-cell state. Various authors have described a certain degree of embryonation (up to the morula stage) in both anisakid eggs taken from the uterus and those passed in faeces of the host (Thomas 1937, Grabda 1976, Brattey 1990). However, Adroher et al. (2004) reported that the eggs spontaneously liberated in culture by females of *Hysterothylacium aduncum*, a gastrointestinal anisakid of fish, were not embryonated. The eggs collected in the present study measured approximately 53 × 43 µm. Vidal-Martínez et al. (1994) reported eggs measuring 53 × 38 µm, Huizinga (1967) reported 65 × 58 µm, and Lucker (1941) reported ca. 60 × 50 µm. Although the last 2 measurements are greater than those found in the present study, they are similar to those for the eggs with the developed larvae inside, which measured up to 66 × 55 µm, probably due to the lack of rigidity and elasticity of the egg shell associated with the movement of the larva inside the egg, as suggested by Adroher et al. (2004). The eggs exhibited negative buoyancy in all solutions, as in other anisakids (Huizinga 1966, Adroher et al. 2004). This could explain why mullet, which are catadromous fish, are usually infected with *Contracaecum* in endemic zones, since they feed on detritus, micro-algae and benthic organisms in sandy- or muddy-bottomed coastal areas where the eggs and larvae of these parasites can be found, either free or within copepod hosts (Salgado-Maldonado & Barquín-Álvarez 1978, Deardorff & Overstreet 1980, Chávez López & Montoya Mendoza 1988, Juárez-Arroyo & Salgado-Maldonado 1989).

According to Huizinga (1967), the shell of the eggs of *C. multipapillatum* is thin with 2 layers: a transparent, adhesive and lightly mamillated outer layer and a thin inner vitelline layer. Although some eggs in the present study had 2 apparently smooth layers, many seemed to have 3 layers, the outermost layer being slightly rough (Fig. 3). Among anisakid eggs, a double covering, with a smooth or slightly undulating outer layer is frequently present (see Anderson 2000 for references); however, a triple covering with a rough outer layer has been reported in another anisakid (Adroher et al. 2004).

Many authors, working with different anisakids, have shown that the development of the eggs is directly related to the maintenance temperature, such that they develop more rapidly with increasing temperature, with a maximum around 13–20°C. Temperature has also been shown to affect the infectivity of the larvae for the first host, at least in the anisakid *Pseudoterranova decipiens* (see Palm 1999 for references). The salinity of the medium has also been shown to have an effect in *Anisakis simplex*, with increasing numbers of eggs hatched when

salinity was 10‰ or greater (Højgaard 1998). In the present study, hatching of *C. multipapillatum s.l.* was greatest at 15°C, tending to increase slightly with salinity, both at 15°C and at 24°C (Table 2). The fact that we found only slight differences with temperature could be due to working within a range of high temperatures (15–24°C). The hatching percentages reported for different species of anisakids vary greatly, not only due to experimental conditions but also to the species and strains employed (see Bier 1976 for references). Hatching was high in the present study (61–98%).
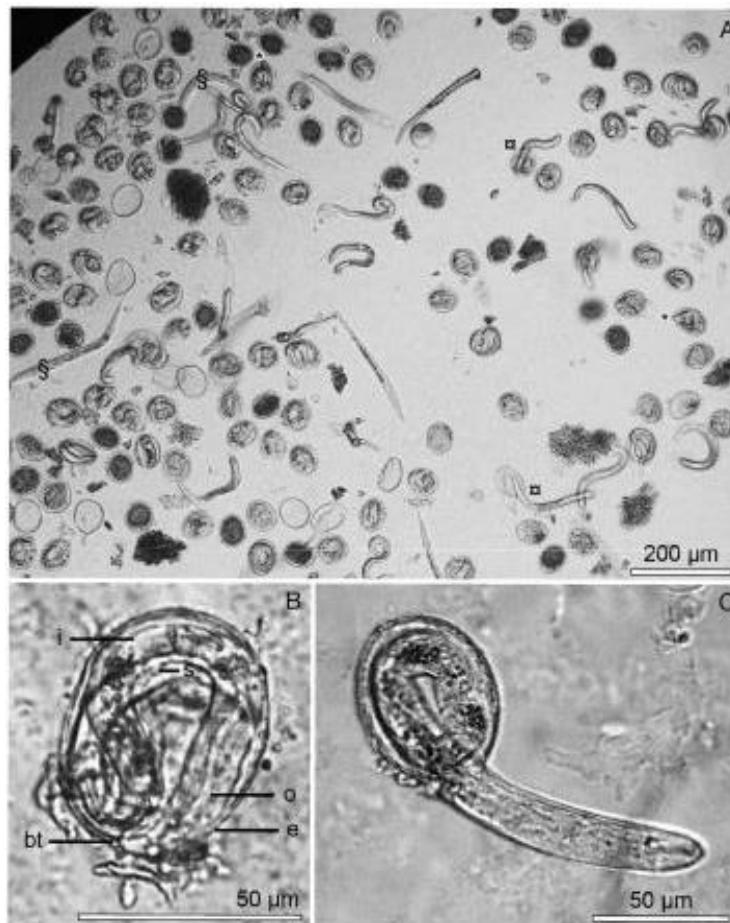


Fig. 4. Eggs and larvae of *Contracaecum multipapillatum s.l.* during development. (A) Larvae within eggs, with larvae pushing the eggshell to hatch (∗), larvae hatching (¤), empty eggs, larvae with sheath and some exsheathed larvae (§); note that these are larger than ensheathed larvae. (B) Larva in an egg, with the boring tooth (bt), oesophagus (o), intestine (i) and sheath (s) visible. (C) Larva during hatching

The decrease in the percentage of live larvae at the end of the experiment at 24°C compared to at 15°C and its relationship with salinity at 24°C may be due to a more rapid development/metabolism of the larvae as a result of the higher incubation temperature and the efforts of the larvae to maintain osmotic equilibrium. Both would require greater energy expenditure by the larvae, still ensheathed, resulting in their exhausting their energy reserves within the saline medium, which lacks exogenous energy substrates. Similar results were

obtained for C. rudolphii s.l. collected from cormorants (Dziekonska-Rynko & Rokicki 2010). The high rate of hatching and survival of the larvae in all of the saline solutions used in our study (Table 2), particularly at 15°C, suggests that the life cycle of this parasite can take place both in the sea and in fresh or brackish water. This has also been suggested for other anisakids such as C. osculatum baicalensis, *H. aduncum* and *P. decipiens* (Sudarikov & Ryzhikov 1951, Yoshinaga et al. 1987, Burt et al. 1990). The presence or absence of light during culture at 24°C had no significant effect on the results (not shown).

Table 2. Effect of salinity and temperature on development and hatching of eggs and survival of hatched larvae of *Contracaecum multipapillatum s.l.* Data are mean (range) for eggs from 2 different females, except where marked. Experiments were performed in triplicate

| Temp (°C) | NaCl (‰) | Unhatched eggs (%) | Hatched eggs (%) | Larvae alive (%) |
|---|---|---|---|---|
| 15 | 9[a] | 12 | 88 | 75 |
| | 28 | 19 (12–27) | 80 (73–88) | 82 (74–90) |
| | 35 | 3 (2–5) | 97 (95–98) | 81 (79–82) |
| 24 | 9 | 29 (19–39) | 71 (61–81) | 55 (48–62) |
| | 28 | 25 (22–28) | 75 (72–78) | 40 (36–44) |
| | 35 | 20 (18–21) | 80 (79–82) | 30 (12–47) |

[a]Eggs from a single female
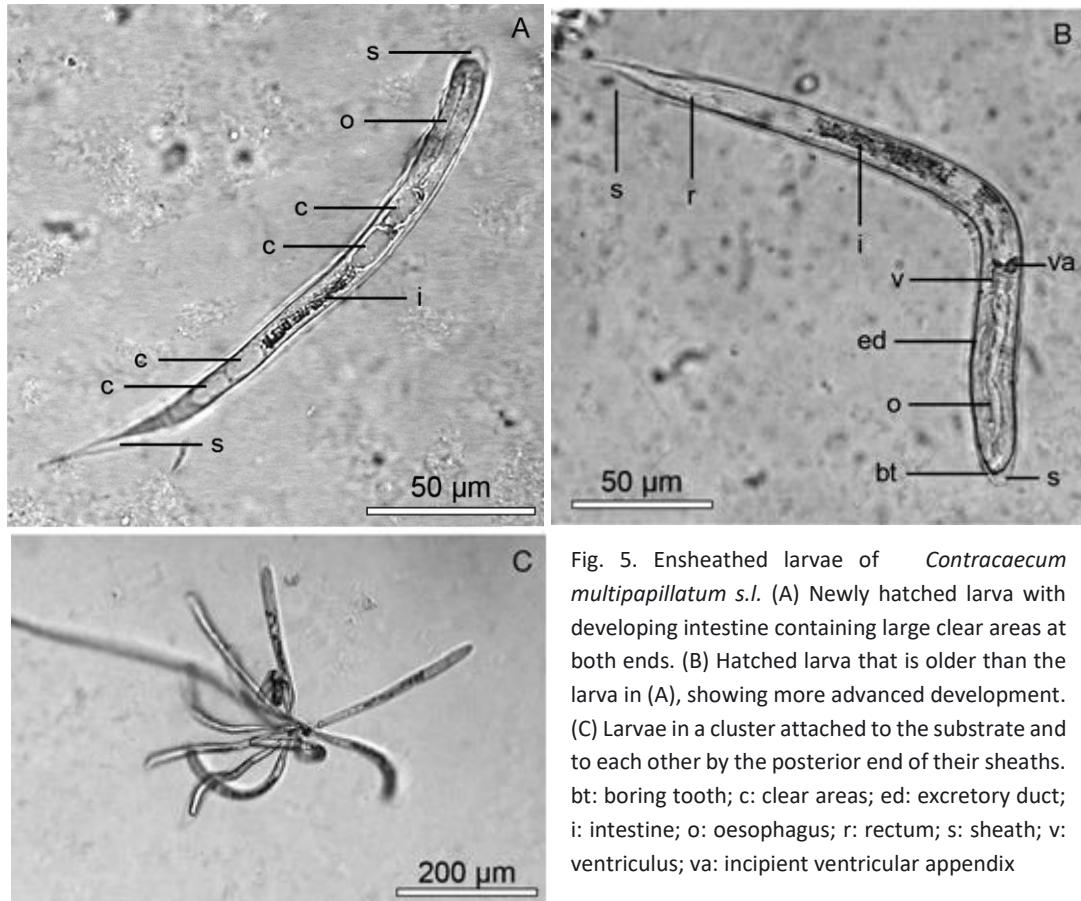
Fig. 5. Ensheathed larvae of *Contracaecum multipapillatum s.l.* (A) Newly hatched larva with developing intestine containing large clear areas at both ends. (B) Hatched larva that is older than the larva in (A), showing more advanced development. (C) Larvae in a cluster attached to the substrate and to each other by the posterior end of their sheaths. bt: boring tooth; c: clear areas; ed: excretory duct; i: intestine; o: oesophagus; r: rectum; s: sheath; v: ventriculus; va: incipient ventricular appendix

Much debate surrounds the larval stage which hatches from anisakid eggs. Køie (1993), Køie & Fagerholm (1993) and Køie et al. (1995) found that a larva is enclosed by 2 cuticles before hatching and is consequently third-stage, at least in *H. aduncum*, *C. osculatum*, *A. simplex* and *P. decipiens.* Smith et al. (1990) successfully infected trout intraperitoneally with larvae of *C. osculatum* which had recently been hatched (~0.4 mm in length), and they studied the development of the larvae up to 13 mm and with L3 characteristics, without observing any moulting. Thomas (1937) had already shown that it was the L3 that emerged from the egg of *C. spiculigerum* (=*C. rudolphii)* after 2 moults inside it. Similar results were reported by Moravec (2009). In this respect, in the other anisakids mentioned it has not been possible so far to demonstrate the existence of new moults after hatching and before reaching the final host, suggesting that, at least in most anisakids, the L3 hatches from the egg. However, other authors believe that it is the L2 that hatches from the egg (Huizinga 1966, 1967, Measures & Hong 1995, Bartlett 1996).
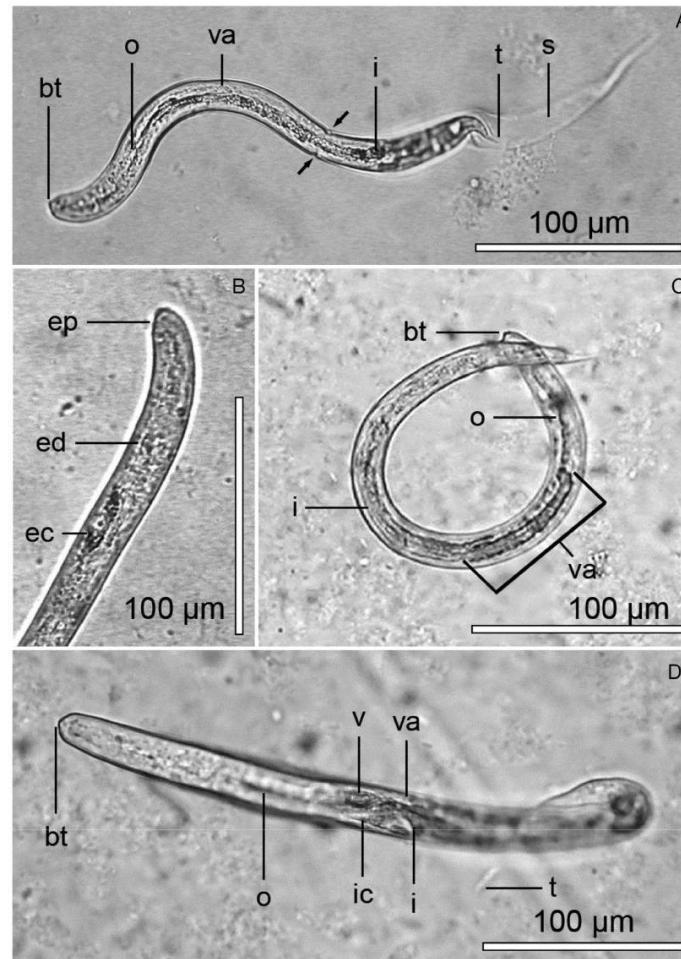
Fig. 6. (A) Exsheathing larva and (B–D) exsheathed larvae of *Contracaecum multipapillatum s.l.* bt: boring tooth; ec: excretory cell; ed: excretory duct; ep: excretory pore; i: intestine; ic: incipient intestinal caecum; o: oesophagus; s: sheath; t: tail; v: ventriculus; va: ventricular appendix. The arrows in panel (A) indicate the strangulation caused by the sheath during larval exsheathment

To our knowledge, our study is the first to attempt the laboratory culture of hatched *C. multipapillatum* larvae in a nutritive medium. If the proposal of Huizinga (1967) is correct, then the larvae obtained from hatching the eggs of *C. multipapillatum s.l.* must be L2, despite the belief of many authors that the larvae hatching from the eggs of the anisakids studied are L3 (see above). The size of the recently hatched larvae (261 × 16 µm) is smaller than the 362 × 13 µm reported by Huizinga (1967). These larvae exhibited a flicking motion, which has also been described in other species of anisakids (Davey 1969, McClelland & Ronald 1974a, Adroher et al. 2004), and they were often attached to each other by the posterior end of their sheaths, forming clusters, or to eggshells or to substrates in the medium (Video S1), in a similar way to that described for hatched larvae of *C. spiculigerum* (= *C. rudolphii*), *P. decipiens* or *A. simplex* (Thomas 1937, Huizinga 1966, McClelland & Ronald 1974b, Højgaard 1998). The culture

medium, at 24°C, permitted some growth in the larvae, leading to exsheathment and a subsequent increase in diameter up to 22 μm as well as a change to a slower, less vigorous serpentine swimming movement. This change has been observed in other anisakids such as *H. aduncum* (R. Benítez & F. J. Adroher unpublished) and *C. osculatum* (Davey 1969). As other authors have also shown, an increase in temperature results in more rapid development of the eggs and larvae (McClelland 1990, Measures 1996). This would explain the exsheathment in the medium at 24°C, but not at 15°C, in the 25 d of the experiment, although, at the latter temperature, this was observed some days after completing data collection. However, in no case were additional moults observed.

According to Huizinga (1967), exsheathment takes place in the intestine of the copepod, the first host of the parasite, as has also been observed in *C. osculatum* (Davey 1969). The larvae grow within the sheath, often filling it completely, before freeing themselves of it. We observed larvae in the process of exsheathing in which the pressure exerted by the sheath on the body of the larva at the point of exit can be seen to be strangling it (Fig. 6A). In fact, in the cultures some larvae were seen to be unable to exsheathe completely. Although it is not known whether digestive enzymes of the copepod are needed to facilitate or stimulate exsheathment, proteases have been shown to be involved in hatching, exsheathment and moulting in nematodes (reviewed by Malagón et al. 2013). Finally, the description of recently ex sheathed larvae (Fig. 6) is very similar to that of L3 found in the fish hosts of *C. multipapillatum s.l.*, although less developed and smaller. This suggests that it could be the L3 larval stage that hatches from the egg in this nematode, as occurs in other anisakids (see above). These larvae appear to have a functional intestine, since, on exsheathing, intense movement of the intestinal content was observed (Video S2). This was probably a result of entering into direct contact with the medium on escaping from the sheath, although it is not known whether the larvae at this stage were able to ingest food.

The high prevalence of infection by *Contracaecum* spp. in mullet (95% in *Mugil curema* and 100% in *M. cephalus)* and pelicans (83% in *Pelecanus occidentalis)* in the area of La Paz, Mexico (Iglesias et al. 1998, Valles-Vega 2011, 2014), which probably maintains the life cycle of *C. multipapillatum s.l.* in this geographical zone (Fig. 7), could be explained by the following: the density of the eggs of *C. multipapillatum s.l.*, which is greater than that of seawater, their ability to develop and the survival of the larvae at both temperate (15°C) and high temperatures (24°C) and at different salinities (9, 28 and 35‰), and, finally, on the one hand, the catadramous behaviour and benthic feeding habits of the mullet, and, on the other hand, the frequent consumption of these fish by pelicans (Humphrey et al. 1978). Cannon (1977) noted that *Contracaecum* and *Thynnascaris* (=*Hysterothylacium)* occur principally in bottom feeders.

Regarding the possible first crustacean host of the parasite in this zone, Valles-Vega (2014) made several unsuccessful attempts to experimentally infect omnivorous marine copepods such as *Acartia clausi*, *A. lilljeborgi* and *Centropages furcatus* from Bahía de La Paz or *Calanus helgolandicus*, *Centropages typicus* and *Acartia* sp. from Motril waters (northern

Alborán Sea in the western Mediterranean Sea), with recently hatched larvae. However, Huizinga (1967) was able to experimentally infect the freshwater copepod *Cyclops vernalis*.
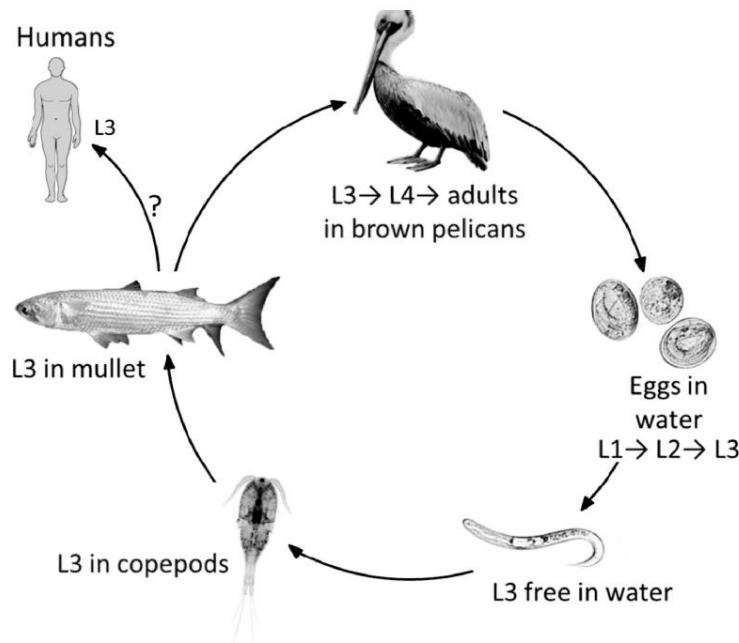


Fig. 7. Tentative life cycle of *Contracaecum multipapillatum s.l.* in the geographical area of Bahía de La Paz, Gulf of California, Mexico

Previous studies in Bahía de La Paz showed that surface water temperature varies during the annual cycle from a minimum average of 20.5°C in winter to an average maximum of 31°C in summer, not significantly varying until 100 m depth (Obeso-Nieblas et al. 2008, Guevara-Guillén et al. 2015). Salinity shows minimal annual changes depending on river inputs and evaporation, ranging between averages of 34.5‰ in winter and 39‰ in summer (Chávez-Sánchez 2012). Finally, in the study area, Palomares-García (1996) showed that the copepods, intermediate hosts of *Contracaecum*, occur throughout the year, and their abundance is related to the seasonal hydrographic changes, with up to 129 species of copepods documented (Aceves-Medina et al. 2007 for references), the most abundant being *A. clausi*, *A. lilljeborgi* and *C. furcatus* (S. Hernández-Trujillo unpublished data). There are also mullet (intermediate/ paratenic hosts) and pelicans (final hosts) throughout the year (I. Valles-Vega unpublished data). These data show that the life cycle of *C. multipapilatum* s.l. is likely to occur throughout the year in this area of the Gulf of California, since, as we have shown, *C. multipapilatum* L3 from the mullet and *C. multipapilatum* adults from the brown pelican correspond to the same species (K2P = 0).

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Compliance with ethical standards

Conflicts of interest: the authors declare that they have no conflict of interest.

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# A scanning electron microscopy study of early development *in vitro* of *Contracaecum multipapillatum s.l.* (Nematoda: Anisakidae) from a brown pelican (*Pelecanus occidentalis*) from the Gulf of California, Mexico

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**a b s t r a c t**

Eggs obtained from the uteri of female nematodes, genetically identified as *Contracaecum multipapillatum s.l.*, found in a brown pelican (*Pelecanus occidentalis*) from Bahía de La Paz, Gulf of California, Mexico, were used to study the early developmental stages of this anisakid by scanning electron microscopy (SEM). Egg dimensions were approximately 54 × 45 μm measured by SEM. Observation of the eggs revealed an outer surface of fibrous appearance. The newly hatched larvae were ensheathed and highly motile. Observation with SEM showed that the sheaths of the larvae were striated and revealed an excretory pore and a cleft near the anterior end of the sheath, presumably to facilitate the opening of the sheath for the emergence of the larva. The hatched larvae were placed in nutritive culture medium, where they grew within their sheath, some exsheathing completely 2 weeks later. The surface patterns of the sheath and the cuticle of the exsheathed larvae were clearly different. Although they did not moult during culture, SEM revealed a morphology typical of third-stage larvae of *Contracaecum* from fish, as previously observed by optical microscopy. Thus, we suggest that newly hatched larvae from eggs of *C. multipapillatum* are third larval stage but with sheath of the second larval stage, as occuring in other anisakids.

## 1. Introduction

The anisakids (family Anisakidae) are a group of nematodes which parasitize the digestive system of fish, fish-eating birds and marine mammals. After fertilization by the male, the female releases eggs which are passed into the water with the faeces of the definitive host. The eggs then develop at ambient temperature. It is still unclear which larval stage hatches from the egg: while some authors believe it to be the second larval stage, L2 (Huizinga 1966; Huizinga 1967; Measures and Hong 1995; Anderson 2000), others believe it to be L3 (Thomas 1937; Køie 1993; Køie and Fagerholm 1993; Køie et al. 1995; González 1998; Adroher et al. 2004; Moravec 2009). Furthermore, although in some species, the larva appears to hatch in the water where it is ingested by the first intermediate host, usually an aquatic invertebrate (copepods, euphasiids,…), for other species, it has been suggested that the larva hatches when the egg is broken by the mouthparts of these invertebrates during ingestion (Køie 1993; González 1998). The larvae grow in these intermediate hosts and become infective for the next intermediate hosts: fish and squid, although, in *Contracaecum* rudolphii, the direct experimental infection of fish with recently hatched larvae has been demonstrated (Thomas 1937; Huizinga 1966; Dziekońska-Rynko et al. 2008; Moravec 2009). When L3 is sufficiently developed, it is then able to infect the final host and complete the cycle. This life cycle is complex, involving one or more paratenic hosts (invertebrates, fish and squid).

In the genus *Contracaecum*, copepods seem to be the first intermediate hosts, fish the second intermediate/paratenic hosts and fish-eating birds or marine mammals the definitive hosts. In *Contracaecum multipapillatum s.l.*, the fish are often mullet (several species) and the definitive hosts are birds, generally of the family Pelicanidae (Humphrey et al. 1978; Deardorff and Overstreet 1980; Iglesias et al. 1998; Valles-Ríos et al. 2000; Mattiucci et al. 2010; Iglesias et al. 2011; Valles-Vega et al. 2017). Humans can accidentally interfere with the anisakid life cycle if they ingest live L3 with raw fish or that which has not undergone suitable heating or freezing processes. These larvae can cause digestive problems resulting in anisakiasis. Although approximately 97% of these cases are due to *Anisakis* spp. and 3% to *Pseudoterranova* spp. (Rello-Yubero et al. 2004), at least five cases involving *Contracaecum* spp. have been reported (see Valles-Vega et al. 2017 for references) and at least one for an immature female of *Hysterothylacium aduncum* (Yagi et al. 1996).

The present study describes for the first time the eggs from a female uterus of *C. multipapillatum s.l.*, the larval stages hatching from these eggs and early developmental stages in *in vitro* culture using scanning electron microscopy.

## 2. Materials and methods

2.1. Collection and culture of parasites

The uteri of two females of *C. multipapillatum s.l.* obtained from the digestive tract of one brown pelican (*Pelecanus occidentalis*) from Bahía de La Paz, BCS, Mexico, were dissected to extract the eggs. These eggs were processed for *in vitro* maintenance in physiological saline solution and the hatched larvae were cultured in nutritive modified Grace's medium

supplemented with 20% (v/v) fetal bovine serum at 24 °C, as described by Valles-Vega et al. (2017). The larvae obtained after egg hatching and at different stages of development in culture were processed for SEM, as was a uterus with eggs of a third female of the parasite from the same pelican.

## 2.2. Molecular identification

The three females of the nematode were processed for genetic identification using the sequence of ITS1-5.8-ITS2 from nuclear ribosomal DNA. This procedure has been described previously (Valles-Vega et al. 2017). Briefly, DNA extracted from the nematode specimens was amplified with primers described by Zhu et al. (2000) and then purified and sequenced by MacroGen (South Korea). Sequences were aligned with the Clustal X software. Neighbour Joining analysis, based on Kimura-2-parameters (K2P) distance (Kimura 1980) values, was used to construct phenetic trees, using the MEGA 5.05 software (Tamura et al. 2011) and reliability of the measure of stability of the branches was tested by 10,000 bootstrap replications. The comparison of the ITS1 sequence with another 15 from *Contracaecum* from fish-eating birds, and the sequence of *Ascaris suum* as an outgroup, deposited in the GeneBank was carried out (Fig. 1).
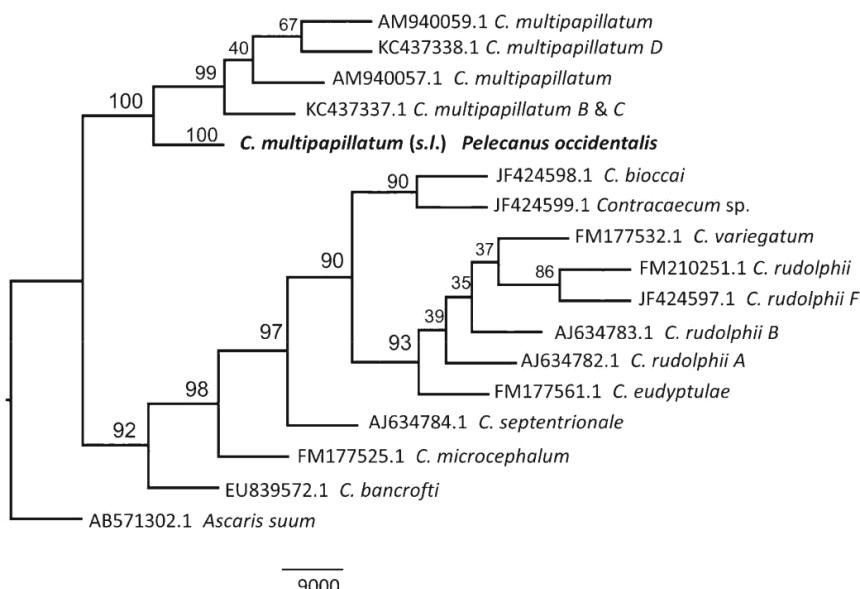


Fig. 1 Neighbour Joining reconstruction between sequences of *Contracaecum multipapillatum* collected from brown pelican (in bold, indicating the host) to this study and sequences of *Contracaecum* species of birds from the NCBI database, with the tree inferred from the ITS1 data set. The numbers on the tree branches represent the percentage of bootstrap resampling (with 10,000 replicates). *Ascaris suum* was used as an outgroup. The GenBank accession nos. are in front of species names

## 3. Results

The females of *Contracaecum* from the brown pelican were genetically identified as *C. multipapillatum s.l.* (K2P < 0.07 and Fig. 1) and markedly separated from other species of *Contracaecum* from fish-eating birds (K2P > 0.30 and Fig. 1). Also, all three females showed an identical ITS1-5.8-ITS2 sequence. The eggs from the uteri of these females (Fig. 2), examined using SEM, were ovoid, measuring ± standard deviation $54.0 \pm 2.9 \times 44.8 \pm 2.0$ µm (n = 13). Using SEM, the external layer of these eggs appeared smooth (Fig. 3a), but, at higher magnification, the surface was seen to consist of a fibrous structure (Fig. 3b). This was laid out in plateaux surrounded by incipient ridges (Fig. 3c), although some eggs also exhibited eggshell areas with more or less parallel ridges (Fig. 3d). Furthermore, a rounded area, differentiated from the rest of the surface, was observed on some eggs. This may be an opercular region (Fig. 4).
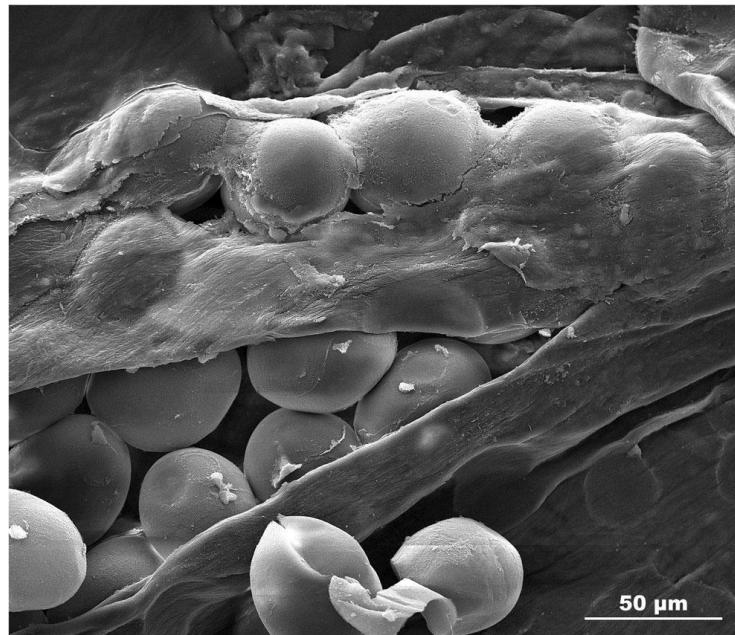


Fig. 2 Uterus with eggs of a gravid female of *Contracaecum multipapillatum s.l.* viewed with SEM

Using SEM, the recently hatched larvae in saline solution measured $209 \pm 21 \times 14.7 \pm 0.9$ µm (n = 5) excluding the sheath. They showed a striated sheath (Fig. 5) with annuli occasionally subdivided into two (Fig. 6), the sheath corresponding to the cuticle of the previous larval stage. An oval excretory pore of ca. $0.14 \times 0.24$ µm diameter (Fig. 5b, inset) was observed at its anterior end. This pore was ~ 0.7 µm from the first striation of the sheath and 1.4–1.7 µm from a cleft situated between the first and second annulus (Fig. 5b). The posterior end gradually narrow, finishing in a blunt point (Fig. 5c) with adhesive properties (Fig. 5d). During preparation of these larvae for SEM, the sheath contracted, emphasizing the body of the larva within it. Although the sheath appeared to have a tooth (Fig. 5b), the sheath was actually covering the boring tooth of the L3 below. Consequently, the position of the excretory pore was not related to the position of the tooth, as is usually the case in descriptions of L3. These ensheathed larvae were placed in nutritive culture medium and allowed to grow to fill their sheaths. After 2 weeks, when the larvae measured on average $273 \pm 31$ µm (n = 5) by SEM, some larvae began to exit from the

sheath (Fig. 7), apparently as a result of a rupture of the sheath at the above-mentioned cleft, near (~ 3 µm) the anterior end (Fig. 5b). The larvae freed themselves from the sheath at different times using rapid movements. The freed larvae also adhered to substrates by the tail, although less frequently than ensheathed larvae. No new moulting in the culture medium was observed during the duration of the experiments (2 months).
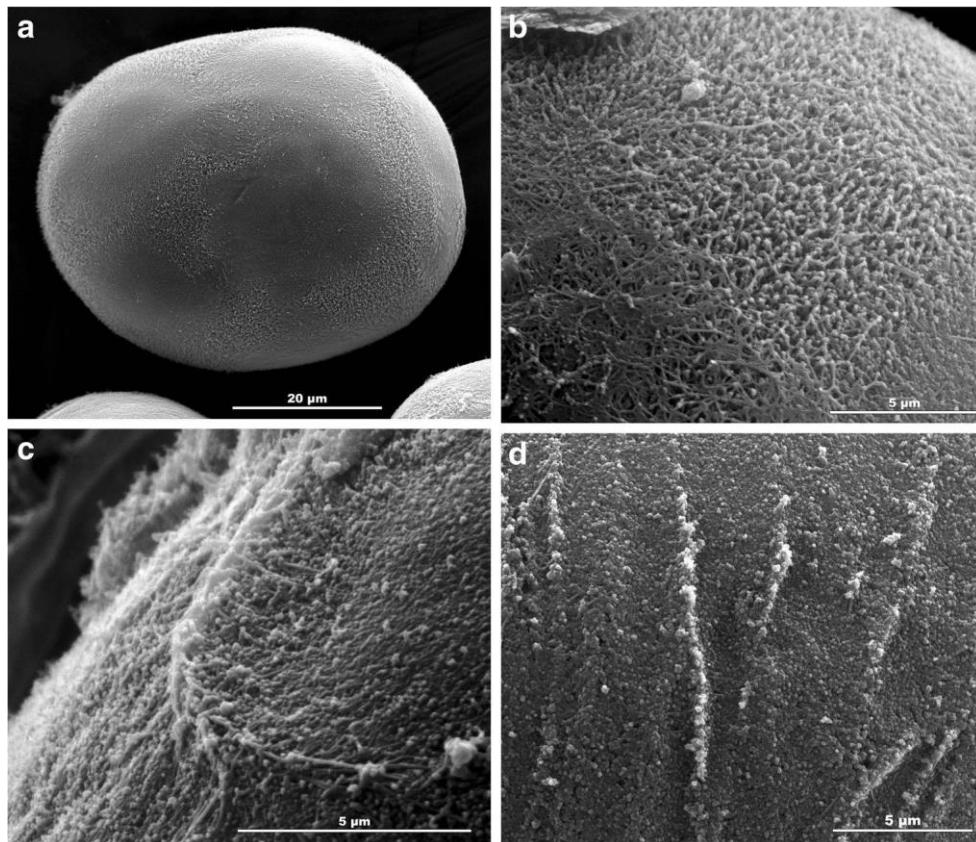


Fig. 3 Eggs of *Contracaecum multipapillatum s.l.* (SEM). a Egg with smooth covering with plateaux and ridges. b Detail of the fibrous surface. c Detail of ridges around plateaux. d Egg surface ridges more or less parallel

Observed with SEM, the exsheathed larvae exhibited a striated cuticle (Fig. 6b), except at the anterior end, where it was smooth (Fig. 7b). The first striation was observed some 3–4 µm from the anterior end (the width of the larva at this point ~ 9–10 µm). The mean measurement of the annuli was 0.64 µm (range 0.46–0.85 µm) with non-uniform parallel vertical bands (Fig. 6b). These larvae have a boring tooth (height ~ 1.5 µm) with an excretory pore located at its base (Fig. 7b inset) between the ventrolateral lips, which appears oval in shape. Delimiting the mouth, which appeared as a groove, are the lips, relatively undeveloped, with papillae (2 dorsolateral papillae on the dorsal lip and 1 papilla on each of the ventrolaterals, Fig. 7b), incipient interlabium, mouth, and lateral cuticular suture, starting with the first cephalic striation (Fig. 7b) and running along the body (Fig. 7c) on both sides, almost as far as the tail of the larva, which finishes in a slightly thickened, unornamented, blunt point (Fig. 7d).
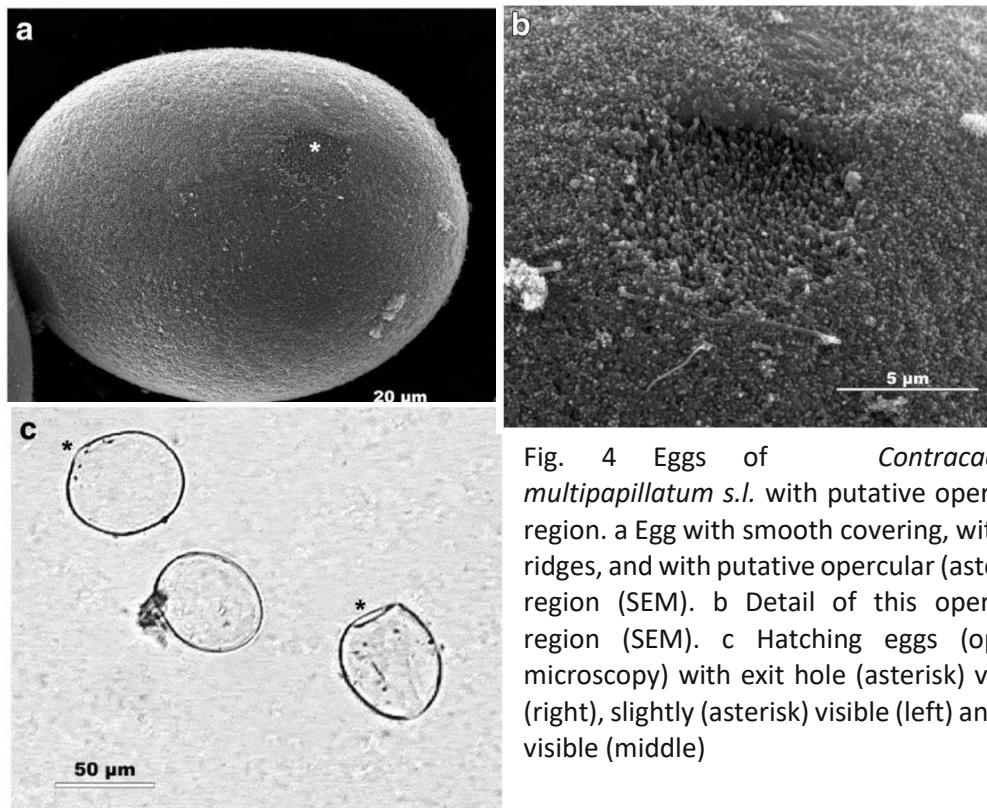
Fig. 4 Eggs of *Contracaecum multipapillatum s.l.* with putative opercular region. a Egg with smooth covering, without ridges, and with putative opercular (asterisk) region (SEM). b Detail of this opercular region (SEM). c Hatching eggs (optical microscopy) with exit hole (asterisk) visible (right), slightly (asterisk) visible (left) and not visible (middle)

## 4. Discussion

The eggs of *C. multipapillatum s.l.* collected in the present study measured approximately 54 × 45 μm by SEM. These values are similar to those obtained by optical microscopy by Vidal-Martínez et al. (1994) 53 × 38 μm (parasites from the southern Gulf of Mexico), and Valles-Vega et al. (2017) who reported 53 × 43 μm for the eggs of the females, molecularly identified, from the same brown pelican host (parasites from the southern Gulf of California). Other authors reported higher values such as 60 × 50 μm (Lucker 1941) and 65 × 58 μm (Huizinga 1967). These differences may be due to intraspecific variability or that they correspond to different species from the same sibling species complex.

According to Huizinga (1967), the eggshell of *C. multipapillatum* had a lightly mamillated outer surface, although Valles-Vega et al. (2017) reported eggs with a smooth or slightly rough surface by optical microscopy. Observed with SEM, the outer layer of the eggs was smooth (Figs. 2, 3a, and 4a). However, at higher magnification, it was seen to be generally formed of plateaux surrounded by incipient ridges with a fibrous appearance (Fig. 3b, c), although, in some eggs, the outer surface appeared uniform (Fig. 4a). The plateaux may be due to the pressure exerted on the eggs in the uterus, forcing them close together (Fig. 2), which could create these plateaux and their corresponding ridges. This gives these areas the appearance of a crater with wilted fibres oriented towards the outside of the plateaux (Fig. 3c). The greater or lesser development of these ridges may determine whether the outer surface appears slightly rough or smooth (Fig.

3), as described previously in *C. multipapillatum* (Huizinga 1967; Valles-Vega et al. 2017) and other anisakids (see Anderson 2000). The fibrous material of the eggshells (Figs. 3 and 4) has been described previously in the uterine layer (the outermost layer) of the eggs of other ascaridoids and may be composed of proteins and mucopolysaccharides (Cruthers et al. 1974; see Wharton 1980 for review). Moreover, in some eggs, a rounded zone, differentiated from the rest of the surface, was observed, which could serve as an exit during hatching (Fig. 4). This is also described as an opercular region in some ascaridoids (Ubelaker and Allison 1975).
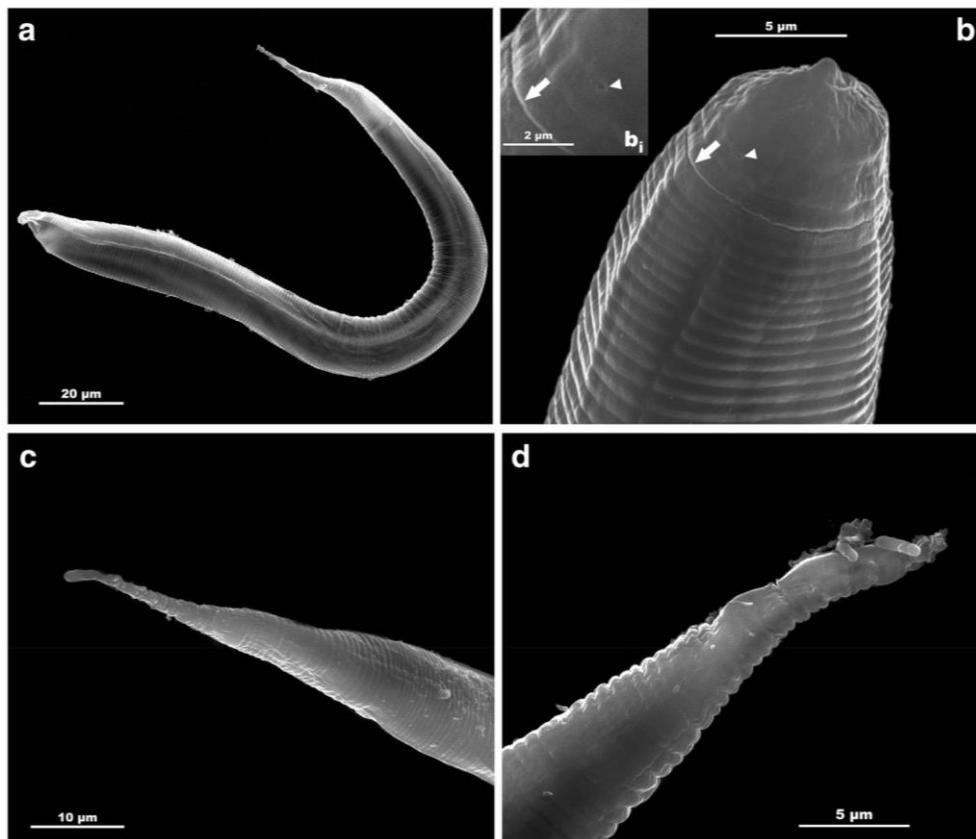


Fig. 5 Ensheathed larvae of *Contracaecum multipapillatum s.l.* a Larva. b Anterior end with boring tooth under the sheath; note the cleft in the sheath (arrow) where it could break and allow the larva to emerge. Inset (b$_i$) shows the excretory pore of the L2 on the sheath (arrowhead). c, d End of tail of larvae with adhesive blunt point; note material adhered in d

The SEM photos show hatched larvae in which the sheath covering them is seen to be striated. In some parts of the sheath, the annuli can be seen to subdivide in two, which may be a growth process of the cuticle (Fig. 6a). Near the anterior end of the sheath is a cleft (Fig. 5b) where it can be supposed that the sheath breaks, thus allowing the larva to emerge from the sheath at the anterior end. Huizinga (1967) described it thus: the anterior end of the sheath became swollen and a cap separated from the remainder of the sheath, permitting the larva to escape, which is consistent with the cleft detected in the present study (Fig. 5b). According to this author, exsheathment takes place in the intestine of the copepod, the first host of the

parasite, prior to the larva entering the haemocoel, as has also been observed in other species of *Contracaecum* both those parasitizing birds and those parasitizing seals (Huizinga 1966; Davey 1969; Moravec 2009). It is still not known whether this exsheathment is simply a physical action resulting from the pressure of the larva on the sheath or whether substances secreted by the larvae are involved. The role of nematode proteases, not only in the nutrition and development of the parasite, but also in hatching, exsheathment and moulting has recently been examined (Malagón et al. 2013). Live larvae observed by optical microscopy measured a mean of 260 μm (Valles-Vega et al. 2017) while those measured by SEM were only 210 μm. The same occurred with larvae measured at the start of exsheathment (315 vs. 270 μm, respectively). This discrepancy of 15–20% may be a consequence of the method used to prepare the larvae for observation by SEM. In any case, the increase in size from hatching to exsheathment is still ~ 55–60 μm. It is likely that this increase in size of the larva is involved in the breaking of the sheath at the cleft (Fig. 5b), although the release of substances by the larva to aid escape from the sheath should not be discounted.

The characteristics observed in the exsheathed larva (Fig. 7) coincide with those of a typical L3 of *Contracaecum* from a fish host, as described previously with SEM (Valter et al. 1982; Weerasooriya et al. 1986; Fernández-Bargiela 1987; Valles-Vega and Gómez del Prado-Rosas 2014). Also, no cephalic collar, described by Chandler (1935) thus: BJust behind head cuticle conspicuously marked with annulations, which are very close together and end rather abruptly, was observed, nor was a spine at the posterior end (Fig. 7d), described by some authors in the most well-developed L3 (~ 2–3 cm) found in host fish (Fernández-Bargiela 1987; Valles-Vega 2011; Valles-Vega and Gómez del Prado-Rosas 2014), although Chandler (1935) described it as a Bdemarcated conical lobe rather than a spine. The cuticle does not show subdivided annuli like the sheath, probably because they are recently formed. Different superficial patterns have been observed in the cuticle of other anisakids, depending on the species and developmental stage (Valter et al. 1982; Weerasooriya et al. 1986).
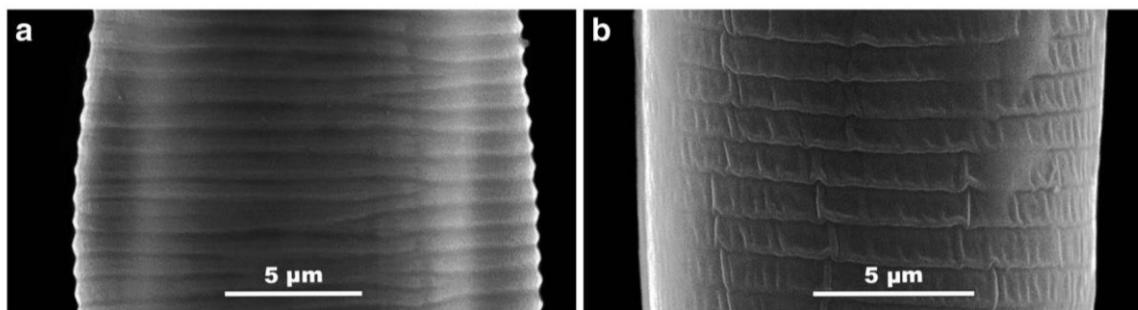


Fig. 6 Cuticle of *Contracaecum multipapillatum s.l.* a Sheath from ensheathed larva, note division of some annuli. b Cuticle of exsheathed larva, note the vertical bands and the thickening of the lower edge of the annuli. As shown in Fig. 7b, it appears that when the larva is curved, the upper part of an annulus is introduced into the lower part of the previous one on the inner side of the curve, while on the outer side, the annuli are fully expanded

Finally, Moravec (2009), in C. rudolphii, which parasitizes cormorants, observed that the development of the oesophageal appendix and the appearance of the boring tooth did not take place until moulting to L3. Smith et al. (1990) did not observe moulting during the development of newly hatched larvae of C. osculatum until they had attained a length of more than 13 mm in the visceral cavity of an experimental host fish, identifying them as L3 both morphologically and morphometrically. The SEM description of L3 of *Contracaecum* collected from host fish (Valter et al. 1982 and references therein; Weerasooriya et al. 1986) concurs with the data from the present study, except, of course, in the size of the larvae. Consideration of these previous studies, those of Køie with different anisakids including C. osculatum (Køie 1993; Køie and Fagerholm 1993; Køie et al. 1995) and our own results (Valles-Vega et al. 2017; and present study) leads the present authors to suggest that the larva hatching from the egg in *C. multipapillatum s.l.* is the third stage larva since development of the boring tooth, the oesophageal appendix and other structures typical of L3 of *Contracaecum* was observed without any moulting. As development is still not complete, the characteristic collar and spine of L3 of *C. multipapillatum s.l.* are still not observed.
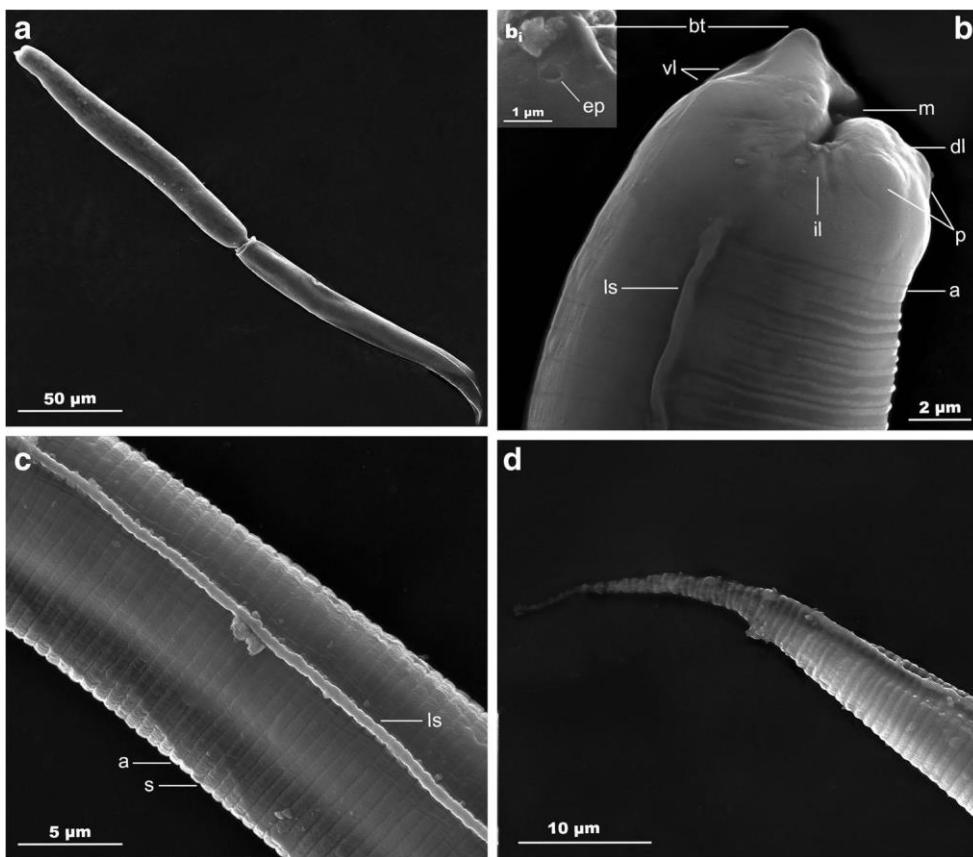


Fig 7 Exsheathing (a) and exsheathed (b, c, and d) larvae of *Contracaecum multipapillatum s.l.* a Note the point of strangulation caused by the sheath during larval exsheathment. b Cephalic end, note mouth as a slit and, in inset (b$_i$), excretory pore in the base of the boring tooth. c Lateral suture in mid body. d Tail ending in blunt point. a annulus, bt boring tooth, dl dorsal labium, ep excretory pore, il incipient interlabium, ls lateral suture, m mouth, p papillae, s striations of cuticle, vl ventrolateral labium

Further work is required in order to achieve *in vitro* culture of these anisakids which would then enable us to study their development and identify the differences between stages and species

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Compliance with ethical standards

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

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# A scanning electron microscopy study of *Anisakis physeteris* molecularly identified: from third stage larvae from fish to fourth stage larvae obtained *in vitro*

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**abstract**

The development of the fourth larval stage (L4) of *Anisakis physeteris* was studied using scanning electron microscopy (SEM), comparing it with third larval stage (L3) recently obtained from the host fish, blue whiting (*Micromesistius poutassou*), from the western Mediterranean Sea (east coast of Spain, zone FAO 37.1.1). After molting to L4, samples of the parasite were examined at different times in order to observe their development. Following collection of the L4, a small portion was taken from the middle of the larva for molecular identification, confirming in all cases that it was *A. physeteris*. The anterior and posterior sections of the larvae were prepared for morphological study by SEM. The development of a row of denticles on each of the three prominent lips, almost reaching the buccal commisures, was observed in the L4. Pores of unknown function were found in the upper external part of each lip. Clearly developed cephalic papillae, amphids, and deirids were also observed in L4, while, although present in L3, these were beneath the cuticle. Phasmids were detected in L4 but not in L3. The L4 tail finished in a conical lobe with a blunt point, absent in L3. In the oldest L4, some preanal papillae were observed beneath the cuticle in males, while, in females, the vulva could be seen by light microscopy, apparently still covered by the cuticle.

## 1. Introduction

Nematodes of the genus *Anisakis* are parasites of the digestive tract of cetaceans. They are present in all the oceans and have a complex life cycle which also includes crustaceans, squid, and fish. The eggs, which are released into the sea in the feces of the definitive host, develop by hatching into the third larval stage (L3), which infects marine invertebrates, particularly euphausiids, the first intermediate hosts of *Anisakis* spp. These crustaceans are then ingested by squid and/or fish, which act as intermediate/paratenic hosts. The L3 then grow until they are infective for the definitive host, but without changing stage. The ingestion of these infected fish or squid by suitable cetaceans allows the L3 to develop into adults in their digestive tract. In the case of *Anisakis physeteris*, squid seem to have an epidemiological role as intermediate/ paratenic hosts as they are an important source of food for sperm whales (95% or more of the diet; Santos et al. 2001 and references therein), the definitive host of this species (Baylis 1923; Kagei et al. 1967; Mattiucci and Nascetti 2008).

In L3 of the genus *Anisakis*, there are two morphological types of larva. Berland (1961) classified them as type I (elongated ventriculus with oblique join to the intestine, tail with mucron) and type II (shorter, thicker ventriculus with straight join to the intestine, conical tail), with the species within each type being morphologically indistinguishable. Molecular studies are gradually clarifying the taxonomy of these and other anisakids. Mattiucci et al. (2018) have divided the genus *Anisakis* into four clades. The species with type II L3 were included in clade 3 with three species identified to date: *A. physeteris*, *A. paggiae*, and *A. brevispiculata*.

Although anisakid L3 and their adult forms have been accurately described, the descriptions of their fourth larval stages are frequently lacking in detail. The aim of the present study is to fill in these gaps, using SEM to study the differences between L3 collected from the host fish and the L4 of *A. physeteris*, obtained *in vitro*, and their evolution during development. In spite of most cases of human anisakiasis being caused by larvae of the *A. simplex* s.l. complex (Rello Yubero et al. 2004), this study is of particular interest since *A. physeteris* is also able to cause it. Both L3 (Asato et al. 1991) and L4 (Clavel et al. 1993) have been collected from patients, in addition to L3 molting to L4 (Kagei et al. 1978).

The present study describes the third and fourth larval developmental stage of *A. physeteris* using scanning electron microscopy (SEM) and aims to improve understanding of the morphological characters of biological and taxonomical significance of this anisakid.

## 2. Materials and methods

2.1. Collection of parasites

L3 of *Anisakis* spp. were collected from blue whiting landed at the ports of Villajoyosa, Castellón and Gandía (western Mediterranean Sea, east coast of Spain, zone FAO 37.1.1). The fish were transported to the laboratory under refrigerated conditions and then dissected. The larvae, encapsulated in the visceral cavity, were collected and placed in a cold solution of NaCl

0.9%. They were then classified morphologically as type I or II sensu Berland (1961) using optical microscopy.

## 2.2. Cultivation of parasites

Following morphological identification of the type II larvae of *Anisakis*, these were axenized in antibiotic-antimycotic solution (Iglesias et al. 1997) and individually placed in culture. The L3 measured between 2 and 3 cm in length. Both the culture medium and the procedure were described previously by Iglesias et al. (2001) for *A. simplex*. The parasites remained in culture until attaining the level of development required for the study, with any larvae failing to complete the molt to L4 being discarded. The larvae were examined daily by optical microscope to monitor their degree of development, mobility, and the sterility of the culture.

## 2.3. Collection of larvae for SEM

After completing the molt to L4, the larvae were removed from the culture for the SEM study of 1–9 weeks. In addition, L3 recently collected from the fish were also examined by SEM for comparison with the L4. All larvae collected were fixed in hot 70% (v/v) ethanol and preserved for preparation for examination by SEM. The fixed larvae underwent critical point drying and cut into 3 sections, thus avoiding the distortion of the larvae which occurs if cut when fresh or only fixed. The anterior and posterior sections were separated for SEM preparation while a small cylindrical part of the central section was used for molecular identification.

## 2.4. Molecular identification

The extraction of the genomic DNA from the central section of each larva was carried out using the RealPure (REAL) kit, according to the manufacturer's instructions. Amplification of the region ITS1-5.8S-ITS2 of the ribosomal DNA was performed using the primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (1998). The polymerase chain reaction procedure (PCR) was carried out as previously described in Molina-Fernández et al. (2015). The expected size of the amplicon was around 1000 bp. Next, a restriction fragment length polymorphism (RFLP) of the amplified DNA was carried out using the restriction enzymes HinfI (final concentration 0.5 U/µl, temperature 37 °C for 10 min) and TaqI (0.5 U/ µl, 65 °C for 10 min) (Fast Digest, Thermo Scientific). To identify the species, electrophoresis with 3% agarose gel was performed to visualize of the band patterns of the larvae. The controls of the digestion by TaqI of the DNA amplicon of *A. physeteris* produced 3 fragments of 300, 280, and 140 bp. When digestion was with HinfI, the fragments were of 380, 290, and 270 bp, according to D'Amelio et al. (2000), Romero et al. (2014) and Molina-Fernández et al. (2018).

## 3. Results and discussion

All larvae prepared for SEM (2 L3 and 8 L4) were analyzed molecularly and identified as *Anisakis physeteris* (Baylis, 1923). The L4 were obtained after 15–20 days of culture.

3.1. Anterior or cephalic end

At the anterior end of anisakid L3 larvae, it is traditional to describe the presence of 3 lips or fairly pronounced labial protuberances with papillae or papilla-like structures, 2 on the dorsal lip and 1 on the 2 subventral lips. In *A. physeteris*, 3 incipient lips could be seen with 2 protuberances on each (Fig. 1a), as in *Anisakis* type I (Valter et al. 1982; Weerasooriya et al. 1986), covered by the cuticle and corresponding to the cephalic papillae and amphids (Fig. 1b, c) which are observed after molting to L4. The observation that these sense organs are covered by the cuticle in L3 poses the question as to whether they are functional or not and whether their development in L3 is only preparation for L4. However, the observation of undifferentiated papilla-like structures on the labial protuberances of recently hatched L3 of other anisakids (McClelland and Ronald 1974; Molina-Fernández et al. 2017) suggests that these structures may have some function in L3. Jones (1994) proposed that at least the amphids of L3 of *A. simplex* (*s.l.*) are functional since their internal structure is well-developed and this may also be true for the papillae. The mouth (Fig. 1a, d) is triradiate but opens to form a triangular aperture, as also occurs in L4 (Fig. 2). The boring tooth (Fig. 1a, d) is found surrounding these radia, between the two subventral lips. Below the boring tooth, in a ventral position, is an oval excretory pore with a maximum diameter of 7–10 mμ with its mouth pointing forwards (Fig. 1a, e) and appearing to empty towards the exterior of the larva. The deirids (also called body papillae, cervical papillae or lateral cervical papillae) are covered by the cuticle but visible in L3 (Fig. 1f, g), as are the papillae and amphids. These deirids are in a subdorsal position, close to the lateral lines running along the larva. Davey (1971) considered deirids to be present in specimens of the genus *Anisakis*, at least from the L3 stage, as found in the present study.

▷ Fig. 1 *Anisakis physeteris* L3. a Anterior end. b–g Detail of the b structures: b Cephalic papilla. c Amphid. d Mouth (m), boring tooth (bt) and excretory pore (ep). e Excretory pore. f Location of deirid and lateral line in anterior end of larva. g Deirid and lateral line
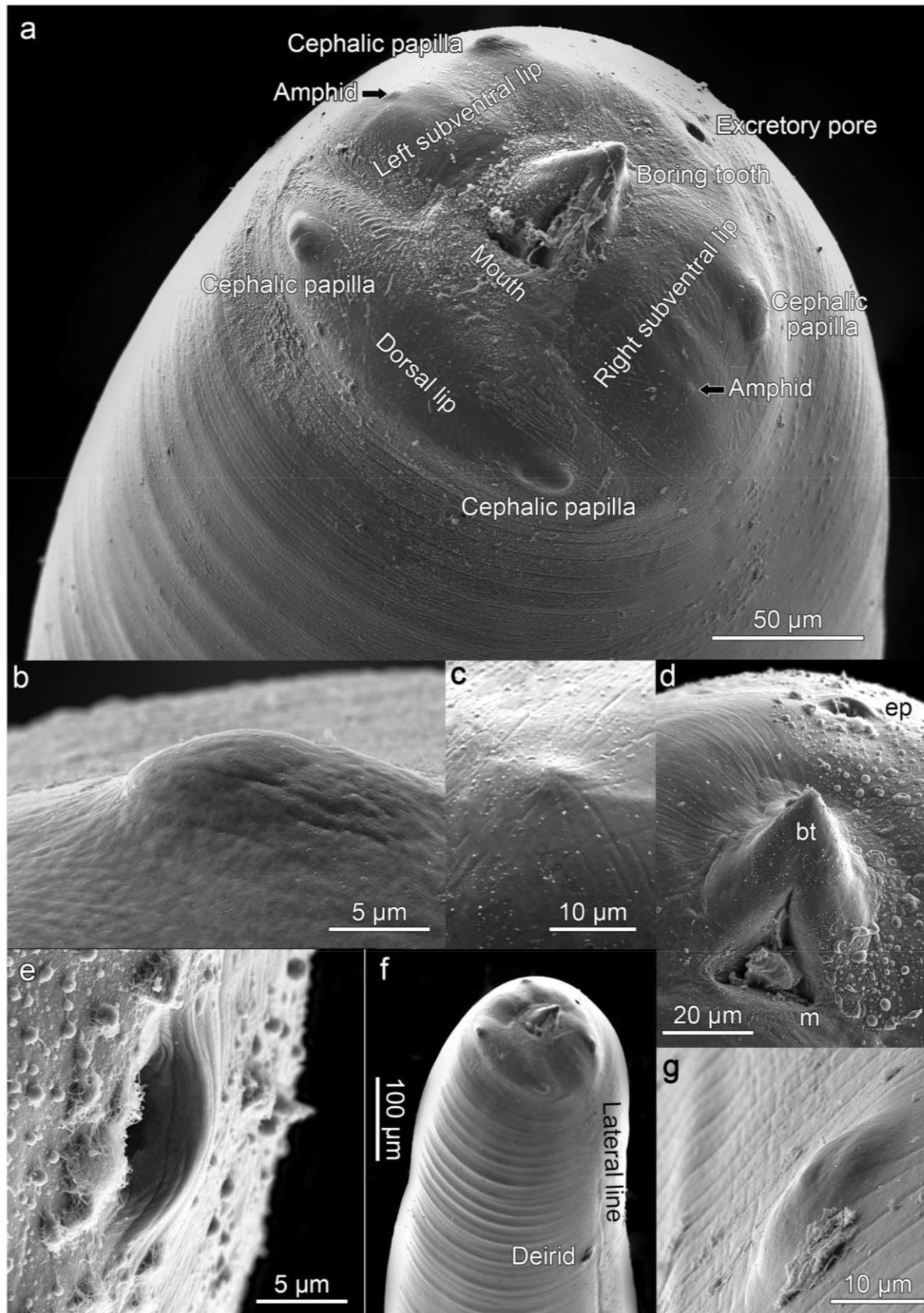
The L4 does not have a boring tooth but has 3 developed lips, the dorsal lip being clearly larger than the 2 subventral lips (Fig. 2a). A large, elongated cephalic papilla (Fig. 2a, b) is located laterally (towards the other subventral lip) at the base of each subventral lip. The structure of the papillae is similar to that described in the adults of other *Anisakis* spp. (Weerasooriya et al. 1986; Abollo and Pascual 2002; Di Azevedo et al. 2015). There is also an amphid (4.3–6.3 µm diameter), with an annular structure, on each subventral lip, on the side close to the dorsal lip (Fig. 2a, c). The amphid is surrounded by irregular cuticle. The dorsal lip shows 2 elongated cephalic papillae, opposite each other at the base and has no amphids (Fig. 2a). These papillae (Fig. 2b) are of a similar size to those of the subventral lips, their length increasing with larval development time (from 18.2 to 32.8 µm) and maintaining the width (10.4–12.7 µm). They are surrounded by a more regular cuticle than that surrounding the amphid (Fig. 2b, c). At the anterior end, other papillae-like structures of unknown function, varying between individuals, can occasionally be observed (Fig. 2a, d). At the external upper part of the three lips, small pores of unknown function can be seen (Fig. 2e, f). This character does not appear to have been described previously in anisakids. As the L4 develops, the upper part of the lips can be seen to become more bilobed, clear in type I L4 of *Anisakis* (Weerasooriya et al. 1986) and in adults of the genus *Anisakis* (Davey 1971), shown by a cleft which is not always visible (Fig. 2a, e, h). The lobes are symmetrical in the dorsal lip but not in the subventral lips, allowing them to fit together (Fig. 2a, e).

Fig. 2 *Anisakis physeteris* L4. a Anterior end. Note the upper part of the b lip, bilobed but symmetric in the dorsal lip and asymmetric in the subventral lips. b–j Detail of the structures of cephalic end: b Cephalic papilla. c Amphid. d Papillalike structures located between the base of the dorsal and subventral lips (see in a). e Face view of the L4 with the three denticulate lips (dorsal (dl) and right (rsl) and left (lsl) subventral lips), observe the shape of the lips that allows them to fit; note pores in lips. f Pore of the right subventral lip marked with arrowhead in e, magnified. g Row of denticles, continuation of denticulate ridge, along side of upper part of lip and following the line of the mouth; (ex) external and (in) internal lip surfaces. h Upper part of dorsal lip (viewed from above), bilobed, with denticulate ridge, note rough external (ex) and smooth internal (in) surfaces. i Mouth commissure between subventral lips, note excretory pore (ep) opening towards mouth aperture and row of denticles reaching almost to the commisure. j Small hemispherical denticles located at end of rows
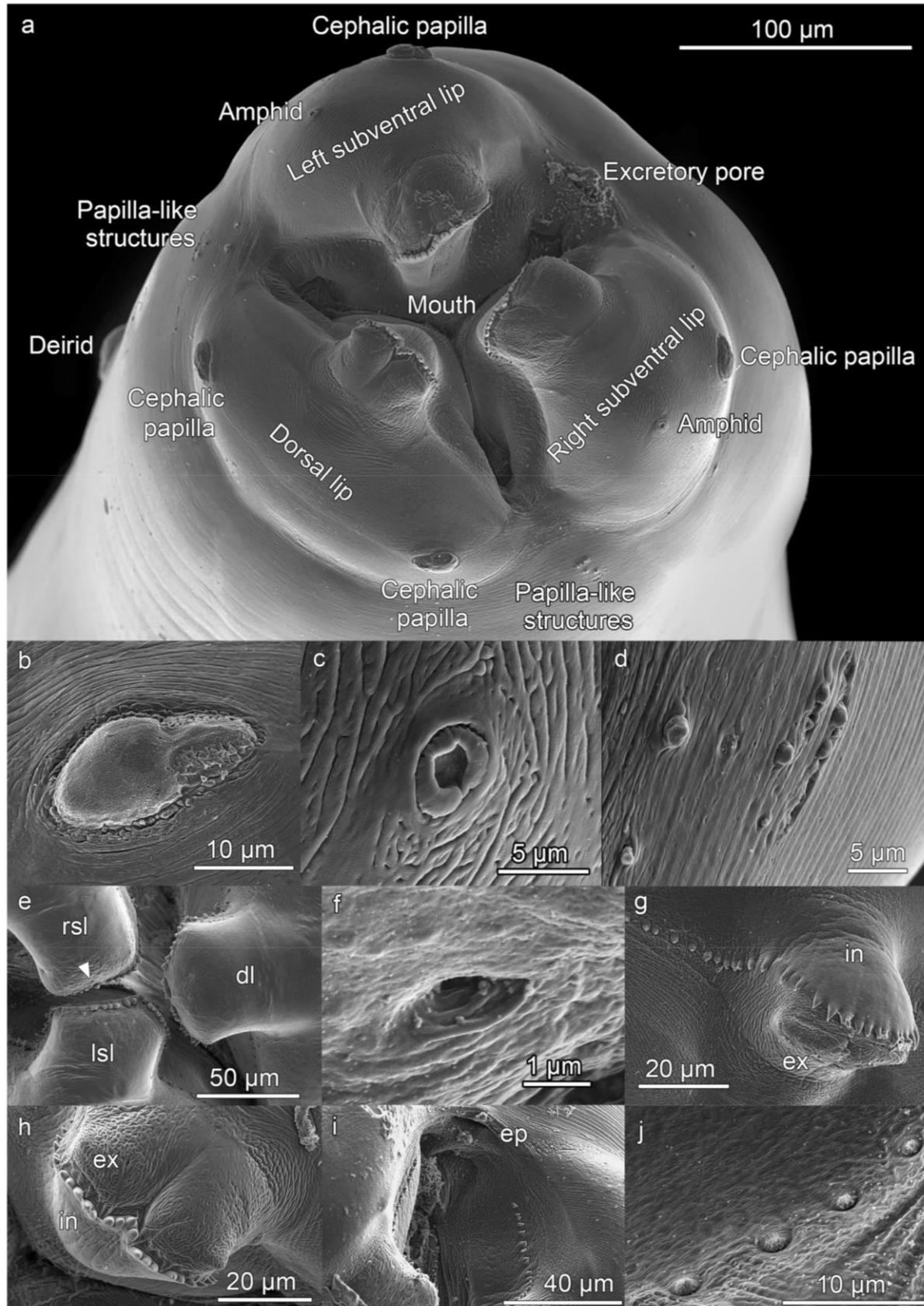
The lips are crowned by a ridge of denticles, as in type I *Anisakis* (Weerasooriya et al. 1986), which continues along the side of the lips with the denticles becoming progressively smaller and more rounded (possibly due to their being less developed) and almost reaches the commisures of the triradiate mouth (Fig. 2a, e–j). The number of denticles seems to vary between lips and individuals, roughly ranging from 40 to 65 per labium, the largest being those of the ridge. Weerasooriya et al. (1986), for *Anisakis* type I, reported between 35 and 40 only in the ridge on the lip and did not mention the presence of denticles outside the ridge. In the ridge of *A. physeteris*, between 13 and 19 simple denticles were counted, blunt or pointed, and, occasionally, double, while in type I *Anisakis*, the denticles were saw-toothed. In this sense, it has been observed that *A. pegreffii* only has denticles in the ridge, many forming a saw with no separation between them (personal observation). A total of 278 denticles (90–94 per lip) were counted in adults of *A. physeteris* (Kikuchi 1974), although the species identification was only morphological and it is not known whether this parameter varies between the 3 species of clade 3. The oval excretory pore is located between the subventral lips, as in L3, although in L4, it is within the area of influence of the mouth, thus allowing it to empty either towards the mouth or to the exterior (Fig. 2a, i).

Near the anterior end of the L4, there are a pair of deirids, positioned symmetrically and subdorsally next to the lateral suture of the cuticle (Fig. 3a, b). These project from the cuticle and are oval and flat with a central button. They are surrounded by structured cuticle which emphasizes them. Their maximum diameter appears to increase slightly as the L4 develops, from around 16 mµ (1 week after molting) to around 27 mµ (9 weeks after molting). No centrids were observed in either of the larval stages. The vulva was observed by optical microscopy (Fig. 3c) in the first third of the body of some of the L4 females which had spent most time in culture. However, it was not possible to examine it by SEM, probably due to it being hidden beneath the cuticle.

3.2. Posterior or caudal end

The posterior end of L3 and L4 is similar (Fig. 4). In the former, it is conical with a rounded point with a C-shaped anus with the ends pointing towards the posterior end (Fig. 4a, b). No other structures were observed in L3. L4 showed an anus and tail similar to L3 although terminating in a conical lobe with a blunt point (Fig. 4c, d, e). The plectanes described in adults of *A. physeteris* (Davey 1971) were not observed. Phasmids are other sensory organs located at the caudal end of adult nematodes of the class Secernentea. These have not yet been detected in L3 of *Anisakis* although we do not rule out that they could be formed beneath the cuticle, like the other sensory structures mentioned above. But one pair of phasmids were observed here throughout the L4 stage (Fig. 4f, g), as in other anisakids (Fagerholm 1991). These are symmetrical and located laterally at each side of the tail, unlike in other members of clade 3, such as *A. paggiae* where they are subventral (Di Azevedo et al. 2015) and A. brevispiculata where they have been reported as sublateral (Abollo and Pascual 2002). They are found closer to the point of the tail than to the anus and are separated from the surrounding cuticle by a

groove (Fig. 4g). In some cases, a depression can be seen (Fig. 4f) but this may be due to the technique of sample preparation for SEM. Their diameter, around 5 mµ, does not seem to vary significantly during the development of L4. In some of the most welldeveloped L4, papillalike bumps, beneath cuticle, could be observed in the preanal ventral zone. These may be the preanal papillae of the adult male (Fig. 4h, i).
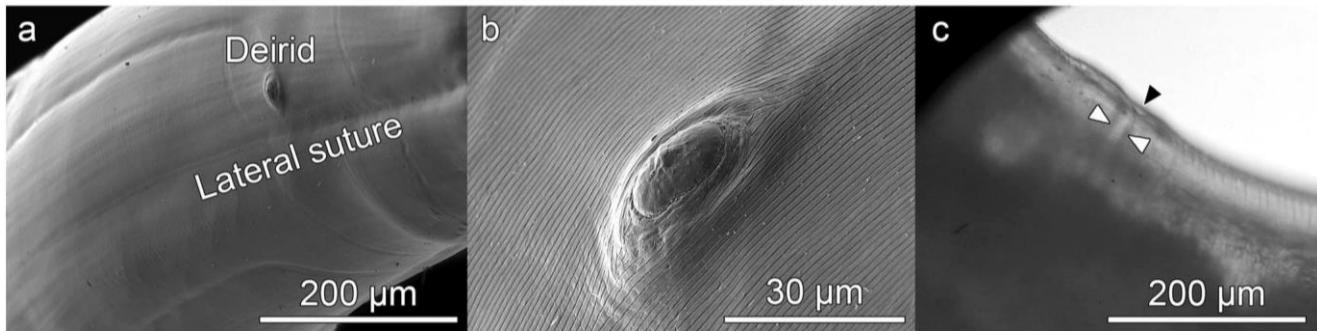


Fig. 3 *Anisakis physeteris* L4. a Location of deirid and lateral suture near anterior end of larva. b Deirid. c Vagina (white arrowheads) and vulva (black arrowhead) by optical microscopy, located in anterior third of body

### 3.3. Cuticle

At the anterior end of the larva, the cuticle has an irregular structure. In the L3, it is smooth (Fig. 1) with some slight, irregularly distributed striations (Valter et al. 1982). In the L4, the surface is rougher, although appearing structured (Fig. 2). The internal face of the upper zone of the lips is smooth while the external face is rough (Fig. 2g). On the body of the L3 and L4 larvae, below the lips, the cuticle is striated and a cephalic collar is absent (Figs. 1a, f, and 2a). The striations are across the body and parallel to each other, distributed more or less regularly throughout the length of the worm (Figs. 1g, and 5). Occasionally, the cuticular annuli are subdivided (Fig. 5b), probably as part of the growth process of the cuticle (MolinaFernández et al. 2017). Vertical bands can also be observed in them (Figs. 4b, and 5b). These annuli are similar to those described for type I L4 of *Anisakis* but are different to the irregular striations shown in type I L3 (Shiraki 1974; Weerasooriya et al. 1986). While in type I *Anisakis*, the width of the cuticular annuli increases from L3 to L4, in *Pseudoterranova decipiens*, it decreases (Shiraki 1974; Weerasooriya et al. 1986). In type II *Anisakis*, the width of the annuli at the anterior end of L3 is similar to that of L4. However, while the width of the annuli remains the same throughout the body of L4, in L3, the annuli at the posterior end are wider (7.3–9.0 mµ), similar to the case described by Tongu et al. (1990) (6.4–9.3 mµ). In L4, up to and including the third week, the annuli measure 1.0–1.1 mµ (tail zone) but later increase in width up to 1.4–1.9 mµ (> 3 weeks), although, in the zone closest to the conical lobe, they decrease to ~ 0.7 mµ. Although no lateral alae can be observed, a lateral line in the cuticle, without structural changes, can be observed in L3 (Fig. 5c) and a lateral suture in L4 (Fig. 5d) separating the dorsal portion from the ventral portion throughout the length of the nematode (Figs. 1f, 3a, and 5), except at the tail (Fig. 4a), where they are not visible in L3, although in L4 they reach the base of the conical lobe (Fig. 4c,

d). The annuli at the end of the tail of the L3 exhibit a distinctive pattern (Fig. 5a), while, in the L4, the lobe and the blunt point have an irregular cuticle (Fig. 4d) which is clearly different from that of type I *Anisakis* which shows Ba large number of spherical elevations (Weerasooriya et al. 1986). The usefulness of cuticle structure for the identification of anisakid species and their developmental stages has been widely debated. Thus, while some authors such as van Thiel (1966) or Davey (1971) rejected its use, others such as Shiraki (1974) or Weerasooriya et al. (1986) considered that it could be useful, particularly for deteriorated specimens collected from a human patient where the morphological identification would be difficult. This problem has since been resolved by the development of molecular techniques (Zhu et al. 1998).

It is noteworthy that the sensory structures of one larval stage develop during the previous stage, remaining beneath the cuticle until molting, when they become visible. As they are sensory organs, they may not become functional until exposed to the external medium following molting, although, as mentioned previously, Jones (1994) suggested that they are already functional. This is certainly the case of cephalic papillae, amphids, and deirids of the L4, formed beneath the cuticle of the L3 or of the caudal papillae of the males, formed beneath the L4 cuticle. Further study will be required in order to answer these questions.

▷ Fig. 4 *Anisakis physeteris* larvae caudal end. a Anus and tip of L3. b b Detail of end of tail of L3, note appearance of cuticle. c Anus and conical end of tail with blunt point (white arrowhead) of L4, note lateral suture (black arrowheads). d Conical end of tail of L4 with blunt point (white arrowhead), note lateral suture (black arrowheads). e Detail of blunt point at end of tail of L4, note appearance of cuticle. f View from above of end of tail of L4 showing the two phasmids (black arrowheads), lateral and symmetric. g Detail of a phasmid of L4, note the papilla (black arrowhead) surrounded by cuticle (white arrowhead). h Papillalike structures in the ventral preanal zone of L4, beneath the cuticle as seen in i
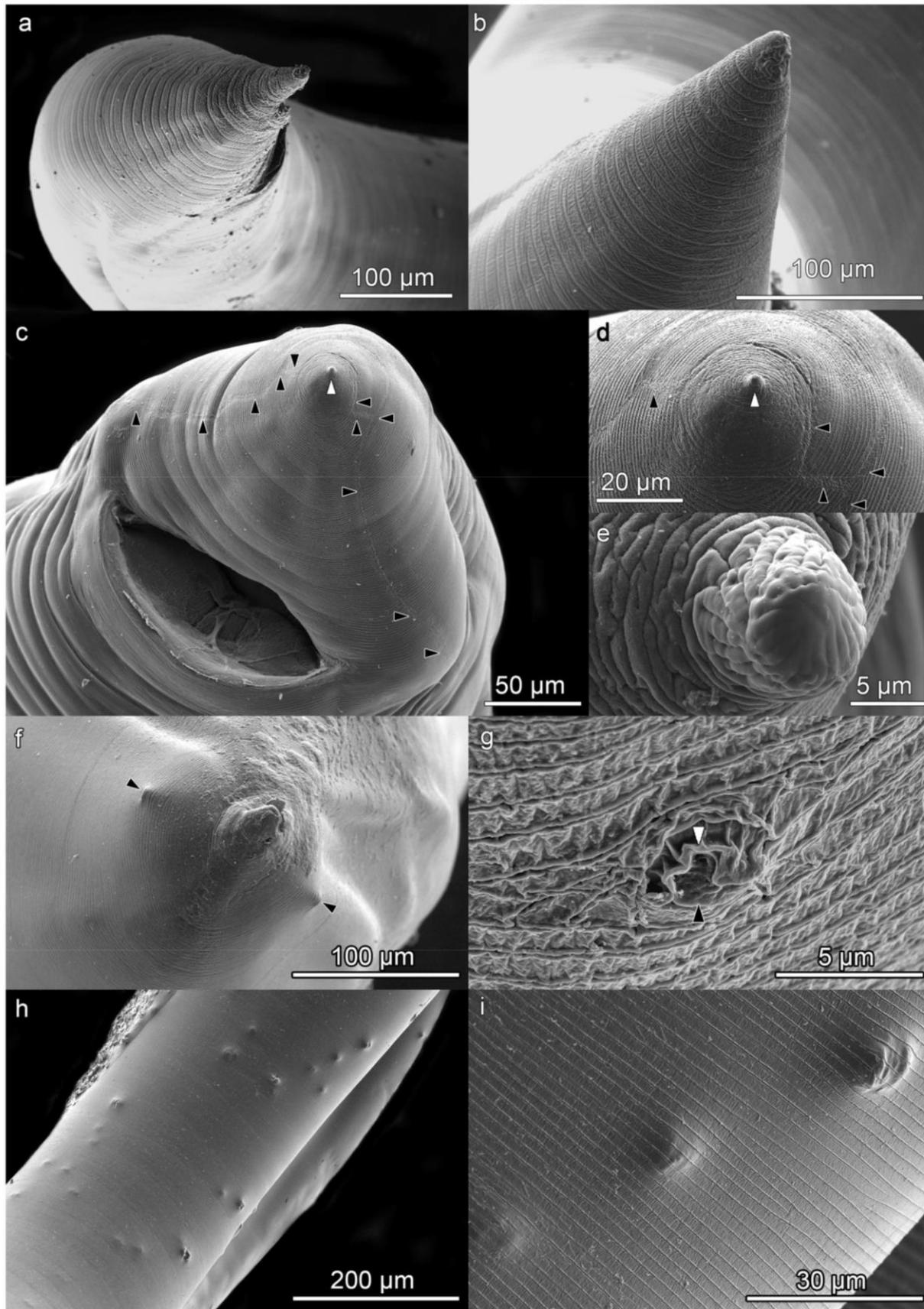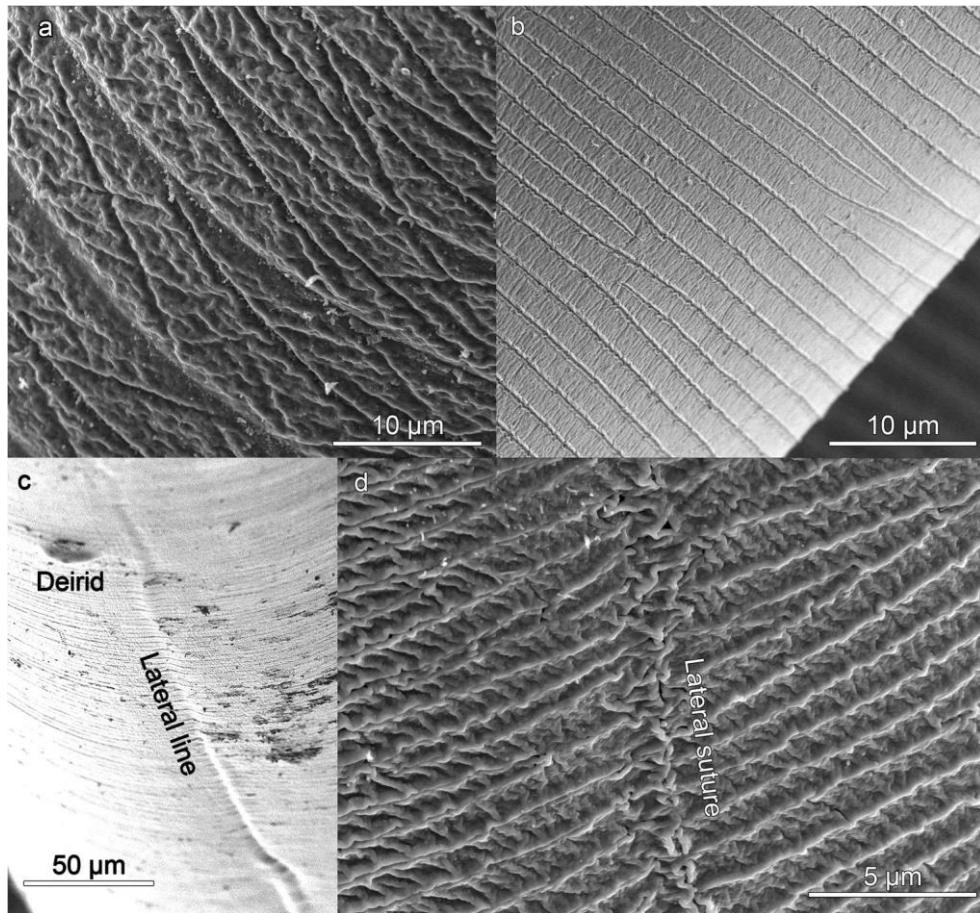
Fig. 5 *Anisakis physeteris* cuticle. a Cuticle structure of posterior end of L3. b Cuticle structure of L4 in ventral preanal zone. c Detail of lateral line, in anterior zone of L3. d Detail of lateral suture, in posterior zone of L4

Compliance with ethical standards

Conflicts of interest: the authors declare that they have no conflict of interest.

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# 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

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## Abstract

Proteolytic activity was studied in two sibling species of *Anisakis* (Nematoda: Anisakidae), *A. simplex s.s.* and *A. pegreffii*, throughout their *in vitro* development from third larval stage (L3) from the host fish (L3-0h) to fourth larval stage (L4) obtained in culture. Proteases have a significant role in the lifecycle of the parasite and in the pathogen-host relationship. Proteolytic activity peaks were detected at pH 6.0 and 8.5. Protease activity was detected in all the developmental stages of the two species studied at both pH values. These pH values were used for assaying with specific inhibitors which permitted the determination of metalloprotease activity, and, to a lesser extent, that of serine and cysteine protease. Aspartic protease activity was only detected at pH 6.0. At this pH, L4 larvae showed higher proteolytic activity than L3 larvae in both species (p<0.001), the majority of activity being due to metalloproteases and aspartic proteases, which could be related to nutrition, especially the latter, as occurs in invertebrates. At pH 8.5, proteolytic activity was higher in *A. pegreffii* than in *A. simplex s.s.* (p<0.01). At this pH, the majority of activity was due to metalloproteases in all developmental phases of both species, although in L3-0h, the activity of these proteases was significantly higher (p<0.03) in *A. simplex s.s.* than in *A. pegreffii*. This could be related to the greater invasive capacity of the former. Serine proteases have frequently been implicated in the invasive capacity and pathogenicity of some parasites. This may be related to the significantly higher activity (p≤0.05) of serine protease in all the larval stages studied of *A. simplex* at pH 6.0. In summary, there are interspecific differences in proteases that have been related to pathogenesis in nematodes. These differences could thus be contributing to the previously reported differences in pathogenicity between these two *Anisakis* species.

## 1. Introduction

Anisakidosis is an illness caused by the third larval stages (L3) of anisakid nematodes. Although often undiagnosed, it is common in countries where fish or squid are typically consumed raw or only lightly cooked, such as Japan, where there are thought to be between 2000 and 3000 cases annually (Umehara et al., 2007). There are also significant numbers of cases in other countries with high consumption of fish, including Spain, Italy and South Korea (González Quijada et al., 2005; Im et al., 1995; Lim et al., 2015; Pampiglione et al., 2002; Repiso Ortega et al., 2003), as well as in many other countries throughout the world.

More than 97% of cases of anisakidosis are caused by the larvae of the *Anisakis simplex s.l.* complex (Rello Yubero et al., 2004), for which reason the infection is also known as anisakiasis. This complex comprises 3 species whose L3 are morphologically indistinguishable but can be differentiated molecularly. The two most frequent species are *A. simplex sensu stricto* and *A. pegreffii*, with studies to date suggesting that the former is more pathogenic than the latter (Arai et al., 2014; Jeon and Kim, 2015; Quiazon et al., 2011; Rello Yubero, 2003; Romero et al., 2013; Suzuki et al., 2010). Molecular diagnosis has permitted the identification of human anisakiasis cases caused by both species (Arai et al., 2014; Arizono et al., 2012; Lim et al., 2015; Mattiucci et al., 2013; Umehara et al., 2007; Yera et al., 2016; and others), although use of this type of diagnosis is still uncommon.

Proteases participate in important biological processes in parasitic nematodes, being directly involved in their growth and survival, embryonic development, digestion of protein for nutrients, moulting and numerous metabolic processes (Britton and Murray, 2002; Hashmi et al., 2002; Pratt et al., 1992; Ray and McKerrow, 1992; Williamson et al., 2003b; Yu et al., 2014). They also play a vital part in host-parasite interaction such as invasion of the host, migration through host tissues, protection of the parasite against the host's immune system and activation of inflammatory processes (Malagón et al., 2013; McKerrow et al., 2006). It would thus seem that the proteolytic activity of the parasites has a crucial role in their pathogenicity, as reported in the nematodes *Strongyloides stercoralis* (McKerrow et al., 1990), *Anisakis simplex* (Jeon et al., 2014; Sakanari and McKerrow, 1990), *Onchocerca volvulus* (Lustigman, 1993), *Trichinella spiralis* (Criado-Fornelio et al., 1992), and *Ancylostoma caninum* (Hawdon et al., 1995).

Despite their biological importance and their implications for health, the proteases of *Anisakis* have not been studied in depth. As has been suggested for other pathogenic agents, some proteases could act as targets for chemotherapy or vaccines, as is the case of the cathepsins, which are well conserved in nematodes (Britton and Murray, 2002; Sallé et al., 2018). At present, the treatment of anisakiasis is invasive and involves the extraction of the larvae by endoscopy in hospital, while there is still no effective pharmacological treatment. Consequently, further study of nematode biology is necessary if new, effective drug targets are to be found.

Proteases or peptidases can be divided into two large groups according to their mechanism of action: one, including the cysteine- and serine-proteases, which form covalent enzymatic complexes with the substrate, and the other, including aspartic and metallo-proteases, which do not form these covalent complexes (Rawlings et al., 2012; Rawlings and

Barrett, 1993), with each group then having different inhibition strategies. These 4 groups of proteases are the most widely-studied in nematodes (Caffrey et al., 2013; Malagón et al., 2013; McKerrow et al., 2006; Sajid and McKerrow, 2002; Sakanari and McKerrow, 1990), although there are also other groups such as the glutamic or threonine proteases.

The aim of the present study was to perform a general characterization of proteolytic activity and to observe its variation during *in vitro* development of two sibling species of the complex *Anisakis simplex s.l.: A. simplex s.s.* and *A. pegreffii*. Since both species can cause anisakiasis, any differences observed may be due to their different pathogenicity.

## 2. Material and methods

### 2.1. Sample collection and *in vitro* culture

The L3 were extracted, as described previously (Molina-Fernández et al., 2018b), from the gut cavity of blue whiting, *Micromesistius poutassou*, caught in the Cantabrian and Mediterranean Sea and landed at Spanish ports. Immediately after extraction the larvae were placed in cold 0.154 M NaCl solution in an ice bath to prevent their development. The following larval stages were chosen for study: L3 recently extracted from the fish (L3-0h), L3 after 24 hours culture (L3-24h), L4, 24 hours after moulting (L4-24h), and L4 after 14 days culture (L4-14d: 10 days after moulting to L4). In the case of L3-0h, the larvae were frozen immediately after removal from the fish, having been first washed in cold sterile saline solution. For the other stages, the larvae were axenized and individually placed in culture as described previously (Iglesias et al. 1997, 2001). After attaining the stage desired, the larvae were washed with the cold, sterile saline and frozen at -20 ºC until required. To obtain the necessary larvae of each species of *Anisakis*, fish were chosen from different geographical areas according to the predominant species of *Anisakis* in each. Fish landed at Ondarroa (Cantabrian Sea, Northern Spain, zone FAO VIIIc) were used for larvae of *A. simplex s.s.* and from Villajoyosa and Gandía (Mediterranean Sea, Eastern Spain, zone FAO 37.1.1) for *A. pegreffii*. Molecular identification was carried out as described previously (Molina-Fernández et al., 2018a, 2018b, 2015). Following DNA extraction from 28-30% of the collected larvae from each of the fishing grounds, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the ribosomal DNA fragment 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 F*ast Digest (Thermo Scientific) at 65 °C and 37 °C for 10 min, respectively. The results were visualized by electrophoresis in 3% agarose gel, which permitted the sibling species of *A. simplex* complex to be identified according to the band pattern (D'Amelio et al., 2000; Martín-Sánchez et al., 2005a). Some larvae showed a mixed banding pattern between *A. simplex s.s.* and *A. pegreffii* with one or other restriction enzyme and were thus classified, for the purposes of this study, as hybrid type I larvae.

## 2.2. Preparation of *Anisakis* extracts

The larvae of each stage were homogeneized separately in a small volume of Tris/HCl 50 mM buffer with glycerine 20% w/v at pH 7.8, in order to stabilize the proteins and prevent their rapid degradation (Gianfreda and Scarfi, 1991; Iyer and Ananthanarayan, 2008), obtaining a final volume of 500 µl. The homogenates were then centrifuged at 19000 x *g* for 20 min, at 4 °C. The cell pellets were discarded and the supernatant fractions were used for the enzymatic activity assays. These fractions were diluted with the same buffer to a protein concentration of 2.5 mg/ml (Bradford, 1976). The larvae and the extract were kept in an ice bath for the entire process to prevent their degradation.

## 2.3. Proteolytic activity assays

Soluble extract of L3-0h of *A. simplex s.s.* was used to determine the effect of pH on proteolytic activity. A discontinuous system of buffers was employed, with pH increments of 1: citrate/HCl and citrate/NaOH from pH 2 to 7, Tris/HCl from 7 to 9 and glycine/NaOH from 9 to 11; always at a final concentration of 50 mM. The effects of dithiothreitol (DTT) (final concentration 0-10 mM) and $CaCl_2$ (final concentration 0-20 mM) on proteolytic activity were assayed. The use of DTT to reduce total proteolytic activity was ruled out, while the use of $CaCl_2$ at 1 mM stabilized this activity. Changing ionic strength with NaCl did not improve activity. Activity was determined by measuring the fluorescence emitted following the degradation of the fluorogenic bodipy FL casein substrate (Thermo Fisher) with $\lambda_{ex}$ 490 nm/$\lambda_{em}$ 510 nm, using a fluorimeter (Fluostar Optima-BMG Labtech), where enzymatic activity was monitored at 37 ºC for 60 min and measured every 1 min, in black microtiter plates. The final concentrations in the reaction mixture were: 50 mM buffer, 50 µl extract/ml (125 µg protein/ml), 1 mM $CaCl_2$ and 5 µg substrate/ml, for a final volume of 200 µl (Malagón et al., 2011). The enzymatic reaction was started with the substrate. To standardize the procedure, the area of greatest stability of the measurement was selected –corresponding to that between 10 and 30 minutes – for calculation of proteolytic activity. Enzymatic activity was expressed as a variation ($\Delta$) of the fluorescence relative units (FRU) $min^{-1}mg^{-1}$ protein. After determination of the pH values where peaks of maximum activity were observed, specific buffers were used to cover pH±1 to improve determination of optimum pH. The buffer Tris-maleic/HCl was used for pH 5.0, 5.5, 6.0, 6.5 and 7.0 and glycine/NaOH for pH 8.0, 8.5, 9.0, 9.5 and 10.0. After establishing the optimum pH values, the proteolytic activity of each of the larval stages of *A. simplex s.s.* and *A. pegreffii* was determined.

2.4. Inhibition assays

To assess the contribution of the different protease classes to hydrolysis of the substrate, protease inhibitors were assayed as previously described (Malagón et al., 2011). Inhibition assays were carried out at the two pH values where maximum proteolytic activity had been detected. The following control enzymes and specific inhibitors were used for each group of proteases, with their final concentration in the reaction mixture shown: pepsin (64 U/ml) and pepstatin A (0.02mM), respectively, for the aspartic proteases; thermolysin (0.2 U/ml) and 1,10-phenanthroline (2 mM), for the metalloproteases; papain (0,24 U/ml) and l-trans-epoxysuccinyl-leucylamide-(4-guanidine)-butane (E64, 0.05 mM), for the cysteine proteases; chymotrypsin (0.1 U/ml) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 2 mM), for the serine proteases. The quantity of each inhibitor was sufficient to totally inhibit the control enzyme from time 0. A solvent (0.5% methanol, used for pepstatin A and 1,10-phenanthroline) control was also performed. Inhibition assays were carried out at pH 6.0 and pH 8.5. The final concentrations in the reaction mixture were: buffer 50 mM, $CaCl_2$ 1 mM, enzymes and inhibitors as described above, 50 µl of extract/ml (125 µg protein/ml), 5 µg of substrate/ml. The addition of reducing agents (such as DTT or L-cysteine) to the reaction mixture did not significantly affect cysteine protease activity. The ideal incubation time for the stabilization of the interaction between the extract and the inhibitor was established as 5 minutes. The reaction was initiated by adding the substrate bodipy FL casein. Measurements were taken every minute for 60 minutes, at 37 ºC. The most stable part of the activity curve, between 10 and 30 minutes, was selected for data processing. The effect of inhibitors was expressed as percentage of inhibition (%I), determined as: %I = 100 − [(mean $\Delta$FRU min$^{-1}$ mg$^{-1}$ protein in presence of the inhibitor/mean $\Delta$FRU min$^{-1}$ mg$^{-1}$ protein in absence of the inhibitor) × 100].

2.5. Statistical study

The aim of the statistical study was to compare the proteolytic activity of *A. simplex s.s.* and *A. pegreffii* in each of the developmental stages studied and the evolution of this activity during the development of each species, while also analyzing the differences in the contribution of of each type of protease to this activity. This study was carried out using the program SPSS version 22.0 for Windows. Variance analysis (ANOVA) was also performed, after determining that the residuals of the variables followed a normal distribution using the Shapiro-Wilk test (p>0.05 in all cases) and with the aid of Q-Q plots. Following ANOVA, a *post hoc* study was carried out using the Bonferroni test for the variables which had shown significant differences. The significance level was designated as p<0.05.

## 3. Results and discussion

3.1. Molecular identification

A total of 270 larvae from the port of Ondarroa (Cantabrian Sea) were used, of which 75 were analyzed by PCR-RFLP (27.8% of the larvae), 90.7% (68 larvae) being identified as *A. simplex s.s.*, 8.0% (6 larvae) as *A. pegreffii* and 1.3% (1 larva) as a type I hybrid of the two species. A total of 215 larvae from the Mediterranean Sea ports of Villajoyosa and Gandía were employed, of which 65 were analyzed (30.2% of the larvae), 90.8% (59 larvae) being identified as *A. pegreffii*, 1.5% (1 larva) as *A. simplex s.s.* and 7.7% (5 larvae) as type I hybrids of the two species. These data coincide with the known distribution of these species of *Anisakis*, with *A. pegreffii* more frequent in the western Mediterranean Sea and *A. simplex s.s.* in NE Atlantic Ocean, although with certain sympatric zones in the seas to the south and west of the Iberian Peninsula (Martín-Sánchez et al. 2005; Mattiucci and Nascetti 2008; Molina-Fernández et al. 2015, 2018b; Mattiucci et al. 2018).

3.2. Proteolytic activity

Activity of L3-0h de *Anisakis simplex s.s.* tended to be maximum at pH 6.0 and 8.5. It has also been observed that $CaCl_2$ (1 mM) favours proteolytic activity (Gianfreda and Scarfi, 1991; Iyer and Ananthanarayan, 2008; Kocher and Sood, 1998) and helps to maintain the stability of the proteases (Morris and Sakanari 1994). The use of reducing agents (such as DTT) in the reaction mixture may facilitate the action of the cysteine proteases, although, under the experimental conditions of the present study, it reduced total proteolytic activity. This may be due to the ability of the sulfhydryl groups of the reducing agents to form a complex with the metal ion of some proteases, thus inhibiting their action (Coombs et al., 1962; Rufo et al., 1990) and affecting its measurement.

At pH 6 (Fig. 1), the proteolytic activity throughout the development of the two species was similar, although, the L4 showed significantly greater activity than L3 in the two species (p<0.001). It has been suggested that L3 of *Anisakis* do not ingest food orally until moulting to L4 (Sommerville and Davey, 1976; Yasuraoka et al., 1967). This change in the uptake of nutrients may require the action of different proteases for digestion in the intestine. Dziekońska-Rynko et al. (2003) reported greater activity of several digestive hydrolases in L4  *A. simplex* than in L3, relating it to changes in feeding mechanisms taking place after moulting to L4.
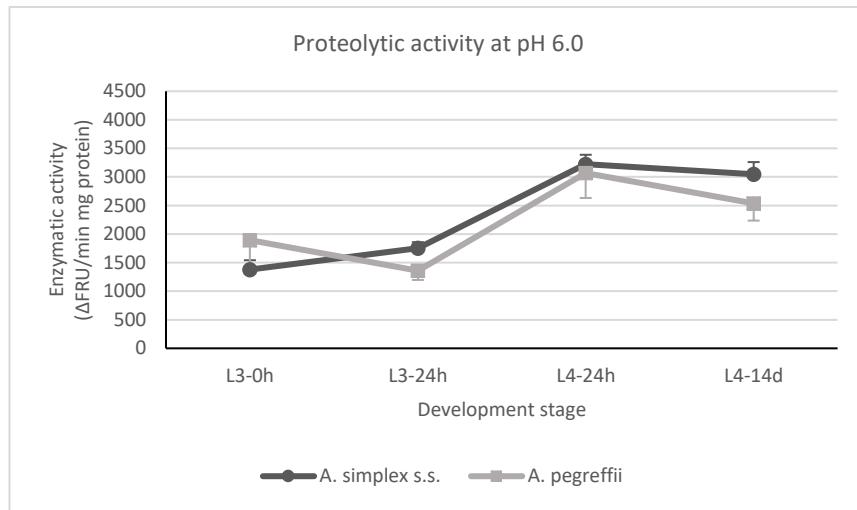
Figure 1.- Proteolytic activity measured as optimal bodipy FL casein cleaving activity at pH 6.0 in *Anisakis simplex s.s.* and *A. pegreffii*, during its *in vitro* development. Each point is the mean ± standar deviation of three to five experiments in triplicate.

These data suggest that the proteolytic activity detected at this pH may be related, in particular, to digestive processes involving the parasite's nutrition. The inhibition assays (Fig. 3) show that most of this activity during the development of *A. simplex s.s.* and *A. pegreffii* was due to aspartic (34-54%) and metallo-proteases (40-52%). The former are digestive proteases mainly associated with nutrition, in both parasites and free-living nematodes (Brown et al., 1995; Chang et al., 2011; Geldhof et al., 2000; Hawdon et al., 1989; Hwang et al., 2010; Williamson et al., 2003a, 2003b; Yang et al., 2009), and, according to Delcroix et al. (2006), aspartic and cysteine proteases assume the same role in invertebrate digestion as trypsins in that of vertebrates. Iglesias et al. (2001) showed that the development of L4 to adulthood in *A. simplex* is determined by the presence of pepsin (an aspartic protease), at least in culture. The pepsin aids availability of predigested peptides in the medium, facilitating their assimilation by the parasite. This can also occur *in vivo* through the pepsin from the glandular chamber of the stomach in cetaceans, the definitive hosts of *Anisakis* spp., as has also been suggested for the related nematode *Ascaris suum* (Rhoads and Fetterer, 1998; Rhoads et al., 1998). In *Hysterothylacium aduncum*, the addition of pepsin to the culture medium was found to significantly reduce aspartic protease activity in the parasite, making use of the exogenous contribution from either the culture medium or the digestive system of the fish, the definitive hosts of this nematode. The greater expression of aspartic proteases in the absence of pepsin suggests that these type of proteases have a digestive function in these nematodes (Malagón et al., 2011).

Furthermore, it has been observed that infective L3 of *A. simplex* stimulate pepsin expression in the stomach of guinea pigs, an experimental host of this anisakid (Dziekońska-Rynko et al., 1997). At pH 8.5 total proteolytic activity was greater in *A. pegreffii* than in *A. simplex s.s.* (p<0.01), although showing a similar trend in both species (Fig. 2). Maximum activity was found in L4-24h in both species. However, the differences between larval stages were only significant in *A. pegreffii* (p<0.03). No aspartic proteases were detected at this pH since they are only active at acidic pH values.
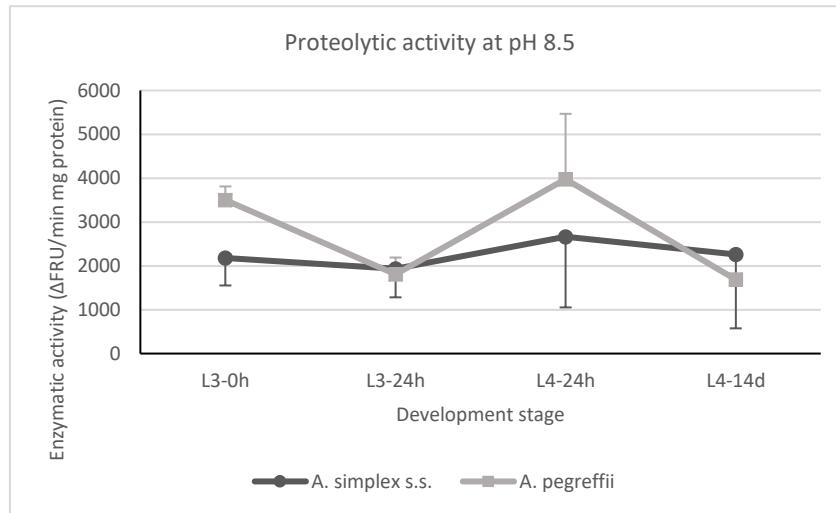


Figure 2.- Proteolytic activity measured as optimal bodipy FL casein cleaving activity at pH 8.5 in *Anisakis simplex s.s.* and *A. pegreffii*, during its *in vitro* development. Each point is the mean ± standar deviation of three to five experiments in triplicate.
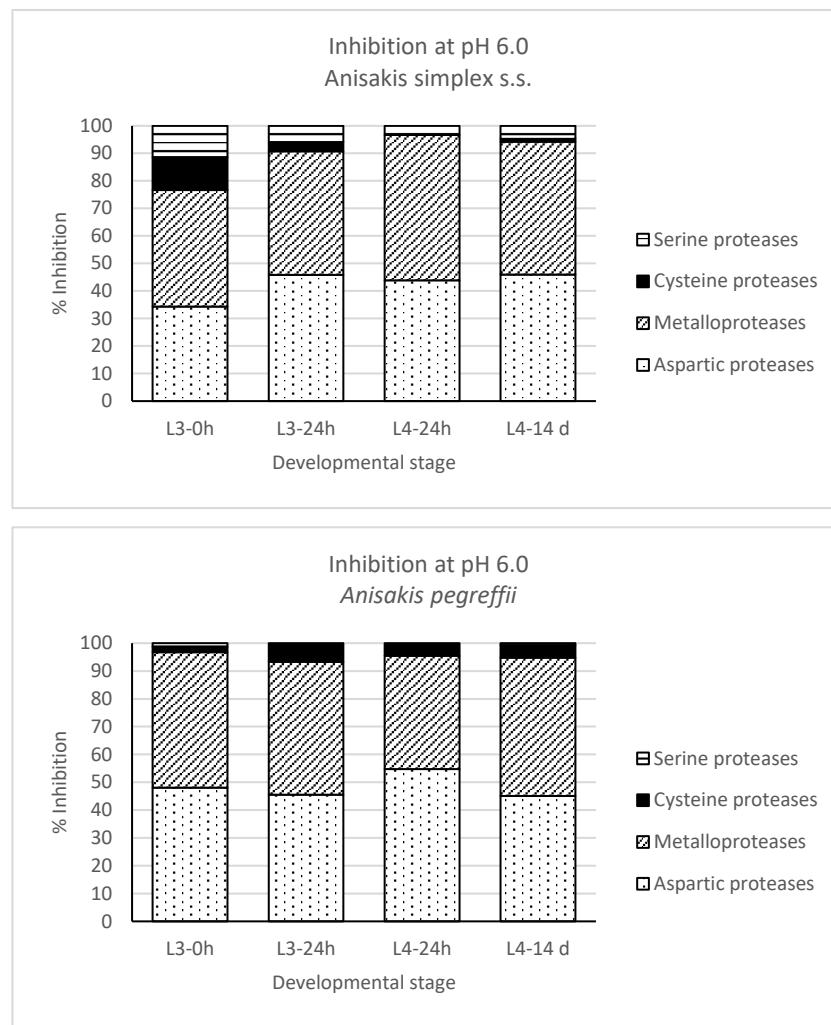
Figure 3.- Inhibition percentage of optimal bodipy FL casein cleaving activity at pH 6.0 in *Anisakis simplex* complex, during its *in vitro* development. Each inhibition percentage is the mean of three to five experiments in triplicate. Top: *A. simplex s.s.*. Bottom: *A. pegreffii*.

While, at pH 6.0, the metalloproteases shared most of the proteolytic activity (40-52%) with the aspartic proteases (Fig. 3), at pH 8.5 they accounted for the greater part of the activity (40-75%; Fig. 4). In nematodes these proteases are involved in resisting the host's immune system (Culley et al., 2000), in the moulting process (Hong et al., 1993; Rhoads et al., 1997) and in nutrition (Rhoads and Fetterer, 1998). Metalloaminopeptidases have been described in *Ascaris suum* (en L3, L4 and adults) and in the sheep stomach nematode *Haemonchus contortus*, suggesting that they have an important role in digestion (Newton, 1995; Rhoads and Fetterer, 1998). However, other aminopeptidases detected in both *A. simplex* and in the anisakid *Pseudoterranova decipiens* have not been implicated in digestion but in the activation of biological molecules (proenzymes, prohormones) and in moulting (Davey and Sommerville, 1974; Sakanari and McKerrow, 1990). Another Zn-protease has been implicated in the eclosion

and moulting of *H. contortus* (Gamble et al., 1989; Rogers, 1982). This functional diversity might explain their high level of activity in all larval stages and at the two pH values studied (Figs. 3 and 4). In *H. aduncum*, the metalloproteases also showed the greatest proteolytic activity (60-90%), at least at pH 5.5 (Malagón et al., 2011). It has been suggested that in the gastrointestinal tract of the final host the metalloproteases of the nematode parasite complete the digestion initiated by the pepsin and other enzymes from the host (Rhoads et al., 1998). However, in the L3-0h, collected from the host fish, the contribution of the metalloproteases to total activity was significantly higher (p<0.03) in *A. simplex s.s.* (70%) than in *A. pegreffii* (41%). These metalloproteases may contribute to the significantly greater capacity for penetration of the gut wall of laboratory animals by L3 of *A. simplex s.s.* (Romero et al., 2013).

As can be observed in the results (Figs. 3 and 4), the activity of cysteine protease in the species of *Anisakis* studied was low in almost all developmental stages at both pH values. It is noteworthy that, at pH 6.0, the L3-0h from the host fish showed greater activity in *A. simplex s.s.* (6-fold; p=0.05) than in *A. pegreffii*, while the opposite occurred (>2-fold in *A. pegreffii*) in the subsequent developmental stages (in the cetacean host). However, these data are difficult to interpret, particularly in view of their low activity (<12% of total proteolytic activity) and the great diversity of cysteine proteases reported from parasitic nematodes (Jasmer et al., 2001; Pratt et al., 1992; Rehman and Jasmer, 1999; Shompole and Jasmer, 2001) with a wide variety of, often vital, functions. These enzymes are present in both larvae and adults, suggesting involvement in nutrition, tissue penetration and defense against the host's immune system, in moulting, in development and maturation or in the adaptation of the nematode to the definitive host (Lustigman et al., 1996; Malagón et al., 2010b; McKerrow, 1995; M. L. Rhoads et al., 1998; Tort et al., 1999).
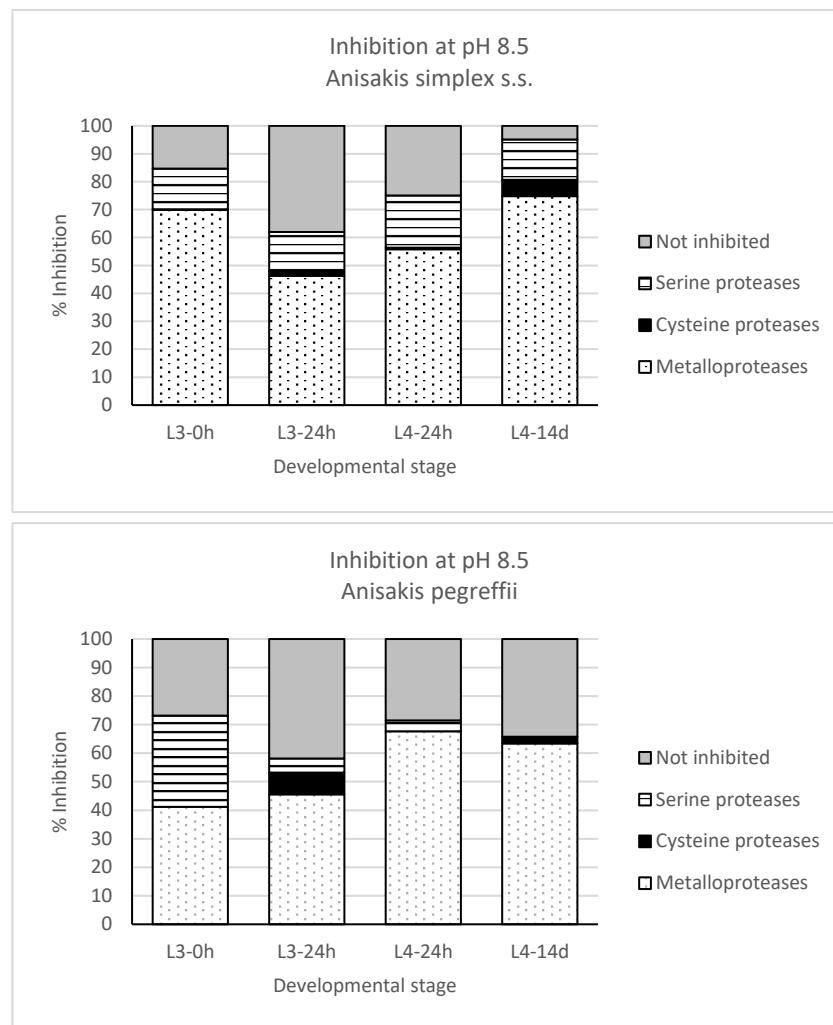
Figure 4.- Inhibition percentage of optimal bodipy FL casein cleaving activity at pH 8.5 in *Anisakis simplex* complex, during its *in vitro* development. Each inhibition percentage is the mean of three to five experiments in triplicate. Top: *A. simplex s.s.*. Bottom: *A. pegreffii*.

At pH 8.5 (Fig. 4), the activity of serine protease in *A. pegreffii* was <5% in all larval stages except L3-0h, in which it accounted for almost a third of total activity (32%), while, during the development of *A. simplex s.s.*, activity remained between 14-19% of the total. The difference in activity between the two species is significant at all stages ($p<0.05$) except in L3-0h ($p=0.09$). At pH 6.0 (Fig. 3), activity was even lower in both parasites (<12%) while statistical comparison of the species showed that activity was greater in *A. simplex s.s.* ($p≤0.05$), except in the most highly-developed stage (L4-14d; $p=0.20$). This type of enzyme is involved in the pathogenicity of the parasite and has a vital role in penetration of the host tissues. Sakanari and McKerrow (1990), in their examination of the products of excretion/secretion of L3 of *A. simplex*, found, in addition to metalloaminopeptidase, a trypsin-like serine protease (previously reported by Matthews, 1984), accounting for 80% of total secretory activity, which they implicated in the

pathogenicity of the parasite, attributing to it the penetration of the larva in the host tissues. Morris and Sakanari (1994), working with L3 extracts, identified two serine proteases: one, trypsin-like, 89% similar to porcine trypsin and of the same Mw as that secreted, and, another, 85% similar to one secreted by the pathogenic bacteria *Dichelobacter nodosus*, which is able to degrade the elastin, keratin and collagen of cell tissues. Collagenolytic activity associated with serine proteases and metalloproteases has previously been described in *H. aduncum*, in stages L3, L4 and adults (Malagón et al., 2010a). It is thus clear that several workers have implicated the serine proteases, including those of L3 of *A. simplex*, in the invasion and penetration of host tissues. As reported previously, *A. pegreffii* is less pathogenic than *A. simplex*. This may also be due to the significantly lower serine protease activity of the former at pH 6.0, particularly in L3, the stage which must establish itself in the definitive host, at 37 ºC.

At pH 6.0, all proteolytic activity was inhibited by the joint activity of the inhibitors used (Fig. 3). However, at pH 8.5, a part of the activity (4-42%) was not inhibited by any of the inhibitors (Fig. 4). In similar studies on *H. aduncum* at pH 5.5, the uninhibited activity was minimal (<5%), while, at pH 4.0 it was high (10-60%) (Malagón et al., 2011). This suggests that these nematodes may have special proteases which are not sensitive to general inhibitors or may have unusual types of protease, such as threonine or glutamic proteases. This study has found interspecific differences in some aspects related to pathogenesis in these and other nematodes and which could feasibly contribute to the differences in pathogenicity previously observed in these two species of *Anisakis*. Further research is required to clarify these questions.

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Compliance with ethical standards.

Conflicts of interest: the authors declare that they have no conflict of interest.

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# Anisakis simplex s.s. and A. pegreffii: two sibling species major etiological agents of anisakiasis, with different pathogenicity are showing differential cathepsin-like activity

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**a b s t r a c t**

Humans can contract anisakiasis by eating fish or squid containing live larvae of the third stage (L3) of the genus *Anisakis*. 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. Cathepsins B and L 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 extracted from fish and decreased during development, which could be related to virulence, invasion of host tissues and/or intracellular digestion. Cathepsin B activity was not identified with either of the substrates used (Z-RR-AMC and Z-FR-AMC). With Z-RR-AMC, 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 (90% inhibition with AEBSF). These results show differential serine protease activity between sibling *Anisakis* species, regulated by larval development, at least in *A. simplex s.s.*

## 1. Introducción

Anisakiasis is a parasitic 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. Cases have been described for the larvae of 4 genera of Anisakidae: *Anisakis*, *Contracaecum*, *Pseudoterranova* and *Hysterothylacium*, although more than 97% of these cases are caused by larvae of the genus *Anisakis*, principally belonging to the complex *Anisakis simplex s.l.*, in which case the infection is known as anisakiosis (Audícana and Kennedy, 2008). However, the most common name for an infection by any of the anisakid larvae is anisakiasis. It has been estimated that in Japan alone there are between 2000 and 3000 cases of anisakiasis each year (Umehara et al. 2007), although it is also often reported in Asian countries such as South Korea (Lim et al. 2015) or European countries such as Spain or Italy (Arenal Vera et al. 1991; López-Vélez et al. 1992; Pampiglione et al. 2002; Fumarola et al. 2009; and others). Although some cases have been reported for anisakiasis in humans caused by *A. physeteris* or *Anisakis* larvae type II sensu Berland (1961), (Asato et al. 1991; Clavel et al. 1993; Kagei et al. 1978), the etiologic agent is usually the larva of *Anisakis* type I, generally named *A. simplex*. The larvae of each type can only be differentiated molecularly. The few human cases diagnosed molecularly, to date, have been caused by *A. simplex s.s.* or *A. pegreffii*, two species of the complex *A. simplex s.l.* (Arai et al., 2014; Arizono et al., 2012; Lim et al., 2015; Mattiucci et al., 2013; Umehara et al., 2007; Yera et al., 2016; and others). 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 (Arai et al., 2014; Jeon and Kim, 2015; Quiazon et al., 2011; Rello Yubero, 2003; Romero et al., 2013; Suzuki et al., 2010).

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 and other processes such as digestion, embryogenesis and 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). 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 causing plant diseases. They may also be useful for diagnosis and development of vaccines since some cathepsins are highly conserved in nematodes (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.

## 2. Material y methods

2.1. Sample collection and *in vitro* culture

The L3 of *Anisakis* 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 on ice to our laboratory, where they were immediately processed to collect the larvae of these nematodes as indicated elsewhere (Molina-Fernández et al., 2018). Briefly, the larvae were placed in a petri dish, with 0.9% w/v NaCl solution, on a bed of ice, 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, washed in sterile saline, axenized and placed in individual culture as previously 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 washed in sterile saline and immediately 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 and Nascetti 2008; Molina-Fernández et al. 2015, 2018b; Mattiucci 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 (Mediterranean Sea, eastern Spain, zone FAO 37.1.1) for larvae of *A. pegreffii*.

2.2. Genetic identification

The genetic identification of the larvae was carried out as previously described (Molina-Fernández et al 2015, 2018a, 2018b). Briefly, we first proceeded to the individual extraction of larval DNA using the RealPure kit following the manufacturer's instructions. Next, an amplification of this DNA was carried out by polymerase chain reaction (PCR) using NC5 (forward) and NC2 (reverse) primers described by Zhu et al. (1998), obtaining an amplicon of about 1000 bp corresponding to the ITS1-5.8S-ITS2 region of the rDNA. Then, a restriction fragment length polymorphism (RFLP) of that rDNA fragment was carried out using two restriction enzymes, independently, *TaqI* and *HinfI* (Fast Digest, Thermo Scientific) at 65 °C and 37 °C for 10 min, respectively, using a final enzyme concentration of 0.5 U/µl. The band pattern generated was visualized by 3% agarose gel electrophoresis and compared to a control for each species. The digestion with *TaqI* showed, in *A. simplex s.s.*, a characteristic pattern of 3 bands of 430, 400 and 100 bp, while, in *A. pegreffii*, the pattern showed 3 bands of 400, 320 and 150 bp. With *HinfI*, *A. simplex s.s.* showed a 3-band pattern of 620, 250 and 100 bp, while, in *A. pegreffii*, the pattern showed 3 bands of 370, 300 and 250 bp. The larvae that showed the sum of band patterns of the two controls, with any of the restriction enzymes, were considered as L3 type I hybrid larvae for the purpose of this work.

## 2.3. Preparation of protein extracts

The extracts of the parasites were prepared extemporaneously on the day of the assay 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. The button was discarded. The method of Bradford (1976) was used to calculate the protein concentration of the extract.

## 2.4. Cathepsin assays

For the determination of the cathepsin L- and B-like activity of the soluble extract, the operation 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 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-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 excitation λ at 355 nm and λ emission at 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, in order 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.

2.5. Statistical study

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.

## 3. Results and discussion

3.1. Genetic identification

A total of 101 larvae of *Anisakis* type I from blue whiting from the port of Ondarroa (Cantabrian Sea) were analyzed using the PCR-RFLP technique, identifying 89.1% as *A. simplex s.s.*, 3.0% as *A. pegreffii* and 7.9% as hybrids of the two species. A further 55 larvae of *Anisakis* type I from the Mediterranean ports of Villajoyosa and Gandía were identified as 90.9% *A. pegreffii*, 1.8% *A. simplex s.s.* and 7.3% as hybrids. 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 and Nascetti 2008; Molina-Fernández et al. 2015, 2018a, 2018b; Mattiucci et al. 2018).

3.2. 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). A wide variety of functions have been proposed for them, including intra- and extracellular digestion, embryogenesis, moulting, tissular migration and modulation of the host immune system (Caffrey et al., 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 low (acidic) pH, with statistically significant differences between pH values (p<0.001), with pH 5.0 the most favourable (Fig. 1).
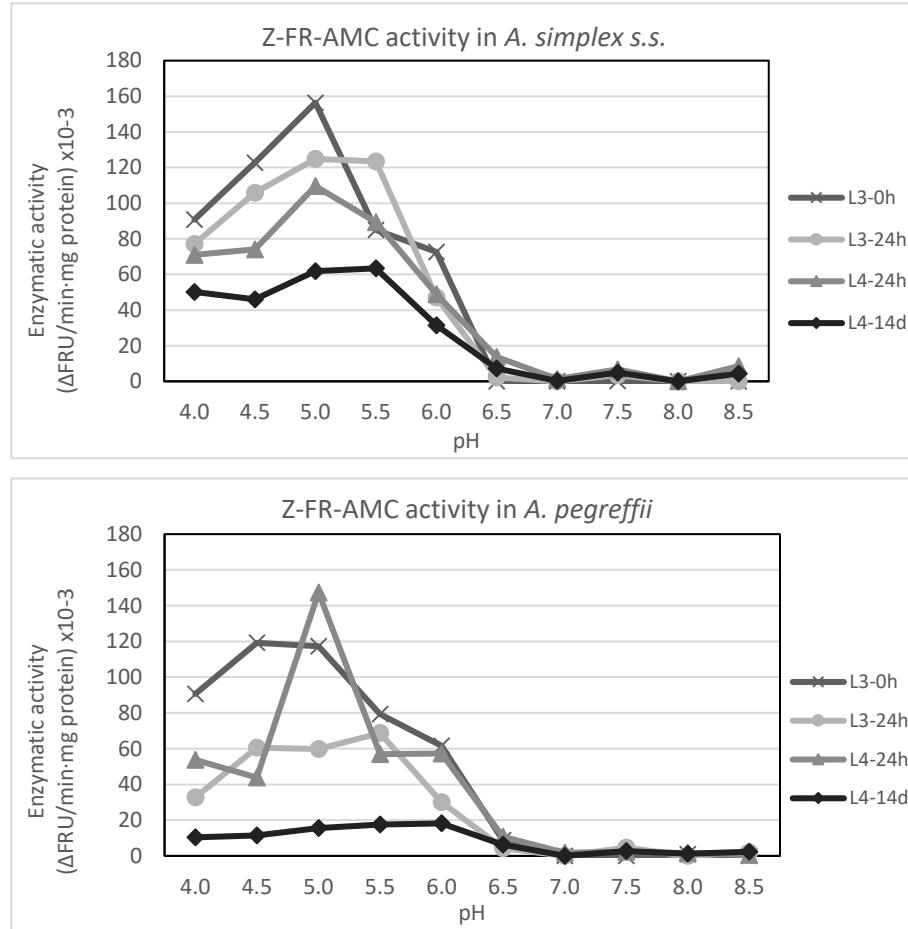


Figure 1. Profile of cathepsin L-like activity measured by Z-FR-AMC cleavage in *Anisakis* spp. during its *in vitro* development, depending on pH. Each point is the mean of two or three experiments in triplicate. Upper panel: *A. simplex s.s.* Bottom panel: *A. pegreffii*.

The cleaving of substrate Z-FR-AMC occurred between pHs 4.0-6.0 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. mansoni* 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.

With substrate Z-FR-AMC, maximum activity was detected at pH 4.5-5.5 at all developmental stages in both species of parasite (Fig. 1). 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 in 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 (Day et al. 1995; Neveu, 2003; Stack et al. 2008), 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 (Dávila et al., 2006; Iglesias et al., 1997; Sommerville and Davey, 1976; Yasuraoka et al., 1967). 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.
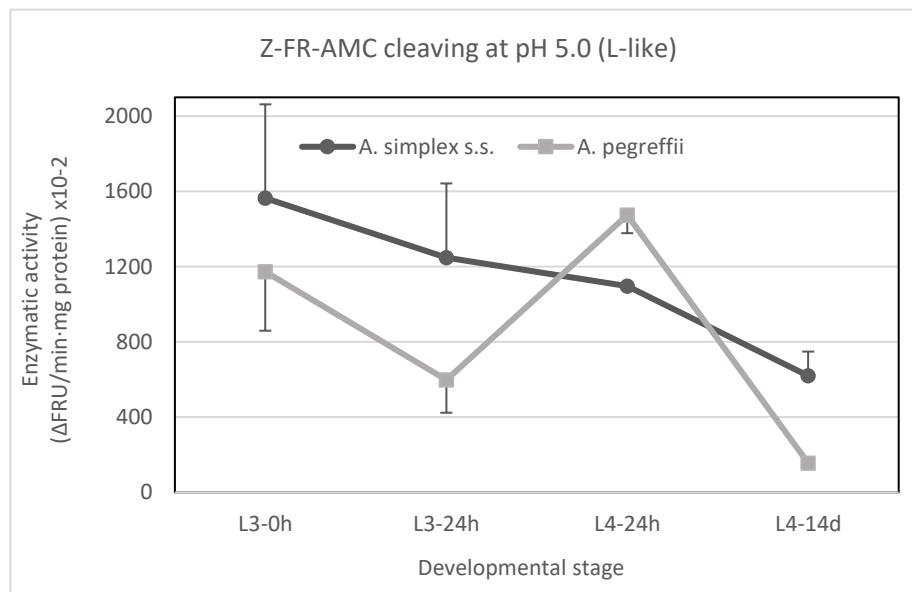


Figure 2.- Profile of cathepsin L-like activity as measured by cleavage of substrate Z-FR-AMC at pH 5.0 in *Anisakis simplex s.s.* (●) and in *A. pegreffii* (■) during its in vitro development. Each point is the mean $\pm$ standard deviation of two or three experiments in triplicate.

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 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 (Malagón et al., 2010; Sajid y McKerrow 2002) 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 other anisakids such as *H. aduncum* (Malagón et al., 2010), and perhaps also in processes related to attachment and moulting in an acidic medium associated with the gut 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).

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 (Fig. 3). In *A. pegreffii* activity was lower throughout all developmental stages, although somewhat higher at alkaline pH, especially in L4. Similarly, L3 of *A. simplex s.s.* showed very low activity. However, L4 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 within an optimum pH of 7.5 for cleaving Z-RR-AMC (Malagón et al. 2010). However, when the inhibition assays were carried out to determine the type of activity of the *Anisakis* extracts, no inhibition with E64 was observed under our experimental conditions, thus proving that it was not a cysteine protease and the activity was therefore not cathepsin B-like. Assays with other class-specific inhibitors revealed inhibition of around 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.
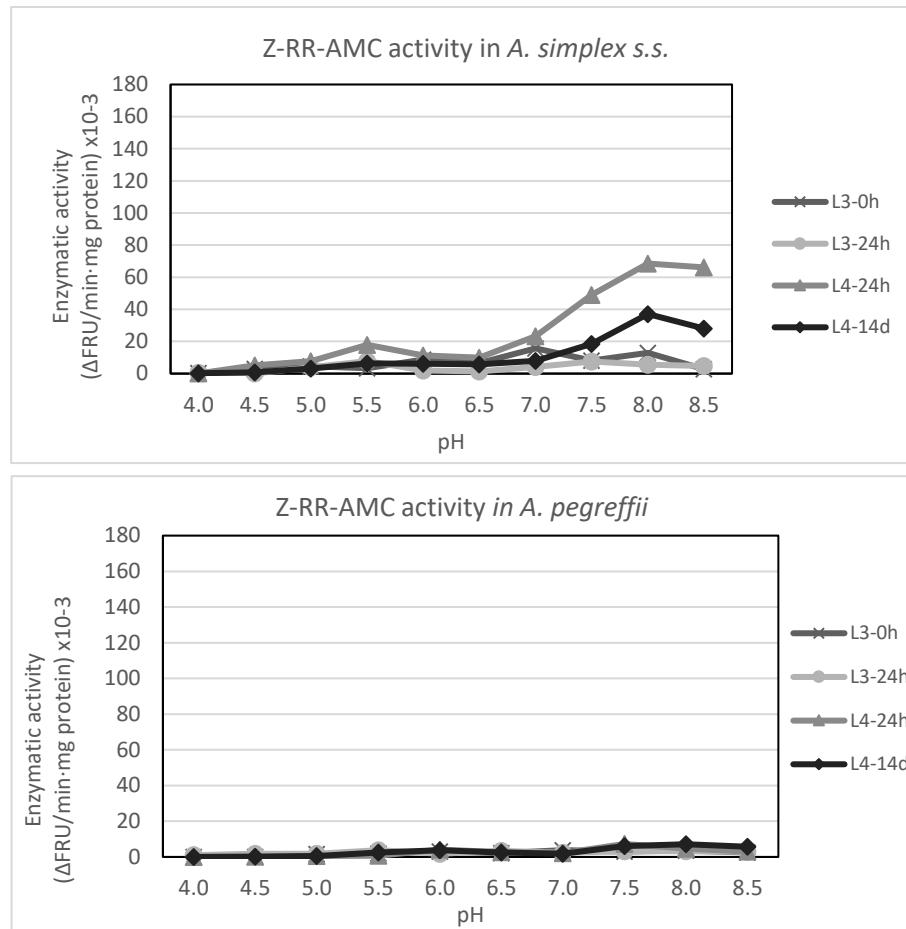
Figure 3.- Profile of enzymatic activity measured by cleavage of Z-RR-AMC in *Anisakis* spp. during its *in vitro* development, depending on pH. Each point is the mean of two or three experiments in triplicate. Upper panel: *A. simplex s.s.* Bottom panel: *A. pegreffii*.

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 y McKerrow, 1990). At a later date, 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 digestión 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.
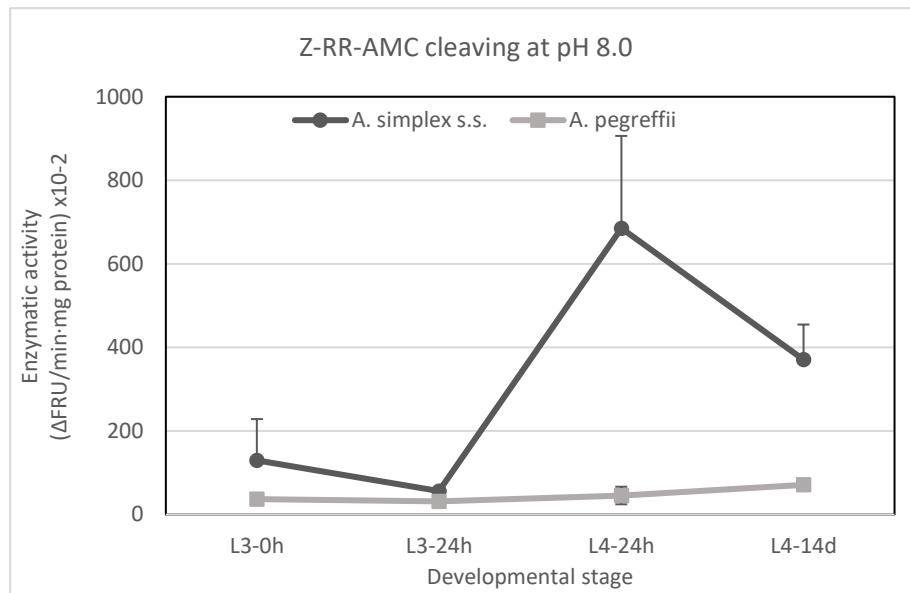
Figure 4.- Profile of enzymatic activity measured by cleavage of substrate Z-RR-AMC at pH 8.0 in *Anisakis simplex s.s.* (●) and in *A. pegreffii* (■) during its in vitro development. Each point is the mean $\pm$ standard deviation of two or three experiments in triplicate.

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*. It should also be noted that Molina-Fernández et al. (2018a) 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 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* (Dowd 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. 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 uncinaria in humans, with encouraging results so far (Robinson et al. 2009; Knox 2012; Hotez et al. 2013; Figueiredo et al. 2015).

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Compliance with ethical standards.

Conflicts of interest: the authors declare that they have no conflict of interest.

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*Anisakis simplex s.s.* and *A. pegreffii*: two sibling species major etiological agents of anisakiasis, with different pathogenicity are showing differential cathepsin-like activity

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# 6.
# DISCUSIÓN GENERAL

6.1. Epidemiología

Hasta la fecha se han descrito en España al menos 8 casos de anisakidosis asociados al consumo de sardinas: 4 casos de anisakiasis, 2 de ellos gastroalérgica, y 4 casos de alergia ligada al consumo de sardinas enlatadas (López-Vélez et al. 1992; López-Serrano et al. 2000; Audícana y Kennedy 2008). También se han diagnosticado varios casos en Japón debido al consumo de este pescado (Ishikura y Kikuchi 1990; Shirahama et al. 1992; Ishida et al. 2007). La sardina es un pescado con una amplia distribución, de elevado consumo en numerosos países, y además en algunas regiones se consume crudo, por lo que se debe evaluar el posible riesgo de anisakiasis debido a su consumo.

En nuestro estudio realizado en sardinas capturadas en distintos puntos de las costas españolas, no se han encontrado larvas de *Anisakis* en el pescado procedente de las costas del Mar Mediterráneo (Artículo I), como anteriormente se había reportado por otros autores (Rello et al. 2008; Gutiérrez-Galindo et al. 2010), aunque se halla otro anisákido, *Hysterothylacium aduncum*. Otros autores encuentran parasitación en esta área, pero en general la prevalencia es muy baja, siendo menor del 5% (Ruiz-Valero et al. 1992). Conforme nos alejamos de la costa española, en zonas cercanas a Cerdeña, Italia, la prevalencia se va incrementando hasta alrededor del 20% (Meloni et al. 2011; Angelucci et al. 2011; Piras et al. 2014). Esta prevalencia, sin embargo, es mucho menor que la de otros peces capturados en esta misma zona, como la caballa, el jurel o la bacaladilla, donde se han encontrado prevalencias cercanas al 100% (Angelucci et al. 2011; Piras et al. 2014).

Según otros estudios, no se ha encontrado parasitación por *Anisakis* en sardinas procedentes de las costas españolas en general (Viu et al. 1996; De la Torre Molina et al. 2000; Abollo et al. 2001). En algunas zonas de España, como en Galicia, se ha reportado una prevalencia del 10% (Sanmartín et al. 1994). La prevalencia más alta que hemos detectado en las costas españolas son las de las sardinas procedentes del puerto de La Coruña, 28,3% (Artículo I) (Molina-Fernández et al. 2015) siendo similar a la hallada en las costas del norte de Portugal, 28,1% (Silva y Eiras 2003). La prevalencia de larvas en musculatura encontrada por estos autores fue de 10,7% (Silva and Eiras 2003), valor similar a la que encontramos en sardinas procedentes de Galicia (9,1%). La presencia de larvas en la musculatura conlleva a un riesgo para la salud humana, pues esta es la parte del pescado que se suele consumir.

A diferencia de las sardinas, la prevalencia por *Anisakis* en bacaladillas suele ser alta. Varios autores han reportado prevalencias de 90-100% en bacaladillas procedentes de distintas zonas del Atlántico Nororiental (ver Tabla 1 de Artículo II). En las costas Atlánticas peninsulares la prevalencia oscila entre 53 % en el Golfo de Cádiz (Ojeda

Torrejón et al. 2001; Gómez-Mateos et al. 2016) y el 100% en aguas de Galicia (Chía et al. 2010). En cambio, en aguas del Mediterráneo peninsular la prevalencia por *Anisakis* oscila entre el 9% en el norte del mar de Alborán (Valero et al. 2000), el 30% en el golfo de Valencia (Cuéllar et al. 1991), pasando por el 25,1% en aguas de Tarragona (Osanz Mur 2001). En bacaladillas, además de varias especies de *Anisakis*, se ha reportado con frecuencia las L3 de *H. aduncum* y ocasionalmente L3 de otros anisákidos como *Hysterothylacium* sp., *Contracaecum* sp., *C. osculatum* y *Pseudoterranova decipiens*, así como L4 y adultos de *H. aduncum* (Karasev 1990).

Las aguas del Mediterráneo español muestran una menor prevalencia por *Anisakis*, tanto en sardinas como en bacaladillas, que las costas Atlánticas peninsulares. Sin embargo, la prevalencia aumenta conforme nos adentramos en el Mar Mediterráneo, por ejemplo, en las zonas cercanas a Cerdeña, con prevalencias de más del 80% en bacaladillas y de alrededor del 20% en sardinas (Angelucci et al. 2011; Meloni et al. 2011). Uno de los posibles factores que pueden justificar la diferente prevalencia de *Anisakis* en el Mediterráneo occidental es la diferente distribución de hospedadores definitivos (cetáceos) y abundancia de zooplancton, potenciales primeros hospedadores del ciclo de vida de los anisákidos. En las aguas de Córcega, incluido el mar de Liguria, y la costa norte de Cerdeña, se ha estimado que existen unas mil ballenas y más de 25.000 delfines, y se destaca la importancia de esta zona como hábitat para estos mamíferos marinos (Forcada et al. 1995; Forcada y Hammond 1998; Notarbartolo di Sciara et al. 2008). Se estima, por ejemplo, que hay >0,30 delfines por kilómetro cuadrado en el mar de Liguria, cuando en el mar Balear por ejemplo esta cifra se sitúa entre 0,08 y 0,09, según si hablamos de la región sur o norte (Forcada y Hammond 1998). La densidad de delfines en el mar Balear es un 63% menor que en las otras zonas del Mediterráneo noroccidental, estimándose que existen menos de 6.000 delfines en esta zona. Esta diferencia de densidad de mamíferos marinos se puede explicar por las características del agua del mar de Liguria, donde la presencia de masas de aguas frías, acompañadas de fuertes vientos, frecuentes en dicha zona, provocan el afloramiento de aguas profundas, ricas en nutrientes y zooplancton. Esto supone una fuente de alimento para los delfines y otros depredadores marinos (Jacques 1989; Orsi Relini et al. 1992) que pueden actuar como hospedadores definitivos de *Anisakis*.

Además de todo lo mencionado anteriormente, se suma el hecho de que en esa zona se encuentra un Área Marina Protegida (conocidas como MPAs), creada para la conservación de especies en peligro y de sus hábitats (Agardi, 1997 y 2001). Este tipo de áreas se establecen para proteger hábitats acuáticos importantes, incluyendo zonas de parto y áreas de alimentación (Hoyt 2005). Esta zona protegida ocupa el noroeste del Mar Mediterráneo, limita en el norte con Francia, Italia y Mónaco, y en el sur con la isla

de Cerdeña, abarca Córcega y las aguas internacionales adyacentes y se conoce como Santuario de Pelagos (Figura 13). Tiene una superficie de 90.000 km² y se caracteriza por los altos niveles de producción primaria, gracias a la interacción de factores oceanográficos, climáticos y geomorfológicos. Además, la presencia de una corriente ciclónica dominante crea una barrera entre la costa y el mar adentro, que intensifica la actividad biológica de las zonas próximas y además facilita los afloramientos de aguas profundas y ricas. La mayor actividad primaria en esta zona hace que se genere una gran cantidad de biomasa y un zooplancton de enorme diversidad biológica, lo que atrae a depredadores como mamíferos marinos (Tethys Research Institute).



Figura 13. Santuario de Pelagos.

En otras regiones, como en la parte sur del mar Mediterráneo Occidental, las surgencias o afloramientos de nutrientes no suelen ser grandes, y no contribuyen por tanto a la producción de zooplancton (Forcada y Hammond 1998), por lo que la presencia de mamíferos marinos en esta zona es menor. Sin embargo, hay mayor densidad de delfines en el mar de Alborán que en el mar Balear. Esto puede deberse a la intensa corriente que ocurre cuando el agua del Altántico ingresa por el estrecho de Gibraltar, sobre todo por su parte norte, que se asocia a un fenómeno de afloramiento muy intenso (Le Vourch et al. 1992). Por lo tanto, la zona del estrecho de Gibraltar sería más rica en nutrientes que las adyacentes y fomentaría la afluencia de vida marina. Esto podría ser uno de los factores que expliquen la mayor prevalencia de *Anisakis* en peces

del Golfo de Cádiz que en las regiones procedentes del norte del Mar de Alborán (Gómez-Mateos et al. 2016). El golfo de Cádiz, además, se presenta como un lugar de gran interés ecológico, ya que muchas especies de mamíferos marinos que pueden actuar como hospedadores definitivos de *Anisakis* viven en esta zona o la utilizan como zona de paso durante sus migraciones (Raga y Pantoja 2004; Mattiucci y Nascetti 2008).

El mar Atlántico que baña la Península Ibérica se describe, a diferencia del Mediterráneo español, como una zona con gran densidad de mamíferos marinos, estimándose que la habitan unos 30.000 delfines y marsopas (Santos et al. 2014). Además, la mayor presencia de eufáusidos que pueden actuar como primeros hospedadores intermediarios para *Anisakis*, junto con la mayor densidad de hospedadores definitivos comentada, podrían facilitar el mantenimiento del ciclo de vida de *Anisakis* en el Atlántico (Raga y Pantoja 2004; Papetti et al. 2005; Rello et al. 2008), al igual que ocurre en el Santuario de Pelagos. De hecho, los estudios epidemiológicos sobre la infección de *Anisakis* spp. en peces de aguas del Atlántico peninsular, como ya se ha comentado, muestran una clara mayor prevalencia que en los de su misma especie procedentes del Mediterráneo español.

Por otro lado, se observa una mayor prevalencia de *Anisakis* en el pescado de mayor tamaño y peso. Esto ocurre tanto en bacaladillas como en sardinas, y este hecho había sido previamente descrito por diversos autores como Bussmann y Ehrich (1978) o Valero et al (2000) para bacaladillas, Takao et al (1990) para sardinas del género *Sardinops* , arenques  (Van Thiel et al., 1960), bacalaos (Wootten y Waddel, 1974), jureles (Adroher et al. 1996), etc. Esto podría deberse a que las bacaladillas y las sardinas de menor tamaño se alimentan mayoritariamente de fitoplancton y copépodos (Palomera et al. 2007), que, aunque pueden infectarse directamente mediante la ingestión de L3 de *Anisakis*, las larvas no se desarrollan correctamente en ellos (Køie 2001). Cuando los peces aumentan de tamaño incorporan a su dieta otros componentes del plancton marino, como eufáusidos, que sí son unos adecuados hospedadores intermediarios de *Anisakis* (Cunha 2005, Palomera et al. 2007). Por otra parte, la mayor intensidad que se halla en los peces de mayor tamaño podría deberse a la acumulación de parásitos en el hospedador (Valero et al. 2000), ya que las larvas pueden vivir hasta 3 años en los peces (Smith 1984; Strømnes y Andersen 2003). Además de estos factores, no hay que olvidar otros como la dieta de los peces, pues cuanto mayor es un pez más presas consume y de mayor tamaño serán éstas, con lo que se incrementa la probabilidad de ingerir presas parasitadas.

Tanto en bacaladillas como en sardinas (Artículos I y II), hemos descrito la presencia de larvas de *Anisakis* del tipo I, y en bacaladillas además la presencia de Anisakis del tipo II (según Berland, 1961). Del clado I son las larvas de *Anisakis simplex*

*s.s.*, de *A. pegreffii* y los individuos recombinantes o híbridos entre ambas especies. Del clado II, la única especie encontrada es *A. physeteris*, aunque ésta sólo se ha encontrado en bacaladilla del Mediterráneo.

La especie predominante en nuestros estudios, tanto en bacaladillas como en sardinas fue *A. pegreffii*. Las bacaladillas procedían de la costa oriental española. Este dato concuerda con lo esperado, ya que *A. pegreffii* es la especie predominante en el mar Mediterráneo occidental (Mattiucci y Nascetti 2008). De hecho, la probabilidad de encontrar L3 de *A. pegreffii* en bacaladillas del Mediterráneo es 6 veces mayor que la de encontrarlas en las bacaladillas del Atlántico (Romero et al. 2013). Un 5,4% de las larvas halladas en bacaladillas (4 larvas) se encontraron en la musculatura con una prevalencia del 2,1%, mientras que en sardinas estos datos supusieron el 3,3% de las larvas (3 larvas) y el 1,1% de prevalencia. Estas larvas son las más importantes desde el punto de vista sanitario, pues la musculatura es la región del pescado que se suele consumir, y, por tanto, la que supone un mayor riesgo de producir anisakiasis en el caso de que contenga larvas. De las 7 larvas en total halladas en esta parte del pescado, 5 fueron identificadas como *A. pegreffii*, una como *A. simplex s.s.* y una como recombinante entre ambas especies. Esto concuerda con la mayor prevalencia hallada por *A. pegreffii* en nuestros estudios. Se ha demostrado que, aunque *A. pegreffii* presenta una menor capacidad que *A. simplex s.s.* de penetrar en la musculatura del pez y en la pared gastrointestinal de animales de experimentación, también es capaz de hacerlo (Suzuki et al. 2010; Quiazon et al. 2011; Romero et al. 2013), infectando también al hombre. Prueba de ello son los casos de anisakiasis producidos por *A. pegreffii* que se han descrito en humanos (D'Amelio et al. 1999; Umehara et al. 2007; Fumarola et al. 2009; Mattiucci et al. 2011, Arizono et al. 2012; Mattiucci et al. 2013; Lim et al. 2015; Mladineo et al. 2016; Yera et al. 2016; Mattiucci et al. 2017).

En cuanto a *A. physeteris*, aunque se ha descrito parasitando bastantes especies de peces, en nuestro ámbito geográfico es más frecuente en el Mediterráneo que en el Atlántico, aunque depende también de la especie de pez. Por ejemplo, en la bacaladilla es frecuente en el Mediterráneo y muy rara en el Atlántico (ver Tabla 1 del Artículo II), pero en otros peces, como la merluza o el pez espada, presentan este parásito los procedentes tanto los de unas aguas como de las otras (Matiucci et al. 2018). No se ha descrito la presencia de este nematodo en sardinas hasta la fecha. Esto podría deberse a la dieta de estos peces que no deben incluir los hospedadores intermediarios/paraténicos de *A. physeteris* y/o al hábitat de los mismos. El primer estudio llevado a cabo en bacaladillas capturadas en el Mediterráneo que incluyera una identificación molecular de las larvas halladas fue llevado a cabo por Orecchia et al. en 1989 en aguas italianas (Orecchia et al. 1989). En él se describe la presencia de *A.*

*pegreffii* y de *A. physeteris*, con una prevalencia del 62% y del 2,7% respectivamente. Valero et al. (2000) también muestran la presencia de *Anisakis* del tipo II en el mar de Alborán. Otros autores han descrito en aguas de Cerdeña la presencia de *A. physeteris*, de *A. pegreffii* y de híbridos *A. pegreffii/A. simplex s.s.*, donde curiosamente destaca la ausencia de *A. simplex s.s.* a pesar de existir individuos híbridos/recombinantes (Meloni et al. 2011; Tedde et al. 2011; Angelucci et al. 2011; Piras et al. 2014).

## 6.2. Desarrollo y morfología de *A. physeteris* y de *C. multipapillatum*

### *6.2.1. Contracaecum multipapillatum s.l.*

El primer cultivo *in vitro* con *Contracaecum multipapillatum* se llevó a cabo en 1967 por Huizinga (1967). Estos fueron los primeros estudios para tratar de aclarar el ciclo de vida de este anisákido. Hasta ese momento sólo se conocía, y de manera parcial, algunos aspectos de la biología de las especies del género *Contracaecum* gracias a los estudios de Thomas (1937, 1940) realizados en cormoranes, y de Penner (1941), que tomaron en consideración la importancia de los copépodos como hospedadores intermediarios de este parásito. Fue Huizinga (1967) quien hizo los avances más significativos para comprender mejor su biología, analizar sus características morfológicas y dilucidar su ciclo de vida. Para ello, extrajo huevos directamente del útero de hembras de *C. multipapillatum* obtenidas del proventrículo del pato-aguja americano (*Anhinga anhinga*). Estudió el desarrollo de estos huevos en distintos medios como agua de mar, de lagos o de grifo, jugo gástrico artificial o formalina. El autor describe que el desarrollo de los huevos hasta L1 tiene lugar en 5 días a 21 ºC, y que tras una muda que ocurre en el interior del huevo, eclosiona la L2. Según Huizinga, esta L2 sería ingerida por copépodos, donde no ocurren más mudas, y posteriormente pasaría a los peces cuando éstos ingieren copépodos infectados, y por último a aves piscívoras.

En nuestros estudios realizados sobre el desarrollo larvario de este mismo parásito (Artículos III y IV), trabajamos a partir de huevos de hembras maduras y fecundadas del anisákido obtenidas de *Pelecanus occidentalis* o pelícano pardo procedente del Golfo de California. Se llevó a cabo la identificación molecular de las hembras de los nematodos y se identificaron como *C. multipapillatum*, pero sin poder adscribirse a ninguna de las especies ya descritas pertenecientes a este complejo (ver artículo III). Mientras el grupo mexicano que trabaja en la caracterización de esta especie no asigne un nombre a la misma, la seguimos denominando *C. multipapillatum s.l.* Con los huevos de dicha especie hemos probado soluciones con distinta salinidad, así como diferente temperatura. Huizinga (1967) indica en su trabajo que el tamaño de los huevos era entre 65 y 58 µm, un dato muy similar al obtenido por nosotros, ya que observamos que los huevos, aunque en las primeras etapas de desarrollo, miden entre 53,4 y 43,1 µm, cuando la larva se desarrolla dentro del huevo el tamaño de éste aumenta hasta los 65,5 x 54,8 µm. Vidal Martínez et al. (1994) reportan medidas de 53 x 38 µm, y Lucker (1941) de 60 x 50 µm (Lucker 1941). Vemos por tanto que existe una variabilidad de medidas, debido probablemente a la falta de rigidez de la cubierta del huevo y a la elasticidad que presenta cuando la larva se encuentra en su interior, que se asocia a la movilidad de ésta (Adroher et al. 2004), además de la propia variabilidad intraespecífica.

Uno de los aspectos sobre el ciclo de vida de los anisákidos que más debate genera está relacionado con el desarrollo que ocurre dentro del huevo y el estadio larvario que se libera tras la eclosión. Hay autores que defienden que es el segundo estadio larvario (L2) el que sale del huevo, ya que en su interior solo detectan una muda, como describe Huizinga (1966, 1967), en dos especies del género *Contracaecum*: *C. spiculigerum* y *C. multipapillatum*; Bartlett (1996) en *C. rudolphi*; Measures y Hong (1995) en *P. decipiens*, Yoshinaga et al. (1987) en *H. aduncum* o Smith (1983) en *A. simplex*. Otros autores, de los cuales el primero fue Thomas (1937), quien observó el desarrollo de los huevos de *C. spiculigerum* en placas Petri, describe la presencia de 2 mudas en el interior del huevo, por lo que de éste saldría la L3 y no la L2. Del mismo modo, el grupo de Køie en 1993 y 1995 llegan a la conclusión de que ocurren 2 mudas dentro del huevo y que es la L3 la que se libera tras la eclosión, al menos en los anisákidos que ellos estudian: *C. osculatum*, *P. decipiens* y *A. simplex* (Køie y Fagerholm 1993; Køie et al. 1995). Para tratar de aclarar estas cuestiones sobre el ciclo de vida de *C. multipapillatum*, se estudiaron las larvas obtenidas tras la eclosión de los huevos de este nematodo, como se describe en el Artículo III, y se observó que presentan las características típicas de las L3 que se encuentran en peces, tanto en microscopía óptica como electrónica, aunque lógicamente de menor tamaño y menos desarrolladas, por lo que proponemos que el estadio que se libera del huevo en este anisákido, como en los antes mencionados, sería la L3 y no la L2. Smith y colaboradores en 1990 (Smith et al. 1990) infectaron trucha arcoíris con larvas recién eclosionadas de *C. osculatum* y *P. decipiens* y siguieron su desarrollo en los peces, llegando a la conclusión que las larvas recién salidas del huevo muestran las mismas características morfológicas que las larvas que se desarrollaron en los peces, y que no se observa ninguna muda entre las primeras y las segundas. Esto apoyaría la nuestra propuesta de que las larvas que eclosionan de los huevos serían L3 (Figura 14).

El ciclo de vida de *C. multipapillatum* que proponemos para la bahía de La Paz sería el siguiente:
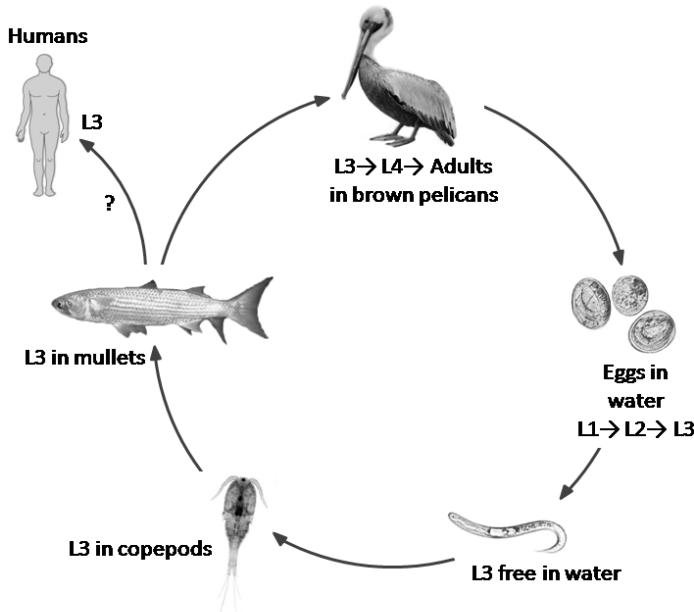


Figura 14. Ciclo de vida de *Contracaecum multipapillatum* propuesto para la Bahía de la Paz, México.

Se trata de una zona geográfica donde habitan pelícanos, que son hospedadores definitivos de *Contracaecum*. Las lisas (*mullets*), que forman parte de la dieta de los pelícanos, actuarían como segundos hospedadores intermediarios/paraténicos. El diagnóstico molecular de las larvas halladas en las lisas coincide con el de las hembras adultas recogidas en el pelícano (Artículo III). Los copépodos actuarían como primeros hospedadores intermediarios, ingiriendo las L3 que se encuentran libres en el agua.

6.2.2. *Anisakis physeteris*

Tradicionalmente, las larvas del género *Anisakis* se han agrupado en dos tipos, según su morfología (Berland 1961). Las especies pertenecientes al tipo I presentan un ventrículo más alargado, su unión con el intestino es oblicua, y el final del extremo posterior termina en una pequeña espina o mucrón. Las especies del tipo II tienen un ventrículo más corto, con unión recta con el intestino, y el final de la cola no presenta una espina terminal. Dentro del tipo II se han descrito, molecularmente, 3 especies: *A. physeteris*, *A. paggiae* y *A. brevispiculata*. Aunque la mayoría de los casos de anisakiasis que se han descrito hasta la fecha han sido causados por especies del tipo I, como *A. simplex s.s.* y *A. pegreffii*, también se han reportado casos producidos por *A. physeteris*, tanto por L3 (Asato et al. 1991), como L4 (Clavel et al. 1993) y L3 en proceso de muda a L4 (Kagei et al. 1978). Por lo tanto, se deben tener en consideración las especies del tipo II como posibles causantes de anisakiasis, ya que se ha demostrado, por ejemplo, que tanto *A. physeteris* como *A. paggiae* pueden provocar lesiones en animales de experimentación y ambas son, por tanto, potencialmente patógenas para los humanos (Romero et al. 2014). Además, el hecho de que se haya encontrado en humanos no solo la L3 sino también la L4 hace necesario conocer sus características morfológicas, de modo que permitan una correcta identificación de la larva en cualquiera de sus estadios juveniles. Son abundantes los trabajos que describen las L3 y los adultos de anisákidos, pero las descripciones de las L4 suelen ser escasas e incompletas. Además, el conocimiento de las características morfológicas es útil para un mejor entendimiento de la biología y la taxonomía de las especies. Para ello, L3 recogidas de peces e identificadas molecularmente como *A. physeteris*, fueron usadas para el estudio al microscopio electrónico de barrido (Artículo V).

La L3, que es la que se encuentra en los peces y por tanto el estadio que puede producir anisakiasis en el hombre, presenta en su extremo anterior estructuras sensoriales como papilas cefálicas, ánfidos y deiridios pero hemos observado que todos ellos se encuentran recubiertos por la cutícula, por lo que no sabemos si son funcionales en este estadio, o lo serán tras la muda a L4, cuando quedan expuestos al medio externo. En este sentido, Davey (1971) indica que los deiridios ya están formados en las L3, como podemos observar en nuestro estudio gracias a las imágenes de microscopía electrónica. Jones (1994) propone que los ánfidos de las L3 de *A. simplex* sí son funcionales, y sugiere que también lo son las papilas, ya que observa que la estructura interna está desarrollada. Si analizamos estudios realizados con otros anisákidos, vemos que ya aparecen estructuras tipo papila en la L3 recién salida del huevo, como hemos visto además en *C. multipapillatum* en el artículo IV (McClelland y Ronald 1974; Molina-Fernández et al. 2017), a diferencia de otras estructuras que se forman cuando la larva

se desarrolla y madura, lo que podría indicar que estas estructuras mencionadas anteriormente podrían ser, de algún modo, funcionales en las L3.

En las L4 destaca la aparición de dentículos en las crestas de los labios, que se habían descrito ya en las L4 de tipo I (Weerasooriya et al. 1986) y de tipo II (Kikuchi 1974). Estas crestas denticuladas continúan por el lateral de los labios, disminuyendo de tamaño y aparentando un menor grado de desarrollo. Weerasooriya y colaboradores en 1986 (Weerasooriya et al. 1986) no hacen referencia a que existan dentículos fuera de las crestas de los labios en la L4 de tipo I, pero en nuestro laboratorio se ha podido comprobar que, al menos *A. pegreffii*, sólo tiene dentículos en las crestas de los labios, a diferencia de lo encontrado para *A. physeteris*.

En cada labio subventral de la L4 hay una papila cefálica y un ánfido. La papila es alargada y grande, dirigida hacia el otro labio subventral, con estructura similar a la descrita para los adultos de otros anisákidos (Weerasooriya et al. 1986; Abollo y Pascual 2002; Di Azevedo et al. 2015). El ánfido es anular y está localizado hacia el labio dorsal. A diferencia de los labios subventrales, el labio dorsal presenta dos papilas cefálicas, similares en forma y tamaño a las presentes en los labios subventrales, pero ningún ánfido. En la parte superior de los tres labios, cerca de la cresta denticulada, se observa un pequeño poro no descrito previamente, cuya función desconocemos. Conforme aumenta el desarrollo, los labios se van haciendo bilobulados, separándose los dos lóbulos por una hendidura más o menos marcada, como se describe en la L4 de *Anisakis* tipo I (Weerasooriya et al. 1986) y en adultos del género *Anisakis* (Davey 1971). Otro órgano sensorial hallado en las L4 de *A. physeteris*, pero no encontrado en la L3, son los fásmidos en la parte posterior, descritos previamente en adultos, y que están, pareados y simétricos, cerca del extremo de la cola. Finalmente merece la pena comentar que bajo la cutícula de las L4 más desarrolladas y que van a dar lugar a los machos, se observó unas elevaciones que deben corresponder a las papilas preanales de los machos adultos. Como se observa en este trabajo, las estructuras, al menos las superficiales, que se necesitan para una fase de desarrollo determinada, se van formando en la fase anterior para ser probablemente funcionales inmediatamente tras la muda de una fase a la siguiente (ver artículo V). También destaca la presencia de la vulva en las hembras L4, que se encuentra cubierta por la cutícula y por tanto no se ha podido observar mediante microscopio electrónico de barrido pero sí en microscopio óptico.

6.3. Actividad proteolítica en especies gemelas de *A. simplex s.l.*

El interés sobre la actividad proteolítica en larvas de *Anisakis* comenzó en los años 80 cuando Matthews (1982, 1984) encontró en sus L3 una enzima proteolítica que presentaba propiedades parecidas a la tripsina. Más adelante, Kennedy et al. (1988) estudiaron los productos de excreción/secreción y los antígenos somáticos de esas L3 y mediante SDS-PAGE revelaron 2 bandas con actividad proteasa, con un peso molecular estimado de 23.400 y 46.100 daltons, planteándose si se trataba de una molécula dímera o de dos proteasas diferentes.

Sakanari y McKerrow (1990) encuentran dos tipos de proteasas en los productos de excreción/secreción de la L3 de *Anisakis* a pH 7,8, que se correspondían con metaloproteasas y serín proteasas, siendo mayoritaria la última. Esta serín proteasa es tipo tripsina con un peso molecualr de 25 kD, y ellos sugieren que puede ser la misma que encontraron Kennedy et al. (1988) y que podría estar relacionada con la patogenicidad del parásito. Más tarde, Morris y Sakanari (1994) detectan actividad por metaloproteasas y cisteín proteasas en los extractos de L3 de *Anisakis* durante el aislamiento de dos serín proteasas: una resultó ser idéntica a la secretada, descrita anteriormente, y otra presentaba una similitud del 85% con la secuencia de otra serín proteasa secretada por una bacteria patógena, *Dichelobacter nodosus*, que es capaz de degradar la elastina, la queratina y el colágeno de los tejidos celulares. En este sentido, *H. aduncum* ha mostrado actividad colagenolítica *in vitro*, tanto en la L3 como en la L4, debida, al menos en parte, a serín proteasas, aunque también a metaloproteasas (Malagón et al. 2010a).

En el artículo VI que presentamos en esta memoria hemos investigado la actividad proteolítica, tratando de caracterizarla parcialmente, de dos especies gemelas del complejo *A. simplex s.l.*: *A. simplex s.s.* y *A. pegreffii*, principales agentes etiológicos de la anisakiasis, ya que, como se ha descrito anteriormente, parecen mostrar distinta patogenicidad. Esta actividad se ha seguido a lo largo del desarrollo *in vitro* de los parásitos desde L3 hasta L4 a lo largo de 2 semanas de cultivo, con el fin de observar la posible evolución diferencial, no solo entre especies, sino entre las fases de desarrollo, y tratar de relacionar las actividades y sus cambios, si se producían, con una posible función biológica. Se han detectado dos picos de máxima actividad a pH 6,0 y 8,5 con L3-0h de *A. simplex s.s.* A pH 6,0 los dos estadios de L4 presentan una actividad significativamente mayor que los dos de L3 (p<0,01) en ambas especies. Esto podría estar relacionado con el hecho de que las L3 no parecen ingerir nutrientes por vía oral (Yasuraoka et al. 1967; Sommerville y Davey 1976) hasta que mudan a L4, nutriéndose probablemente mientras tanto a través de la cutícula. Así, la digestión externa y absorción de aminoácidos a través de la cutícula podrían constituir un mecanismo de

adaptación y supervivencia de la L3, al igual que la elongación de las cadenas carbonadas de los intermediarios de la glucolisis a través de la incorporación de $CO_2$ por fijación, gracias a la fosfoenolpiruvato-carboxikinasa (PEPCK) demostrada tanto en *A. simplex* como en *H. aduncum* (Dávila et al., 2006; Malagón et al., 2009), contribuyendo al metabolismo del parásito. Es tras la muda a L4 cuando el parásito comienza a alimentarse de manera oral, y este cambio en la forma de adquirir los nutrientes puede hacer necesaria la expresión de distintas proteasas de función digestiva. Dziekónska-Rynko y colaboradores, trabajando con L3 y L4 de *A. simplex*, observaron que las L4 muestran mayor actividad proteolítica con función digestiva que las L3 (Dziekónska-Rynko et al. 2003), reportando que algunas hidrolasas solo se detectan en L4, y las que encuentran en ambos estadios larvarios, presentan mayor actividad en las L4 (a excepción de la fosfatasa ácida). Los autores sugieren que estos cambios en la actividad de las hidrolasas digestivas estudiadas podrían estar relacionados con diferencias metabólicas que tienen lugar tras la muda a L4, producidos por el cambio en la forma de alimentación de la larva. Así, los cambios que se detectan en nuestro estudio al pasar de L3 a L4 podrían también estar relacionados sobre todo con procesos digestivos y metabólicos, ligados a la nutrición del parásito, ya que una parte importante (34-54%) de la actividad proteolítica detectada se debe a aspártico proteasas, con frecuencia implicadas en procesos digestivos en nematodos parásitos (*Angiostrongylus cantonensis, Necator americanus*) y de vida libre como *Caenorhabditis elegans* (Hawdon et al. 1989; Brown et al. 1995; Geldhof et al. 2000; Williamson et al. 2003a, 2003b; Yang et al. 2009; Chang et al. 2011).

A pH 8,5 la actividad proteolítica es mayor en *A. pegreffii* que en *A. simplex s.s.*, con diferencias estadísticamente significativas, aunque en ambas especies sigue una tendencia similar, siendo máxima en las L4-24h. La actividad mayoritaria se debe a metaloproteasas (40-75%), enzimas que se han relacionado con numerosos procesos como la evasión del sistema inmune del hospedador, la muda o la nutrición de los nematodos (Hong et al. 1993; Rhoads et al. 1997; Rhoads y Fetterer 1998; Culley et al. 2000). En anisákidos tales como *A. simplex*, *P. decipiens* e *H. aduncum*, las metaloproteasas se han relacionado con la activación de moléculas biológicas, con la muda y con la digestión (Davey y Sommerville 1974; Sakanari y McKerrow 1990; Malagón et al. 2011). Esta diversidad de funciones puede explicar que aparezcan como actividad mayoritaria, tanto a pH 8,5 como a 6,0 (40-52%). En *H. aduncum*, también aparecen como actividad principal a pH 5,5 (Malagón et al. 2011). En la fase L3-0h recién extraída del hospedador, la actividad de las metaloproteasas es mayor en *A. simplex s.s.* que en *A. pegreffii*, con diferencias estadísticamente significativas. Esta diferencia podría estar relacionada con la mayor capacidad de penetración en la pared digestiva del hospedador de las L3 de *A. simplex s.s.* como se ha comentado anteriormente.

Malagón et al. (2011) relacionan estas metaloproteasas de la L3 infectiva con la invasión del hospedador. Las serín proteasas y cisteín proteasas presentan una baja actividad en las dos especies de *Anisakis* estudiadas, aunque cabe destacar que existe una diferencia estadísticamente significativa (p<0,05) en la actividad las serín proteasas en todas las fases, excepto en L3-0h (p=0,09), entre estas dos especies, siendo mayor en *A. simplex s.s.* Esto podría estar relacionado con su mayor patogenicidad ya que se ha sugerido que estas enzimas tienen un papel en la patogenicidad de los parásitos y en la invasión de los tejidos del hospedador en algunos nematodos y se han relacionado con la patogenicidad de *A. simplex* (Sakanari y McKerrow 1990; Morris y Sakanari 1994).

Además, en el artículo VII, sobre actividad catepsina, se detecta más actividad serín proteasa en *A. simplex. s.s.* que *en A. pegreffii* con el sustrato Z-RR-AMC. Esta actividad se detecta a un máximo entre 7,5 y 8,5 y el pico de mayor actividad aparece a pH 8,0. La mayor actividad se observa en las L4 de *Anisakis simplex s.s.*, siendo la L4-24h la fase con más actividad, seguida de la L4-14 días, y siendo la actividad prácticamente impercepsible en *A. pegreffii*. A pesar de emplearse un sustrato específico de catepsinas B, cuando se han realizado ensayos con inhibidores, se observa que la actividad que estamos detectando no se corresponde con estas catepsinas ya que no se inhibe con E64, inhibidor de cisteín proteasas, sino que mayoritariamente está compuesta por serín proteasas y en parte por metaloproteasas. Casi el 90% de la actividad en las L4 de *A. simplex s.s.* se inhibe con AEBSF (inhibidor de serín proteasas). Según se ha demostrado, las serín proteasas también son capaces de degradar los sustratos Z-RR-AMC y Z-FR-AMC. Morris y Sakanari (1994) detallan una serín proteasa tipo tripsina purificada a partir de extractos de la larva infectiva de *A. simplex*, que es capaz de degradar ambos sustratos de catepsinas, aunque el primero de manera más eficiente. Igualmente, en *A. simplex* se ha identificado una serín proteasa cuyo pH óptimo es 7,5 (Matthews 1982, 1984; Sakanari y McKerrow 1990), muy cercano al pH óptimo detectado en nuestro estudio. La actividad detectada con este sustrato es mayor en *A. simplex s.s.* que en *A. pegreffii*, con diferencias estadísticamente significativas, al igual que ocurre en el estudio realizado sobre la actividad proteolítica. En *A. pegreffii* la actividad es muy reducida en todas las fases de desarrollo, así como en las L3 de *A. simplex s.s.*, mientras en las L4 de esta especie, la actividad es claramente mayor que en las L3 (p<0,05). En este sentido, Cavallero et al. (2018) realizan un estudio comparativo entre las especies *A. simplex s.s.* y *A. pegreffii* y detectan mayor presencia de transcritos de serín proteasas del tipo de la tripsina en la primera especie que en la segunda.

Con el otro sustrato empleado en nuestros estudios sobre catepsinas (Artículo VII), Z-FR-AMC, que es degradable por catepsinas B y L, se detecta actividad entre pH 4,0 y 6,0, siendo indetectable a partir de pH 6,5. Esta actividad se inhibe en su totalidad

con E64, inhibidor de cisteín proteasas, por lo que suponemos que estamos detectando catepsinas, que serían de tipo L, ya que a este pH apenas se detecta actividad con el sustrato específico de catepsinas B (Z-RR-AMC) y la poca que se detecta no se inhibe con E64. En general la actividad es mayor en las L3-0h y decrece a medida que aumenta el desarrollo larvario. La actividad es mayor en *A. simplex s.s.* que en *A. pegreffii* con diferencias que consideramos estadísticamente significativas por el método estadístico empleado (Kruskal-Wallis p=0,06). Esto podría estar relacionado con la mayor patogenicidad que presenta la primera especie y con la invasión de los tejidos del hospedador por la L3-0h, al igual que se ha descrito para otros helmintos (Day et al. 1995; Neveu et al. 2003; Stack et al. 2008).

Debido a que no se ha podido detectar actividad catepsina B bajo nuestras condiciones experimentales, serían necesarios más ensayos para comprobar si este parásito presenta dicho tipo de actividad o carece de ella, como se ha reportado en otros nematodos. Además, el hecho de que, mediante las dos técnicas empleadas para detectar la actividad proteolítica, se hayan encontrado serín proteasas, que otros autores ya han relacionado con la patogenicidad de *A. simplex*, y que además éstas presenten una mayor actividad en *A. simplex s.s.* que en *A. pegreffii*, hace necesario el planteamiento de nuevos estudios para identificar y caracterizar las proteasas que hemos detectado y comprobar su potencial patógeno mediante estudios específicos.

# 7.
# CONCLUSIONES

1. La prevalencia por *Anisakis* está relacionada con el peso y/o la talla de bacaladillas y de sardinas: al aumentar el tamaño (edad) del pescado, aumenta la probabilidad de que éste se encuentre infectado por larvas de *Anisakis*. Así mismo, en bacaladillas del Mediterráneo se ha observado que no suelen infectarse por *Anisakis* antes del año de edad y que se infectan antes con *Anisakis* tipo I que con *Anisakis* tipo II, lo que probablemente está relacionado con la variación de la dieta del pescado con su edad, así como con la abundancia de presas en la zona.

2. Las sardinas muestran una prevalencia por *Anisakis* más baja que otros peces de interés comercial como la bacaladilla, por lo que el riesgo de contraer anisakiasis por consumo de sardinas es bajo. Aun así, existe una relación directa entre la zona de captura del pescado y la prevalencia por larvas *Anisakis*. La mayor prevalencia hallada en sardinas tiene lugar en Galicia (28,3%) seguida de Ondarroa (5%). No se ha detectado infección por *Anisakis* en sardinas procedentes del Mar Mediterráneo. Resultados que ponen de manifiesto un diferente riesgo de anisakiasis en el hombre dependiendo, además de la edad del pescado, de la especie de pescado consumida y de la procedencia del mismo.

3. *A. simplex s.s.* es la especie predominante en las costas atlánticas del norte de España, en el Golfo de Vizcaya, mientras que *A. pegreffii* predomina en el mar Mediterráneo que baña la costa oriental de España. La única especie de *Anisakis* perteneciente al tipo II encontrada fue identificada molecularmente como *A. physeteris*, hallándose sólo en las bacaladillas del Mediterráneo. Así mismo se encontraron individuos híbridos o recombinantes entre *A. simplex s.s.* y *A. pegreffii* tanto en el Atlántico como en el Mediterráneo. Estos datos avalan la distribución conocida hasta el momento de las especies de *Anisakis* en nuestro entorno geográfico.

4. Se ha observado que es el tercer estadio larvario de *Contracaecum multipapillatum* el que eclosiona del huevo, envainado con la cutícula de la L2, y que, tras la pérdida de la vaina, presenta las mismas características que las L3 que se encuentran en el pescado.

5. Se han detectado estructuras sensoriales en el extremo anterior de las L3 de *A. physeteris*, cubiertas con la cutícula, que quedan expuestas tras la muda a L4, lo que deja en cuestión la funcionalidad de las mismas en las L3. Los dentículos que se desarrollan en las crestas de los labios de las L4, descienden por los laterales de los

labios hasta las comisuras de la boca, disminuyendo de tamaño conforme se alejan de la cresta. También, se ha descrito por primera vez la presencia de unos poros en la cara exterior de los 3 labios de la L4 cuya función es desconocida. En esta fase L4 se comienzan a desarrollar las estructuras propias de los adultos habiéndose observado la vulva de la hembra y las papilas preanales del macho, siempre bajo su cutícula.

6.  Se detecta actividad proteolítica en todas las fases de desarrollo de *A. simplex s.s.* y *A. pegreffii*. En esta actividad proteolítica predomina la expresión de aspártico proteasas y metaloproteasas a pH ácido (6,0) y de metaloproteasas a pH alcalino (8,5). Esta situación es compatible con la fisiología del parásito, ya que esta actividad proteolítica parece tener una función digestiva, especialmente útil al pH ácido de las cámaras estomacales de sus hospedadores definitivos, como al ligeramente alcalino de los tejidos, que pueden ser invadidos y digeridos, de sus hospedadores intermediarios/paraténicos.

7.  La actividad serín proteasa es mayor en *A. simplex s.s.* que en *A. pegreffii*, con diferencias estadísticamente significativas a pH 6,0 y 8,5. Este tipo de proteasas en nematodos suelen estar implicadas en la patogenicidad por lo que las diferencias significativas entre las dos especies estudiadas podrían relacionarse con ella.

8.  La actividad que se detecta con el sustrato fluorogénico Z-FR-AMC a pH ácido corresponde exclusivamente a catepsina tipo L, ya que se inhibe totalmente en presencia de inhibidores de las cisteín proteasas y a que no hay actividad en este rango de pH con el sustrato Z-RR-AMC, específico de catepsinas B. Esto ocurre tanto en *A. simplex s.s.* como en *A. pegreffii*. La ausencia de actividad catepsina tipo B bajo nuestras condiciones experimentales, no descarta que exista esta actividad en las especies mencionadas.

9.  La actividad determinada con el sustrato Z-RR-AMC no corresponde a catepsina B ya que no se inhibe por inhibidores específicos de cisteín proteasas. Esta actividad es significativa solo en la fase L4 de *A. simplex s.s.*, siendo casi inactiva en *A. pegreffii* y se corresponde mayoritariamente a serín proteasas. Esto pone de manifiesto claramente una expresión diferencial entre las especies gemelas estudiadas que es regulada por el desarrollo.

10. Se ha constatado que existe una expresión diferencial de actividad proteolítica entre fases y especies del complejo *Anisakis simplex s.l.*, que es consistente con la diferente patogenicidad observada entre *A. simplex s.s.* y *A. pegreffii*, lo cual apoya la hipótesis de que ambos hechos están directamente relacionados.

# 8.
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# 9.
# SUMMARY

## 1. Introduction and justification

It is known as anisakidosis the pathology produced by anisakid larvae, which are parasitic nematodes of the Anisakidae family. Larval third stage (L3) of this family can be found in fish and squid, which act as intermediate/paratenic hosts, and larval fourth stage (L4) and adults in marine mammals and piscivorous birds, which are definitive hosts of the parasites of the subfamily Anisakinae. Human can suffer anisakidosis when consuming raw or undercooked fish that is infected by the L3 of any of the four genera that have been related to anisakidosis: *Anisakis*, *Pseudoterranova*, *Contracaecum* or *Hysterothylacium* (Myers 1975; Ishikura et al. 1993; Fernández-Caldas et al. 1998; Takahashi et al. 1998).

This pathology usually occurs with digestive and/or allergic symptoms, and despite being an underdiagnosed infection (Navarro Suárez et al. 2014), it is estimated that there have been more than 30,000 cases worldwide, most of which have been described in Japan (Takahashi et al. 1998; Audícana et al. 2003). In addition, it is estimated that in this country between 2,000 and 3,000 new cases are described every year (Umehara et al. 2007). In Spain, a large number of cases have also been described (Arenal Vera et al. 1991; Repiso Ortega et al. 2003; del Rey-Moreno et al. 2008), being the European country where most cases have been diagnosed, most of them due to the consumption of anchovies in vinegar (Bao et al. 2017). The larvae of the genus *Anisakis* are the most important because of its clinical importance: it is estimated that around 97% of all the cases described worldwide are caused by this type of larvae (Audícana et al. 2003). The pathology produced larvae of the genus *Anisakis* is known specifically as anisakiasis/anisakiosis, and there are several species involved: *A. simplex sensu stricto*, *A. pegreffii* and, rarely, *A. physeteris s.l.* The first two belong to a complex of sibling species morphologically indistinguishable in L3 stage, and although it has been demonstrated by studies both in experimental animals and *in vitro*, that *A. simplex s.s.* is more pathogenic than *A. pegreffii*, both are capable to produce anisakiasis (Quiazon et al. 2011; Arizono et al. 2012; Romero et al. 2013).

A possible way to address this difference in pathogenicity would be through the study of the proteolytic activity. Proteases are a type of protein of great importance in parasitic nematodes, where they have been related to their pathogenicity and they have a crucial role in the invasion of host tissues (McKerrow et al. 2006), as well as participating in various biological functions, such as nutrition, moulting or embryonic development (Malagón et al. 2013). The study of the proteolytic activity of the

previously described species could help to identify the possible differences among them, justifying this difference in pathogenicity. In addition, changes in proteolytic activity that occur during the development process could be related to some biological function. Also of interest are cathepsins, which are a type of proteases of special importance in parasitic nematodes. These molecules have been postulated as possible therapeutic targets, being of great importance in the development of vaccines (Dalton et al. 1996; Renard et al. 2000; Robinson et al. 2008; Knox 2012).

On the other hand, it is interesting to analyze the presence of anisakid larvae in fish of commercial and culinary interest, which allows to take preventive measures to avoid anisakidosis. There are several species that can affect humans, so it is also relevant to know which species are parasitizing the fish we consume. It is also important to deep the knowledge into the biology and development of these parasites, and also to identify the characteristics that allow to distinguish between the different species with clinical importance. In addition, the life cycle of several species of anisakids is not elucidated and there are different opinions about the embryonic development that occurs inside the egg. Knowing thoroughly the life cycle of a parasite allows to design strategies to control it, which is fundamental in any parasitosis. In the case of anisakids, which have complex life cycles and with many species not yet well differentiated involved, much research is still needed.

## 2. Materials and Methods

2.1. Sample collection for epidemiological and life cycle studies

Sardines (*Sardina pilchardus*) and blue whiting (*Micromesistius poutassou*) landed in several harbors of Spain were transported in ice flakes to lab and then immediately measured and weighed, after which they were dissected. Larvae removed from the abdominal cavity were maintained on ice bath until their morphological identification (Berland 1961). The viscera and the musculature of the fish were subjected to a pepsin digestion for 2-8 hours separately. All the extracted larvae were morphologically identified and then frozen at -20 °C until molecular identification using PCR-RFLP technique, for which NC2 and NC5 primers described by Zhu et al. (1998) and the restriction enzymes Taq1 and Hinf1 Fast Digest, following the method used by other authors (Martín-Sánchez et al. 2005), were employed.

For life cycle studies, female adults from *Contracaecum multipapillatum s.l.* obtained from brown pelican (*Pelecanus occidentalis*) were dissected and eggs were taken from the most external part of the uteri. Then, they were axeniced and placed in

saline solution at different concentrations. The development of the eggs was monitored to distinguish the different stages and hatching and survival of the larvae in the saline solutions, which were renewed weekly, was recorded. After 1 month under these conditions, eggs and larvae were moved from the saline solution to a Grace's insect medium solution supplemented with 2% v/v basal medium Eagle's vitamins (100×) solution, 1 mM L-cysteine, 1 g/L glucose, 20% v/v heat-inactivated foetal bovine serum and 1% v/v RPMI-1640 amino acid solution (50×), adjusting pH to 7.2.

The *C. multipapillatum s.l.* larvae obtained inmediately after egg hatching and others at different development stages were processed for scanning electron microscopy (SEM) without any further preparation. Likewise, larvae of *A. physeteris* collected from blue whiting from Mediterranean Sea and L4 obtained from *in vitro* culture (Iglesias et al. 1997, 2001) were fixed in hot 70% (v/v) ethanol and preserved for preparation for examination by SEM. The fixed larvae underwent critical point drying and cut into 3 sections, to avoid the distortion of the larvae which occurs when fresh or only fixed are cut. The anterior and posterior sections were separated for SEM preparation while a small cylindrical part of the central section was used for molecular identification.

## 2.2. *In vitro* culture for protease and cathepsin assays and preparation of *Anisakis* extracts

The following larval stages of *Anisakis* type I from blue whiting from Cantabrian Sea and Mediterranean Sea, were taken to study: L3 freshly collected from fish (L3-0h), L3 after 24 hours in culture (L3-24h), larvae from fourth larval stage (L4) 24 hours after moulting (L4-24h), and L4 after 14 days in culture (L4-14d). For the L3-0h stage, the larvae immediately after its extraction from the fish and washing in sterile cold saline solution were frozen. For the rest of the stages, the larvae were axenized and individually placed in culture as indicated in other works (Iglesias et al. 1997, 2001). Once the desired stage was obtained, the larvae were washed and frozen at -20 °C until use.

For extract preparation, larvae were separately homogenized in a small volume of 50 mM Tris/HCl buffer with 20% glycerin w/v at pH 7.8, in order to stabilize the proteins and avoid its rapid degradation (Gianfreda and Scarfi 1991; Iyer and Ananthanarayan 2008), finally completing upto 500 µl. They were subsequently centrifuged at 19,000 x *g* for 20 minutes at 4 ºC, and the supernatant was used for enzymatic assays.

2.3. Proteolytic and cathepsin assays

To determine the proteolytic activity, soluble extract of L3-0h of *A. simplex s.s.* and discontinuous system of buffers of pH 2 to 11 were used. The proteolytic activity was determined by measuring the fluorescence emitted after the degradation of the fluorogenic substrate bodipy FL-casein with excitation λ 490 nm and excitation λ 510 nm. The final concentration into well was: 50 mM of buffer, 50 µl of extract/ml (equivalent to 25 µg of protein), 1 mM of $CaCl_2$ and 5 µg of substrate/ml, for a final volume of 200 µl (Malagón et al. 2010b).

Inhibition tests were carried out at the two pHs in which maximum proteolytic activity was detected (Malagón et al. 2011). The following control enzymes and inhibitors specific to each group of proteases were used: pepsin (64 U/ml) and pepstatin A (0.02mM), respectively, for aspartic proteases; thermolysin (0.2 U/ml) and 1,10-phenanthroline (2 mM), for metalloproteases; papain (0.24 U/ml) and E64 [trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane] (0.05 mM), for the cysteine proteases; chymotrypsin (0.1 U/ml) and AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] (2 mM), for serine proteases. The final concentration into well was: 50 mM buffer, 1 mM $CaCl_2$, enzymes and inhibitors as described above, 50 µl of extract/ml (equivalent to 25 µg of protein), 5 µg of substrate/ml. The substrate was the same, i.e. bodipy FL casein.

For the determination of cathepsin L and B activities, procedures of Malagón et al. (2010) were employed. Ionic strength was maintained constant at 0.6 M by addition of NaCl as necessary. Dithiothreitol (DTT) was added to buffers for 1 mM final concentration into well. Two fluorogenic substrates were used: Z-FR-AMC (N-α-benciloxicarbonil-L-fenilalanil-L-arginina-7-amido-4-metil-cumarina) to determine cathepsins B and L, and Z-RR-AMC (N-α-benciloxicarbonil-L-arginil-L-arginina-7-amido-4-metil-cumarina) to determine cathepsin B. Fluorescence was detected using a fluorometer with excitation at λ 355 nm and emission at 460 nm. The final concentration in well was: 100 mM of tris-maleic buffer with DTT at 1mM, 50 µl of extract/ml (equivalent to 10 µg of protein), 150 µl of substrate (100 µM)/ml (final concentration 15 µM), and bidistilled water to complete the final volume upto 200 µl.

2.4. Statistical study

SPSS 20.0 was used for the analysis of epidemiological risk factors in fish. The following variables were used: size, weight, sex, condition factor, origin of the fish and month of capture. Univariate model was designed using *Anisakis* infection as a

dependent variable, and those variables that had shown a statistically significant association were selected to perform a multivariate model.

For the study of proteases and cathepsins in *A. simplex s.s.* and *A. pegreffii*, SPSS 22.0 was also used. An analysis of the variance (ANOVA) was carried out, after checking that the residuals of the variables followed a normal distribution by the Shapiro-Wilk test ($p>0.05$ in all cases) and with the support of Q-Q graphics. After ANOVA, a *post hoc* study was carried out using the Bonferroni test for the variables that had shown significant differences. The level of significance was set at $p<0.05$. When the residuals of the variables did not follow a normal distribution, Kruskal-Wallis test was performed and a *post hoc* study using the Mann-Whitney U test in the variables that had shown statistically significant differences was carried out.

## 3. Results

### 3.1. Epidemiological and life cycle studies

Three *Anisakis* genotypes were identified in the Atlantic Ocean: *A. simplex sensu stricto*, *A. pegreffii* and a hybrid genotype between these two species; and four genotypes were found in the Mediterranean Sea: the same three previously mentioned and *A. physeteris*. Sardine and blue withing infection was associated with fish length/weight, which agree with the results of other authors (Adroher et al. 1996; Valero et al. 2000; Rello et al. 2008). The public health authorities must continue to emphasize the need for suitable thermal treatment (freezing or cooking) of the fish prior to consumption.

In *Contracaecum multipapillatum s.l.*, hatching and survival of the larvae were greater at 15 °C than at 24 °C, and increased salinity resulted in a slight increase in hatching but seemed to reduce survival at 24 °C, but not at 15 °C. The newly hatched larvae were ensheathed and highly motile. When placed in culture medium, the hatched larvae grew within their sheath, and a small percentage exsheathed completely 2 weeks later. Although they did not moult during culture, SEM and optical microscopy revealed a morphology typical of third-stage larvae. Thus, we suggest that newly hatched larvae from eggs of *C. multipapillatum s.l.* are third larval stage but with sheath of the second larval stage, as occuring in other anisakids. We suggest a life cycle of *C. multipapillatum* in which L3 hatch from the egg, copepods are involved as first intermediate hosts, mullets as intermediate/paratenic hosts and brown pelicans as final hosts in the geographical area of Bahía de La Paz, Mexico

In *A. physeteris* the development of a row of denticles on each of the three prominent lips, almost reaching the buccal commisures, was observed in the L4. Pores of unknown function were found in the upper external part of each lip. Clearly developed cephalic papillae, amphids, and deirids were also observed in L4, while, although present in L3, they were beneath the cuticle. Phasmids were detected in L4 but not in L3. In females the vulva could be seen by light microscopy, apparently still covered by the cuticle.

## 3.2. Proteolytic assays: proteases and cathepsins

Two peaks of maximum proteolytic activity were detected at pH 6.0 and 8.5. Activity was detected in all developmental stages in *A. simplex s.s.* y *A. pegreffii*. At pH 6.0, L4 larvae showed higher proteolytic activity than L3 larvae in both species ($p<0.001$), the majority of which was due to metalloproteases and aspartic proteases, that could be related to nutrition (Dziekońska-Rynko and Rokicki 2005). At pH 8.5, proteolytic activity was higher in *A. simplex s.s.* than in *A. pegreffii* ($p<0.01$). At this pH, most of the activity was due to metalloproteases in all developmental phases of both species, although in L3-0h, the activity of these proteases was significantly higher ($p<0.03$) in *A. simplex s.s.* than in *A. pegreffii*. This could be related to the greater invasive capacity of the former: some authors relate metalloproteases with pathogenicity of some anisakids (Malagón et al. 2011). Serine proteases, which have also been implicated in the pathogenicity of *A. simplex* (Sakanari and McKerrow 1990; Morris and Sakanari 1994), show a higher activity ($p<0.05$) in *A. simplex s.s.* than in *A. pegreffii*. These differences in metalloproteases at L3-0h and in serine proteases could be contributing to the previously reported differences in pathogenicity between these two *Anisakis* species.

The cathepsin-like activities in *A. simplex s.s.* and *A. pegreffii*, were also studied. With Z-FR-AMC, substrate for cathepsin L, activity at acid pH, with a peak at pH 5.0, was detected. This activity was fully inhibited with E64, a cysteine proteases specific inhibitor, and might be involved in digestive processes, among others. The activity of *A. simplex s.s.* is higher than the one shown by *A. pegreffii* ($p=0.06$). With Z-RR-AMC, substrate from cathepsin B, a peak at pH 8.0 in L4-stages of *A. simplex s.s.* is detected. This activity was not inhibited with E64 but with AEBSF, showing to be composed by 90% with serine proteases and with some contribution of metalloproteases. These characteristics suggest that the detected activity might be produced by a trypsin-like serine protease, previously described in the scientific literature (Sakanari and McKerrow 1990; Morris and Sakanari 1994), although further research is needed to confirm this

hypothesis. In any case, it is noticeable that the activity is concentrated within phase L4 of *A. simplex s.s.* and that it is very low in *A. pegreffii* (p=0.001).

## 4. Conclusions

1. There is a correlation between both sardines and blue whiting length/weight and the *Anisakis* infection. Risk of infection increases with fish size. The greater prevalence in sardines were from La Coruña, NW Spain (28,3%) followed by Ondarroa, northern Spain (5%). The species found in this study were *A. simplex s.s.*, *A. pegreffii*, hybrids genotypes between them, and *A. physeteris* (this only in the Mediterranean blue whiting).

2. Third larval stage (L3) of *Contracaecum multipapillatum s.l.* hatches from the egg ensheathed with L2 cuticule and become infective without any further moulting, being the morphology of newly hatched larvae identifiable to the L3 found in fish. The SEM studies of the L3 and L4 stages of *Anisakis physeteris* have allowed the identification of diverse structures in them, highlighting the sensory ones.

3. 3. Aspartic- and metalloprotease activities predominate at pH 6.0 while, the activity is mostly produced by metalloproteases at pH 8.5, both in *A. simplex s.s.* and *A. pegreffii*. Serine proteases show higher activity in the former than in the latter (p<0.05) and they might be contributing to the differential pathogenicity showed by these two species. Metalloproteases activity is greater in *A. simplex s.s.* than in *A. pegreffii* in the L3 just obtained from fish (L3-0h), that could be related to their different tissue penetration capacity. No cathepsin B-like have been detected under our experimental conditions in any of the studies species. Cathepsin L-like shows a peak at pH 5.0 and decreasing with development and it could be related to nutrition.

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