Prevalence of *Anguillicoloides crassus* (Nematoda, Dracunculoidea) in wild European eels (*Anguilla anguilla* L.) from Mar Menor lagoon (Western Mediterranean, Spain)

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SUMMARY

Anguillicoloides crassus infection in wild European eels (*Anguilla anguilla*) from Mar Menor, a hypersaline coastal lagoon situated on the shores of the Mediterranean in south-east Spain, was evaluated. As well, an artificial digestion is described for the detection, isolation and counting of larval stages in the swimbladder wall. A total of 109 eels were collected between November 2008 and March 2009 and adult worms were recovered from the swimbladders of infected eels. The detected prevalence was 7.34%. Second-stage larvae (L2) numbers ranged from one to thousands per swimbladder. This developmental stage was the most prevalent parasite stage detected in infected eels. L2 were even found in eels harbouring neither pre-adult nor adult nematodes, which could indicate that infected eels are chronically infected. The lack of a suitable intermediate host for this nematode or the recent introduction of *A. crassus* into this environment could be the causes of the low prevalence of this parasite in eels from Mar Menor lagoon.

Keywords: European eel, *Anguillicoloides crassus*, Nematode, Mediterranean Sea.

RÉSUMÉ

Prévalence de *Anguillicoloides crassus* (Nematoda, Dracunculoidea) chez l´anguille européenne sauvage (*Anguilla anguilla* L.) dans la Mer Menor (sud-ouest Méditerranée, Espagne)

L'objectif de la présente étude était d'évaluer la prévalence de l'infection par le nématode *Anguillicoloides crassus* chez l´anguille européenne sauvage (*Anguilla anguilla*) dans la Mer Menor, qui est une lagune côtière hypersaline située sur le littoral sud-oriental du Mediteranée espagnol. En plus, nous décrirons une méthode de digestion artificielle pour la détéction, isolement et décompte des différents stades larvaires localisés dans la paroi de la vessie natatoire Au total, 109 anguilles on été collectés pendant novembre 2008 et mars 2009. Durant l´autopsie on a collecté tous les nématodes adultes situés dans la lumière de la vessie natatoire des poissons parasités. L´examen parasitologique a révélé une prévalence total de 7,34 %. Le rang des larves de deuxième stade (L2) était compris entre une et milliers par vessie parasité. Ce stade larvaire a été le plus prevalent de tous les parasites isolés dans les anguilles parasitées Les L2 ont été présents même dans les anguilles dont la vessie n´était parasitée par stades préadultes ou adultes du nematode; ce résultat semble indiquer qu´il s´agit d´une infection chronique par *A. crassus* dans les animaux. L´absence d´un approprié hôte intermédiaire, ou bien la récente introduction de *A. crassus* dans l´environnement de la Mer Menor peut être la cause de la basse prévalence de ce nematode dans les anguilles capturées dans cette lagune littoral.

Mots clés : Anguille européenne, *Anguillicoloides crassus*, Nématode, Mer Méditerranée.

Introduction

Nematodes of the genus *Anguillicoloides* (Nematoda: Dracunculoidea) are widely distributed parasites of eel swimbladders. *Anguillicoloides crassus* was accidentally translocated from Asia to Europe in the early 1980s. Today, this nematode is also found in eels along the eastern seaboard of North America [4], as well as in several imported and indigenous eel species on the remote island of Reunion [30]. Eels are teleosts classified in the Red Book of threaten species, and since March 2009 have been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. They are capable of active osmotic and ionic regulation of their body fluids while moving through gradients. This ability enables juvenile yellow eels to live in fresh or tidal waters and migrate between these environments [33], or to live solely in marine waters [35] until they mature into silver eels and migrate to spawning areas near the Sargasso Sea [33]. *Anguillicoloides crassus* can cause extensive damage to the swimbladder of its host, resulting in changes in gas composition [39] and a decline in swimming performance [31]. Both effects may impair the success of the eel when migrating to spawn [5, 31]. In fact, *A. crassus* infection has been identified along with factors such as pollution, overfishing and ocean warming as a possible cause of the depletion of European eel stocks.

After the first description of *A. crassus* in Spain [34], several studies have demonstrated that the parasite is widespread in this country [22], although there are still some Spanish rivers that have yet to be colonized by this nematode. No data are available regarding the prevalence of this parasite in ecosystems such as Mar Menor lagoon, a hypersaline coastal lagoon in south-east Spain. Sea water was originally thought to act as a barrier preventing the dissemination and reproduction of this parasite [36], although subsequent studies have reported infected eels in brackish costal waters, in the open sea [13, 21] and even in hypersaline waters [24].

Therefore, the aim of this work was to study the prevalence of *A. crassus* in wild European eels (*Anguilla anguilla* Linnaeus) from Mar Menor, a Mediterranean coastal lagoon whose ecology, fisheries and tourism are of great significance, and which figures in several international conservation agreements.

Materials and Methods

SAMPLING AREA

Mar Menor lagoon lies in the SE of the Iberian Peninsula (37°38' N, 0°42' W), Spain (Fig. 1). This ecosystem is one of the largest coastal lagoons on the Mediterranean coast (135 km^2 area, average depth 4 m, maximum depth 6.5 m) and is isolated from the sea by a 24-km-long sand-bar known as La Manga that is breached in five places by channels linking the lagoon to the sea. The lagoon is hypersaline, with salinity levels (43–46.5 g/l) greater than the adjacent Mediterranean Sea because of low precipitation (around 300 mm per year) and high evaporation rates (mean annual temperature 18°C).

EEL SAMPLING PROCEDURE

A total of 109 wild European eels *Anguilla anguilla* were collected from Mar Menor lagoon between November 2008 and March 2009 by local fishermen in winter months when eel catches are allowed (Table 1). Eels were transported to the laboratory, kept in sea water aquaria and sampled the same day as they were caught. Eels were killed with an overdose of the anesthesic MS222 (Sigma Chemical Co., St. Louis, MO, USA), and their weight and length were recorded. The swimbladder of each eel was removed and examined for parasites.

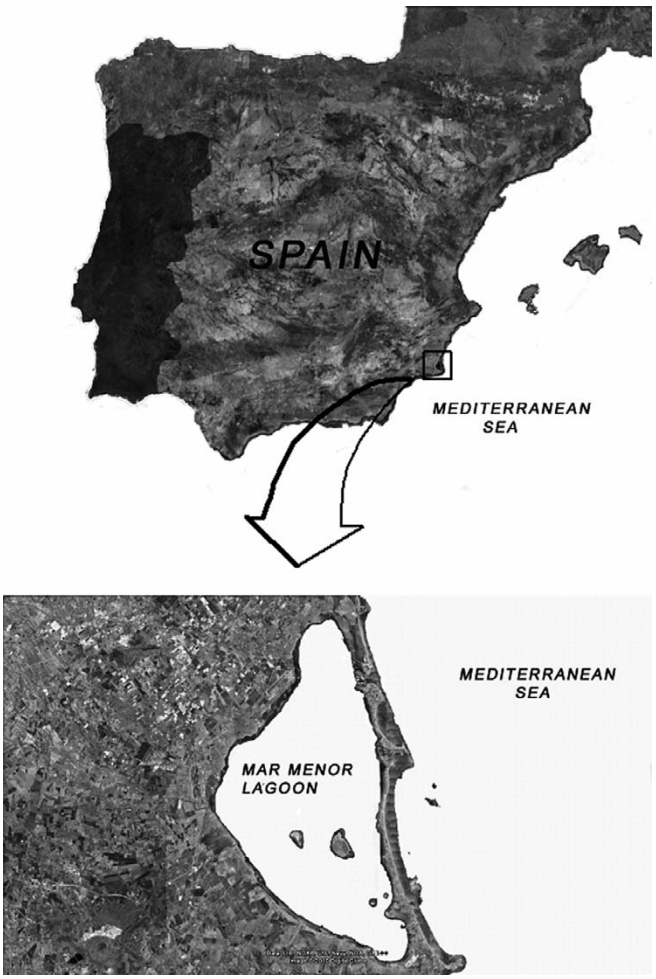

FIGURE 1: Map of Spain showing the location of Mar Menor lagoon.

PARASITE SAMPLING PROCEDURE

Pre-adult and adult *Anguillicoloides crassus* were removed from the swimbladder lumen of each infected eel, washed in distilled water, preserved in 70% ethanol and later examined under a light microscope for morphometric study and sex determination according to MORAVEC and TARASCHEWSKI [25]. Swimbladders were then frozen for subsequent examination for larval stages in the wall.

For this purpose, swimbladders were thawed, digested in freshly prepared 1.5% (w/v) pepsin (1: 10 000 activity) and 1.5% (w/v) chlorhydric acid in distilled water. Digestion was carried out at 40 ºC for 1h under gentle shaking. The digested

Period of time	Number of eels	Prevalence (%)				
		L2	L3	L4	Adults	Total
December 2008	34	11.76	2.94	0	2.94	14.7
January 2009	54	1.85	0	0	3.7	5.55
March 2009	21	0	0	0	0	0

TABLE I: Distribution of the number of *Anguilla anguilla* specimens sampled in each period of time and the prevalence of different life stages of *Anguillicoloides crassus* detected.

		Mar Menor (N =109)
Adults	Total Prevalence (%)	3.67
	Female prevalence (%)	54.54
	Male prevalence (%)	27.27
	Undetermined sex (%)	18.18
	Mean Intensity (range)	3 (1-9)
	Mean abundance (SD)	0.11 (5.3)
L2[a]	Prevalence (%)	4.59
	Mean Intensity (range)	8 666 (1- 42 960)
	Mean abundance (SD)	397.52 (22 232.92)
L3	Prevalence (%)	0.92
	Mean Intensity (range)	1 (1)
	Mean abundance (SD)	0.01 (0)
L4	Prevalence (%)	0
	Mean Intensity (range)	0
	Mean abundance (SD)	0
Total parasite burden	Prevalence (%)	7.34
	Mean Intensity (range)	5 417.87 (1-42 969)
	Mean abundance (SD)	397.64 (19 007)

[a]*Eggs containing L2 or hatched L2, SD = standard deviation.*

TABLE II: Prevalence, infection intensity and mean abundance of *Anguillicoloides crassus* in Mar Menor lagoon.

material was washed by centrifugation at 500 g for 5 min and the sediment was resuspended in 1ml of distilled water. The number of L3 and L4 *A. crassus* larval stages in the complete volume of resuspended pellet (1ml) was microscopically observed in a Favatti chamber based on the morphometric criteria indicated by ROLBIECKI [29], while dilutions were performed for counting of eggs. Prevalence (number of infected eels divided by total number of eels sampled), intensity (nematodes per infected eel) and abundance (total parasite number divided by total number of eels collected) [6] were calculated.

Results

Table 2 summarises the prevalence of *Anguillicoloides crassus*, the intensity of infection and the mean abundance detected. The prevalence in wild eels from Mar Menor was 7.34% with a mean intensity of almost 5 418 worms per infected eel. Adult nematode prevalence was 3.67% with a mean intensity of 3. Although the sex of some adult worms could not be determined due to damage, a higher proportion of *A. crassus* females than males was detected. No fourth stages (L4) were recovered from infected fish and the prevalence of the third stage (L3) was very low. After artificial digestion, microscopic examination showed that the second stage (L2) larval cuticular sheath of most eggs had been digested and so they were examined under the microscope and counted as hatched L2. This stage had the highest prevalence and its intensity ranged from one to 42 960 in individual swimbladders, with a mean intensity of 8 666. These developmental stages were found even in eels not harbouring any other *A. crassus* developmental stages.

Discussion

As powerful homeosmotic regulators, European eels are able to adapt to short-term fluctuations occurring during an estuarine tidal cycle or to longer-term changes in salinity during seasonal migrations [14, 20]. Therefore, to survive in parasitized eels, *Anguillicoloides crassus* must be able to tolerate changes in the osmolarity of the blood plasma of the definitive host. Although the sea was previously thought to act as a barrier to dissemination [36], experimental studies have in fact demonstrated that osmoconformation with the blood plasma of the eel host enables most of the *A. crassus* population to survive and complete its life cycle in both marine and brackish waters [18]. Although field studies have shown that *A. crassus* occurs in eels living in all kind of waters, observations suggest that *A. crassus* prevalence and the intensity of infection decrease with increased salinity [13, 27, 32]. Nevertheless, although infection levels are thought to be lower in marine environments than in fresh water, a high prevalence (46.4%) has recently been reported in eels from a marine environment in Sweden [38], and even in eels from an hypersaline estuary (46.77% salinity) in which an *A. crassus* prevalence of 71.87% was detected [24].

In the present study, an artificial digestion of the swimbladder was performed in order to examine all the larvae (second stage L2, third stage L3 and fourth stage L4). As the quantity of eggs containing L2 and hatched L2 in the swimbladder of an infected eel is very large [10], their number is not included in most *A. crassus* prevalence studies. In a recent study, ROLBIECKI [29] reported the presence of eggs containing L2 or hatched L2 in 87.3% of *A. crassus*-infected

eels. Nevertheless, due to their great quantity, their number was not counted. Artificial digestion is a common method for recovering parasitic nematode larvae from mammal [37], reptile [3], gastropod [28] and fish [9] tissue. Artificial digestion has previously been used to recover L3 of *A. crassus* from the flesh of fish as a tool for studying fish paratenic hosts [1], although, to our knowledge, it has never been used for *A. crassus* prevalence studies. The artificial digestion method used in the current study allows a rapid and easy quantification of L2: the L2 cuticular sheath of most eggs is digested by pepsin-clorhidric acid and so can be seen under a microscope and counted as hatched L2 stage. Our results corroborate the idea that some eels have large amounts of L2 (up to 42 960). Furthermore, 2.75% (n = 3) of total eels sampled only had L2 in the wall, and no adults or any other larval stages. Similar results were reported by PALÍKOVÁ and NAVRÁTIL [26]. Most studies do not include the L2 stages and so could have underestimated prevalences.

In this study low *A. crassus* prevalence was detected. Nevertheless, higher prevalences have been previously reported in European eels from Spain: ESTEVE and ALCAIDE [11] reported 11.5% *A. crassus* prevalence in wild eels from L'Albufera, a brackish lake on the Mediterranean coast, while KORTA and DIAZ [22] reported a prevalence of up to 70% in freshwater environments. In our opinion, the low prevalence detected in eels from Mar Menor lagoon could be due either to the recent arrival of this exotic nematode in this environment or to the lack of adequate intermediate hosts. A long-term survey monitoring *A. crassus* prevalence over two decades in an oligohaline canal in southern France (La Camargue, Mediterranean coast) [23] showed that *A. crassus* infection rates rapidly increase in the first few years following its appearance and then stabilize at a prevalence of around 60-70%. A similar pattern of *A. crassus* spread has been reported by other authors [2, 15]. Although it is difficult to follow the sequence of the introduction of *A. crassus* into Spanish environments, the low prevalence detected in Mar Menor could be due to its recent arrival in this environment.

According to KIRK [19], the only currently known factors that directly limit the dispersal of this parasite are temperatures below 4°C and a lack of suitable intermediate hosts in the environment. In fact, one of the keys to the success of *A. crassus* as a colonizer is its adaptability to a wide range of common intermediate hosts [15]. In European freshwater many calanoid and cyclopoid copepods, as well as ostracods, are accessible for *A. crassus* [19]. The ubiquitously distributed marine copepod *Eurytemora affinis* has been identified as a potential key intermediate host in estuarine and brackish waters [17]. Nevertheless, although *E. affinis* shows strong osmoregulatory abilities [16], previous studies have demonstrated that the optimal physiological range for this copepoda is between 5 and 10 g l^{-1} [7]. According to GILABERT [12], copepods are mainly represented in Mar Menor lagoon by three species, *Oithona nana*, *Centropages ponticus* and *Acartia* spp., none of which have been described as a possible intermediate host of *A. crassus. E. affinis* has not been found in Mar Menor lagoon. Further studies should be conducted in order to detect any possible intermediate hosts of *A. crassus* in the hypersaline seawater of Mediterranean environments.

L2 was the most prevalent larval stage detected in infected eels. The small L3 prevalence and the absence of L4 suggest that it is a chronic infection; probably, eels were infected after ingesting an intermediate host containing L3, which, after moulting and sexual maturation, commenced reproduction in the eel swimbladder cavity and produced eggs containing L2. In the absence of re-infection, L2 persist in the final host until they are eliminated into the environment in faeces or when eels die. A similar pattern has previously been described for other nematodes [8].

Further studies should be undertaken to monitor the hypothetical spread of *A. crassus* in Mar Menor lagoon and to find the potential intermediate host operating in this particular environment.

Acknowledgements

This work was supported by La Fundación Séneca, Coordination Centre for Research (grant 04538/GERM/06). The authors wish to thank E. ROMERO for his assistance with the eel sampling and D. RIQUELME for his technical assistance with swimbladder parasite processing.

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