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RESEARCH ARTICLE

Monitoring for *Anguillicoloides crassus*, Anguillid herpesvirus 1, aquabirnavirus EVE and rhabdovirus EVEX in the European eel population of southern Spain

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Abstract

European eel is critically endangered in Europe. Among other stressors, pathogens are well-known to harm eels' fitness. One hundred and eighty-two eels were captured in three Eel Management Units in Andalucía (SE Spain) and analysed for *Anguillicoloides crassus*, Anguillid herpesvirus 1 (AngHV1), the rhabdovirus Eel Virus European X (EVEX) and the aquabirnavirus Eel Virus European (EVE). *A. crassus* adults and preadults were isolated and morphometrically identified, and the eel swimbladders were artificially digested to count *A. crassus* larvae. Also, eel tissues were examined by PCRs for the presence of viruses. EVEX and EVE were not detected in any of the eels. The estimated prevalence (95% confidence limits) was 71 (64–78)% for *A. crassus* and 35 (28–42)% for AngHV-1, varying these prevalences significantly between and within EMUs. Moreover, *A. crassus* prevalence was highest in smaller eels, in sites closest to the sea and eels sampled in the autumn. By contrast, AngHV-1 prevalence was highest in biggest eels, in sites far from the sea and sampled in the summer or winter. However, in mixed effects logistic models including site as a random variable, the risk of infection was associated with distance to the sea in both *A. crassus* and AngHV-1 infections and also to winter sampling in the case of AngHV-1 and not to other variables. These results are evidence that both pathogens are highly endemic in eels from Andalusian habitats. Further studies are needed to better understand the risk factors associated with these pathogens on eel populations.

KEYWORDS
Anguillicoloides crassus, Anguillid herpesvirus 1, European eel, EVE, EVEX, Spain

1 | INTRODUCTION

Over the last decades, native European eel (*Anguilla anguilla* L.) stocks have declined sharply in the European Union. The ICES/EIFAC Working Group on Eels determined in October 2002 (ICES, 2003) that the stock was outside safe biological limits, and, thus, *A. anguilla* was considered critically endangered. The causes of the decline are not completely elucidated but most authors agree that they should

be multiple and synergistic (Wirth & Bernatchez, 2003), including habitat loss, overfishing, environmental pollutants, physical barriers for eels' migration and oceanic variations (Dekker, 2003; Durif et al., 2011; Henderson et al., 2012; ICES, 2011; Muñoz et al., 2019; Schneebauer et al., 2017; Sures, 2001). In addition, pathogens that affect eels must also be considered among the factors causing the population decline of eels (Haenen et al., 2010; Henderson et al., 2012).

Infectious agents are well-known to harm eels' fitness (Esteve & Alcaide, 2009; Van Ginneken et al., 2004). In this regard, the introduction to Europe of the Japanese eel (*Anguilla japonica*) for commercial purposes into Germany (Taraschewski et al., 1987) and into the Netherlands (Van Banning & Haenen, 1990) was particularly detrimental, causing the spread of the nematode *Anguillicola crassus* Kuwahara, Niimi and Itagaki, 1974, Moravec & Taraschewski, 1988 syn. *Anguillicoloides crassus* (Laetsch et al., 2012), along with its original Asiatic host. Since its introduction, this allochthonous parasite has been described in several countries (Djebbari et al., 2009; Dzido et al., 2020; Gargouri & Maamouri, 2006; Jakob et al., 2016; Kantzoura et al., 2021) infecting six eel species: *A. japonica*. and *A. anguilla* (see in Knopf, 2006), *A. rostrata* (Johnson et al., 1995), *A. marmorata*, *A. mossambica* and *A. bicolor* (Sasal et al., 2008). Several studies have shown that *A. crassus* causes oedema and hyperplastic and fibrotic changes in the swimbladder mainly due to the hematophagous activity of the adult nematodes and the migration of the L3 larvae through the swimbladder wall (Haenen et al., 1989; Kirk, 2003). This leads to an increase in stress parameters leading to immunosuppression and a reduction in the body condition of eels (Abdelmonem et al., 2010; Barry et al., 2017; Costa-Dias et al., 2010; Dezfuli et al., 2021; Honka & Sures, 2021). The consequence of this health loss is that the eels' success in completing their migration to the Sargasso Sea for spawning may be compromised (Kirk, 2003). The introduction of this nematode in its new European host is possibly an important contributing factor for the European eel decline (Taraschewski, 2006).

Furthermore, several viruses have been described in European eel populations (Haenen et al., 2010; Jakob et al., 2016), which may also play a role as pathogenic, inhibiting factors for the eel during its spawning migration. In this sense, Jørgensen et al. (1994) isolated the rhabdovirus Eel Virus European X (EVEX) from wild diseased eels from Denmark, Davidse et al. (1999) recorded the first isolation of Anguillid herpesvirus 1 (AngHV1) in the Netherlands and Haenen et al. (2001) described the aquabirnavirus Eel Virus European (EVE), among other viruses, in the same area. The distribution of EVE and EVEX seems to be limited; instead, AngHV1 is present in most eel populations nowadays, and it is recognized to be the most dangerous virus for European eels due to its high pathogenicity under stress conditions (Davidse et al., 1999; Hangalapura et al., 2007; van Beurden et al., 2012).

In view of the significant decline of the European eel population as a result of a wide range of factors and their complex interrelationship, the European Union asked member countries to develop management programmes focussing on the reduction in eel mortalities related to anthropogenic factors, highlighting the need to monitor the eel population health in relation to contaminants and pathogens, and assess the general health condition indices (Armitage et al., 2014; Capoccioni et al., 2020; European Commission, 2014a, 2014b; Haenen et al., 2012; ICES, 2011, 2015a, 2015b, 2016; Peeler et al., 2011; Van Ginneken et al., 2004; Walker et al., 2009). In this context, the aim of this study was to evaluate the presence of the nematode *A. crassus* as well as the viruses AngHV1, EVE and EVEX in different rivers from southern Spain as part of a wider European effort to complete the distribution map of these pathogens, improve our knowledge of the epidemiological factors that determine their expansion and advance in the design of management plans for this threatened eel species.

2 | MATERIALS AND METHODS

2.1 | Sampled animals and study area

Between May 2013 and December 2014, a total of 182 European eels, captured in freshwater rivers and lagoons in Andalusia (southern Spain), were analysed for the presence of the nematode *A. crassus* and the viruses AngHV1, EVE and EVEX. Among them, 34 eels (18.7%) were captured in the Mediterranean Eel Management Unit (EMU) area (17.964 km^2 of eastern Andalucía, including Antas, Guadalfeo, Guadalhorce and Palmones rivers, which flow into the Andalusian Mediterranean slope), 93 (51.1%) came from the Guadalquivir EMU area (57.014 km^2 inland wetlands of eastern Andalusia, near the Guadalquivir river course, including Isla Mayor, PIMSA and Los Tollos lagoons) and 55 (30.2%) from the Atlantic EMU area (10.698 km^2 of western Andalucía, including Almodóvar, Iro, Piedras, Tinto and Zurraque rivers, which flow into the Andalusian Atlantic slope) (Figure 1). Eels were caught by local fishermen by means of traditional fishing gear and collected by technicians of the 'Junta de Andalucía' (Andalusia's autonomous government). At the laboratory, animals were anaesthetised and then euthanized with a lethal dose of tricaine methanesulfonate (MS222) at 100 mg/L, after which per eel the length and weight were recorded. Eel gender was determined only in 53 individuals, being all of them females. During eel necropsy, samples of gills, liver, spleen and kidney were collected per eel. Each tissue sample was placed in five volumes of RNA stabilizing buffer (RNAlater®, Sigma-Aldrich, St. Louis, MO), incubated for 24 h at 4°C and then stored at −80°C until further processing. The swimbladder was carefully removed and placed in labelled bags per eel and stored at −20°C until examination. Only the swimbladder of 179 eels could be obtained.

The geographical coordinates of the sampling site were used to calculate distance to the sea following the course of the river. Moreover, during the days of eel capture, water samples were taken to measure salinity (% NaCl concentration) at approximately the same time of the day on two consecutive days.

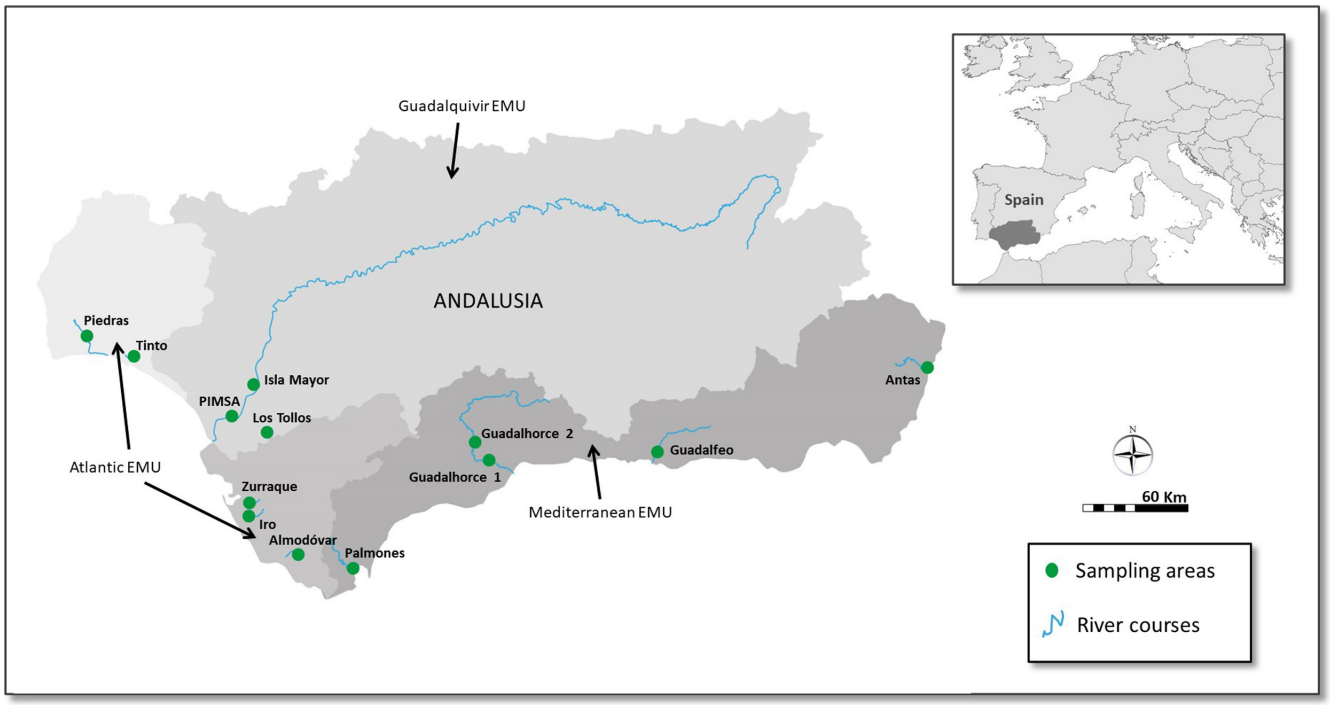

FIGURE 1 Sampling points of the 182 European eels caught in rivers and lagoons of Andalusia (southern Spain).

2.2 | Parasitological analysis

Once defrosted, the swimbladder of each eel was longitudinally opened, and the inner surface of the organ was examined to collect nematode adults and preadults; when detected, specimens were washed with distilled water and preserved in 70% ethanol until their morphometrical identification according to descriptions by Moravec and Taraschewski (1988). The detection of macroscopic alterations in the swimbladder (degree of opacity and thickening of the wall, presence of pigmentation and exudates in the lumen of the organ) led to the establishment of two categories (bladders with lesions or without macroscopic alterations), although the degree of lesion was not quantified as proposed by Lefebvre et al. (2002). Subsequently, the swimbladder was artificially digested as described by Martínez-Carrasco et al. (2011) to isolate and count *A. crassus* larval stages. Briefly, the swimbladder was incubated (1 h/40°C) in soft agitation, with a 1.5% (weight/volume) pepsin solution and 1.5% (weight/volume) hydrochloric acid in distilled water. The resulting homogenate was centrifuged at 800*g* for 5 min, and larvae present in the sediment were counted under the microscope using Favatti counting chambers for L3 and L4 stages (Rolbiecki, 2008) and graduated glass slides for L2 (which in most cases appeared free as a result of the digestion of the egg shells which contained each one larva).

Prevalence and intensity of infection for each parasite stage were defined according to Bush et al. (1997). Specifically, the prevalence was the number of hosts infected with *A. crassus* (irrespective of the parasite stage of development) divided by the number of hosts examined, and the value was expressed as a percentage, and the intensity was the median of a particular stage of the parasite among the infected eels.

2.3 | Virologic analysis

To determine the presence of the eel viruses AngHV-, EVEX and EVE, spleen, kidney, liver and gill fragments from each individual eel were pooled and homogenized, and 30 µg was subjected to DNA and RNA extraction (NucleoSpin Mini Kit, Macherey-Nagel). A single-step real-time PCR protocol was used for the DNA virus AngHV1, while for the RNA viruses EVEX and EVE, the reverse transcriptase (RT)-dependent conversion of RNA into cDNA was followed by the PCR amplification of the cDNA in a two-step real-time RT-PCR assay, according to van Beurden et al. (2011, 2016), using Ambion® Path-ID™ qPCR Master Mix. Positive genomic control DNA from cultured AngHV1 and cDNA from EVEX and EVE were kindly provided by Dr Engelsma's Laboratory (Wageningen Bioveterinary Research, the Netherlands); additionally, TaqMan™ Exogenous Internal Positive Control (ThermoFisher Scientific) was used to test for the presence of PCR inhibitors.

2.4 | Statistical analysis

Parasite distributions were investigated, and normality was assessed using Shapiro–Wilk test. The proportion of positive eels and parasite median infection intensity were compared across independent variables, including eel's weight and length, the capture site, the distance

from this site to the sea and its salinity, using Yates-corrected chi squared test (or Fisher's exact test when required) and Kruskal–Wallis non-parametric tests, respectively. Larval distributions were $\log_{10}$ transformed prior to comparing medians and explanatory numerical variables were categorized in equidistant levels. Differences between multiple median comparisons following the Kruskal–Wallis test were carried out using Dunn's test, with *p*-values adjusted with the Benjamini–Hochberg method. Random effects logistic regression models were developed to assess the independent contribution of explanatory variables associated with the presence or absence of *A. crassus* and AngHV-1 infection, respectively, in the bivariate analysis with a significance value lower than 10% ($p < .10$). In these models, sampling site (river/lagoon) was fitted as a random variable, and other explanatory variables were incorporated as categorical fixed effects. Models were estimated using the maximum likelihood method, and Akaike's information criterium (AIC) was used to select the most parsimonious models. For all statistical comparisons, differences were considered significant and marginally significant for $p < .05$ and $p < .10$, respectively, for a double-sided test. All the analyses and graphics were performed in the R software program (R Core Team, 2022).

3 | RESULTS

3.1 | *Anguillicoloides crassus* prevalence

Swimbladders were recovered and examined for parasites from 179/182 (98%), and 127 of them were parasitized by *A. crassus*. The estimated prevalence (95% confidence limits: CL) of infected eels was 71 (64–78)% (Table 1). This prevalence included the swimbladders with adult and/or preadult stages (52%, 93/179 eels), and those with larval stages (L3 and L4) detected after digestion (59%, 105/179 eels). Macroscopic alterations were detected in seven of 179 (4%) eels, including 7 (6%) *A. crassus* infected animals (one with adults and larvae, and six with larvae only) and one (2%) non-infected eel ($p > .05$).

The estimated prevalence (95% CL) of *A. crassus* (adults-preadults and/or larvae) differed according to sampling sites and EMU areas, ranging between 8 (0–24)% in Los Tollos (Guadalquivir EMU) and 100% in three of the five sampling sites in the Mediterranean EMU (Table 1). Prevalence over 70% was detected in every sampling site except in those in the Guadalquivir EMU area and in Almodóvar river (Mediterranean EMU). Overall, the estimated prevalence was significantly lower in the Guadalquivir area (58%) than the Atlantic (77%) and Mediterranean (97%) areas, and in the Atlantic compared with the Mediterranean ($p < .05$) (Table 1).

Differences in the estimated prevalence of infection between EMUs were mostly associated with larval infections, as the overall prevalence of adult–preadult infections was similar across EMUs, ranging between 51% in the Guadalquivir and Atlantic areas and 58% in the Mediterranean area ($p > .05$). By contrast, prevalence of larval infection in EMUs was 43% in the Guadalquivir area (0% in Los Tollos), 68% in the Atlantic area and 88% in the Mediterranean EMU ($p < .05$) (Table 1). The prevalence was highest in smaller, lighter eels, in those captured in the autumn and in sites closest the sea ($p < .05$) (Table 2). The prevalence was numerically highest in water with the highest salinity, but differences were not significant ($p > .05$) (Table 2).

In the most parsimonious multivariable logistic regression model, the risk of *A. crassus* infection was not associated with distance to the sea, but that it was significantly lower in the site not connected to the sea (Los Tollos lagoon) compared with other sites. Moreover, it was marginally significantly higher in the summer than in spring, and there was almost no remaining random variability associated with site (Table 4).

3.2 | *Anguillicoloides crassus* intensity

Adult–preadult and larvae in infected eels were not normally distributed ($p < .05$). The mean, median, range and interquartile range number of parasites in infected eels were 5.8, 4, 1–23 and 2–8 adults–preadults/eel, respectively. For larvae, these values were 69,166, 200, 1–3,290,000 and 11–14,606 larvae/eel, respectively.

The distribution of *A. crassus* adult–preadult and $\log_{10}$-transformed larvae intensity in infected eels in sampling sites is shown in Figures 2 and 3, respectively. Median adult–preadult parasite intensity was numerically highest (8 nematodes per eel) in Zurraque river (Atlantic EMU) and lowest (one nematode per eel) in Los Tollos lagoon and Almodóvar river (Figure 1), but differences in medians in pairwise comparisons between sites and EMUs were not statistically significant ($p < .05$). Similarly, the $\log_{10}$-transformed median larvae intensity did not differ significantly between rivers/lagoon ($p > .05$) and ranged between 1.2 larvae/eel in Antas and Almodóvar and 4.8 larvae/eel in Zurraque river (Figure 3).

Adult–preadult *A. crassus* intensity was marginally significantly higher in eels with lesions in the swimbladder, those sampled in the autumn and closest to the sea ($p < .10$) (Table 3). The infection intensity of *A. crassus* larvae was not associated with any of the explanatory variables analysed (Table 3).

3.3 | Virus infection prevalence and spatial distribution

The DNA extracted from a pool of different tissues analysed by real-time PCR revealed a total of 64/182 eels positive for AngHV1 Herpesvirus (Table 1), while none were positive for EVE and EVEX. Hence, the estimated AngHV1 prevalence (95% CI) was 35 (28–42)%, and varied significantly between EMUs, being 48 (38–59)%, 23 (11–36)% and 19 (7–31)% in the Guadalquivir, Atlantic and Mediterranean EMUs, respectively ($p < .05$) (Table 1). Moreover, there were significant differences in the prevalence of AngHV1 within the Guadalquivir and Mediterranean areas, ranging in the former between 40% (PIMSA) and 100% (Los Tollos lagoon), and in the

TABLE 1 Prevalence (%) and 95% confidence interval (CI) of *Anguillicoloides crassus* (adults and/or larvae) and Anguillid herpesvirus 1 (AngHV1) in European eels from Andalusia (southern Spain)

			A. crassus							
			Adult/larvae		Adult		Larvae		AngHV1	
EMU area	River/lagoon	No. eels	Prevalence	95% CI	Prevalence	95% CI	Prevalence	95% CI	Prevalence	95% CI
Atlantic	Tinto	21	90	78–100	48	26–69	90	78–100	29	0–48
	Piedras	8	88	65–100	63	29–96	88	65–100	20	0–45
	Zurraque	5	80	45–100	60	17–100	60	17–100	20	0–55
	Iro	11	73	46–99	55	25–84	45	16–75	18	0–41
	Almodóvar	8	38	4–71	38	4–71	25	0–55	25	0–55
	All sampling areas	53	77	66–89	51	37–64	68	55–80	24	12–35
Guadalquivir	PIMSA	50	68	55–81	58	44–72	60	46–74	40	26–54
	Isla Mayor	31	61	0–78	55	37–72	32	16–49	42	25–59
	Los Tollos	12	8	0–24	8	0–24	0	0	100	100
	All sampling areas	93	58	48–68	51	40–61	43	33–53	48	38–59
Mediterranean	Palmones	6	100	100	67	29–100	83	54–100	17	0–46
	Guadalhorce 1	3	100	100	67	13–100	100	100	75	33–100
	Guadalhorce 2	4	100	100	75	33–100	75	33–100	50	1–99
	Guadalfeo	10	90	71–100	70	42–98	80	55–100	0	0
	Antas	10	100	100	30	2–58	100	100	0	0
	All sampling areas	33	97	91–100	58	41–74	88	77–99	18	5–30
All EMUs		179	71	64–78	52	45–59	59	51–66	35	28–42

Note: A cross-sectional epidemiological study in 13 river and lagoon sampling areas in 2013–2014.

TABLE 2 Prevalence (%) and 95% confidence interval (CI) of *Anguillicoloides crassus* (adults and/or larvae) and Anguillid herpesvirus 1 (AngHV1) infections in European eels from Andalusia (southern Spain) according to explanatory variables

		A. crassus				AngHV1			
Variable	Level	No. eels	Prevalence	95% CI	*p* value	No. eels	Prevalence	95% CI	*p* value
Eel weight (g)	40–230	126	77	70–84	<.0001	129	29	21–36	<.0001
	231–419	29	72	56–89		29	31	14–48	
	420–609	16	56	32–81		16	63	39–86	
	610–798	8	0	0		8	100	100	
Eel length (cm)	31–44	72	83	75–92	<.0001	75	24	14–34	.0002
	45–57	71	70	60–81		71	37	25–48	
	58–69	28	57	39–75		28	43	25–61	
	70–82	8	13	0–35		8	100	100	
Macroscopic lesions in eel's swimming bladder	No	171	70	63–77	.4408	174	34	27–41	.1316
	Yes	8	88	65–100		8	63	29–96	
Sampling season	Spring	24	63	43–82	.0151	24	21	13,636	.0417
	Summer	47	70	57–83		49	43	29–57	
	Autumn	27	96	89–100		28	18	11,780	
	Winter	81	65	55–76		81	41	30–51	
Site distance to the sea (m)	1897–14,045	26	96	89–100	.0183[a]	26	4	0–11	.0031[a]
	14,046–26,193	106	74	65–82		109	33	24–42	
	38,342–50,489	35	66	50–81		35	43	26–59	
	Not connected	12	8	0–24		12	8	0–24	
Water salinity (%)	0	49	71	59–84	.2820	49	39	25–52	.0569
	0.0–0.4	58	64	51–76		58	38	25–50	
	0.8–1.7	46	72	59–85		49	41	27–55	
	2.4–13	26	85	71–98		26	12	0–24	

[a]The not connected Los Tollos lagoon was excluded in this analysis.

latter between 0% (Antas and Guadalfeo rivers, with a sample size of 10 eels each) and 63% (Guadalhorce) ($p < .05$).

As shown in Table 2, AngHV1 prevalence increased with eel's body weight and length and was highest in the summer and winter, increasing with distance to the sea, but negatively associated with water salinity (Table 2). In multivariable random effects modelling, the risk of AngHV1 infection was positively associated with distance to the sea, significantly higher in the winter than in the autumn, and there was no substantial remaining variation between sampling sites (Figure 3).

3.4 | AngHV1 and *A. crassus* co-infections

Of 179 eels tested for both pathogens, we detected 38 eels (21%) co-infected with AngHV1 and *A. crassus*, 24 eels (13%) infected only with AngHV1, 89 eels (50%) infected only with *A. crassus* and 28 eels (16%) free of both pathogens. Infection with AngHV1 was marginally associated with *A. crassus* infection ($p = .058$).

Prevalence of co-infections was 15% (6/41), 18% (8/45) and 26% (24/93) in the Mediterranean, Atlantic and Guadalquivir EMU ($p > .05$). However, there were significant differences in the prevalence of co-infections within the Mediterranean area, ranging from no co-infections in Antas and Guadalfeo rivers and 57% in Guadalhorce river ($p < .05$). Overall, co-infections were positively associated with increasing distance to the sea ($p < .05$), but no to other eel, site, season or water variables tested.

4 | DISCUSSION

Globalization has dramatically increased worldwide trade of goods and live organisms, often entailing the introduction of allochthonous species into new habitats, including pathogens (Dangel et al., 2015), resulting in new host–parasite combinations between autochthonous and non-native hosts (Galli et al., 2005). The arrival of invasive species often affects the fitness of the native populations (Honka & Sures, 2021) and biodiversity, causing important changes

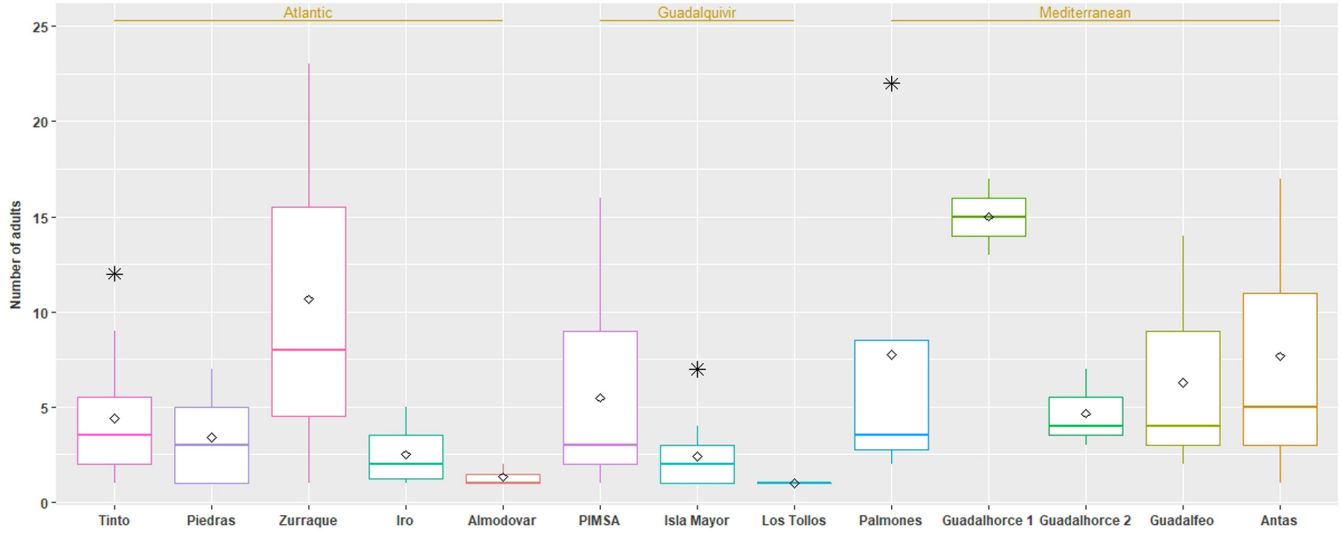

FIGURE 2 Distribution of the number of adult *Anguillicoloides crassus* in infected European eels from Andalusia (southern Spain). A cross-sectional epidemiological study carried out during 2013–2014 in 13 sampling areas of rivers and lagoons. Box and whiskers plots represent percentiles excluding outlying values (asterisk), and diamonds are the arithmetic means.

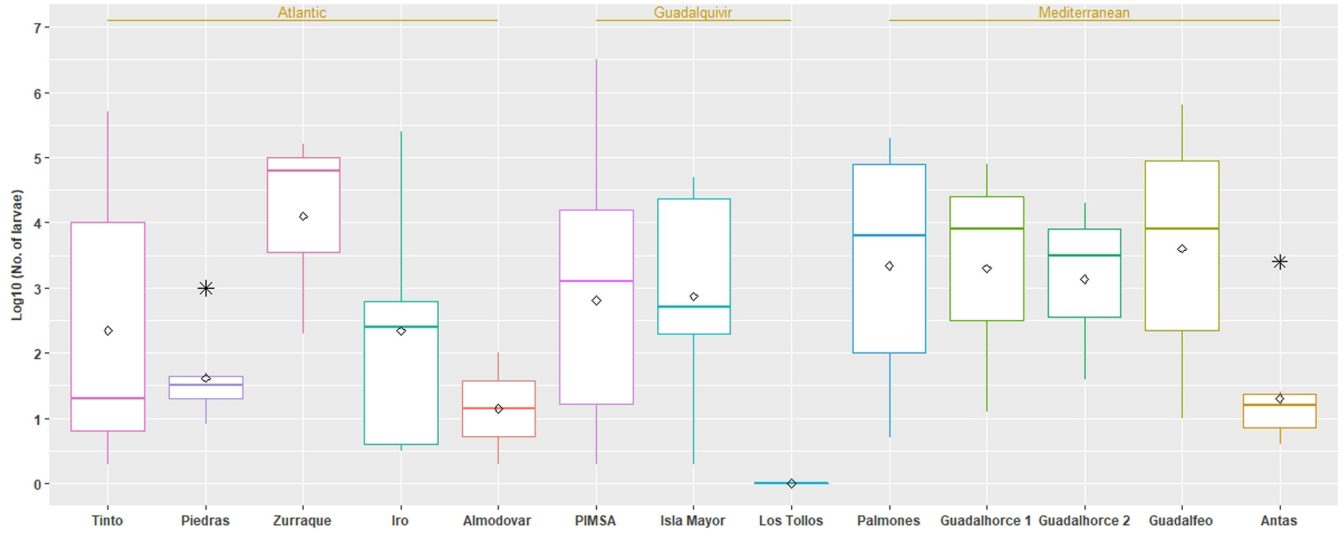

FIGURE 3 Distribution of the decimal logarithmic transformation of the number of *Anguillicoloides crassus* larvae detected in the swimbladder of infected European eels from Andalusia (southern Spain). A cross-sectional epidemiological study (2013–2014) based on 13 sampling areas of rivers and lagoons in 2013. Box and whiskers plots represent percentiles excluding outlying values (shown as asterisk), and diamonds are the arithmetic means.

in the structure of populations (Hatcher & Dunn, 2011; McGeoch et al., 2010). An illustrative example is the introduction of the nematode *A. crassus* through Japanese eels (*Anguilla japonica*) imported into Europe for commercial purposes (eel farming and consumption) depleted stocks of the native European eel (*A. anguilla*). The parasite expanded throughout Europe, because of the spread from infected farms or by the movement of live eels from infected areas (Honka & Sures, 2021).

The prevalence of *A. crassus* in its native host and natural distribution area was usually around 17%–56% (Knopf, 2006). However, within a short period, infection rates in European eel populations in Western and Central European rivers reached over 90% (Sures & Streit, 2001; Taraschewski et al., 1987; van Banning & Haenen, 1990; Wielgoss et al., 2008). However, both the prevalence and the *A. crassus* infection intensity in new infected areas in Europe declined in recent years (Knopf, 2006). It has been postulated that the rapid spread experimented by *A. crassus* is linked to the high number of intermediate (copepods) and paratenic hosts and the short life cycle of *A. crassus* (Haenen, 1995). Moreover, the pathological lesions in the eels shortly after introduction of *A. crassus* would result from the absence of a specific immune response of the European eel against this parasite (Knopf et al., 2000; Knopf & Lucius, 2008), as well as to the lack of physical obstacles to their expansion in the aquatic environment and their long-term survival outside the host (Haenen

TABLE 3 Intensity of *Anguillicoloides crassus* adults and larvae (log_{10}-transformed) infection (No. parasites in infected eels) in European eels from Andalusia (southern Spain) according to explanatory variables

		Adults					Larvae				
Variable	Level	No. eels	Mean	Median	Range	*p* value	No. eels	Mean	Median	Range	*p* value
Eel weight	40–230	68	4.7	3.0	1–22	.8438	81	2.6	2.3	0.3–6.5	.1051
	231–419	16	6.2	4.0	1–23		17	2.0	1.3	0.3–4.8	
	420–609	9	4.1	3.0	1–12		7	3.5	3.6	1.4–5.4	
Eel length	31–44	40	4.8	3.0	1–22	.8567	52	2.4	1.7	0.3–5.7	.3849
	45–57	38	4.9	3.0	1–23		39	2.9	2.3	0.5–6.5	
	58–82	15	5.1	3.0	1–14		14	2.6	2.7	0.3–5.7	
Macroscopic lesions in eel's swimbladder	No	92	4.7	3.0	1–22	.0828	98	2.7	2.3	0.3–6.5	.1277
	Yes	1	23.0	23.0	23–23		7	1.6	1.3	0.6–4.8	
Sampling season	Spring	12	4.3	2.0	1–23	.0706	10	2.6	2.4	0.3–5.4	.7454
	Summer	20	4.7	3.0	1–22		31	2.3	1.4	0.3–5.7	
	Autumn	15	7.4	5.0	1–17		24	2.5	1.5	0.6–5.8	
	Winter	46	4.3	3.0	1–16		40	2.8	3.1	0.3–6.5	
Site distance to the sea (m)	1897–14,045	14	7.0	4.0	1–22	.0588	23	2.5	1.4	0.6–5.8	.7182
	14,046–26,193	58	5.2	3.0	1–23		69	2.6	2.0	0.3–6.5	
	38,342–50,489	20	2.8	2.5	1–7		13	2.9	3.1	0.3–4.7	
	not connected	1	1.0	1.0	1–1		0	–	–	–	
Water salinity (%)	0	22	5.5	4.0	1–22	.5574	32	2.8	2.4	0.3–5.8	.5767
	0.0–0.4	32	5.1	3.0	1–16		32	2.7	2.8	0.3–6.5	
	0.8–1.7	27	3.8	3.0	1–17		23	2.6	2.3	0.3–4.9	
	2.4–13	12	5.8	3.0	1–23		18	2.1	1.4	0.5–5.4	

TABLE 4 Results from the random effects logistic regression investigating the risk of eel's infection with *Anguillicoloides crassus* adults and larvae and Anguillid herpesvirus 1 (AngHV1), expressed as odds ratios (OR)

Variables	Levels	OR	95% CI	*p* value
A. crassus				
Fixed components				
Distance to the sea (m)	1897–14,045	1.00		
	14,792–22,317	0.59	(0.25–73.7)	.7061
	44,308–50,489	0.69	(0.04–11.7)	.8001
	Not connected	0.01	(0.00–0.17)	.0027
Season	Spring	1.00		
	Summer	3.80	(0.94–15.4)	.0619
	Autumn	6.95	(0.42–114)	.1742
	Winter	0.52	(0.20–1.39)	.1949
Random effect	Standard deviation			
Site	<0.001			
AngHV1				
Fixed components				
Distance to the sea (m)[a]	1897–14,045	1.00		
	14,792–22,317	26.37	(2.43–286)	.0071
	44,308–50,489	18.88	(1.73–206)	.0161
Season	Spring	1.00		
	Summer	1.24	(0.44–3.47)	.6878
	Autumn	3.44	(0.72–16.4)	.1206
	Winter	2.68	(1.04–6.93)	.0415
Random effect	Standard deviation			
Site	<0.001			

[a]The not connected Los Tollos lagoon was excluded in this analysis.

& Van Banning, 1990; McCallum et al., 2003). This parasite has reached even habitats considered to be suboptimal such as marine environments (Wielgoss et al., 2008) or hypersaline environments (Loukili & Belghyti, 2007; Martínez-Carrasco et al., 2011). However, since encapsulation of the nematode in the tissues of the European eel allowing parasite persistance has not been demonstrated, and non-acquired immunity against *A. crassus* has not been verified (Knopf, 2006), different hypothesis for the regulation of *A. crassus* intensity in the host has been proposed, including density-dependent regulation mechanisms in infrapopulations (Fazio, Sasal, et al., 2008; Kantzoura et al., 2021), the fibrosis and sclerotization of the swimbladder wall in previously infected animals hindering reinfections (Abdelmonem et al., 2010; Würtz & Taraschewski, 2000), reduced host abundance (Schabuss et al., 2005), acceleration of the silvering processes (Fazio, Moné, et al., 2008) or, more likely, the co-evolution of *A. crassus* and *A. anguilla* after the introduction of the parasite (Weclawski et al., 2013). Since the infection was first described in eels caught in the Iberian Peninsula (Ría de Aveiro in Portugal) (Silva et al., 1992), the distribution area in Europe has expanded. In Spain, *A. crassus* has been reported from rivers and estuaries throughout the country at prevalences ranging between 7.8% and 66% (Aguilar et al., 2005; Esteve & Alcaide, 2009; Gallastegi et al., 2002; Maíllo et al., 2005; Martínez-Carrasco et al., 2011; Muñoz et al., 2019). Therefore, the prevalence of *A. crassus* in eels described in previous studies carried out in Spain is slightly lower than those found in our study (71%), but in agreement with the hypothesis that prevalence reaches values of 60%–90% once the infection has stabilized in an area (Hohenadler et al., 2018; Lefebvre & Crivelli, 2004). On the contrary, in our study, the intensity of adults and preadults found in the swimbladders of the eels was 4 (1–23), which is consistent with previous studies reporting 3–7 worms per eel (Knopf, 2006). Based on previous studies, the average intensity of *A. crassus* found in the Andalusian eel population could limit the swimbladder's functionality by reducing its gas secreting capability and altering its elasticity (Schneebauer et al., 2017). It is worth noting that 16% of the swimbladders analysed contained 10 or more adult and/or preadult nematodes, resulting in a very high parasite intensity. In fact, adult–preadult *A. crassus* infection intensity was marginally significantly higher in eels with lesions in the swimbladder. As mentioned, *A. crassus* causes fibrosis in the swimbladder tissue due to larvae penetrating (L3) and developing towards L4 within the wall of this organ, and adults drilling through the inner tunica epithelium to feed on host blood (Kantzoura et al., 2021). These pathological issues occasionally result in high mortality rates, as reported in Hungary and the Czech Republic (Barus et al., 1996; Palíková & Navrátil, 2001). In any case, pathological alterations occurring in the swimbladder lead to a reduction in body condition of infected eels, which are thought to have a reduced capacity to successfully migrate to the Sargasso

Sea and, consequently, to complete their reproductive cycle (Palstra et al., 2007).

The high infection prevalence and intensity of L3 and L4 gives evidence to the fact that *A. crassus* has adapted to Andalusian river and lagoon habitats, where there is abundance of intermediate and paratenic hosts, mainly copepods (Dangel et al., 2015), amphibians, small fish (Haenen & Van Banning, 1990), some of them harbouring acanthocephalans as internal parasites (Hohenadler et al., 2018; Weclawski et al., 2013), are an important part of the eel diet. This high biodiversity (Szabolcs et al., 2022), together with the fact that eels are predators of a wide variety of prey (Tesch, 2003), is the reason why *A. crassus* has been able to adapt to fluvial and lagoon ecosystems of Andalusia.

Factors positively associated with *A. crassus* prevalence were being a small eel, sampling in summer and autumn and decreasing distance to the sea, although only distance to the sea and season remained significant in the multivariable model. This result was consistent with the pattern described by Costa-Dias et al. (2010), who described how the larger individuals are usually located upstream, where *A. crassus* presence is low, whereas smaller eels live downstream, where the infection rate is markedly higher. With that in mind, Barry et al. (2017) postulated that smaller eels, feeding on invertebrates, would consume more intermediate and/or paratenic hosts than larger fish, which have a more piscivorous diet increasing their chance of infection. Contrary to what has been reported by other authors (Costa-Dias et al., 2010; Kirk et al., 2000; Nielsen, 1997), water salinity has not been found to be a determining factor for the prevalence and intensity of infection with *A. crassus* in the rivers analysed. *A. crassus* was more prevalent in eels captured in summer and autumn than in other months, which contrasts with many studies not registering any infection seasonality (Kennedy & Fitch, 1990; Möller et al., 1991; Molnár et al., 1994; Thomas & Ollevier, 1992; Würtz et al., 1998). However, Lefebvre et al. (2002) found that the prevalence of *A. crassus* in eels from two French lagoons reached maximum values mainly in early summer. Moreover, Benajiba et al. (1994) recorded a biannual prevalence peak (spring and autumn) in a lagoon in the South of France and justify their results by associating a fast life cycle (De Charleroy et al., 1990) to variations in the planktonic biomass. In our case, and following the hypothesis of Benajiba et al. (1994), the infection of eels in spring would cause a peak of adult presence 3–4 months later, that is in summer and autumn. In any case, the temperatures recorded in the sampling areas ranged between 11.6 and 24.3 °C, so they were always above 10°C, which is the hatching limit of the parasite eggs (Nagasawa et al., 1994). It should be noted that most studies analysed host size and parasite seasonality separately, and Lefebvre et al. (2002) stated that correlation between both factors may generate confounding effects and prevent the identification of seasonality. In addition, the great differences in the climate between the areas in which the seasonality data of this parasite are available might also being generating confounding effects. Although the sampling effort in our study was similar in all sites, with the same number of days of capture and number of nets, it was not possible to increase the number of animals sampled at some of the sampling points. So, further studies involving a greater number of sampling areas are needed for a more robust identification of *A. crassus* infection seasonality and risk factors.

Concerning the viral infections investigated in this study, AngHV1, and to a lesser extent EVE and EVEX, are the viruses most frequently found in European eels (van Beurden et al., 2012), although other ones, such as Eel Picornavirus 1 (EPV-1) have been also detected (Danne et al., 2022). AngHV1 has been recorded in eels caught in different European countries such as the Netherlands, Germany and Greece (Haenen et al., 2010; Jakob et al., 2009; Van Ginneken et al., 2004; Varvarigos et al., 2011), and is thought to play a relevant role in the decline of the wild European eel stocks (Haenen et al., 2012). Eel Virus European X is probably cosmopolitan though only rarely diagnosed (Caruso et al., 2014), whereas EVE has been mostly detected in European eels held in captivity (Van Beurden et al., 2012). Co-infections with EVE and AngHV1 have been reported in the Netherlands (Haenen et al., 2002) and Greece (Varvarigos et al., 2011), while cases of EVE–EVEX co-infection have been described in Germany (Ahne & Thomsen, 1985), Italy (Van Ginneken et al., 2004) and the United Kingdom (McConville et al., 2018). EVEX was isolated in eels from France, United Kingdom, Denmark, Sweden and the Netherlands (Jørgensen et al., 1994; Van Ginneken et al., 2005). There are few studies of viral infections of eels in Spain. In the Albufera of Valencia, eels have been found to be carriers of AngHV1 with prevalences above 83% (Bandín et al., 2014), and co-infections with EVEX and AngHV1 have been described in eels from Mar Menor Lagoon (Muñoz et al., 2019). Our results revealed that 35.2% of the sampled eels harboured AngHV1 and it was between 10 and 40% in some areas with the highest prevalence in Los Tollos lagoon (100%) and the other rivers in Guadalquivir EMU area (Table 1). Instead, the virus was not detected in Guadalfeo (n = 10) and Antas (n = 10) rivers in the Mediterranean EMU areas, indicating that there may be river basins where this virus is not yet present or, if it is present, it has a very low prevalence. Efforts should be made to avoid anthropogenic spreading of AngHV1, EVEX and EVE by restocking into these areas as described elsewhere (ICES, 2015a; Kullmann et al., 2017; Van Beurden et al., 2012; Van Ginneken et al., 2004). Eels with AngHV1 may present injuries affecting the skin, gills and liver, usually showing haemorrhages and necrosis (Davidse et al., 1999; Haenen et al., 2002; Hangalapura et al., 2007; Lepa & Siwicki, 2012). Also, AngHV1 has been shown to induce immunosuppression by limiting phagocytosis and decreasing B and T lymphocyte proliferation (Schulz et al., 2019). However, persistent viral infections have been described in eels without clinical signs, which may act as a reservoir of infection (Van Nieuwstadt et al., 2001). Moreover, Tuan et al. (2016) highlighted the risk of virus transmission by other fish species to eels.

It has been proven that stressors such as high water temperatures, low oxygen concentrations, contamination with toxic substances such as polychlorinated biphenyls (PCB's) or inorganic elements, silvering, exhaustion from migration to spawning grounds in the Sargasso Sea (Haenen et al., 2010; Kullmann et al., 2017; Lortholarie et al., 2021; Muñoz et al., 2019; van Beurden et al., 2012;

Van Ginneken et al., 2005) and especially, infection with *A. crassus*, might reactivate AngHV1 infection in persistent, non-clinical carriers, causing high mortalities (Haenen et al., 2010; van Beurden et al., 2012; Van Nieuwstadt et al., 2001). In this sense, several authors demonstrated high cortisol levels in the blood derived from the presence of *A. crassus* (Honka & Sures, 2021; Sures & Streit, 2001). Even, Kullmann et al. (2017) suggested that *A. crassus* infected eels are able to spawn, but the egg quality might be low, and off-spring might also be infected. In this regard, the existence of 21.2% of the eels that carried both the parasite *A. crassus* and AngHV1, an association that was marginally significant, must be considered a relevant result of this research. The presence of co-infection of both pathogens reached 57% of the animals from the Guahalhorce river and Isla Mayor lagoon (Guadalquivir EMU). Nowadays, authors stress the importance of studying co-infections and multiple stressors rather than single primary or relevant fish pathogens (Gorgoglione et al., 2020), independently from when the impact of infections is evaluated (Serrano & Millán, 2014), and the severity of disease in the hosts may depend on interactions between co-infecting pathogens (Pedersen & Fenton, 2006).

In accordance with Lefebvre et al. (2002) and Kullmann et al. (2017), we found an association between AngHV1 prevalence and eel size. The prevalence of AngHV1 in the present study was highest in summer and winter, which is partially in line with the data provided by Bandín et al. (2014), who suggested that AngHV1 showed an increase in the months when the water reaches higher temperatures (18–22,5°C). The optimal temperature for AngHV1 replication is between 20 and 26°C, although when fishes swim in waters outside this temperature range, the virus may persist in fish tissues (Davidse et al., 1999; Smail & Munro, 2012). Nevertheless, when interpreting our data, we should consider that almost half of the samples of this study (47.6%) were collected during the winter season. So, it would be desirable to further analyse in future studies the possible fluctuation of prevalence in different seasons of the year.

As far as EVE and EVEX viruses are concerned, they have not been detected in our study, coinciding with authors showing that these viruses are more frequently found in eel farms (Haenen et al., 2012; ICES, 2016; McConville et al., 2018; van Beurden et al., 2012; Van Ginneken et al., 2005). When present, EVEX causes a decrease in eels' fitness and EVE induces renal damage (Van Beurden et al., 2012; van Ginneken et al., 2004, 2005). So, follow-up studies of infection with these eel viruses should continue to be conducted, because these viruses might negatively influence the spawning migration of the eel (Van Ginneken et al., 2005).

5 | CONCLUSIONS

Our results showed that *A. crassus* is widely distributed in Andalusia. Therefore, this parasitic nematode of European eel should be considered as one of the negative factors in the decline of the wild eel stock. It is not easy to recommend possible management measures to improve this situation since the life cycle of the parasite is shown to be perfectly established in this habitat, and actions to control the intermediate and paratenic hosts of *A. crassus* are extremely difficult to carry out. Restocking and translocation of eels from one area to another should be controlled to avoid the introduction of this nematode into new areas. In this sense, it would be advisable to evaluate the presence of *A. crassus* larvae from faecal samples in eels destined for reintroduction, although this does not provide security on eel's infection since larvae are not detected in the prepatent period. Surveillance also needs to be performed to avoid AngHV1, EVEX and EVE introduction and spread. We hope that information provided in the present study will be useful to administrations responsible for elaborating such surveillance and control programmes.

AUTHOR CONTRIBUTIONS

M. Rocío Ruiz de Ybáñez: Conceptualization, Investigation, Data curation, Writing—original draft. **Laura del Río**: Investigation, Validation, Writing—review and editing. **César Flores-Flores**: Methodology, Validation, Writing—review and editing. **Pilar Muñoz**: Investigation, Writing—review and editing. **Eduardo Berriatua**: Formal statistical analisis, Methodology, Writing—review and editing. **Silvia Rubio**: Investigation, Writing—review and editing. **Carlos Martínez-Carrasco**: Conceptualization, Project administration, Supervision, Funding acquisition, Investigation, Data curation, Writing—review and editing.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Authors declare the absence of shared data.

ANIMAL WELFARE STATEMENT

The Ethical Committee for Animal Experimentation of the University of Murcia reports that, following the basic rules applicable for the protection of animals used in experimentation and other scientific purposes (described in RD 53/2013), all applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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