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Gastrointestinal helminths in brown trout (*Salmo trutta* Linnaeus, 1758) captured in Galician rivers (NW Spain)

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ABSTRACT

Specimens of *Salmo trutta* ($n = 613$) captured by local anglers in different rivers in Galicia (NW Spain) during the 2015 fishing season (15 March–15 August) were examined. In total 1479 adult helminths were recovered from the gastrointestinal tracts of 221 fish. Moreover, the microscopic observation of the sediments obtained, previous diphasic concentration, revealed the presence of helminth eggs in 485 trout specimens. The following species were identified by morphological and molecular analysis: *Crepidostomum metoecus* (8.97%) (Trematoda); *Salmonema ephemeridarum* (16.97%), *Raphidascaris acus* (9.46%) and *Pseudocapillaria* sp. (2.12%) (Nematoda); and *Echinorhynchus truttae* (8.48%) (Acanthocephala). The prevalence, mean intensity and mean abundance of each helminth species were determined in relation to size/age of the fish. The helminth infracommunity comprised a maximum of four species and the species richness was $S = 5$. The biological cycles of most of the helminth species recovered are dependent on benthic macroinvertebrate fauna, which, in turn, is influenced by the water quality. Therefore, any changes that take place in the aquatic ecosystem (due to anthropogenic activities or climate change) may be reflected in the helminth composition.

1. Introduction

Parasitic organisms are found worldwide and represent a high proportion of the biodiversity on Earth. Helminth fish parasites are an essential part of the aquatic environment and are more common and diverse in wild than in farmed hosts [1–3]. Numerous studies on diverse fish parasites and their ecology have been conducted in the last decade, as fish parasites are also of interest from an environmental perspective [4]. Several ecological factors and host characteristics influence the number and diversity of parasites that infect hosts at the individual level. In fish, these factors may include size/age, the number and type of prey consumed and also prey selectivity, habitat and season [5–7]. Helminth parasites transmitted via the trophic chain usually have one or more intermediate hosts, and the abundance of parasites in fish will depend on the importance of different prey species in the fish diet [8,9]. Information about the prevalence of helminth infections in wild fish will help to clarify the roles of these parasites in ecosystems, e.g. in regulating the abundance or density of hosts or stabilizing food webs [10].

The brown trout (*Salmo trutta* Linnaeus, 1758) is the most widely distributed salmonid species in the world [11]. The wide geographical distribution of this species can be explained by its ecological variability and excellent ability to spread and colonize new water courses. However, the primary factors affecting the establishment of natural populations are water temperature, precipitation and the availability of suitable spawning grounds [12]. Thus, trout mainly live in upland streams and rivers with cold, well-oxygenated waters, although they are also found in some lakes and lowland rivers. The diet of brown trout includes benthic macroinvertebrates, while the adults also consume amphibians and small fish [13–15]. This species is indigenous to Spain, where two populations occur: a migratory community with a northern distribution (Galician and Cantabrian Mountains), and a sedentary population that inhabits the remaining rivers, although is absent from several rivers in southern and eastern Spain [13,16]. The brown trout is not cultured for commercial purposes in Spain, but is an important angling species and consequently is socio-economically important [17]. In Galicia (NW Spain), a region with exceptional hydrological

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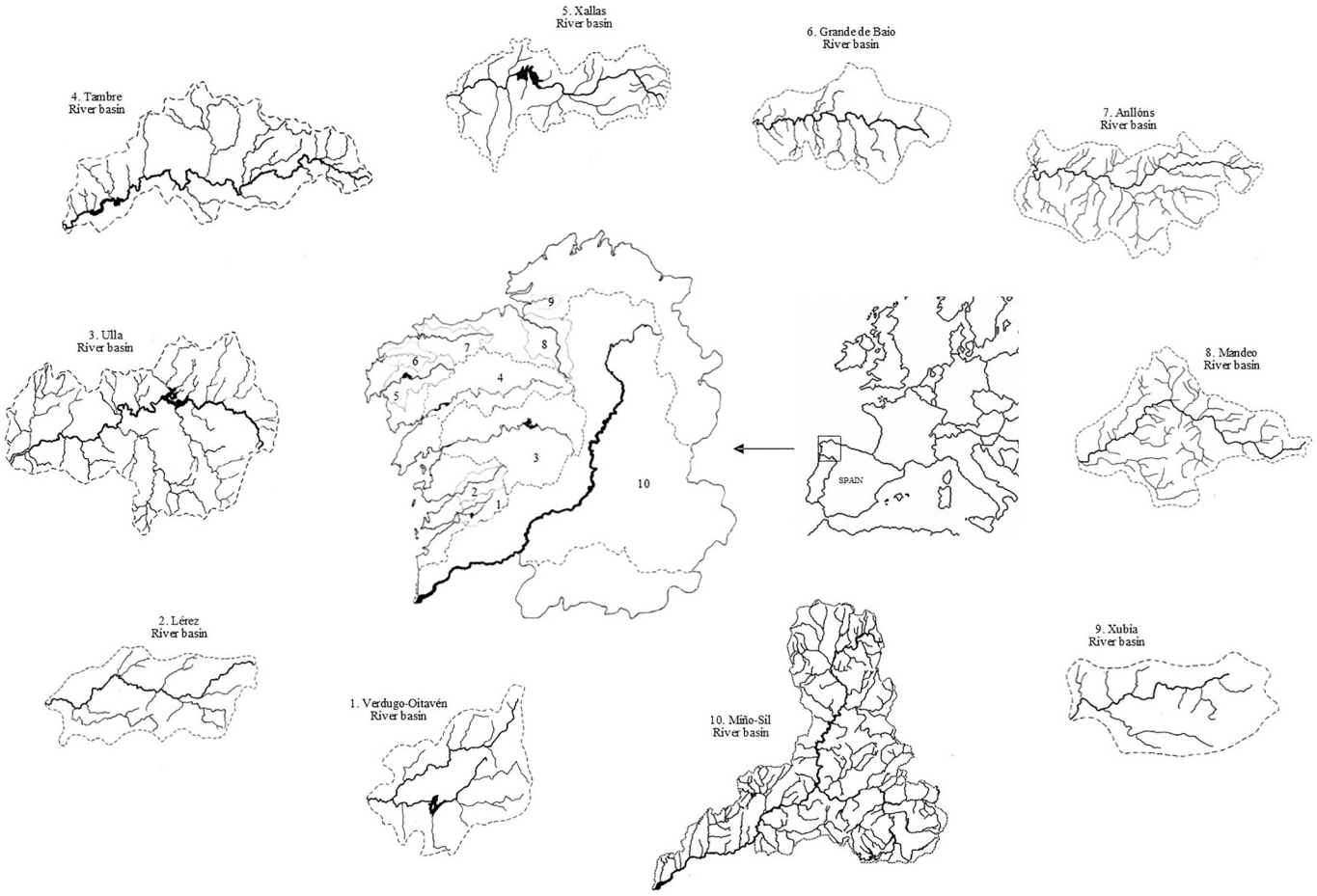

Fig. 1. Geographical location of the river basins (Galicia, NW Spain) where specimens of brown trout (*Salmo trutta*) were captured by local anglers and subsequently examined for detection of gastrointestinal helminth parasites.

characteristics and through which thousands of small waterways flow as a consequence of the rugged relief and high rainfall, the brown trout is one of the most representative species because of its abundance and wide distribution [18,19].

Information on the helminth parasites of this wild freshwater fish in Spain is scarce and out of date. The aim of the present study was therefore to obtain further information on the gastrointestinal helminths of brown trout and provide, for first time in Spain, preliminary data on the parasite community in this host.

2. Material and methods

2.1. Study area and fish sampling

The study was carried out in the region of Galicia (NW Spain), which covers a total area of 29,574 Km^2 (43° 47′ N - 41° 49′ N, 6° 42′ W - 9° 18′ W) (Fig. 1). In total, 613 specimens of brown trout (*S. trutta*) were captured by local anglers during the 2015 fishing season (15 March–15 August) in 44 rivers belonging to 10 river basins Verdugo-Oitavén (n = 16); Lérez (n = 52); Ulla (n = 220); Tambre (n = 123); Xallas (n = 9); Grande de Baio (n = 3); Anllóns (n = 5); Mandeo (n = 2); Xuvia (n = 5) and Miño-Sil (n = 178) (Fig. 1). The anglers removed the complete gastrointestinal tracts from the fish and stored them in hermetically sealed plastic bags at approximately −20 °C until sending them to the Parasitology Laboratory of the Faculty of Pharmacy (University of Santiago de Compostela) for their processing and analysis. The anglers also provided data on the river, date of capture and length of the fish. The specimens were classified according to Sánchez-Hernández et al. [20], as follows: 19.0 (minimum legal size) –19.1 cm (<2 years; n = 160); 19.2–25.9 cm (2–3 years; n = 355) and 26.0–50.6 cm (>3 years; n = 98).

2.2. Parasite screening

The gastrointestinal tracts were defrosted, differentiated into stomach, pyloric caeca and intestine and placed in Petri dishes with 0.9% saline solution. They were then opened longitudinally and examined individually under a stereomicroscope (Z45 E, Leica Inc., Buffalo, NY, USA). Helminths were removed, washed with 0.9% saline solution and preserved in 70% ethanol. For purposes of morphological identification, trematodes were stained with hydrochloric carmine; the remaining parasites were stained with lactophenol cotton blue. Specimens were identified to the lowest taxon possible by microphotographs obtained with an optical microscope equipped with a digital camera (AX70, Olympus Optical Co., Ltd., Tokyo, Japan) following the descriptions provided by Moravec [21,22], Gibson [23] and Buchmann and Bresciani [24]. Moreover, the species identification was confirmed by molecular analysis as described in section 2.3.

Once all helminth adult forms were recovered, the pyloric caeca samples were individually homogenized in 0.04 M phosphate buffered saline (PBS) pH 7.2, in an Ultra-Turrax® T10 homogenizer (IKA®-Werke GmbH and Co., KG, Staufen, Germany). Similarly, the fish intestinal contents were removed by scraping with a scalpel blade and ground with 0.04 M PBS pH 7.2 in a mortar. Both homogenates thus obtained were individually subjected to diphasic concentration in 0.04 M PBS pH 7.2/diethyl ether (2:1) by centrifugation at 1250*g*, 4 °C, for 15 min, after

Table 1
Prevalences, mean intensities and mean abundances of adult forms and prevalences of eggs for gastrointestinal helminth species identified in brown trout (*Salmo trutta*) captured in rivers in Galicia (NW Spain) grouped according to the estimated age of the fish.

Helminth parasite		Parameter	Trout age			Overall
			<2 years	2–3 years	>3 years	
Crepidostomum metoecus	Adults	P	4.40	10.44	10.27	8.97
		MI (95% CI)	2.86 (1.62–4.00)	5.03 (2.77–7.76)	3.60 (2.00–5.31)	4.48 (2.85–6.42)
		MA (95% CI)	0.13 (0.04–0.22)	0.52 (0.26–0.85)	0.37 (0.14–0.66)	0.39 (0.23–0.59)
	Eggs*	P	51.22	65.09	75.36	63.13
Salmonema ephemeridarum	Adults	P	17.45	18.92	9.15	16.97
		MI (95% CI)	4.29 (2.92–5.90)	6.79 (4.53–9.84)	4.22 (2.00–6.50)	5.89 (4.38–7.86)
		MA (95% CI)	0.75 (0.44–1.10)	1.28 (0.78–1.95)	0.39 (0.13–0.68)	1.00 (0.70–1.42)
	Eggs	P	35.57	49.27	55.14	46.66
Raphidascaris acus	Adults	P	5.06	10.11	14.35	9.46
		MI (95% CI)	1.25 (1.00–1.55)	2.56 (1.82–3.29)	3.43 (2.00–5.05)	2.59 (1.97–3.25)
		MA (95% CI)	0.06 (0.02–0.11)	0.26 (0.16–0.38)	0.49 (0.22–0.81)	0.24 (0.17–0.33)
	Eggs	P	1.96	3.42	5.05	3.26
Pseudocapillaria sp.	Adults	P	3.75	1.63	0.98	2.12
		MI (95% CI)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	2.00 (2.00–2.00)	1.08 (1.00–1.25)
		MA (95% CI)	0.04 (0.01–0.07)	0.02 (0.01–0.03)	0.02 (0.00–0.06)	0.02 (0.01–0.03)
	Eggs	P	–	0.81	0.98	0.65
Echinorhynchus truttae	Adults	P	6.85	7.83	18.43	8.48
		MI (95% CI)	5.45 (2.60–8.75)	8.68 (5.52–12.61)	12.00 (3.75–23.75)	8.83 (5.95–12.65)
		MA (95% CI)	0.38 (0.14–0.67)	0.68 (0.37–1.12)	1.59 (0.29–3.62)	0.75 (0.44–1.18)
	Eggs	P	8.65	6.52	10.28	7.67

P = prevalence; MI = mean intensity (adult specimens/infected trout); MA = mean abundance (adult specimens/total examined trout): CI = confidence interval; **Crepidostomum* spp.

filtration of the samples through a set of 2 sieves (mesh size 150 and 45 μm). The supernatants were carefully discarded and the pellets were resuspended in 500 μL of 0.04 M PBS pH 7.2 and stored at −20 °C. Aliquots (10 μL) of the sediments thus obtained were examined in triplicate under bright field microscopy (×200 magnification) (AX70, Olympus Optical Co., Ltd.) for detection of helminth eggs.

2.3. Molecular characterization

Genomic DNA was extracted from representative adult forms of each helminth taxon by using the Stool DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The DNA thus extracted was stored at -20 °C until use. New PCR techniques were used to amplify fragments of the larger subunit of the rRNA (LSU-rRNA) gene of *Crepidostomum* and the smaller subunit of the rRNA (SSU-rRNA) genes of *Salmonema*, *Raphidascaris*, *Pseudocapillaria* and *Echinorhynchus*. For that, primers targeting hypervariable regions of these loci were designed by aligning sequences of the mentioned helminth parasites available in the GenBank® database (National Institute of Health, Bethesda, MD, USA).

For the LSU-rRNA gene of *Crepidostomum*, a fragment of ~850 bp was amplified using the forward primer C28sF (5'-GAC ACT GCT CCT CTC TAA GTC CTA-3′) and the reverse primer C28sR (5'-CCT TAG ACT GGA CAA GCC AGA CCT-3′). The reaction mixture contained 2.0 μL template DNA, 0.1 μM each primer, 12.5 μL EmeraldAmp® MAX PCR Master Mix 2× (Takara Biotechnology, Kusatsu, Japan) and molecular grade water up to a final volume of 25 μL. The PCR conditions consisted of initial denaturation of 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min, with a final extension at 72 °C for 7 min.

For the SSU-rRNA locus of the remaining helminths, a PCR product of ~650 bp was amplified using the following degenerate primers: H18sF (5′-AAG GCA GCA GGC RCG CAA ATT A-3′) and H18sR (5'-TGC AAC CAT ACT RCC CCC GGA A-3′). The reaction mixture contained 2.0 μL template DNA, 0.1 μM each primer, 12.5 μL EmeraldAmp® MAX PCR Master Mix 2× (Takara Biotechnology) and molecular grade water up to a final volume of 25 μL. The PCR conditions consisted of initial denaturation of 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min.

The amplicons were electrophoresed on 2% agarose gels and stained with Real Safe (Real Laboratory S.L., Paterna, Valencia, Spain). Positive PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN®, Hilden, Germany) and sequenced in both directions. The sequencing reactions were assembled using the SeqMan™ 7.0 (DNASTAR®, Madison, WI, USA) and the resulting sequences were compared with those deposited in GenBank® (National Institute of Health), by using the public web interface of the BLAST® 2.12.0 program (https://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information).

2.4. Analysis of the parasite populations

Prevalence (percentage of hosts infected with a particular parasite taxon), mean intensity (number of individuals of a parasite taxon found in a host, divided by the number of hosts infected with this taxon) and mean abundance (total number of specimens of a parasite taxon in the host divided by the total number of hosts examined, including both infected and uninfected hosts) were calculated for each helminth species according to Bush et al. [25]. Furthermore, the gastrointestinal helminth associations were studied in relation to the estimated age of all fish from which adult forms were recovered. Moreover, several ecological parameters were used to measure the helminth community structure in those watersheds where >50 trout were analysed (Lérez, Ulla, Tambre and Miño-Sil basins; Fig. 1), including 573 of the 613 specimens analysed. Thus, following to Magurran [26], species richness (*S*), defined as the total number of parasitic species recorded, and infracommunity, which refers to all individuals of all parasite taxa found in a single host, were determined, and the mean number of parasites and mean number of species were thus calculated for the four river basins previously considered. In order to study the spatial variations among the watersheds considered, the frequencies (number of fish) of co-occurrences of different helminth species were determined and the observed frequencies were compared with the expected frequencies.

2.5. Statistical analysis

Statistical tests were performed using Statgraphics® Centurion XVI v.16.2.04 Statistical Software (©1982–2013 StatPoint Technologies, Inc., Warrenton, VA, USA) and IBM® SPSS Statistics v.28.0.1.1 (©1989–2021 International Business Machines Corporation, Endicott,

Table 2
Gastrointestinal helminth infracommunities detected in brown trout (*Salmo trutta*) captured in rivers in Galicia (NW Spain) according with the helminth species identified.

Gastrointestinal helminth infracommunity (n/% infected trout)	Species*	n samples
Single (174/28.38%)	Cm	32
	Se	66
	Ra	36
	Pc	6
	Et	34
Double (38/6.20%)	Cm-Se	9
	Cm-Ra	2
	Cm-Et	5
	Se-Ra	11
	Se-Pc	4
	Se-Et	5
	Ra-Pc	1
	Ra-Et	1
Triple (8/1.31%)	Cm-Se-Ra	3
	Cm-Se-Et	2
	Cm-Ra-Et	1
	Se-Ra-Et	1
	Se-Pc-Et	1
Quadruple (1/0.16%)	Cm-Se-Ra-Pc	1

* Cm = *Crepidostomum metoecus*; Se = *Salmonema ephemeridarum*; Ra = *Raphidascaris acus*; Pc = *Pseudocapillaria* sp.; Et = *Echinorhynchus truttae.*

NY, USA). According to Reiczigel et al. [27], differences in the prevalence rates of parasites between the different age groups of fish were determined using Fisher's exact test. The Shapiro-Wilk test was used to test the data fit to a normal distribution. Mean intensities and mean abundances were compared by the bias-corrected and accelerated (BCa) bootstrap-ANOVA method at 95% confidence intervals (CI) using 1000 replicates. Regarding the trout from the four river basins in which >50 specimens were analysed, observed and expected frequencies were compared by χ^2-test. Moreover, Spearman's rank correlation coefficients were used to assess for relationships between fish length and 1) intensity of infection by each common parasite species, 2) total helminth abundance, or 3) species richness. Pairwise associations between the intensity of most common helminth species were computed using Spearman's rank correlation coefficient, excluding fish that did not harbour at least one of the two species in a pair. Mean fish length and the mean number of parasites and species for the four river basins considered were compared using an ANOVA test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Gastrointestinal helminth species

In total, 1479 adult specimens of five helminth species were recovered from 221 of the 613 brown trout examined. The following species were identified by morphological and molecular analysis: *Crepidostomum metoecus* (Trematoda) in 55 samples (8.97%; GenBank® accession number: OL588598); *Salmonema ephemeridarum* (syn. *Cystidicoloides tenuissima*) (Nematoda) in 104 specimens (16.97%; GenBank® accession number: OL588599); *Raphidascaris acus* (Nematoda) in 58 specimens (9.46%; GenBank® accession number: OL588600); *Pseudocapillaria* sp. (Nematoda) in 13 specimens (2.12%; unsuccessful molecular analysis); and *Echinorhynchus truttae* (Acanthocephala) in 52 specimens (8.48%; GenBank® accession number: OL588601) (Fig. S1). Adult forms of helminths fixed in 70% ethanol have been deposited in the collection held in the Natural History Museum of the University of Santiago de Compostela (Santiago de Compostela, Spain) under the following catalogue numbers: MHN USC 25203 for *C. metoecus*; MHN USC 25204 for *S. ephemeridarum*; MHN USC 25205 for *R. acus*; MHN USC 25206 for *Pseudocapillaria* sp. and MHN USC 25207 for *E. truttae*. The prevalence, mean intensity and mean abundance of adults of each helminth species in relation to the different age groups of fish are shown in Table 1.

The prevalence increased with the age of the fish, except for *S. ephemeridarum* and *Pseudocapillaria* sp. infections. This increase was statistically significant for *C. metoecus* (<2 vs. 2–3 years; $P = 0.02$) and for *R. acus* (<2 vs. >3 years; $P = 0.01$). However, the prevalence of *S. ephemeridarum* in specimens >3 years was significantly lower than for specimens in the other age groups ($P = 0.02$). Considering the mean intensities and mean abundances of infection, no significant differences were observed, except for *R. acus* infection, in which the mean abundance was significantly lower in fish <2 years old than in fish >3 years old ($P < 0.01$).

On the other hand, microscopic observation of the sediments obtained from pyloric caeca and intestinal homogenates revealed the presence of helminth eggs in 485 of the 613 trout specimens. Eggs of the following helminths were detected: *Crepidostomum* spp. ($n = 387$; 63.13%); *S. ephemeridarum* ($n = 286$; 46.66%); *R. acus* ($n = 20$; 3.26%); *Pseudocapillaria* sp. ($n = 4$; 0.65%) and *E. truttae* ($n = 47$; 7.67%). The prevalence again tended to increase with the age of the fish and was statistically significant higher for *Crepidostomum* spp. (all groups; $P < 0.05$) and *S. ephemeridarum* (<2-year-old fish vs. >2-year-old fish; $P < 0.05$) (Table 1).

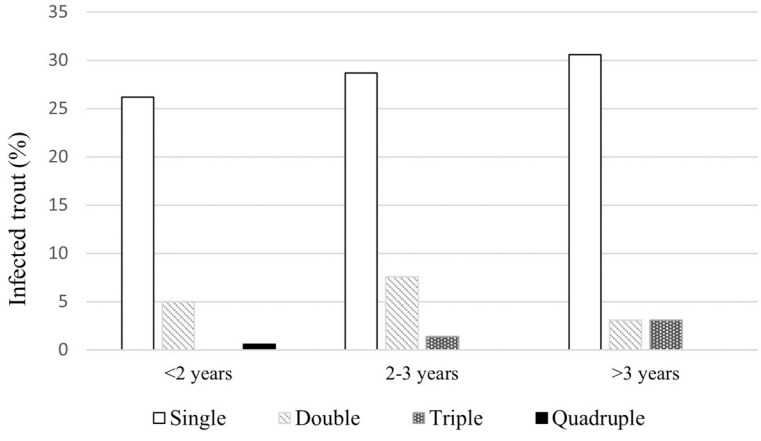

Fig. 2. Percentage of brown trout (*Salmo trutta*), captured in rivers in Galicia (NW Spain), infected by different gastrointestinal helminth infracommunities according to the estimated age of the fish.

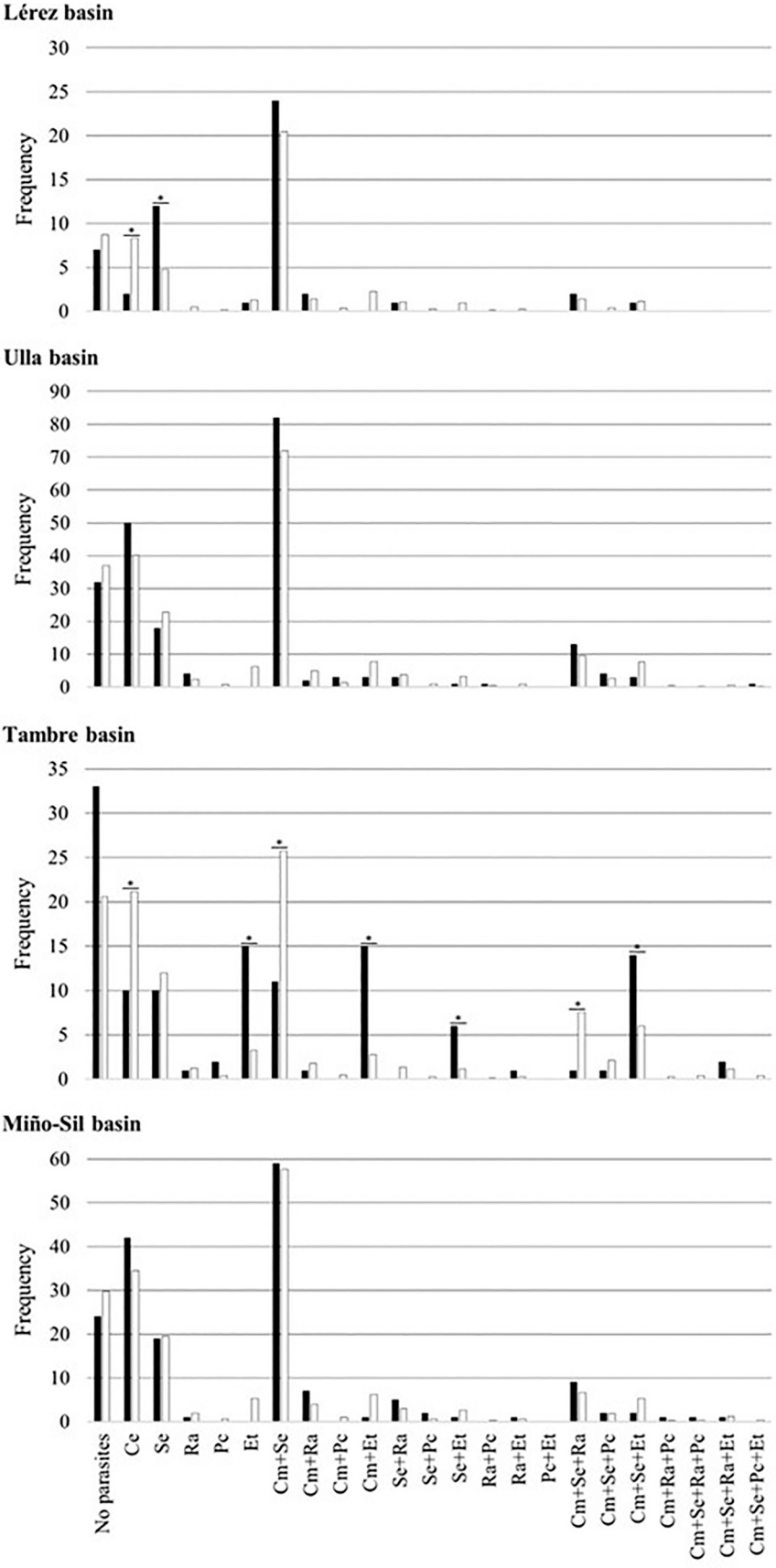

Fig. 3. Observed (black bars) and expected (white bars) frequencies of different co-occurrences of helminth species found in brown trout (*Salmo trutta*) from four Galician basins (NW Spain). Cm = *Crepidostomum metoecus*; Se = *Salmonema ephemeridarum*; Ra = *Raphidascaris acus*; Pc = *Pseudocapillaria* sp.; Et = *Echinorhynchus truttae*; $^{*}P < 0.05$.

Table 3
Relationships (Spearman's rank correlation coefficients) between length of the brown trout (*Salmo trutta*) and the number of worms per specimen for the four common helminth species, the total abundance of all helminth species per specimen, and the richness of species per fish determined in four Galician basins (NW Spain).

	Lérez basin	Ulla basin	Tambre basin	Miño-Sil basin
Crepidostomum metoecus	−0.579	0.139	−0.127	−0.157
Salmonema ephemeridarum	−0.621	0.013	0.038	0.251
Raphidascaris acus	−0.06	0.083	0.295	0.152
Echinorhynchus truttae	–	−0.363	−0.007	–
Total abundance	−0.572*	0.203	0.220	−0.014
Richness	0.148	0.266*	0.104	−0.227

* $P < 0.05$.

3.2. Helminth infracommunity and ecological parameters

In those trout specimens from which adult parasitic forms were recovered, the helminth infracommunity comprised no more than four species. Thus, of the 613 trout examined, 174 (28.38%) were parasitized by a single helminth; 38 (6.20%) by two helminths; 8 (1.31%) were parasitized by three helminths; and one specimen (0.16%) was parasitized by four helminth species (Table 2). The complexity of the helminth infracommunities increased with the age of the fish, as parasitisation by two helminth species was less frequent in trout >3 years and parasitisation by three helminth species was statistically significant more common in trout specimens >3 years than in trout <2 years ($P < 0.02$) (Fig. 2).

The observed frequencies of co-occurrence (based on presence/absence) of the helminth species (*C. metoecus*, *S. ephemeridarum*, *R. acus* and *E. truttae*) in the four basins in which >50 specimens were analysed (Lérez, Tambre, Ulla and Miño-Sil watersheds) did not show statistically significant differences except in single parasitisms in trout from the Lérez basin and in single, double and triple parasitisms in specimens from Tambre watershed (Fig. 3). The co-occurrence of *C. metoecus* and *S. ephemeridarum* was the most frequent in all watersheds, except in the Tambre basin, where the prevalence of *E. truttae* was high. Thus, in this watershed, single parasitism by *E. truttae* and co-occurrence of *C. metoecus* and *E. truttae* were most frequently observed parasitisms (Fig. 3).

Regarding the correlation analyses, trout length was significantly correlated with the total abundance of helminths per fish in specimens from Lérez basin and the trout length of the trout was also significantly correlated with the species richness in fish from Ulla basin (Table 3). Moreover, all the 24 pairwise correlations computed between the intensity of infection by two parasite species were negative and 15 of them were statistically significant. It was remarkable that all the correlations computed in the Ulla basin were statistically significant and that the infections by *C. metoecus* and *S. ephemeridarum* were significantly correlated in trout from all four watersheds (Table 4).

The parameters corresponding to the helminth community in the four river basins where the highest numbers of trout were captured (Lérez, Tambre, Ulla and Miño-Sil watersheds) are summarized in Table 5. The mean length of fish specimens captured in the Miño-Sil watershed was significantly greater than that of specimens captured in the other three river basins sampled ($F = 12.65$; $P < 0.01$). The proportions of infected trout captured in Lérez, Ulla and Miño-Sil basins (86.54%, 85.45% and 86.52%, respectively) were statistically significantly higher than the corresponding proportion in the Tambre basin (73.27%; $P < 0.01$). The highest values of species richness ($S = 5$) were determined in Ulla and Miño-Sil basins and the highest mean number of adult parasites by trout and mean number of species per fish were found in specimens captured in the Ulla watershed, although the differences relative to the trout captured in the other three basins were not statistically significant (Table 5).

4. Discussion

To our knowledge, this is the most complete study on gastrointestinal helminths in *S. trutta* carried out to date in Spain, in which 613 specimens captured in 44 rivers belonging to 10 river basins were analysed. The existing data on the helminths of the digestive tract of brown trout in this country date from 1979, when Álvarez Pellitero published the results of the analysis of 1205 fish captured in 9 rivers of León, a region bordering Galicia [28]. Furthermore, a review of the scientific literature showed that most studies focusing on this host date from the 20th century and more recent studies are scarce.

Macroscopic examination of the gastrointestinal tracts of 613 brown trout revealed the presence of adult helminths in 221 of the specimens. However, in the previous study carried out by Álvarez Pellitero [28], a higher number of the brown trout examined harboured adult helminths, specifically 1187 of the 1205 fish. Possible reasons for this difference may be the time taken between evisceration of the fish by local anglers and processing of the samples, which may have led to deterioration of the most delicate parasitic forms. In addition, we cannot rule out geographical and temporal differences in the distribution of helminth parasites and their hosts that may have occurred due to the environmental variations derived from agricultural and industrial development and climate change during the 40 years (that had elapsed between both works). Thus, studies on the parasitic helminth community are important to provide information to help understand ecosystem changes, as the composition of helminths may differ between regions and over time [29–31].

Microscopic analysis of the sediments obtained after the application of a diphasic concentration method to the homogenized gastrointestinal samples enabled the detection of helminth eggs in a greater number of samples (485 of the 613 trout examined). Interestingly, eggs belonging to smallest and most fragile helminths (i.e. *Crepidostomum*) were detected in a significantly greater number of samples than the corresponding adult forms (see Table 1; $P < 0.05$). Although the protocol applied to the pyloric caeca and intestinal contents is more laborious, requires more time and does not allow identification of samples to species level, it provides additional epidemiological data.

Five gastrointestinal helminths were identified in the Galician brown trout: one trematode (*C. metoecus*), three nematodes (*S. ephemeridarum*, *R. acus* and *Pseudocapillaria* sp.) and one acanthocephalan (*E. truttae*). All of these helminths have previously been reported to occur in this host in Europe by several authors [28,32–34]. Although García Pérez et al. [35] detected a cestode species (*Cyatocephalus* sp.) in trout from northern Spain, no cestodes were found in the present study or in the previous study by Álvarez Pellitero [28], suggesting that gastrointestinal cestodes are very scarce in brown trout in northwest Spain.

The brown trout is known to harbour two trematode species of the genus *Crepidostomum*: *C. metoecus* (pyloric caeca location) and *Crepidostomum farionis* (intestinal location), with *C. metoecus* being more frequently reported than *C. farionis* [33,34]. In this study, adults of *C. metoecus* were identified in 8.97% of the trout analysed. However, microscopic examination of the homogenized gastrointestinal samples led to detection of *Crepidostomum* spp. eggs at a higher rate (63.13%). These differences can be explained by the small size (2–6 mm) and the fragility of adult forms. Moreover, the infection tended to increase with the size/age of the trout ($P < 0.05$) (see Table 1). Álvarez Pellitero [28] reported prevalence rates of 79.0% and 63.0% for *C. metoecus* and

Table 4
Matrix of pairwise associations (Spearman's rank correlation coefficients) between the intensity of infection of the four common helminth species found in brown trout (*Salmo trutta*) from four Galician basins (NW Spain). Double zeros (fish that did not harbour worms of either species in a pairwise association) were excluded in the correlation analyses. Actual sample sizes are the number of specimens harbouring at least one of the two species in a pair and are indicated below the diagonal.

	Cm	Se	Ra	Et
Lérez basin				
Crepidostomum metoecus	–	−0.867*	−0.682*	−0.424
Salmonema ephemeridarum	17	–	−0.729*	−0.539
Raphidascaris acus	11	12	–	−0.725
Echinorhynchus truttae	8	10	5	–
Ulla basin				
Crepidostomum metoecus	–	−0.344*	−0.741*	−0.733*
Salmonema ephemeridarum	72	–	−0.343*	−0.323*
Raphidascaris acus	38	74	–	−0.724*
Echinorhynchus truttae	24	64	28	–
Tambre basin				
Crepidostomum metoecus	–	−0.579*	−0.401	−0.158
Salmonema ephemeridarum	26	–	−0.406	−0.478*
Raphidascaris acus	11	23	–	−0.420*
Echinorhynchus truttae	36	48	38	–
Miño-Sil basin				
Crepidostomum metoecus	–	−0.540*	−0.809*	−0.377
Salmonema ephemeridarum	28	–	−0.788*	−0.323
Raphidascaris acus	40	31	–	−0.369
Echinorhynchus truttae	21	12	22	–

Cm = *Crepidostomum metoecus*; Se = *Salmonema ephemeridarum*; Ra = *Raphidascaris acus*; Et = *Echinorhynchus truttae*
* $P < 0.05$.

C. farionis, respectively, and the same tendency for the infection to increase with the length/age of the fish. Different prevalence rates of *Crepidostomum* were also found in trout in Europe (27.1–90.0%) [33,34], indicating that these trematode species are widely distributed, with *C. metoecus* again being more prevalent than *C. farionis*. However, it is remarkable that Dezfuli et al. [32] did not detect any trematode species in trout captured in rivers in northern Italy.

Salmonema ephemeridarum was the most prevalent nematode species (16.97%; see Table 1). Although this helminth was previously described in the Iberian Peninsula by Álvarez Pellitero [28], with a prevalence of 79.5%, it has scarcely been reported in other parts of Europe. Thus, Hartvigsen and Kennedy [36] detected prevalence rates of between 0% and 24.1% in 245 trout captured in 10 reservoirs in SW England, and Byrne et al. [33] reported prevalence rates of 0–10% for the same host in Lough Feeagh (West Ireland).

Regarding *R. acus*, prevalence and mean intensity values similar to those obtained in the present study (9.46%; 2.59 adults/trout; see Table 1) were reported by Dorucu et al. [37] for trout in Scotland (2.8–10.0%; 1–3 adults/trout) and by Dezfuli et al. [32] for trout in Italy (2.2–4.4%; 1–13 adults/trout). However, in the early study carried out by Álvarez Pellitero [28], high values of prevalence and mean intensity (44.1%; 8.3 adults/trout) were detected and again tended to increase with age and length of the fish. We observed the same trend in the present study, with statistically significant differences between specimens of age < 2 years and the older fish (>3 years; $P = 0.01$). This finding can be explained by the fact that trout may ingest invertebrate hosts (oligochaetes or crustaceans) or other fish harbouring larval stages (paratenic hosts), as suggested by Moravec [38,39].

Finally, *Pseudocapillaria* sp. was the least prevalent nematode (2.12%; see Table 1) which conferring to this species a rare category in the helminth community of Galician brown trout according to the terms defined by Valtonen et al. [40]. Parasites of this genus are considered not frequent in the brown trout [32–34,41,42], although Álvarez Pellitero [28] reported a prevalence rate of 25.9% for *Capillaria coregoni* (syn. *Pseudocapillaria salvelini*). Unfortunately, molecular analysis did not enable identification of the helminths to species level, possibly due to the low quality or degradation of the DNA extracted.

Adult forms of the acanthocephalan *E. truttae* were observed in 8.48% of the fish analysed (see Table 1). This prevalence rate is within the ranges described for the same host in different areas of Central Scotland [37] and in northern Italy [32] (5.5–93.3% and 1.9–47.1%, respectively). The presence of this acanthocephalan species in brown trout implies ingestion of gammarid crustaceans infected with the cystacanth form, which acts as an intermediate host [43]. Thus, different prevalence rates in *E. truttae* suggest that the presence and/or abundance of the infected gammarid may differ among the various river basins as previously suggested by Couso-Pérez et al. [44] and Wendt et al. [45].

The standard length or size of fish is known to be positively associated with the prevalence and/or intensity of parasitic infections, and large fish tend to harbour heavier worm burdens because they have more opportunities to become infected: large fish are older than smaller fish and have thus had more time to accumulate parasites; they also have a higher feeding rate and are able to eat more parasitized prey; and they also provide more internal and external surface area for parasites to occupy [46–50]. Thus, the results obtained in the present study allow us to confirm that the prevalence and intensity of infection tend to increase with the size/age of the trout, with this tendency being statistically significant for *C. metoecus* and *R. acus*. Our results also show that the complexity of the helminth infracommunity increased with the age of the trout, as infracommunity of two helminth species were less frequent in trout >3 years and those comprising three helminth species were statistically significant more common in trout specimens >3 years than in those <2 years ($P < 0.02$; see Fig. 2).

The correlation analyses performed in trout from the four basins where >50 specimens were analysed (Lérez, Tambre, Ulla and Miño-Sil) revealed statistically significant positive correlation between the length of the fish and the species richness only in the Ulla watershed, probably due to the high number of fish host examined in that basin ($n = 220$; see Table 3). Moreover, all correlations between pairs of helminth species from the four watersheds considered showed negative correlations, being 15 of them statistically significant (see Table 4). This finding suggests that some form of antagonistic interaction is occurring between many pairs of helminths in the trout gastrointestinal tract. Consistent negative interactions are strong evidence of competitive interactions between species [32,51,52]. The high number of negative correlations detected in the present work could be indicative of competitive interactions in shaping the helminth communities as previously reported by several authors [32,52,53]. These correlation results can also be seen from the perspective of shared or different intermediate hosts. The

Table 5
Gastrointestinal helminth communities from populations of brown trout (*Salmo trutta*) captured in rivers belonging to four Galician basins (NW Spain).

	Lérez basin	Ulla basin	Tambre basin	Miño-Sil basin
Capture data				
Area (km^2)	449.5	2803.0	1530.0	12,307.0
Number examined fish / % infected fish	52 / 86.54	220 / 85.45	123 / 73.27	178 / 86.52
Mean fish length (cm ± SD)	21.88 ± 3.79	21.72 ± 3.58	20.97 ± 5.00	24.01 ± 5.67
Community data				
Total number of adults	92	554	390	250
Species richness (*S*)	4	5	4	5
Infracommunity data				
Mean number of parasites (range)	4.84 (1–31)	6.52 (1–91)	7.80 (1–45)	5.32 (1–48)
Mean number of species (range)	1.16 (1–2)	1.28 (1–3)	1.36 (1–3)	1.15 (1–3)

SD = standard deviation.

negative relationships appeared to be stronger in pairs of helminths that used different intermediate hosts, but are also found between pairs of helminths that share the same intermediate host [32]. Thus, pair associations of *C. metoecus-R. acus*, *C. metoecus-E. truttae* and *R. acus-E. truttae*, helminths species in which life cycles gammarid species can act as intermediate host [24,39,54,55], were frequently statistically negative correlated in the four watersheds considered. Also, the association constituted by the pair *C. metoecus-S. ephemeridarum* was statistically negative correlated in all basins (see Table 4). In addition, with the exception of the Tambre watershed, in which *E. truttae* was high prevalent (see Couso-Pérez et al. [44]), the co-occurrence by *C. metoecus* and *S. ephemeridarum* was the most frequently detected in the basins considered (see Fig. 3). This may be because both parasites have common intermediate hosts in their biological cycles, i.e. Ephemeroptera insects. Both gammarid crustaceans and ephemeropteran insects are part of the food chain of trout. The diet, including the feeding habitat and the food preferences, plays a major role in the composition of gastrointestinal parasite communities of the fish [56–58]. Fish feed on prey that can act as intermediate or paratenic hosts for helminth parasites with complex life cycles, causing variations in the degree of parasite association [7,59,60].

The gastrointestinal helminth community of brown trout captured in the four river basins considered (Lérez, Tambre, Ulla and Miño-Sil) is characterized by low species richness ($S = 5$) (see Table 5) in comparison with the richness of gastrointestinal helminth species observed in the early study carried out by Álvarez-Pellitero [28] in wild trout in Spain ($S = 8$). Nevertheless, similar species richness has been reported for *Salmo trutta trutta* captured in German rivers ($S = 5$), and higher value was reported for trout captured in the Baltic Sea ($S = 12$), demonstrating that the anadromous life facilitates the acquisition of new parasite taxa [61].

Finally, the biological, physical and chemical conditions of aquatic environments are being modified by pollution associated with anthropogenic activities [62]. Pollution also affects the biodiversity, community structure and the developmental stages of intestinal helminths, which have been suggested to act as bioindicators of water quality by several authors [63–68]. Moreover, the presence of trophically-transmitted parasites with complex life cycles in an ecosystem implies that their respective intermediate hosts must also be present. The findings of the present study show that the gastrointestinal helminth community of *S. trutta* captured in Galician rivers is mainly constituted by helminths with biological cycles that depend on the benthic macroinvertebrate fauna, considered one of the most important bioindicators of the ecological status of the water bodies in the European Union Water Framework Directive (Directive 2000/60/EC) [69,70]. Consequently, and although further studies on other environmental parameters are required, the composition of the gastrointestinal helminth community of this salmonid fish would provide complementary information about the ecological status of the rivers.

In summary, the results of the present study show that the gastrointestinal helminth community of brown trout (*S. trutta*) captured in 44 rivers in Galicia, a region with exceptional hydrological characteristics located in the Northwest Spain, is low richness ($S = 5$) and is mainly constituted by helminths with biological cycles that depend on the benthic macroinvertebrate fauna, which, in turn, is influenced by the water quality. Therefore, any changes that take place in the aquatic ecosystem where the hosts live (due to anthropogenic activities or climate change) may be reflected in the helminth composition.

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Declaration of Competing Interest

None.

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