Digenean trematode infections in the endangered fish species *Apricaphanius iberus*

Master's degree in Terrestrial Ecology and Biodiversity Management

Specialization in Biodiversity Management

Author

Carmen Amelia Ampuero Gonzales

Directors

Maria Constenla Matalobos

Anna Soler Membrives

Academic Tutors

Maria Constenla Matalobos

Anna Soler Membrives

Departament de Biologia Animal, de Biologia Vegetal i d'Ecologia (BABVE),
Facultat de Veterinària (UAB)

September 9th, 2023

Student's contribution to the project: (A) entirely by the student; (B) partially by the student; (C) entirely by others.

This work has been conducted from February 2022 to August 2022.

Experimental design: B

- The main study design was developed by SEAaq investigation team. The student contributed to designing protocols for assessing *Hydrobia* snails and cercariae emergence.

Field sampling: B

- SEAaq investigation team conducted samplings in 2021. The student participated in 2022 samplings of *Apricaphanius iberus* and *Hydrobia* snails.

Laboratory analysis: B

- SEAaq investigation team conducted partial laboratory assessments of 2021 samplings. The student participated in laboratory analysis for both 2021 and 2022 samplings.

Data processing: B

- SEAaq investigation team partially handled data processing for 2021 samplings. The student processed data from both 2021 and 2022 samplings.

Statistical analysis: A

- The student was involved in statistical analysis under supervision, with additional environmental statistical information provided by the Director for the "Materials and Methods" section.

Writing: A

- The student has authored project with the guidance and support of the tutors.

The journal format aligns with the guidelines set forth by **Aquatic Conservation: Marine and Freshwater Ecosystems**, a journal specialized in publishing original research on preserving aquatic environments, encompassing freshwater, brackish water and marine ecosystems. It provides a platform for the presentation and discussion of aspects related to conservation of aquatic resources.

Barcelona, 9th September 2023

Dr. María Constenla Matalobos	**Dr. Anna Soler Membrives**

ABSTRACT

The Spanish toothcarp, *Apricaphanius iberus*, is an endemic and endangered fish species found in coastal ecosystems on the Iberian Peninsula, which have encountered challenges including environmental fluctuations and parasitism by digenean trematodes. The study focuses on *La Pletera*, a protected area with diverse conditions, where metacercariae infestations were found in the fish's gills, indicating potential respiratory and osmoregulatory impacts. The research aims to assess parasite descriptors in fish, considering factors like sex and length of the host, identify the digenean species, to detect larval stages in potential intermediate host (*Hydrobia* specimens), estimate cercariae emerging from the snails, and explore connections between lagoon conditions and infection rates.

A. iberus specimens, cohabiting *Hydrobia* snails and *Gambusia holbrooki* were collected from three lagoons with different microecosystems. Analysis identified a total of 21,256 digenean metacercariae parasitizing *A. iberus* gills, resulting in only one non-parasitized individual. A high abundance of metacercariae was found encysted on one side gills in each individual, with differing results among lagoons. *G. holbrooki* exhibited no metacercariae, highlighting host specificity. Cercariae emergence confirmed *Hydrobia* snails as first intermediate host of this digenean. Histological studies detected metacercariae in various fish tissues including pharynx, intestines, jaw, spinal cord, and fins, among others, but no larval stages were found in snails.

Key words: *A. iberus*, cercariae, environmental conditions, *Hydrobia* snails, intermediate host, metacercariae abundance.

1. INTRODUCTION

Apricaphanius iberus, aka Spanish toothcarp, is a small fish from the family Cyprinodontidae, endemic of brackish and hypersaline coastal water lagoons and salt marshes in the Iberian Peninsula (Alcaraz & García-Berthou, 2007). Its distribution range is restricted to three biogeographical areas along the eastern Mediterranean coastline: Catalonian, Levantine and Murcian regions (*sensu* Doadrio et al., 1996; Oliva-Paterna et al. 2006). Its habitat distribution is considered among the most fluctuating ecosystems in the world since they depend on the amount of freshwater input, climate and human activities that affect their nutrient dynamics and water regime (Gedan et al., 2011; Beer & Joyce, 2013; Casamitjana et al., 2019).

Coastal lagoons are categorized as opened or closed depending on their connection to the sea, and each of them is characterized by microecosystems (Casamitjana et al., 2019). Although *A. iberus* specimens have adapted to the fluctuating conditions of their habitat, a decline on its populations has been recorded, mainly due to the introduction of the invasive *Gambusia holbrooki* (aka mosquitofish), water pollution and wetland fragmentation (Oliva-Paterna et al., 2006), leading the species to be listed as endangered by the IUCN (IUCN, 2006).

In addition, parasitism of digenean trematodes has also been a persistent threat in *Aphaniidae* specimens of the Mediterranean coastline. Digenean metacercariae have been found encysted in the head, visceral regions and other tissues of an *Aphanius* species (Kalantan et al., 1987). Specifically, metacercariae of the family Heterophyidae Leiper, 1909, have been detected parasitizing the serosa of the visceral organs (peritoneum) of *Aphanius fasciatus* (Valenciennes) from the northern Mediterranean coast of Italy, resulting in high parasite prevalence and intensity (Gustinelli et al., 2022).

Heterophyid digenean have an indirect life cycle that usually use prosobranchiate snails as first intermediate host and fish as second one, while fish-eating birds or mammals serve as definitive hosts (Cribb et al., 2003). Heterophyid encysted metacercariae have been found to cause detrimental effects, as hyperplasia and cell proliferations in tissue sections, especially in gills but also in muscle and other organs, following different patterns among fish tissues (Abdallah et al., 2009; Santos et al., 2013; Attia et al., 2021). These parasites are often organ-specific in the intermediate host (Paperna & Dzikowski, 2006) and many species of this family have been

reported to encyst in gill tissues, and even causing proliferation of gill cartilage (Blazer & Gratzek, 1985; Olson & Pierce, 1997).

In a specific study based on the project *Aphanius* (PID2019-103936GB-C21/C22), several specimens captured from the wild (*La Pletera*, *Costa Brava*, Catalonia) were tested for metabolic performance at different salinities and, surprisingly, unexpected mortalities occurred during the experiments. An extremely large number of metacercaria were detected in the gills of these specimens, suggesting a relevant impairment of respiratory and osmoregulatory functions and allostatic capacity in these fish. The structure and morphology of the metacercaria highly suggested an Heterophyidae as the most likely family of digenean trematodes.

The study site, *La Pletera*, is a protected area composed of wetlands and some closed coastal lagoons that receive water input from the sea during sudden intense flooding events, but also go through long periods of confinement (Casamitjana et al., 2019), with higher salinity and lower water levels (Quintana et al. 1998, 2018). Furthermore, these lagoons are the habitat of the invasive *Gambusia holbrooki.* Mud snails of the genus *Hydrobia* are also very common in brackish water environments of Mediterranean coastal regions (Giusti et al., 1998), as in *La Pletera*, and can be good candidates to be studied as potential intermediate hosts for digeneans after these are released through feces of the definitive host into aquatic environments (Bordalo et al., 2011). Therefore, *La Pletera* gathers suitable variables to study digenean infections in *A. iberus* specimens.

Taking into account that these infections are a threaten and a new challenge for the management and conservation of this endangered species, the main aim of this work is to characterize the digenean trematode populations in *A. iberus* at three nearby coastal lagoons with different environmental conditions in *La Pletera*. Specifically, 1) to assess parasite descriptors of this digenea in fish (second intermediate host) and inferred depending on the sex and length of the host, 2) to detect larval stages in the potential first intermediate host (*Hydrobia* specimens) and estimate the cercariae emerging from the snails, and 3) to infer the probable relation between environmental conditions of each lagoon with different degrees of infection.

2. MATERIALS AND METHODS

2.1 Ecological characterization of three lagoon systems

Three samplings of *A. iberus* were carried out in *La Pletera* (*L'Estartit*, *Costa Brava*, Girona, Catalonia, NW Mediterranean) in three different lagoon systems (M01, L04, M03) (Figure 1). Physiochemical parameters, such as electrical conductivity, dissolved oxygen and temperature were measured from lagoons L04 and M03 *in situ*, during monthly campaigns conducted from February 2016 to August 2019 (Menció et al., 2023), and from May 2022 to April 2023 (Quintana X., personal communication, 2023) (Figure 2). No M01 was reported to have intermediate conditions of conductivity and dissolved oxygen (Quintana X., personal communication, 2023).

For purposes of this study, conductivity is used to estimate salinity dynamics in the mentioned lagoons. This is feasible as salinity and conductivity are directly proportional (Rusydi, 2018).

FIGURE 1 Map of sampling area *Apricaphanius iberus*. *La Pletera* (*L'Estartit*, *Costa Brava*, Girona, Catalonia, NW Mediterranean).

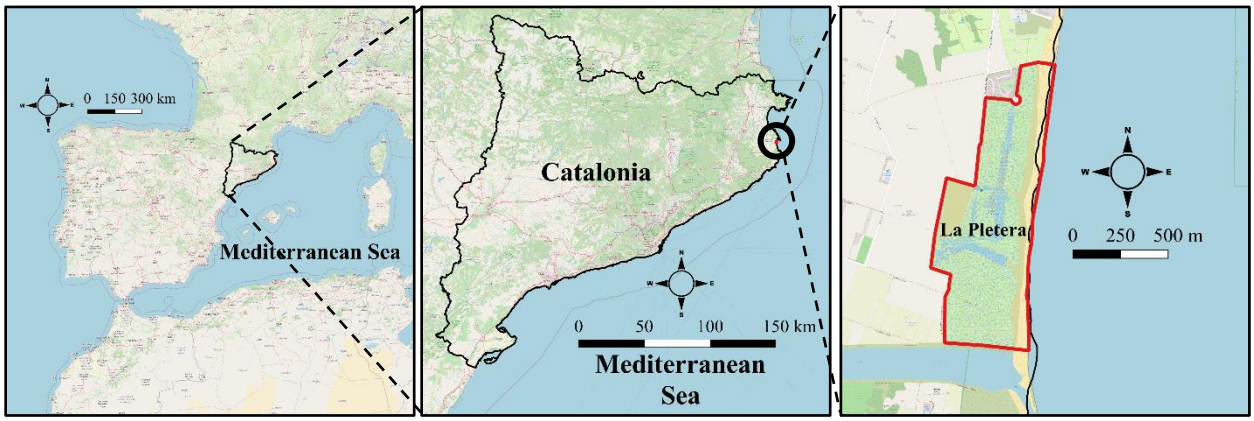
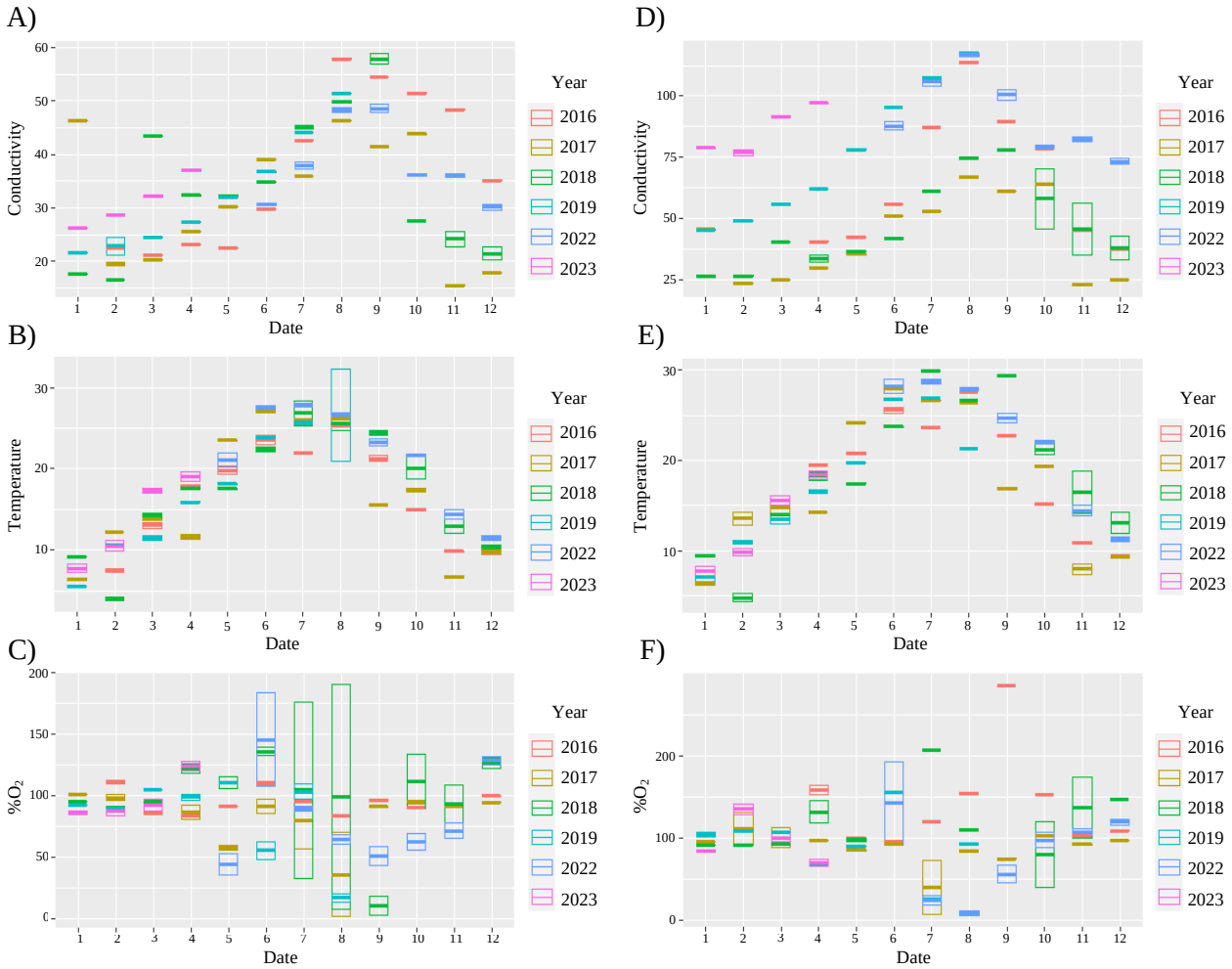


FIGURE 2 Environmental conditions recorded from lagoon L04 (A, B and C) and M03 (D, E and F) in *La Pletera* (Catalonia, NW Mediterranean) from 2016 to 2019 and 2022 and 2023. A and D) Conductivity (mS); B and E) Temperature (Celsius); C and F) Dissolved oxygen (%O_2). Numbers from 1 to 12 represent months from January to December, respectively.



2.2 *Apricaphanius iberus* collection

Samplings of *A. iberus* were carried out on May 3rd, 2022, and March 21st and June 20th, 2023. Fish net traps were set up the day before to the collection in the three selected lagoons coded as M01, L04 and M03. Spanish toothcarps were sexed and counted. When possible, twenty *A. iberus* were collected and kept alive from the three lagoons, i.e., ten males and ten females. The remaining ones were immediately returned to their lagoons. A total of 94 fish specimens (49 females and 45 males) were collected throughout the three samplings.

Specimens from the invasive *G. holbrooki* were counted and euthanized *in situ* by using an overdose of eugenol, except in the March 21st and June 20th samplings, where ten mosquitofish specimens were also taken alive for further analysis.

Fishes were carefully transported in tanks to ensure survival. Once in the lab, fishes were kept in aquariums until euthanized with overdose of phenoxyethanol. Sex, standard length (SL), total length (TL), and total weight (TW) data of each individual were recorded.

2.3 Metacercaria quantification and identification

Fish were fixed individually in 10% buffered formalin. After 48 hours, gills from one side of each individual were taken out and observed under the stereomicroscope to look for metacercariae. Metacercariae abundance was quantified and recorded from the four gill arches and filaments of each individual. The rest of the specimen was kept in the same container with 10% buffered formalin for further histological analysis.

Half of each individual of *A. iberus* (n = 10 of each lagoon and sampling, when possible) kept in 10% buffered formalin were processed by routine histological techniques and sections were stained with hematoxylin and eosin. Metacercariae quantification per organ section was recorded.

Gills from one side of ten individuals were analyzed in fresh. Measurements of five metacercariae were taken from images at 40× magnification by a Leica camera model CTR5000 attached to a Leica DM500B microscope and an image-processing software (ProgRes® C3). Measurements are presented as the range expressed in micrometers (µm). After metacercariae quantification, they were preserved in 100% ethanol for further molecular studies.

Parasites were morphologically identified at a potential family level following the descriptions given by Scholz et al. (2001), Elsheikha and Elshazly (2008), Pearson (2008), Masala et al. (2016) and Gustinelli et al. (2022).

2.4 *Hydrobia* collection and cercariae release

On March 21st and June 20th, 2023, *Hydrobia* specimens along with two liters of water were collected from lagoons M01, L04 and M03. A total of 300 snails were kept alive. Three replicates of 20 snails per lagoon were kept in 20 mL containers with its respective water lagoon,

which was previously observed under the stereomicroscope to prevent the presence of cercariae. For M01, three additional replicates of 40 individuals were also included.

Three heat lamps were placed surrounding the twelve containers to warm them up and stimulate the release of cercariae. Water temperature was recorded using a thermometer inside an additional container with a random lagoon water. After 24, 48 and 168 hours, water from each container was poured into medium glass plates and observed under the stereomicroscope for detection and counting of cercariae. Measurements of three small cercariae were taken from captured images. Additionally, six larger cercariae were preserved in 70% ethanol before measurements were obtained. To account for potential size reduction, the reduction ratio of the small cercariae was calculated from fresh and ethanol-preserved ones at different sections (length and width of head and tail), and then applied proportionally to the larger specimens to obtain their estimated measures.

Ten *Hydrobia* specimens from each replicate were also fixed in 10% buffered formalin for the detection of larval stages by histological techniques. Specimens were processed without the shell by routine histological techniques to further identification of digenean larval stages. Sections were stained using Ziehl-Neelsen, and hematoxylin and eosin.

2.5 Data analysis

All variables were tested for normality and homoscedasticity using the Shapiro-Wilk and the Levene's test, respectively. Data distribution was also plotted for visual assessment. Statistical analyses were performed in R Studio software (version: 4.1.3), and significant level was set at $p < 0.05$. Parasite prevalence (P%) and mean abundance (MA) were calculated following previous studies (Bush et al., 1997).

Fulton's body condition factor K was calculated as

$$K = \frac{10{,}000 \times TW}{SL^3},$$

where TW is total weight (g) and SL is standard length (mm).

Standard length (SL), Fulton's body condition (K) and total weight (TW) were tested for differences among sex, lagoons and seasons using Kruskal-Wallis chi-squared test. When necessary for further analysis, SL was used as a covariate.

Correlations between SL, K, TW, and total abundance of metacercariae in fish were tested in order to detect any possible relationships among them. This process was carried out using non-parametric Spearman's correlation tests. Differences in metacercariae abundance and sexes, lagoons and seasons were tested using Generalized Lineal Models (GZMs, Poisson distribution). Differences in metacercariae abundance, and gills, gill arches and filaments were tested using Mixed Effects Models (using individuals as random factors).

Cercariae release data was calculated using emergence ratios per snail. Mean abundance and standard deviation were also determined using these ratios.

3. RESULTS

Total length (TL) of assessed *A. iberus* ranged from 24 – 40 mm (31.53 (SD = 4.35)). Biometrical data and body condition index of fish are shown in Table 1. All three variables (SL, TW and K) were significantly higher in females (SL: KW = 40.0, p-value < 0.05; TW: KW = 40.38, p-value < 0.05; K: KW = 4.46, p-value < 0.05).

TABLE 1 Biometrical data of sampled *Apricaphanius iberus* in *La Pletera* (Catalonia, NW Mediterranean). Mean and standard deviation (SD) of standard length (SL, mm), total weight (TW, g) and Fulton's body condition (K). Differences between males and females are represented by superscript letters (a and b) and their absence means no significant differences.

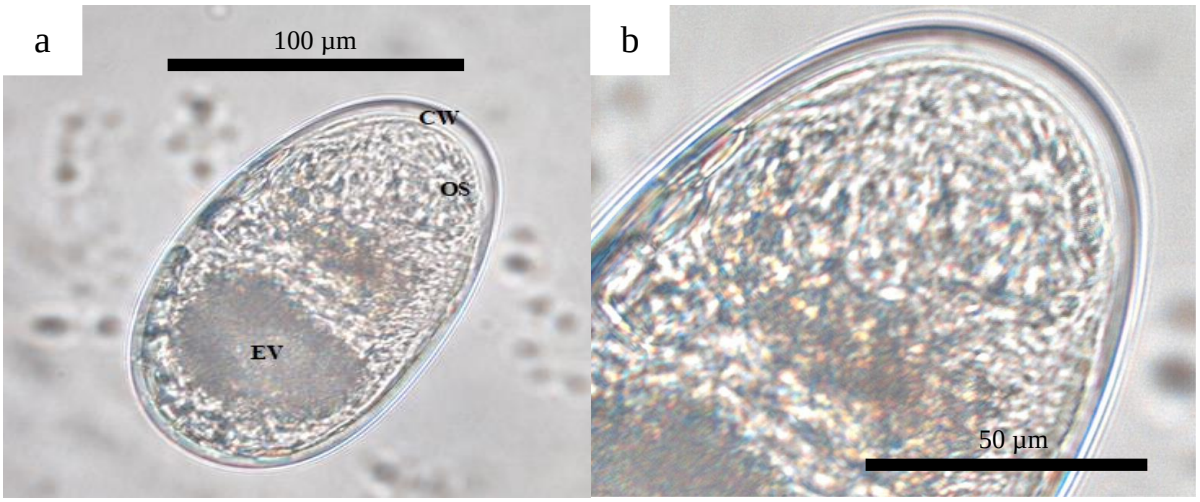
Lagoon / **Sex**	**N° of fish examined**	**SL (SD)**	**TW (SD)**	**K (SD)**
M01	36	25.36 (4.43)	0.52 (0.29)	2.95 (0.41)
Female	20	27.55 (4.61)	0.67 (0.30)	3.02 (0.44)
Male	16	22.63 (2.16)	0.34 (0.11)	2.86 (0.37)
L04	9	26.78 (4.35)	0.67 (0.32)	3.32 (1.08)
Female	4	30.50 (3.11)	0.85 (0.24)	2.96 (0.54)
Male	5	23.80 (2.39)	0.53 (0.33)	3.60 (1.37)
M03	49	25.91 (3.39)	0.55 (0.24)	2.99 (0.52)
Female	25	28.30 (2.35)	0.72 (0.21)	3.14 (0.57)
Male	24	23.42 (2.36)	0.37 (0.10)	2.83 (0.40)
Total	94	25.78 (3.89)	0.55 (0.27)	3.00 (0.56)
Female	49	28.17 (3.52)[a]	0.71 (0.25)[a]	3.08 (0.51)[a]
Male	45	23.18 (2.28)[b]	0.37 (0.15)[b]	2.92 (0.60)[b]

Individuals from lagoon L04 exhibited a marginal greater SL compared to the other lagoons, however, it did not result in a statistical significance, likely attributed to the low number of specimens assessed. A similar pattern appeared during the assessment of females, with those in spring exhibiting higher SL and TW compared to those in summer and winter, presumably due to the same constraint of sample size. Consequently, no statistically significant differences were discerned in SL, TW, or K among the three sampled lagoons and across seasons, whether considering all sampled specimens or examining males and females separately.

3.1 Digenean metacercariae infection in fish

A total of 21,256 digenean metacercariae were found parasitizing the gills of 94 *A. iberus*, resulting in only one non-parasitized individual (Table 2). Encysted metacercariae were ovoidal in shape, composed by a thin and clear cyst wall (3.50 (SD = 0.43) µm in the narrower region; 4.72 (SD = 0.17) µm in the wider one), and a transparent inner layer. Cysts measurements resulted in 155.52 (SD = 4.10) µm long and 94.25 (SD = 2.28) µm wide. The oral sucker (35.96 (SD = 3.32) µm wide; 41.78 (SD = 2.99) µm long), which bears circumoral spines, and the excretory vesicle full of dark granules could be identified (Figure 3).

FIGURE 3 Encysted digenean metacercariae found in *Apricaphanius iberus* in *La Pletera* (Catalonia, NW Mediterranean). a) entire cyst where different structures can be seen: CW, cyst wall; OS, oral sucker; EV, excretory vesicle; and b) detail of the oral sucker

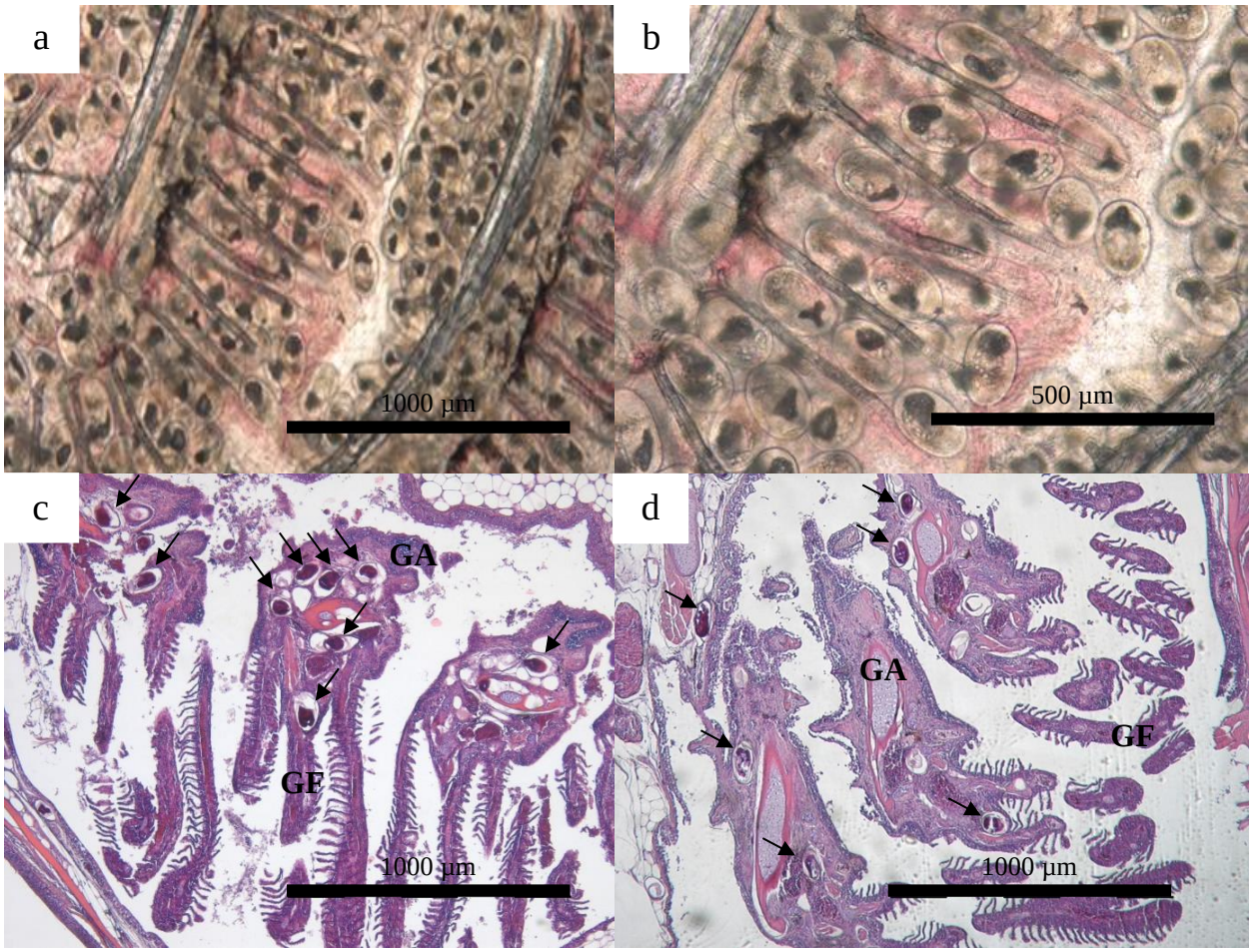


High abundance of metacercariae were found encysted on one side of the gills in each individual (Figure 4), ranging from 0 up to 2036. No significant correlation between SL, TW and K, and metacercariae abundance was identified, nor with sex taking into account SL as covariate.

TABLE 2 Prevalence and mean abundance of metacercariae in *Apricaphanius iberus* from in *La Pletera* (Catalonia, NW Mediterranean) for the three sampled lagoons. P%, prevalence; MA, mean abundance; SE, standard error.

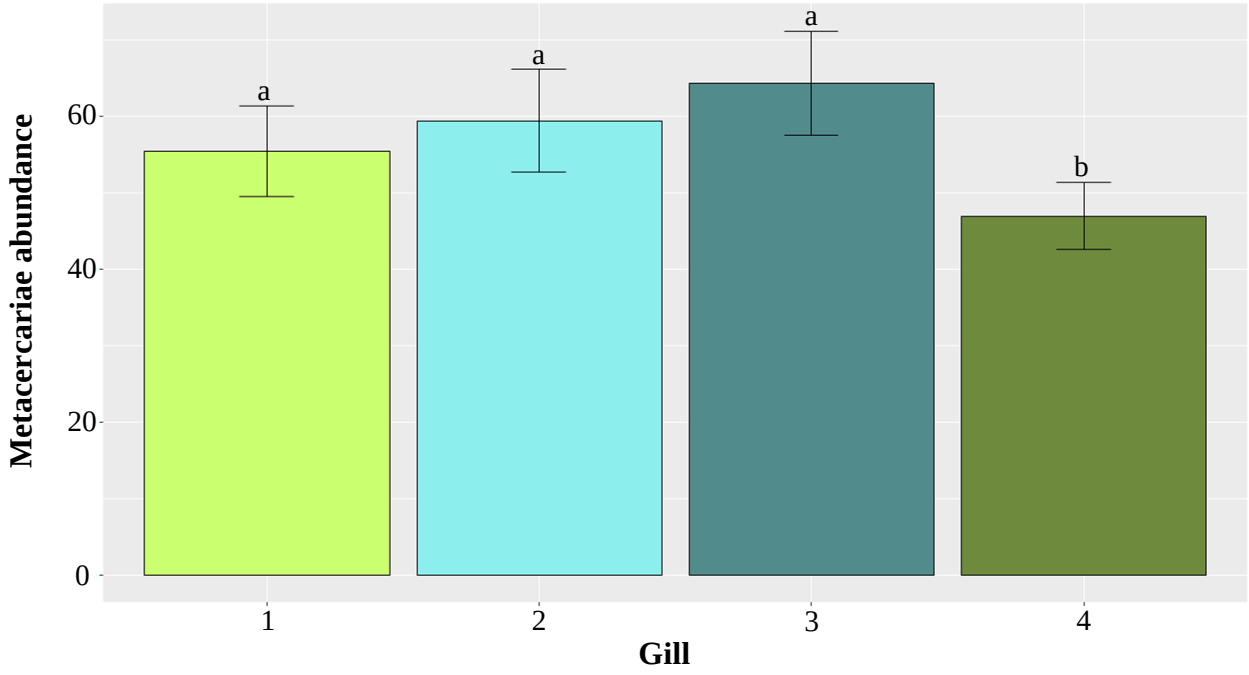
Lagoon / **Season**	**P%**	**MA ± SE**
M01		
Spring 2022	100%	53.67 ± 11.59
Winter 2023	100%	308.37 ± 115.93
Summer 2023	100%	113.50 ± 43.50
L04		
Spring 2022	100%	723.00 ± 107.22
Winter 2023	-	-
Summer 2023	-	-
M03		
Spring 2022	100%	92.14 ± 20.63
Winter 2023	100%	291.40 ± 33.75
Summer 2023	95%	109.85 ± 23.98
Total	98.94%	226.13 ± 32.79

FIGURE 4 Encysted metacercariae found in *A. iberus* gill arches in *La Pletera* (Catalonia, NW Mediterranean). Fresh mounts (a and b) and histological sections (c and d) of gill arches (GA) and filaments (GF) with high numbers of encysted metacercariae. Arrows (in c and d) indicate encysted metacercariae.



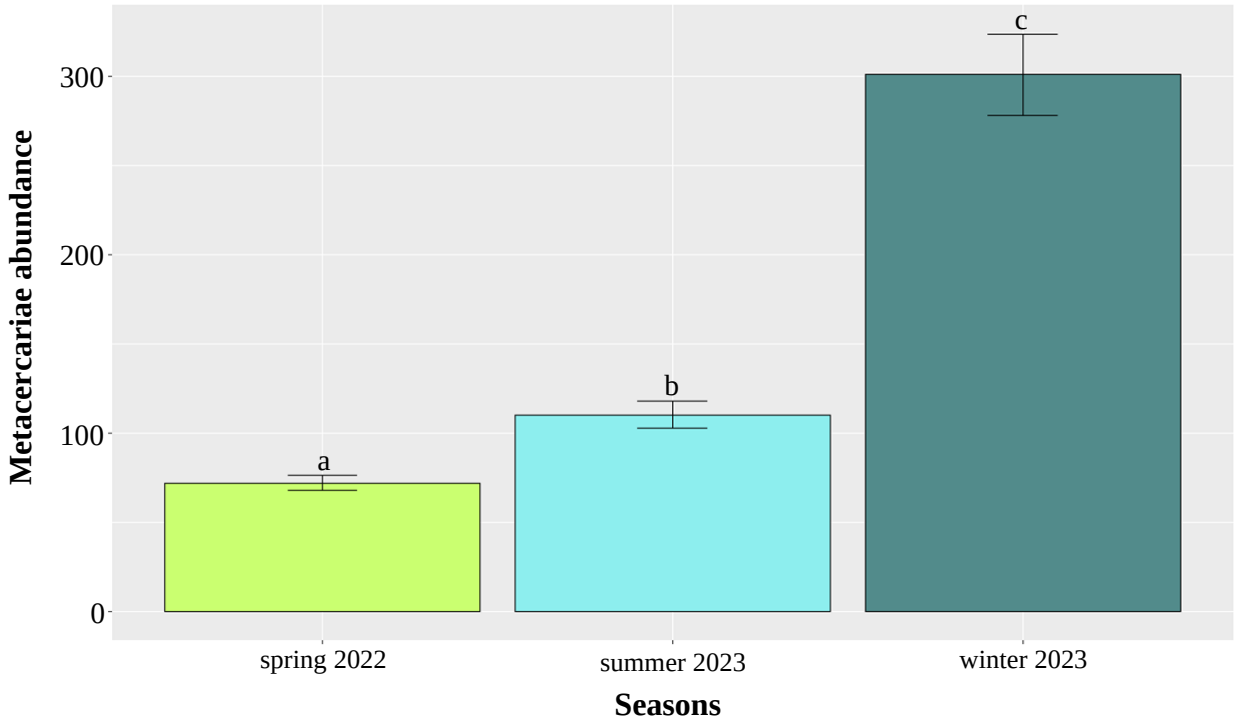
Fourth gill has less significant metacercariae abundance compared to the other gills (G1-G4: *t* -ratio= 2.26, p-value < 0.05; G2-G4: *t*-ratio= 2.41, p-value < 0.05; G3-G4: *t*-ratio= 4.03, p-value < 0.05), being the third gill the one with the highest abundance (Figure 5). No differences were found in the distribution of metacercariae between gill arches and filaments.

FIGURE 5 Mean abundance of metacercariae in the *A. iberus* gills in *La Pletera* (Catalonia, NW Mediterranean). Letters (a and b) means significant differences among the four gills.



Regarding differences among lagoons, the single sampling in L04 presented the highest MA (723.00 ± 107.22), compared to the other two lagoons (M01-L04: t-ratio = -5.17, p-value < 0.01; M03-L04: t-ratio = 5.63, p-value < 0.01). Taking into account seasonal samplings (excluding L04), significant differences of metacercariae MA were found, being winter the season with the highest values, and spring, the lowest ones in both lagoons, M01 and M03 (spring-summer: z-ratio = -40.02, p-value < 0.01; spring-winter: z-ratio = -168.27, p-value < 0.01; summer-winter: z-ratio = -125.79, p-value < 0.01) (Figure 6).

FIGURE 6 Metacercariae abundance per season (excluding lagoon L04) found in *A. iberus* gill arches in *La Pletera* (Catalonia, NW Mediterranean). Letters (a, b and c) means significant differences among seasons.



Although most metacercariae were detected encysted in gill arches and filaments (Figure 4), by histological analysis they were also found encysted in digestive tract (especially in in pharynx but also in intestines), near pseudobranch, liver, spleen, kidney, pancreas, gonads, spinal cord, and in muscular, adipose and connective tissues (Figure 7). Externally, metacercariae were observed in upper and lower jaw, nasal region, operculum, and dorsal, ventral and caudal fins (Figure 8).

FIGURE 7 Histological sections showing digenean metacercariae (indicated by black arrows) in different tissues and internal organs of *Apricaphanius iberus* from *La Pletera* (Catalonia, NW Mediterranean). a) pharynx; b) intestine; c) adipose tissue surrounding the pseudobranch; d) gonads. Arrows indicate encysted metacercariae.

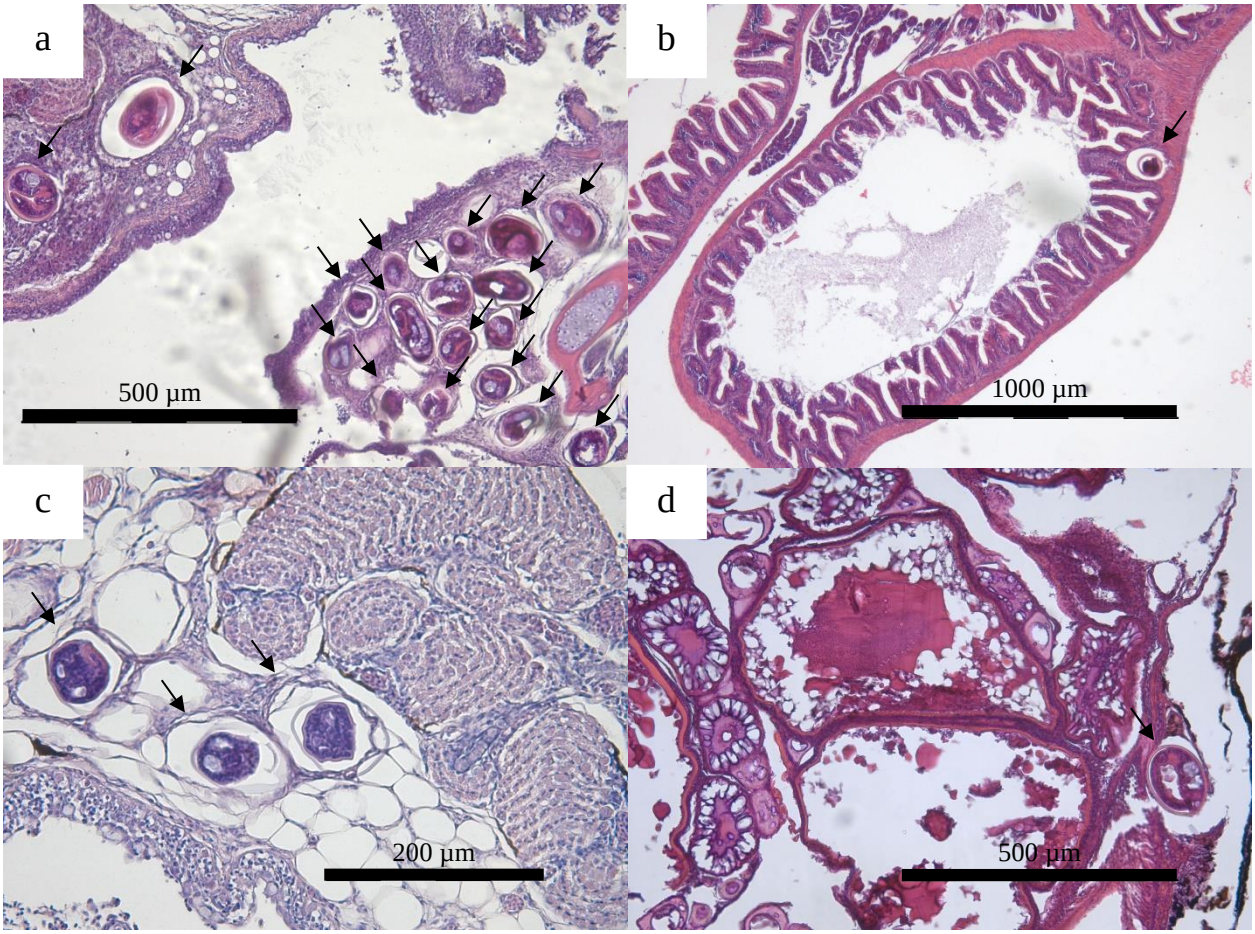
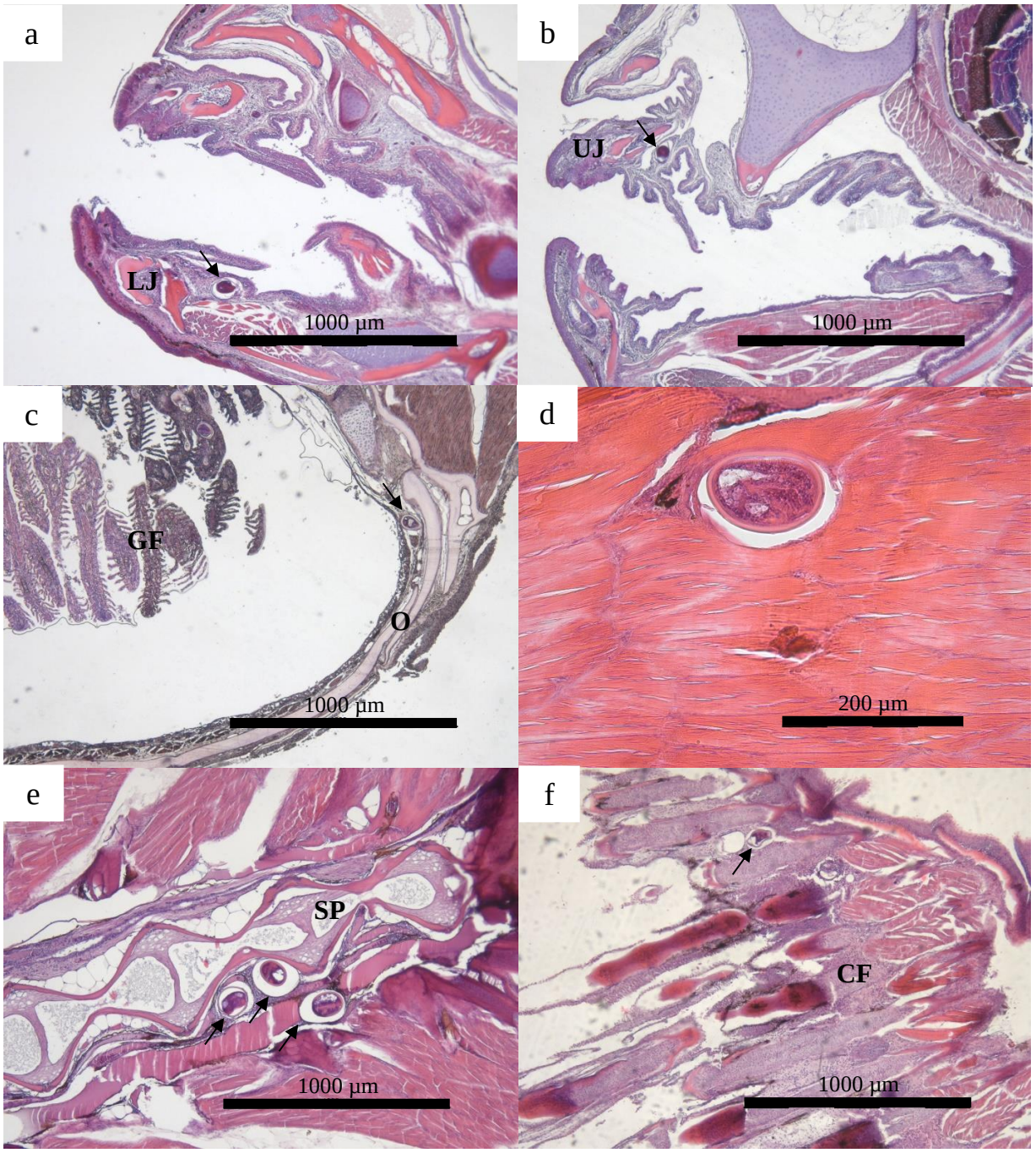


FIGURE 8 Histological sections showing external digenean metacercariae in: a and b) jaws (a: LJ, lower jaw and b: UJ, upper jaw); c) operculum (O); d) muscle tissue; e) spinal cord (SC); and f) fins (CF, caudal fin); of *Apricaphanius iberus* from *La Pletera* (Catalonia, NW Mediterranean). Arrows indicate encysted metacercariae.



No metacercariae were found in mosquitofish gills.

3.2 Digenea cercariae in snails

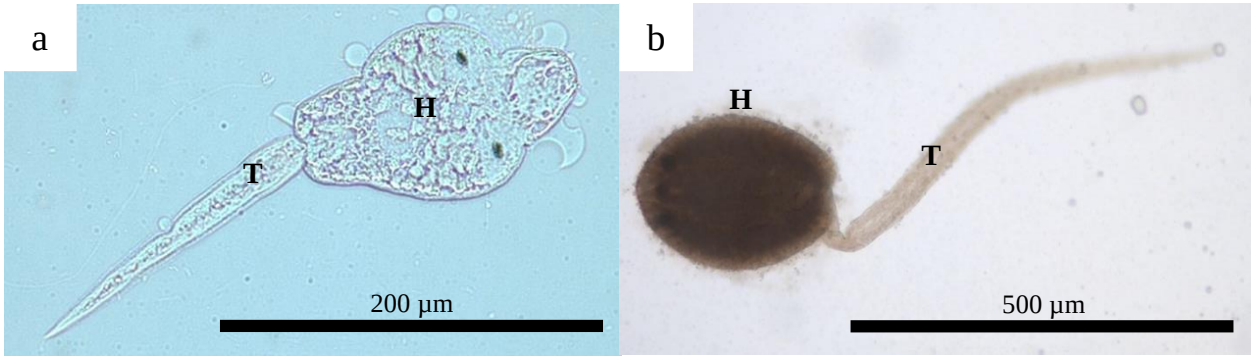
A total of 60 digenean cercariae could be observed emerging from *Hydrobia* specimens from different lagoons and seasons. Among those parasites, cercariae of two sizes were identified (small: mean emerging ratio = 0.29 (SD = 0.27); big: mean emerging ratio = 0.15 (SD = 0.28)). Table 3 displays emergence ratio values of both cercariae sizes. They emerged from snails of L04 lagoon during winter and summer sampling assessments, whereas they only emerge from lagoons M01 and M03 during winter assessment. All captured snails during summer sampling from lagoon M03 were dead.

TABLE 3 Number of cercariae released per *Hydrobia* snail from the different lagoons from *La Pletera* (Catalonia, NW Mediterranean). Not displayed times (L04: 168 hours; M03: 24 y 168 hours) had zero cercariae emergence. Dash (-) means no alive snails.

	Lagoons	**M01**				**L04**			**M03**	
	Hours	24	48	168	**Total**	24	48	**Total**	48	**Total**
Season	**Size**									
Winter	Small	0	0.2	0	0.2	0.45	0.15	0.6	0.1	0.1
	Big	0.03	0.6	0.03	0.65	0	0.05	0.05	0.05	0.05
Summer	Small	0	0	0	0	0.55	0	0.55	-	-
	Big	0	0	0	0	0	0	0	-	-
Total	Small	0	0.2	0	0.2	1.0	0.15	1.15	0.1	0.1
	Big	0.03	0.6	0.03	0.65	0	0.05	0.05	0.05	0.05
	Total	0.03	0.8	0.03	0.85	1.0	0.2	1.2	0.15	0.15

Cercariae of two sizes were observed only during winter sampling analysis as in summer only small cercariae could be observed. Small cercariae were composed by an oval head measuring 101.22 (SD = 52.00) µm x 158.29 (SD = 38.24) µm, and with a pair of visible eyespots. A single stem tail, transversely divided in two regions, was attached to the head. Tail had a total length of 175.55 (SD = 1.83) µm and a width of 19.81 (SD = 2.07) µm in the widest section (Figure 9a). Larger cercariae possessed the same morphological features (Figure 9b). Estimation measures were head 326.82 (SD = 58.74) µm x 383.40 (SD = 38.72) µm; estimated tail measure was 830.60 (SD = 65.55) µm and 47.52 (SD = 8.94) µm width in the widest section. Both cercariae types were very active and swam by rapidly and repetitively beating their tails.

FIGURE 9 Fresh mount of digenea cercariae released from *Hydrobia* snails in *La Pletera* (Catalonia, NW Mediterranean). a) Small size cercariae; b) big size cercariae (image captured after storage in 70% ethanol). H, head; T, tail.



No sporocyst nor redia could be identified through histological studies of snails from any of the lagoons.

4. DISCUSSION

This research provides relevant information of important parasitic infections in *A. iberus* from Catalan lagoons. The study reveals a high prevalence (98.94%) and abundance (226.13 ± 32.79) of parasites located in gills of one side (approximately 450 metacercariae per fish). As mentioned before, this might indicate a significant negative impact on the respiratory and osmoregulatory functions, as well as allostatic capacity of these fish species, potentially enhancing the challenges faced by this endangered fish species. In addition, the fact that no metacercariae have been found in specimens of *G. holbrooki* strongly suggests the high specificity of the parasite in his second intermediate host, *A. iberus*. Such types of infections have been investigated in distinct species within the *Aphanius* genus across various geographical regions.

In a seasonal incidence study of metacercariae in *Aphanius dispar* specimens from Saudi Arabia, Kalantan et al. (1987) found 86.6% parasite prevalence, with up to 41 metacercariae per fish. Although such results were not classified as “high” by the authors, it was found that metacercariae cysts caused degeneration of tissues and blood vessels congestion (Kalantan et al., 1987). More recently, Gustinelli et al. (2022) found an 83% digenean prevalence in *Aphanius fasciatus* specimens from coastal lagoons in northeastern Italy, whose metacercariae mean abundance was up to 83, being these outcomes qualified as extraordinarily high for this species.

Furthermore, metacercariae has also been documented in species of other fish families. Masala et al. (2016) reported a total of 17,899 Heterophyidae metacercariae encysted in muscle tissue of 57 examined *Mugil cephalus* fish with a total prevalence of 95%, which yielded a mean abundance of around 314 metacercariae per fish.

Metacercariae abundance in *A. iberus* is notably higher, with values approximately ten times greater than those reported by Kalantan et al. (1987) and approximately five times higher than those observed in the study conducted by Gustinelli et al. (2022). Moreover, Masala et al. (2016) research yielded a mean abundance result that aligns more closely with the findings of the present study. However, it is essential to consider that *M. cephalus* exceeds *A. iberus* in both size and weight. Therefore, it can be inferred that observed parasite load in the gills of this smaller species could become exceptionally burdensome and detrimental to them.

Although most metacercariae infections are classified as benign, if the infection is substantial, it can severely affect or even kill the host, mainly the ones living in small bodies of water (Cribb, 2005). Thus, the parasite load identified in this research may result in a reduction of respiratory surface area and cause detrimental effects on fish at times of stress or when dissolved oxygen is low (Blazer & Gratzek, 1985). This scenario appears to be exemplified by lagoon L04, where exceptionally metacercariae abundance is recorded during the spring 2021 sampling, followed by the inability to capture any Spanish toothcarp specimen in subsequent samplings. The likely reduction or disappearance of its population in this lagoon could be attributed directly to the negative effects of the significant parasite loads encountered and/or to the vulnerabilities induced by these infections that hinder its population's ability to adapt to environmental changes.

Furthermore, morphological characteristics noted in this study closely resemble those documented for the Heterophyidae family in previous descriptions (Scholz et al., 2001; Elsheikha and Elshazly, 2008; Pearson, 2008; Masala et al., 2016; Gustinelli et al., 2022). The oral sucker bearing circumoral spines, the size of the oval head and the main structures such as the excretory vesicle, which is full of dark granules, and the outer cyst wall are general metacercariae characteristics that give the first clues to suggest categorization into said family. The majority of Heterophyidae parasites exhibit a site-specificity, often encysting muscle tissue, fins, adipose tissue around the internal organs, and gills (Waikagul & Thaenkham, 2014). In this study, the high

numbers of metacercariae in gills point to this organ as the main target organ in *A. iberus*, with equal affinity for gill arches and filaments, but less in the fourth gill.

Considering the observation and measurements of cercariae in the present work, their morphology is also taken into account to identify their potential taxonomic categorization. In this case, the two types of cercariae exhibit resemblances to characteristics observed in members of Heterophyidae family, such as the oval head with a pair of visible eyespots and, specially, the single stem tail (Waikagul & Thaenkham, 2014). However, cercariae belonging to closely related species share similarities in their physical structure, making it challenging to differentiate them solely based on their morphology (Waikagul & Thaenkham, 2014).

Although no larval stages could be identified in *Hydrobia* snails through histological analyses, the emergence of cercariae from these snails confirm this mollusk as the first intermediate host of the parasite. These mud snails inhabit brackish water environments of all Mediterranean coastal lagoons (Giusti et al., 1998), where they can live and reproduce in a wide range of temperatures and salinities (Barnes, 2005). Nevertheless, according to Pascual and Drake (2008), exposure to water with extremely high salinity and low oxygen levels, regardless of water temperature, could indicate negative effects on the normal activity (speed of movement, browsing rate, etc.) of some *Hydrobia* species. Thus, this could be related to mortality of mud snails from lagoon M03 during spring sampling since this lagoon is characterized by high salinity levels and fluctuating oxygen levels that may have exceeded the environmental thresholds of the unknown *Hydrobia* species.

Environmental factors of the assessed lagoons play an important role in metacercariae abundance. Specifically, lagoon L04, which exhibits the lowest salinity levels, is the lagoon where snails have been most readily encountered during samplings, and it is also the location where the highest metacercariae abundance has been observed. This finding is in accordance with results described by Koprivnikar and Poulin (2009a), in which the trematodes *Maritrema novaezealandesis* (Microphallidae) and *Philophthalmus* sp. (Philophthalmidae) exhibited increased cercarial emergence at the lowest salinity tested (30 PSU), thus maximizing the chance of successful transmission to the next host.

On the contrary, in lagoon M03, characterized by higher salinity levels, it has been more challenging to locate snails in its shores and the parasite abundance has been found to be lower. Thus, it can be assumed that the digenean abundance depends on the first intermediate host and its responsiveness to environmental conditions. Limited emergence of cercariae might be attributed to host stress caused by altered salinity conditions (Berger & Kharazova, 1997), as numerous mollusks close their operculum in response to changes until they acclimatize (Cheung & Lam, 1995).

Although, those results differ from previous studies in which cercariae emergence increased with higher salinity (Sindermann, 1960; Sindermann & Farrin, 1962; Mouritsen, 2002; Studer & Poulin, 2012), the overall relationship between cercariae emergence and salinity of lagoons is likely context-dependent and influenced by various ecological factors. Contradictory findings may be attributed to variations in experimental conditions versus natural real conditions, host-parasite interactions and the specific trematode species being studied.

Seasonal variations are also relevant because it involves changes in temperatures, a well-studied factor that influences parasite infections. Several research studies have suggested that elevated temperatures establish suitable conditions for parasite transmission (Lyholt & Buchmann, 1996; Mouritsen, 2002; Studer et al., 2010; Koprivnikar et al., 2014). However, findings of this study present a contrasting proposition: the highest metacercariae abundance is observed during winter season. This outcome aligns with the research conducted by Koprivnikar and Poulin (2009b), which documented a higher cercariae emergence rate at lower temperatures (15°C) rather than higher temperatures (25°C), suggesting potential interspecific variations in trematode responses to temperature fluctuations.

Nevertheless, it is important to highlight that parasite abundance may be primarily influenced by factors such as oxygen and salinity rather than temperature. During the summer, L04 and M03 lagoons exhibit elevated salinity levels and significant oxygen fluctuations, occasionally resulting in very low oxygen levels. In contrast, parasites appear to proliferate more readily under lower salinity conditions and in environments with stable to higher oxygen levels. A notable example of this is the extremely high parasitization observed in *A. iberus* specimens from lagoon L04 during the spring.

The complex water dynamics of *La Pletera*, influenced by seasonal changes that impact lagoons' salinity, oxygen and overall temperature, might contribute to the escalation of parasite infection. During periods of rainfall and storms, its water inflow is a mixture of groundwater and surface water, while drier seasons predominantly entail subterranean sources (Menció et al., 2017). Unfortunately, *La Pletera* salt marshes are currently experiencing extended periods of confinement, which are exacerbated by elevated temperatures associated with global warming.

It is plausible to assert that climate change could potentially expedite evaporation rates, induce diminished water levels, and increase water salinity. While this scenario might initially seem advantageous for *A. iberus* specimens due to the apparent preference of the digenean parasite to lower temperatures and salinities, coupled with low interaction with *G. holbrooki* specimens that struggle in high-salinity environments, it can yield counterproductive outcomes. Although *A. iberus* specimens can withstand significant environmental fluctuations, certain biological factors and this heavy parasitization may make them more susceptible to shifts in its surroundings.

Even though parasitization appears to be a threat for *Apricaphanius iberus* specimens, changes in temperature, salinity and oxygen levels within its habitat present a more substantial menace to its survival. Facca et al. (2020) proposed *A. iberus* as one of six ecological bioindicators of lagoon environmental conditions, providing relevant information of ecosystem health via their presence and abundance. In this context, the decline in its populations has predominantly been attributed to abrupt shifts in its habitat. Seasonal and interannual fluctuations in population have shown closer correlation with temperature variations, particularly low winter temperatures, as well as heavy rain and flooding events, which incite stress due to rapid shifts in salinity and prey availability (Prado et al., 2017). While the issue posed by the digenean parasites requires immediate attention, a broader scope of efforts is necessary to conserve a suitable habitat for this species, encompassing anticipated shifts arising from the impacts of climate change.

5. CONCLUSIONS

In conclusion, while metacercariae infections in *Apricaphanius iberus* do not appear to cause massive mortality, they still have a discernible impact on the population. This is exemplified by the case of L04 lagoon, where a high metacercariae abundance has been recorded and subsequent samplings failed to yield any Spanish toothcarp individuals. This suggests that the parasitization weakens this species, rendering them more susceptible to stressors such as the

invasive *G. holbrooki* and impairing their ability to adapt to environmental fluctuations in factors like salinity and oxygen levels.

The heavy parasitization observed in the endangered *A. iberus* populations inhabiting the salt marshes and lagoons along the Catalan coast raises significant concerns regarding the species' survival and overall health. When coupled with the pressures exerted by *G. holbrooki* fish and the uncertainties associated with climate change, the situation of *Apricaphanius iberus* specimens becomes increasingly precarious, pushing them closer to the brink of extinction.

ACKNOWLEDGMENTS

I would like to thank my project directors, María Constenla and Anna Soler, for their guidance throughout these last months. Special thanks to Anna Vila, Xavier Quintana and the University of Girona team for their assistance with sampling and data.

I am also thankful to my mother Rose Marie, my family and my partner for their constant encouragement and unconditional love. Finally, I thank my friends from Peru, the three R's and my lab mates for their unwavering support.

REFERENCES

Abdallah, K. F., Hamadto, H. H., El-Hayawan, I. A., Dawoud, H. A., Negm-Eldin, M., & Wel-A, A. (2009). Metacercariae recovered from fresh-water fishes in the vicinity of Qualkyobia Governorate, Egypt. *Journal of the Egyptian Society of Parasitology*, 39(2), 467-477. PMID: 19795754.

Alcaraz, C., & García-Berthou, E. (2007). Food of an endangered cyprinodont (Aphanius iberus): ontogenetic diet shift and prey electivity. *Environmental Biology of fishes*, 78, 193-207. https://doi.org/10.1007/s10641-006-0018-0.

Attia, M. M., Elgendy, M. Y., Abdelsalam, M., Hassan, A., Prince, A., Salaeh, N. M., & Younis, N. A. (2021). Morpho-molecular identification of *Heterophyes heterophyes* encysted metacercariae and its immunological and histopathological effects on *farmed Mugil cephalus* in Egypt. *Aquaculture International*, 29(3), 1393-1407. https://doi.org/10.1007/s10499-021-00708-3.

Barnes, R. S. K. (2005). Interspecific competition and rarity in mudsnails: feeding interactions between and within Hydrobia acuta neglecta and sympatric Hydrobia species. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 15(5), 485-493. https://doi.org/10.1002/aqc.677.

Beer, N. A., & Joyce, C. B. (2013). North Atlantic coastal lagoons: conservation, management and research challenges in the twenty-first century. *Hydrobiologia*, 701, 1-11. https://doi.org/10.1007/s10750-012-1325-4.

Berger, V.J., & Kharazova A. D. (1997). Mechanisms of salinity adaptations in marine mollusks. *Hydrobiologia* 355:115–126. https://doi.org/10.1023/A:1003023322263.

Blazer, V. S., & Gratzek, J. B. (1985). Cartilage proliferation in response to metacercarial infections of fish gills. *Journal of Comparative Pathology*, 95(2), 273-280. https://doi.org/10.1016/0021-9975(85)90013-1.

Bordalo, M. D., Ferreira, S. M., Jensen, K. T., & Pardal, M. A. (2011). Trematode fauna of Hydrobia ulvae (Gastropoda: Prosobranchia) in a eutrophic temperate estuary. *Journal of the Marine Biological Association of the United Kingdom*, 91(4), 913-921. https://doi.org/10.1017/S0025315410002006.

Bush, A. O., Lafferty, K. D., Lotz, J. M., & Shostak, A. W. (1997). Parasitology meets ecology on its own terms: Margolis et al. revisited. *The Journal of parasitology*, 575-583. https://doi.org/10.2307/3284227.

Casamitjana, X., Menció, A., Quintana, X. D., Soler, D., Compte, J., Martinoy, M., & Pascual, J. (2019). Modeling the salinity fluctuations in salt marsh lagoons. *Journal of hydrology*, *575*, 1178-1187. https://doi.org/10.1016/j.jhydrol.2019.06.018.

Cheung, S. G., & Lam, S. W. (1995). Effect of salinity, temperature and acclimation on oxygen consumption of Nassarius festivus (Powys, 1835) (Gastropoda: Nassariidae). *Comparative Biochemistry and Physiology Part A: Physiology*, 111(4), 625-631. https://doi.org/10.1016/0300-9629(95)00051-8.

Cribb, T. H., Bray, R. A., Olson, P. D., & Littlewood, D. T. J. (2003). Life cycle evolution in the Digenea: a new perspective from phylogeny. *Advances in parasitology*, 54(1), 197-254. https://doi.org/10.1016/S0065-308X(03)54004-0.

Cribb, T. H. (2005). Digenea (endoparasitic flukes). In *Marine Parasitology* (Rohde, K., ed.), pp. 76-87, CSIRO Publishing.

Crivelli A. J. (2006). *Aphanius iberus*. *The IUCN Red List of Threatened Species 2006*: e.T1846A8299534. https://doi.org/10.2305/IUCN.UK.2006.RLTS.T1846A8299534.en.

Doadrio, I., Perdices, A., & Machordom, A. (1996). Allozymic variation of the endangered killifish Aphanius iberus and its application to conservation. *Environmental Biology of Fishes*, *45*, 259-271. https://doi.org/10.1007/BF00003094.

Elsheikha, H. M., & Elshazly, A. M. (2008). Preliminary observations on infection of brackish and fresh water fish by heterophyid encysted metacercariae in Egypt. *Parasitology research*, 103, 971-977. https://doi.org/10.1007/s00436-008-1043-z.

Facca, C., Cavraro, F., Franzoi, P., & Malavasi, S. (2020). Lagoon resident fish species of conservation interest according to the habitat directive (92/43/CEE): A review on their potential use as ecological indicator species. *Water*, 12(7), 2059. https://doi.org/10.3390/w12072059.

Gedan, K. B., Kirwan, M. L., Wolanski, E., Barbier, E. B., & Silliman, B. R. (2011). The present and future role of coastal wetland vegetation in protecting shorelines: answering recent challenges to the paradigm. *Climatic change*, 106, 7-29. https://doi.org/10.1007/s10584-010-0003-7.

Giusti, F., Manganelli, G., & Bodon, M. (1998). A proposed neotype for Hydrobia acuta (Draparnaud, 1805). *Journal of Conchology*, 36(3), 1-8.

Gustinelli, A., Caffara, M., Scaravelli, D., Fioravanti, M. L., & Scholz, T. (2022). The first new species of European *Ascocotyle* Looss, 1899 (Digenea: Heterophyidae) described in more than half a century. *International Journal for Parasitology: Parasites and Wildlife*, 17, 327-334. https://doi.org/10.1016/j.ijppaw.2021.10.003.

Kalantan, A. M., Arfin, M., & Nizami, W. A. (1987). Seasonal incidence and pathogenicity of the metacercariae of *Clinostomum complanatum* in *Aphanius dispar*. *Jpn J Parasitol*, 36(1), 17-23.

Koprivnikar, J., & Poulin, R. (2009a). Effects of temperature, salinity, and water level on the emergence of marine cercariae. *Parasitology Research*, 105, 957-965. https://doi.org/10.1007/s00436-009-1477-y.

Koprivnikar, J., & Poulin, R. (2009b). Interspecific and intraspecific variation in cercariae release. *Journal of Parasitology*, 95(1), 14-19. https://doi.org/10.1645/GE-1582.1.

Koprivnikar, J., Ellis, D., Shim, K. C., & Forbes, M. R. (2014). Effects of temperature and salinity on emergence of *Gynaecotyla adunca* cercariae from the intertidal gastropod *Ilyanassa obsoleta*. *The Journal of Parasitology*, 100(2), 242-245. http://www.jstor.org/stable/24624662.

Lyholt, H. C. K., & Buchmann, K. (1996). *Diplostomum spathaceum*: effects of temperature and light on cercarial shedding and infection of rainbow trout. *Diseases of Aquatic Organisms*, 25(3), 169-173. https://doi.org/10.3354/dao025169.

Masala, S., Piras, M. C., Sanna, D., Chai, J. Y., Jung, B. K., Sohn, W. M., Garippa, G., & Merella, P. (2016). Epidemiological and molecular data on heterophyid trematode metacercariae found in the muscle of grey mullets (Osteichthyes: Mugilidae) from Sardinia (western Mediterranean Sea). *Parasitology Research*, 115, 3409-3417. https://doi.org/10.1007/s00436-016-5101-7.

Menció, A., Casamitjana, X., Mas-Pla, J., Coll, N., Compte, J., Martinoy, M., Pascual, J., & Quintana, X.D., 2017. Groundwater dependence of coastal lagoons: the case of La Pletera salt marshes (NE Catalonia). *Journal of Hydrology*, 552, 793–806. https://doi.org/10.1016/j.jhydrol.2017.07.034.

Menció, A., Madaula, E., Meredith, W., Casamitjana, X., & Quintana, X. D. (2023). Dataset for analyzing and modelling the eutrophication processes in groundwater-coastal lagoon systems: The La Pletera lagoons case study (NE Spain). *Data in Brief*, 48, 109197. https://doi.org/10.1016/j.dib.2023.109197.

Mouritsen, K. N. (2002). The Hydrobia ulvae–Maritrema subdolum association: influence of temperature, salinity, light, water-pressure and secondary host exudates on cercarial emergence and longevity. *Journal of helminthology*, 76(4), 341-347. https://doi.org/10.1079/JOH2002136.

Oliva-Paterna, F. J., Mar, T., & Fernández-Delgado, C. (2006). Threatened fishes of the world: Aphanius iberus (Cuvier & Valenciennes, 1846) (Cyprinodontidae). *Environmental Biology of Fishes*, *75*(3), 307-309. https://doi.org/10.1007/s10641-006-0016-2.

Olson, R. E., & Pierce, J. R. (1997). A trematode metacercaria causing gill cartilage proliferation in steelhead trout from Oregon. *Journal of wildlife Diseases*, 33(4), 886-890. https://doi.org/10.7589/0090-3558-33.4.886.

Paperna, I., & Dzikowski, R. (2006). Digenea (Phylum Platyhelminthes). In Fish diseases and disorders. Volume 1: Protozoan and metazoan infections (pp. 345-390). Wallingford UK: Cabi. https://doi.org/10.1079/9780851990156.0345.

Pascual, E., & Drake, P. (2008). Physiological and behavioral responses of the mud snails Hydrobia glyca and Hydrobia ulvae to extreme water temperatures and salinities: implications for their spatial distribution within a system of temperate lagoons. *Physiological and Biochemical Zoology*, 81(5), 594-604. https://doi.org/10.1086/588173.

Pearson, J. (2008). Family Heterophyidae Leiper, 1909. In *Keys to the Trematoda*, Volume 3 (pp. 113-141). Wallingford UK: CABI. https://doi.org/10.1079/9780851995885.0113.

Prado, P., Alcaraz, C., Jornet, L., Caiola, N., & Ibáñez, C. (2017). Effects of enhanced hydrological connectivity on Mediterranean salt marsh fish assemblages with emphasis on the endangered Spanish toothcarp (Aphanius iberus). *PeerJ*, 5, e3009. https://doi.org/10.7717/peerj.3009.

Quintana, X. D., Moreno-Amich, R., & Comin, F. A. (1998). Nutrient and plankton dynamics in a Mediterranean salt marsh dominated by incidents of flooding. Part 1: Differential confinement of nutrients. *Journal of Plankton research*, 20(11), 2089-2107. https://doi.org/10.1093/plankt/20.11.2089.

Quintana, X., D. Boix, X. Casamitjana, A. Colomer, J. Compte, D. Cunillera-Montcusí, S. Gáscon, F. Gich, A. Menció, M. Martinoy, J. Montaner, J. Pascual, J. Sala, J. Solà & I. Tornero. (2018). Management and restoration actions of confined Mediterranean coastal lagoons in the Empordà and Baix Ter wetlands. *Management and restoration of Mediterranean coastal lagoons in Europe*, 10, 173-192.

Rusydi, A. F. (2018, February). Correlation between conductivity and total dissolved solid in various type of water: A review. In *IOP conference series: earth and environmental science* (Vol. 118, p. 012019). IOP Publishing. https://doi.org/10.1088/1755-1315/118/1/012019.

Santos, C. P., Lopes, K. C., da Silva Costa, V., & dos Santos, E. G. N. (2013). Fish-borne trematodosis: potential risk of infection by *Ascocotyle* (Phagicola) longa (Heterophyidae). *Veterinary Parasitology*, 193(1-3), 302-306. https://doi.org/10.1016/j.vetpar.2012.12.011.

Scholz, T., Aguirre-Macedo, M. L., & Salgado-Maldonado, G. (2001). Trematodes of the family Heterophyidae (Digenea) in Mexico: a review of species and new host and geographical records. *Journal of Natural History*, 35(12), 1733-1772. https://doi.org/10.1080/00222930152667087.

Sindermann, C.J. (1960). Ecological studies of marine dermatitis producing Schistosome larvae in Northern New England. *Ecology*, 41 (4), 678–684. https://doi.org/10.2307/1931801.

Sindermann, C. J., & Farrin, A. E. (1962). Ecological studies of Cryptocotyle lingua (Trematoda: Heterophyidae) whose larvae cause" pigment spots" of marine fish. *Ecology*, 69-75. https://doi.org/10.2307/1932041.

Studer, A., Thieltges, D. W., & Poulin, R. (2010). Parasites and global warming: net effects of temperature on an intertidal host–parasite system. *Marine Ecology Progress Series*, 415, 11-22. https://doi.org/10.3354/meps08742.

Studer, A., & Poulin, R. (2012). Effects of salinity on an intertidal host–parasite system: Is the parasite more sensitive than its host? *Journal of Experimental Marine Biology and Ecology*, 412, 110-116. https://doi.org/10.1016/j.jembe.2011.11.008.

Waikagul, J., & Thaekham, U. (2014). Approaches to research on the systematics of fish-borne trematodes. Academic Press. https://doi.org/10.1016/C2012-0-02622-1.