

# *Monorchis parvus* and *Gymnophallus choledochus*: two trematode species infecting cockles as first and second intermediate host

## Review

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## Abstract

The most deleterious stage of a trematode life cycle occurs in the first intermediate host where the parasite penetrates as a miracidium and asexually multiplies in sporocysts or rediae. When infection advances, other organs can be occupied with severe effects on host individual health and population dynamics. Existing studies focused on these host/parasite systems are still scarce due to the usual low prevalence in ecosystems. Using cockles (*Cerastoderma* spp.) and two trematode species (*Monorchis parvus* and *Gymnophallus choledochus*) infecting these bivalves as first and second intermediate host, the present work aimed to (1) summarize the most relevant literature and (2) provide new information regarding this host/parasite system, taking advantage of a 21-year monthly database from Banc d'Arguin (France). This long-term monitoring showed that different trematode species display varying host size range preference (6–38 and 31–36 mm for *M. parvus* and *G. choledochus*, respectively). The occurrence of coinfection was lower than expected, raising some questions related to parasite interspecific competition. This review improved our understanding of the processes shaping the prevalence and distribution of parasitism. This study highlighted that beyond constant trematode assemblage monitoring, there is a need to identify the main predictors of rediae/sporocysts infection, such as the definitive host dynamics and miracidium infection processes, for future better management of host severe disease and mortality episodes.

## Introduction

Trematodes are ubiquitous in coastal waters being among the most common and abundant parasites (Lauckner, 1983; Schmidt and Roberts, 2000). The life cycle of a typical trematode usually involves three hosts and three transmission stages. Adult trematodes live and reproduce within the definitive host releasing eggs in its intestinal tract. Then, the definitive host emits feces containing these eggs from which the free-living stage miracidium hatches and infects, usually a mollusc, as the first intermediate host. After maturation into rediae and/or sporocysts, the free-living cercariae stage is formed and emerges to actively seek a suitable second intermediate host where it settles as a metacercaria. Alternatively, metacercariae can settle immediately upon contact with appropriate aquatic substrata, i.e. encysting on rocks or vegetation (Morley, 2015). For the life cycle to be completed, the parasitized second intermediate host (or the metacercaria itself) must be consumed by a suitable definitive host. Nevertheless, there are some examples of abbreviated life cycles, facultative or not, which are reduced to two hosts or even to one host possibly driven by evolutionary processes (Galaktionov and Dobrovolskij, 2003).

Trematodes are intimately linked to molluscs as first intermediate hosts. Gastropods are the most commonly infected group, with only very few trematode lineages using exclusively bivalves as first intermediate hosts (i.e. during the life cycle they do not infect gastropods). Nevertheless, bivalves can act as the first, but more often as the second intermediate host or both; however, evidence reveals that bivalves as trematode hosts came later in the patterns of evolution (Cribb *et al.*, 2003). The impact of trematodes on its molluscan host survival is species specific, depending on parasite abundance but is essentially related to the parasitic stage. The sporocyst (or redia) stage of a trematode life cycle was adopted in an evolutionary perspective, to successfully invade the host tissues, through asexual multiplication, resulting in a short-term rapid population growth and then expansion due to cercariae dispersal. As a result of this strong interaction between the parasite and vital organs of the host, sporocyst represents the most deleterious stage of a trematode life cycle with known effects at the metabolism, reproduction, growth and behaviour levels (Galaktionov and Dobrovolskij, 2003; Longshaw and Malham, 2013). Trematode effects at the individual level can be reflected on population performance (e.g. Friesen *et al.*, 2017) and community structure (Poulin, 1999). On the other hand, sporocyst/host relationship dynamics are much harder to fully understand

due to intrinsic low prevalence (e.g. Thieltges *et al.*, 2008; Magalhães *et al.*, 2017) or sudden prevalence outbreaks followed by mass mortalities (Jonsson and André, 1992; Thieltges, 2006).

In the present review, besides literature compilation, the authors take a unique opportunity to identify patterns or trends in a trematode/first intermediate host relationship using a long-term database. Cockles (*Cerastoderma* spp.) are the main focus of this review, known for being a key-species due to their role in ecosystem engineering (Donadi *et al.*, 2014), carbon sequestration (Morgan *et al.*, 2013) and an important connection between trophic levels (Rakotomalala *et al.*, 2015). Cockle reproduction and development includes (Malham *et al.*, 2012) a gametogenesis process occurring in Spring (February–March) followed by a rapid gonad development (April–May). Its sexual maturity is usually reached when cockle shell length reaches 12–14 mm, although it may be strongly driven by seasonality and other external environmental variables. Spawning can occur from May to July/August when mean temperatures are around 13°C (Boyden, 1970). Following fertilization, eggs are pelagic and the planktonic larval stage lasts from 2 to 5 weeks (approximately 6 mm shell length). After settlement, a cohort lifespan can reach up to 40 months, 5 cm in length and span a geographically variable range (Magalhães *et al.*, 2016). Cockles are the habitat for 16 different trematode species (de Montaudouin *et al.*, 2009) and therefore an obvious choice to study these species dynamics. In this regard, the main objective of the present work is to review the literature to date on *Cerastoderma* spp. infection by *Monorchis parvus* and *Gymnophallus choledochus*, two of the four trematode species that use cockles as the first intermediate host (de Montaudouin *et al.*, 2009). This work also represents the first long-term (i.e. 21 years) description of these host/parasite systems phenology with the following specific aims: to understand infection relationship to host shell length, to examine seasonal and year effects, to depict correlations to temperature and host density, to assess co-infection with metacercariae and other sporocysts and finally to provide a molecular identification for both parasites as a quality control of the stereomicroscope morphological identification.

## Material and methods

### Literature review

All information gathered in this review was collected from an extensive search of the literature published before September 2019. A complete search on the Web of Science was performed using several combinations of keywords such as '*Cerastoderma* (or *Cardium*) *edule*', '*Monorchis parvus*', '*Gymnophallus choledochus*', among others. The reference list of relevant papers and some grey literature was also consulted. The inclusion in the present manuscript was constrained to those studies that clearly identified the occurrence and description of both species under review with a total of 97 gathered publications (39 for *M. parvus* and 57 for *G. choledochus*).

### Long-term monitoring

#### Sampling site description

The sampling station was located in Banc d'Arguin (44.60°N, 1.25°W), Arcachon Bay, France. Arcachon bay is a 180 km<sup>2</sup> macrotidal lagoon situated on the Atlantic southwest coast of France. This lagoon opens to the Atlantic Ocean through a wide channel. The junction with the Atlantic is characterized by the presence of several sand banks, including Banc d'Arguin. Cockles (*Cerastoderma edule*) are distributed along the semi-sheltered, intertidal part of this bank. The sediment is largely dominated by medium sands [350 µm of median grain size (de

Montaudouin *et al.*, 2000)] with organic matter content not exceeding 2% (Baudrimont *et al.*, 2003). Salinity is 32–35 year round, while mean surface water temperature fluctuates between 9.5°C in winter and 21.1°C in summer (de Montaudouin *et al.*, 2000). Since 1972, Arguin (43.6 km<sup>2</sup>) is a national reserve which is largely protected from anthropogenic activity. Sampling was performed under strict authorization.

#### Cockle sampling and parasite identification

During 21 years (between October 1997 and September 2018), cockles were collected monthly by sampling 6 quadrats (0.25 m<sup>2</sup> each) and sieving them through a 1 mm mesh sieve. Cockle shell length was measured to the nearest mm with a caliper. Ten cockles per cohort (identified by size-frequency histograms) were dissected and squeezed between two glass slides for trematode observation under a stereomicroscope. All digenean trematodes were identified to the species level following several authors' descriptions (Bowers, 1969; Bowers *et al.*, 1996; Bartoli *et al.*, 2000; Desclaux *et al.*, 2006; de Montaudouin *et al.*, 2009). Trematodes found in the metacercariae stage (using cockles as the second intermediate host) were counted to assess parasite abundance, i.e. the number of metacercariae per cockle. For trematodes using cockles as the first intermediate host, it was not possible to count sporocysts and/or cercariae due to the dense mass created. Therefore, only prevalence was calculated, i.e. the percentage of infected cockles (Bush *et al.*, 1997). During the whole study period, a temperature probe (HOBO® Water Temp Pro v2-U22-001) was settled in the sediment of the sampling area with temperature recorded every hour.

#### Molecular identification

##### DNA isolation, amplification and sequencing

Samples for DNA analysis were removed from sporocysts found in infected cockles. For both species, three replicates were collected. Sporocysts were placed in microtubes and frozen at –20°C. Extraction of DNA was done with QIAamp DNA Micro Kit (QIAGEN) following the protocol supplied by the manufacturer. About 530 bp of 18S (small subunit ribosomal RNA gene), 600 bp of ITS1 (Internal Transcribed Spacer 1) and 300 bp of COI (cytochrome c oxidase subunit I) genes were amplified using primers Bb18S and Bb18AS for 18S (de Montaudouin *et al.*, 2014), BbITS and BbITAS for ITS1 (de Montaudouin *et al.*, 2014), TremCOIS2 and TremCOIAS2 for COI were designed. All polymerase chain reaction (PCR) and sequencing primers are described in Table 1. The PCR was performed with Gotaq G2 Flexi DNA Polymerase (PROMEGA), with 50 µL mixtures containing: 10 µL of 5X Colorless GoTaq® Reaction Buffer (final concentration of 1X), 1.5 µL of MgCl<sub>2</sub> solution (final concentration of 1.5 mM), 1 µL of PCR nucleotide mix (final concentration of 0.2 mM each dNTP), 0.5 µL of each primer (final concentration of 1 µM), 0.2 µL of GoTaq® G2 Flexi DNA Polymerase (5 U µL<sup>-1</sup>), 1 µL template DNA and 33.8 µL of nuclease-free water. The temperature profile was as follows for 18S and ITS1: 94°C/10 min – (94°C/60 s–59°C/30 s–72°C/90 s)×40 cycles – 72°C/10 min – 4°C, for COI: 95°C/10 min – (95°C/60 s–43°C/30 s–72°C/60 s)×40 cycles – 72°C/10 min – 4°C. Amplified PCR products were analysed by electrophoresis in a 1% p/v agarose gel stained with ethidium bromide and were sent to Eurofins Company for complete double strain sequencing, using the same set of primers as used for the PCR. Overlapping sequence (forward and reverse) fragments were merged into consensus sequences and aligned using Clustal Omega. For COI, the sequences were translated into amino acid alignment and checked for stop codons to avoid pseudogenes. All sequences obtained in this study have been deposited in GenBank. Accession numbers for *M. parvus*

**Table 1.** Nucleotide sequences of specific primer pairs used in this study

Primer code	Used for	Sequence 5'-3'	Reference
Bb18S	PCR, sequencing	5'-ACTGGAGGGCAAGTCTGGTGC-3'	de Montaudouin <i>et al.</i> (2014)
Bb18AS	PCR, sequencing	5'-CAGCTTTGCAACCATACTTCCC-3'	de Montaudouin <i>et al.</i> (2014)
BbITS	PCR, sequencing	5'-GACCGAAGCTTGATCATTTAGAGG-3'	de Montaudouin <i>et al.</i> (2014)
BbITAS	PCR, sequencing	5'-CTTAAGTTCAGCGGGTAATCACG-3'	de Montaudouin <i>et al.</i> (2014)
TremCOIS2	PCR, sequencing	5'-TGTTYTTTAGKTCTGTAKAC -3'	This study
TremCOIAS2	PCR, sequencing	5'-AATGCATMGGRAAAAAACA -3'	This study

are MN544855 for 18S, MN592817 for ITS and MN547970 for COI. For *G. choledochus*, accession numbers are: MN544854 for 18S, MN592818 for ITS and MN547969 for COI.

### Data analysis

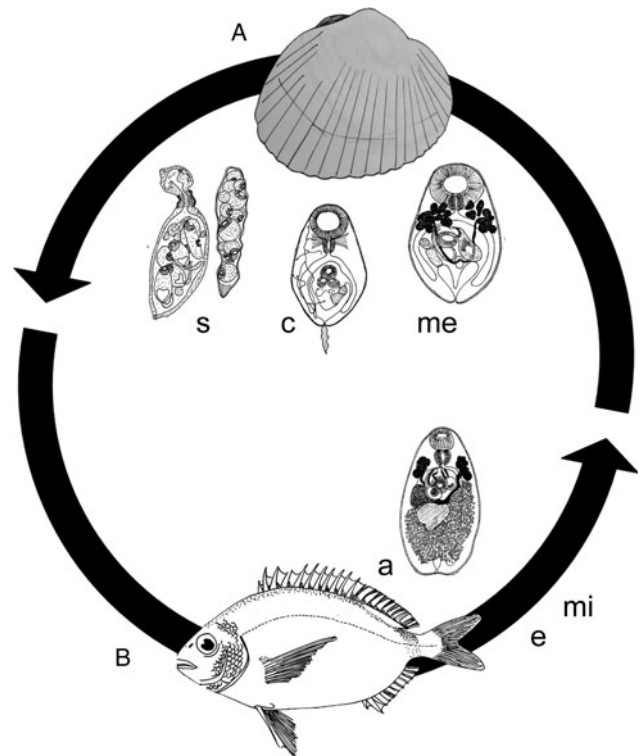
Correlation between cockle shell length classes and each associated trematode species prevalence was tested using non-parametric Spearman analysis. To test the effect of seasonality on the prevalence of *M. parvus* and *G. choledochus*, a Kruskal-Wallis ANOVA on ranks and a one-way ANOVA (selected based on the result of the Cochran test for homogeneity of variance) were, respectively, performed to compare the different months, using the 21 years as replicates. Then, to test the effect of years on trematode prevalence (*M. parvus* and *G. choledochus*), two one-way ANOVAs were performed to compare the different years using the 12 months as replicates. One year was defined as the 12 months between October *N* and September *N*+1. These analyses were narrowed to the cockle size range susceptible to be parasitized by each trematode species (6–38 and 31–36 mm for *M. parvus* and *G. choledochus*, respectively) to avoid the dilution of prevalence values related to seasonal juvenile dominance. Mean prevalence of *M. parvus* and *G. choledochus* per year was correlated with yearly cockle density, mean surface sediment temperature of each month, each year and each previous year using non-parametric Spearman analysis. Two paired Wilcoxon tests were performed to compare the abundance of trematode species that use cockles as the second intermediate host and trematode species richness in infected *vs* non-infected cockles with the sporocysts of *M. parvus* and *G. choledochus* (separately analysed). For each infected cockle, a pair was constituted by randomly selecting a non-infected organism of the same shell length and same sampling date. The 21-year data matrix was checked for the occurrence of cockle coinfection by *Bucephalus minimus*, *G. choledochus* and *M. parvus*, three out of the four trematode species known to use this bivalve as the first intermediate host (de Montaudouin *et al.*, 2009), through proportion comparisons of the expected double infection *vs* the real double infection (Difference test).

## Results

### *Monorchis parvus* Looss, 1902

#### Literature review

**Description and life cycle** (Fig. 1): *Monorchis parvus* was originally described in 1902 (Looss, 1902) infecting teleost fishes (*Diplodus* spp.) of the Adriatic Sea. It is a parasite from the Platyhelminthes phylum, Trematoda class, Digenea subclass and Monorchidae family. The typical life cycle of monorchiid trematodes includes a bivalve as the first intermediate host, the same or other bivalves as the second intermediate host and teleost fish as the definitive host (Lauckner, 1983).



**Fig. 1.** Schematic representation of the *Monorchis parvus* life-cycle with parasite life stages and respective intermediate hosts. (A) *Cerastoderma* spp., the host of sporocysts (s), cercaria (c) and metacercaria (me); eggs (e) and miracidia (mi) are not represented and (B) *Diplodus* spp., hosts of the adult (a) form.

Over time, *M. parvus* was found infecting cockles (*Cerastoderma* spp.) but for a long time was misidentified as *Distomum* sp., *Lepodora rachiaea*, *Lepidapedon rachion* and received the synonym *Cercaria cerastodermæ* I. Firstly, this species was found infecting *C. edule* (the edible cockle) and wrongly assigned to *Distomum* sp. (Lebour, 1905, 1907b) and then to *L. rachiaea* (Lebour, 1907a, 1912). Later on, this trematode was found infecting *C. edule* and *C. glaucum* (the lagoon cockle) but was incorrectly identified as *L. rachion* (the former *L. rachiaea*) (Bowers, 1965; Boyden, 1970) from the Lepidapedidae family, which usually also infects teleost fish as a definitive host. The mother sporocyst, the daughter sporocyst, the fully formed cercariae and the encysted metacercariae of this species were first described in 1978, infecting cockles, and renamed as *C. cerastodermæ* I (Sannia *et al.*, 1978). This species was finally assigned to the correct family (Monorchidae) maintaining the name *C. cerastodermæ* I for 15 years (Sannia and James, 1978; Sannia *et al.*, 1978; Lauckner, 1983; Jonsson and André, 1992; Russell-Pinto, 1993). It was only at the end of the 20th century, with morphological and molecular data (Bartoli *et al.*, 2000), that *C. cerastodermæ* I (until then reported to infect cockles as

**Table 2.** Review of the literature regarding *Monorchis parvus* and *Gymnophallus choledochus* infection

Reference	Study location	Intermediate hosts (size, mm: infected tissue)			Prevalence in the first host (%)	Main findings
		First	Second	Definitive		
<i>Monorchis parvus</i>						
Looss (1902)	Adriatic Sea	–	–	DA and DS	–	First description
Lebour (1905)	Budle Bay, UK	CE	–	–	0.5	Occurrence in March; wrongly assigned to <i>Lepodora rachiaea</i>
Lebour (1907a)	Holy Island, UK	CE	–	–	1	Occurrence in April; wrongly assigned to <i>Lepodora rachiaea</i> that usually uses <i>Melanogrammus aeglefinus</i> as definitive host; cercariae shape and size description
Lebour (1907b)	Morecambe Bay, UK	CE	–	–	0.5	Occurrence; wrongly assigned to <i>Distomum</i> sp.; visceral mass of the cockle completely riddled with sporocysts specially in the gonad location; sporocyst, cercaria and cyst size and description
Lebour (1912)	Review	CE	–	–	–	Wrongly assigned to <i>Lepodora rachiaea</i> that usually uses <i>Melanogrammus aeglefinus</i> as definitive host; cercariae shape and size description
Bowers (1965)	Kyle of Tongue, Scotland	CE	–	–	0.5	Occurrence; wrongly assigned to <i>Lepidapedon rachion</i>
Boyden (1970)	Several estuaries in the UK	CE <sup>a</sup> and CG <sup>b</sup>	–	–	a: 0.07–7.7 b: 0.12–2.4	Occurrence; wrongly assigned to <i>Lepidapedon rachion</i> ; considered a rare species; indication that the definitive host must be a marine fish; young stages found in late winter-spring possibly related to the definitive host arrival
Sannia et al. (1978)	Kyle of Tongue <sup>a</sup> and Thames estuary <sup>b</sup> , UK	CE	–	–	a: 0.5 b: 1.18	Species renomination and first description of the mother sporocyst, daughter sporocyst, cercaria and encysted metacercariae; considered a rare occurrence; possible seasonal behaviour
Sannia and James (1978)	Thames estuary, UK	CE	–	–	1.18	Description of life cycle seasonality; random distribution of infection among size groups
Lauckner (1983)	Review	CE	–	–	–	Compilation of knowledge on this species to date
Jonsson and André (1992)	Tjärnö, Sweden	CE	–	–	buried:17–20 surface:50–81	Severe tissue damage (+foot), cause of mass mortality, impairment of the burrowing capacity
Russell-Pinto (1993)	Ria de Aveiro, Portugal	CE [gill] CG [digestive gland and gonad]	CE [gill] CG [digestive gland and gonad]	–	1.17–1.71	Occurrence and life cycle stages description (named as <i>Cercaria cerastodermae</i> I); higher prevalence in October
Bartoli et al. (2000)	Ria de Aveiro, Portugal and experimental infection	CE	CE	DS and DA	0.47	Renamed as <i>Monorchis parvus</i> ; life cycle description
de Montaudouin et al. (2000)	Arcachon Bay, France	CE [19: whole tissue]	–	–	0.2	Occurrence
Jousson et al. (2000)	Mediterranean Sea	–	–	DS, DV and DA	–	Cryptic diversity among <i>Monorchis parvus</i> species
Desclaux et al. (2002)	Arcachon Bay, France	CE	–	–	3	Occurrence only in cockles found in the surface
Jousson and Bartoli (2002)	Mediterranean Sea	–	–	DS, DV and DA	–	Presence of a host-associated species complex
Desclaux (2003)	Arcachon Bay, France	CE	–	–	0.1–1.3	Occurrence

Figus <i>et al.</i> (2004)	Sardinia, Italy	CG	–	–	0.8–1	Occurrence
Levron <i>et al.</i> (2004)	Corsica, France	–	–	DA	–	Ultrastructural characteristics of spermiogenesis and the spermatozoon
Levron <i>et al.</i> (2005)	Corsica, France	–	–	DA	–	<i>Nosema monorchis</i> , a microsporidium parasitizing <i>Monorchis parvus</i>
Ternengo <i>et al.</i> (2005)	Corsica, France	–	–	DA	–	Occurrence
Bartoli <i>et al.</i> (2005)	Corsica, France	–	–	DA, DS and DV	–	Occurrence
Russell-Pinto <i>et al.</i> (2006)	Ria de Aveiro, Portugal	CE [whole tissue]	–	–	0.95	Occurrence; s.e.m. diagnosis
Thieltges and Reise (2006)	Wadden Sea, Germany	CE [adults]	–	–	0.1 ± 0.2	First occurrence in the Wadden Sea
Thieltges <i>et al.</i> (2006a, 2006b)	Wadden Sea, Germany	CE	–	–	0.5 ± 1.3	Occurrence
Iglesias (2006)	Galicia, Spain	CE	CE	–	–	Occurrence as sporocysts and encysted metacercariae
Bartoli and Gibson (2007)	Western Mediterranean	CG	CG	DA and DV	–	Synopsis of the life cycle
Gam <i>et al.</i> (2008)	Merja Zerga, Morocco	CE	–	–	0–3	Occurrence
Thieltges <i>et al.</i> (2008)	Wadden Sea, Germany	CE	–	–	–	Occurrence
Gargouri Ben Abdallah and Maamouri (2008)	Tunisian Coast	–	–	DA, DS and DV	–	Occurrence
de Montaudouin <i>et al.</i> (2009)	Review	CE	–	–	–	Inclusion in a digenean trematodes identification key; microhabitat description (gonads)
Dubois <i>et al.</i> (2009)	Arcachon Bay, France	CE [22–36: digestive gland and gonad]	–	–	–	Represent 4–22% of the host tissue; no influence on stable isotope signatures; causes C depletion
Antar and Ben Abdallah (2013)	Bizerte Lagoon, Tunisia	–	–	DA, DS and DV	–	Occurrence
Freitas <i>et al.</i> (2014)	Ria de Aveiro, Portugal	CE	–	–	0.2 ± 1	Occurrence
García-Souto and Pasantes (2015)	Pontevedra, Spain	CE	–	–	–	Cytogenetic study
Magalhães <i>et al.</i> (2015)	Arcachon Bay, France	CE [19–40]	–	–	–	Occurrence
Magalhães <i>et al.</i> (2017)	Arcachon Bay, France	CE	–	–	1	Occurrence
COCKLES Project (2017–2020)	Arcachon Bay, France	CE	–	–	1	Occurrence
<i>Gymnophallus choledochus</i>						
Huet (1888)	–	CE	CE	–	–	First description without name assignment, however with wrong observation of cercariae leaving the mother sporocyst and the consequent existence of an intermediate host
Odhner (1900)	Sweden	–	–	Tt	–	Adult first description
Odhner (1905)	Greenland	–	–	SM and SSSs	–	Adult size and species re-description
Johnstone (1905)	Lancashire coast, UK	CE	–	–	–	Wrongly assigned to <i>Cercaria fissicauda</i>
Lebour (1908)	Northumberland, UK	CE and TT	–	–	2	Observation of 'tailless cercariae' (in fact, metacercariae) inside sporocysts; wrongly assigned to <i>Cercaria strigata</i>
Nicoll (1923)	United Kingdom	–	–	Shorebirds	–	Included in a reference list
Isaitchikow (1924)	Crimea, Ukraine	–	–	AF	–	Occurrence; extension of the distribution from Scandinavia to the Black Sea

(Continued)

Table 2. (Continued.)

Reference	Study location	Intermediate hosts (size, mm: infected tissue)			Prevalence in the first host (%)	Main findings
		First	Second	Definitive		
Hutton (1952)	Plymouth, UK	CE [gonad, digestive gland and foot]	–	–	1.2	Awareness of discrepancies in the species description; species was renamed as <i>Cercaria fulbrighti</i> ; conclusion that 2 types of life histories may exist
Stunkard and Uzmann (1958)	Boothbay Harbor, Maine, USA	–	–	SM	–	Occurrence
James (1964)	–	–	–	Shorebirds	–	Review of the family
Bowers (1965)	Wales, UK	CE	–	–	0.3–0.4	Occurrence; wrongly assigned to <i>Cercaria dichotoma</i>
Prévot (1965)	Experimental approach	–	HD	LAM [experimental infection]	–	Experimental infection
Cheng (1967)	Review	CE	–	–	–	Description of sporocyst and cercariae; wrongly renamed as <i>Cercaria hueti</i>
James and Bowers (1967)	Gower Peninsula, Wales	CE	–	–	–	Occurrence; wrongly assigned to <i>Cercaria dichotoma</i>
Bowers and James (1967)	Gower Peninsula, Wales	CE	NC	–	0.3	Occurrence; wrongly assigned to <i>Cercaria dichotoma</i>
Pascoe et al. (1968)	Gower Peninsula, Wales	CE	–	–	0.25	Occurrence; wrongly assigned to <i>Cercaria dichotoma</i> ; higher oxygen consumption when the sporocyst contains more cercariae
Loos-Frank (1969)	North Sea	CE	CE, HD, NH or AM	Shore duck [gall-bladder]	0.8–7.0	Occurrence; alternative cycle description
Frank (1969)	North Sea	CE	CE or polychaetes	Shorebirds	–	Alternative life cycle description
Richards et al. (1970)	Gower Peninsula, Wales	CE	–	–	0.1	Occurrence; wrongly assigned to <i>Cercaria dichotoma</i> ; sporocysts containing metacercariae have more stored carbohydrate in relation to their metabolic needs than those containing cercariae; lipid is the principal source of energy
Lauckner (1971)	Baltic Sea, Germany	CE	–	–	–	Occurrence
Loos-Frank (1971)	North Sea	CE	–	–	–	Occurrence
Margolis (1973)	Note	–	HD, NC, NH and AM	–	–	Note on helminths using polychaetes as intermediate host
Bartoli (1971)	Camargue, France	CE	CG and HD	–	–	Occurrence
Bartoli (1974)	Camargue, France	–	HD [ventral acicule]	–	–	Competitive exclusion of <i>Gymnophallus choledochus</i> by <i>Gymnophallus nereicola</i>
Boyden (1970)	Several estuaries in the UK	CE <sup>a</sup> and CG <sup>b</sup>	HD or NC	Shore duck	a: 0.2–11 b: 0.25–1.8	Wrongly assigned to <i>Cercaria dichotoma</i> ; youngest sporocysts occurring from October until March; final cercarial stages only visible between May and August; low prevalence; possibility of definitive host being a duck winter migrant
Bozhkov and Odening (1979)	Review	–	–	–	–	Helminths life cycles with a variable number of hosts

Lauckner (1983)	North Sea, Germany	CE	–	–	0.15	Higher incidence on surface cockles; viscera and foot heavily bloated; disappearance of gonadal tissue; loss of burrowing capacity; reduced resistance to thermal stress
Bartoli (1984)	Mediterranean Sea	CG	–	–	–	Occurrence
Bartoli and Combes (1986)	Brusc lagoon, France	CG	HD	Charadriiforms birds	–	Occurrence; referred as a rare species
Russell-Pinto (1993)	Ria de Aveiro, Portugal	CE <sup>a</sup>	CE and HD <sup>b</sup>	–	a: 2.24 b: 1.6	Occurrence and description of the life cycle stages
Ching (1995)	Review	CE	HD	–	--	Evaluation of some characters of the family
de Montaudouin <i>et al.</i> (2000)	Arcachon Bay, France	CE [33]	–	–	0.2	Occurrence
Poulin and Cribb (2002)	Review	–	–	–	–	Review on abbreviated life cycles
Desclaux (2003)	Arcachon Bay, France	CE	–	–	0.26–0.36	Occurrence
Figus <i>et al.</i> (2004)	Sardinia, Italy	CG	–	–	5	Occurrence
Russell-Pinto <i>et al.</i> (2006)	Ria de Aveiro, Portugal	CE [gonad and digestive gland]	CE	–	2.24	Cercariae and metacercariae taxonomic details (s.e.m.)
Thieltges (2006)	Wadden Sea, Germany	CE	CE	–	71	Probable cause of high summer mortality
Thieltges and Reise (2006)	Wadden Sea, Germany	CE [2+]	–	–	6.5 ± 5 and 2.4 ± 1.6	Occurrence; only found in adult cockles
Krakau <i>et al.</i> (2006)	Wadden Sea, Germany	CE	–	–	5	Occurrence
Thieltges <i>et al.</i> (2006a, 2006b) <sup>a</sup>	Wadden Sea, Germany	CE	–	–	5.2 ± 3.9	Occurrence
Thieltges <i>et al.</i> (2006a, 2006b) <sup>b</sup>	Wadden Sea, Germany	–	–	SM	–	Occurrence
Bartoli and Gibson (2007)	Western Mediterranean	CG	HD	LCM	–	Life cycle synopsis
Gam <i>et al.</i> (2008)	Merja Zerga coastal lagoon (Morocco)	CE [27 ± 1]	–	–	7	Occurrence only in large cockles and in the outer area of the lagoon
Rangel and Santos (2009)	Ria de Aveiro, Portugal	–	DN [branchial segments]	–	–	First record in <i>Diopatra neapolitana</i> (unencysted form)
Derbali <i>et al.</i> (2009)	Gulf of Gabes, Tunisia	CG [visceral mass]	–	–	–	Occurrence
de Montaudouin <i>et al.</i> (2009)	North-eastern Atlantic shoreline	CE	CE	–	–	Inclusion in an identification key; microhabitat
Fermer <i>et al.</i> (2011)	South coast of Ireland	CE	–	–	5	Occurrence
Morgan <i>et al.</i> (2012)	Cork, Ireland	CE	CE	–	–	Occurrence
Peoples (2013)	Review	–	HD, NC, NH and AM	–	–	Review of helminths using polychaetes as hosts
Feis <i>et al.</i> (2015)	Wadden Sea, Germany	CE	–	–	0.05–10.1	Lack of genetic population structure
Skirnisson (2015)	Skerjafjörður, Iceland	–	–	SS	–	Occurrence
Cremonte <i>et al.</i> (2015)	Review	–	–	–	–	Review of the family with morphological and molecular data

(Continued)

Table 2. (Continued.)

Reference	Study location	Intermediate hosts (size, mm: infected tissue)			Prevalence in the first host (%)	Main findings
		First	Second	Definitive		
Magalhães <i>et al.</i> (2017)	Arcachon Bay, France	CE	-	-	-	Occurrence
Correia <i>et al.</i> (2020)	Portugal and Morocco	CE	CE*	-	0-5 (50*)	Occurrence
COCKLES Project (2017-2020)	Bay of Somme, France	CE	-	-	8	Occurrence
COCKLES Project (2017-2020)	Formosa, Portugal	CE	-	-	1	Occurrence
COCKLES Project (2017-2020)	Texel, The Netherlands	CE	-	-	4	Occurrence

DA, *Diplodus annularis*; DS, *D. sargus*; CE, *Cerastoderma edule*; CG, *C. glaucum*; DV, *D. vulgaris*; TT, *Tellina tenuis*; HD, *Hediste diversicolor*; NC, *Nephtys caeca*; NH, *Nephtys hombergi*; AM, *Arenicola marina*; DN, *Diopatra neapolitana*; Tt, *Tadorna tadorna*; SM, *Somateria mollissima*; SS, *S. spectabilis*; AF, *Aythya ferina*; LAM, *Larus argentatus michahellis*; LCM, *L. cachinnans michahellis*. Data are chronologically exhibited according to published year and separated by trematode species. Reference; study location; intermediate host species [with respective mention to host size (mean and/or range of the shell length or age) and infected tissues when available]; prevalence of infection in the first intermediate host (mean, range or both) and main findings are specified.

the first and second intermediate host) was identified as being the same species described almost 100 years before by Looss in its adult stage (Looss, 1902), i.e. *M. parvus*.

Bartoli *et al.* (2000) provided thus, the first full description of *M. parvus* life cycle (all references gathered in Table 2): (1) the sporocysts invariably start to occur in the gonad, dispersing then to the gills, foot and visceral mass of *C. edule* or *C. glaucum* presenting a variable size range [from  $445 \times 143$  to  $919 \times 162 \mu\text{m}$  (Bartoli *et al.*, 2000)] that depends on the sporocyst content (cercariae or metacercariae, respectively); (2) the cercariae [body length ranging from 91 to  $120 \mu\text{m}$  (Bartoli *et al.*, 2000; de Montaudouin *et al.*, 2000)] do not leave the first intermediate host, remaining inside the daughter sporocyst; (3) the metacercariae [variable diameter between 65 and  $298 \mu\text{m}$  (Bartoli *et al.*, 2000; de Montaudouin *et al.*, 2009)] encystment occurs inside the first intermediate host, which is therefore also the second intermediate host; (3) the adult stages were found in wild *Diplodus annularis*, in wild and experimentally infected *D. sargus* and in wild *D. vulgaris* with maximum length varying between 480 and  $629 \mu\text{m}$  (Bartoli *et al.*, 2000).

**Distribution and prevalence:** As mentioned before, *M. parvus* was observed for the first time in the Adriatic Sea infecting the definitive host *Diplodus* spp. (Looss, 1902). Since then, it was described infecting cockles from Tjärnö, in Sweden (Jonsson and André, 1992) to Merja Zerga, in Morocco (Gam *et al.*, 2008), including several estuaries and coastal lagoons of the European Atlantic coast [Germany (e.g. Thieltges and Reise, 2006), UK (e.g. Lebour, 1905), France (e.g. de Montaudouin *et al.*, 2000), Spain (e.g. Iglesias, 2006), Portugal (e.g. Russell-Pinto, 1993)] and Mediterranean Sea (Bartoli and Gibson, 2007).

*Monorchis parvus* was often referred to as a rare trematode (Boyden, 1970; Lauckner, 1983), whose prevalence in cockles ranged most commonly of low values (0.07% registered in the Crouch estuary, UK by Boyden (1970) to rare episodes of high values (81% registered in Tjärnö, Sweden by Jonsson and André (1992)). The shell length of these infected cockles was rarely specified in the literature. Nevertheless, there is some evidence of a *M. parvus* random distribution within age and size groups of the first intermediate host (Sannia and James, 1978). This indicates that all age groups are equally susceptible to infection by this parasite which combined with the usual low prevalence may protect the host population as a whole. Some authors agree that *M. parvus* presents a seasonal behaviour with young stages most commonly found in late winter and spring (Boyden, 1970) and large number of developing cercariae during the summer (Sannia *et al.*, 1978), possibly related to definitive host seasonal migratory movement that arrives to cockles beds in late winter and leaves after spring (Boyden, 1970; Sannia and James, 1978).

**First host effects:** *Monorchis parvus* can occupy 4-22% of the total cockle tissue (Dubois *et al.*, 2009); therefore, old mature infections (i.e. well-developed sporocysts containing mainly fully developed cercariae and metacercariae) exert an excessive destruction of the host tissues and visceral mass, consuming the host energy, inhibiting shell growth, impairing the cockles burrowing capacity, increasing the susceptibility to infection by other digenaeans species and consequently resulting in cockles mass mortality (Sannia and James, 1978; Jonsson and André, 1992; Dubois *et al.*, 2009).

#### Long-term monitoring

Shell length of cockles infected by *M. parvus* ranged from 6 to 38 mm. The maximal prevalence was 5.00% for the 38 mm shell length class (Fig. 2). Cockle shell length and *M. parvus* prevalence showed a positive correlation ( $R = 0.37$ ;  $P = 0.02$ ).

From the total number of sampled cockles, 5377 (i.e. 92%) were positioned in the 6-38 mm shell length range and therefore



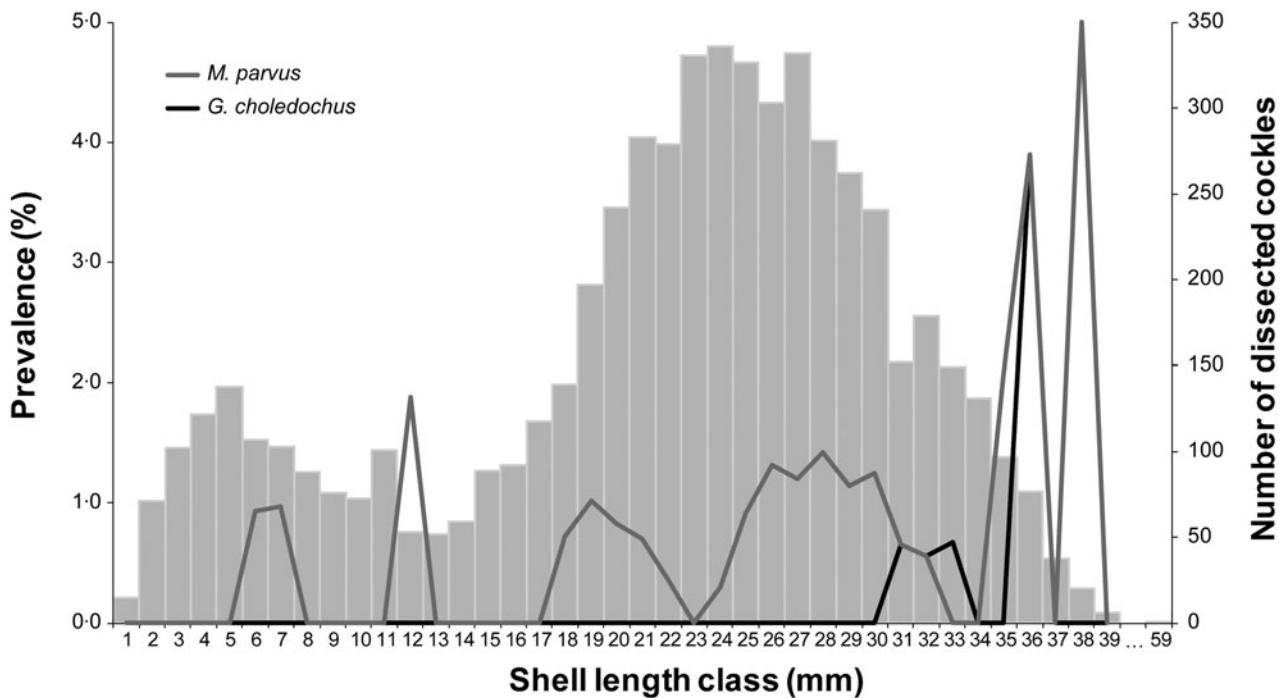


Fig. 2. Prevalence of *Monorchis parvus* (grey line) and *Gymnophallus choledochus* (black line) by shell length class and a respective number of dissected cockles (bars).

susceptible to be infected by *M. parvus*. Out of these cockles, 41 were infected by *M. parvus* with an overall prevalence of 0.76%.

The prevalence of *M. parvus* ranged between 0.08% in September and 1.32% in October (Fig. 3A,  $N = 21$  years) without significant differences (Kruskal–Wallis ANOVA on ranks,  $P = 0.71$ ). The prevalence of *M. parvus* was different among years (one-way ANOVA,  $P < 0.001$ ). The Tukey *post hoc* test discriminated 4 years as different from the others: 1999–2000, 2006–2007, 2014–2015 and 2015–2016 with 2.48, 2.55, 3.33 and 1.67%, of prevalence, respectively (Fig. 3B).

There were no significant correlations between the mean prevalence of *M. parvus* per year and the mean temperature of each month, each year, each previous year and yearly cockle density.

The 41 pairs of cockles infected and non-infected by *M. parvus* were analysed considering the abundance and species richness of the other trematode species using *C. edule* as the second intermediate host. Species richness was the only variable that showed to be slightly but significantly lower (paired Wilcoxon test,  $P = 0.04$ ) when cockles were found positive for *M. parvus* infection (Table 3).

#### Molecular identification

Sequences of 18S, ITS1 and COI were obtained for specimens previously identified with morphological criteria. The amplified product of 18S, ITS1 and COI for *M. parvus* presented 525, 889 and 329 bp, respectively. The ITS1 sequences were identical to those stored in Genbank (KM268112) but no identical sequences were found for 18S and COI.

#### *Gymnophallus choledochus* Odhner, 1900

##### Literature review

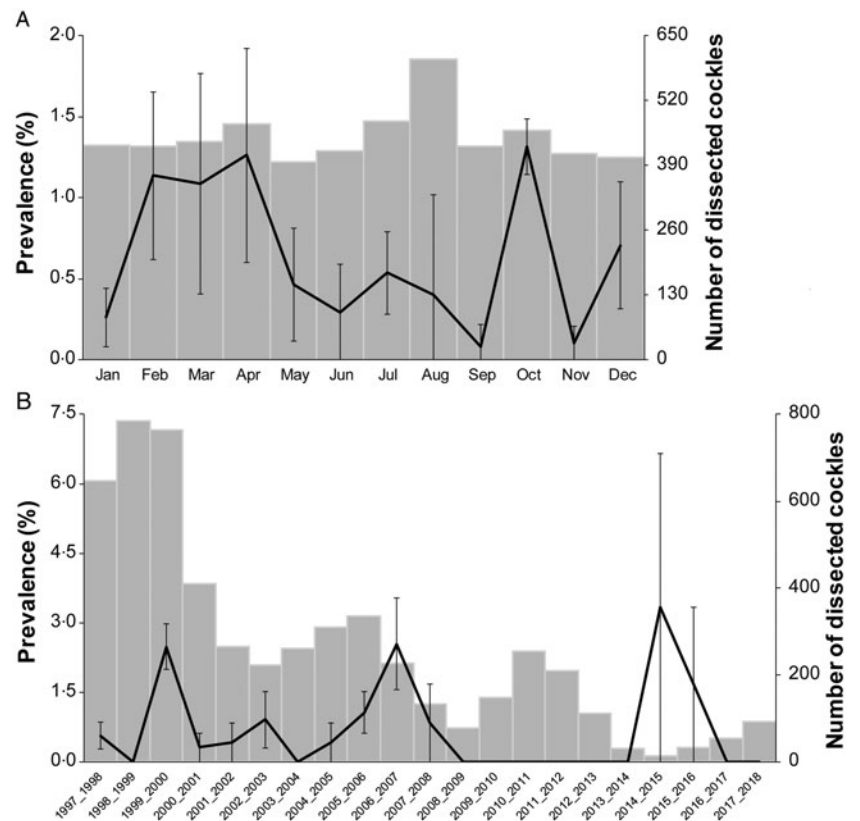
**Description and life cycle** (Fig. 4): *Gymnophallus choledochus* was originally described by Odhner (1900) infecting its definitive host *Tadorna tadorna* (the common shelduck) in Sweden. It is a parasite from the Platyhelminthes phylum, Trematoda class, Digenea subclass and Gymnophallidae family, known by its furcocercous cercariae. Gymnophallids are a small group of digeneans found

infecting a narrow group of marine birds as the definitive host whose typical life cycle includes bivalves as the first intermediate host and the same or other bivalves and polychaetes as the second intermediate host (Scholz, 2002).

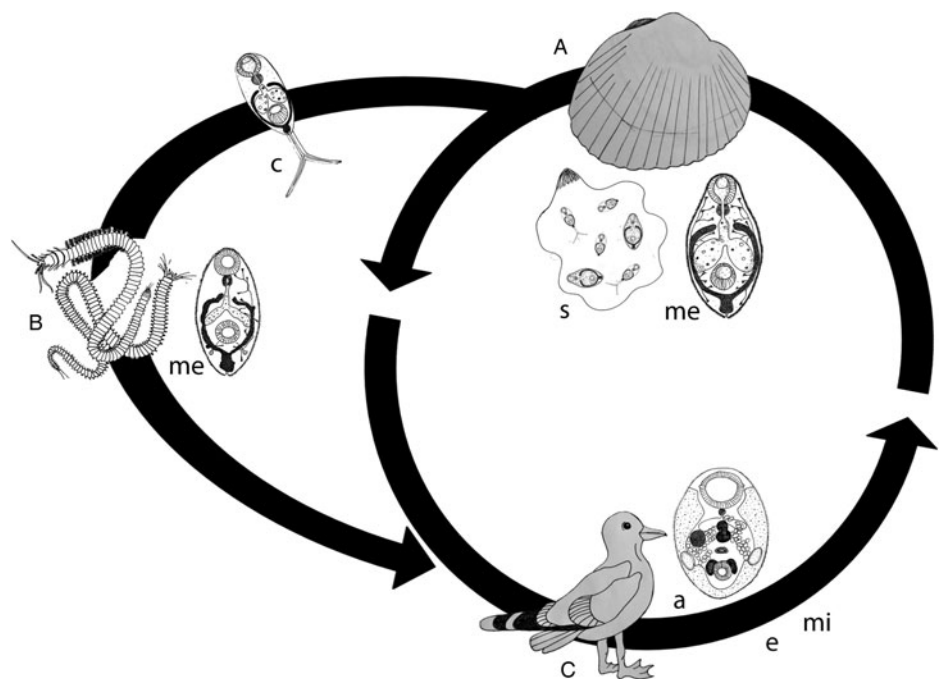
In fact, the first observation of *G. choledochus* infecting *C. edule* was made by Huet (1888), whose work included a complete description of sporocyst and cercaria stages but without assigning a name. Since then, *G. choledochus* was found infecting cockles of the genus *Cerastoderma* but wrongly assigned to *Cercaria fissicauda* (Johnstone, 1905), *C. strigata* (Lebour, 1908), *C. dichotoma* (Bowers, 1965; James and Bowers, 1967; Pascoe *et al.*, 1968; Boyden, 1970; Richards *et al.*, 1970) and *C. hueti* (Cheng, 1967). *Gymnophallus choledochus* was also previously named by its synonyms *C. fulbrighti* and *G. fulbrighti* (Hutton, 1952) and its life cycle was only fully described for the first time in 1969 (Loos-Frank, 1969).

The sporocysts and cercariae of *G. choledochus* start to occupy the gonad of *C. edule* and *C. glaucum* (all references gathered in Table 2). Polychaetes such as *Hediste diversicolor*, *Nephtys hombergii*, *N. caeca*, *Arenicola marina* and *Diopatra neapolitana* are usually infected as the second intermediate host by unencysted metacercariae. This parasite can also follow an alternative pathway and settle as metacercariae in the first intermediate host (*Cerastoderma* spp. cockles), inside the sporocyst. This host alternation particularity of the *G. choledochus* life cycle was attributed either to low temperatures during the cold season (Frank, 1969; Loos-Frank, 1969) or due to evolutionary suppression of the polychaete second intermediate host (e.g. Russell-Pinto *et al.*, 2006). The adults of *G. choledochus* mature in the gall-bladder of gulls, ducks and wading birds.

**Distribution and prevalence:** *Gymnophallus choledochus* was found infecting cockles from the North Sea (e.g. Loos-Frank, 1969) to the northern African coast [Morocco (Gam *et al.*, 2008), Tunisia (Derbali *et al.*, 2009)] and other areas of the Mediterranean Sea (e.g. Bartoli, 1984). However, it was mainly reported in estuaries and coastal lagoons of the European Atlantic coast: Germany (e.g. Lauckner, 1971), UK (e.g. Lebour, 1908), France (e.g. Bartoli, 1971) and Portugal (e.g. Russell-Pinto, 1993).



**Fig. 3.** Prevalence of *Monorchis parvus* ( $\pm$ standard error) and a respective number of dissected cockles placed in the parasite infection size range (6–38 mm), (A) per month gathering all sampled years and (B) per year.

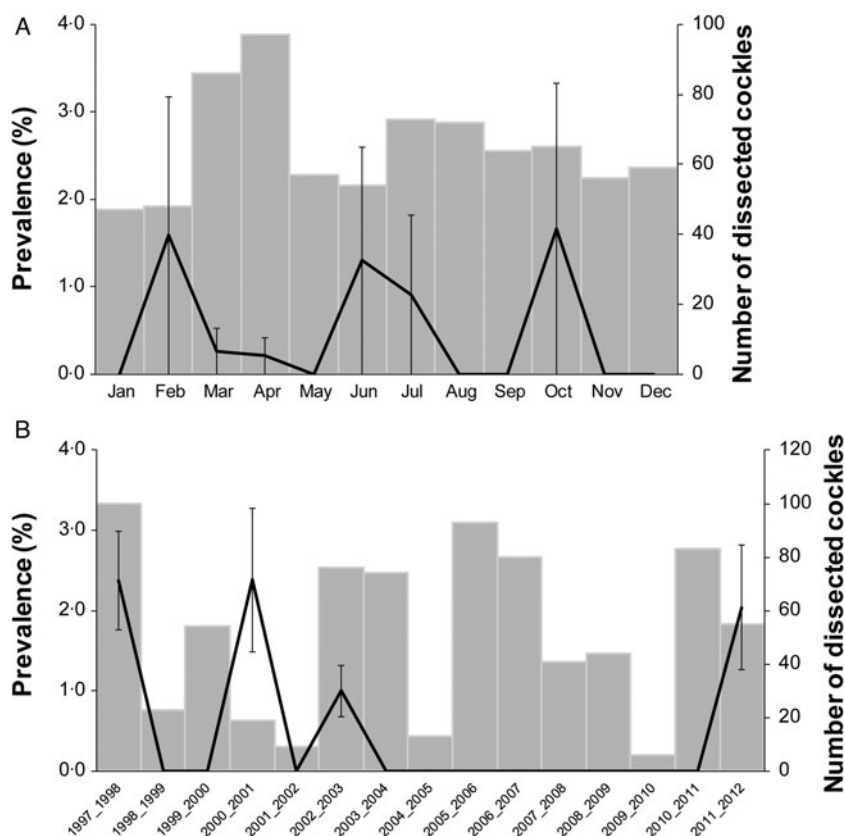


**Fig. 4.** Schematic representation of the *Gymnophallus choledochus* life-cycle with parasite life stages and respective intermediate hosts. (A) *Cerastoderma* spp., the host of sporocysts (s) and metacercariae (me); (B) Several polychaete species, hosts of unencysted metacercariae (me); eggs (e) and miracidia (mi) are not represented and (C) shorebirds, hosts of the adult (a) form.

The prevalence of *G. choledochus* in the first intermediate host is often low, ranging between 0.05 and 10%, both reported for the Wadden Sea, Germany (Feis et al., 2015), but can also reach high values [71% registered also in the Wadden Sea by Thieltges (2006)]. The few references that indicate the infected cockles size range reveal a preference of the parasite for adult hosts (Thieltges and Reise, 2006; Gam et al., 2008). Similarly to what was observed with other trematode species infecting cockles as the first intermediate host (Magalhães et al., 2015), *G. choledochus*

is considered a rare species (Boyden, 1970; Bartoli and Combes, 1986) which consequently makes difficult to study patterns such as infection seasonality. Nevertheless, Boyden (1970) noted the youngest sporocyst stages of *G. choledochus* occur from October until March with final cercarial stages only visible between May and August and Thieltges (2006) reported a *G. choledochus* outbreak in summer.

*First host effects:* The sporocyst (or redia) stage of a trematode life cycle multiplies asexually, ultimately invading all host tissues



**Fig. 5.** Prevalence of *Gymnophallus choledochus* ( $\pm$ standard error) and a respective number of dissected cockles placed in the parasite infection size range (31–36 mm), (A) per month gathering all sampled years and (B) per year.

and therefore being the most deleterious stage (Lauckner, 1983), with the ability to impair some of the basic functions of its host. *Gymnophallus choledochus* is not an exception, reported as causing bloating of the viscera and foot of cockles, the disappearance of gonadal tissue, being even able to reduce or disrupt their burrowing capacity and resistance to thermal stress (Lauckner, 1983). *Gymnophallus choledochus* was reported as the probable cause of cockles occurring on the sediment surface (Lauckner, 1983). Mass mortalities (Thieltges, 2006) can be caused directly by the tissue destruction described above or indirectly by energy depletion. Accordingly, some authors demonstrated that mature sporocysts, i.e. containing cercariae and eventually metacercariae, present higher oxygen consumption (Pascoe *et al.*, 1968) and higher metabolic needs (Richards *et al.*, 1970).

#### Long-term monitoring

*Gymnophallus choledochus* infected cockles within a narrow shell length range (31–36 mm). The maximal prevalence was 3.90% for the 36 mm shell length class (Fig. 2). Cockle shell length and *G. choledochus* prevalence showed a positive correlation ( $R = 0.36$ ;  $P = 0.02$ ).

From the total number of sampled cockles, 785 (i.e. 13%) were positioned in the susceptibility range for *G. choledochus* infection. Out of these cockles, six were found positive for this parasite infection with an overall prevalence of 0.76%.

The prevalence of *G. choledochus* ranged between 0.21% in April and 1.67% in October (Fig. 5A) without significant differences (one-way ANOVA,  $P = 0.78$ ). The prevalence of *G. choledochus* ranged between 1% in 2002–2003 and 2.38% in 2000–2001 (Fig. 5B) without significant differences (one-way ANOVA,  $P = 0.52$ ).

A significant positive correlation ( $P < 0.05$ ) between *G. choledochus* mean prevalence and mean monthly temperature was

only found at two occasions in April ( $R = 0.59$ ) and in July ( $R = 0.57$ ).

The six pairs of cockles infected and non-infected by *G. choledochus* were analysed considering the abundance and species richness of the other trematode species using *C. edule* as the second intermediate host and showed no significant differences (paired Wilcoxon test,  $P > 0.05$ ) (Table 3).

#### Molecular identification

Sequences of 18S, ITS1 and COI were obtained for specimens previously identified with morphological criteria. The amplified products of 18S, ITS1 and COI for *G. choledochus* presented 554, 554 and 284 bp, respectively. The ITS1 sequences were identical to those stored in Genbank (Y18937) but no identical sequences were found for 18S and COI.

#### Trematode species found and sporocyst coinfection

During the whole study period (October 1997–September 2018), 5833 cockles were dissected (with shell length ranging between 1 and 59 mm) and 12 different trematode species were identified: one species using cockles exclusively as the first intermediate host – *B. minimus* (Bucephalidae); two species using cockles both as the first and second intermediate host – *G. choledochus* (Gymnophallidae) and *M. parvus* (Monorchiiidae); and nine species using cockles exclusively as the second intermediate host – *Curtuteria arguinae*, *Himasthla continua*, *H. elongata*, *H. interrupta* and *H. quissetensis* from the Himasthliidae family, *Parvatrema minutum*, *Psilostomum brevicolle*, *Renicola roscovitus* and *Diptherostomum brusinae* from the families Gymnophallidae, Psilostomidae, Rencolidae and Zoogonidae, respectively.

The number of double infections found was significantly lower than the expected probability ( $P < 0.001$ ). Out of the 41 cockles

found infected by *M. parvus*, one presented a double infection with *B. minimus*. From the six cockles infected by *G. choledochus*, one was simultaneously infected by *B. minimus*. *Monorchis parvus* and *G. choledochus* were never found infecting the same cockle.

## Discussion

### Low prevalence as first intermediate host

This present first long-term data analysis of the relationship between *M. parvus* and *G. choledochus* and their first (and second) intermediate host confirms that low prevalence is the most natural scenario. Poulin and Mouritsen (2003) showed that among 54 species of gastropods, only 16.9% ( $\pm 20.3$ ) were infected by several trematode species (mean species richness = 3); Thieltges *et al.* (2008) refer to a mean prevalence of 6.8% ( $\pm 10.31$ ) concerning 16 different trematode species using gastropods as the first intermediate host; and Magalhães *et al.* (2015) reviewing the literature on a single trematode species infecting cockles as the first intermediate host showed that low prevalence is frequent. Being an outcome of long-term monthly monitoring, this study departs high prevalence outbreaks and subsequent host mass mortality as the cause for this constant low prevalence. At least in the studied area, low prevalence is more likely to be the consequence of a complex and rare interaction between abiotic and biotic factors.

It is clear in trematode ancestry studies, the close association between molluscs and parasites within which gastropods are often considered the plesiomorphic form (Galaktionov and Dobrovolskij, 2003). In this sense, bivalve appearance in trematode life-cycle, especially as the first intermediate host, is later in the trematode/host coevolution and results from gastropod host-switching (Cribb *et al.*, 2003). This evolutionary pattern associated with the narrow specificity of miracidium infection may be responsible for the commonly observed low prevalence. On the other hand, with evolutionary theories aside, bivalve sedentary habit (living near in the same place all their life) and the related restricted diet [focused mainly on suspended organic material (Iglesias *et al.*, 1992)] can also contribute to the low prevalence of sporocysts. Convergent ideas were exposed by Bartoli (1981) when discussing a limited penetration of gymno-phallid cercariae associated with limited host mobility. Simultaneously, different hosts, even belonging to the same species, may present different behaviours or phenotypes making them more or less susceptible to a given infection. As an example, trematode infection in different snails of the same species was found to be dependent not on the miracidium abundance but on each snail ecotype (Bojko *et al.*, 2017).

### Size-dependent infection

The majority (86% of the total prevalence) of *M. parvus* infection occurred in cockles with shell length >18 mm, indicating a preferential development of the sporocysts in mature cockles. In fact, this parasite tends to establish first and to feed in the cockles gonads (de Montaudouin *et al.*, 2009; Dubois *et al.*, 2009), a more favourable microhabitat [e.g. gonads store most lipids (Fernandez-Reiriz *et al.*, 2015)]. Therefore, the 'adults preference' referred by some authors concerning trematode infection (Magalhães *et al.*, 2015 and references within) could be no more than a different time scale between miracidium penetration and sporocysts maturation among species.

Conversely to what was found with the *M. parvus* infection profile, *G. choledochus* was only registered as infecting cockles in a narrow size range (>31 mm). Miracidia of the genus *Gymnophallus* emerge only after the eggs reach the digestive gland of the first intermediate host (passive infection) (Stunkard and Uzman,

1958). Thus, assuming that *G. choledochus* eggs have the theoretical potential to infect the cockles of any size class through filtration activity, this larger shell length 'preference' can be interpreted as a longer (compared to *M. parvus*) period between eggs entrance and sporocysts development and/or as a different level of pathogenicity among the two trematode species. However, this result could be a sign that *G. choledochus* has a preference for adult cockles, probably related to gonad development in the host (Thieltges and Reise, 2006; Gam *et al.*, 2008).

Finally, both trematode species were significantly more prevalent in larger cockles. Beyond what was previously stated, overall it is assumed that larger (and consequently older) cockles are more likely to be infected because they were longer exposed to parasite pressure and filter more intensively. This same positive correlation between cockles size and trematode prevalence was previously recorded (Thieltges, 2008; Magalhães *et al.*, 2015, *et cetera*).

### Infection seasonality

It was expected to find a seasonal pattern in cockle infection, at least in the case of *G. choledochus* due to the seasonal variation of its definitive host (marine birds), which in practice was not verified. This lack of seasonal influence on the prevalence of these two trematodes can be ascribed to constant favourable conditions for miracidium infection regardless of seasonal abiotic and biotic environmental factors. Notwithstanding, data from small samples (in this case, 10 cockles/cohort/month) must be interpreted with caution, even though they are issued over 21 years. Besides, the seasonality pattern should be preferentially assessed through evaluation over time of the larvae maturation stage rather than only assessing the presence/absence. *Monorchis parvus* prevalence did not follow any seasonal pattern and the same conclusion as for *G. choledochus* can be suggested. *Monorchis parvus* prevalence showed to be significantly higher in 1999–2000, 2006–2007, 2014–2015 and 2015–2016 (the latter with less expression). However, temperature does not explain these higher prevalence values, suggesting the influence of other abiotic or biotic factors is still to be identified.

### Interspecific parasite co-occurrence

Neither *M. parvus* nor *G. choledochus* infection influenced the prevalence and abundance of other trematode species using cockles as the second intermediate host, contrary to what was observed in the same study area but concerning *B. minimus* infection (Magalhães *et al.*, 2015). Nonetheless, those cockles infected by *M. parvus* presented lower trematode species richness compared to non-*M. parvus*-infected cockles. Interspecific competition may potentially be determining this dominance, meaning that the presence of *M. parvus* is able to exclude the other trematode species either by consuming the entire space (priority of occupancy) or even by direct competition. Observations of sporocysts antagonistic interaction have already been reported in a freshwater snail host (Basch *et al.*, 1969). Lower trematode species richness in cockles infected by *M. parvus* can also result from physiological alterations induced by this parasite invasion, such as the decrease in the host filtration capacity making it less likely to be infected by other species (de Montaudouin *et al.*, 1998). However, the literature available on cockles infection by *M. parvus* showed an opposite trend (§ 3.1.1), caution is needed when interpreting these results because the lower species richness found in *M. parvus*-infected cockles may just be the result of the difficulty to determine other species presence masked by the dense mass of *M. parvus* sporocysts that can represent up to 22% of the total cockle tissue (Dubois *et al.*, 2009).

**Table 3.** Results of Wilcoxon pairwise test for comparison between infected (+) or not infected (–) cockles in terms of trematode species abundance and species richness

	Total abundance	Species richness
<i>Monorchis parvus</i>		
+	208 ± 356	3.41 ± 1.86
–	227 ± 408	3.95 ± 1.61
Z	1.143	2.047
P	0.253	0.041
N	41	41
<i>Gymnophallus choledochus</i>		
+	78 ± 85	3.8 ± 2.4
–	60 ± 69	3.5 ± 3.2
Z	1.363	0.524
P	0.173	0.600
N	6	6

Similar to other mollusc hosts (Kuris and Lafferty, 1994), cockles presented no or rare double infection by parasites using this bivalve as the first intermediate host. Again, it can be the result of general low prevalence, making it less likely to detect a coinfection in the sample. However, some authors loss of host chemical attractiveness (Baiocchi *et al.*, 2017), physiological or behavioural alterations (Binning *et al.*, 2017) or changes in the immunity (Loker, 1994) that prevent the development of later infections.

#### Molecular identity of *G. choledochus* and *M. parvus*

Finally, molecular identification was performed for both species using 18S, ITS1 and COI sequences. Concerning *M. parvus*, ITS1 sequence matched with other sequences stored in GenBank. Surprisingly, our sequence also matched completely with a sequence of *M. monorchis* (Accession number: Y18937) (Bartoli *et al.*, 2000). However, confusion occurs because the sequence is associated with *M. monorchis* but details refer to *M. parvus*. The ITS1 sequence used for *G. choledochus* in the present study matched with two other sequences stored in GenBank. Our study provides for the first time 18S and COI sequences for *G. choledochus*. Our COI sequence showed no match with a GenBank stored sequence (Feis *et al.*, 2015) due to correspondence to different parts of the same gene. These data and species identity confirmation provides on the one hand a quality control of the stereomicroscope morphological identification and on the other hand will allow further confirmation (or not) of the identity of sporocysts found infecting cockles in other parts of their distribution area. Parasites found to be morphologically similar to *M. parvus* or *G. choledochus* can therefore be more accurately identified in the future.

#### Conclusion

Overall, this study on the occurrence of two trematode species in their first intermediate host improves our understanding of the relationship between trematode parasites and their bivalve hosts and of the processes shaping the prevalence and distribution of parasitism. By confirming that low prevalence is often the rule, this study highlighted that beyond constant trematode assemblage monitoring, there is a need to identify the main predictors of sporocysts infection, such as the definitive host presence and

abundance and eggs/miracidium infection processes, for improved future management of severe host disease and mortality episodes. *Diploodus* spp. (Bauchot, 1987) are dispersed along the whole climate gradient, although differing in abundance and diversity according to the depicted territory. In its turn, wading birds are known as long distance travellers, but fish species chose certain geographic locations that present better survival conditions (UNEP/CMS, 2014). Hence, changes in the distribution of these definitive trematode hosts during the last decades due to climate-related migration (Howard *et al.*, 2018; Morley *et al.*, 2018) may lead to new records of these parasites in other regions or to changes in the current prevalence patterns. Future studies are encouraged, as there are certainly numerous parasite species remaining to be described and many infection modelling processes to be identified.

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