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Parasites, pathological conditions and resistance to *Marteilia cochillia* in lagoon cockle *Cerastoderma glaucum* from Galicia (NW Spain)

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ABSTRACT: A histopathological survey revealed parasites and pathological conditions affecting lagoon cockles *Cerastoderma glaucum* along the Galician coast; serious pathological threats were not detected because the potentially pathogenic conditions (infections with a *Marteilia*-like parasite and bucephalid sporocysts, disseminated neoplasia and a condition involving large foci of heavy haemocytic reaction) were rare, while more prevalent parasites had negligible or limited pathogeny. Considering that *C. edule* and *C. glaucum* are sympatric in some Galician rias, it is remarkable that *C. glaucum* was not seriously affected by *Marteilia cochillia* while *C. edule* suffered an intense outbreak of this parasite associated with massive mortality. Comparison of the digestive gland between cockle species showed co-occurrence of digestive tubules in different phases, with abundant disintegrated tubules, in the case of *C. glaucum,* while *C. edule* showed synchronicity and absence of fully disintegrated tubules; these differences could influence their susceptibility to *M. cochillia* because the main location of this parasite in common cockles is the epithelia of the digestive gland. Moreover, the observation of histological sections through the digestive gland easily allows differentiating the 2 cockle species.

KEY WORDS: *Rickettsia*-like organism · *Nematopsis* · *Pseudoklossia* · Ciliates · *Steinhausia* · *Paravortex* · Trematoda · Disseminated neoplasia

INTRODUCTION

Populations of 2 commercially exploited cockle species, viz. *Cerastoderma edule* and *C. glaucum*, occur along the European coasts. The common cockle *C. edule* occupies a continuous range in tidal shallow water areas from northern Africa along the eastern Atlantic coasts to northern Norway and Murmansk in the Arctic and is not found in the Mediterranean and Baltic Seas (Krakau et al. 2012). The lagoon cockle *C. glaucum* has a wide distribution around European coasts, ranging from the northern Baltic Sea to the Black Sea, the Caspian Sea and even the Aral Sea (Nikula & Väinölä 2003). The distribution area of *C. glaucum* is very fragmented, as it cannot survive in loose-sediment habitats exposed to currents and waves (Brock 1979), and typically inhabits closed brackish-water lagoons and estuaries (Tarnowska et al. 2010). Both cockle species overlap part of their range, and there are several areas where the 2 cockle species live in

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sympatry (Tarnowska et al. 2012). In those overlapping areas, distinguishing the 2 species based on morphological traits is quite difficult but possible (Brock 1978, Machado & Costa 1994). A reliable discrimination between the 2 species can be performed on the basis of molecular methods (Malham et al. 2012). Furthermore, ecological, physiological and biochemical differences have been shown (Boyden 1970, Malham et al. 2012). Reise (2003) summarised those differences as follows: *C. edule* has higher burying ability and is better adapted to air exposure than *C. glaucum*; the former produces more, smaller, pelagic eggs, with longer larval longevity and dispersal, while the eggs of the latter are benthic; the tolerance ranges of temperature (3–25°C) and salinity (10–35 psu) of *C. edule* are narrower than those of *C. glaucum* (0–32°C, 4–60 psu), with optimum temperature values of 10 and 20°C, respectively. Literature on parasites and pathological conditions of *C. edule* and *C. glaucum* was reviewed by Longshaw & Malham (2013).

In Galicia, both species co-occur in some exploited beds, but they occupy different ecological niches. Historically, *C. edule* has largely been more abundant and has been the main component of exploited stocks, although both species appeared mixed in the official statistics of captures, simply named 'cockles'. In 2012, mass mortality of the common cockle *C. edule*, caused by the protistan parasite *Marteilia cochillia*, occurred in the Ría de Arousa, one of the most productive cockle-growing areas of Galicia, leading to cockle fishery collapse in this ria and, thus, a huge decrease in Galician cockle landings (Villalba et al. 2014). Since then, newly recruited common cockles have also become infected with *M. cochillia*, which has impeded *C. edule* fishery recovery (authors' unpubl. data). Interestingly, *C. glaucum* was not affected by mortality in those Galician marteiliosis-affected beds where both species lived in sympatry. As a consequence, 'cockles' as shellfish have attained a much higher price, and the presence of *C. glaucum* has recently increased in the fresh market. *M. cochillia* infects the digestive gland of the cockle, causing dysfunction of this organ (disrupting food absorption) and fatal outcome. The digestive gland is affected by cyclic morphological changes linked to cycles of feeding and digestion (Morton 1970, Mathers 1976, Mathers et al. 1979). Most studied bivalve species show a monophasic cycle by which all digestive tubules of an individual have the same appearance at any one time, while in some other species, the digestive tubules act in a diphasic cycle and 2 distinct forms occur simultaneously within an individual. One of the most obvious differences between monophasic and diphasic cycles is the occurrence of disintegrated tubules in the latter, whereas tubules never fully disintegrate in the former (Mathers 1976, Mathers et al. 1979). Various studies have been published on different pathological issues of *C. edule* in Galicia (Villalba et al. 2001, 2014, Carballal et al. 2001, 2003, Ordás & Figueras 2005); conversely, only 1 article has been published regarding *C. glaucum* in Galicia, devoted to a case of disseminated neoplasia in the Ría de Vigo (Rodríguez et al. 1997).

Here we report on a histopathological survey performed from 2012 to 2015 in sympatric cockle populations of Galicia, where *C. edule* have been decimated by marteiliosis; the survey was originally envisaged to assess whether lagoon cockles *C. glaucum* were affected by *M. cochillia*. Particular attention was paid to the digestive gland, the organ most seriously affected by marteiliosis. Furthermore, this survey provided new information on the parasites and pathological conditions affecting *C. glaucum* and allowed identification of reliable histological features of the digestive gland, enabling discrimination between the 2 cockle species. The information presented here could provide important data for future epidemiological assessment including comparison studies between congeneric *C. edule* and *C. glaucum*.

MATERIALS AND METHODS

Samples

The survey involved collecting a total of 520 market-sized (>25 mm in length) lagoon cockles *Cerastoderma glaucum* from 10 intertidal beds along the Galician rias of Arousa, Pontevedra and Vigo (Fig. 1), in the period 2012 to 2015. Sampling dates and sample sizes in the different localities are shown in Tables 1 and 2. Identification of the cockle species was performed using previously described diagnostic characters based on shell morphology, including shell shape, ligament length to shell width ratio, valve profile, shape of the ventral valve junction and the amount of periostracum (Boyden 1971, Brock 1978, Machado & Costa 1994, Chao 2002). The fact that *C. glaucum* was the only cockle species occurring in most study beds at the sampling time, as a result of mass mortality of *C. edule* caused by marteiliosis, made species identification easier.

Additionally, cockles from 2 beds were collected to compare the histological features of the digestive gland between *C. edule* and *C. glaucum*. Thus, in May 2015, 30 common cockles *C. edule* (28.9 ±

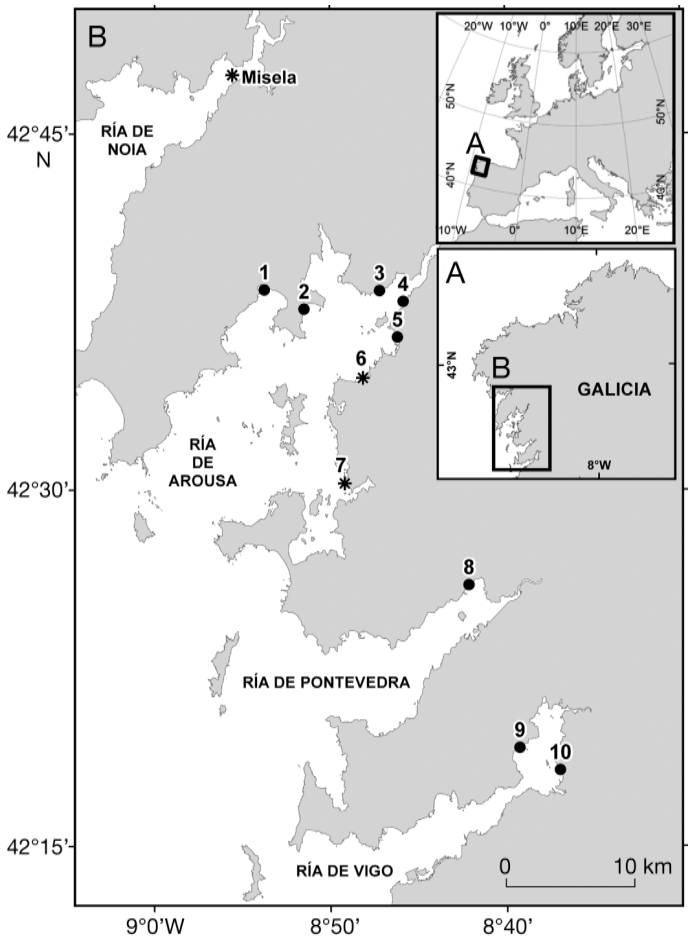

Fig. 1. Locations of the sampled shellfish beds in Galicia, NW Spain. Numbered sites: cockles *Cerastoderma* spp. sampled for pathological analysis (1: Praia de Barraña; 2: Praia de Mañóns; 3: Rianxo; 4: Punta Remuíño; 5: Praia de Compostela; 6: Corón; 7: Sarrido; 8: Combarro; 9: Praia Maior; 10: Praia de Cesantes). Stars: cockles sampled for comparison of digestive gland histological features between *C. edule* and *C. glaucum*

1.8 mm; mean ± SD) from the bed of Misela (Fig. 1) and 30 lagoon cockles *C. glaucum* (29.2 ± 2.3 mm) from the bed of Corón (Fig. 1) were taken and processed histologically. In this case, cockle species identification was performed based on the morphological criteria mentioned above and further confirmed with the 1-step PCR procedure developed by Freire et al. (2011).

Furthermore, cockles of both species were taken at the same time from the bed at Sarrido (Fig. 1) in May 2016. We collected 30 market-sized (>25 mm) common cockles and 9 market-sized (>25 mm) lagoon cockles; all common cockles and 4 lagoon cockles were taken to the laboratory, purged in aerated seawater and processed for histology as explained below, while the remaining 5 lagoon cockles were opened and a piece of meat was separated and immersed in fixative solution immediately after collection in the field, to assess whether purging cockles in water for ca. 24 h (see below) could affect the histological structure of the digestive gland. Finally, 30 market-sized lagoon cockles imported from the Mediterranean coast in January 2014 were also processed to assess whether the histological structure of the digestive gland of *C. glaucum* was influenced by geographical location.

Histology

Each cockle sample was kept in a tank with aerated seawater for ca. 24 h to purge cockles, thus favouring the release of sediment particles that could interfere with tissue sectioning. A piece of meat (5 mm thick) containing visceral mass, foot, mantle and gills was taken from each cockle, fixed in Davidson's solution and embedded in paraffin; samples were then deparaffinised, 5 µm thick sections were stained with Harris's haematoxylin and eosin (Howard et al. 2004) and examined by light microscopy for the presence of parasites and pathological conditions. Two additional histological sections were processed with Feulgen staining (Bancroft & Stevens 1996) to test for the presence of DNA in the cytoplasm of digestive gland cells as a possible sign of infection with DNA viruses.

Transmission electron microscopy (TEM)

Small pieces of digestive gland from 2 lagoon cockles were processed to examine the ultrastructure of the disintegrated digestive tubules that had been observed in histological sections, particularly to look for microorganisms that could be responsible for tubule disintegration. The pieces were fixed in 2% glutaraldehyde in filtered sea water for 2 h at 4°C. They were then postfixed in 2% tetroxide osmium (2 h at 4°C) and embedded in EPON. Ultrathin sections were cut (70–90 nm) and tissues were stained with uranyl acetate and lead citrate and observed in a JEOL 100 CX2 transmission electron microscopy.

PCR procedure for cockle species identification

Small pieces (ca. 5 × 5 mm) of gills of the cockles collected for digestive gland comparison, from Misela and Corón, were preserved in dimethyl sulphox-

ide (DMSO) storage buffer (25 mM EDTA, 20% v/v DMSO and saturated NaCl; Moss et al. 2008) for molecular analysis. DNA extractions were performed with the commercial Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. DNA quality and quantity were checked in a spectrophotometer (Nanodrop®ND-1000, Nanodrop Technologies). DNA was adjusted to 200 ng μl^{-1} with deionised water. The PCR assay described by Freire et al. (2011) was employed for cockle species identification using the universal primer ITS-forward, which targets DNA of both *C. edule* and *C. glaucum*, and the species-specific reverse primers ITS-Ce-R and ITS-Cg-R, which specifically target the ITS1 and ITS2 region of each species, respectively. PCR assays were performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM of each primer and 1.25 U of *Taq* DNA polymerase (Roche Applied Science). A negative control (no DNA) was used in each PCR assay. The amplifications were carried out in a Tgradient thermocycler (Biometra) according to PCR cycling conditions described by Freire et al. (2010, 2011). PCR products were electrophoresed in a 2% agarose gel, stained with RedSafeTM Nucleic Acid Staining (Biotechnology) and visualised in a ChemiDoc™ MP System with Image™ Lab software (BioRad). A 100 bp ladder (Bioline) was used as a molecular mass marker.

Statistical and epidemiological analysis

The percentage of cockles affected by each parasite and pathological condition was determined for each sampled bed included in the survey. Overall prevalence (and 95% confidence interval, CI) of each parasite and pathological condition were calculated for the whole study period using EpiCalc 2000 software Version 1.02 (Gilman & Myatt 1998).

Table 1. Prevalence (%) of prokaryote and protistan parasites of lagoon cockles *Cerastoderma glaucum* from different localities (see Fig. 1). Dates are given as yyyy/mm/dd. RLC: *Rickettsia*-like colonies

Locality	Sampling date	Sample size	Prokaryotes				Protists					
			Bacteria-like colonies[a]		Intracellular RLC		Unidentified apicomplexan	*Nematopsis* sp.	Coccidia		Ciliates	*Marteilia*-like parasite
			Gills	Mantle	Gills	Digestive gland			Kidney	Gills		
Praia de Barraña	2013/06/25	30	3	0	27	53	0	93	0	7	93	0
Praia de Mañóns	2014/04/14	30	0	0	10	7	0	87	3	0	90	0
Rianxo	2013/03/04	45	29	0	33	31	0	71	2	2	89	0
	2014/04/22											
	2015/05/04											
Punta Remuíño	2013/06/05	30	7	13	70	63	0	93	0	0	100	0
Praia de Compostela	2013/06/04	30	3	0	50	60	0	87	0	0	100	7
Corón	2014/04/28	30	0	0	10	13	0	73	3	0	93	0
Sarrido	Monthly	186	2	5	0	0	0	68	7	2	81	0
	Dec 2012 to Dec 2013											
Combarro	2013/12/12	21	0	0	0	10	48	91	0	10	91	0
Praia Maior	2013/09/02	90	67	45	16	12	0	97	0	13	70	0
	2013/11/20											
	2015/06/02											
Praia de Cesantes	2013/05/28	28	32	0	11	25	0	96	0	18	86	0
Overall prevalence (95% CI)		520	17.3 (14.2–20.9)	10.4 (8.0–13.4)	17.1 (14.0–20.7)	17.9 (14.7–21.5)	1.9 (1.0–3.6)	81.0 (77.3–84.2)	2.9 (1.7–4.8)	5.0 (3.4–7.3)	84.6 (81.2–87.6)	0.4 (0.1–1.5)

[a]Surrounded by a fibrous eosinophilic cover

Table 2. Prevalence (%) of fungal and metazoan parasites and other pathological conditions of lagoon cockles *Cerastoderma glaucum* from different localities (see Fig. 1). Dates are given as yyyy/mm/dd. LFHRR: large foci of heavy haemocytic reaction

Locality	Sampling date	Sample size	Fungi	Metazoa					Other pathological conditions	
					Trematodes			Copepods		
			Stein-hausia-like cysts	*Para-vortex* sp.	Bucephalidae sporocysts	Encysted metacercariae	Gymnophallidae-like metacercariae	Gills – pallial cavity	Intestine	Disseminated neoplasia	LFHHR
Praia de Barraña	2013/06/25	30	3	0	0	3	13	3	10	0	0
Praia de Mañóns	2014/04/14	30	0	0	0	0	0	0	0	0	0
Rianxo	2013/03/04	45	0	31	2	0	0	0	0	0	0
	2014/04/22										
	2015/05/04										
Punta Remuíño	2013/06/05	30	0	3	0	0	3	0	0	0	0
Praia de Compostela	2013/06/04	30	0	3	0	3	0	10	0	0	0
Corón	2014/04/28	30	0	3	0	0	0	0	0	0	0
Sarrido	Monthly	186	5	40	0	10	0	0	0	3	0.5
	Dec 2012 to Dec 2013										
Combarro	2013/12/12	21	0	14	0	43	0	5	0	5	0
Praia Maior	2013/09/02	90	0	28	17	3	0	0	3	2	3
	2013/11/20										
	2015/06/02										
Praia de Cesantes	2013/05/28	28	0	11	4	0	0	0	0	4	18
Overall prevalence (95% CI)		520	1.9 (1.0–3.6)	23.6 (20.1–27.6)	3.3 (2.0–5.3)	6.2 (4.3–8.7)	1.0 (0.4–2.4)	1.0 (0.4–2.4)	1.2 (0.5–2.6)	1.9 (1.0–3.6)	1.7 (0.8–3.4)

RESULTS

The examination of histological sections of the lagoon cockles *Cerastoderma glaucum* collected in the survey showed the occurrence of prokaryote, fungal, protistan and metazoan parasites as well as other pathological conditions (Tables 1 & 2).

Prokaryotes

Colonies of tightly packed basophilic bacteria-like organisms, with a fibrous eosinophilic cover, 24 to 42 µm long (n = 10; Fig. 2A), were observed in the gills of cockles from 7 localities (Table 1). Similar colonies 32 to 74 µm long (n = 10) were also observed in the mantle of cockles from 3 localities (Table 1); in cockles from Praia Maior, these types of enveloped colonies were also observed in palps. Few colonies per histological section were observed, and an associated host haemocytic reaction was not detected.

Lagoon cockles from most localities showed intracellular, round, basophilic *Rickettsia*-like colonies in epithelial cells of gills (Table 1); their diameter ranged from 7.1 to 16.7 µm (n = 10; Fig. 2B). Similar intracellular colonies, 9.5 to 14.3 µm long (n = 10), were also seen in digestive tubules (Fig. 2C) and, less frequently, in digestive ducts of cockles from most localities (Table 1). The intensity of infection was light, and no haemocytic response was observed. Various cockles showed *Rickettsia*-like colonies in gills and digestive epithelia.

Protists

Unidentified apicomplexa, 12 to 17 µm in length and 7 to 11 µm in width (n = 10), were observed in intestinal epithelium and in the surrounding connective tissue (Fig. 2D) of cockles from Combarro; infection intensity was low and haemocytic reaction was not observed.

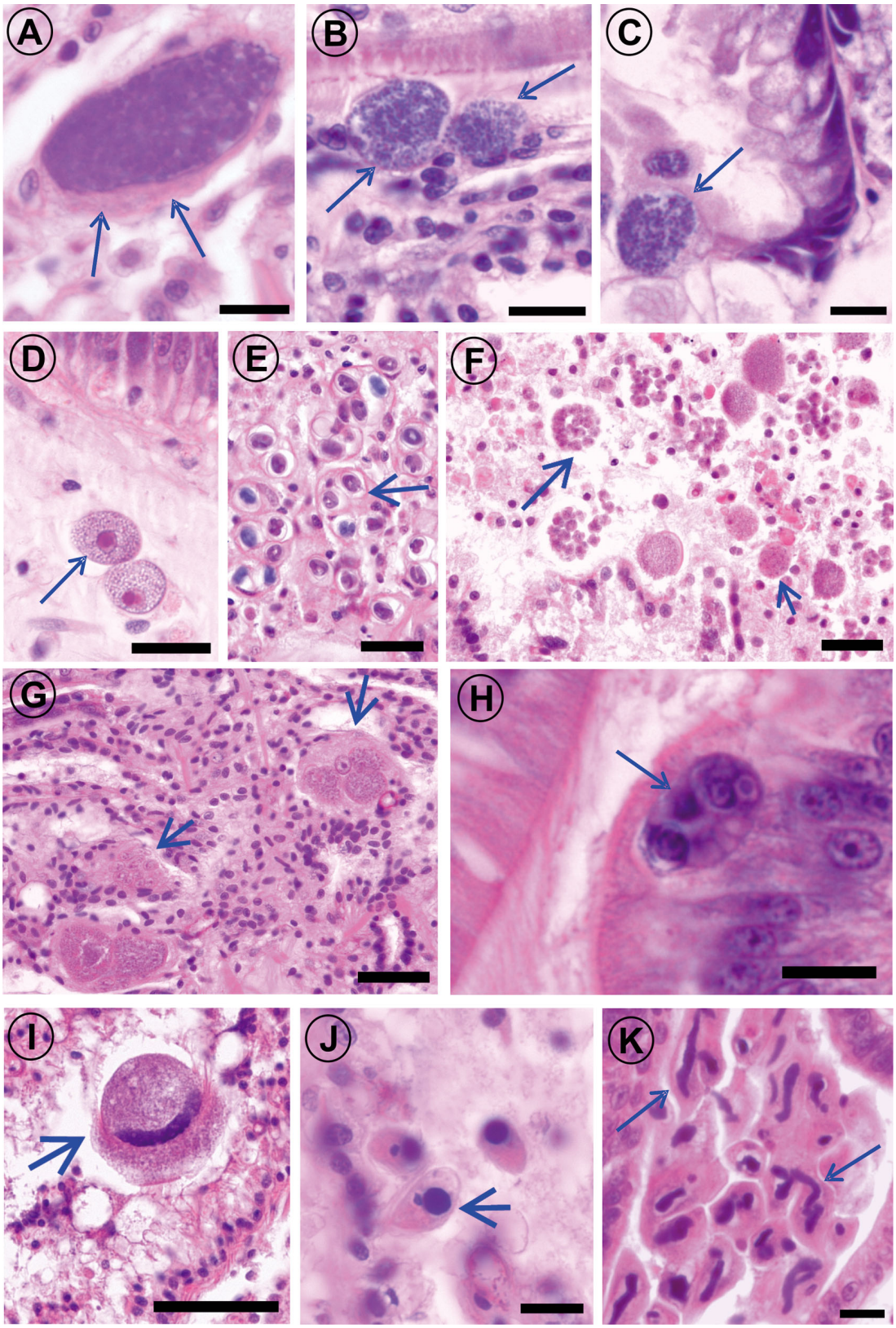

Fig. 2. Histological sections showing parasites of *Cerastoderma glaucum*. (A) Bacteria-like colony surrounded by a fibrous eosinophilic cover (arrows) in the gills. (B) *Rickettsia*-like colonies (arrows) in epithelial cells of the gills. (C) *Rickettsia*-like colonies (arrows) in epithelial cells of a digestive tubule. (D) Unidentified apicomplexa (arrow) in the connective tissue surrounding the intestine. (E) Oocysts of *Nematopsis* sp. (arrow) in the connective tissue of the gills. (F) Sporocysts (arrow) and gamonts (arrowhead) of a *Pseudoklossia*-like coccidian in the nephridium. (G) Unidentified coccidian-like organism (arrows) in the gills. (H) *Marteilia*-like plasmodium (arrow) in the stomach epithelium. (I) Ciliate *Trichodina* sp. (arrow) in the gills. (J) Ciliates *Hypocomella* sp. (arrow) in the gills. (K) Ciliates *Sphenophrya* sp. (arrows) in the gills. Scale bars = (A–C,H,J,K) 10 µm, (D–F) 20 µm, (G) 30 µm, (I) 40 µm

Oocysts of a gregarine *Nematopsis* sp. were detected in every locality with high prevalence (Table 1). Intrahaemocytic oocysts were mostly seen in connective tissue of the gills but also in mantle, digestive gland, gonad and heart. One or 2 oocysts haemocyte^{-1} were frequently observed but up to 4 oocysts haemocyte^{-1} were seen. The oocysts were oval (6 to 10 µm in length and 6 to 8 µm in width, n = 10) and usually contained 1 sporozoite (Fig. 2E). The intensity of infection was generally light, with oocysts appearing isolated or in small foci. Exceptionally, some cockles had more oocysts associated with light lesions, involving haemocyte foci with disrupted *Nematopsis* sp. and brown deposits, mainly in gills.

Pseudoklossia-like coccidians (macro- and microgamonts, oocysts, sporocysts and sporozooites) were observed in the kidney of cockles from 4 localities (Table 1) with light infection intensity (Fig. 2F). Macrogamonts had a rounded shape (about 16 µm in diameter) with refringent cytoplasm, large nucleus and prominent nucleolus; this was the most frequent stage, which appeared inside nephridial cells or in the lumen, some of them attached to the epithelium by a delicate stalk. Microgamonts were rounded and refringent (about 18 µm in diameter) and had numerous microgametes; they were seen unattached in the kidney lumen. Oocysts (about 26 µm in diameter) enclosing sporocysts (about 5 µm in diameter) were seen in the nephridial lumen; 2 sporozoites were observed inside the sporocysts.

Unidentified coccidian-like organisms were seen in the gills of cockles from 6 localities (Table 1). Macrogamonts (12 to 27 µm long, n = 10) and microgamonts (17 to 27 µm mean long, n = 10) were located within host cells (Fig. 2G). The intensity of infection was light and a local haemocytic response was only observed in the cockles with more coccidians.

Some plasmodia resembling immature stages of the sporulation process of a *Marteilia*-like parasite, 7 to 14 µm in length (n = 12), were observed in the apical border of the stomach epithelium of 2 lagoon cockles from 1 locality, Praia de Compostela (Table 1). Some of those plasmodia only enclosed non-divided secondary cells, while others included some secondary cells enclosing non-divided tertiary cells (Fig. 2H). Plasmodia were not detected in the digestive diverticula.

Ciliates were seen in every locality with high prevalence (Table 1). Three kinds of ciliates were observed, viz. *Trichodina* sp., 27 to 47 µm in length (n = 10), which were seen unattached in the pallial cavity close to mantle, labial palps or gill surface (Fig. 2I); *Hypocomella* sp. ciliates, 10 to 12 µm in length (n = 10), pear-shaped with 2 nuclei (macro- and micronucleus; Fig. 2J); and *Sphenophrya* sp. ciliates, 17 to 37 µm in length (n = 10), with a single long nucleus (Fig. 2K). The number of ciliates per histological section was generally low; some cockles showed a higher number of ciliates but a host haemocytic response was not detected.

Fungi

Cysts, 11 to 20 µm long (n = 10), with spores, 1 to 2 µm in diameter, of a *Steinhausia*-like microsporidian were seen infecting ovocytes (Fig. 3A) in cockles from 2 locations (Table 2). Cysts were mostly seen in the ovocyte cytoplasm but sometimes in the nucleus. Ovocytes contained mostly 1 cyst but some of them had 2 or more cysts. Few ovocytes per histological section appeared parasitised and a haemocytic response was not observed.

Metazoans

Turbellarians *Paravortex* sp. were seen in the intestinal lumen and, less frequently, in digestive ducts of cockles from most localities (Fig. 3B, Table 2). One or 2 flatworms per histological section were frequently seen but up to 20 turbellarians per histological section were observed in lagoon cockles from Sarrido. Neither host injury nor host haemocytic reaction were observed.

Urosporidium-like haplosporidians were observed hyperparasitising flatworms in 1 lagoon cockle from Sarrido; haplosporidian cysts with spores (about 4 µm in length) in different degrees of maturity were observed in the connective tissue and external epithelium of the flatworms (Fig. 3B). No cockle haemocytic reaction was observed in association with the hyperparasites.

Different larval stages of digenean trematodes were observed. Sporocysts of a bucephalid trematode containing developing cercariae were detected in 3 localities (Table 2, Fig. 3C). Sporocysts were located mostly in the gonad, mantle and gills but also in digestive gland and foot. A light haemocytic reaction was observed in 1 of the affected cockles. The intensity of infection was mostly heavy, causing host tissue disruption and castration. Two types of trematode metacercaria were also detected in lagoon cockles. Encysted, rather spherical metacercariae were detected in foot, gills and digestive gland but also in palps, mantle and kidney of cockles from

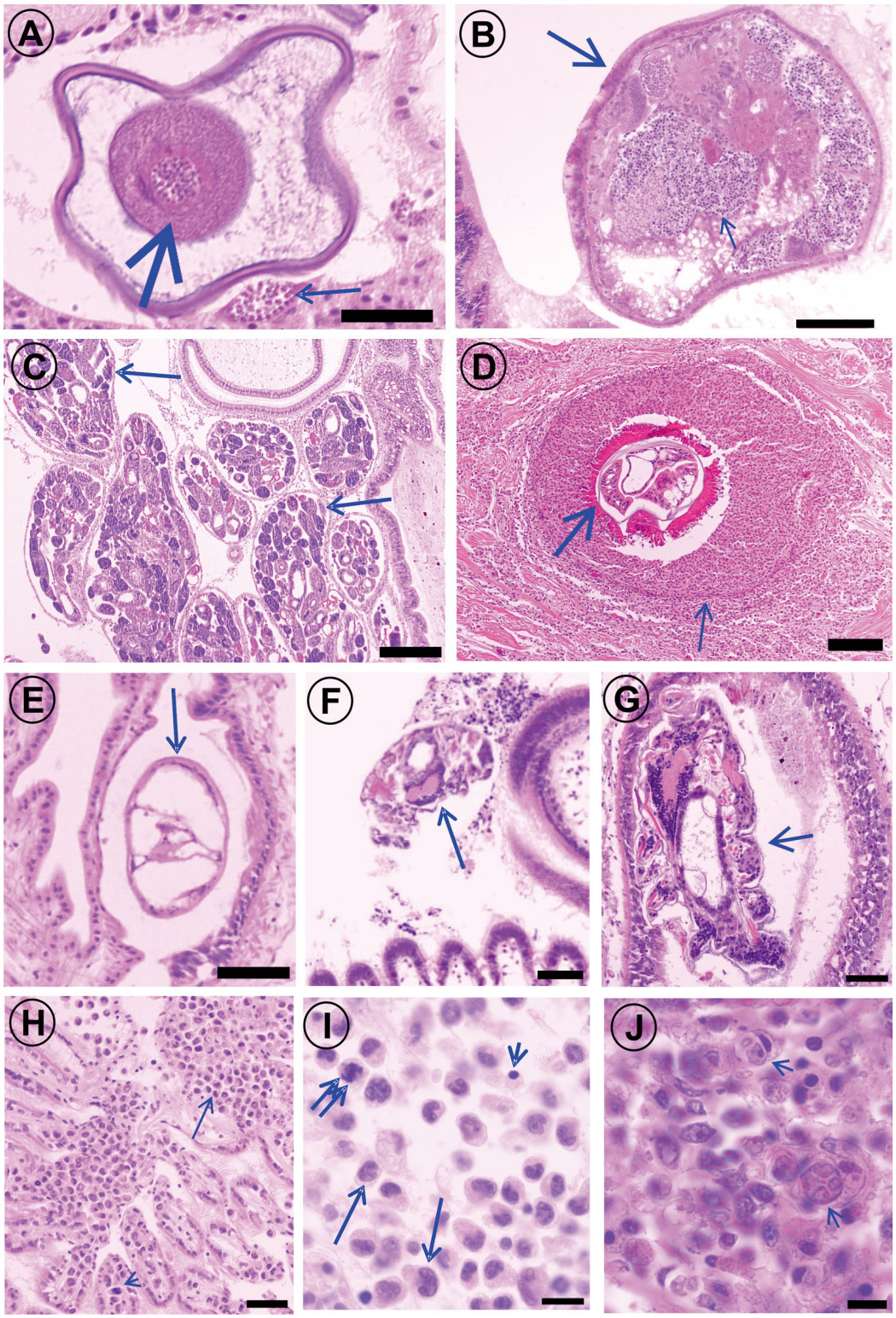

Fig. 3. Histological sections showing parasites and pathological conditions of *Cerastoderma glaucum*. (A) Sporocysts of a *Steinhausia*-like microsporidian within an ovocyte (arrowhead) and in the wall of the gonad follicle (arrow). (B) *Paravortex* sp. turbellarian (large arrow) hyperparasitised with *Urosporidium*-like spores (small arrow), in the intestinal lumen. (C) Sporocysts of a bucephalid trematode (arrows) in the visceral mass. (D) Encysted metacercaria (large arrow) surrounded by heavily accumulated host haemocytes (small arrow), in the foot. (E) Unencysted Gymnophallidae-like metacercaria (arrow) in the extra-pallial space surrounded by mantle. (F) Copepod (arrow) close to the gills. (G) Copepod (arrow) in the intestinal lumen. (H) Branchial area of a cockle affected by disseminated neoplasia; the connective tissue is heavily infiltrated with neoplastic cells (arrow), and a mitotic figure (arrowhead) is shown. (I) Higher magnification of neoplastic cells (arrows), which are larger and enclose larger nuclei than normal haemocytes (arrowhead); a mitotic figure (double arrow) can be seen. (J) Unidentified cells (arrowheads) within haemocytes occurring in a large focus of haemocytic reaction in the visceral mass; the unidentified cells could be either protistan parasites or damaged host cells. Scale bars = (A,H) 30 µm, (B,D) 100 µm, (C) 200 µm, (E–G) 50 µm, (I,J) 10 µm

5 localities (Table 2); cyst diameter ranged from 97 to 226 µm (n = 12) and, normally, 1 to 2 cysts per histological section were observed, sometimes causing local host inflammatory reaction involving heavy accumulation of haemocytes surrounding the cysts (Fig. 3D). Unencysted, oval Gymnophallidae-like metacercariae were observed in the extra-pallial space beside the mantle margins (Fig. 3E) in cockles from 2 localities (Table 2). Few metacercariae per histological section were detected, and they did not cause any significant damage.

Unidentified copepods were located close to the gills and in the mantle cavity of cockles from 3 localities (Table 2, Fig. 3F). Few copepods per histological section were seen and they did not cause any significant damage. Copepods were also detected in the intestinal lumen of cockles from 2 localities (Table 2, Fig. 3G); few intestinal copepods per histological section were found, and a host haemocytic reaction was not observed.

Other pathological conditions

Cases of disseminated neoplasia were found in cockles from 4 localities (Table 2). Neoplastic cells were round and measured 7.1 ± 0.4 µm (mean ± SD) in diameter (n = 10). Their nuclei were enlarged, 5.5 ± 0.2 µm in diameter (n = 10), pleomorphic and occupied most of the cytoplasm; nucleoli were observed in nuclei of some cells. Neoplasia was light in most affected cockles, with few neoplastic cells that were located in the connective tissue, mainly in gills. Some cockles were heavily affected by neoplasia, showing intense infiltration of neoplastic cells in the connective tissue of most organs and abundant mitotic figures (Fig. 3H,I).

A pathological condition characterized by the presence of large foci of heavy haemocytic reaction (LFHHR) was observed in cockles from 3 localities (Table 2). The haemocytic foci were mainly observed in connective tissue of gills and mantle but also in gonad, kidney and digestive gland. Some haemocytes occurring in the foci enclosed 1 to 4 unidentified cells (3–5 µm in length; Fig. 3J). Necrotic cells and brown deposits were observed within the foci.

Comparison of the digestive gland between *C. edule* and *C. glaucum*

Histological examination of the digestive gland showed that every lagoon cockle *C. glaucum*, regardless of where it was collected, had a significant proportion of the digestive tubules in breakdown phase while the remaining tubules were in other phases (holding, absorptive, digestive or excretory; Fig. 4A–C). The proportion of tubules in each phase was variable among *C. glaucum* individuals. No difference was detected between lagoon cockles from Sarrido fixed in the field and those from the same bed fixed in the laboratory after being held in aerated seawater for 24 h. The tubules in breakdown phase showed loss of epithelial integrity; identification of individual cells was difficult, most nuclei had disappeared, and brownish material (sometimes in spherical arrangement) and cell debris could be observed in the decomposing cells (Fig. 4C). Some tubules, apparently in a transition stage, showed an area with entire cells and another area with decomposing cells (Fig. 4B). No Feulgen-positive staining was detected in the digestive tubules in breakdown phase except in some remaining nuclei. Viral particles or other microorganisms were not detected by examination of ultrathin sections of digestive tubules in breakdown phase with TEM.

In contrast to *C. glaucum*, the digestive gland of every common cockle *C. edule* showed mostly synchronic digestive tubules in digestive phase, with tall digestive cells, some nests of young basophilic cells and reduced lumen (Fig. 4D); digestive tubules with a broken epithelium were not observed.

Regarding cockle species discrimination by PCR, amplification products of 185 and 470 bp were obtained from every common cockle *C. edule* and every lagoon cockle *C. glaucum*, respectively, thus confirming species identification.

DISCUSSION

This histopathological survey revealed the occurrence of different parasites (including prokaryotes, protists, fungi and metazoans) and pathological conditions affecting lagoon cockles *Cerastoderma glaucum* along the Galician coast. Regarding prokaryotes, bacteria-like colonies surrounded by a fibrous eosinophilic cover were detected in gills, mantle and labial palps with no serious histological host damage. Similar enveloped bacterial colonies have been observed in gills and mantle of common cockles *C. edule*, some of them with a high number of such colonies per section, which were associated with local lesions (Carballal et al. 2001). Enveloped bacterial colonies have also been reported from other bivalve species (Joly 1982, Le Gall et al. 1988, Goggin

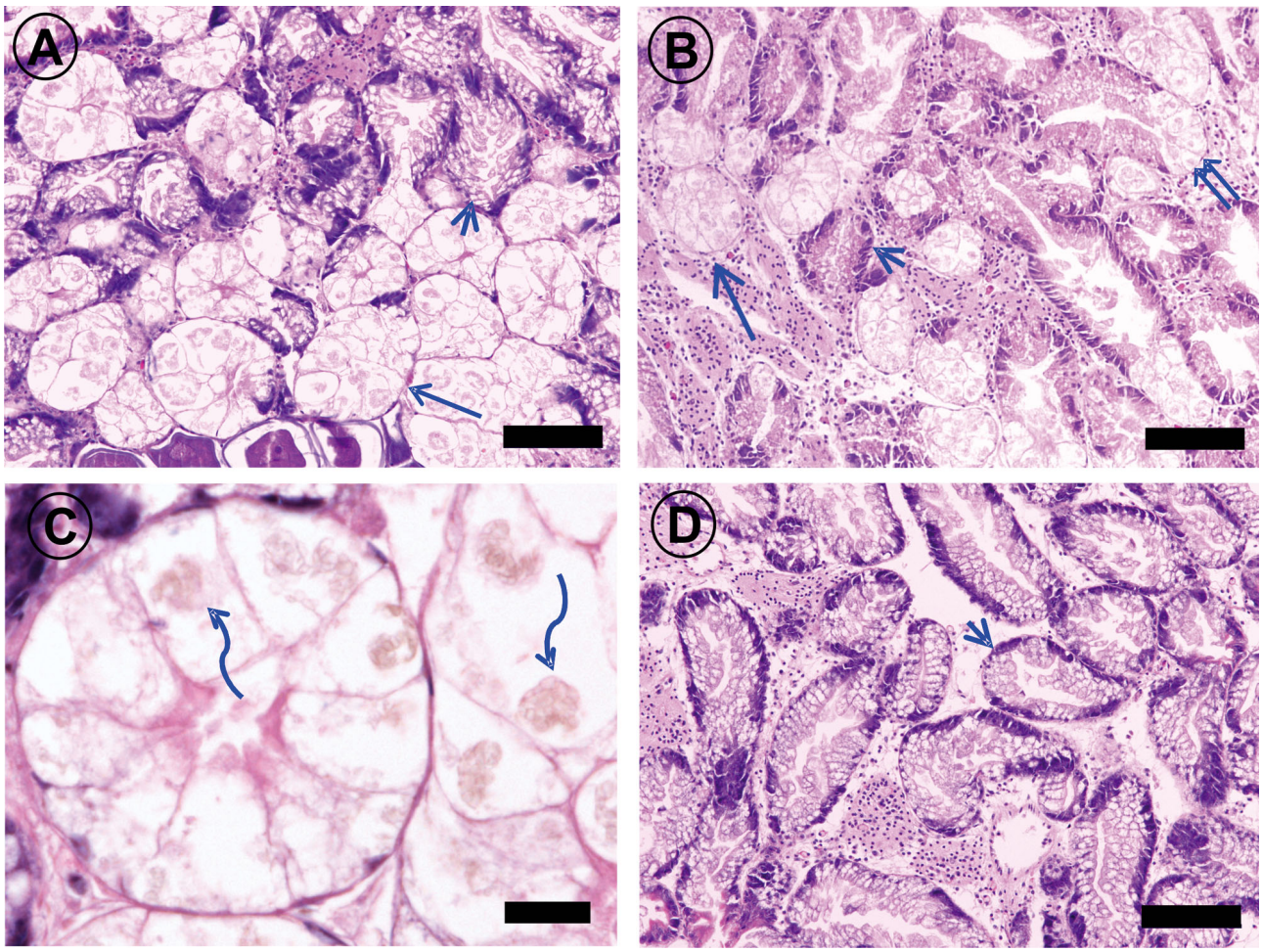

Fig. 4. Histological sections through the digestive gland of cockles (A–C) *Cerastoderma glaucum* and (D) *C. edule*; digestive tubules in breakdown (disintegration) phase are pointed out with arrows and tubules in other phases with arrowheads. (A) Digestive gland in which disintegrated digestive tubules are dominant. (B) Digestive gland in which disintegrated digestive tubules are the minority; some tubules are in transition to breakdown phase (double arrow). (C) Detail of disintegrated digestive tubules, in which identification of individual cells is difficult, with brownish deposits (sinuous arrows) and other cell debris and without distinguishable nuclei. (D) Digestive gland; disintegrated digestive tubules are not observed. Scale bars = (A,B) 100 µm, (C) 20 µm, (D) 50 µm

& Lester 1990, Villalba et al. 1999, Costa et al. 2012, Ruiz et al. 2015).

Rickettsia-like colonies that caused no important lesions were observed in lagoon cockles at most localities. *Rickettsia*-like organisms have also been detected at low infection intensity in common cockles *C. edule* from Galicia (Carballal et al. 2001, Ordás & Figueras 2005). Observation of intracellular basophilic colonies of prokaryotic organisms is frequent in histological sections of marine bivalve molluscs, mostly in branchial and digestive epithelia; such structures have been reported as *Rickettsia*-like, *Chlamydia*-like or *Mycoplasma*-like organisms (Cajaraville & Angulo 1991, Darriba et al. 2012, Ruiz et al. 2013, 2015); determining the type of microorganism involved usually requires examination with TEM. Most reports have not been associated with serious host damage; however, *Rickettsia*-like infections have been linked with mortalities in some bivalve species (Le Gall et al. 1988, Norton et al. 1993, Villalba et al. 1999), and even infection with the rickettsia '*Candidatus* Xenohaliotis californiensis' causes mass mortalities of abalones (gastropod molluscs, *Haliotis* spp.) in many countries (Crosson et al. 2014).

Regarding protists, the unidentified apicomplexan observed in the intestinal epithelium and the surrounding connective tissue resembled the unidenti-

fied gregarines observed by Carballal et al. (2001) in the same anatomical location in *C. edule* from Galicia and those reported by Bower et al. (1992) in Manila clams *Ruditapes philippinarum*, but also the unidentified coccidians described by Joly (1982) and Navas (2008) in chequered carpet-shell clams *R. decussatus*. The observation of the stage of this parasite occurring in the histological sections, without further analyses, does not allow appropriate identification, thus it is referred to here as an unidentified apicomplexan.

The gregarine *Nematopsis* sp. was highly prevalent, detected in every locality without relevant pathogenic effects. Histological analysis does not allow species identification. Two species of this genus, *N. portunidarum* and *N. incognito*, have been described from *C. glaucum* (Longshaw & Malham 2013). *Nematopsis* sp. has been reported in *C. edule* from Galician rias without causing important damage (Carballal et al. 2001, Ordás & Figueras 2005). Azevedo & Cachola (1992) considered that a mortality event of *C. edule* in Portugal was probably caused by *Nematopsis* sp. without providing much support. Coccidians were observed in the kidney of lagoon cockles without causing important damage to the host. Organ location, absence of merogonic stages and the number of sporozoites per sporocyst would support their allocation into genus *Pseudoklossia* (Desser & Bower 1997). Similar coccidian stages were reported in the kidney of common cockles *C. edule* from Galicia (Carballal et al. 2001). Another unidentified coccidian-like organism that caused no serious histological damage was observed in the gills of lagoon cockles; this organism was found in more sampled beds than were the organisms infecting the kidney. No reports of coccidians infecting gills of cockles were included in the review by Longshaw & Malham (2013); a coccidian-like protist was seen in the gills of razor clams *Ensis siliqua* (Ruiz et al. 2013).

As usual in bivalve molluscs, a high prevalence of ciliates was detected in lagoon cockles from each locality, and they did not cause evident damage. *Trichodina* ciliates observed in *C. edule* from Galician rias also did not cause apparent damage (Carballal et al. 2001, Ordás & Figueras 2005). Nevertheless, mortalities attributed to *Trichodina* sp. have been reported in *C. edule* and *Crassostrea angulata* (Lauckner 1983); alterations of gill structure and haemocytic response have been described in *Crassostrea gigas* (Boussaïd et al. 1999). *T. cardiorum* has been reported from *C. glaucum* and both *T. cardii* and *T. polandiae* from *C. edule* (Longshaw & Malham 2013). *Rinchodida* ciliates have been described from *C. glaucum*, including *Hypocomella rabeii*, *Sphenophyra cardii* and *Hypocomidium fabious* (Lauckner 1983, Longshaw & Malham 2013).

Species identification of the plasmodia resembling immature stages of a *Marteilia*-like parasite was not possible because morphological characters with taxonomic value for this group of organisms appear late in the sporulation process (Feist et al. 2009, Villalba et al. 2014). Three species of the genus *Marteilia,* viz. *M. refringens* (Villalba et al. 1993), *M. cochillia* (Villalba et al. 2014) and *M. octospora* (Ruiz et al. 2016), occur in Galicia, including the Ría de Arousa where the 2 lagoon cockles infected with a *Marteilia*-like parasite were found. Even if *M. cochillia* was the species infecting those 2 lagoon cockles, *C. glaucum* should be considered resistant to *M. cochillia* since no case of advanced infection with fully developed parasites was detected (only 2 out of 520 individuals were lightly infected with immature parasite stages). The comparison of susceptibility to *M. cochillia* between the 2 cockle species, *C. glaucum* and *C. edule*, was enlightening in the most thoroughly sampled location, Sarrido, where the prevalence of the parasite in *C. edule* was 92% in December 2012 and reached 100% in February 2013 (authors' unpubl. data), leading to the disappearance of live common cockles *C. edule* from that shellfish bed; conversely, this parasite was not found in lagoon cockles *C. glaucum* in any monthly sample, from December 2012 to December 2013, in that bed. The epidemic outbreak of *M. cochillia* in 2012 caused the common cockle *C. edule* fishery to collapse in the Ría de Arousa (Villalba et al. 2014), with subsequent outbreaks in the rias of Pontevedra and Vigo, devastating common cockle *C. edule* beds there as well (authors' unpubl. data). Similarly, immature stages of *M. refringens* were detected in the stomachs of a few Pacific oysters *C. gigas* cultured in areas where the parasite was prevalent in flat oysters *Ostrea edulis* (Cahour 1979) and mussels *Mytilus galloprovincialis* (Montes et al. 1998); likely, those parasite cells observed in *C. gigas* did not correspond to viable infections in that oyster species reputed as resistant to marteiliosis (Berthe et al. 2004). The resistance of *C. glaucum* to *M. cochillia* is highly relevant, since understanding the basis of that resistance could be useful to design cockle production recovery strategies.

Fungal infections with *Steinhausia* spp. have been observed, with microsporidians parasitising ovocytes of marine bivalves, including *C. edule* (Lauckner 1983, Bower et al. 1994, Longshaw & Malham 2013) but they have not been reported before in *C. glaucum*. Host lesions are not usually observed.

Metazoan parasites detected in this survey included flatworms, larval stages of digenean trematodes and copepods. *Paravortex* sp. turbellarians have been observed in the digestive lumina of multiple bivalves; they are considered as commensals and do not cause relevant problems to the host (Lauckner 1983, Longshaw & Malham 2013). *Paravortex cardii* has been reported in both *C. edule* and *C. glaucum*, and *P. karlingi* in *C. edule*; the former flatworm species is restricted to the digestive gland and the latter occurs in the intestine (Pike & Burt 1981). Hyperparasitisation of flatworms by a *Uroporidium*-like haplosporidian was detected in 1 lagoon cockle; *Urosporidium* sp. have been reported infecting flatworms (reported as *P. cardii*) located in the intestine of *C. edule* (Carballal et al. 2005).

Results showed that lagoon cockles *C. glaucum* are first and second intermediate hosts of trematodes in Galicia, because sporocysts and metacercariae, respectively, were observed during the histological survey. The sporocysts of a bucephalid trematode seemed pathogenic, causing host tissue disruption and castration. *Bucephalus* spp. have been reported to infect *C. glaucum* and *C. edule*, causing castration and even death of the cockles (reviewed by Longshaw & Malham 2013). The limited geographic distribution and the low overall prevalence recorded prevents us from considering the infestation with bucephalid sporocysts as a serious threat for *C. glaucum* in Galicia, but its pathogenicity should not be ignored. Two types of trematode metacercariae were observed in the survey, suggesting that *C. glaucum* is second intermediate host of more than 1 trematode species. Regarding encysted metacercariae, the wide range of cyst diameter and the variety of microhabitats within the host suggested the occurrence of larvae of the families Echinostomatidae, Renicolidae and Psilostomatidae in the Galician lagoon cockles (Lauckner 1983, de Montaudouin et al. 2009, Longshaw & Malham 2013). Regarding unencysted metacercariae, both host species (*C. glaucum*) and anatomical location (extra-pallial space beside the mantle margin) suggested that the Gymnophallidae-like metacercariae observed in Galician lagoon cockles corresponded to *Meiogymnophallus fossarum* (Lauckner 1983, de Montaudouin et al. 2009). High loads of metacercariae have been reported to cause damage to bivalve molluscs, including impaired growth rate, reduced capacity to burrow and increased mortality (Lauckner 1983). The low prevalence and light infection intensity suggested that trematode metacercariae are not a serious threat for *C. glaucum* in Galicia; however, examination of histological sections is not the best diagnostic procedure to detect trematode metacercariae, and both prevalence and intensity could have been underestimated.

Copepods were detected in different anatomical locations without causing evident lesions. The copepod *Herrmannella rostrata* has been reported in the mantle cavity and gills of common cockles *C. edule* from various European countries (Longshaw & Malham 2013), including Galicia (Díaz et al. 2011); moreover, copepods *Mytilicola intestinalis* have been reported in the intestine of various bivalve mollusc species (Lauckner 1983), including *C. edule* (Carballal et al. 2001, Longshaw & Malham 2013). Thus, the copepods found in lagoon cockles in our survey could correspond to *H. rostrata* and *M. intestinalis*, according to their respective anatomical location.

Disseminated neoplasia is a cancerous disease reported from multiple bivalve mollusc species (Carballal et al. 2015). High mortality linked with neoplasia has been reported from Galician common cockle *C. edule* beds (Carballal et al. 2001, Villalba et al. 2001, Ordás & Figueras 2005, Díaz et al. 2016); this disease was also reported in cockles *C. edule* from Ireland (Twomey & Mulcahy 1988) and France (Poder & Auffret 1986, Le Grand et al. 2010). In lagoon cockles, this disease had been reported in a single cockle *C. glaucum* from the Ría de Vigo (Rodríguez et al. 1997). Both the prevalence and the severity of this neoplastic condition detected in *C. glaucum* through this survey were low, suggesting that this disease is not a serious threat for lagoon cockles in Galicia.

The pathological condition referred to as LFHHR has been reported to affect common cockles *C. edule* from various natural beds of Galicia (Carballal et al. 2001). Mortality of common cockles *C. edule* associated with both disseminated neoplasia and LFHHR was detected in northern Galician rias (Villalba et al. 2001). This condition had null or low prevalence except at 1 locality; the intensity was light in the affected lagoon cockles of this survey, thus it did not seem to cause serious problems. Regarding its aetiology, the unidentified cells that were seen within haemocytes occurring in the foci could be either protistan parasites or damaged host cells (Villalba et al. 2001). Picornavirus-like particles were linked with this disease, but they could be a secondary infection (Carballal et al. 2003).

Serious pathological threats for the lagoon cockle *C. glaucum* populations in Galicia were not detected through this survey because the potentially pathogenic conditions (infections with *Marteilia* sp. and

bucephalid sporocysts, disseminated neoplasia and LFHHR) were rare (low overall prevalence), while more prevalent parasites (overall prevalence higher than 10%, i.e. ciliates, *Nematopsis* sp., *Paravortex* spp., enveloped bacterial colonies and *Rickettsia*-like organisms) had negligible or limited pathogeny (at the light intensity they were detected). The recorded prevalence data serve as the first baseline prevalence dataset of parasites and pathological conditions affecting Galician lagoon cockles, which should be useful as a reference for future health surveys. Comparison of these results with information on the health condition of common cockles *C. edule* from the same rias (even the same localities in the same year; Carballal et al. 2001, Díaz et al. 2016, our internal reports from multiple surveys) shows that diseases associated with mortality of common cockles, namely infection with *M. cochillia*, disseminated neoplasia and LFHHR, do not seem to be a real threat for *C. glaucum*.

The morphological comparison of the digestive gland showed evident differences between the 2 cockle species. The synchronicity of the digestive tubules in every examined common cockle *C. edule* was consistent with a monophasic digestive cycle, as it had been reported for this species (Morton 1970). In contrast, the co-occurrence of digestive tubules in different digestive phases within the digestive gland of every lagoon cockle *C. glaucum*, with abundant tubules in breakdown phase, resembled descriptions from other bivalve mollusc species like *Pecten maximus* (Mathers 1976) and *Chlamys varia* (Mathers et al. 1979), for which a diphasic digestive cycle was proposed. According to Mathers et al. (1979), the digestive tubules never fully disintegrate in molluscs with a monophasic digestive cycle (including *C. edule*), whereas total disintegration of tubules is observed in molluscs with a diphasic digestive cycle. A result of this study is that observation of histological sections through the digestive gland easily allows differentiating the 2 cockle species. The occurrence or absence of full disintegration of digestive tubules could influence the differences in susceptibility to infection with *M. cochillia* between the cockle species because the main location of this parasite in common cockles is in the epithelia of the digestive gland. Nevertheless, understanding the basis of the resistance requires further study. Moreover, the differences in digestive patterns between the 2 cockle species could be linked with their different ecological, behavioural and ecophysiological patterns described in the literature (Hummel et al. 1994, Mariani et al. 2002, Tarnowska et al. 2012).

Carrasco et al. (2011) reported the occurrence of disintegrated digestive tubules due to 'digestive epithelial virosis' in all the examined cockles (n = 30) in a study envisaged to determine the cause of cockle mortality, using standard histology as the only analytical procedure; viral infection was not confirmed with any other procedure. The 'condition' is shown in 2 micrographs of a histological section through the digestive gland of a cockle showing disintegrated digestive tubules that resembled the tubules in breakdown phase reported in *C. glaucum* in the present study. No sign of viral infection in disintegrated digestive tubules of *C. glaucum* was detected in our survey with TEM and Feulgen staining. Carrasco et al. (2011) collected cockles from the Delta de l'Ebre (Spanish Mediterranean coast) and considered them to be *C. edule,* although this species rarely occurs in the Mediterranean Sea (Krakau et al. 2012, Longshaw & Malham 2013) while *C. glaucum* is widely distributed (Nikula & Väinölä 2003). Carrasco et al. (2011) also detected infection with a *Marteilia*-like parasite in 40% of those cockles, some of them with heavy infection intensity, with fully mature parasites, which were later identified as the new species *M. cochillia* (Carrasco et al. 2013). If cockles from the Delta de l'Ebre were *C. glaucum*, then the type host of the parasite would be *C. glaucum*, which would contrast with the resistance of *C. glaucum* to *M. cochillia* supported by results from our survey. Therefore, confirming species identification of cockles infected with *M. cochillia* from the Delta de l'Ebre seems very important, using both morphological and molecular characters with taxonomic value.

Paradoxically, the resistance of the Galician lagoon cockles *C. glaucum* to *M. cochillia* and the occurrence of heavy infections with this parasite in Mediterranean lagoon cockles *C. glaucum* could be both true and linked with divergence and diversity within the widespread *C. glaucum* complex (Nikula & Väinölä 2003). The high level of differentiation found in recent studies between the main genetic groups suggested that the taxonomy of *Cerastoderma* needs revision (Tarnowska et al. 2010). Support for a presumed subdivision into a Mediterranean and an Atlantic–Baltic subspecies or species (*C. glaucum* and *C. lamarcki*, respectively) has been claimed from immunological (Brock 1987), chromosomal DNA (Brock & Christiansen 1989) and isoenzyme (Hummel et al. 1994) differences. Recent studies of *C. glaucum* populations from the Mediterranean and Atlantic coasts using ITS and COI markers have also shown significant genetic differentiation (Ladhar-Chaabouni et al. 2010). In this context, differences in

susceptibility to *M. cochillia* between cockles *C. glaucum* from Atlantic and Mediterranean coasts is a hypothesis that should be assessed.

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