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ORIGINAL PAPER

Two enigmas may solve each other: the oocyte coat and atresia in the common cockle, *Cerastoderma edule* (Linnaeus, 1758)

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Abstract

Two co-occurring, enigmatic aspects of bivalve reproduction were investigated in the common cockle *Cerastoderma edule*: the oocyte coat and oocyte atresia. Qualitative histology and transmission electron microscopy (TEM) of cockles collected on the French Atlantic coast revealed not only the fine structure of the oocyte coat, but also confirmed that it is secreted by the oocyte itself and composed of acid mucopolysaccharides (AMPS), known to be viscous and adhesive. Quantitative histology showed that at the peak of oogenesis, oocyte coats occupy the largest fraction (approx. 40%) of the gonad acinal volume, representing both a significant sacrifice of female gamete capacity, and a non-gamete energetic investment. Potential benefits of the coat include protection from mechanical abrasion, predation, and opportunistic microbes. Atresia (oocyte degeneration) was a known second source of reduced fecundity, with a minimum impact of approximately 50% of the total oocyte volume. It is suggested that this high proportion of atresic oocytes is related to the previously-documented genetic inviability of early post-fertilization life stages. The qualitative histological and TEM observations revealed atresic debris adhering to the exterior surface of the oocyte coats. Such an arrangement would isolate adjacent oocyte coats, enabling the oocytes to be spawned individually, rather than as an egg mass, and therefore to undergo planktonic development and dispersion. Oocyte atresia and the oocyte coat of *Cerastoderma edule* therefore appear to be linked in the first indication of an adaptive function in bivalves.

Introduction

The presence of a thick, non-living coat surrounding the oocytes of several marine bivalve species has been periodically reported in the scientific literature, often with astonishment, and then collectively forgotten (see Collin and Giribet 2010; Beninger and Chérel 2019 for references). Various authors have believed themselves to be the first to document such a feature (Belding 1931; Loosanoff and Davis 1950; Lutz et al. 1981, 1982), while most have simply not reported/observed it; this may be partly due to its poor staining with the common topological histology stains (Martínez-Castro and Vásquez 2012; Kandeel et al. 2013). The coat has been given different names in the relatively few studies in which it has been identified: 'gelatinous egg capsule' (Creek 1960; Gustafson and Reid 1986; Gustafson and Lutz 1992), 'gelatinous membrane' (Ansell 1961), 'albuminous sheath' (Kingston 1974), 'egg capsule' (Lutz et al. 1982) or 'jelly coat' (Hodgson and Burke 1988; Gros et al. 1997).

Various types of oocyte coats have been reported in the Mollusca, and grouped according to their layering: primary, secondary, and tertiary (Wourms 1987; Ponder et al. 2019). 'Jelly-like' primary coats have been well documented in the polyplacophoran basal group, and this character may thus be pleisiomorphic in the Bivalvia (Buckland-Nicks and Reunov 2010). The occurrence of this coat in Bivalvia is enigmatic, as it does not appear to be taxonomically-related, and no major ecological variable appears to explain its presence (Beninger and Chérel 2019). Its implications for bivalve ecology, fisheries, and aquaculture are nonetheless intriguing and potentially important. Much the same has been written

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about the phenomenon of bivalve oocyte atresia (degeneration of oocytes within the gonad), very largely overlooked in the literature (Beninger 2017). Although it has recently been brought to the fore in several papers, this phenomenon remains poorly-understood and rarely identified, despite the fact that it is responsible for most of the early life-stage mortality in both wild and cultured populations (Smolarz et al. 2017; Chérel and Beninger 2017, 2019). Its causes and exact cellular metabolomic nature remain unknown.

In the course of investigating the reproductive impact of oocyte atresia in the common cockle *Cerastoderma edule*, a species presenting a well-developed oocyte coat (Chérel and Beninger 2019), it became evident that atresia also impacted the formation and structure of the oocyte coat. The present study is the first to report on the detailed structure of the oocyte coat in *C. edule* or any bivalve, and its relationship to ongoing oocyte atresia.

Materials and methods

Species, sites and sampling

Cerastoderma edule is an intertidal endobenthic bivalve found on the North-East Atlantic coast, from Mauritania to Norway. Fishing and farming of this species are important to the economies of the Netherlands, the United Kingdom, and France (FAO, https://www.fao.org/fishery/species/3543/en). The study was carried out at two sites on the French Atlantic coast within 50 km of each other (Fig. 1), as part of a program to investigate the impact of oocyte atresia in this species (Chérel and Beninger 2019). Adult cockles (15–30 individuals, > 2 cm SL) were haphazardly sampled monthly from July 2010–May 2012 at the first site, and bi-weekly from January–November 2018 at the second site. Only gametogenic females were included in this study ($n = 301$).

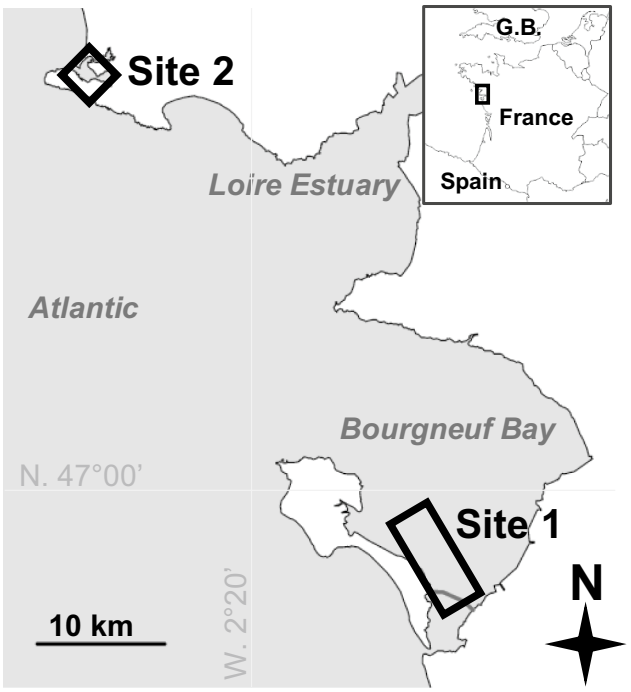

Fig. 1 Location of the two sampled sites

Histology and stereology

Sampled cockles were shucked and fixed in Bouin's solution, embedded and sectioned at 7 µm as per Chérel and Beninger (2017, 2019). Slides were first stained with Alcian blue in order to reveal the oocyte coat (Beninger and Chérel 2019): acetic acid 1 min, dry 3 min, Alcian blue 30 min), followed by a modified Masson's trichrome topological protocol as per Chérel and Beninger (2019). Observations and photomicrographs were performed using an Olympus Provis light microscope, and Olympus cellSens Standard software.

Stereological counts were carried out using an 11 × 11 grid at 100 × on five features of interest: atresic oocytes (AO), immature healthy oocytes (IO), mature healthy oocytes (MO), oocyte coat (C) and intra-acinal lumen (IAL) (Chérel and Beninger 2019).

A periodic acid-Schiff stain was performed, on additional histological sections, as per Cannuel and Beninger (2007), to determine whether any neutral mucopolysaccharides were also present in the oocyte coat.

The following volume fractions were calculated as per Chérel and Beninger (2017, 2019):

$$\text{Total Oocyte Volume Fraction : OVF} = \frac{\text{AO} + \text{IO} + \text{MO}}{\text{C} + \text{IAL} + \text{AO} + \text{IO} + \text{MO}} \times 100$$

$$\text{Atresic oocyte Volume Fraction : AVF} = \frac{\text{AO}}{\text{AO} + \text{IO} + \text{MO}} \times 100$$

$$\text{Mature oocyte Volume Fraction : MVF} = \frac{\text{MO}}{\text{AO} + \text{IO} + \text{MO}} \times 100$$

$$\text{Immature oocyte Volume Fraction : IVF} = \frac{\text{IO}}{\text{AO} + \text{IO} + \text{MO}} \times 100$$

$$\text{Intra} - \text{acinal Lumen Volume Fraction: LVF} = \frac{\text{IAL}}{\text{C} + \text{IAL} + \text{AO} + \text{IO} + \text{MO}} \times 100$$

in addition to the oocyte coat volume fraction:

$$\text{Coat Volume Fraction : CVF} = \frac{\text{C}}{\text{C} + \text{IAL} + \text{AO} + \text{IO} + \text{MO}} \times 100$$

Transmission electron microscopy

Three tissue pieces of approx. 3 mm^3 each were removed from the dorsal region of the visceral mass, where previous histological observations confirmed that gonad acini were most likely to be found. The presence of oocytes was verified under a dissecting microscope prior to proceeding with the subsequent processing steps. The tissue pieces were immediately fixed in cold 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (made with filtered seawater from the sampling sites to ensure appropriate pH 7.4 and osmolarity) for at least 2 h. The tissue pieces were then rinsed in 0.2 M cacodylate buffer and cut to obtain 1-mm^3 pieces. Post-fixation was performed in 0.2 M cacodylate buffer/1% osmium tetraoxide at 4° for 1 h. After dehydration in a graded ethanol series and propylene oxide bath (2 × 15 min), samples were transferred to EPON resin/propylene oxide (1:1) at room temperature, for 1.5 h. After embedding in pure resin for 1 h, polymerization was effected at 60 ℃ for 12 h.

Semi-thin sections were cut at 1 µm using a LEICA EMUC7 ultramicrotome and stained with toluidine blue for light microscopic observation. Thin sections were cut at 80–90 nm, collected on uncoated 300-mesh copper/rhodium grids (MAXTAFORM HR25), contrasted with uranyl acetate (Bozzola and Russel 1992), and examined using a JEOL JEM-1400 transmission electron microscope (TEM).

Spawning and fertilization

Several spawning induction techniques were attempted and abandoned due to poor results: mechanical stimulation, temperature shock, addition of scraped/stripped gonad material, and addition of spawned spermatozoa from male cockles. Acceptable results were obtained by adapting the technique of Honkoop and Van der Meer (1998), using cockles and seawater collected during the most likely period of maximal gamete maturity (February–July). Cockles were stored overnight at 4 ℃, out of water. Groups of three to five individuals were then placed in separate containers filled with site sea water at 15 ℃. Oocyte spawning occurred within 10 min to several hours, depending on the maturation state of the cockles. Additional thermal stimulation with 30 ℃ sea water occasionally improved gamete release. Oocytes and spermatozoa were collected using Pasteur pipettes, and allowed to fertilize in a small container of site seawater.

Results

Qualitative characteristics of oocyte coat

The oocyte coat may be invisible or scarcely visible using traditional methods of topological histological staining, and therefore very difficult to identify (Fig. 2a). This problem was solved with the Alcian blue staining step (Fig. 2b), which also revealed the coat to be composed of acid mucopolysaccharides (AMPS–viscous mucus). Similarly, the use of toluidine blue, which is orthochromatic with most ooplasmic compounds, yet metachromatic with AMPS, allowed the coat to be clearly distinguished in semi-thin sections (Fig. 3).

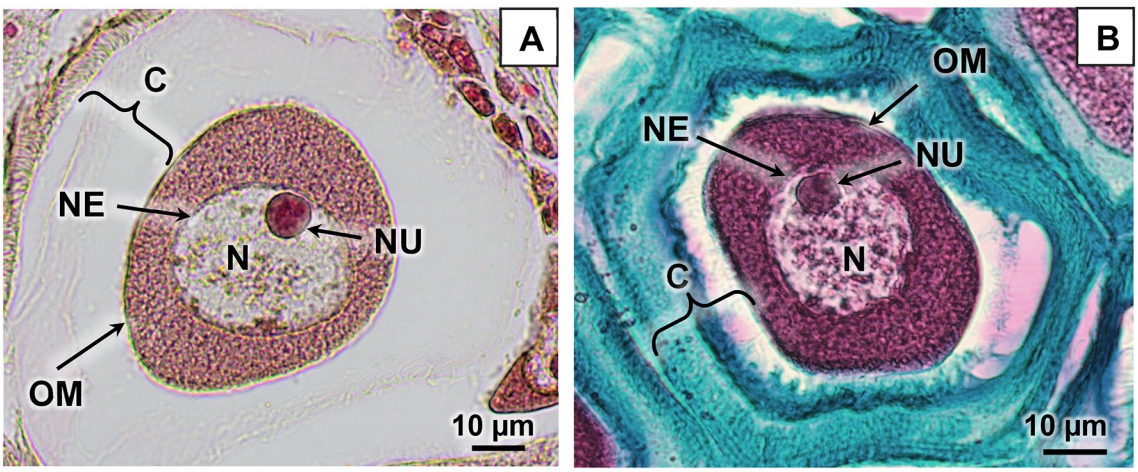

Fig. 2 *Cerastoderma edule*. Mature healthy oocytes stained with **a** modified Masson's trichrome and **b** with Alcian blue added. Nuclear envelope (NE), nucleus (N), nucleolus (NU), oocyte coat (C), and oolemma (OM)

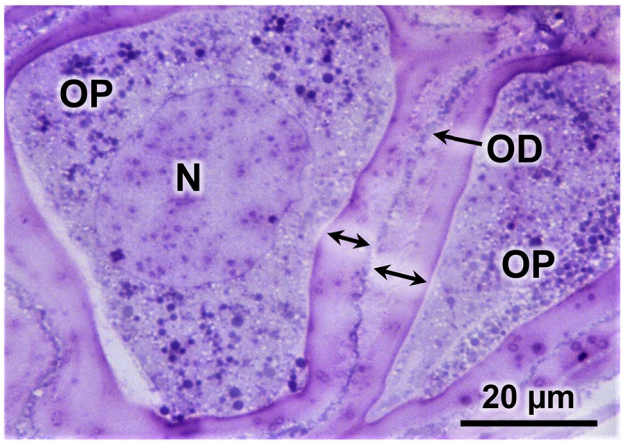

Fig. 3 *Cerastoderma edule* oocytes. Semi-thin section stained with toluidine blue. Ooplasm (OP) and cellular debris (OD) stain dark blue, oocyte coats (double arrows) stain purple. Nucleus (N)

Transmission electron microscopy revealed the oocyte coat to be formed of two distinct layers. The inner layer was more electron-dense and homogenous than the outer layer; a thin (0.5–2 µm) electron-lucent zone was present between the oolemma and the inner coat layer; its position, thickness, and TEM aspect corresponded to the zona pellucida (Figs. 4a and 6a)

Origin and development of the oocyte coat

Young oocytes developed from the acinal wall, in contact with auxiliary cells (Fig. 5a). The oocyte peduncle formed as the young oocyte grew and began vitellogenesis (Fig. 5b–e). The oocyte coat was absent in very young oocytes (Fig. 5a–c), first appearing as a very thin layer closely appressed to ~ 20-µm diameter oocytes (Fig. 5c, d). The oocyte coat thickened concomitantly with oocyte growth, becoming very large around late pedunculated and mature oocytes (Figs. 5e and 2b). Atresic oocytes, recognizable by their irregular shape and/or the change in appearance of the chromatin (Chérel and Beninger 2019) often presented a slightly thinner coat than that of healthy oocytes (Fig. 5f, g). Of the 301 females sampled over the study period, only 16 showed no signs of oocyte atresia in the sections examined, and these individuals all showed a lack of gametogenetic activity.

Compared to the young oocyte, vitellogenic oocytes contained numerous mitochondria, electron-dense yolk vesicles, and clear vesicles in the ooplasm (Fig. 6a). Clear vesicles were often located close to the oolemma (Fig. 6b), and fusion with the envelope was observed, accompanied by exocytosis (Fig. 6c). These observations, together with the growing oocyte coat, strongly suggest that these vesicles are filled with AMPS, which are exocytosed to form the layers of the coat.

Cellular debris and the oocyte coat

Cellular debris was observed in all semithin and thin sections examined ($n = 6$ individuals), adhering to the exterior surface of the oocyte coat (Fig. 3), and most conspicuously between the oocyte coats of adjacent oocytes. Close examination of the debris under TEM revealed distinct, naked mitochondria and yolk granules (Fig. 4).

Features of spawned oocytes

Spawned *Cerastoderma edule* oocytes were visible to the naked eye; they did not adhere to each other and were negatively buoyant (Fig. 7a). The newly-spawned, coated oocytes measured approximately 135 µm in diameter (oocyte 66 µm + 2 × 35 6 µm coat thickness); the coat was present on both unfertilized and fertilized oocytes (the latter characterized by the disappearance of the nuclear envelope) (Fig. 7b). The coat increased in thickness to approximately 47 µm as the zygote developed, probably due to hydration of the mucopolysaccharides. The coat persisted up to at least the early veliger (pre-pediveliger) stage (rearing was not attempted) (Fig. 7c). Veligers swam within the confines of the coat, whose outer reaches appeared much more viscous and resistant to the mechanical stress of the swimming (Online resource 1).

Oogenesis and oocyte coat quantification

Oogenesis was continuous, with no apparent inter-individual synchronicity (Fig. 8; Chérel and Beninger 2019). At the population level, oocyte coats were observed throughout the year. The volume fraction of the coat (CVF) was at least 20−50% of the total acinal volume (Fig. 8).

A CVF mean of 33.6% ± 1.9 (95% confidence interval) was calculated for all individuals together (site 1 + 2, $n = 301$). During active gametogenesis, defined as MVF > 20% ($n = 165$), the mean CVF increased to 40.5% ± 2.1 (95% confidence interval).

Overall, the coat volume fraction varied directly with respect to the mature and atresic volume fraction, and inversely with respect to immature volume fraction (Fig. 9).

Discussion

Qualitative characteristics of oocyte coat

The results of the Alcian blue, toluidine blue, and PAS staining confirm the exclusively acid mucopolysaccharide composition of the oocyte coat, as was originally hypothesized for *Thyasira gouldi* by Blacknell and Ansell (1974), and subsequently demonstrated in *Codakia orbicularis* by Gros

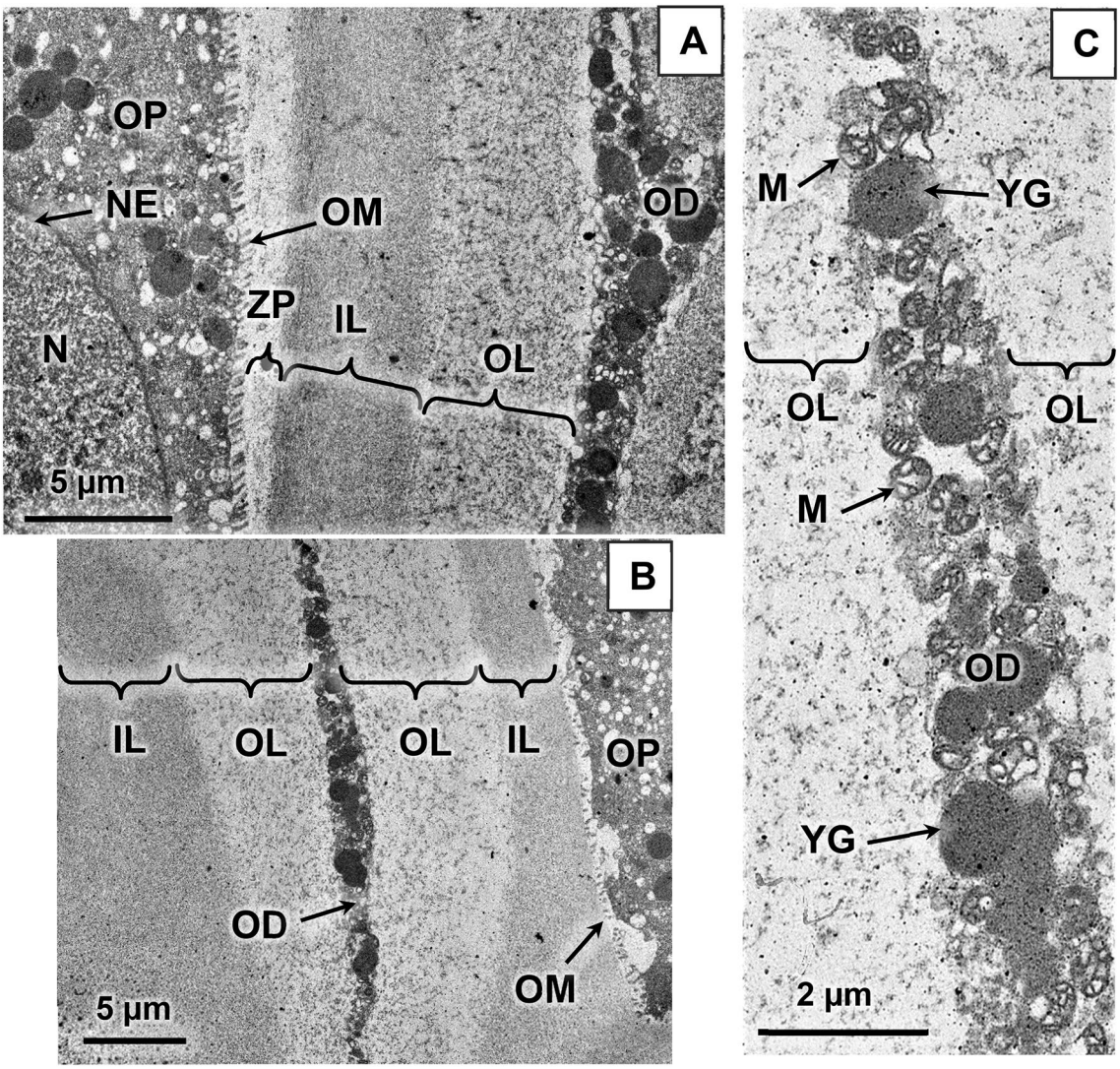

Fig. 4 *Cerastoderma edule* oocytes. Transmission electron micrographs. **a** Part of mature oocyte with coat. Nucleus (N), nuclear envelope (NE), ooplasm (OP) and oolemma (OM) with short microvilli adjacent to zona pellucida (ZP). **b** Inner layer (IL) and outer layer (OL) of coats from 2 oocytes separated by oocyte debris (OD). **c** Oocyte debris containing mitochondria (M) and yolk granules (YG) between adjacent coat outer layers (OL). Note similarity between the mitochondria and yolk granules in Fig. 6

et al. (1997) and in *Cerastoderma edule* by Beninger and Chérel (2019).

The clear space between the oolemma and the oocyte coat, visible in the histological sections, was not observed in the electron microrographs; this feature is thus most probably an artefact of the histological preparation (most likely the dehydration sequence). Within the coat itself, two AMPS layers were evident: an inner, finely-clumped layer, and an outer, more coarsely-clumped layer – this may correspond to increased clumping in the oldest AMPS secretions. The presence of such a double layer has also been shown in *Codakia orbicularis* under phase-contrast optics (Gros et al. 1997).

Origin and development of the coat

Although Raven (1966) stated that molluscan oocyte coverings external to the oolemma are secreted either by the auxiliary ('follicle') cells or by the oviduct, the contemporary consensus is that the primary envelope (all coats immediately exterior to the oolemma) is secreted by the oocyte itself (see Wourms 1987, Ponder et al 2019 for reviews). Gros et al. (1997) stated that the thick oocyte coat of *Codakia orbicularis* is secreted by the oocyte itself (their unpublished observations). This conclusion is supported by the thinner coat around many atresic oocytes, and by the acinus stereological data of the present study, which clearly show an

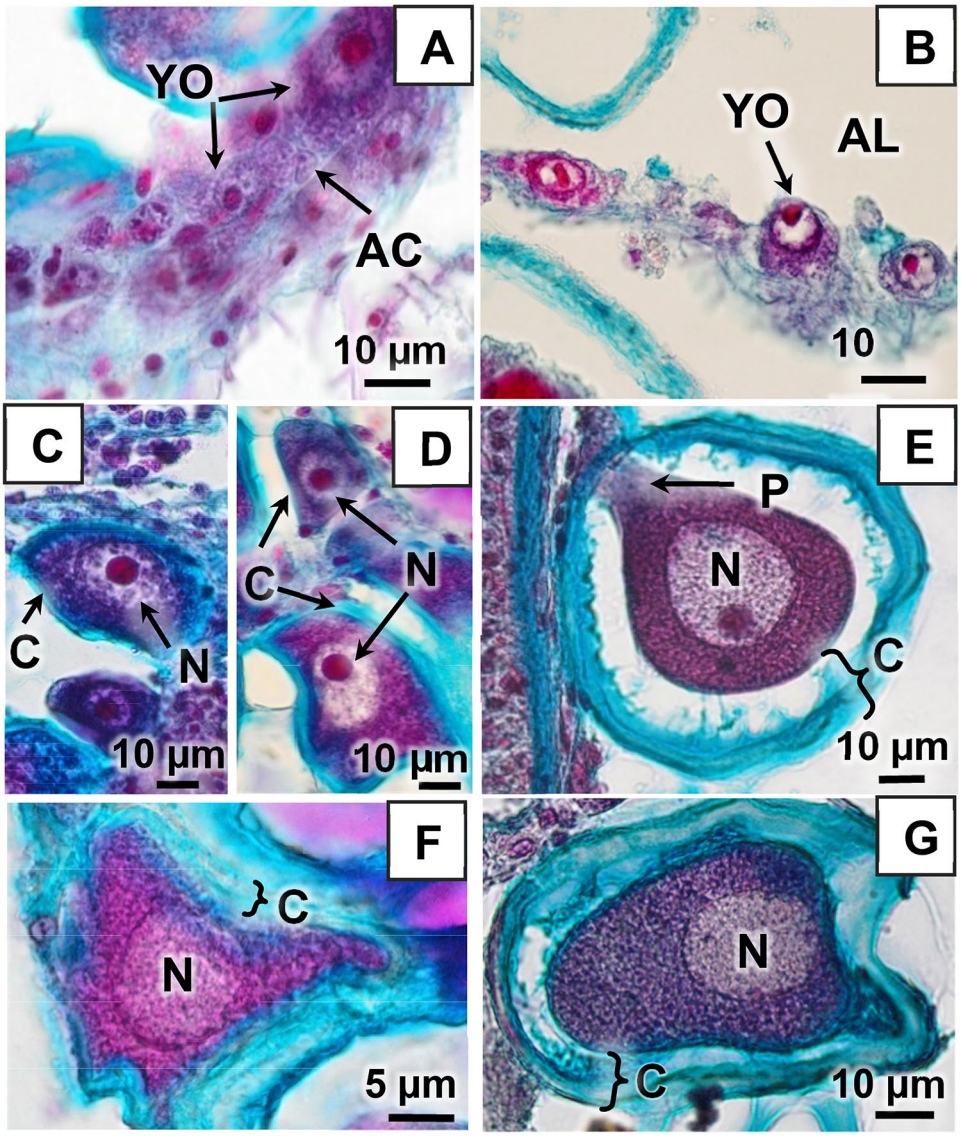

Fig. 5 *Cerastoderma edule*. **a** Young oocyte (YO) and adjacent auxiliary cell (AC); **b** YO beginning growth toward acinal lumen (AL). **c**, **d** Thin coat (C) around very young oocytes. **e** Thick coat around pedunculated (P) oocyte. **f** Young atresic oocyte and **g** mature atresic oocyte. Nucleus (N)

inverse relationship between the immature oocyte volume fraction and the coat volume fraction. The light and electron microscopic data of the present study are also consistent with an oocytic origin for the coat secretions: Mucus (i.e. mucopolysaccharide) vesicles were present in the ooplasm of vitellogenic oocytes, but absent from the ooplasm of pre-vitellogenic oocytes, which is also consistent with this interpretation. These mucus vesicles have not been observed in bivalve species whose oocytes lack an oocyte coat (Pipe 1987a; Dorange and Le Pennec 1989; De Gaulejac et al. 1995; Eckelbarger and Davis 1996; Chung et al. 2007, 2008; Lee and Chung 2008; Camacho-Mondragón et al. 2015). The TEM micrographs of the present study show that the cellular mechanism of coat secretion is merocrine, with large membrane-bound vesicles releasing their contents at the oocyte cell surface. This mode of coat secretion is common in the Metazoa (Buckland-Nicks and Reunov 2010).

The sharp distinction between the inner and outer oocyte coat layers, with no gradation, as observed in the TEM micrographs, argues for discrete secretion at different times: first the outer, and later the inner layer. The electron-lucent *zona pellucida* or 'vitelline coat' is probably the final secretion, characteristic of mature oocytes: a thin layer of glycoprotein, crucial to maturation and fertilization (Focarelli et al. 1990; Focarelli and Rosati 1993). It is conceivable that the same secretory process is responsible for the oocyte coat layers, with qualitatively different mucopolysaccharides/glycoproteins in each layer (Wourms 1987). Differential expression of this character would help to explain why some bivalve species possess thick oocyte coats and others do not (Beninger and Chérel 2019). The lack of merocrine secretory vesicles in the TEM micrographs of the non-coat species may be due to the fact that secretory activity is comparatively slight for the zona pellucida, and in any event terminates in the mature oocyte.

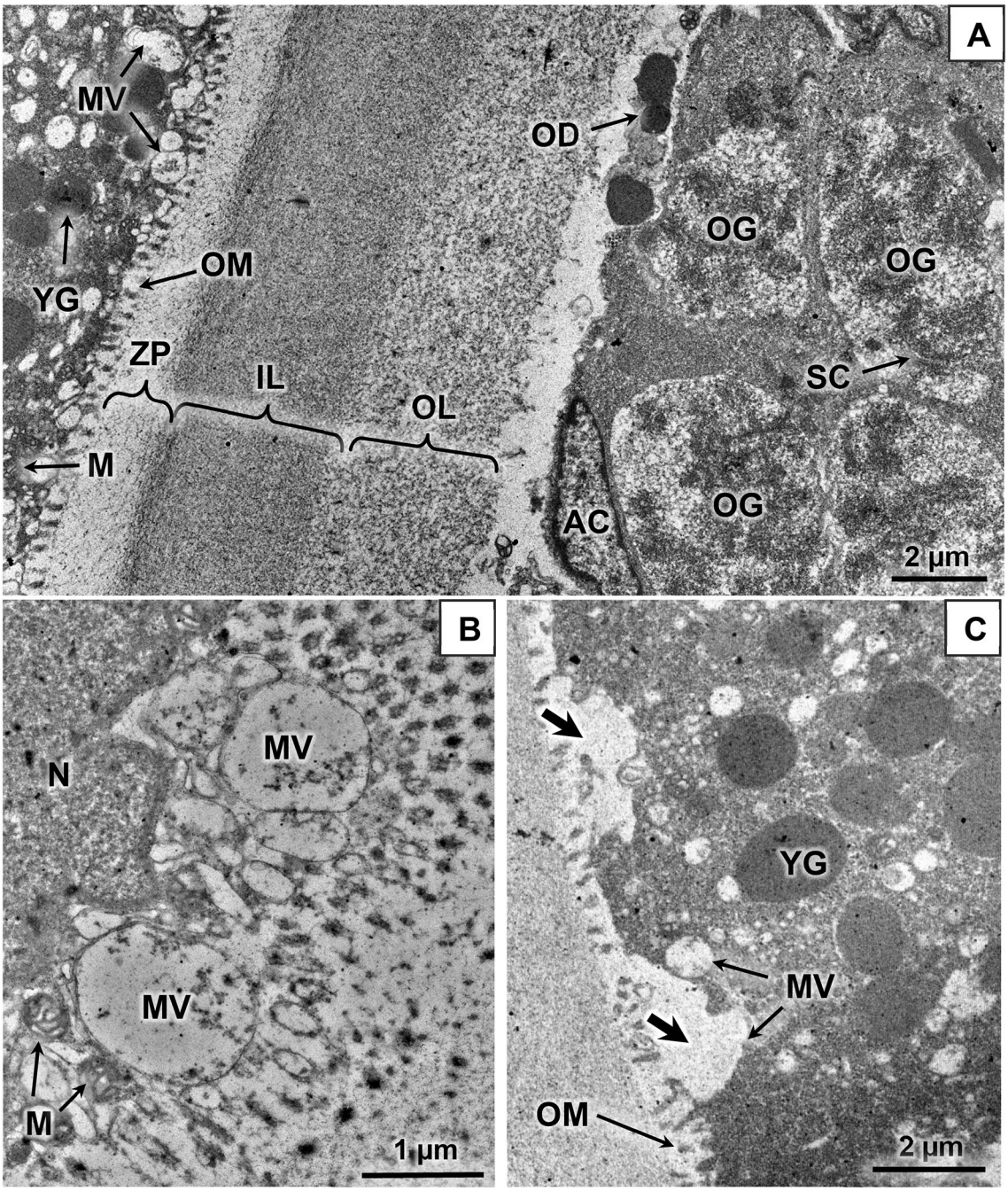

Fig. 6 *Cerastoderma edule*. Transmission electron micrographs**. a** mature oocyte (left) with inner (IL) and outer (OL) layers of oocyte coat (OC) and zona pellucida (ZP) adjacent to oolemma (OM). Oogonia without coat (OG), with accompanying auxiliary cell (AC). Note synaptonemal complex (SC) in nucleoplasm. Putative mucus vesicles (MV) and yolk granule (YG) in mature oocyte, oocyte debris (OD) on external surface of oocyte coat. **b** Mucus vesicles and mitochondria close to oolemma, N, Nucleus. **c** mucus vesicle exocytosis (bold arrows)

Costs and benefits of the cockle oocyte coat

The oocyte coat occupied a large part of the acinal volume in the cockles of the present study (~40% at peak oogenesis), thereby reducing the number of oocytes per acinus. Although the intra-acinal degree of hydration is unknown, secretion of the coat invariably entails energy expenditure. The possible benefits of the oocyte coat were proposed in Beninger and Chérel (2019): protection from abrasion, predation and opportunistic microbes. Other authors have

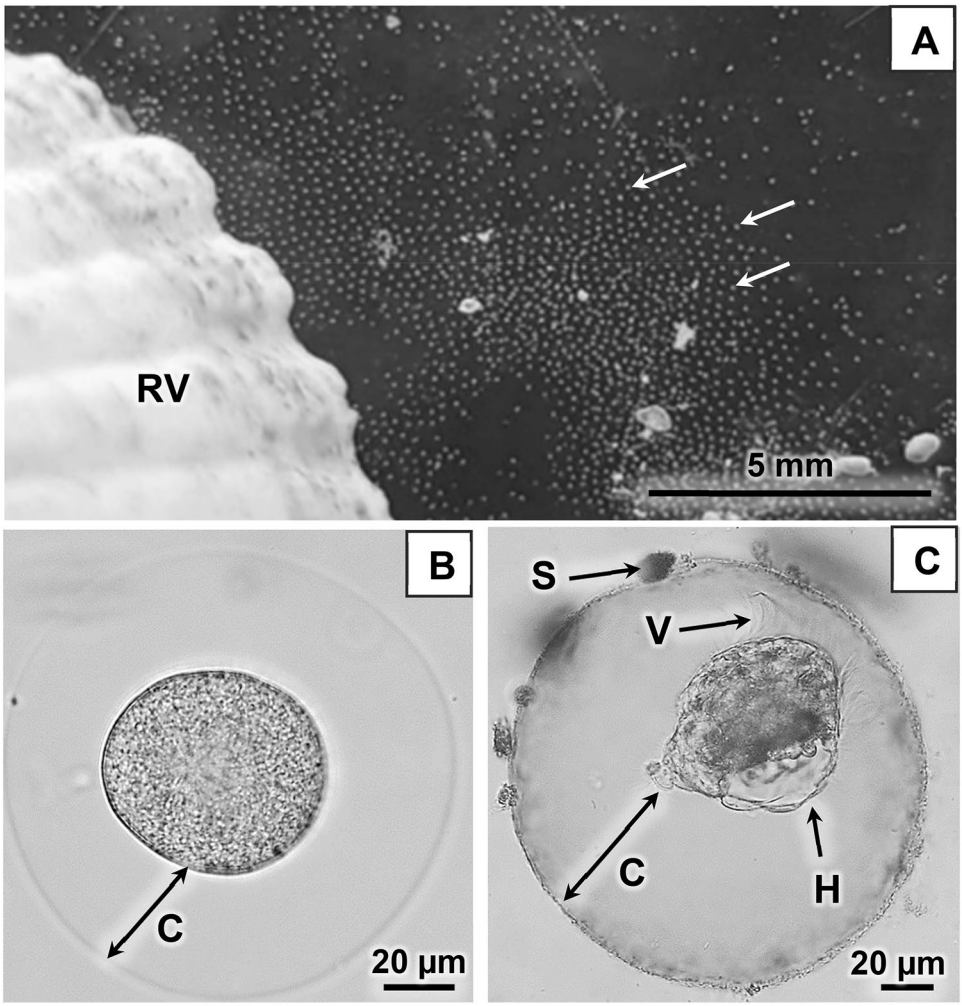

Fig. 7 *Cerastoderma edule*. **a** female cockle spawn, non-adhering oocytes (white arrows); RV, right valve; **b** spawned, fertilized, unstained live oocyte surrounded by oocyte coat (C). **c** veliger larva in coat (C), shell hinge (H) and velum (V) composed of ciliary tracts. S, seston particles adhering to coat

variously suggested that such a coat might minimize polyspermia (in sea urchins) (Hagström 1959) and increase the probability of an encounter with spermatozoa (Farley and Levitan 2001; Levitan 2006). Indeed, the coat doubles the size of the uncoated oocyte (Honkoop and Van der Meer 1998; Pronker et al. 2015; present study). Such an increase in size might also offer a size refuge from some zooplanktonic and benthic suspension-feeding predators.

Relation between oocyte coat and atresia: can two enigmas solve each other?

'For what purpose?' is usually the first question that comes to mind when we learn that a large proportion of energetically costly oocytes is routinely 'self-destroyed'. Both the causes and potential benefits of oocyte atresia are very poorly-understood (Beninger 2017; Chérel and Beninger 2017, 2019). The sacrifice of healthy or impaired female gametes for nutrient transfer to vitellogenic oocytes or developing embryos is well-documented in many animal taxa, including the Mollusca (Webber 1977; De Jong-Brink et al. 1983; Wourms 1987; Ponder et al. 2019). Several authors have proposed that auxiliary cells resorb metabolites from oocyte degeneration for recycling in future vitellogenesis (Pipe 1987a,, 1987b; Dorange and Le Pennec 1989; Le Pennec et al. 1991; De Gaulejac et al. 1995; Chung 2007, 2008; Chung et al. 2007, 2008; Lee and Chung 2008; Kim and Chung 2014; Kim et al. 2014; Kim 2016). We have not observed such a process in *Cerastoderma edule*; indeed, the quantity of available oocyte debris is too great to be resorbed by the auxiliary cells alone–and no masses of macrophages have been observed in any of the individuals examined.

The lack of inter-oocyte adhesion, despite the presence of a viscous and sticky acid mucopolysaccharide coat (Beninger and St-Jean 1997; Smith and Morin 2002), is intriguing. The cellular debris observed on the external surface of all oocyte coats within the acini would effectively isolate the mucopolysaccharides from neighboring oocytes prior to and during spawning, thus enabling the oocytes to be released individually, rather than as a large, negatively-buoyant egg mass, which would be subjected to desiccation and osmotic

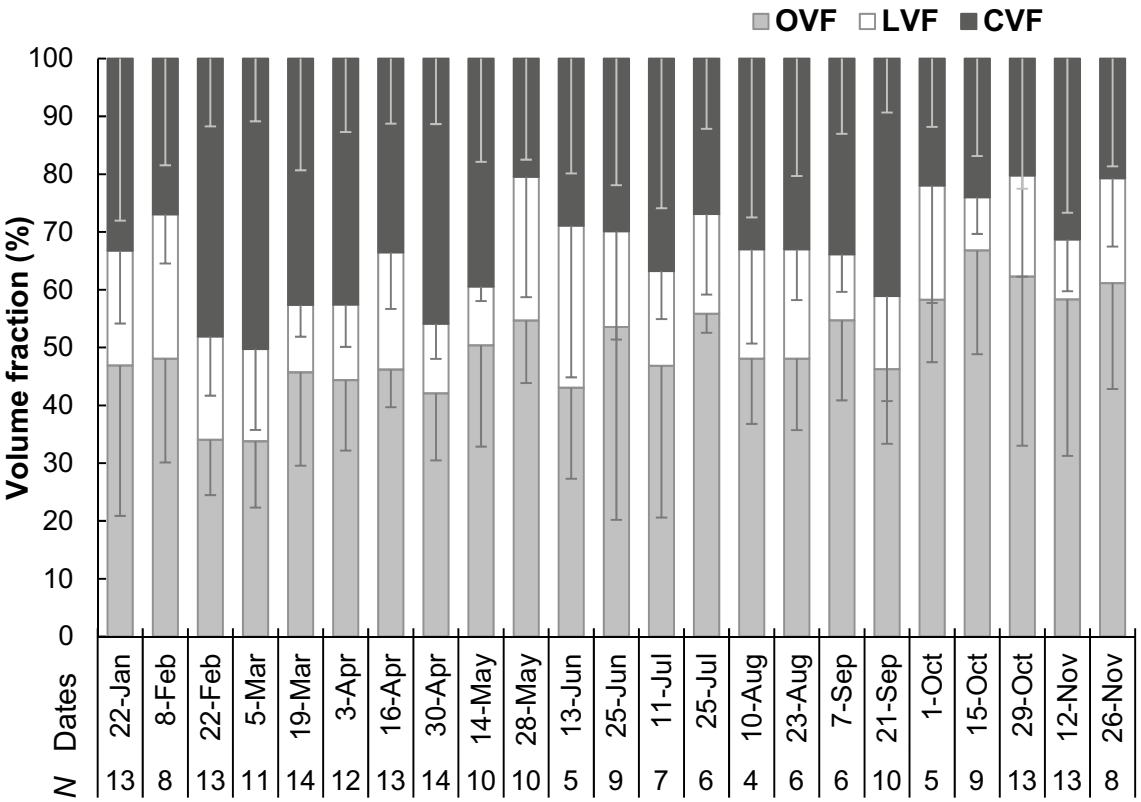

Fig. 8 *Cerastoderma edule* females pooled from site 2. Total oocyte (OVF), intra-acinal lumen (LVF), and oocyte coat (CVF) volume fractions of acini. Error bars represent half the range of values. *N*, number of females used for counts

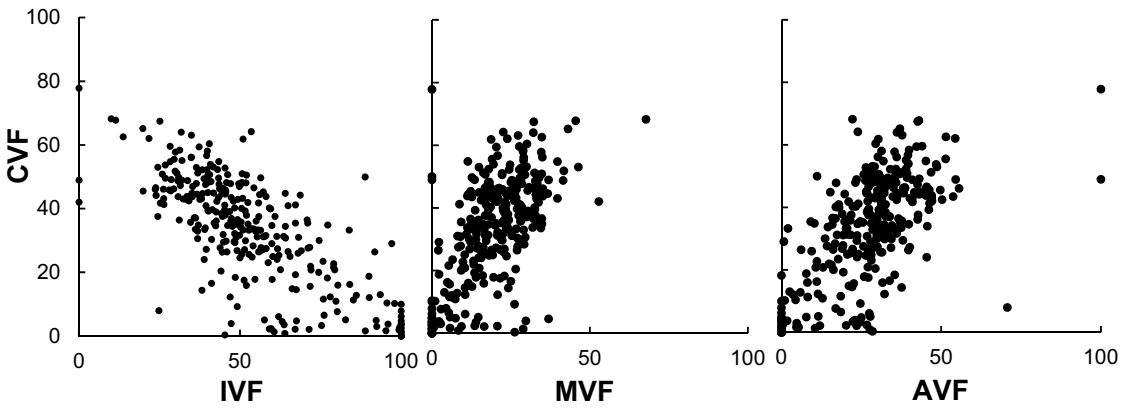

Fig. 9 *Cerastoderma edule* pooled from site 1 and 2. Coat volume fraction (CVF) as a function of oocyte volume fraction: IVF, MVF and AVF

stress in the intertidal habitat of this species. Cockles can thus accrue the advantages of the oocyte coat without sacrificing the necessity of broadcast spawning in this habitat. Additionally, the non-sticky state would allow the oocytes to be spawned individually in small numbers, resulting in the extended 'dribble spawning' strategy previously reported for this species (Chérel and Beninger 2019). The presence of atresic oocytes in all oogenic individuals is consistent with this interpretation.

We are unaware of any other study which shows the sacrifice of germ line cells (i.e. atresia) resulting in the elaboration of a 'non-stick' surface on coated, healthy oocytes. The data of the present study suggest that these two otherwise unrelated processes, oocyte atresia and oocyte coat

production, are functionally linked in *Cerastoderma edule*. High-fecundity organisms, including bivalves, are characterized by a large proportion of genetically-inviable propagules (Plough et al. 2016; Plough 2018); oocyte atresia may thus be an early manifestation of inviability. Utilization of the debris of these inviable oocytes may be an evolutionary mitigation of a gametogenic 'Red Queen' state [see Strotz et al. (2018) for a review of the Red Queen hypothesis].

The findings of the present study augment the already considerable avenues for future research on the consequences of both oocyte atresia and the oocyte coat in marine bivalves. It would be of immediate interest to replicate these investigations in some of the other bivalves known to have both oocyte coats and planktonic development (Beninger and Chérel 2019).

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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