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The seminal receptacle and implications for reproductive processes in the invasive gastropod *Crepidula fornicata*

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ABSTRACT

The calyptraeid gastropod *Crepidula fornicata* is the object of considerable research attention, due to its invasive status in the North-Eastern Atlantic, its introduction to habitats throughout the Northern hemisphere, and its scientific interest as a model organism for the study of developmental and reproductive processes in the Metazoa. Since the knowledge concerning the structural foundations for its reproductive processes is surprisingly weak, we investigated the seminal receptacle, a key structure in the reproductive biology of other metazoans, using histology, scanning electron and transmission electron microscopy. The seminal receptacle consists of 9–11 lobes, each subdivided into small, narrow lobules. The inner epithelium of the lobules appears to be highly dynamic, characterised by the perforation and attachment of received spermatozoa, the progressive degeneration of this epithelium, and the concomitant detachment of the spermatozoa. The allocation of spermatozoa to many different lobules, in different phases, may explain the extended reproductive season of *C. fornicata*, and thereby contribute to its colonizing and invasive success. The same compartmentalisation, as well as the complete covering of the inner epithelium of the lobules by spermatozoa and the large amount of spermatozoan debris in the lumina, suggest that the *C. fornicata* seminal receptacle may be a site of sperm competition in this polyandrous species.

1. Introduction

While indigenous to a wide latitudinal range in the western North Atlantic, in the past century the calyptraeid gastropod *Crepidula fornicata* has been introduced to coastal regions from the Black Sea through the Mediterranean, along the European Atlantic as far as Norway and Sweden, to the Irish and British Isles, the North Sea, the Baltic, and even the Pacific coast of North America (World Registry of Marine Species, 2015). It is a major invasive species (*sensu* Davis and Thompson, 2000) along the European Atlantic, with extreme densities in the highly human-impacted intertidal and subtidal zones of France and the Netherlands. Although some positive consequences such as increased epifaunal biodiversity have been suggested (De Montaudouin and Sauriau, 1999; Thieltges et al., 2006), considerable, and mostly negative ecological and economic consequences of these invasions have been reported, such as competition for food and space with commercially important species, fouling of fishing and aquaculture gear, etc. (Blanchard, 1997, 2009; Soulas et al., 2001; Blanchard et al., 2001; Le Pape et al., 2004; Beninger et al., 2007; Decottignies et al., 2007a,b; Martin et al., 2007; Arbach Leloup et al., 2008).

Concomitant to the practical scientific and economic interest in *C. fornicata*, theoretical interest has also developed recently, with the recommendation of this species as a model for the study of deep developmental processes in one of the three major metazoan superclades, the Lophotrophozoa (Henry et al., 2010). It has also been proposed as a particularly appropriate model system for the study of sex change in the Metazoa, and has recently contributed to the understanding of sexual selection, and in particular sperm competition, in the major metazoan phylum Mollusca (Proestou et al., 2008). Since *C. fornicata* is a protandric species in which adults form sessile stacks, with females (usually only one, and at the most two) at the bottom and several males above, it is indeed an interesting model in which to study sperm competition.

Whether the goal is to control *C. fornicata* invasions, or to use this species as a model for the study of sex change, sexual selection, and development, it is clear that a sound understanding of its reproductive biology is essential. Much of the literature concerning this aspect focusses either on gross structural observations such as sex frequencies, penis presence, brooding periods, and egg masses (Conklin, 1898; Coe, 1936, 1938a,b; Collin, 1995; Richard et al., 2006), or on molecular-genetic aspects (Gaffney and McGee,

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1992; Dupont et al., 2006; Proestou et al., 2008). Until recently, virtually no documentation of the gonadal events (gametogenesis, pre- and post-oviposition atresia, structural dynamics) was available, especially over entire seasonal cycles (Beninger et al., 2010a,b). Similarly, in contrast to the spermatozoa of this species (Kohnert and Storch, 1984a,b), no detailed study of the *C. fornicata* seminal receptacle has been performed to date, and very few such studies have been performed in the Caenogastropoda (formerly a prosobranch grouping) as a whole (Giusti and Selmi 1985; Voltzow, 1994).

Where the seminal receptacle has been studied in detail, this structure is known to be much more than its name implies, constituting a central element in the dynamics of reproductive activity and sexual selection (Beninger et al., 1993; Elner and Beninger 1995; Lanteigne et al., 1996; Baur, 1998; Neubaum and Wolfner, 1999; Simmons, 2001). Indirect evidence for its importance in metazoan reproductive biology is provided by the relatively rapid gene evolution associated with these structures (Swanson et al., 2004; Kelleher et al., 2007; Prokupek et al., 2008, 2010). Given that *C. fornicata* is capable of storing sperm in its seminal receptacles for up to 1 year (Hoagland, 1978), it is evident that a detailed knowledge of this structure is crucial to understanding the reproductive biology of this species. We therefore present a morphological and ultrastructural study of the *C. fornicata* seminal receptacle, with a view to elucidating the structural foundations upon which the reproductive processes of this species are based.

2. Materials and methods

2.1. Terminology

Due to the complexity of the gastropod reproductive system, and the divergent anatomical terminology found in the literature, the nomenclature of some parts of the system is rather confusing – especially with respect to the sperm reception and storage organs (Beeman, 1977; Tompa, 1984). Compounding the problem is the fact that in many other taxa, the terms 'seminal receptacle' and 'spermatheca' are synonymous, since there is only one sperm storage structure in the female; however, in the Gastropoda, there are two sperm-holding structures in the female. For clarity, we define the seminal receptacle (= *receptaculum seminalis*) as the structure in which spermatozoa are stored, as opposed to the spermatheca (= copulatory bursa, *bursa copulatrix*), in which they are initially received at copulation (Beeman, 1977). Similarly, we use the terms 'lobe' and 'lobule' to designate the subdivisions of the seminal receptacle, rather than 'ampulla', which has been used for several other structures of the gastropod reproductive system. Finally, we use the term 'inner epithelium' rather than 'endothelium', since the latter is mainly used to designate the inner lining of vertebrate (and not invertebrate) blood vessels, and even here it is often considered a type of epithelium (Muñoz-Chápuli et al., 2005).

Table 1
Measurement ranges (length and width) of the component parts of the *Crepidula fornicata* euspermatozoon; $n = 10$ spermatozoa.

Spermatozoan region	Length (μm)	Width (μm)
Acrosome[a]	1.89–1.93	0.12–0.14, 0.38–0.43
Nucleus	16.65–16.81	0.64–0.68
Midpiece	27.88–30.86	0.58–0.62
Flagellum[b]	96.62–101.59	0.41–0.46[1], 0.148–0.153[2]
Spermatozoa	146.59–146.80	

[a] Since the acrosome is conical, two width measurements are given: from the apical bleb (smallest width) to the basal plate (largest width).
[b] Flagellum length = glycogen piece (1) + end piece (2); flagellum width is given for both pieces separately (1 and 2).

2.2. Sampling and processing

Stacks of *C. fornicata* were hand-collected on March 19, 2007 in the intertidal zone of Bourgneuf Bay (French Atlantic coast, 46–47°N, 1–2°W), a slipper limpet-invaded ecosystem located south of the Loire estuary (Valdizan et al., 2009; Beninger et al., 2010a,b), placed in a cooler and transported to the laboratory within 1 h, where the seminal receptacles were immediately dissected out and fixed. The seminal receptacles of 10 mature females (characterised by orange-coloured gonads) were used for light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) preparations. Histological fixation and processing was performed as described by Beninger et al. (2010a). Due to the sponge-like nature of the seminal receptacles, it was necessary to dehydrate them in an ascending ethanol/Roti-Histol (Roth, Karlsruhe, Germany) series and embed them in paraffin before sectioning, to expose the interior for SEM. The 7-μm histological sections were then deparaffinated in Roti-Histol, rinsed three times in 100% ethanol, and bone-dried in hexylmethyldisilazane (Cannuel and Beninger, 2006). The resulting sections were mounted on SEM stubs, sputter-coated with gold–palladium, and observed using a JEOL JSM 6400F (JEOL, Tokyo, Japan). For biometric measurements of intact spermatozoa, the seminal receptacle contents of one female were pressed into a watch glass, fixed as above, and several drops were placed on Whatman anodisc membranes (Thermo Fisher Scientific, Waltham, MA, USA) in Petri dishes containing agar at 37 °C for 1 h. The anodisc membranes and adjoined spermatozoa were rinsed with filtered phosphate buffer (pH 7.3) and dehydrated in a graded series of ethanol, with trichlorofluoromethane as the final medium. The anodisc membranes were mounted on stubs, sputter-coated and observed as above.

For thin-section and TEM observations, fresh seminal receptacle lobes were sectioned, cut into approx. 1 mm^3 pieces, and immediately fixed in slightly hyperosmotic 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3; 1100 milliosmoles; 4 °C for 2 h). The tissue pieces were then rinsed in 0.2 M cacodylate buffer, post-fixed in cacodylate buffer/1% osmium tetroxide at 4 °C for 1 h, dehydrated in a graded ethanol series, then soaked in a 50:50 (V:V) solution of Spurr resin and propylene oxide for 1.5 h, and finally embedded in pure Spurr resin for 12 h. Polymerisation was induced at 60 °C for 48 h. Thin sections were cut on a Reichert-Jung SuperNova ultramicrotome at 1 μm, stained with toluidine blue, and examined under a light microscope, whereas ultrathin sections were cut at 600 nm, collected on uncoated 300 mesh copper/rhodium grids, contrasted with uranyl acetate and lead citrate (Reynolds, 1963), and examined with an HF2000-FEG transmission electron microscope (Hitachi, Tokyo, Japan).

3. Results

3.1. Structure and ultrastructure of the seminal receptacle

The seminal receptacle of *C. fornicata* is located to the right of the transverse branch of the gonad, and is composed of 9–11 olive-shaped lobes, each containing a number of lobules 150–200 μm in width (Fig. 1A and B). A single lobe wall encloses all of the lobules (Fig. 1B and C). Each lobule is comprised of a stratified outer and a cuboidal inner epithelium, separated by a common, well-developed, fibrous basal lamina (Fig. 2A–C).

Spermatozoa from previous copulation(s) lined the inner side of the inner epithelium of the lobule, oriented with the acrosomes toward the epithelium (Figs. 1B, C, 2A and B). Isolated spermatozoa showed the typical antero-posterior arrangement of acrosome, nucleus, mid-piece, and the somewhat thinner flagellum (Fig. 1F). The mean dimensions showed a very homogeneous

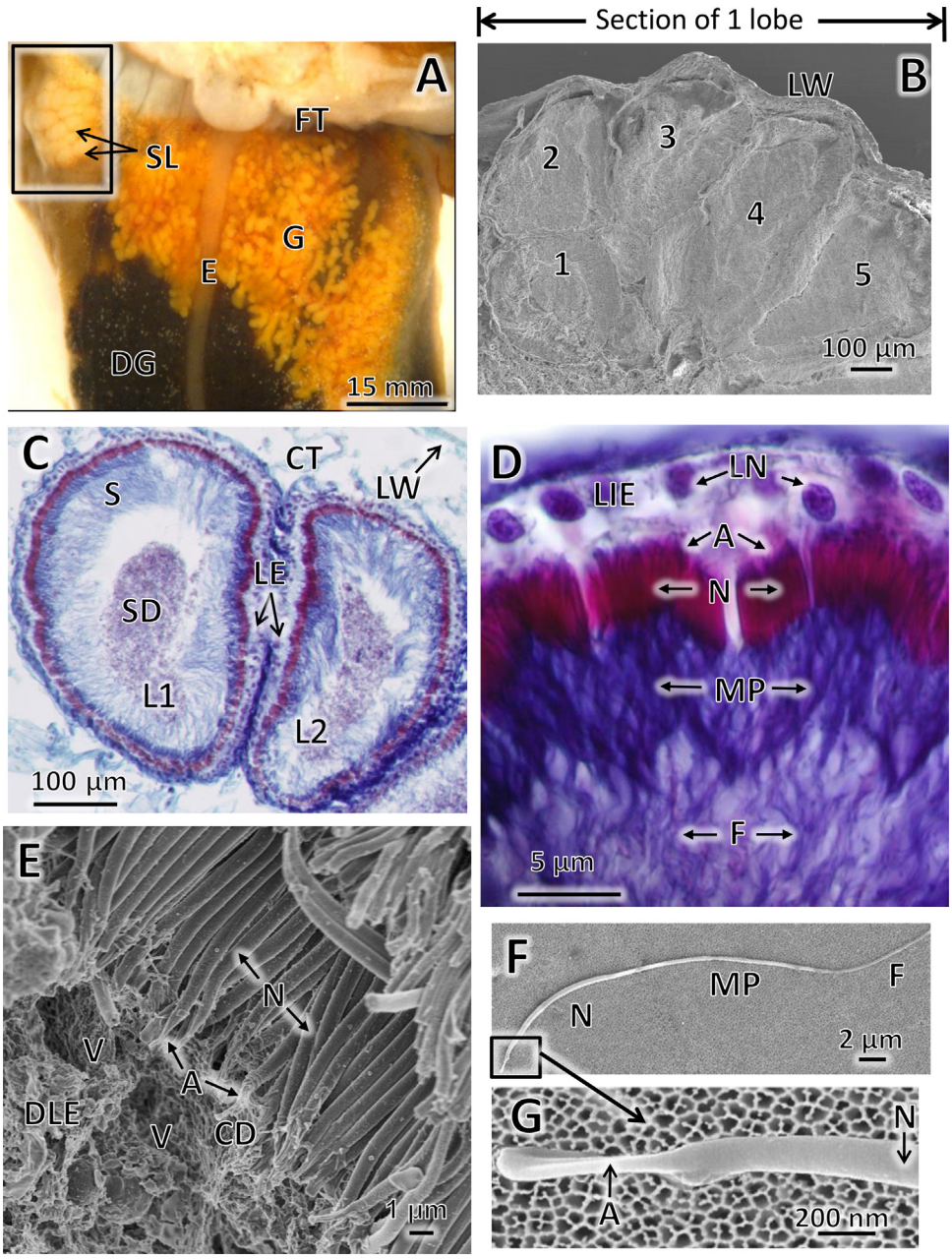

Fig. 1. General anatomical relationships, histology, and scanning electron microscopy of the *Crepidula fornicata* seminal receptacle. (A) Location and general aspect of the seminal receptacle (enclosed in rectangle). (B) SEM of a single lobe, showing 5 numbered lobules. (C) Transverse histological section through two lobules, showing general tissue organisation and spermatozoan debris in lumina. (D) Detail of inner epithelium of the lobule, showing attached spermatozoa. (E) SEM showing relation between the spermatozoan and the lobule epithelium. (F and G) SEM detail of *C. fornicata* spermatozoon. Abbreviations: A, acrosome; CD, cytoplasmic debris; CT, loose connective tissue; DG, digestive gland; DLE, degenerating, detaching inner epithelium of the lobule; E, esophagus; F, flagellae; FT, foot; G, gonad; LE, lobule epithelia; L1, L2, lobules 1 and 2; LIE, inner epithelium of the lobule; LN, lobule nucleus; LW, lobe wall; MP, mid-piece; N, nucleus; S, spermatozoa; SD, spermatozoan debris; SL, seminal receptacle lobes; V, vacuole.

spermatozoan length (146.59–146.80 µm; see Table 1). SEM and TEM micrographs show that the acrosomes actually penetrated the cytoplasm of the inner epithelium as far as the basal nuclei, which they were observed to contact, but not to penetrate (Figs. 1E, 2D and E). The acrosomes embedded in the inner epithelium presented ruffled acrosomal membranes, and the epithelial cell cytoplasm was partially withdrawn around the acrosome (Fig. 2E).

The lumina of the lobules were filled with spermatozoan debris (Figs. 1C and 2B). No paraspermatozoa were observed in any of the lobules examined, either attached to the inner epithelia or in the lumina.

3.2. Dynamics within lobules

Within a given individual, and indeed a given lobe, lobules could be observed in several states, which appeared to be sequential. The reasoning for this conclusion was that the lobules presented states in which the inner epithelium was intact, states where it was variously degraded, and a state where it was

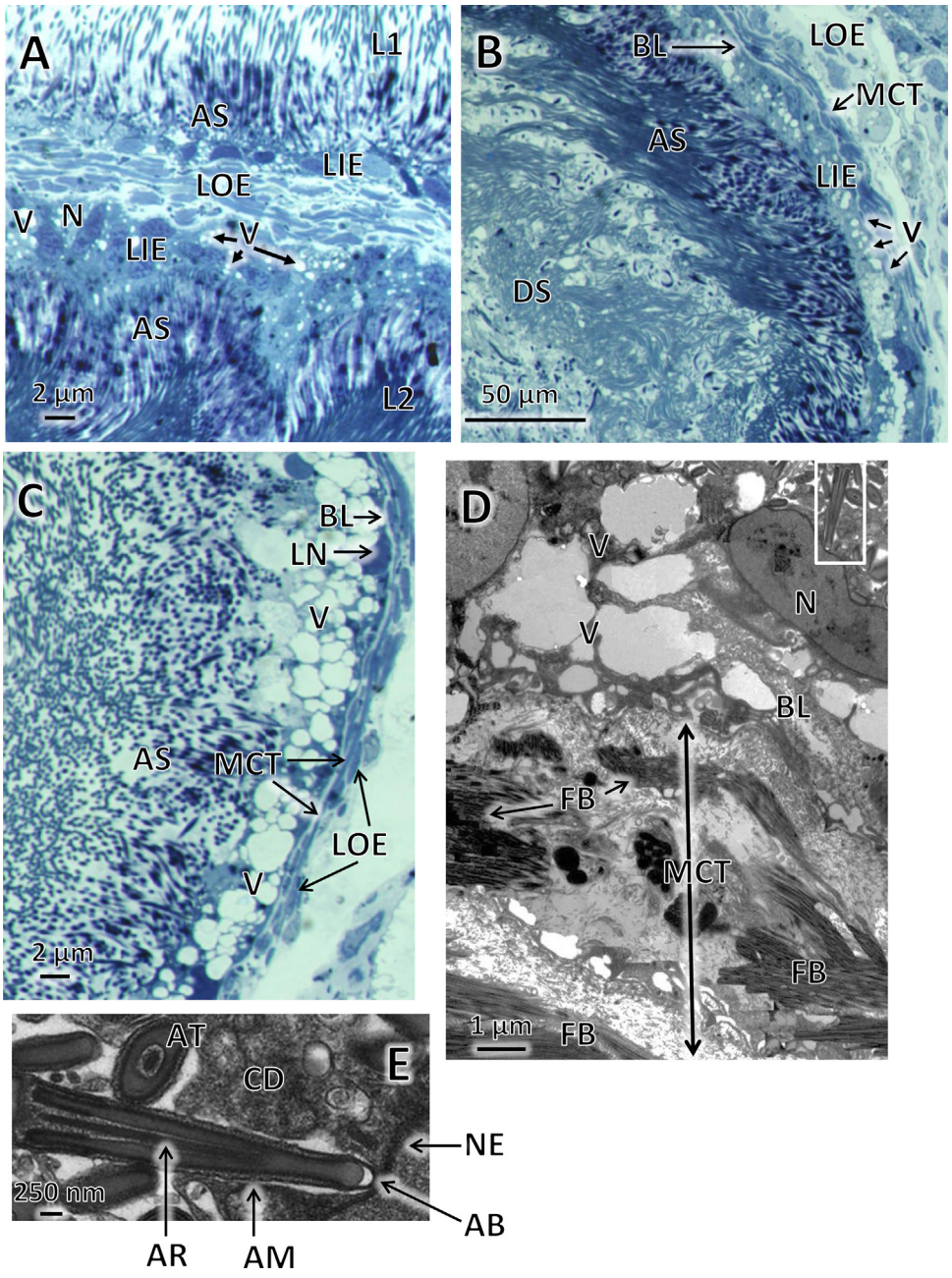

Fig. 2. Ultrastructure of *Crepidula fornicata* seminal receptacle lobule. (A) Thin section through the epithelia of two lobules (L1 and L2), showing fusion of the outer epithelia, and scattered vacuoles in the inner epithelium of L2 but not L1. (B) Thin section through the epithelia of another lobule, showing greater prevalence of vacuoles in the inner epithelium of the lobule. (C) Thin section through the epithelia of yet another lobule, showing extreme vacuolation of the inner epithelium of the lobule. (D) TEM detail of the basal region of the inner epithelium of the lobule. Note vacuolation and tearing of the cytoplasm and basal lamina. White rectangle delimits region shown in detail in (E). (E) Detail of an acrosome which has penetrated the epithelial cell cytoplasm down to the basal nucleus. Note ruffled acrosomal membrane and withdrawal of lobule epithelial cell cytoplasm from acrosome vicinity. Abbreviations: AB, acrosomal bleb; AM, acrosomal membrane; AR, acrosomal rod; AS, attached spermatozoa; AT, acrosome transverse section; BL, basal lamina; CD, cytoplasmic debris; DS, detached, degenerating spermatozoa; FB, myofibrilla; LIE, inner epithelium of lobule; LN, nucleus of inner epithelium of lobule cell; LOE, lobule outer epithelium; MCT, musculo-connective tissue; N, nucleus; NE, nuclear envelope; V, vacuole.

completely degraded. We assume that (i) a tissue must first be intact before it can be degraded, and (ii) tissues are not formed pre-degraded.

The dominant feature in this sequence was the progressive proliferation and expansion of irregularly shaped vacuoles in the inner epithelium cytoplasm (Fig. 2A–C), accompanied by the formation of cytoplasmic debris. Vacuoles also appeared in the basal lamina, which showed signs of tearing (Fig. 2D). In some lobules the extent of the vacuoles was so great that the inner epithelial layer ceased to exist as a recognizable structure, resulting in a de facto release of the previously anchored spermatozoa (Fig. 2C).

4. Discussion

4.1. Gross structure

Although the general organization of the seminal receptacle into lobes has been documented for all studied *Crepidula* species, to our

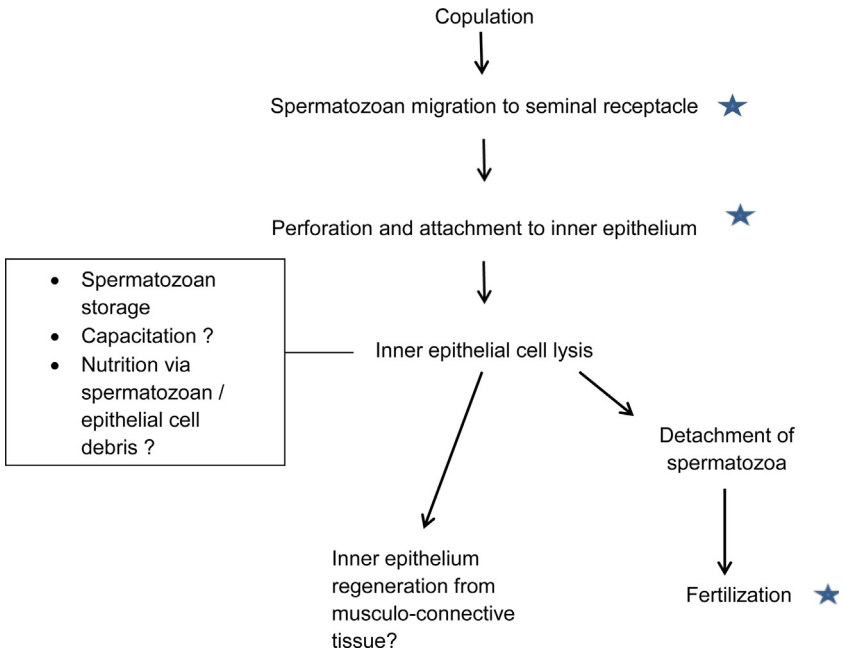

Fig. 3. Proposed sequence of events leading to fertilisation in *Crepidula fornicata*. ★ = steps at which sperm competition may intervene.

knowledge no previous study has reported the lobe subdivision into numerous small lobules in this or any other caenogastropod taxon. Such an organization allows the compartmentalisation of many different 'packages' of spermatozoa, with at least two ecologically and evolutionarily important consequences: (i) the different dynamic stages of individual lobules could mediate the extended oviposition period of *C. fornicata* (Beninger et al., 2010a,b), itself a probable factor in the colonization and invasion success of this species; (ii) the sub-lobular organization could substantially contribute to the interacting processes of sperm competition.

4.2. Spermatozoan penetration, storage, and detachment

The remarkable arrangement of spermatozoa within the gastropod seminal receptacle has been reported and speculated upon for well over a century (Conklin, 1898; Beeman, 1977; Silberzahn, 1978; Hadfield and Switzer-Dunlap, 1984; Voltzow, 1994; Beninger et al., 2010a). Although there are numerous references which state that various gastropod species present spermatozoa 'embedded' in the epithelium of the seminal receptacle, it is unclear whether, as in other metazoans, the spermatozoa merely insert between epithelial cells, within cell plicae/microvilli or cellular 'pockets', or actually penetrate these cells (Beeman, 1977; Hadfield and Switzer-Dunlap, 1984; Giusti and Selmi, 1985; Voltzow, 1994). The data of the present study clearly show not only cell penetration in the inner epithelium of the seminal receptacle of *C. fornicata,* as has been reported for the polychaete *Spirorbis spirorbis* (Daly and Golding, 1977), but also demonstrate that this penetration is accompanied by cellular damage and consequent degeneration. Together with the spermatozoan debris which fills the seminal receptacle lumina, ample trophic resources are thus available to the embedded spermatozoa, which may be stored for up to one year (Hoagland, 1978), and in particular to the mid-piece containing the mitochondrial arrays.

In the heterobranch genera *Aplysia* and *Phyllaplysia,* the ruffled acrosomal membranes of embedded spermatozoa have been shown to vesiculate and eventually disappear altogether; this has been interpreted as a capacitation step for the acrosomal reaction (Beeman, 1977). However, in vitro experiments have shown that *C. fornicata* oocytes can be fertilised by sperm stripped directly from the testis (Valdizan et al., 2009), precluding capacitation. We therefore suggest that in this caenogastropod, acrosomal membrane degeneration facilitates the time-delayed detachment of the spermatozoa from the spermathecal inner epithelium.

4.3. Dynamics within lobules

The combination of complex seminal receptacle division into small lobules, each fully lined with embedded spermatozoa, and each lobule displaying a different state of inner epithelial degeneration and spermatozoan detachment, probably contribute to the observed wide range of potential fertilization periods in *C. fornicata* (Beninger et al., 2010a,b); in effect, females could use lobules iteratively, allowing 'dribble breeding' throughout most of the year, as has been observed for this species (Beninger et al., 2010a,b). In turn, the extended period of release of larvae and recruitment of juveniles could allow *C. fornicata* to take advantage of more periods of favourable environmental conditions, as well as periods of lower planktonic and benthic competition, than if the reproductive periods were limited to one or two per year. This aspect of their reproductive biology, summarised in Fig. 3, may be a major contributor to their success as a coloniser and as an invasive species. Further study of the possible regeneration of the inner epithelium of the lobule from the muscular/connective tissue is warranted, since this type of tissue is known to be pluripotent in other molluscs, in which it may periodically form the walls and germ cells of the gonad acini or tubules (e.g., Beninger and Le Pennec, 2006).

4.4. Sperm competition

Sperm competition is a frequent dimension of sexual selection, and the final phase of male competition for access to female gametes (Parker, 1970; Birkhead and Møller, 1998). Sperm competition is greatly accentuated in animals possessing sperm storage organs such as the seminal receptacle. Like many caenogastropods, not only can *Crepidula* spp. be fertilised by several males (Gaffney and McGee, 1992; Dupont et al., 2006; Proestou et al., 2008; Brante et al., 2011; Xue et al., 2014), but the presence of a large num-

ber of variously developed seminal receptacle lobules enhances the probability of both cryptic female choice and intense sperm competition. Indeed, since male gastropods typically provide no post-zygotic investment in offspring (Bramachary, 1989; Kamel and Grosberg, 2012), sexual competition will take place essentially through sperm competition. Molecular genetic evidence suggests that sperm competition could be a driving force in the determination of *C. fornicata* male reproductive success (Proestou et al., 2008). The anatomical and ultrastructural data of the present study elucidate the structural basis for such sperm competition.

The division of the seminal receptacle into multiple narrow lobules may also explain the extremely filiform shape of the *C. fornicata* spermatozoon. The large volume of spermatozoan debris found in the lumina of the lobules shows that the number of spermatozoa competing for attachment sites is far higher than the available number of such sites. Furthermore, the limited available space in the inner epithelium of the lobules will favour the evolution of thin, 'spaghetti-like' spermatozoa, such as those observed in *C. fornicata.* Such a form would not only compete most effectively for attachment space, but also impede the arrival of rival spermatozoa, similar to the competitive principles operating in diving beetles (Higginson et al., 2012).

The results of the present study not only support the idea that seminal receptacle morphology is a contributing factor to the complexity of sperm morphology in internally versus externally fertilizing species (Miller and Pitnick, 2002; Higginson et al., 2012), but also suggest that such evolutionarily driven complexity may be a hallmark of all species which possess such sperm storage organs, found in a great many taxa across the Metazoa.

Fig. 3 summarises the sequence of events involved in sperm transfer, storage, and relations within the seminal receptacle, shown or suggested by the results of the present study. It is clear that the *C. fornicata* seminal receptacle is much more than a simple sperm storage organ; the spermatozoan–seminal receptacle relations have important implications for the reproductive, evolutionary, and invasion success of this gastropod.

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