Chitin distribution in the *Oithona* digestive and reproductive systems revealed by fluorescence microscopy

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ABSTRACT

Among copepods, which are the most abundant animals on Earth, the genus *Oithona* is described as one of the most important and plays a major role in the marine food chain and biogeochemical cycles, particularly through the excretion of chitin-coated fecal pellets. Despite the morphology of several *Oithona* species is well known, knowledge of its internal anatomy and chitin distribution is still limited. To answer this problem, *Oithona* nana and *Oithona* similis individuals were stained by WGA-FITC and DAPI for fluorescence microscopy observations. The image analyses allowed a new description of the organization and chitin content of the digestive and reproductive systems of *Oithona* male and female. Chitin microfibrils were found all along the digestive system from the stomach to the hindgut with a higher concentration at the peritrophic membrane of the anterior midgut. Several midgut shrinkages were observed and proposed to be involved in fecal pellet shaping and motion. Amorphous chitin structures were also found to be a major component of the ducts and seminal vesicles and receptacles. The rapid staining protocol we proposed allowed a new insight into the *Oithona* internal anatomy and highlighted the role of chitin in the digestion and reproduction. This method could be applied to a wide range of copepods in order to perform comparative anatomy analyses.

INTRODUCTION

Copepods are the most abundant animals on Earth ahead of insects and nematodes [1] and inhabit all aquatic niches: groundwater, vernal ponds, glaciers, lakes, rivers and oceans [2]. Among marine copepods, *Oithona* has been described as the most important marine planktonic genus in terms of abundance [3]. A recent study, based on the Tara Oceans metagenomic data, has shown the global distribution of *Oithona* in coastal and open ocean waters [4], which highlighted its key role as a major secondary producer of the marine food chain [5, 6]. The important contribution of copepods in the biological carbon pump has also been demonstrated [7], in particular through the excretion of fecal pellets [8] that sink, provide organic and inorganic compounds to microplankton [9, 10], and deposit on the sediments where they could remain as fossils for several thousand years [11, 12]. The biochemical analysis of the copepod fecal pellets have revealed a high amount of chitin [13], a beta-1-4 N-acetylglucosamine polymer, the most abundant biopolymer in nature after celluloses [13], and mostly known in copepods as a component of the exoskeleton. Besides the role of copepods in the carbon pump, the abundance of chitin in the fecal pellets also points out the implication of copepods in the global nitrogen cycle [14].

Morphological traits of more than forty *Oithona* species are well known [15], especially the structure

of the antenules, the oral appendages, the swimming legs and the caudal rami [16]. However, such morphological traits are only accessible through finical dissections under microscope that need expertise and are time consuming. Recently, also molecular tools have proven their usefulness in species identification [4, 17].

The external anatomy of copepods has been analyzed through Congo red fluorescence [18] and electronic microscopy that allowed the identification of *Oithona nana* (Giesbrecht, 1892 [19]) female sexual orifices with attached male spermatophores [2]. The internal anatomy of *Oithona similis* (Claus, 1863 [20]) has been recently described using phase contrast microscopy and provided the first insight into the organization of the female reproductive system [21]. For the digestive system, to our knowledge, only diagrams of freshwater cyclopoids exist [22–24]. Electron microscopy revealed that chitin microfibrils are present in the anterior and posterior midgut peritrophic membrane (PM) of free-living and in the posterior PM of parasitic copepods [16], but no *Oithona* species have been included in the study.

For a better understanding of the ecological success of *Oithona*, a detailed knowledge of its internal anatomy is crucial. Fluorescence microscopy based on a double staining coupling Wheat Gamma Agglutinin-Fluorescein IsoThioCyanate (WGA-FITC) and DiAmidino-2-PhenylIndole (DAPI) was used to elucidate the internal anatomy. FITC is a green fluorescent protein that can be conjugated with a wheat lectin that has an affinity and specificity to N-acetyl-β-D-glucosamine [23]. WGA-FITC staining is widely used for chitin detection by fluorescence, in a liquid medium containing lysed cells or directly on whole organisms [25–28]. On copepods, WGA-FITC was used only once before; but after dissolution of the soft tissues which did not allow the investigation of the internal anatomy [29]. In the present study, the WGA-FITC and DAPI staining was used to provide a new insight into the internal anatomy and chitin content of *Oithona nana* and *Oithona similis* with a focus on their digestive and reproductive systems.

MATERIAL AND METHODS

Biological materials samples

Oithona nana and *O. similis* specimens were sampled at two locations of the Toulon harbor, France, at the East of the little harbor of Toulon (Lat 43° 06' 52.1" N and Long 05° 55' 42.7" E) and the North of the great harbor of Toulon (Lat 43° 06' 02.3" N and Long 05° 56' 53.4" E). Sampling took place in November 2016, January 2017 and March 2017. The samples were collected from the upper water layers (0-10m) using zooplankton nets with a mesh of 90μm and 200μm. Samples were preserved in 70% ethanol and stored at -4°C.

Individual staining

Protocol adapted from the method of Farnesi *et al.*. After gently mixing the ethanol preserved samples (about 20 reversals), 100μL were sampled in a 1.5mL tube. After two minutes, the ethanol was removed, and 100μL of Phosphate Buffered Saline (PBS) at 1X and 10μL of WGA-FITC at 2mg/ml (Sigma-Aldrich [30]) were added for chitin staining. After mixing, the sample was incubated for 30 minutes protected from light before supernatant removing. To stain the DNA, 100μL of PBS at 1X and 10μL of DAPI (Sigma-Aldrich [31]) at 10X were added. The microscopy observations were done directly after mixing.

Microscopy

The stained individuals were placed between slide and coverslip and observed under a reflected fluorescence microscope Olympus BX43. WGA-FITC was excited with the 460/495nm line from a 100W mercury lamp with an interference excitation filter (BP460), and collected with a 505nm dichroic mirror (DM505) and a 510nm interference barrier filter (BA510IF). DAPI fluorescence was excited with the 340/390nm line from a 100W mercury lamp with an interference excitation filter (BP340), and collected with a 410nm dichroic mirror (DM410) and a 420nm interference barrier filter (BA420IF). Selected *Oithona* individuals were photographed with a sixteen-megapixel camera using the ToupView software (v.3.7). For each individual, three photographs were taken: one in polarized light, one with the WGA-FITC fluorescence and one with the DAPI fluorescence. Some color adjustments were made with the ImageJ software [32].

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RESULTS

Oithona morphology with WGA-FITC microscopy

The *Oithona* chitin was labelled with WGA-FITC directly on the individuals and observed by fluorescence microscopy. The setae and spines of the exopod segments of the five leg pairs could be identified and counted on *O. nana* (Figure 1.A). These first results revealed the chitinous structure of the setae and the spines, and could provide a rapid method for taxonomical identification. However, because of the individuals and setae position on the plate, we were not able to identify and count the setae of all tested individuals. Chitinous elliptic or spherical structures of unknown function and larger than 6 micrometers (Figure 1.A) were also visible at the exopods of the swimming legs. These structures may be smaller, or absent in other individuals observed.

Chitin distribution in the *Oithona* digestive system

Chitin was detected along the digestive system, from the stomach to the hindgut of the nauplius (Figure 1.B) and adults (Figure 1.C, 1.D, 1.E) of the two species. The exoskeleton chitin was also stained by the WGA-FITC, which allowed a clear identification of the stomach in the prosome, of the midgut in the prosome and of the urosome and the hindgut in the urosome. Along all the digestive system, the chitin had a microfibrilar structure aligned along the antero-posterior axis with regions showing higher microfibrils density, especially the anterior midgut and some stomach areas (Figure 1.C, 1.D, 1.E, 1.F). Some individuals contained in their anterior and posterior midgut one or several elliptical fecal pellets completely engulfed by chitin (Figure 1.E, 3.B), however, no fecal pellets were found in the nauplius. In the anterior and posterior midgut, we observed several shrinkages at different interval distances corresponding to midgut contractions. In certain cases, several shrinkages (up to four) were separated by less than five micrometers (Figure 1.D), while other individuals showed more distant shrinkages (Figure 1.F).

Chitin distribution in the *Oithona* reproductive system

The DAPI staining on *Oithona* females allowed the identification of the ovaries and the oviducts that presented a heart shape in the middle of the prosome (Figure 2.A), as previously described by Mironova et al. [21]. The oviducts start from each lateral side of the gonads to the seminal receptacle in the genital double somite (the first two segments of the urosome). Comparing to the microfibrilar structure of the chitin found in the digestive system, the chitin staining in the reproductive system was mainly amorphous. Besides, its distribution was discontinuous along the ducts, altering chitin rich and poor areas (Figure 2.B).

In the female, we distinguished two parts forming the seminal receptacle (Figure 2.C). The first part was chitin rich and located in the anterior region of the receptacle. The chitin distribution between the anterior receptacle and the oviduct was discontinuous. The second part was located in the posterior receptacle and contained less and sparser chitin, presenting a mix of microfibrilar and amorphous structures. In some females, the presence of the DNA rich material in the posterior region of the seminal receptacle was observed by DAPI staining and was likely to be male semen.

In the male, the chitin staining allowed the identification of the spermiducts, which presented the same chitin pattern observed in the oviducts (Figure 3.A, 3.C). The spermiducts probably start from each side of the male gonads (not visible on the pictures) to the seminal vesicles, in the sexual somite (Figure 3.B). As for the female seminal receptacle, the male seminal vesicle can be divided in two parts (Figure 3.D, 3.E). The first part of the vesicles is chitin-rich, located in the anterior region of the vesicle. The distribution of the chitin from this upper part of vesicle to the spermiduct was not continuous. The second part located in the posterior region of the vesicle was DNA-rich corresponding likely to male semen.

DISCUSSION

Biological materials samples

The use of WGA-FITC revealed chitinous structures in the exopods of the swimming legs, which were not known before. We hypothesize that these could be luminous glands [33]. However, luminescence is not conspicuous in *Oithona*.

The WGA-FITC staining allowed also the identification of the chitin distribution in the *Oithona* organs, which provides a high quality view of the external and internal anatomy and pointed out the major role of

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chitin in the *Oithona* digestion and reproduction. According to insect studies, the distribution of chitin in the digestive system is limited to midgut [34, 35]. The same chitin distribution was observed in decapods [36, 37]. In both *Oithona* species, we detected chitin throughout the digestive system, which distinguish it from other arthropods. In the PM of some insects [34, 38, 39], chitin plays a role in protection (chemical, mechanical and against viruses, bacteria and pathogens) and in digestion [35]. As the synthesis of chitin has a significant metabolic cost for the organism, we hypothesized that, like the insects and decapods PM, the formation of a chitin coat around fecal pellets help to protect against toxins and pathogens that were not degraded during the digestion.

In copepods, no evidence of midgut contraction has previously been described although the phenomenon has been suggested at several instances [40]. We suppose that the midgut shrinkages observed in this study could play a key role in the formation and motion of the fecal pellets to the anus. However, we observed no correlation between the presence of intestine shrinkages and fecal pellets. As proposed by Yoshikoshi and Kô for other copepods [41], we also hypothesize that, in *Oithona*, the formation of chitin coat around the fecal pellets can be produced by engulfing digested food in chitin microfibrils present in the PM of the anterior midgut (Figure 4).

The presence of chitin along the oviducts and spermiducts walls validates the cuticular appearance of the ducts described by Cuoc et al. [42]. The bipartite structure of the seminal receptacles and vesicles found in *O. nana* and *O. similis* males and females were very similar. In male, we hypothesized that the chitinous structure of the vesicle plays a role in the holding of the spermatophores during their formation.

Likewise, in the females, we hypothesized than this structure would play a role in the holding of the ovisac but also in the opening and closing of the oviduct to release ovocytes in the seminal receptacle.

CONCLUSION

With this study, we adapted and tested a simple and rapid double staining protocol that can help to the taxonomic identification of copepods, and enable new studies on copepod comparative anatomy at a larger scale. The application of the method to *Oithona* extended the knowledge of the biochemistry and structure of its digestive and reproductive systems. Considering the important role of copepods in the carbon and nitrogen sequestration through chitin synthesis, more efforts should be done to understand the molecular and physiological mechanisms involved in fecal pellets formation. For instance, laser dissection of the digestive system and muscle staining could be performed to determine if the midgut shrinkages are due to muscular contractions, or other physicochemical mechanisms.

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AUTHORS' CONTRIBUTION

KS and JLJ collected the samples. BV developed the double staining protocol. KS, BV and MAM performed the microscopy analysis, KS, MAM, AC, JLJ and PW contributed to the manuscript. MAM supervised the study.

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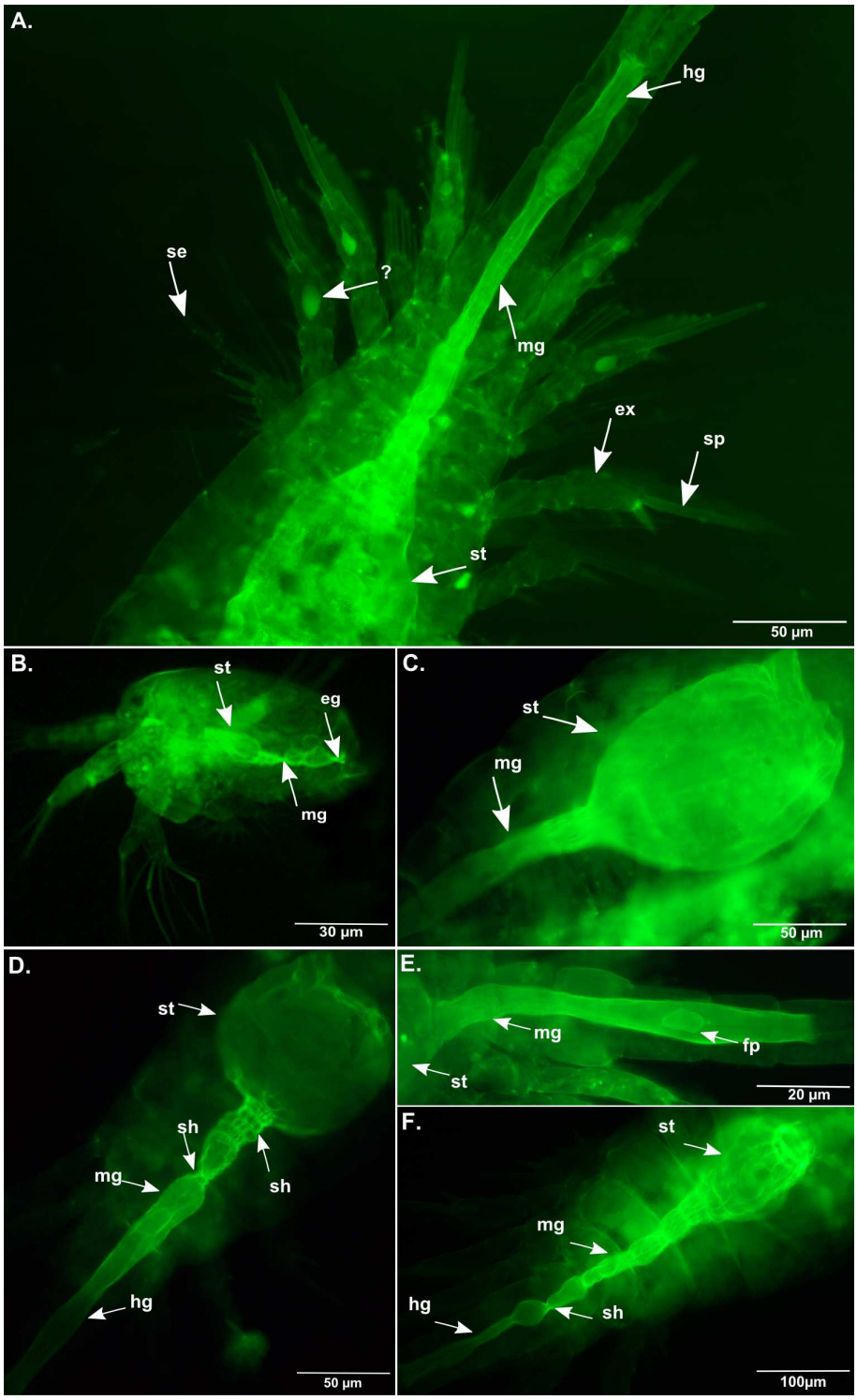

Figure 1. ***Oithona*** **appendages morphology and digestive system by WGA-FITC fluorescence microscopy. st:** stomach, **mg:** midgut, **hg:** hindgut, **sh:** shrinkage **ex:** exopod, **se:** setal, **sp:** spine, **fp:** fecal pellet. **A.** Dorsal view of the *O. nana* female swimming appendages. **B.** Lateral view of the *Oithona* nauplius digestive system. **C.** Lateral view of the *O. nana* female stomach. **D.** Dorsal view of the *O. nana* female stomach. **E.** Lateral view of an *O. nana* male gut. **F.** Dorsal view of an *O. similis* female adult stomach.

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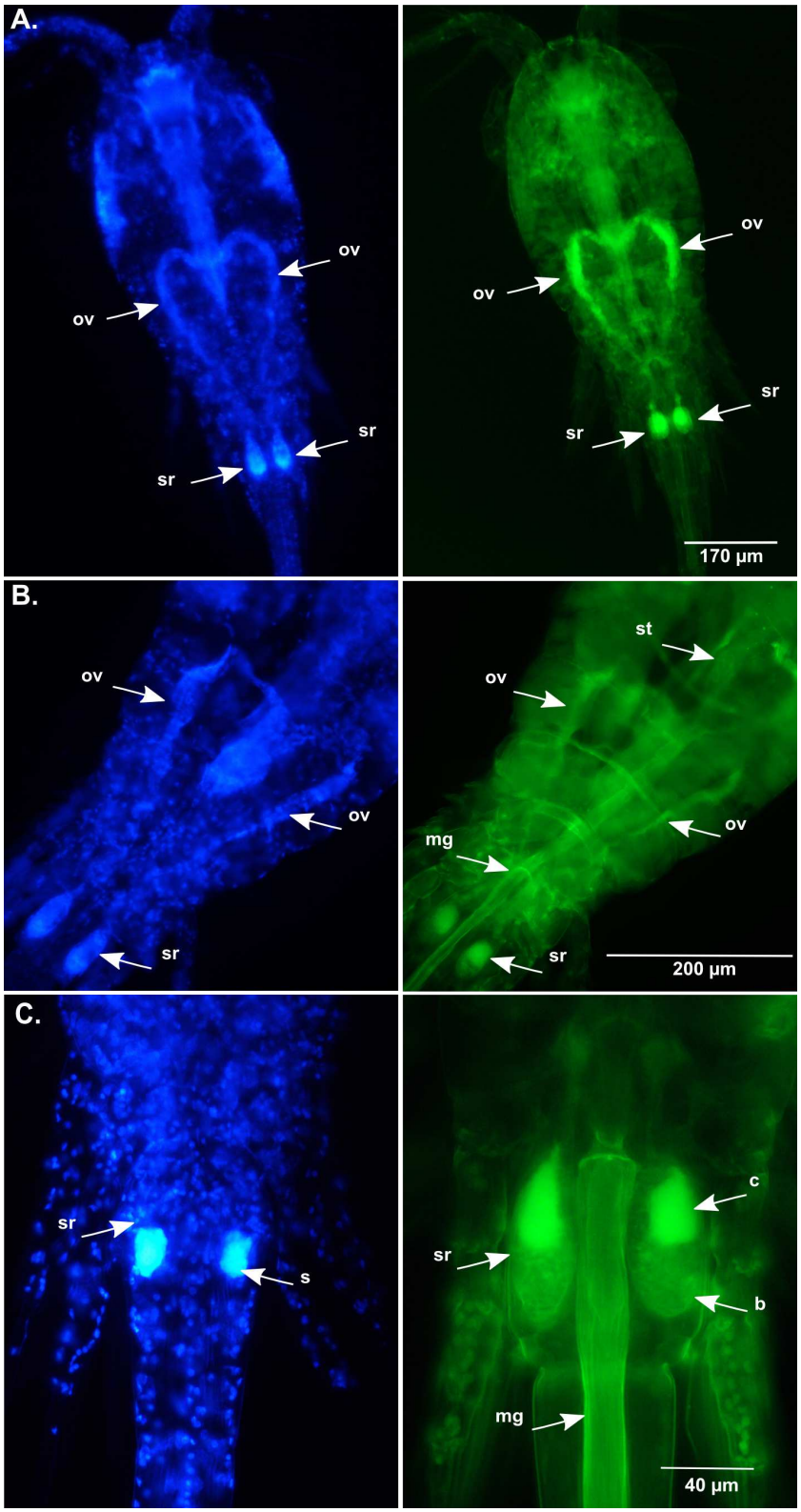

Figure 2. ***Oithona*** **female reproductive system by DAPI and WGA-FITC fluorescence microscopy. mg:** midgut, **sr:** seminal receptacle, **s:** semen, **ov:** oviduct, **hg:** hindgut, **b:** diffuse chitin region, **c:** chitin rich region. **A.** Dorsal view of the *O. nana* female reproductive system. **B.** Dorsal view of the *O. similis* female reproductive system. **C.** Dorsal view of the *O. nana* female double sexual somite.

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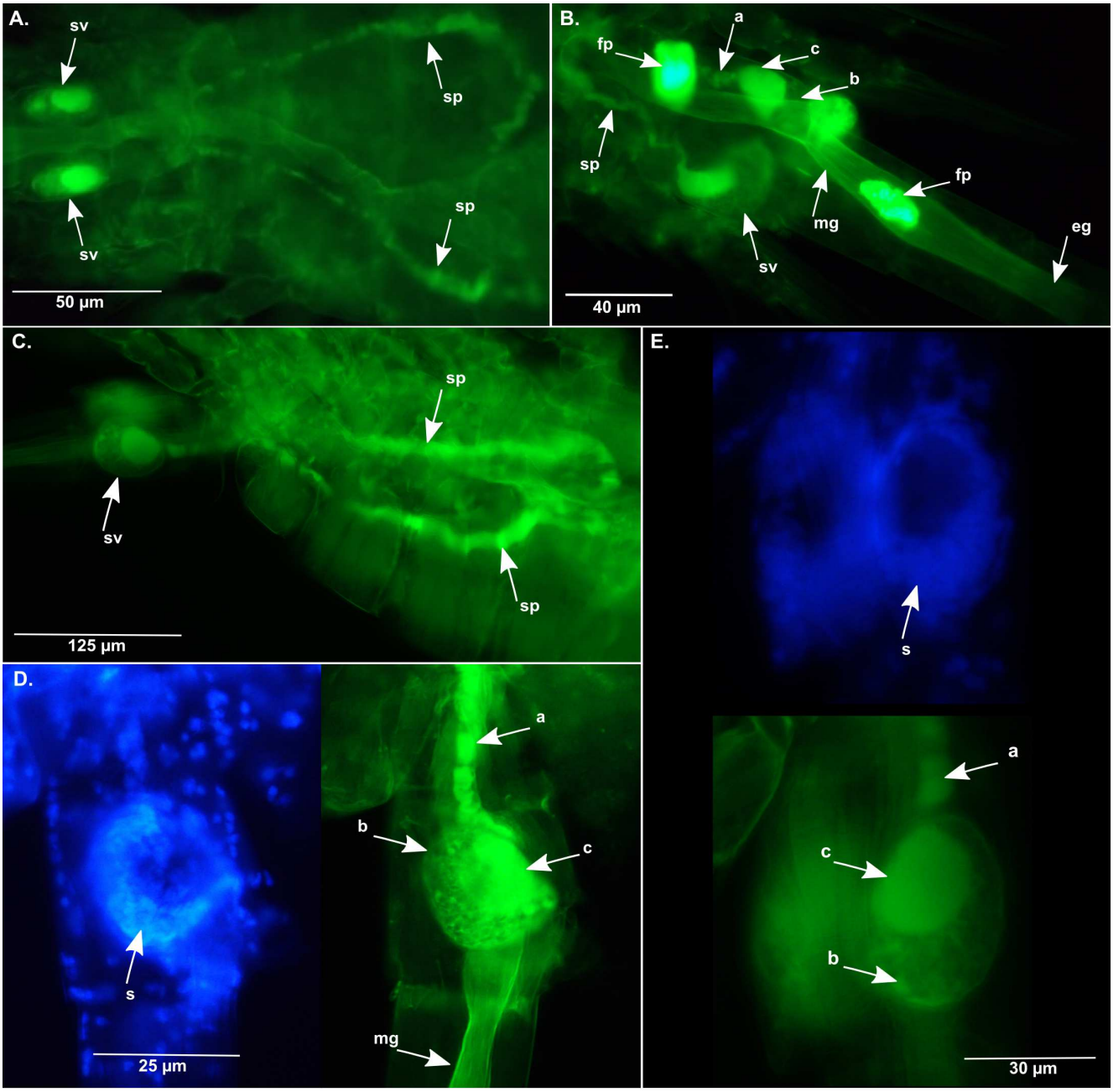

Figure 3. ***Oithona*** **male reproductive system by DAPI and WGA-FITC fluorescence microscopy.** **mg:** midgut, **fp:** fecal pellet, **sv:** seminal vesicle, **sp:** spermiduct, **a:** heterogeneous chitin, **b:** diffuse chitin, **c:** chitin rich region. **A.** Dorsal view of the *O. nana* male reproductive system. **B.** Dorso-lateral view of the *O. nana* seminal vesicle. C. Lateral view of the male O. similis reproductive system. D. Lateral view of the O. nana male double sexual somite. **E.** Lateral view of the *O. similis* male double sexual somite.

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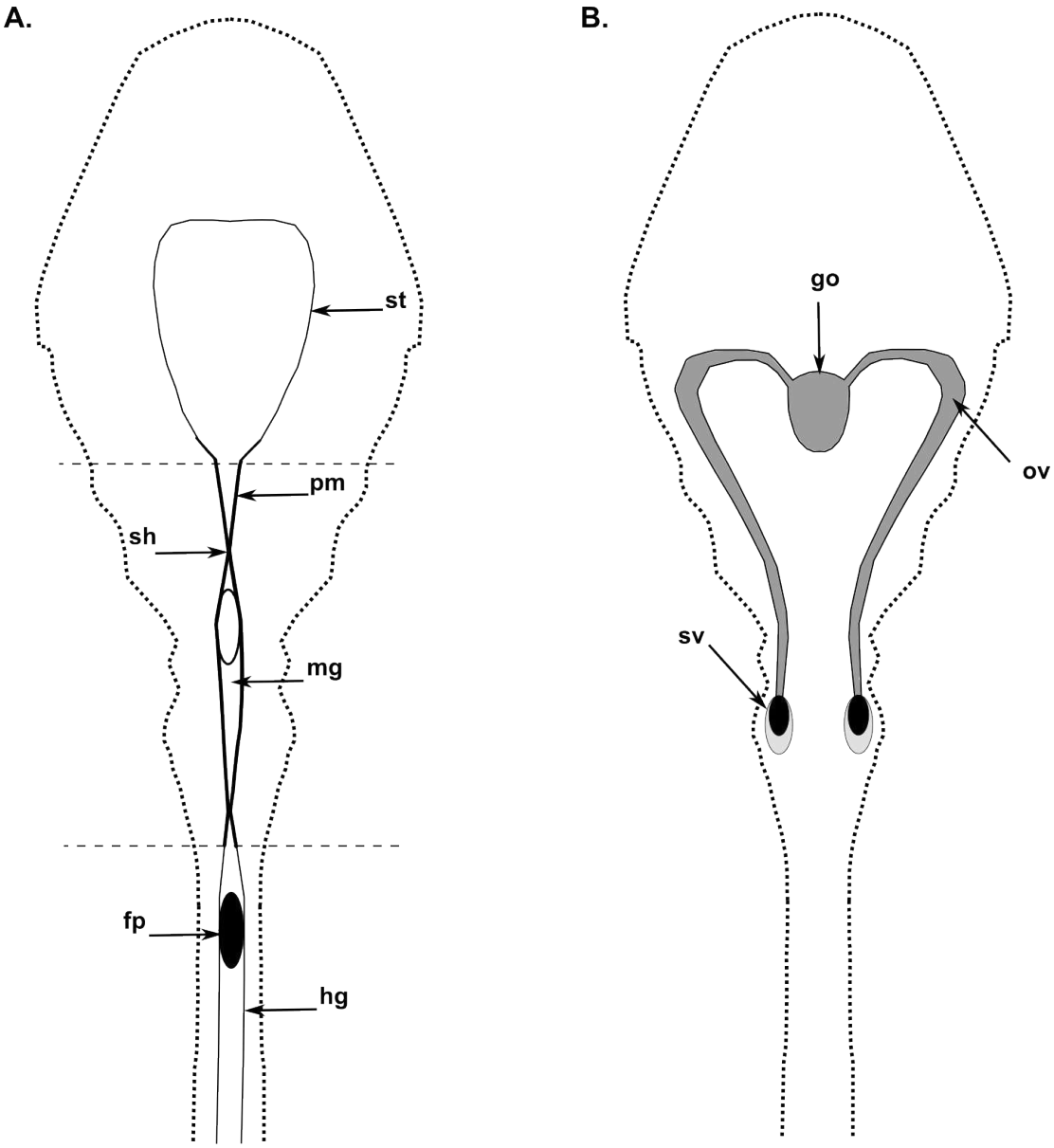

Figure 4. The diagram of the internal anatomy of a female *Oithona nana*. st: stomach, **mg:** midgut, **pm:** peritrophic membrane **sh:** shrinkages, **fp:** fecal pellet, **hg:** hindgut, **sr:** seminal receptacle, **ov:** oviduct. Thick black zones correspond to chitin rich areas. Dark gray zones correspond to heterogeneous chitin area. Light grey zones correspond to amorphous chitin areas. **A.** Diagram of the dorsal view of the digestive system **B.** Diagram of the dorsal view of the reproductive system.

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