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

Kevin Sugier [Corresp.,] [1, 2, 3, 4] , **Benoit Vacherie** [1] , **Astrid Cornils** [5] , **Patrick Wincker** [1, 2, 3, 4] , **Jean-Louis Jamet** [6] , **Mohammed-Amin Madoui** [1, 2, 3, 4]

[1] Commissariat à l'Energie Atomique (CEA), Institut François Jacob, Genoscope, Evry, France

[2] Centre National de la Recherche Scientifique (CNRS) UMR8030, Evry, France

[3] Université d'Evry Val d'Essonne, Evry, France

[4] Université Paris-Saclay, Evry, France

[5] Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Polar Biological Oceanography, Bremerhaven, Germany

[6] Université de Toulon, Aix Marseille Universités, CNRS/INSU, IRD, MIO UM 110, Mediterranean Institute of Oceanography, La Garde, France

Corresponding Author: Kevin Sugier
Email address: ksugier@genoscope.cns.fr

Among copepods, which are the most abundant animals on Earth, the genus *Oithona* is described as one of the most numerous 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.

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Kevin Sugier[1,2,3,4], Benoit Vacherie[1], Astrid Cornils[5], Jean-Louis Jamet[6], Patrick Wincker[1,2,3,4],

Mohammed-Amin Madoui[1,2,3,4]

[1] Commissariat à l'Energie Atomique (CEA), Institut François Jacob, Genoscope, Evry, France

[2] Centre National de la Recherche Scientifique (CNRS) UMR8030, Evry, France

[3] Université d'Evry Val d'Essonne, 2 rue Gaston Crémieux, 91057 Evry, France

[4] Université Paris-Saclay, Evry, France

[5.]Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Polar Biological

Oceanography, Bremerhaven, Germany

[6.] Université de Toulon, Aix Marseille Universités, CNRS/INSU, IRD, MIO UM 110, Mediterranean

Institute of Oceanography, La Garde, France.

Abstract

21 Among copepods, which are the most abundant animals on Earth, the genus *Oithona* is described

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25 distribution is still limited. To answer this problem, *Oithona nana* and *Oithona similis*

26 individuals were stained by WGA-FITC and DAPI for fluorescence microscopy observations.

27 The image analyses allowed a new description of the organization and chitin content of the

28 digestive and reproductive systems of *Oithona* male and female. Chitin microfibrils were found

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30 peritrophic membrane of the anterior midgut. Several midgut shrinkages were observed and

31 proposed to be involved in faecal pellet shaping and motion. Amorphous chitin structures were

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Introduction

36

37 Copepods are the most abundant animals on Earth ahead of insects and nematodes (Humes 1994)

38 and inhabit all aquatic niches: groundwater, vernal ponds, glaciers, lakes, rivers and oceans

39 (Huys & Boxshall 1991). Among marine copepods, *Oithona* has been described as the most

40 important marine planktonic genus in terms of abundance (Gallienne & Robins 2001). A recent

41 study, based on the *Tara* Oceans metagenomic data, has shown the global distribution of *Oithona*

42 in coastal and open ocean waters (Madoui *et al*. 2017), which highlighted its key role as a major

43 secondary producer of the marine food chain (Beaugrand *et al*. 2003; Zamora-Terol *et al*. 2014).

44 The important contribution of copepods in the biological carbon pump has also been

45 demonstrated (Jonasdottir *et al*. 2015), in particular through the excretion of faecal pellets

46 (Steinberg & Landry 2017) that sink, provide organic and inorganic compounds to

47 microplankton (Steinberg *et al*. 2002; Valdés *et al*. 2017), and deposit on the sediments where

48 they could remain as fossils for several thousand years (Bathmann *et al*. 1987; Haberyan 1985).

49 The biochemical analysis of the copepod faecal pellets has revealed a high amount of chitin

50 (Kirchner 1995), a beta-1-4 N-acetylglucosamine polymer, the most abundant biopolymer in

51 nature after celluloses (Kirchner 1995), and mostly known in copepods as a component of the

52 exoskeleton. Besides the role of copepods in the carbon pump, the abundance of chitin in the

53 faecal pellets also points out the implication of copepods in the global nitrogen cycle (Frangoulis

54 *et al*. 2004).

55 Morphological traits of more than forty *Oithona* species are well known (Razouls *et al*. 2005-

56 2018), especially the structure of the antennules, the oral appendages, the swimming legs and the

57 caudal rami (Nishida 1985). However, such morphological traits are only accessible through

58 finical dissections under the microscope that need expertise and are time-consuming. Recently,

59   molecular tools have proven their usefulness in species identification (Cornils *et al*. 2017;

60   Madoui *et al*. 2017).

61   The detailed external anatomy of copepods has been analyzed through Congo red fluorescence

62   (Michels & Büntzow 2010) and through electron microscopy that allowed the species

63   identification of copepods and the characterization of their external structures (Chang 2013;

64   Cuoc et al. 1997; Marques et al. 2017). Using an electron microscope, *Oithona nana* Giesbrecht,

65   1892 female sexual orifices with attached male spermatophores were observable (Huys &

66   Boxshall 1991). Diagrams of marine and freshwater cyclopoids, which provide the structures of

67   the reproductive and digestive systems (Borradaile & Potts 1935; Dussart & Defaye 2001;

68   Kellogg 1902) were available. Some studies proposed methods to observe the reproductive

69   system of aldehyde-preserved copepods by direct light microscopy observation of individuals

70   (Eisfeld & Niehoff 2007; Niehoff 2003; Niehoff & Hirche 1996; Tande & Hopkins 1981), by

71   staining of gonad with borax carmine (Tande & Gronvik 1983; Tande & Hopkins 1981), with

72   fluorescent polyunsaturated aldehydes (PUAs) probes (Wolfram *et al*. 2014), or with Fast Green

73   (Batchelder 1986). The internal anatomy of *Oithona similis* Claus, 1863 has been recently

74   described using phase contrast microscopy and provided the first insight into the organization of

75   the *Oithona* female reproductive system (Mironova & Pasternak 2017). In the Wolfram *et al*

76   study, some pictures of the calanoid *Acartia tonsa* obtained using fluorescent PUA probes, also

77   allowed to determine the anatomy of the digestive system. Other studies (Bautista & Harris

78   1992; Debes *et al*. 2008) used the chlorophyll fluorescence to determine the ingestion rates and

79   the gut contents, but without providing a clear structure of the digestive organs. Electron

80   microscopy revealed that chitin microfibrils are present in the anterior and posterior midgut

81 peritrophic membrane (PM) of free-living and in the posterior PM of parasitic copepods

82 (Yoshikoshi & Kô 1988), but no *Oithona* species have been included in the study.

83 For a better understanding of the ecological success of *Oithona*, a detailed knowledge of its

84 internal anatomy is crucial. Fluorescence microscopy based on a double staining coupling Wheat

85 Gamma Agglutinin-Fluorescein IsoThioCyanate (WGA-FITC) and DiAmidino-2-PhenylIndole

86 (DAPI) were used to elucidate the internal anatomy. DAPI is a blue fluorescent protein which

87 has an affinity to two nucleoids: adenine and thyrosin (Lin *et al*. 1977). This staining is widely

88 used to detect DNA in eukaryotes, prokaryotes and some viruses, without tissue-specificity.

89 FITC is a green fluorescent protein that can be conjugated with a wheat lectin that has an affinity

90 and specificity to N-acetyl-β-D-glucosamine (Allen *et al*. 1973). WGA-FITC staining is widely

91 used for chitin detection by fluorescence, in a liquid medium containing lysed cells or directly on

92 whole organisms (El Gueddari *et al*. 2002; Farnesi *et al*. 2015; Fones *et al*. 2016; Godoy *et al*.

93 2015). On copepods, WGA-FITC was used only once; but after dissolution of the soft tissues

94 which did not allow the investigation of the internal anatomy (Mravec *et al*. 2014). In the present

95 study, we used WGA-FITC and DAPI staining to provide a new insight into the internal anatomy

96 and chitin content of *Oithona nana* and *Oithona similis* with a focus on their digestive and

97 reproductive systems.

Material and methods

99 **Biological materials samples**

100 *Oithona nana* and *O. similis* specimens were sampled at two locations of the Toulon harbor,

101 France, at the East of the little harbor of Toulon (Lat 43° 06' 52.1" N and Long 05° 55' 42.7" E)

102 and the North of the great harbor of Toulon (Lat 43° 06' 02.3" N and Long 05° 56' 53.4" E).

103   Sampling took place in November 2016, January, March and June 2017. The samples were

104   collected from the upper water layers (0-10m) using zooplankton nets with a mesh of 90μm and

105   200μm. Samples were preserved in 70% ethanol and stored at -4°C. In the samples, individuals

106   of the four different development stages were observable (nauplii, copepodites and adults of both

107   sexes), but the large majority were female adults.

108   **Individual staining**

109   This protocol was adapted from Farnesi *et al.* After gently mixing the ethanol preserved samples

110   (about 20 reversals), 100μL were sampled in a 1.5mL tube. After two minutes, the ethanol was

111   removed, and 100μL of Phosphate Buffered Saline (PBS) at 1X and 10μL of WGA-FITC at 2mg

112   mL^{-1} (Sigmaaldrich.com 2017b) were added for chitin staining. After mixing, the sample was

113   incubated for 30 minutes protected from light before supernatant removing. To stain the DNA,

114   dual staining with DAPI can be performed by adding, 100μL of PBS at 1X and 10μL of DAPI

115   (Sigmaaldrich.com 2017a) at 10X. The microscopy observations were done directly after mixing.

116   This protocol can also be used on living individuals from a seawater sample; in this case, sodium

117   chloride at 39g L^{-1} has to be added to the PBS solution.

118   **Microscopy**

119   The stained individuals were placed between slide and coverslip and observed under a reflected

120   fluorescence microscope Olympus BX43. WGA-FITC was excited with the 460/495nm line

121   from a 100W mercury lamp with an interference excitation filter (BP460), and collected with a

122   505nm dichroic mirror (DM505) and a 510nm interference barrier filter (BA510IF). DAPI

123   fluorescence was excited with the 340/390nm line from a 100W mercury lamp with an

124   interference excitation filter (BP340), and collected with a 410nm dichroic mirror (DM410) and

125   a 420nm interference barrier filter (BA420IF). Selected *Oithona* individuals were photographed

126   with a sixteen-megapixel camera using the ToupView software (v.3.7). For each individual, three

127   photographs were taken: one in polarized light, one with the WGA-FITC fluorescence and one

128   with the DAPI fluorescence. Some colour adjustments were made with the ImageJ software

129   (Schneider *et al*. 2012).

Results

130

Oithona morphology with WGA-FITC microscopy

131

132   The *Oithona* chitin was labelled with WGA-FITC directly on the individuals and observed by

133   fluorescence microscopy. The setae and spines of the exopod segments of the five leg pairs could

134   be identified and counted on *O. nana* (Figure 1.A). These first results revealed the chitinous

135   structure of the setae and the spines, and could provide a rapid method for taxonomical

136   identification. However, because of the individuals and setae position on the plate, we were not

137   able to identify and count the setae of all tested individuals. Chitinous elliptic or spherical

138   structures of unknown function and larger than 6 micrometres (Figure 1.A) were also visible in

139   the exopods of the swimming legs. These globular structures were observed in both sexes of

140   *O. nana* (Figure 1.A, 2.A, 2.B, 2.C, 2.D, 2.E), but only in female individuals of *O. similis* (Figure

141   2.F, 2.G). They may also be smaller (2.F), or absent (Figure 3.A, 4.C) in other individuals. These

142   structures can also be present in other exopod segments (Figure 2.A). Another tubular structure,

143   in the distal part of the exopods 3 of the right third leg, right and left fourth legs and right and left

144   fifth legs were noticeable (Figure 1.A). In other *Oithona* individuals, these tubular structures

145   appear to be attached to the globular structure (Figure 2.B, 2.D, 2.G).

146   **Chitin distribution in the *Oithona* digestive system**

147   Chitin was detected all along the digestive system, from the stomach to the hindgut of the

148   nauplius (Figure 1.B) and adults (Figure 1.C, 1.D, 1.E) of the two species. The exoskeleton chitin

149   was also stained by the WGA-FITC, which allowed a clear identification of the stomach in the

150   prosome, of the midgut in the prosome and in the urosome and the hindgut in the urosome.

151   Along the digestive system, the chitin had a microfibrillar structure aligned along the antero-

152   posterior axis with regions showing higher microfibrils density, especially the anterior midgut

153   and some stomach areas (Figure 1.C, 1.D, 1.E, 1.F). Some individuals contained in their anterior

154   and posterior midgut one or several elliptical faecal pellets completely engulfed by chitin (Figure

155   1.E, 4.B). However, no faecal pellets were found in the nauplius. In the anterior and posterior

156   midgut, we observed several shrinkages at different interval distances corresponding to midgut

157   contractions. In certain cases, several shrinkages (up to four) were separated by less than five

158   micrometres (Figure 1.D), while other individuals showed more distant shrinkages (Figure 1.F).

159   **Chitin distribution in the *Oithona* reproductive system**

160   The DAPI and WGA-FITC stainings on *Oithona* females allowed the identification of the

161   ovaries and the oviducts that presented a heart shape in the middle of the prosome (Figure 3.A,

162   3.B) as previously described by Mironova & Pasternak. The oviducts start from each lateral side

163   of the gonads to the seminal receptacle in the genital double somite (the first two segments of the

164   urosome). Comparing to the microfibrillar structure of the chitin found in the digestive system,

165   the chitin staining in the reproductive system was mainly amorphous. Besides, its distribution

166   was discontinuous along the ducts, altering chitin-rich and poor areas (Figure 3.C, 3.D).

167   In females, we distinguished two parts forming the seminal receptacle (Figure 3.E, 3.F). The first

168   part was chitin-rich and located in the anterior region of the receptacle. The chitin distribution

169   between the anterior receptacle and the oviduct was discontinuous. The second part was located

170   in the posterior receptacle and contained less and sparser chitin, presenting a mix of

171   microfibrillar and amorphous structures. Thanks to DAPI staining, in some females, the presence

172   of the DNA rich material in the posterior region of the seminal receptacle was observed and was

173   likely to be male semen.

174   In males, the chitin staining allowed the identification of the spermiducts, which presented the

175   same chitin pattern observed in the oviducts (Figure 4.A, 4.C). The spermiducts probably start

176   from each side of the male gonads (not visible on the pictures) to the seminal vesicles, in the

177   sexual somite (Figure 4.B). As for the female seminal receptacle, the male seminal vesicle can be

178   divided into two parts (Figure 4.D, 4.E). The first part of the vesicles is chitin-rich, located in the

179   anterior region of the vesicle. The distribution of the chitin from this upper part of the vesicle to

180   the spermiduct was not continuous. The second part, located in the posterior region of the

181   vesicle, was observed by DAPI staining and was likely to be filled by DNA-rich male semen.

182   Discussion

183   Comparing to previous staining methods used to observe the digestive and reproductive systems

184   of copepods (Batchelder 1986; Eisfeld & Niehoff 2007; Mironova & Pasternak 2017; Niehoff

185   2003; Niehoff & Hirche 1996; Tande & Gronvik 1983; Tande & Hopkins 1981; Wolfram *et al*.

186   2014), the protocol proposed here allows a clear insight into the chitin distribution in these

187   systems. Moreover, this protocol is simple and rapid, taking a few minutes of manipulation,

188   thirty minutes of incubation, and can be used on living, but also on alcohol-preserved copepods.

189   The main limit of our method remains in the short-time staining of the WGA-FITC: a picture

190   must be taken, a few minutes after fluorescence excitation to save any microscopic observation

191   without loss of quality. Furthermore, for the reproductive system, the DAPI staining allows only

192   the observation of the gonad structure; while the Mironova and Pasternak protocol allows a

193   better identification of the oocytes.

194   The use of WGA-FITC revealed chitinous spherical structures in the exopods of the swimming

195   legs in *O. nana* males and females and in *O. similis* females, which were not observed in

196   previous studies. The absence of these structures in *O. similis* male individuals may be a bias due

197   to their low presence in our samples. Despite, luminescence is not conspicuous in *Oithona,* these

198   structures could be luminous glands (Herring 1988). The green staining revealed also a chitinous

199   tubular structure in the exopods penultimate segment of the swimming legs. These structures

200   resemble the 'Crusalis organ', an osmoregulatory structure that was described by Johnson *et al* in

201   2014 from the coastal/estuarine copepod *Eurytemora affinis*. Since the globular and the tubular

202   structures seemed attached, we suggest they form only one organ involved either in

203   bioluminescence, osmoregulation or both.

204   The WGA-FITC staining allowed also the identification of the chitin distribution in the *Oithona*

205   organs, which provides a high-quality view of the external and internal anatomy and pointed out

206   the major role of chitin in the *Oithona* digestion and reproduction. According to insect studies,

207   the distribution of chitin in the digestive system is limited to the midgut (Hegedus *et al*. 2009;

208   Terra 2001). The same chitin distribution was observed in decapods (Martin *et al*. 2006; Wang *et*

209   *al*. 2012). In both *Oithona* species, we detected chitin throughout the digestive system, which

210   distinguishes it from insects and decapods, but seems consistent with observations in other free-

211   living cyclopoids made by Yoshikoshi and Kô, in 1988.

212   In the PM of some insects (Hegedus *et al*. 2009; Kelkenberg *et al*. 2015; Lehane 1997), chitin

213   plays a role in protection (chemical, mechanical and against viruses, bacteria and pathogens) and

214   digestion (Terra 2001). As the synthesis of chitin has a significant metabolic cost for the

215   organism, we hypothesized that, like the insects and decapods PM, the formation of a chitin coat

216   around faecal pellets help to protect against toxins and pathogens that were not degraded during

217   digestion.

218   In copepods, no evidence of midgut contraction has previously been described although the

219   phenomenon has been suggested at several instances (Gauld 1957). We suppose that the midgut

220   shrinkages observed in this study could play a key role in the formation and motion of the faecal

221   pellets to the anus. However, we observed intestine shrinkages without the presence of faecal

222   pellets, and vice versa. As proposed by Yoshikoshi and Kô for other copepods, we also suggest

223   that, in *Oithona*, the formation of chitin coat around the faecal pellets can be produced by

224   engulfing digested food in chitin microfibrils present in the PM of the anterior midgut (Figure 5).

225   The presence of chitin along the oviduct and spermiduct walls validates the cuticular appearance

226   of the ducts described by Cuoc *et al*. In all *Oithona* males, we observed a pair of spermiducts,

227   while in *Calanus finmarchicus* one of the two spermiducts disappeared during the male

228   differentiation (Tande & Hopkins 1981). The bipartite structure of the seminal receptacles and

229   vesicles found in *O. nana* and *O. similis* males and females were very similar. In males, we

230   hypothesized that the chitinous structure of the vesicle plays a role in the holding of the

231   spermatophores during their formation. Likewise, in the females, this structure would play a role

232   in the holding of the ovisac but also in the opening and closing of the oviduct to release oocytes

233   in the seminal receptacle.

Conclusion

With this study, we adapted and tested a simple and rapid chitin-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 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 undergone to better understand the molecular and physiological mechanisms involved in faecal pellets formation.

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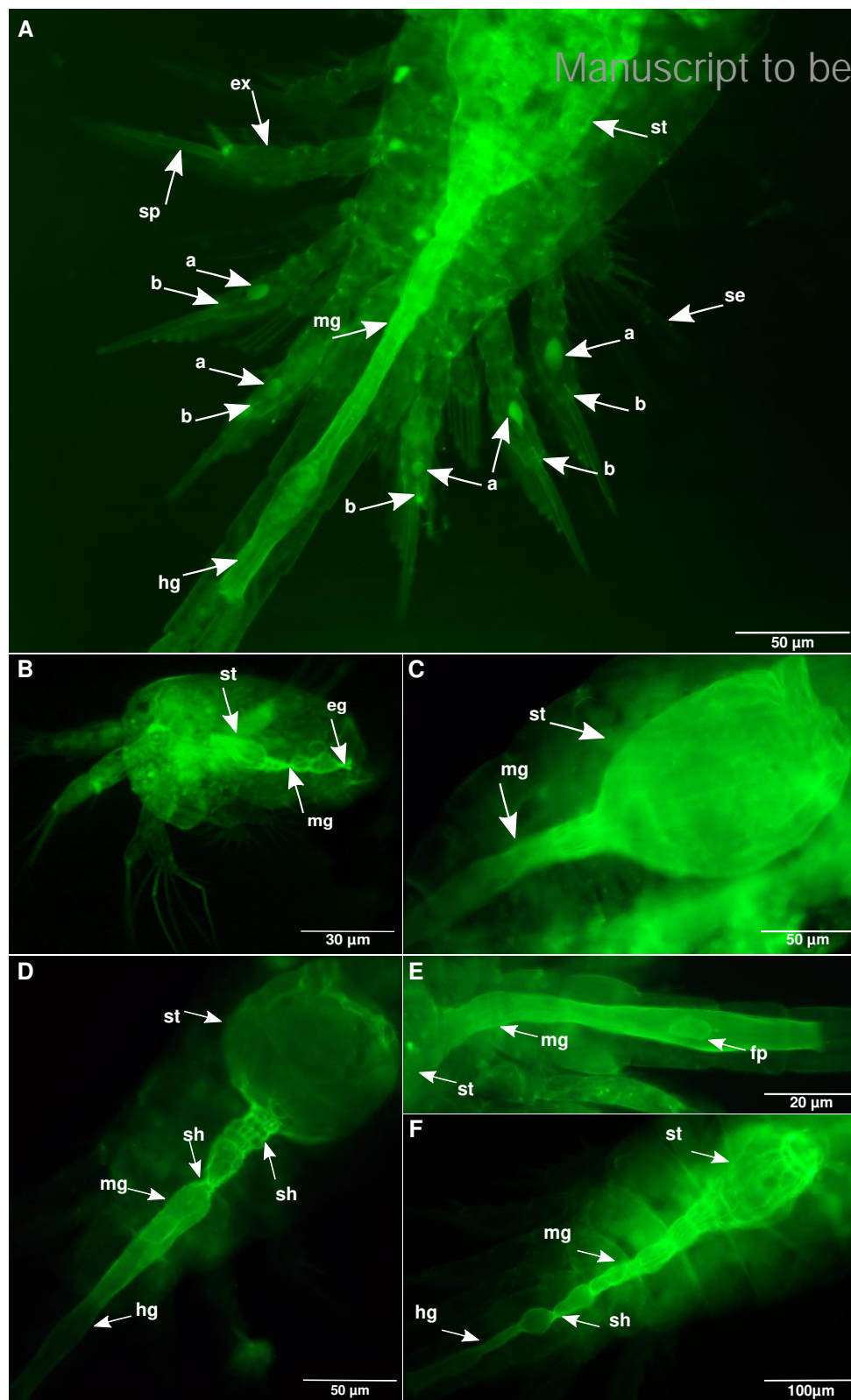
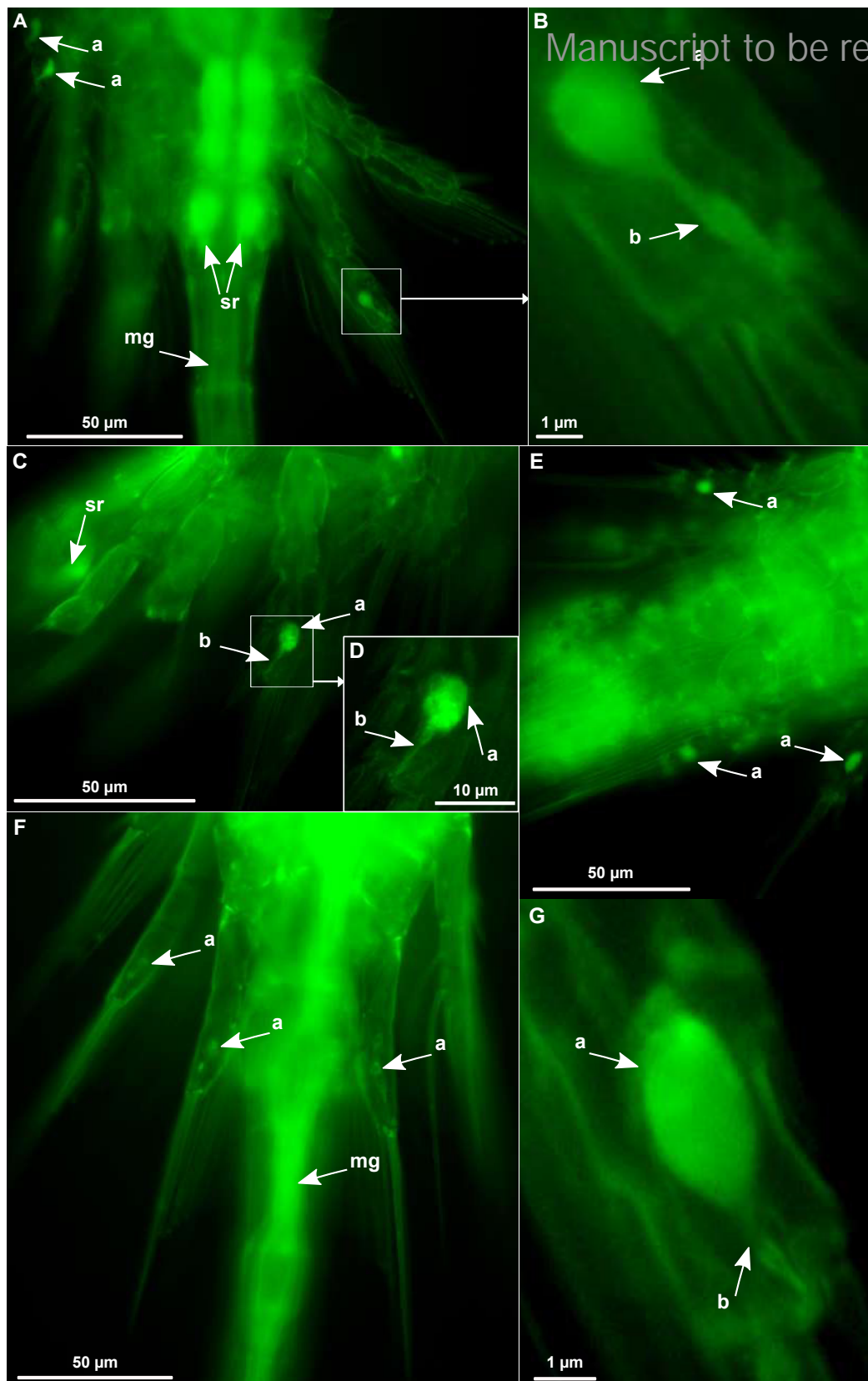
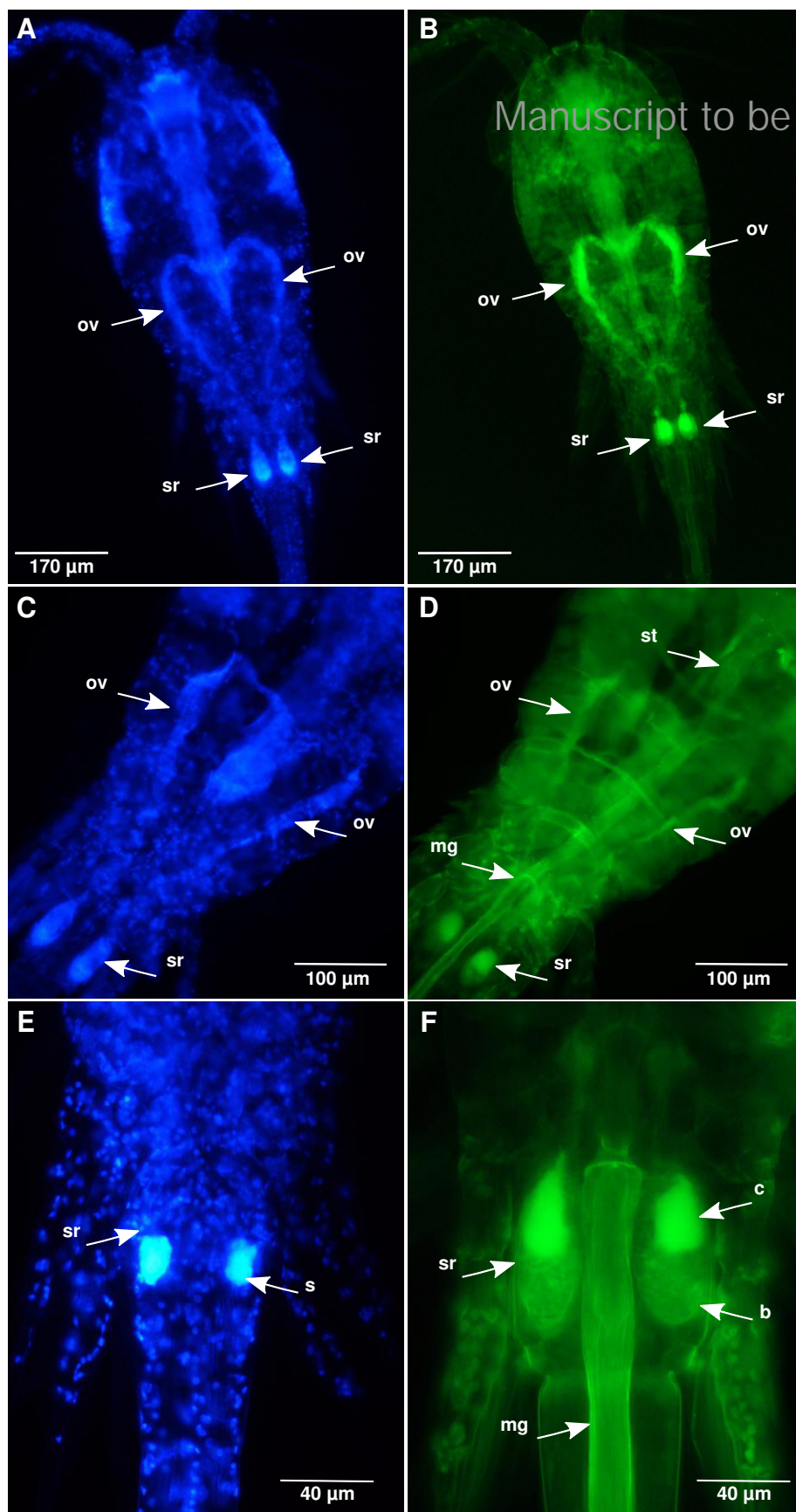
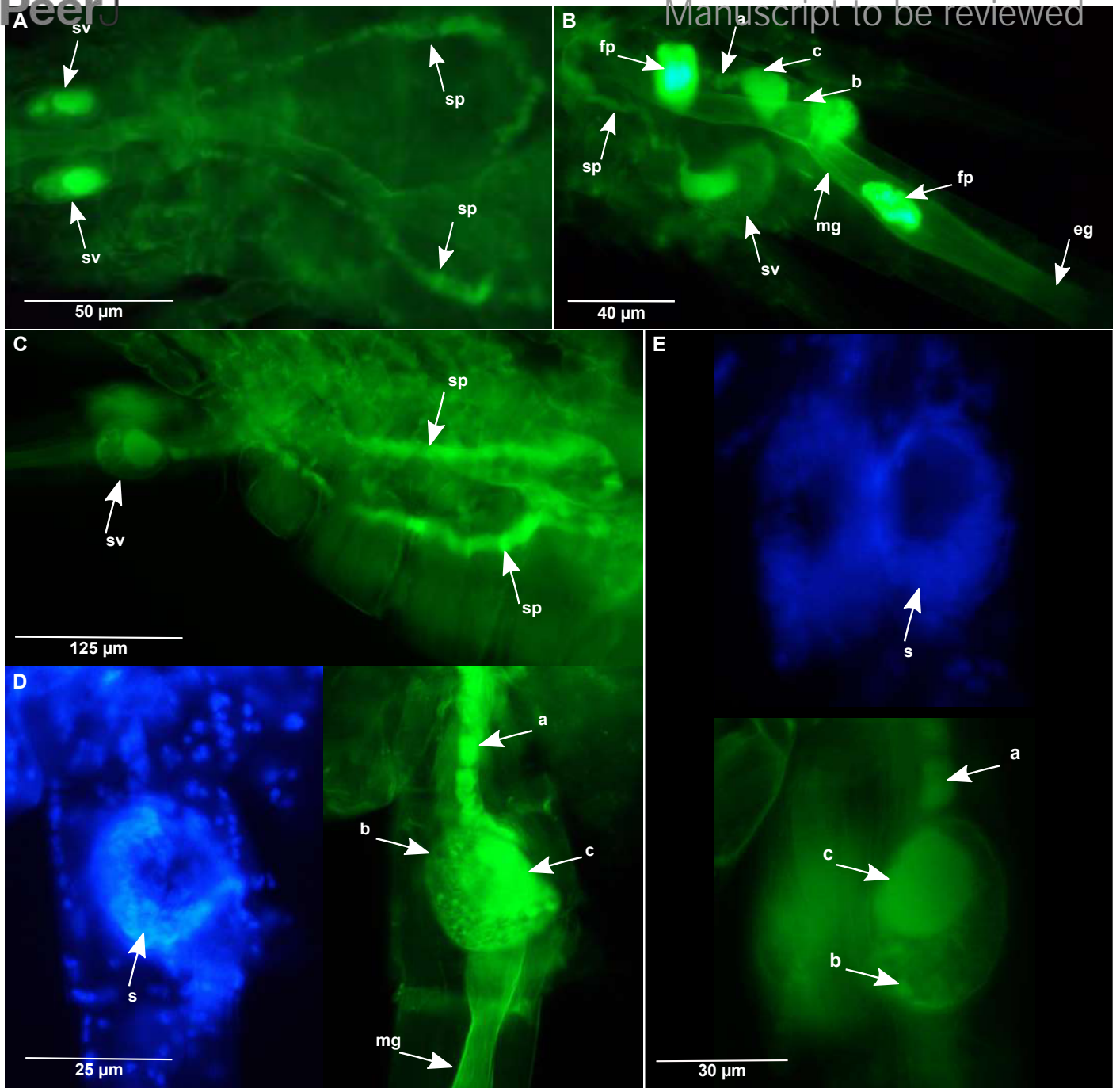
Oithona appendages morphology and digestive system by WGA-FITC fluorescence microscopy.

st: stomach, **mg:** midgut, **hg**: hindgut, **sh**: shrinkage, **ex**: exopod, **se**: setal, **sp**: spine, **fp**: fecal pellet, **a:** globular structure, **b**: tubular structure. **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.

Oithona globular and tubular chitinous structures in the swimming appendages by 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.

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**-**B**.. Dorsal view of the *O.nana* female reproductive system (DAPI staining on the left and WGA-FITC staining on the right). **C**-**D**. Dorsal view of the *O. similis* female reproductive system (DAPI staining on the left and WGA-FITC staining on the right). **E**-**F**. Dorsal view of the *O. nana* female double sexual somite (DAPI staining on the left and WGA-FITC staining on the right).

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.

The diagram of the internal anatomy of a female *Oithona nana*.

st: stomach, **mg**: midgut, **pm**: peritrophic membrane, **go**: gonads, **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.

A

B