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# Zoomorphology

## New data on ultrastructure of proboscis in females of *Bonellia viridis* (Annelida: Bonellinae) --Manuscript Draft--

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<b>Funding Information:</b>	<table border="1"> <tr> <td>российский фонд фундаментальных исследований (рффи) (20-14-00096)</td> <td>Prof. Elena Temereva</td> </tr> <tr> <td>russian science foundation (18-04-00082-P)</td> <td>Prof. Elena Temereva</td> </tr> </table>	российский фонд фундаментальных исследований (рффи) (20-14-00096)	Prof. Elena Temereva	russian science foundation (18-04-00082-P)	Prof. Elena Temereva
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<b>Abstract:</b>	<p>Although <i>Bonellia viridis</i> is the best studied echiurid species, many aspects of its anatomy and ultrastructure are still unclear. In this study, detailed description of proboscis epithelium of females of <i>B. viridis</i> is done and illustrated by accurate schemes. Accordingly to histological specificities, four zones of proboscis stem can be distinguished: dorsal, ventral, and two lateral. These zones differ from each other in ciliation and abundance of bonellin-producing and gland cells. Each zone supplies certain functions: protective for dorsal, transport of food particles for ventral, and agglutination of food particles for lateral. Ciliated cells have been described in epithelium of the dorsal side of the proboscis stem for the first time. These cells, probably, supply slow ciliary movements of proboscis. For the first time, the organization of epithelial cells is described and the ability of epithelial cells to consume nutrients is detected. The absence of continuous basal lamina under the ventral epithelium is ascertained. This unusual state may be explained by activity of bonellin-producing cells, which constantly penetrate between epithelial cells. In bonellin-producing cells, voluminous perinuclear space and contribution of rough endoplasmic reticulum into formation of bonellin-containing granules are detected for the first time. Innervation of epithelial and gland cells occurs via nerve tracts originating from proboscis nerve cords. In general, echiurid proboscis epithelium exhibits many unusual features, which are unknown for any other food collecting apparatuses of annelids. The specificity of organization of proboscis correlates with unique evolutionary transformation, which echiurids underwent from initially segmented ancestor to recent non-segmented state.</p>				

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1 **New data on ultrastructure of proboscis in females of *Bonellia viridis* (Annelida:**  
2 **Bonellinae)**

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23

24 **Abstract**

25 Although *Bonellia viridis* is the best studied echiurid species, many aspects of its anatomy and  
26 ultrastructure are still unclear. In this study, detailed description of proboscis epithelium of  
27 females of *B. viridis* is done and illustrated by accurate schemes. Accordingly to histological  
28 specificities, four zones of proboscis stem can be distinguished: dorsal, ventral, and two lateral.  
29 These zones differ from each other in ciliation and abundance of bonellin-producing and gland  
30 cells. Each zone supplies certain functions: protective for dorsal, transport of food particles for  
31 ventral, and agglutination of food particles for lateral. Ciliated cells have been described in  
32 epithelium of the dorsal side of the proboscis stem for the first time. These cells, probably,  
33 supply slow ciliary movements of proboscis. For the first time, the organization of epithelial  
34 cells is described and the ability of epithelial cells to consume nutrients is detected. The absence  
35 of continuous basal lamina under the ventral epithelium is ascertained. This unusual state may be  
36 explained by activity of bonellin-producing cells, which constantly penetrate between epithelial  
37 cells. In bonellin-producing cells, voluminous perinuclear space and contribution of rough  
38 endoplasmic reticulum into formation of bonellin-containing granules are detected for the first  
39 time. Innervation of epithelial and gland cells occurs via nerve tracts originating from proboscis  
40 nerve cords. In general, echiurid proboscis epithelium exhibits many unusual features, which are  
41 unknown for any other food collecting apparatuses of annelids. The specificity of organization of  
42 proboscis correlates with unique evolutionary transformation, which echiurids underwent from  
43 initially segmented ancestor to recent non-segmented state.

44 **Key words:** echiurids, histology, transmission electron microscopy, epithelium, gland cells,  
45 functional morphology

46

## 47 **1. Introduction**

48 Accordingly to recent echiurid phylogeny (Goto et al. 2020), bonnelids form separate clade of  
49 Bonellinae, which also includes unusual ikedids. Bonellinae is the sister group to all rest  
50 echiurids, which form clade Thalamematinae and include two tribes Thalamematini and Echiurini  
51 (Goto et al. 2020). Echiurids are marine benthic invertebrates, which body is subdivided into  
52 trunk and proboscis (Pilger 1993; Maiorova and Adrianov 2020). The proboscis is usually  
53 extended on the surface of substratum and is used to collect and sort of food particles. Certain  
54 mechanisms of sorting of food particles are studied very poor (Jaccarini and Schembri 1977a).  
55 Moreover, for many echiurids, even position of the proboscis on the substratum is still unclear.  
56 For example, some specific structures were discovered in *Protobonellia zenkevitchi* and allowed  
57 to suggest the certain position of proboscis on the substratum (Temereva et al. 2017).

58 Organization of the proboscis differs in different species. Among echiurids, species from genus  
59 *Urechis* have the shortest proboscis: these echiurids produce the mucous net, which is used for  
60 capture of food particles (Fisher and MacGinitie 1928). Most of echiurids have long language-  
61 like proboscis. Females of *Bonellia viridis* have long proboscis, which terminated by two lobes  
62 (Jaccarini and Schembri 1977a). The proboscis of *B. viridis* is studied in details: there are data on  
63 location of proboscis on substratum (Jaccarini and Schembri 1977b), movements of proboscis  
64 (Jaccarini and Schembri 1977b), the work of cilia of terminal lobes and middle parts of proboscis  
65 (Jaccarini and Schembri 1979), organization of muscles of proboscis (Bosch 1981), the  
66 ultrastructure of integument (Bosch and Michel 1979), and blood vessels extended in the  
67 proboscis (Amor 1973; Bosch 1984). One of specific features of bonnelids is the presence of  
68 bonellin-producing cells, which is located in the connective tissue of body wall and proboscis  
69 and are able to penetrate into the integument (Bosch 1979; Bosch and Michel 1979). Bonellin is  
70 the pigment, which occurs in many echiuran species, but is well known in *B. viridis* because  
71 makes its body green. This unique pigment this unique pigment has been shown to have various

72 toxic and irreversible effects and therefore supplies protective function (Agius and Jaccarini  
73 1981).

74 Most of ultrastructural data, which we know about echiurids are obtained from *B. viridis* and are  
75 extrapolated to other echiurids. At the same time, some of these ultrastructural data seem very  
76 unusual (i.e. syncytial nature of wall of the axial blood vessel (Bosch 1984)) and have to be  
77 reinvestigated.

78 Detailed data on organization of the proboscis may help to understand the echiurid biology and  
79 some aspects of their feeding behavior (Temereva et al. 2017; Kuznetsov et al. 2021).

80 The aim of this study is detailed description of ultrastructure of all organ systems of proboscis of  
81 *B. viridis* with special attention to new data concerning organization of integument and  
82 functional analysis of these data.

83

## 84 **2. Materials and methods**

85 Three specimens of *Bonellia viridis* Rolando, 1822 were collected by SCUBA diving in the  
86 Frioul Archipelago on the coast of Provence, France, 43°16'38"N, 5°18'24"E. The morphology  
87 of all individuals was studied with Leica M165C stereomicroscope equipped with a Leica  
88 DFC420 digital camera (Leica Microsystems GmbH, Wetzlar, Germany). Proboscises were cut  
89 from the trunk and fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing NaCl for  
90 8 hours at +4C. Materials were then washed in the cacodylate buffer (3 times for 4 hours) and  
91 were then post-fixed in 1% osmium tetroxide in the same buffer.

92 The fine morphology of proboscis was studied by scanning electron microscopy (SEM). Pieces  
93 of proboscis and trunk were dehydrated in increasing concentrations of ethanol and acetone; they  
94 were then critical point dried, mounted on stubs, and sputter coated with platinum-palladium.

95 Specimens were examined with JEOL JSM-6380LA scanning electron microscope (JEOL Ltd.,  
96 Tokyo, Japan).

97 For semithin sectioning and transmission electron microscopy (TEM), specimens were  
98 dehydrated in increasing concentrations of ethanol and embedded in Embed-812 resin (Electron  
99 Microscopy Science, USA). Semi-thin and thin sections were prepared with a Leica UC7  
100 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Semithin sections were stained  
101 with methylene blue, observed with a Zeiss Axioplan2 microscope, and photographed with an  
102 AxioCam HRm camera (Carl Zeiss, Oberkochen, Germany). Ultrathin sections were stained with  
103 uranyl acetate (0.5%) and lead citrate (0.4%) and then examined with a JEOL JEM 100B  
104 electron microscope (JEOL Ltd., Tokyo, Japan).

105

### 106 **3. Results**

#### 107 ***3.1. Morphology and histology of proboscis***

108 The body of females of *B. viridis* is subdivided into the trunk and proboscis (Fig. 1A). The  
109 proboscis is long projection of the dorsal side of the trunk, which is flattened in dorso-ventral  
110 direction and terminates with two large lobes (Fig. 1A, B). The proboscis length can exceed the  
111 length of the trunk in several times (Fig. 1A).

112 The margin of each lobe is swollen and is covered by numerous cilia (Fig. 1C, D). The margin is  
113 crossed by vertical grooves, which extend between dorsal and ventral sides of the lobe (Fig. 1C).  
114 The diameter of these grooves is about 20–30  $\mu\text{m}$ . The marginal edge of the lobe bears  
115 longitudinal wrinkle, which extends along the margin and divides it on two parts (ventral and  
116 dorsal). Accordingly to histological data, this wrinkle is formed by two small epithelial folds,  
117 which are associated with huge subepithelial aggregation of gland cells (Fig. 2A). The stem of  
118 proboscis is the longest part, which is able to twist and loop. The ventral side of proboscis stem  
119 bears shallow longitudinal grooves and is covered by cilia (Fig. 1E). At the proximal end, the

120 lateral edges of proboscis are usually directed ventrally and fuse together near the mouth,  
121 forming the short ventral collar (Fig. 1F). Near the mouth, proboscis is crossed by transverse  
122 groves. In some specimens, the large bulb, which is probably form by the foregut, obstructs the  
123 mouth (Fig. 1F).

124 Usually, after fixation, the lateral sides of proboscis bend ventrally (Fig. 2C). Accordingly to  
125 specificities of histology, four epithelial zones may be distinguished in the cross section of  
126 proboscis: ventral, dorsal, and two laterals. In additional, terminal lobes of proboscis also form  
127 specific zone. These zones differ from each other in thickness of epithelium, ciliation, and  
128 abundance of gland cells and bonellin-producing cells (Fig. 3A, B). Thus, ventral epithelium has  
129 14–22  $\mu\text{m}$  in thickness, it is heavily ciliated, and contains many bonellin-producing cells and a  
130 few epithelial glands (Fig. 4A, B). The epithelium of dorsal side has 10–20  $\mu\text{m}$  in thickness,  
131 about lacks cilia, contains a few bonellin-secretory cells and many intraepithelial glands (Fig.  
132 4C). The lateral zones of proboscis look similar to margin of the lateral lobes and are  
133 characterized by presence of numerous subepithelial glands (Fig. 4D). Lateral zones of proboscis  
134 stem bear ciliated grooves, which diameter is about 20  $\mu\text{m}$  (Fig. 2B).

135 The proboscis lacks spacious inner cavity and is filled with extracellular matrix and muscles  
136 (Fig. 2C, D). Muscles are mostly longitudinal and occupy the central and ventral parts of the  
137 proboscis, whereas the dorsal side is about lacks muscle cells (Fig. 2C). Fibers of extracellular  
138 matrix make a grid, which gather muscles in united construction (Fig. 2D). Rare dorso-ventral  
139 muscles are represented by separated muscle fibers. Transverse muscles are not observed on the  
140 dorsal side of proboscis, but form a prominent layer under the ventral epithelium (Fig. 2C). The  
141 proboscis contains the axial blood vessel extending in its center and two lateral blood vessels,  
142 which are incorporated into two lateral coelomic canals (Fig. 2C, D). Two lateral nerve cords  
143 extend along lateral sides of the proboscis above the coelomic canals (Fig. 2C, D). The nerve  
144 cord gives rise to the neurite bundles, which penetrate the connective tissue and extend to the  
145 subepithelial (Fig. 2A) and intraepithelial gland cells (Fig. 4A).



146 **3.2. Ultrastructure**

147 The epithelium consists of epithelial, intraepithelial and subepiermal gland cells of different  
148 types (Fig. 3C). All types of cells are organized similarly in all four zones of the proboscis.

149 **Epithelial cells** of ventral side bear numerous cilia, which form dense cover above the  
150 epithelium (Figs. 4B, 5A). Apical surface of epithelial cells bears branched microvilli and large  
151 apical protrusions, which are covered by flocculent material of cuticle (Fig. 5A, B). The  
152 kinetosome is represented by one centriole (basal body), which connects with two striated  
153 rootlets: transverse and longitudinal (Fig. 5C). Cells connect each other via adherence and  
154 septate junctions, which unite the apical parts of cells (Fig. 5C, D). The middle and basal parts of  
155 cells contact each other loosely; therefore voluminous intracellular spaces are visible (Fig. 5B,  
156 C). The cytoplasm of apical part of cells is electron dense and is armed by transverse bundles of  
157 actin filaments (Fig. 5B). The apical cytoplasm is filled with numerous vesicles of different  
158 diameter and multivesicular bodies (Fig. 5B, C). The middle and basal parts of cell contain large  
159 nucleus of irregular shape, several Golgi apparatuses, and phagosomes (Fig. 5A-C). Epithelium  
160 is not underlined by continuous basal lamina (Fig. 5A).

161 In epithelial cells of lateral sides of proboscis, because of presence of many cilia, the apical  
162 protrusions and microvilli are not such numerous as in the epithelium of ventral side (Fig. 6A).  
163 The epithelium of lateral sides is underlined by thin basal lamina. Epithelial cells of dorsal side  
164 are about lack cilia, nevertheless some ciliated cells can be detected (Fig. 6B). In cells, which  
165 lack cilia, the apical part is substantially protruded and forms wide lobes (Fig. 6C). Because of  
166 presence of apical protrusions, cell junctions are located in subapical zone. Apical membrane  
167 forms thick microvilli, which exposed into the layer of cuticle, and numerous thin canals, which  
168 penetrate the cytoplasm of apical protrusions. These canals terminate by pinocytotic vesicles,  
169 which fill the apical cytoplasm (Fig. 6C). In cells of dorsal epithelium, bundles of actin fibers  
170 extend are not only transverse, but are also longitudinal.

171 **Intraepithelial gland cells** are incorporated into the epithelia of all four zones and do not form  
172 protrusions into extracellular matrix (Figs. 3, 7A, B). There are two types of intraepithelial gland  
173 cells: with electron-lucent vacuoles and with electron-dense flocculent granules. Cells of first  
174 type (with electron-lucent vacuoles) are the most abundant especially in dorsal epithelium (Figs.  
175 4C, 7A). These cells are filled with vacuoles of different diameter, which contain electron-lucent  
176 material and many vesicles of smaller diameter. Apical surface of cells bears thin short microvilli  
177 with electron-dense cytoplasm. Usually, apical surface of gland cells is covered by apical  
178 projections of epithelial cells (Fig. 7A, B). Gland cells of first type connect with the epithelial  
179 cells via adherence and septate junctions (Fig. 7A). Secretory zone of cell is located in middle  
180 part of cell and is represented by Golgi apparatus, which is squeezed between vacuoles (Fig.  
181 7A). Epithelial gland cells of second type contain granules of electron-dense flocculent material,  
182 which diameter is about 0.6–0.8  $\mu\text{m}$  (Fig. 7B). Cells of this type are rare and are usually  
183 occurred in dorsal epithelium.

184 **Subepithelial gland cells** are numerous in terminal lobes and in lateral zones of the proboscis  
185 stem. There are two types of subepithelial gland cells (Figs. 3, 8A, B). Cells of first type contain  
186 large roundish electron-dense granules, which diameter can reach up 1.5  $\mu\text{m}$  (Fig. 8A). The  
187 nucleus is relatively large, is irregular-shaped, and contains nucleolus (Fig. 8A). The secretory  
188 zone contains numerous Golgi apparatuses, which canals are filled with content of middle  
189 electron density (Fig. 8B). Vacuoles, which are produced by Golgi apparatuses, fuse together  
190 forming granules of middle electron density (Fig. 8A); then the density of granules becomes  
191 higher. Subepithelial gland cells of second type are filled with huge vacuole, which contains  
192 electron-lucent material and flocculent fibers (Fig. 8B). The nucleus is located in the central part  
193 of cell: it is relatively small in diameter and contains much heterochromatin and nucleolus. The  
194 secretory zone is also located in the central part of cell and is represented by numerous Golgi  
195 apparatuses, which canals are filled with electron-lucent content (Fig. 8B).

196 **Bonellin-producing cells** are specific type of subepithelial gland cells. These cells contain  
197 electron-dense granules of specific ovoid shape and maximal size 2x1  $\mu\text{m}$  (Fig. 9A). Bonellin-  
198 producing cells can also contain granules of irregular shape and middle electron density. These  
199 cells are mostly located in the connective tissues of the proboscis, but can be also incorporated  
200 into the epithelium (Figs. 3, 4, 9A). These cells have lengthy apical and swollen basal parts.  
201 Apical membrane forms thick short microvilli. Apical part of bonellin-producing cells does not  
202 form typical cells junctions with adjacent cells (Fig. 9A). Apical cytoplasm is filled with  
203 electron-dense granules. The secretory zone is located in the basal part of cell and contains two  
204 main elements: Golgi apparatuses and rough endoplasmic reticulum (RER) (Fig. 9C). Canals of  
205 Golgi apparatuses are filled with electron dense content (Fig. 9C). The RER connects the nuclear  
206 membrane: the perinuclear space is very wide and sophisticatedly lopped, thus, canals of RER  
207 penetrate the perinuclear space (Fig. 9C). The RER produce flocculent electron-dense material,  
208 which is incorporated in the secretory granules. Moreover, secretory granules are located into  
209 space, which is surrounded by RER canals (Fig. 9B). Some areas of cell membrane are very  
210 thick and electron dense (Fig. 9C). Cells are closely contacted the nerve tracts and some certain  
211 neurites (Fig. 9C).

212 Intraepithelial and subepithelial gland cells and bonellin-producing cells are well innervated by  
213 **nerve tracts**, which originate from the lateral nerve cords (Figs. 4A, 5A, 9B). Bundles of  
214 neurites also penetrate between epithelial cells (Fig. 6A). Usually, nerve tracts consist of neurites  
215 of two types. Neurites of first type have large diameter, electron-lucent cytoplasm, and contain  
216 dense-core synaptic vesicles (Figs. 3C, 5A). Neurites of second type are small in diameter, have  
217 electron-dense cytoplasm, and contain synaptic vesicles with electron-lucent content. Neurites  
218 connect each other via synapses (Fig. 9B).

#### 219 **4. Discussion**

220 Most echiurans are deposit-feeders, and the proboscis is used to collect food particles from the  
221 substrate (e.g., Ikeda 1904; Baltzer 1931; Hughes and Crisp 1976; Biseswar 1991; Hughes et al.  
222 1993; Jumars 2015; Kharlamenko et al. 2018). Accordingly to available data, mucus ciliary  
223 mechanism works for capture of food particles: they adhere to the mucus, which is produced by  
224 gland cells, and then transport to the mouth due to activity of cilia (Biseswar 1991). Accordingly  
225 to many observations, the dorsal side of the proboscis usually faces the substratum and ventral  
226 side usually faces the water; the distal edge is submerged into the substratum and collects  
227 substrate particles (Biseswar 1991; Hughes et al. 1993; Goto et al. 2016). The specific position  
228 of the proboscis has been suggested for *Protobonellia zenkevitchi* due to specificities of  
229 morphology and histology of different zones of proboscis (Temereva et al. 2017). *B. viridis* uses  
230 the terminal lobes but not the lateral sides of the proboscis to collect food particles (Jaccarini and  
231 Schembri 1977a). Accordingly to our new data, the margin of terminal lobes of proboscis bears  
232 the huge aggregation of gland cells. The same cells are located along the lateral sides of the  
233 proboscis stem. The presence of similar gland cells in both terminal lobes and lateral sides of the  
234 proboscis stem may evidence the similar function of these zones, i.e. production of mucus for  
235 adherence of food particles. It probably means that the lateral edges of proboscis stem are  
236 involved into collection of food particles.

237 The size of food particles, which echiurids are able to capture, can vary greatly: 2-7  $\mu\text{m}$  in  
238 *Lissomiema mellita* (Kuznetsov et al. 2021), 7-45  $\mu\text{m}$  in *P. zenkevitchi* (Temereva et al. 2017),  
239 90-300 in *B. viridis* (Jaccarini and Schembri 1977a). Usually, the size of food particles is  
240 determined by diameter of ciliary grooves, which extend on certain zones of proboscis  
241 (Temereva et al. 2017; Kuznetsov et al. 2021). In *B. viridis*, ciliary grooves are detected on the  
242 margin of the terminal lobes and on the lateral sides of the proboscis stem. In both parts of the  
243 proboscis, the diameter of ciliated grooves is similar and is about 20  $\mu\text{m}$ . The presence of ciliated  
244 grooves may evidence the sorting of food particles in *B. viridis*. The rejection of particles occurs  
245 on the ventral side of the proboscis and near the mouth (Jaccarini and Schembri 1977b; Pilger

246 1993). In studied specimens of *B. viridis*, the detected protrusion of the foregut may work as an  
247 obstacle for large particles.

248 Distribution of muscles, cells, and fibers in extracellular matrix of proboscis has functional  
249 meaning. Thus, in *P. zenkevitchi*, the terminal part of the proboscis is reinforced by large  
250 vacuolated cells (Temereva et al. 2017). In *L. mellita*, proboscis is very muscular and its ventral  
251 side is reinforced by thick layer of collagenous fibers (Kuznetsov et al. 2021). In *B. viridis*,  
252 muscles are mostly extended in central and ventral parts; moreover, the ventral epithelium is  
253 underlined by layer of transverse muscles. These muscles allow the proboscis to curve on the  
254 ventral side and form a kind of large groove (Fig. 2C).

255 Ultrastructure of epithelium of proboscis is described for *B. viridis* in several papers (Pilger,  
256 1977; Jaccarini and Schembri 1977a; Bosch and Michel 1979; Bosch 1979). These studies are  
257 mostly devoted to the description of gland cells, whereas epithelial cells have never been  
258 described in details. It is known that cells of ventral epithelium bear cilia, whereas cells of dorsal  
259 epithelium lack cilia (the exception is the dorsal side of terminal lobes of *B. viridis*) (Jaccarini  
260 and Schembri 1977a). Accordingly to our data, some cells of dorsal epithelium have cilia, that  
261 allow it to move slowly due to ciliary beating. In general, epithelial cells of all zones of  
262 proboscis are organized in similar way. It is very interesting that all epithelial cells are able to  
263 consume nutrients: the Figure 7B allows to realize the formation of pinocytotic vesicles, their  
264 aggregation into multivesicular bodies, and further transformation into the phagosomes. Because  
265 of pinocytotic activity epithelial cells contain large and well developed Golgi apparatuses, which  
266 produce lysosomes. The consumption activity of epithelial cells is consistent with suggestion that  
267 some intraepithelial glands produce proteins, which may be used for digestion of food prior to  
268 entering the mouth (Bosch and Michel 1979).

269 The subapical location of cells junction in dorsal epithelium allows it to stretch when the  
270 proboscis skirts on the ventral side. Another one interesting and unusual thing is the absence of

271 continuous basal lamina under the ventral epithelium. Strictly speaking, epithelium, which is not  
272 underlined by basal lamina, cannot be regarded as true epithelium (Vracko 1974). The absence  
273 of basal lamina in ventral epithelium may be caused by activity of bonellin-producing cells,  
274 which constantly penetrate between epithelial cells and change the structure of epithelium (see  
275 below).

276 Accordingly to literature, five types of gland cells can be distinguished in proboscis of females  
277 of *B. viridis*: two intraepithelial and three subepithelial (Bosch and Michel 1979). Intraepithelial  
278 gland cells had been detected in ventral epithelium only (Bosch and Michel 1979). As it was  
279 shown in our study, two types of intraepiermal gland cells are scattered in ventral and dorsal  
280 epithelia; moreover, dorsal epithelium contains many mucus-producing cells. The secretion of  
281 these cells is apparently used for protection of dorsal epithelium, which contacts the substratum.

282 Three types of subepithelial gland cells are associated with ventral (one type) and dorsal (two  
283 types) epithelia (Bosch and Michel 1979). Ventral mucus gland cells contain hypertrophied  
284 Golgi apparatus and produce large vacuoles, which then fuse together, forming the united mass  
285 with fibrillary network. The secretion of these cells contains sulfated mucus and is used for  
286 agglutination of food particles (Bosch and Michel 1979). Dorsal mucus cells contain ovoid  
287 granules, which consist of sulfated mucopolysaccharides and are used for protection of dorsal  
288 epithelium. The third type of subepithelial cells is represented by dorsal twisted mucus gland  
289 cells, which contain well developed RER, two Golgi apparatuses, and numerous metachromatic  
290 granules. The secretion of these glands may help to provide traction for the animal as it crawls  
291 over the surface (Bosch and Michel 1979). Accordingly to our data, there are two types of  
292 subepithelial gland cells and both types are associated with the terminal lobes and with the lateral  
293 sides of the proboscis stem. The secretion of these gland cells may be used for agglutination of  
294 food particles.

295 Ultrastructure of bonellin-producing cells has never been described in details (Pilger 1993). It is  
296 known that Golgi apparatus produces the material of bonellin-containing granules and then the  
297 granules are modified in cytoplasm (Kawaguti 1968). Our new data show that the bonellin-  
298 containing granules are produced by two ways (by Golgi apparatuses and by RER) and probably  
299 consist of at least two components. Unusual thing of bonellin-producing cells is the presence of  
300 wide perinuclear space, which is penetrated by canal of RER and contains the bonellin granules.  
301 The dense parts of cell membrane may play role for movements of bonellin-producing cells,  
302 which are able to move in connective tissue and form projections penetrating between epithelial  
303 cells (Bosch 1979). The abundance of bonellin-producing cells in the ventral epithelium is  
304 consistent with light sensitivity of bonellin: it releases due to mechanical or light stimuli.  
305 Because ventral epithelium is faced to the light, it contains much more bonellin-producing cells  
306 in comparison with dorsal epithelium, which is faced to the substratum.

307 For the first time, the innervation of epithelium, gland cells, and bonellin-producing cells has  
308 been shown in our study. In previous researches, the innervation of gland cells and bonellin-  
309 producing cells has not been detected (Pilger 1993).

## 310 **5. Conclusion**

311 For the first time for echiurans, detailed description of epithelium of proboscis is done and  
312 visualized as accurate schemes. Four functional zones are distinguished in proboscis stem:  
313 dorsal, ventral, and two lateral. These zones differ from each other in ciliation and abundance of  
314 bonellin-producing and gland cells. Accordingly to specificities of histology, each zone provides  
315 certain function. Thus, dorsal side works for protection of proboscis, ventral – for transport of  
316 food particles, and lateral for agglutination of food particles. Many unusual and previously  
317 undescribed features are discovered: the capability of epithelial cells to consume nutrients via  
318 pinocytosis, the absence of continuous basal lamina under the ventral epithelium, the presence of  
319 ciliary cells in dorsal epithelium of the proboscis stem, the innervation of epithelial cells and

320 gland cells by nerve tracts, and the specificities of bonellin-producing cells. In sum, epithelium  
321 of proboscis exhibits many unusual features, which are unknown for any food collecting  
322 apparatuses of annelids. The specificity of organization of proboscis correlates with unique  
323 evolutionary transformation, which echiurids underwent from initially segmented ancestor to  
324 recent non-segmented state.

325

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338

### 339 **Conflict of Interest Statement**

340 The authors declare that they do not have any conflict of interest. All authors have read the text  
341 and approved the final version of the manuscript.

342

### 343 **Author contributions**



344 PK performed the histology, prepared the serial sections, made some schemes, and photographed  
345 the sections. AE collected and fixed the animals, helped interpret the results, and helped write  
346 the manuscript. ET prepared all figures, wrote the manuscript, and interpreted the results.

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410 **Figure legends**

411 Fig. 1. Morphology of females of *Bonellia viridis*. **a-b** Photographs of fixed animals; **c-f** SEM. **a**  
412 Whole animal viewed from the left. **b** Terminal lobes of proboscis. **c** A part of one terminal lobe;  
413 longitudinal wrinkle of margin is pointed by arrowheads. **d** A portion of margin of terminal lobe,  
414 which is heavily ciliated. **e** A portion of ventral side of the proboscis stem. **f** Anterior part of the  
415 body viewed from the top; large bulb of foregut obstructs the mouth. *ci* cilia, *cg* ciliated groove,  
416 *ch* chaetae, *ds* dorsal side of terminal lobe, *fb* foregut bulb, *lg* longitudinal groove, *m* mouth, *me*  
417 marginal edge of terminal lobes, *ps* proboscis stem, *tg* transverse groove, *tl* terminal lobe, *tr*  
418 trunk, *vc* ventral collar.

419 Fig. 2. Anatomy proboscis of female of *Bonellia viridis*. **a-c** Photographs of transverse semithin  
420 sections; **d** SEM. **a** A portion of margin of terminal lobe: huge aggregation of subepidermal  
421 glands is visible. **b** Ciliated groove of lateral side of the proboscis stem. **c** Whole proboscis stem.  
422 **d** A portion of proboscis stem: grid, which is formed collagenous fibers, is evident. *abv* axial  
423 blood vessel, *agc* aggregation of gland cells, *bpc* bonellin-producing cells, *cf* collagenous fibers,  
424 *cg* ciliated groove, *dep* dorsal epithelium, *dvm* dorso-ventral muscles, *lbc* lateral blood vessel, *lc*  
425 lateral coelomic canal, *lm* longitudinal muscles, *lnc* lateral nerve cord, *ls* lateral side, *nb* neurite  
426 bundle, *tm* transverse muscles, *vep* ventral epithelium.

427 Fig. 3. Schemes of histological and ultrastructural organization of proboscis of female of  
428 *Bonellia viridis*. **a** Portion of terminal lobe. **b** Portion of the proboscis stem. **c** Ultrastructure of  
429 ventral epithelium of proboscis stem. *bpc* bonellin-producing cells, *ci* cilia, *cu* cuticle, *igc-I*  
430 intraepidermal gland cell type I, *igc-II* intraepidermal gland cell type II, *mc* muscle cells, *mi*  
431 microvilli, *n* nucleus, *nb* neurite bundle, *pha* phagosome, *pns* perinuclear space, *sgc-I*  
432 subepidermal gland cell type I, *sgc-II* subepidermal gland cell type II.

433 Fig. 4. Histological organization of proboscis of female of *Bonellia viridis*; photographs of  
434 transverse semithin sections. **a** Ventral epithelium of terminal lobe. **b** Ventral epithelium of

435 proboscis stem. **c** Dorsal epithelium of proboscis stem. **d** Lateral epithelium of proboscis stem.  
436 *bpc* bonellin-producing cells, *ci* cilia, *ct* connective tissue, *igc-I* intraepidermal gland cell type I,  
437 *igc-II* intraepidermal gland cell type II, *mc* muscle cells, *nb* neurite bundle, *sgc-I* subepidermal  
438 gland cell type I, *sgc-II* subepidermal gland cell type II.

439 Fig. 5. Ultrastructure of ventral epithelium of proboscis stem in female of *Bonellia viridis*; TEM.  
440 **a** General view of epithelium and neurite bundles under the epithelium. **b** Apical parts of  
441 epithelial cells; space between to adjacent cells is marked by asterisks. **c** Organization of  
442 kinetosome and rootlets of cilia; wide spaces between cells are marked by asterisks. **d** Septate  
443 junction between adjacent cells. *af* actin filaments, *aj* adherence junction, *ap* apical projection,  
444 *bpc* bonellin-producing cells, *ce* centriole, *ci* cilia, *ct* connective tissue, *cu* cuticle, *igc-I*  
445 intraepidermal gland cell type I, *lr* lateral rootlet, *mi* microvilli, *m vb* multivesicular body, *n*  
446 nucleus, *nb* neurite bundle, *pha* phagosome, *pv* pinocytotic vesicle, *sj* septate junction, *tr*  
447 transverse rootlet.

448 Fig. 6. Ultrastructure of lateral and dorsal epithelia of proboscis stem in female of *Bonellia*  
449 *viridis*; TEM. **a** General view of epithelium of lateral side. **b** Apical part of ciliated cell of dorsal  
450 epithelium. **c** Apical and middle parts of epithelial cell of dorsal epithelium. *af* actin filaments, *aj*  
451 adherence junction, *ap* apical projection, *bl* basal lamina, *bpc* bonellin-producing cells, *ce*  
452 centriole, *ci* cilia, *cu* cuticle, *G* Golgi apparatus, *igc-II* intraepidermal gland cell type II, *lr* lateral  
453 rootlet, *mc* muscle cell, *mi* microvilli, *m vb* multivesicular body, *n* nucleus, *nb* neurite bundle, *pha*  
454 phagosome, *sj* septate junction, *tr* transverse rootlet.

455 Fig. 7. Organization of intraepidermal gland cells in dorsal epithelia of proboscis stem in female  
456 of *Bonellia viridis*; TEM. **a** Gland cell of first type contains large vesicles with electron-lucent  
457 content. **b** Gland cells of second type contains granules of electron dense flocculent material. *aj*  
458 adherence junction, *ap* apical projection, *cu* cuticle, *G* Golgi apparatus, *igc-I* intraepidermal

459 gland cell type I, *igc-II* intraepidermal gland cell type II, *mi* microvilli, *m vb* multivesicular body,  
460 *n* nucleus, *pha* phagosome, *v* vesicle.

461 Fig. 8. Organization of subepidermal gland cells in lateral zones of proboscis stem in female of  
462 *Bonellia viridis*; TEM. **a** Gland cell of first type contains large electron dense granules. **b** Gland  
463 cell of second type contains mucous material and many Golgi apparatuses. *ecm* extracellular  
464 matrix, *G* Golgi apparatus, *gr* granule of secretion, *ms* mucus secretion, *n* nucleus, *nu* nucleolus,  
465 *ngr* newly formed granule, *sgc-I* subepidermal gland cell type I, *sgc-II* subepidermal gland cell  
466 type II, *sve* secretory vesicles.

467 Fig. 9. Organization of bonellin-producing cells of proboscis stem in female of *Bonellia viridis*;  
468 TEM. Electron dense portions of cell membrane are pointed by arrowheads. **a** Cell incorporated  
469 into ventral epithelium. **b** Cell in connective tissue. *G* Golgi apparatus, *gr* granule of secretion,  
470 *mi* microvilli, *n* nucleus, *nb* neurite bundle, *ngr* newly formed granule, *pha* phagosome, *pns*  
471 perinuclear space, *RER* rough endoplasmic reticulum, *sy* synapse.

472

































