

On the larva and the zooid of the pterobranch *Rhabdopleura recondita* Beli, Cameron and Piraino, 2018 (Hemichordata, Graptolithina)

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Abstract

Hemichordates (Enteropneusta and Pterobranchia) belong to a small deuterostome invertebrate group that may offer insights on the origin and evolution of the chordate nervous system. Among them, the colonial pterobranch Rhabdopleuridae are recognized as living representatives of Graptolithina, a taxon with a rich fossil record. New information is provided here on the substrate selection and the life cycle of *Rhabdopleura recondita* Beli, Cameron and Piraino, 2018, and for the first time, we describe the nervous system organization of the larva and the adult zooid, as well as the morphological, neuroanatomical and behavioural changes occurring throughout metamorphosis. Immunohistochemical analyses disclosed a centralized nervous system in the sessile adult zooid, characterized by different neuronal subsets with three distinct neurotransmitters, i.e. serotonin, dopamine and RFamide. The peripheral nervous system comprises GABA-, serotonin-, and dopamine-immunoreactive cells. These observations support and integrate previous neuroanatomical findings on the pterobranch zooid of *Cephalodiscus gracilis*. Indeed, this is the first evidence of dopamine, RFamide and GABA neurotransmitters in hemichordates pterobranchs. In contrast, the lecithotrophic larva is characterized by a diffuse basiepidermal plexus of GABAergic cells, coupled with a small group of serotonin-immunoreactive cells localized in the characteristic ventral depression. It is envisaged the use of *R. recondita* as a novel and easily accessible hemichordate model organism shed light on the evolution of hemichordates and more generally on the origin of deuterostome developmental mechanisms.

Keywords Pterobranchia · Life cycle · Nervous system · Neurotransmitter · Development · Metamorphosis

Introduction

Hemichordata (Bateson, 1885) is a phylum of marine deuterostome invertebrates with a key phylogenetic position (Nielsen 2012). Indeed, recent molecular analyses recognize this taxon as the sister group of Echinodermata within the Ambulacraria

(Bourlat et al. 2006; Cannon et al. 2009; Satoh et al. 2014a). To date, about 130 hemichordate species have been described and grouped into two classes: the free-living Enteropneusta and the sessile Pterobranchia (Tassia et al. 2016). Although all hemichordates share the tripartite body with the anterior prosome, the intermediate mesosome, and the posterior

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39 metasome, each containing single or paired coelomic cavities
 (Gemmill 1914; Hadfield 1975; Benito and Pardos 1997;
 Q3/Q2 40 Balser and Ruppert 1990), members of the two clades strongly
 Q4 41 differ in several aspects. Enteropneusts, commonly known as
 42 acorn worms, are characterized by a solitary life habit in bur-
 43 rows or under stones from the intertidal to deep-sea zones
 44 worldwide (Cameron 2018). Species like *Saccoglossus*
 45 *kowalevskii* and *Ptychodera flava* are now regarded as model
 46 organisms for the study of deuterostome evolution (Arendt
 47 et al. 2008; Fan and Su 2015; Simakov et al. 2015;
 48 D'Aniello et al. 2015; Tagawa 2016). Much less is known of
 49 pterobranchs, due to their small size and rarity and the limited
 50 number of known species (Beli et al. 2018). Therefore, this
 51 group has been largely overlooked and poorly investigated
 52 (Brown et al. 2008; Smith 2008). However, their occurrence
 53 may be much more common than previously thought, having
 54 been recently recorded from coastal areas at shallow depths
 55 (Beli et al. 2018). Different from the naked free-living
 56 enteropneusts, pterobranch zooids are sessile and have a col-
 57 onial or pseudocolonial life habit (Maletz 2014a), usually
 58 dwelling in proteinaceous tubes (*tubaria*) largely secreted by
 59 the cephalic shields of individual zooids (Dilly 1986; Dilly
 60 1988; Maletz and Steiner 2015). They have one to several
 61 pairs of filter feeding tentaculated arms growing from the col-
 62 lar, a U-shaped gut, ventrally interconnected (in colonial spe-
 63 cies) by a thin, organic stolon system (Maletz 2014b; Maletz
 64 and Cameron 2016; Beli et al. 2018). Pterobranchia tradition-
 65 ally encompassed two orders: Cephalodiscida and
 66 Rhabdopleurida. However, recent phylogenetic analysis
 67 highlighted that extant rhabdopleurids are living representa-
 68 tives of graptolites (Mitchell et al. 2013). This led to the re-
 69 definition of the Pterobranchia into two major clades: the
 70 Graptolithina (including fossil graptolites and extant
 71 rhabdopleurid species) and the Cephalodiscida.

72 The dichotomy between the different life habits of
 73 enteropneust worms and pterobranch zooids is remarkably
 74 paralleled to the distinct neuroanatomical organizations of
 75 the two groups. The nervous system of enteropneusts consists
 76 of intraepithelial neural cells whose neurites develop into a
 77 basiepidermal nerve plexus, with condensed regions forming
 78 the nerve ring in the proboscis, the dorsal nerve cord in the
 79 collar and trunk regions, and the ventral nerve cord in the
 80 trunk region, interconnected to the dorsal one by a
 81 prebranchial nerve ring (Bullock 1945; Knight-Jones 1952;
 82 Kaul-Strehlow et al. 2017). Kaul and Stach (2010) recently
 83 argued that the collar cord of enteropneusts might work as the
 84 centralized nervous system (i.e. where condensed nerve cell
 85 bodies and neurites that arise from them are distinctly inter-
 86 related by synapses and neuronal integration in a more complex
 87 way than elsewhere in the body; see Holland 2003). However,
 88 electrical recordings from nerve cords do not bear this out
 89 (Pickens 1970; Cameron and Mackie 1996). As for
 Q5 90 enteropneusts, a basiepidermal nervous system was early
 91

92 recognized in rhabdopleurid pterobranchs but with a dorsal,
 93 ganglion-like structure, occurring in the mesosome region and
 94 giving rise to distinct nerve branches (Dilly 1975; Benito and
 95 Pardos 1997). More recently, an immunohistochemical inves-
 96 tigation on the nervous system of *Cephalodiscus gracilis*
 97 showed a serotonergic dorsal brain-like condensation at the
 98 base of mesosomal tentacles, associated to peripheral brachial
 99 nerves, tentacle nerves and a ventral stalk nerve (Stach et al.
 100 2012). The dorsal localization of the pterobranch mesosomal
 101 plexus and of the collar cord of enteropneusts has been
 102 interpreted as a symplesiomorphy of hemichordates,
 103 supporting the hypothesis that a centralized nervous system
 104 already occurred in the last common deuterostome ancestor
 105 (Stach et al. 2012). However, the developmental mechanisms
 106 underlying the origin of the dorsal brain of adult pterobranchs
 107 remains to be elucidated.

108 The rarity of pterobranch colonies has led to even rarer
 109 observations on their life cycle (Stebbing 1970; Dilly 1973;
 110 Lester 1988a, 1988b; Sato 2008; Dilly 2014). The discovery
 111 of a new pterobranch species, *Rhabdopleura recondita* Beli,
 112 Cameron and Piraino, 2018, in the Mediterranean Sea (Beli
 113 et al. 2018) offered the opportunity to investigate its life cycle
 114 and to investigate the morphological and neuroanatomical or-
 115 ganization of its two life stages, the larva and the adult zooid.

116 Materials and methods

117 Sampling and in vivo observations

118 *Rhabdopleura recondita* colonies were found associated with
 119 dead and partially dead colonies of calcareous bryozoans.
 120 These were collected by SCUBA diving on rocky pre-
 121 coralligenous substrates, at a depth ranging from 15 to 25 m,
 122 during November 2015 in the locality of Torre del Serpe (40°
 123 8' 23.54" N, 18° 30' 31.23" E) and in March 2016 at Punta
 124 Faci (40° 8' 5.75" N, 18° 31' 2.07" E), Otranto Channel, Italy.
 125 Samples were immediately brought to the laboratory and kept
 126 in aquaria containing natural seawater at 14 °C. From May to
 127 June 2016, additional bryozoan samples were collected in
 128 Tricase Porto (39° 52' 52.48" N, 18° 25' 7.43" E) by demersal
 129 gill nets operated by professional fishermen on submersed
 130 ridges from 60 to 70 m depth in the framework of the project
 131 "Biodiversity MARE Tricase" (Micaroni et al. 2018a, 2018b).
 132 Animals were brought to the laboratory and then kept in nat-
 133 ural seawater aquaria at 21 °C. Dead parts of the collected
 134 bryozoans were fragmented with pliers and pointed tools
 135 and observed at the stereomicroscope and optical micro-
 136 scopes. Naked zooids were isolated from their tubaria and
 137 cleaned, using sharp forceps and acupuncture needles.
 138 Swimming larvae of *R. recondita* were captured by glass pi-
 139 pettes. In vivo images were made using a Sony-RX100-I cam-
 140 era superimposed on a Leica MZ6 ocular stereomicroscope.

141	Light microscopy and scanning electron microscopy	
142	For scanning electron microscopy analysis, zooids and larvae	Anti-Mouse IgG Antibody (Life Technology), Alexa Fluor 191
143	were relaxed in 3.5% MgCl ₂ in sea water and fixed in 2%	488 Goat Anti-Rabbit IgG Antibody (Life Technology), both 192
144	glutaraldehyde in 0.1 M cacodylate buffer and 1.4% NaCl	diluted 1:800 in PBS. In some samples, the antibody anti- 193
145	for 2 h at room temperature. Samples were rinsed in	tyrosinated α -tubulin was omitted and filamentous actin was 194
146	cacodylate buffer solution overnight and post-fixed for 1 h	labeled with TRITC-conjugated phalloidin (Sigma, Italy). 195
147	with 1% osmium tetroxide (OsO ₄). Samples were then	Nuclei were labeled with DAPI (20 mg/ml). Specimens were 196
148	dehydrated in ethanol series (30%, 50%, 70%, 90%, and	mounted with 1,4-diazabicyclo[2,2,2]octane (DABCO, 197
149	100%). Absolute ethanol was gradually substituted with	Sigma, Italy) plus MOWIOL (Sigma, Italy) on microscope 198
150	hexamethyldisilazane (\geq 99% grade; Sigma, Italy). Samples	slides and examined using a Leica SP2 confocal laser scan- 199
151	were left to dry, mounted on stabs, covered by a thin pure gold	ning microscope (Leica Microsystems, Heidelberg, 200
152	layer (Sputter Coater Nanotech), and observed using a ZEISS	Germany), equipped with laser argon/krypton, 75 mW multi- 201
153	LEO-1430 microscope. Standard methods of light microscopy	line. A series of "optical sections" attained by scanning whole- 202
154	were also employed. Samples (adult zooids and larvae) were	mount specimens were projected into one image with greater 203
155	pre-fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer	focal depth. The step size of "optical sections" was 1.5 μ m. 204
156	and 1.4% NaCl for 2 h and, after overnight washing in the	
157	same buffer, post-fixed with 1% solution of OsO ₄ in 0.1 M	Results 205
158	cacodylate buffer. After standard dehydration in ethanol series	
159	(25%, 70%, 90%, and 100%), samples were washed in pro- 206	Behaviour and life cycle of <i>Rhabdopleura recondita</i>
160	pylene oxide and embedded in Epon-Araldite 812 resin. Semi- 207	Colonies of <i>Rhabdopleura recondita</i> were found in associa- 207
161	thin sections (about 0.6 μ m) were cut with a Reichert-Jung	tion with dead skeletal parts of several bryozoan species: 208
162	ULTRACUT E using glass knives, stained with crystal violet	<i>Myriapora truncata</i> (Pallas, 1766), <i>Schizoretopena</i> 209
163	and basic fuchsin, and then mounted with Eukitt (Bio	<i>serratimargo</i> (Hincks, 1886), <i>Celleporina caminata</i> 210
164	Optica). Samples were observed under a Leica light micro-	(Waters, 1879), <i>Pentapora fascialis</i> (Pallas, 1766), 211
165	scope (DMRB model) and photographed using a Leica DFC-	<i>Reptadeonella violacea</i> (Johnston, 1847), <i>Turbicellepora</i> 212
166	320 camera and LAS (Leica Application Suite, Leica)	<i>magnicostata</i> (Barroso, 1919). The tubaria of <i>R. recondita</i> 213
167	software.	are composed of smooth creeping tubes lining the natural 214
		internal cavities of the bryozoan zooecium, and erect, brown- 215
168	Immunohistochemical analysis	ish, and semitransparent tubes, with characteristic annular 216
169	For immunohistochemistry, zooids and larvae were first anes- 217	rings (<i>fuselli</i>), that emerge from the zooecium openings 217
170	thetized as above described and then fixed in 4% paraformal- 218	(Fig. 1a, b). The adult zooids of <i>R. recondita</i> show the typical 218
171	dehyde in 0.1 M PBS for 1 h at room temperature. After	rhabdopleurid body organization: a <i>prosome</i> with cephalic 219
172	triplicate washes in a solution of 0.1% Tween-20 in 0.1 M	shield, a <i>mesosome</i> that encloses the ventral mouth opening 220
173	PBS (PBT), samples were processed for double fluorescent	and two dorsal feeding arms each with two rows of tentacles 221
174	immunolocalization. Samples were permeabilized for 20 min	(mean number of tentacles for each row, 21 \pm 3), and a 222
175	with a solution of 0.25% Triton X, in PBT pH 7.4, and pre-	<i>metasome</i> with the internal U-shaped digestive tract, the 223
176	incubated for 2 h at room temperature in 50% Normal Goat	contractile stalk, and the stolon (Fig. 1c, d). The zooids are 224
177	Serum (NGS) previously inactivated at 55 °C for 30 min.	covered by beating cilia and protrude from their tubes up to 225
178	Samples were separately incubated overnight at 4 °C in a	the mesosome, extending outwards the two dorsal arms and 226
179	solution of 10% NGS in PBT with different primary polyclon-	filter feeding by their ciliated tentacles (Fig. 1e, f). The 227
180	al antibodies: anti-serotonin, anti-dopamine, anti-GABA	pterobranch feeding mechanism has been investigated in de- 228
181	(Sigma, Italy), and anti-RF (clone 1773IIIp, kindly provided	tail (Stebbing and Dilly 1972; Dilly 1972, 1973) with the 229
182	by Prof. Thomas Leitz, Heidelberg) made in rabbit, diluted	identification of a set of feeding characteristics similar to 230
183	1:500. Thereafter, samples were rinsed several times with	those of the lophophore of bryozoans, brachiopods, and 231
184	PBT, pre-incubated in 50% NGS, and incubated overnight at	foronids (Halanych 1993). 232
185	4 °C with anti-tyrosinated α -tubulin antibody (Sigma, Italy)	The pterobranch colony develops from a yellow-coloured, 233
186	made in mouse, diluted 1:250. Samples were washed three	free-swimming larva, spotted with black pigment granules 234
187	times in PBT and then pre-incubated for 2 h in 0.1% bovine	and characterized by a posterior ventral depression (Fig. 1g, 235
188	serum albumin (BSA; Sigma) dissolved in PBS at room tem- 236	h). The distribution of black granules in the larva is not uni- 236
189	perature. Each sample was incubated overnight at 4 °C in the 237	form: two symmetric concentrations occur in the dorso-lateral 237
190	corresponding secondary antibodies: Alexa Fluor 568 Goat 238	area, corresponding to the areas where the morphogenesis of 238
		the dorsal arms will take place, and are absent from the ventral 239

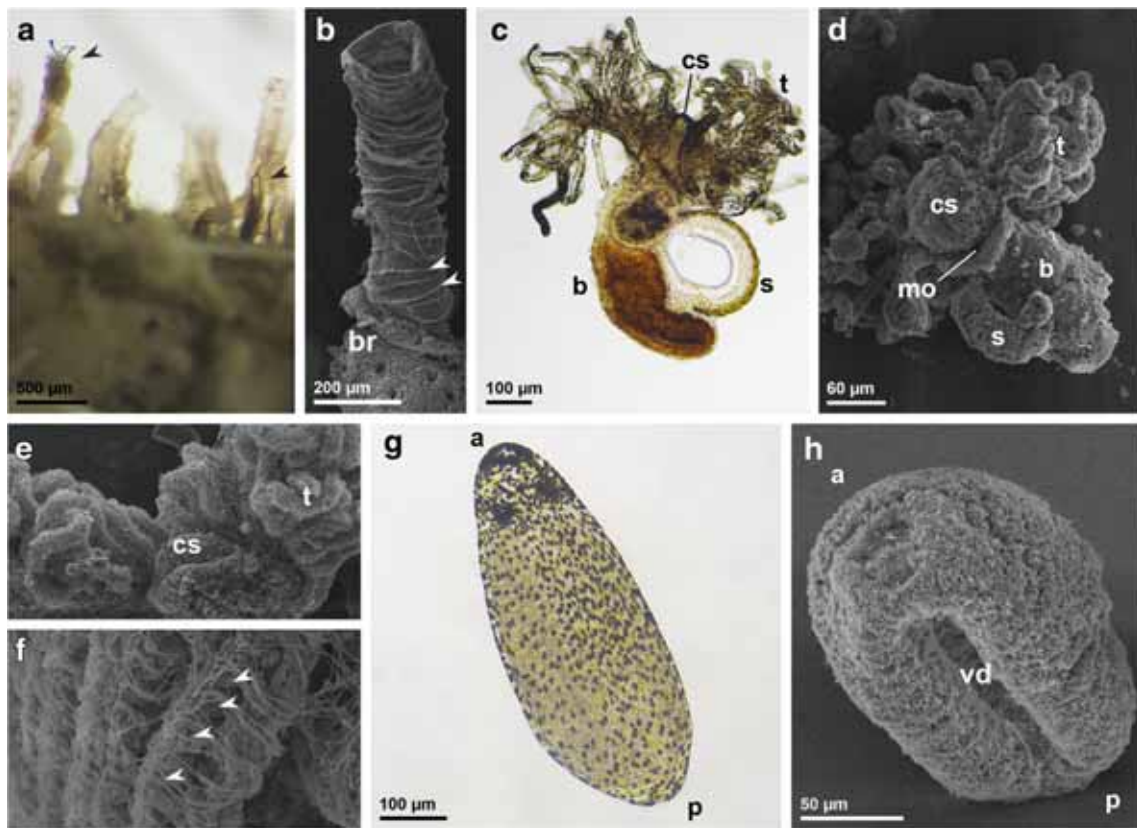


Fig. 1 Overview of *Rhabdopleura recondita* colony, zooid, and larval morphology. **a** In vivo colony picture: the semitransparent tubarium protruding from the calcareous bryozoan zooecia, with protruding visible zooids (black arrowheads). **b** Scanning electron microscope (SEM) image of the tube, characterized by the annular fusellar ridges (white arrowheads). **c** The zooid out of its tube shows two black nontentacled arm tips, cephalic shield, tentacled arms, metasoma, and contractile stalk. **d** SEM image of the zooid: the mouth opening is located in between the cephalic shield and the collar. **e** The two

tentacled arms are placed dorsally to the cephalic shield. **f** Tentacles are lined by longitudinal rows of cilia (arrowheads) for suspension feeding. **g** In vivo swimming larva, mounted on microscope slide (dorsal view): the larva is characterized by yellow and dotted black pigmentation. **h** SEM image of the larva of *R. recondita* (ventral view): the larva is covered by cilia, the characteristic ventral depression appears as a groove. br, bryozoan; b, metasoma; cs, cephalic shield; s, contractile stalk; t, tentacles; a, anterior larval pole; p, posterior larval pole; vd, ventral depression

240 depression. While swimming, the beating cilia rotated around
 241 its (antero-posterior) axis. Occasionally, it remained stationary
 242 for several minutes, and then it glided over the substrate, using
 243 ventral cilia.

244 Once it had found a suitable settlement site inside the cal-
 245 careous bryozoan zooecium, the larva underwent a gradual
 246 metamorphosis through four consecutive stages: (1) secretion
 247 of a transparent protective cocoon, the dome;

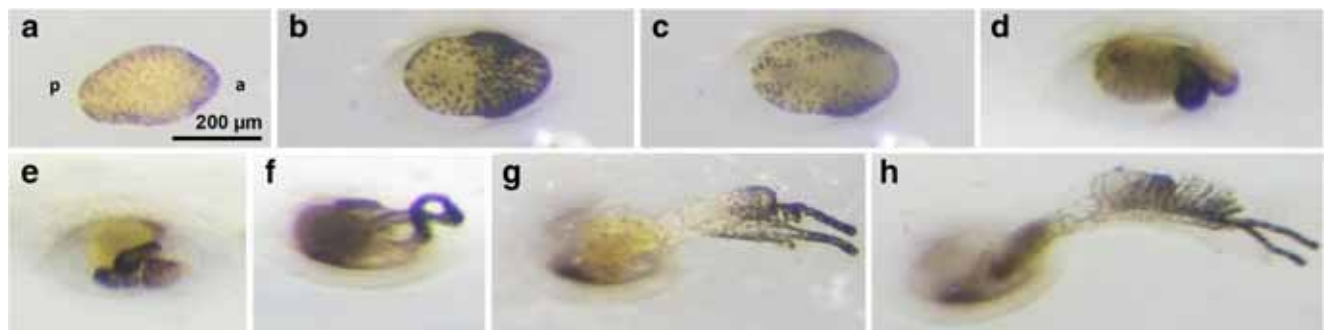
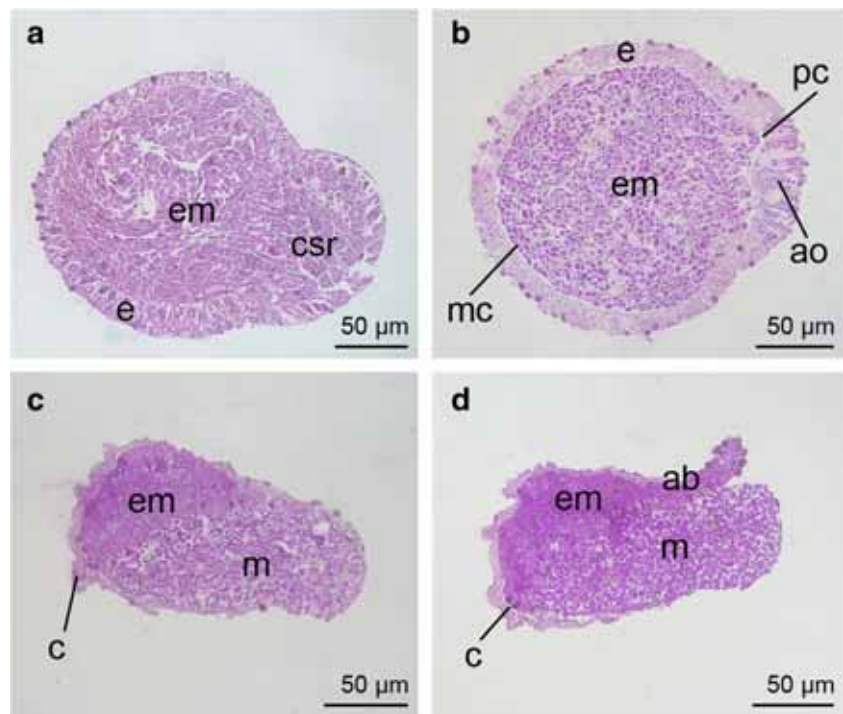


Fig. 2 The metamorphosis of *R. recondita* larva is characterized by four main stages. **a** Free-swimming larva, settling and **b** secreting the dome (arrowhead). At this stage, pigmented cells concentrate at the arm primordia, while **c** the ventral depression remains unpigmented. **d** Second stage: appearance of cephalic shield and initial formations of

arms in dorso-lateral position. **e-g** Third stage: progressive elongation of the black arms and the contractile stalk. **h** Final stage: development of ciliated tentacles on the black arms, differentiation of the tripartite body organization, and formation of the opened tube. a, anterior pole; p, posterior pole

Fig. 3 Histological sections of post-settlement *R. recondita* larvae. **a** Transverse section, median position. The cephalic shield rudiment (csr) is already visible. **b** Transverse section, posterior. A posterior ciliary turf (pc) and an adhesive organ (ao) are formed. A thin mesothelium (mc), surrounded by a thick epidermis (e), lines the inner body portion filled by yolk-rich endomesodermal cells (em). **c, d** Longitudinal sections of advanced post-settlement stage. Endoderm (en) and mesoderm (m) are distinguished as separate tissues. A protective cocoon (c) is secreted (c), paralleled by development of the arms (ab)



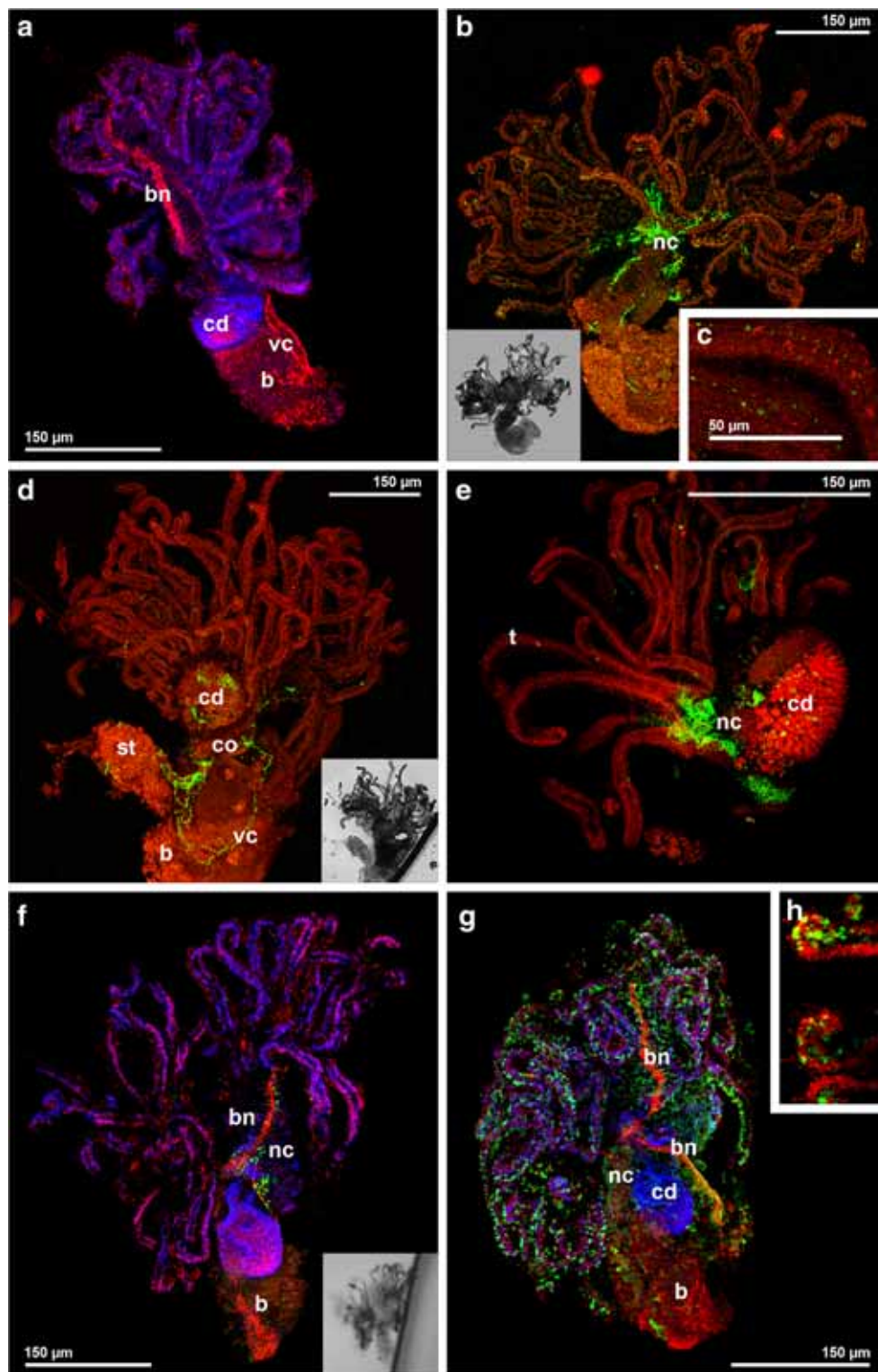
248 the cephalic shield and of the two symmetric dorsal arm buds;
 249 (3) development of the two black tentaculated arms and elongation
 250 of the contractile stalk; (4) completion of the paired
 251 arms and of their ciliated tentacles, differentiation of the tri-
 252 partite body organization, and formation of the tube (Fig. 2).
 253 The whole metamorphosis (from settlement to full zooid differ-
 254 entiation) takes place in 10 days in laboratory conditions
 255 (21 °C). The first pterobranch zooid asexually buds the stolon
 256 and gives origin to all the new colonial zooids. Histological
 257 sections of the competent larva showed the presence of a central
 258 conspicuous mass of endo-mesodermal cells rich in yolk
 259 (Fig. 3a, b) and the absence of a gut, characteristic of its
 260 lecithotrophic nature. A thin muscular mesothelium lines the
 261 coelomic cavity rudiments (Fig. 3a). The epidermis is uni-
 262 formly thick, except in the posterior area, where elongated
 263 mucous-secreting cells constitute the posterior ciliary turf
 264 (Fig. 3b). Shortly after settlement, during the earliest stage of
 265 metamorphosis, the rudiment of the cephalic shield appears:
 266 endodermic derivatives start to differentiate and rise as a mass
 267 of cells thicker than those of the surrounding mesoderm
 268 (Fig. 3c, d).

269 Larval and adult nervous system

270 Whole-mount immunostaining of zooids with an antibody
 271 against tyrosinated α -tubulin showed the general arrangement
 272 of the nervous system (Fig. 4a). Tyrosinated α -tubulin (Tyr-
 273 Tub)-positive cells were stained along the two arms forming
 274 the brachial nerves. These neurite bundles depart from a con-
 275 centration of cells localized in the collar region, which showed

276 positive immunoreactivity for different neurotransmitters 276
 277 (Fig. 4a–h). The Tyr-Tub antibody also stained the epidermal 277
 278 cilia, particularly abundant on the tentacles (Fig. 4a). 278
 279 Serotonin (5-hydroxytryptamine, 5-HT)-immunoreactive 279
 280 (IR) cells were the most conspicuous neurons in the collar 280
 281 region (Fig. 4b), dorsally concentrated at the base of the two 281
 282 arms (Fig. 4c). From the collar region, ectodermal serotonin- 282
 283 IR fibres run along the entire length of the gut, anteriorly 283
 284 reaching the stalk (Fig. 4d). In *R. recondita* zooids, cells pro- 284
 285 ducing neuropeptides with an Arg-Phe-NH₂ carboxy- 285
 286 terminus (RFamide) were concentrated at the base of tenta- 286
 287 cles, in the dorsal region of the collar (Fig. 4e). Dopamine 287
 288 (3,4-dihydroxyphenethylamine)-IR cells are localized in the 288
 289 collar region which were distributed along the brachial axis 289

Fig. 4 Confocal microscope images of the zooids of *R. recondita*. **a** ▶
 Ventral view, antibody against tyrosinated α -tubulin (red) and DAPI-
 stained nuclei (blue). The brachial nerve (bn) and the ventral chord (vc)
 are detected. **b** Lateral view, serotonin immunoreactivity (green) is
 concentrated (nc) at the base of the tentacled arms. Actin counter-
 staining with phalloidin (red). **c** Detail of tentacles (t), with
 regular arrangement of serotonergic cells, (green). **d** Lateral view of
 the zooid, serotonergic cells (green) mark the ventral chord (vc) along
 the body (b) of the zooid, at the base of contractile stalk (st); phalloidin
 counterstaining (red). **e** RFamide-IR cells (green) are concentrated in
 the dorsal collar region (co), behind the cephalic disk (cd); phalloidin
 counterstaining (red). **f** Dopamine-IR cells (green) are concentrated in
 the collar region and along the brachial nerves (bn); DAPI-stained
 nuclei (blue); nervous fibres stained by antibody against tyrosinated
 α -tubulin (red). **g** GABA-IR cells (green) follow the ciliary rows marked
 by the alpha tubulin antibody (red); DAPI-stained nuclei (blue). **h**
 Magnification of the tentacle apices showing the distribution of ciliated
 GABAergic cells



290 of each of the two feeding arms (Fig. 4f). Numerous GABA
 291 (γ -aminobutyric acid)-IR cells occur in the epidermis of the
 292 tentacles (Fig. 4g). GABAergic cells are not present in the
 293 cephalic shield nor in the ventral part of the body, whereas
 294 they are present in the posterior collar and in the dorsal mid-
 295 line of the trunk (Fig. 4g). Magnification of the tentacle apices

shows that GABA signal is present in spots located along the
 lateral rows of ciliated cells (Fig. 4h). In the swimming larva
 of *R. recondita*, basi-epidermal GABA cells are widely distrib-
 uted over the larval body but more concentrated in three areas:
 two symmetrical zones in correspondence to the two dorsal
 pigmented regions and a posterior immunoreactive

296
 297
 298
 299
 300
 301

302 concentration in correspondence to the posterior ciliary turf
 303 (Fig. 5a). Serotonin-IR interconnected cells are detected in
 304 the ventral depression of the larva (Fig. 5b).

305 Discussion

306 *Rhabdopleura* spp. pterobranchs are traced back to the Middle
 307 Ordovician (Sato et al. 2008b; Mitchell et al. 2013; Maletz and
 308 Steiner 2015). However, several aspects of their biology, in-
 309 cluding morphology and lifecycle, are poorly explored (Sato
 310 et al. 2008a). Here, novel information is provided on the new-
 311 ly discovered species *Rhabdopleura recondita* with focus on
 312 species substrate preference, life cycle, larval behaviour, meta-
 313 morphosis, and the nervous system.

314 So far, hemichordate pterobranchs were generally consid-
 315 ered as a rare invertebrate taxon, and for this reason, they
 316 remained poorly investigated, in spite of their potential high
 317 phylogenetic relevance (Röttinger and Lowe 2012). Colonies
 318 of *R. recondita* were found associated with the empty skeletal

parts of at least six bryozoan species, witnessing moderate
 specific requirements in terms of substrate choice preference
 for its larvae. Given the high frequency and abundance of
 these bryozoan species in several coastal areas of the
 Mediterranean Sea (Novosel 2005), it is suggested that
 pterobranchs may be more common than previously thought
 across the whole Mediterranean basin.

By standard immunostaining methods commonly applied to
 marine invertebrates (Pennati et al. 2012), the anatomical com-
 plexity that characterizes the nervous system of *R. recondita* has
 been explored in detail. Three different neuronal subsets char-
 acterized by the neurotransmitters dopamine and serotonin and
 the RFamide peptide were detected in the centralized nervous
 system of *R. recondita*, representing the first evidence of the
 presence of dopamine and RFamide in the central nervous sys-
 tem of Pterobranchia (Fig. 6). GABA-, serotonin-, and
 dopamine-IR cells were also found in the peripheral nervous
 system. Interestingly, GABA-positive cells are present through-
 out the ciliated tentacles, supporting the hypothesis of Dilly
 (1972) who suggested a nervous control of the ciliary beat.
 Accordingly, a basiepithelial net of GABA-positive cells is
 scattered throughout the ciliated ectoderm of the larva, with a
 posterior concentration in correspondence with the ciliary turf.

The ontogenesis and anatomy of the nervous system are
 considered key traits to understand species evolution (Holland
 2003). According to Garstang (1894), the chordate nervous
 system evolved by fusion of the ciliary bands of the deutero-
 stome dipleurula larva with the dorsal migration of the circum-
 oral nerve ring. Bateson (1884, 1885) argued for the homology
 between the enteropneust collar cord formation and the
 chordate neurulation process, also in support of the

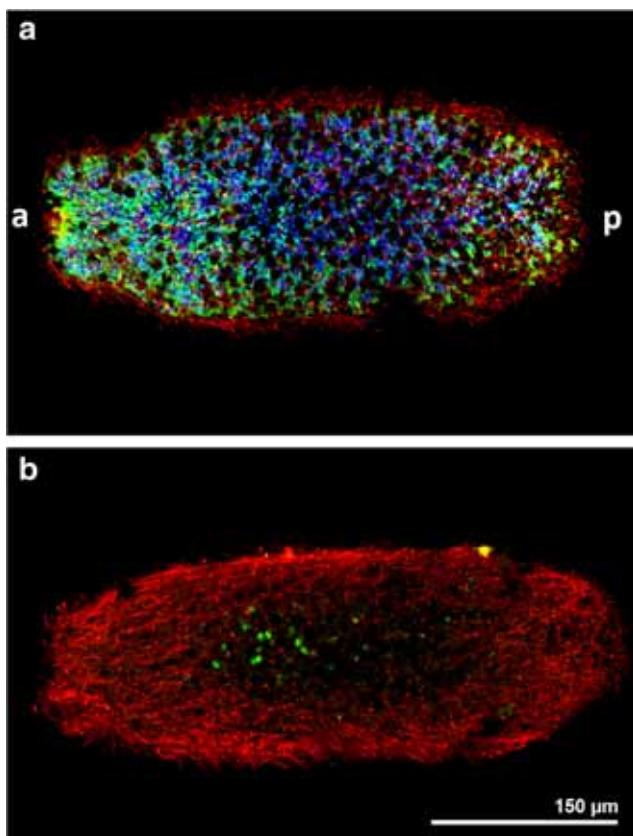


Fig. 5 Confocal microscope images of the larva of *R. recondita*. Posterior part of the larva is on the left. **a** Dispersed GABA-IR cells (green), condensed in correspondence of the posterior ciliary turf; cilia are stained by tyrosinated α -tubulin (red), nuclei are stained by DAPI (blue). **b** Dispersed serotonin-IR cells (green), in correspondence to the ventral groove. The larva is covered by cilia, stained by antibody against tyrosinated α -tubulin (red). Note: the yellow spot in **b** is not significant for the present analysis and it is probably dirt attached to larval cilia

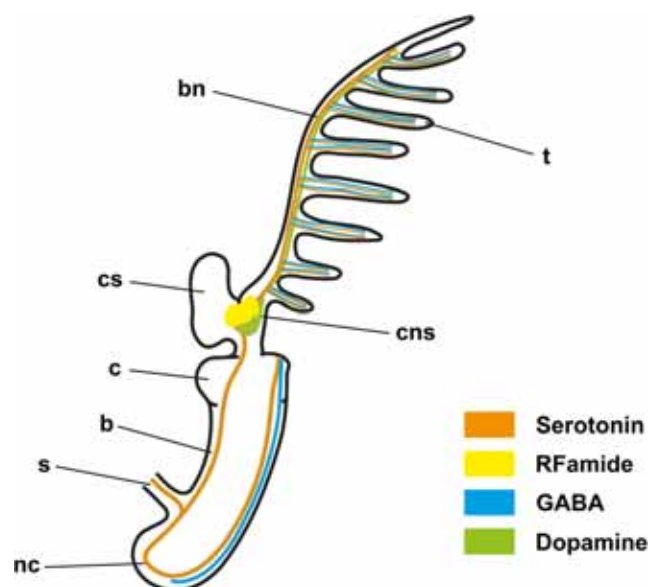


Fig. 6 Schematic overview of the zooid, including the general neuroanatomy, of *R. recondita*, lateral view: cs, cephalic shield; c, collar; s, stalk; t, tentacle; nc, neural cord; CNS, central nervous system; bn, brachial nerve

350 assumption of homology between the acorn worm
351 stomochord and the chordate notochord. However, the diffuse
352 organization of enteropneust nervous system closely resem-
353 bles the basiepidermal echinoderm nervous system (Cameron
354 and Mackie 1996), and it appears the stomochord has a closer
355 evolutionary relatedness to the chordate anterior pharynx rather
356 than notochord (Sato et al. 2014b). In addition, recently,
357 experimental evidence indicates that the neural patterning of
358 enteropneust tubular collar cord is dissimilar to the chordate
359 neural tube (Kaul-Strehlow et al. 2017).

360 In *C. gracilis*, the presence of synaptic vesicles in the brain
361 has been considered indicative of its function as an integrative
362 centre (Stach et al. 2012). Moreover, serotonergic cells are
363 concentrated in the anterior portion of the brain and project their
364 processes posteriorly into the trunk, and anteriorly along the
365 tentacles (Stach et al. 2012). Similarly, a prominent concentra-
366 tion of serotonergic neurons occurs in the dorsal part of the
367 collar, at the base of the feeding arms of *R. recondita*. In this
368 region, the presence of dopaminergic and peptidergic (RFamide
369 immunoreactive) neurons is found. Dopamine is an important
370 neurotransmitter for vertebrate and invertebrate central nervous
371 systems and it is involved in memory and movement (Verlinden
372 2018). RFamide peptides have been demonstrated to be present
373 in the nervous system of vertebrates and invertebrates where
374 they are functional for hormone regulation and behaviour
375 (Osugi et al. 2016). A simple, operational definition of central-
376 ized nervous system may be applied to a prominent condensa-
377 tion of diverse neuronal types (Holland 2003); in this frame-
378 work, the zooid of *R. recondita* shows a centralized nervous
379 system or brain dorsally in the collar, at the base of feeding
380 arms. The evidence of centralized nervous system in the zooid
381 of *R. recondita* corroborates previous findings in *C. gracilis*
382 (Rehkämper et al. 1987; Stach et al. 2012) supporting the hy-
383 pothesis that a centralized nervous system was already present
384 in the last common ancestor of deuterostomes (Burke 2011;
385 Nomaksteinsky et al. 2009). Nonetheless, the biochemical di-
386 versity and anatomical organization of the nervous system in
387 the pterobranch zooids (compared to the solitary, free-living
388 acorn worms) might be also interpreted as an adaptation to
389 the sessile and colonial filter feeding life habit. Overall, the
390 neuroanatomical disparities found between enteropneusts and
391 pterobranchs and between their larval and adult stages do not
392 enable per se reconstruction of the plesiomorphic state of the
393 nervous system of the hemichordate ancestor.

394 The larva of *R. recondita* is characterized by interconnected
395 serotonergic cells localized in a restricted area within the
396 peculiar, unpigmented ventral depression and by the lack of
397 neural centralization. Serotonergic neurons are also known
398 in tornaria larvae of enteropneusts as well as in echinoderm
399 larvae, both considered homologous and evolved from a com-
400 mon ambulacrarian ancestor (Byrne et al. 2007; Kaul-
401 Strehlow and Röttinger 2015). Serotonergic neurons in
402 dipleurula-like larvae are bilaterally associated to the apical

organ, interconnected by a neuropil, and involved in the con- 403
404 trol of ciliary bands (Nezlin and Yushin 2004). Differently, a
405 simple localized network of serotonin-IR cells in the ventral
406 depression of *R. recondita* larva might be related to a morpho-
407 genetic role of serotonin as a paracrine signal. Indeed, seroto-
408 nin is an ancient neurotransmitter found from cnidarians to
409 vertebrates (Hay-Schmidt 2000), known to be involved in
410 mollusc larval metamorphosis (Couper and Leise 1996) and
411 initiation of gastrulation in insects and echinoderms (Hay-
412 Schmidt 2000) and generally involved in cell proliferation,
413 migration, and apoptosis (Azmitia 2001).

414 For several invertebrate taxa, it has been proposed that
415 adults arise from larval set-aside cells with a great proliferative
416 capacity (Peterson et al. 1997), although developmental plas-
417 ticity of differentiated cells is increasingly recognized as a key
418 cellular process contributing to adult formation in indirect
419 developers (Arenas-Mena 2010). The histological sections of
420 the *R. recondita* metamorphosing larva reveal the presence of
421 ectodermic cell proliferation that from the anterior part of the
422 ventral depression gives rise to the cephalic shield (Fig. 3).
423 The observation of the peculiar “stop and go” locomotory
424 behaviour of the larva, with alternation of swimming, resting,
425 and crawling movements over the ventral larval surface, sug-
426 gests that the ventral depression may also play a relevant role
427 in the selection of the settlement substrate, a hypothesis that
428 needs confirmation by means of additional labelling experi-
429 ments and ultrastructure investigations.

430 Overall, it is envisaged that recent advances in genomic
431 resources and developmental genetic tools will increasingly
432 foster the use of pterobranchs, and particularly the highly ac-
433 cessible *R. recondita*, as model organism to add resolution to
434 our understanding of the origin and evolution of hemichordate
435 nervous systems and, more generally, on deuterostome phylo-
436 genetic relationships and the origin of the chordates.

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444

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446 included in this published article.
447

448 **Compliance with ethical standards**

449 **Ethical approval** All applicable international, national, and institutional
450 guidelines for animal testing, animal care, and use of animals were
451 followed by the authors.

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

454 **Sampling and field studies** All necessary permits for sampling and
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457

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