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Developmental Process of a Heterozooid: Avicularium Formation in a Bryozoan, *Bugulina californica*

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In bryozoans (phylum Bryozoa), representative colonial animals mostly found in marine environments, some species possess different types of individuals (heterozooids) specialized in different functions such as defense or structural support for their colonies. Among them, the best-known heterozooids are the avicularia, known to function as defenders. The differentiation processes of heterozooids, including avicularia, should be important keys to understand the evolutionary significance of bryozoans. However, the developmental process of avicularium formation remains to be fully understood. In this study, therefore, in order to understand the detailed developmental process and timing of avicularium formation, extensive observations were carried out in a bryozoan species, *Bugulina californica* (Cheilostomata, Bugulidae), that possesses adventitious avicularia, by performing stereomicroscopy on live materials, in addition to scanning electron microscopy and histological observations. The whole process can be divided into seven stages based on developmental events. Especially notably, at the earlier stages, there are three major budding events that produce proliferating cell masses corresponding to primordial tissues: (1) budding of the peduncle cushion at the outer margin of the distal part of a young autozooid, (2) budding of the head-part primordium from the peduncle cushion, and (3) budding of the polypide inside the head part. Experimental control of temperature showed that 20°C would be the best to maintain *B. californica* colonies.

Key words: avicularia, heterozooid, *Bugulina californica*, Bryozoa, development

INTRODUCTION

Bryozoans are colonial benthic invertebrates that inhabit mostly marine environments. They are characterized by a ciliated tentacle crown (lophophore) in each zooid, which is used for suspension-feeding (Hyman, 1959; Ryland, 1976; Mukai et al., 1997). Including fossil records, the phylum Bryozoa consists of more than 15,000 species (> 6000 extant species), in which the order Cheilostomata is the largest group, with about 5000 species (Gordon, 1999; Bock and Gordon, 2013). Species belonging to the order Cheilostomata with characteristic calcified skeletons exhibit polymorphism among zooids that represent divisions of labor, such as sexually dimorphic zooids, brooding zooids, structural zooids and defensive zooids (Silén, 1977; Reed, 1991; Harvell, 1994; Lidgard et al., 2012; Schack et al., 2019). By the acquisition of zooid polymorphism, these bryozoans have adapted to diverse environments (Schopf, 1973).

Avicularia are strictly found only in cheilostome bryozoans, although analogs are known in the fossil cyclostome family Eleidae. They have a range of functions, including defense and secretion (Darwin, 1872; Nordmann, 1840; Johnston, 1847; Kaufmann, 1968, 1971; Winston, 1984, 1986, 1991; Carter et al., 2010, 2011; Schack et al., 2019). Although lacking functional intestines and a lophophore, avicularia possess a vestigial polypide, which corresponds to tentacles, tentacle sheath, intestine, musculature and nervous system in autozooids (normal individuals: that are capable of feeding using the lophophore), providing evidence that avicularia have been derived from autozooids (Hyman, 1959; Ryland, 1970; Banta, 1973; Silén, 1977; Winston, 1984; McKinney and Jackson, 1989; Reed, 1991; Carter et al., 2011). Nutrients are provided to avicularia through a duct connecting individual zooids, which is termed a "funiculus" (Nitsche, 1871; Huxley, 1856; Lutaud, 1961; Carle and Ruppert, 1983). The morphologies of avicularia are largely transformed from that of autozooids (Fig. 1A), and are diversified among bryozoan species (Carter et al., 2010). Technical terms of bryozoan biology are very distinctive, so they are listed here as a glossary (Table 1).

Based on the budding position and morphology, avicularia are classified into four types: vicarious avicularia, interzooidal avicularia, adventitious avicularia and vibracula
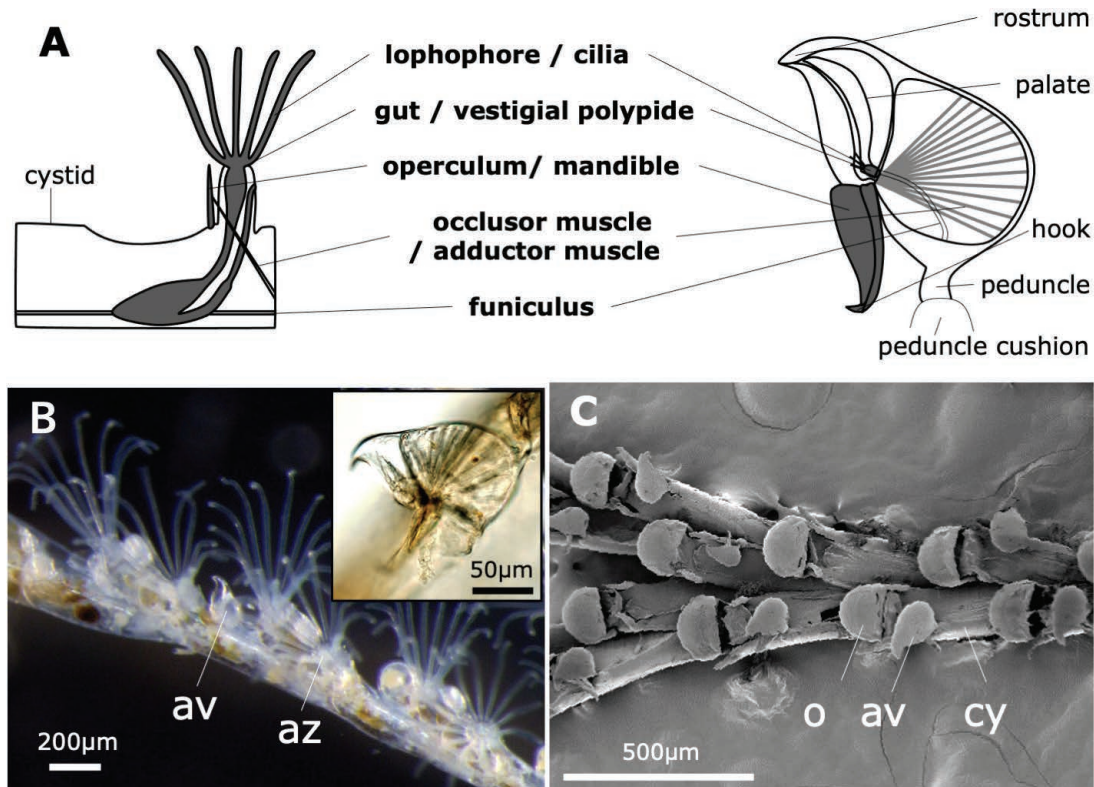
Fig. 1. Structures of autozooids and avicularia in *Bugulina californica*. **(A)** Anatomical structures of an autozooid and an avicularium, showing the homologous body parts. **(B)** A part of a living colony of *B. californica*, with a lateral view of an avicularium (inset). **(C)** SEM image of *B. californica,* showing that avicularia are associated with all autozooids, showing the distinctive features of adventitious avicularia. Abbreviations: *av* avicularium; *az* autozooid; *cy* cystid; *o* ovicell.

(Carter et al., 2010). Vicarious avicularia, which have enlarged opercula and are similar to autozooids in size, occupy the autozooid positions and interact with other zooids. Interzooidal avicularia are budded between autozooids. Their cystids extend to the substratum and they are always smaller than the parent autozooid. Adventitious avicularia (including birds-head avicularia) are budded on the lateral, frontal or basal walls of autozooids. Vibracula show unique morphologies such as setiform mandibles that can rotate, so that they are considered to be specialized types of avicularia for removal of sediments and larvae that are attached to the surface of bryozoan colonies (Ryland, 1970; Schopf, 1973; Cook and Chimonides, 1978, 1985, 1987; Winston, 1984; McKinney and Jackson, 1989).

Aviculiferous fossil bryozoans show that avicularia have been transformed from autozooids, through vicarious avicularia (Cheetham et al., 2006). This was also proved by the evidence, as mentioned above, that avicularia possess homologous body structures and organs to those in autozooids (Fig. 1A; Cook, 1968; Lidgard et al., 2012).

However, the developmental process of the avicularium, especially in relation to environmental cues such as temperature, has not been extensively investigated. Only a few descriptions have been carried out so far in a few species; immature avicularia in five different developmental stages were observed in *Bugula sabatieri* (currently synonymized as *Bugulina simplex*) (Calvet, 1900), and a developmental comparison of polypide and operculum in autozooid and avicularia at four different stages was reported in *Flustra*

foliacea (Silén, 1938).

As for the developmental process of autozooids, only developmental stages of the polypide including the lophophore, digestive tube and ganglion, have so far been described (Borg, 1926; Soule, 1954; Schwaha and Wood, 2011; Schwaha et al., 2011; Schwaha, 2018); the processes of cystid and musculature formation have not been investigated. Besides, the budding process of the avicularium polypide was previously described in an old publication with a few sketches (Calvet, 1900). However, sequential development during avicularium formation has yet to be described.

In this study, to clarify the developmental process of avicularium formation in detail, we established an observation platform in a bryozoan species that constantly produces avicularia and is easy to collect and maintain in laboratories. This study focuses on a bryozoan species, *Bugulina californica* (Cheilostomata, Bugulidae), that produces birds-head avicularia; namely, each autozooid has an avicularium budding on its surface (Fig. 1B, C; Robertson, 1905).

This species was firstly described from California, but is known to be distributed in East Asian regions (Robertson, 1905; Lutaenko et al., 2013). The budding position of the adventitious avicularia is on the distal half of an autozooid, which is on a side wall of the orifice (opening for the extending lophophore) (Robertson, 1905). This indicates that the budding position of avicularia is autonomously defined in the autozooid body; in other words, it is suggested that the developmental program of avicularia formation is incorporated in the developmental process of autozooids. Thus,

Table 1. Glossary of technical terms in byozoan biology.

technical term	definition/explanation
adductor muscle	The muscle closes the mandible and corresponds to occlusor muscle in autozooid.
adventitious avicularia	Avicularium on the surface of zooid budded on the lateral, frontal or basal walls.
autozooid	normal zooid in Bryozoa; feeding and reproduction
basal wall	The basal wall of a zooid is opposite to the frontal wall where lophophore appears.
cystid	Cystid consists of cellular layer and skeletal layer of the zooidal wall. The cellular layer consists of two cellular layers: epidermal layer and peritoneal layer.
funiculus	In gymnolaemates, mesenchymatous strands connecting the polypide with the communication pore on the zooidal wall
heterozooid	A morphologically differentiated zooid, including avicularia, vibracula, rhizoids, kenozooids, zooeciules, and some types of spines.
interzooidal avicularia	Avicularium positioned between autozooids. Their cystids extend to the basal wall and they are always smaller than the parent autozooid.
mandibular hook	Distal part of mandible of an avicularium
operculum	A role in closing the orifice in cheilostomata. It is generally uncalcified.
palate	Part of the avicularium; the mandible closes into a matching space
peduncle	The stalk of a pedunclate (bird's head form) avicularium.
peduncle cushion	Base of peduncle in a pedunclate (bird's head form) avicularium
polypide	Everything except the cystid, such as the organs and tissues of an autozooid
rostrum	Spike-like prolongation of distal part of an avicularium.
tentacle sheath	The membrane that surrounds the lophophore creates a space in which the tentacles are retracted.
vestigial polypide	The remnant of the ciliated tentacles in autozooids. Consists of ciliated cells that have the role of a sensory organ in an avicularium.
vibracula	Vibracula having setiform mandibles that can rotate, so that they are considered as specialized types of avicularia for removal of sediments and larvae
vicarious avicularia	Avicularium having enlarged opercula, which are similar to autozooids in size, occupy the autozooid positions and interact with other zooids.
zooecium	Skeleton of zooid.

since the site of avicularium formation is predetermined, the developmental process can be observed from inception.

Therefore, the developmental stages of adventitious avicularia are defined in this study, based on observations in *B. californica*. The developmental process of zooid budding in Cheilostomata was described in old reports (Nitsche, 1869; Calvet, 1900), in which the budding of autozooids and avicularia was observed. However, only a few immature zooids at a few developmental stages were included in those data.

In this study, the process of avicularia formation was described in detail, and the developmental stages were defined based on observations made by multiple approaches. We firstly defined the budding position and direction of adventitious avicularia, and then observed the process of avicularium formation over time. Based on the defined developmental stages, outer and inner morphologies are described in detail, throughout the process of avicularium formation.

MATERIALS AND METHODS

Field sampling

Colonies of *B. californica* were often found in plastic baskets which were hung at a depth of 2–3 meters from a raft for the aquaculture of *Ciona intestinalis* at the coastal area near the Misaki Marine Biological Station (Moroiso Bay). Stock colonies were kept in the laboratory, in a bucket with running sea water. They were fed marine diatom *Chaetoceros calcitrans* (Marinetech Co Ltd., Aichi).

Rearing conditions

To observe the developmental process of the avicularia, collected colonies were maintained in the laboratory. Collected colonies that were similar in size were kept in an aquarium with 1.5 L filtered sea water. *C. calcitrans* was provided as food. The amount of food was 250–500 µl, frequency of feeding was more than once every 2 days, and water was changed once every 3 days. The colonies were observed every 8 hours using a microscope (Olympus BX51) and images were recorded using the attached camera (Olympus DP74). A colony was put on a slide glass with filtered sea water and covered with a cover slip. Five to 10 immature autozooids without avicularia or with developing avicularia that were located at the tip of a colony were observed to follow the developmental process.

Scanning electron microscopy (SEM)

To elucidate the developmental process of avicularia, morphological and histological features were investigated using SEM and histological observations. For SEM, Bryozoan samples were anesthetized with 7% $MgCl_2$ for longer than 10 min, then fixed in FAA fixative (ethanol: formalin: acetic acid = 16:6:1) for 3 hours, and preserved in 70% ethanol until use. The fixed samples were dehydrated using a graded series of ethanol up to 100%, and hardened in HMDS (hexamethyldisilazane) for 1 hour. After soaking in ethanol and t-butanol, samples were freeze-dried using a Freeze Dryer ES-2030 (Hitachi Global, Tokyo, Japan), and coated with gold ions with an Ion Sputter E-1010 (Hitachi Global, Tokyo, Japan). Samples

were observed with a JSM-5510LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Histological observations

Samples were anesthetized with 7% $MgCl_2$ for longer than 10 min, fixed in FAA fixative for 3 hours, and preserved in 70% ethanol until use. The samples were hydrated in decreasing concentrations of ethanol and then soaked in distilled water, and embedded in agarose gel. After that, the embedded samples were dehydrated in a graded series of ethanol and acetone, then embedded in resin with a kit (Technovit8100, Kulzer GmbH, Hanau). Embedded samples were then sectioned at 5 μm, stained with hematoxylin and eosin, and mounted in Malinol (MUTO Pure Chemicals, Tokyo). Sections were observed using a camera-equipped microscope (Olympus BX51, Olympus DP74).

Examination of rearing temperature

To examine the optimal rearing condition (temperature) for *B. californica*, observations under different temperature conditions were carried out. Collected colonies that were similar in size were kept under three different temperature conditions (15°C, 20°C, 25°C). Other rearing conditions were the same in the three temperature conditions. The period from the stage before the onset of avicularia formation (Stage 0) to the completion of avicularia formation (Stage 7) was recorded for each temperature.

For the statistical test, the homogeneity of variance was examined by the Brown-Forsythe test. The significance of changes in the time for the avicularia formation was examined using one-way analysis of variance (ANOVA) after log-transformation (Zar, 1984). Differences between the means of periods were compared using the Tukey HSD test.

RESULTS AND DISCUSSION

Overall observations on avicularia in *B. californica*

Branches are biserial, with avicularia formed on the outer distolateral surface (Fig. 1B, C; Fig. 2). Avicularium formation occurs concurrently with autozooid development (Fig. 2A). Immature autozooids at branch tips lack avicularium primordia (Fig. 2A, arrowheads). Immature avicularia were seen mostly on the second or third autozooids from branch tips (Fig. 2A). By the time avicularium formation is complete (Stage 7, see below), autozooids are mature and everting lophophores. However, the situation was more complicated at the bifurcating parts of branches (see Supplementary Figure S1). Taken together, these findings strongly suggested that a newly budded autozooid at a colony branch produces the next autozooid as it

develops, and after that, it starts to bud the primordium of an avicularium.

The position where an avicularium was budded in an associated autozooid was, as described in Robertson (1905), on the outer surface of the distal half of an autozooid, at the side of the autozooid orifice (Fig. 1B, C; Fig. 2). The direction of avicularium budding was orthogonal to the autozooid cystid wall (Fig. 2A, B, F), as described in a previous
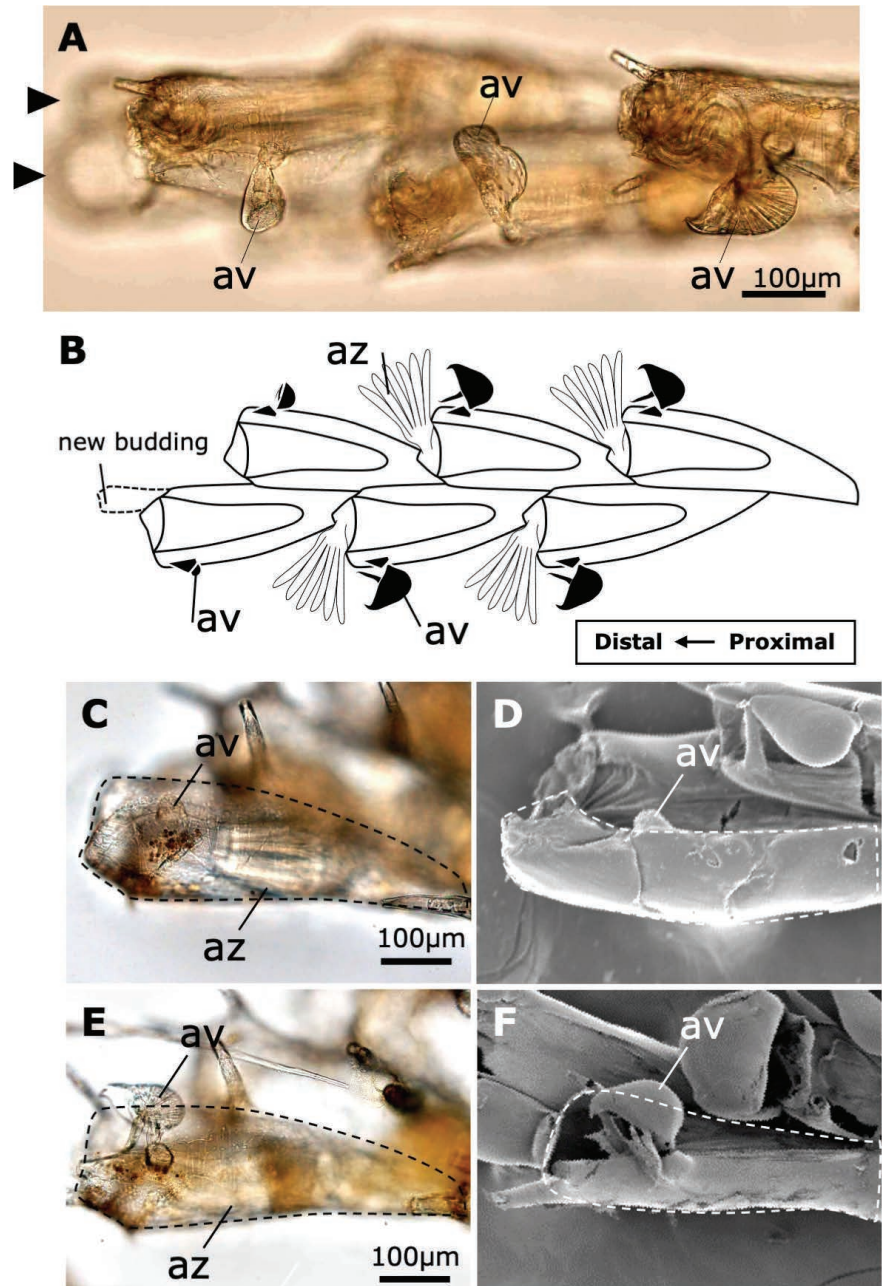

Fig. 2. Position of avicularia formation. **(A)** A distal branch of a colony with various stages of avicularia formation. Note that the more immature avicularia are seen at the more distal part. **(B)** Diagram showing the spatial relationship among developing autozooids and avicularia at the distal branch of a *B. californica* colony. **(C, D)** A newly formed bud is seen on the apical region of an autozooid. The earliest stage is shown; living **(C)** and SEM **(D)** images. **(E, F)** An autozooid with a completed avicularium; living **(E)** and SEM **(F)** images. Abbreviations: *av* avicularium; *az* autozooid; *cy* cystid.

study (Mawatari, 1971). Based on the position of avicularia, it is hypothesized that the avicularia might protect the lophophore of autozooids, although they might have other functions such as waste removal and providing baits (Darwin, 1872; Nordmann, 1840; Johnston, 1847; Kaufmann, 1968, 1971; Winston, 1984, 1986, 1991; Carter et al., 2010, 2011; Schack et al., 2019). To test the above hypothesis, the correlation between the activities of autozooids and those of avicularia should be examined in future studies. Furthermore, since an avicularium is regularly formed on each autozooid in a predetermined position, the developmental program of avicularia should be incorporated into that of autozooids.

Therefore, at the branch tip of the bryozoan colony, immature avicularia in the course of the developmental process were observed along a colony branch; more-developed avicularia are seen at more proximal parts of a branch (Fig. 2A, B). At the beginning of avicularium formation, a small projection was formed at the margin of an autozooid cystid, and was later shown to become the peduncle cushion (PC) of the avicularium (Fig. 2C, D). Thus, various stages of avicularia can be observed at the distal branch portions, so the developmental stages were defined based on the observations on such avicularia.

Developmental stages of avicularium formation

Based on the observations made using stereomicroscopy, scanning electron microscopy (SEM) and histological sections, the developmental process of avicularia formation was classified into seven stages, i.e., Stage 1 to Stage 7 (Fig. 3). Characteristics of each developmental stage are described below (technical terms for bryozoan body parts are defined in Table 1).

Stage 1

When the size of an autozooid reached approx. 100 μm, a small bud was formed at the outer margin (Fig. 2C, Fig. 3A). Based on observations at later stages, it was concluded that this bud developed into a peduncle cushion, not the entire avicularium (see below). At this time, the budding of the main body of an avicularium from the peduncle cushion had not yet occurred. The surface of the peduncle cushion appeared smooth (Fig. 3B). Histological observations also revealed that, at this stage, the peduncle cushion was filled with proliferating cells.

Stage 2

At Stage 2, the primordial avicularium forming the head part of an avicularium was budding from the peduncle cushion, and then elongating at the latter half of Stage 2 (Fig. 3D). The surface of the primordium was wrinkled. Histological observations showed that the primordium was filled with a dense cell mass coming out from the peduncle cushion and no differentiated structures (Fig. 3F). At this stage, the peduncle cushion was still filled with undifferentiated cells (see Supplementary Figure S2B).

Stage 3

At Stage 3, the primordium continued to elongate, and then formed a constriction when it reached over 50 μm in length (Fig. 3G, arrowheads). SEM observations revealed that there were numerous fine wrinkles in multiple directions with major grooves in the proximal-distal direction at the distal part, while at the proximal part the wrinkles looked coarser in the vertical direction to the P-D axis (Fig. 3H). Histological images showed that the epithelial cells and peritoneal cells on the future-mandible side proliferated to become thickened, and from this thickened cell mass a proliferating cell mass budded inside the primordial cavity (Fig. 3I, arrow). This phenomenon in which the cell mass was budded inside a newly formed polypide was also reported in the development of autozooids, suggesting that the bud is the primordium of a polypide (Soule, 1954; Schwaha and Wood, 2011; Schwaha et al., 2011; Schwaha, 2018). Since a previous study suggested that zooid polymorphism was developed by the alteration of pre-existing structures (Silén, 1977), this similarity between autozooid and avicularium development would imply that the avicularia were evolutionarily derived from autozooids.

Stage 4

Until Stage 4, the elongation of the primordial avicularium continued in the direction perpendicular to the autozooid cystid wall. The proximal part under the constriction formed the peduncle, while the distal side became extremely enlarged (Fig. 3J). Mandible formation began at this stage. The wrinkled structures were uniform at the beginning of Stage 2, but this changed at Stage 4 as the primordium elongated (Fig. 3K). SEM observations showed that a deep groove appeared at the boundary between the mandible and the avicularium head (Fig. 3K). Inside the enlarged primordium, a few cells with some fibrous structures came out from the budded cell mass (Fig. 3L, ad). This is suggested to show the onset of adductor muscle formation. Inside the peduncle cushion, fibroblast-like cells were also observed (see Supplementary Figure S2D, arrowhead). This indicates that the muscle formation occurs simultaneously inside the head part and inside the peduncle cushion.

Stage 5

At Stage 5, the apical portion of the enlarged primordium became sharp, to form the rostrum (Fig. 3M, N). The mandibular hook was not formed yet. From Stage 5, the direction of elongation was changed to the direction to the side of the rostrum, i.e., the distal direction of the colony (Fig. 3M–R). SEM observations revealed that some wrinkled structures were seen at the rostrum tip, indicating that the formation of the rostrum tip was not completed, and the calcification was still ongoing at this stage (Fig. 3N). The head part became smooth, and wrinkled structures observed at the previous stages disappeared (Fig. 3N), probably due to calcification that hardened the head structure. It is also possible that the wrinkled epithelial structure enables the abrupt development, as in insect molting/metamorphosis (e.g., Matsuda et al., 2017). Fibers of the adductor muscle that connected the head capsule and the mandible were proliferating, but the connection was not completed (Fig. 3O).

Stage 6

At Stage 6, the mandibular hook was formed and inserted inside the rostrum tip (Fig. 3P). The elongation of the rostrum suggested that the palate, i.e., the distal part of
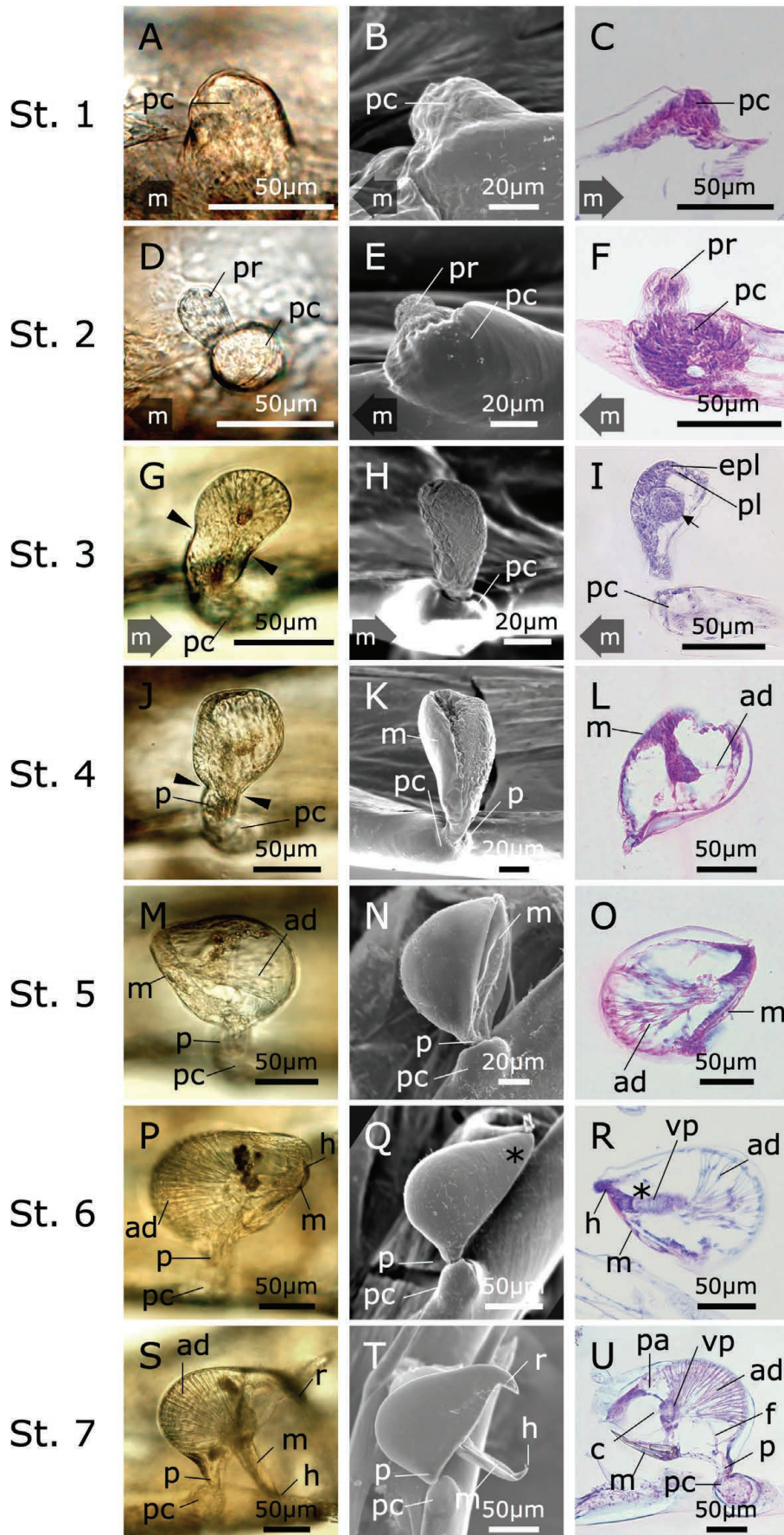
the head part adjacent to the mandible (distal to the vestigial polypide), started to form (Fig. 3Q, asterisk). The formation of this part was also confirmed by histological observations (Fig. 3R, asterisk). The adductor muscle inside the head part was almost completed (see Supplementary Figure S2E, arrow). Also, muscles inside the peduncle cushion were formed, showing fibrous structures (see Supplementary Figure S2F, arrow). Behavioral observations at this stage showed that the mandible opened slightly, but was not completely opened (see Supplementary Movies S1–S4). This is probably because some membranous structure connected the rostrum and the mandible. The membrane is suggested to be torn off as a result of multiple trial openings of the mandible, resulting in the complete opening of the mandible. At the later period of Stage 6, when cilia were formed from the vestigial polypide between the rostrum and the mandible, the avicularia formation was almost completed (Fig. 3Q, R). The position of the cilia is suggested to correspond to that of the lophophore in autozooids, consistently with a previous study (Carter et al., 2010). The budded cell mass that was formed inside at Stage 3 (i.e., the primordial polypide) later

Fig. 3. Developmental stages of avicularia, defined in this study. **(A–C)** Stage 1, **(D–F)** Stage 2, **(G–I)** Stage 3, **(J–L)** Stage 4, **(M–O)** Stage 5, **(P–R)** Stage 6, **(S–U)** Stage 7. Left panels **(A, D, G, J, M, P, S)** live avicularia, middle panels **(B, E, H, K, N, Q, T)** SEM images, right panels **(C, F, I, L, O, R, U)** histological sections stained with hematoxylin and eosin. Detailed characteristics of each stage are described in the text. Thick arrows labelled "m" at the bottom left corner of panels indicate the direction of the future mandible. Arrowheads in **(G)** and **(J)** show the constriction between the peduncle cushion and head part. A small arrow in **(I)** shows proliferating cell mass budded inside the primordial cavity. Abbreviations: *ad* adductor muscle; *av* avicularium; *az* autozooid; *c* cilia; *cy* cystid; *epl* epithelial layer; *f* funiculus; *h* hook; *m* mandible; *p* peduncle; *pa* palate; *pc* peduncle cushion; *pl* peritoneal layer; *pr* primordium; *r* rostrum; *vp* vestigial polypide.
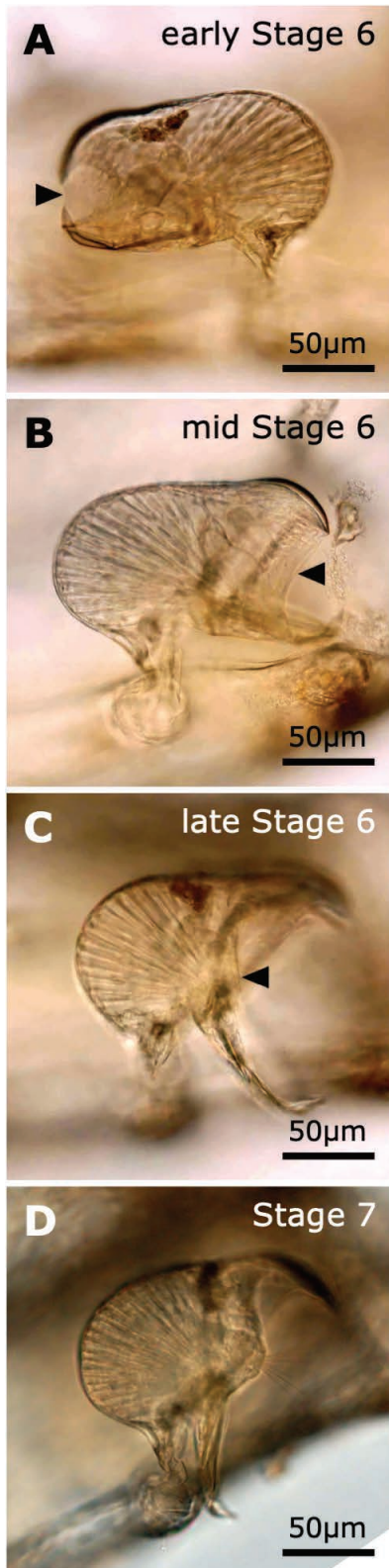
Fig. 4. Transitional changes of mandibular opening during Stage 6. **(A)** Mandible is just slightly open in early Stage 6. Rostrum and mandible connected by membranous tissues (arrowhead). **(B)** Mandible is half open, although it is still connected by membranous tissue (arrowhead). **(C)** Mandible is almost open, but still contains membranous tissues (arrowhead). **(D)** Mandible is completely open in Stage 7.

gradually shrunk to become the vestigial polypide in an avicularium.

Stage 7

At Stage 7, the formation of an avicularium was completed when the mandible was fully opened (Fig. 3S–U). The adductor muscle was fully developed and the funiculus was connected from the associated autozooid to the vestigial polypide (Fig. 3U). Multiple sensory cilia were observed on the outer surface of the vestigial polypide (Fig. 3U).

As described above, the process of avicularium formation can be divided into seven developmental stages. In the course of development, three major budding events play major roles in development: (1) budding of PC primordium from an autozooid (Stage 1), (2) budding of head-part primordium from PC (Stage 2), and (3) internal budding of the polypide inside a head part (Stage 3). Through these steps, with cell proliferation followed by detailed formation of body structures, the avicularium formation is accomplished.

Avicularia formation after autozooid degeneration

Some avicularia were observed to develop even after polypide regression (at 15°C), raising the question of whether avicularium formation requires the presence of a polypide in the parent zooid. In the case that an autozooid with lophophore less than 100 μm was degenerated, before the onset of avicularia formation, the avicularium formation did not start. After that, the autozooid polypide was absorbed although the zoocium/cystid remained (see Supplementary Figure S3). This suggests that the avicularia formation requires the associated polypide, probably because nutrition for the development would be provided from the autozooid via the funiculus. However, in the case that the associated autozooid started to degenerate at Stage 3 of the avicularia formation, and completely died at Stage 6 (the brown body was observed), the avicularia formation was completed, although it took longer (128 hours) than in the normal situation (104.00 $\pm$ 8.64 hours (mean $\pm$ s.e.)) (see Supplementary Figure S4). This indicates that the later stages of avicularia development can proceed, independently of the associated autozooid. This may suggest that dense cells inside the primordium could be incorporated from the parental autozooid until Stage 2, and then at Stage 3, cell differentiation occurred inside the developing avicularium, when the connection with the autozooid was completed. The independent development of avicularia might have been related to the formation of the polypide of the avicularium from Stage 3, although it required a longer time, probably because the nutritional supply was limited due to the degeneration of the autozooid polypide.

After the completion of avicularia formation, i.e., Stage 7, when the associated autozooid was degenerated and absorbed it did not affect the function of the avicularia during a few days (see Supplementary Movie S5). This suggests that completely developed avicularia can survive without an associated polypide, at least for a few days. Alternatively, nutrition could also be supplied by other autozooids in the same colony, through the funicular network. Our observations showed that polypide regression is easily induced by environmental changes and/or nutritional depletion in a colony. In contrast, interestingly, the recycling of avicularian

soft tissues has never been observed, suggesting that unlike autozooids, avicularia cannot be reused as a nutritional source.

Optimal temperature condition for laboratory culture

To examine the preferred temperature for rearing, *B. californica* was incubated at 15, 20 or 25°C (Fig. 5, see Supplementary Figures S5–S7). At 15°C, it took 104.00 ± 8.64 hours (mean ± s.e.) from the onset of budding to the completion of avicularium morphogenesis (Fig. 5, and see Supplementary Figure S5). During this period, even a single stage took more than several tens of hours (see Supplementary Figure S5). Although the observation was continued after the completion, the size was not increased thereafter. Also, at 15°C, many autozooids died during the development, probably because the temperature was too low.

At 20 and 25°C, avicularium morphogenesis took 72.00 ± 1.33 and 46.86 ± 1.14 hours, respectively (Fig. 5, see Supplementary Figures S6, S7). At 25°C, the development sometimes proceeded for two stages during an observation interval of 8 hours (see Supplementary Figure S7). There were statistically significant differences of developmental speed among the three temperature conditions (Fig. 5; $P < 0.05$, Tukey's HSD test). On the other hand, at 25°C, some autozooids died before the avicularium formation due to proliferation of other microorganisms. Among the three temperature conditions, 20°C was optimal for observing all of the developmental stages during the 8-hour observation period (see Supplementary Figure S6).

Developmental comparison between autozooids and avicularia

In the case of autozooid formation by budding, a new cystid is budded from the parent autozooid. The inner surface of the cystid is lined by epithelial and peritoneal layers, both of which are budded into the space inside the cystid to form a polypide (Schwaha and Wood, 2011; Schwaha, 2018).

Stage 3 of avicularia formation resembled this stage of autozooid development (Fig. 3G, H, I), suggesting that the proliferation and budding of the two layers, i.e., epithelial and peritoneal layers, is conserved between autozooids and avicularia. This may also imply that an avicularium is a zooid, and that avicularia may have been acquired by the modification of autozooid development, as suggested in previous studies (Silén, 1977; Schack et al., 2019).

Evolutionary implications and perspectives

In colonial animals, generally, heterozooids show less individuality in comparison with autozooids, in terms of their
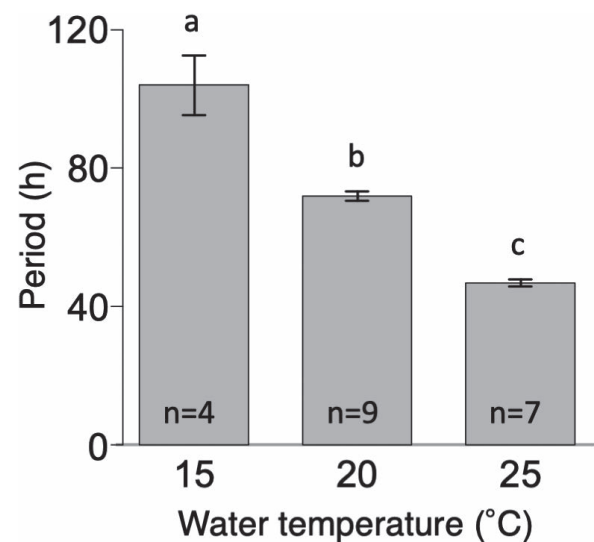

Fig. 5. Period required for avicularia formation, depending on water temperature. The results of developmental observations at 15, 20 and 25°C are shown (mean ± s.e., *N* = 4, 9 and 7, respectively for each condition). Different letters on the bars indicate significant differences (Tukey's HSD test, $P < 0.05$).
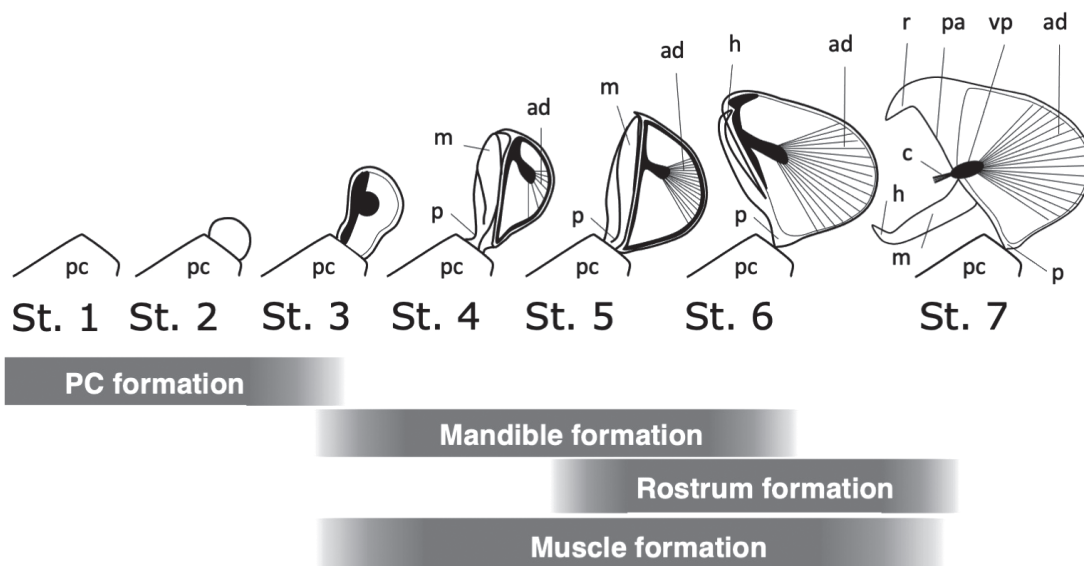

Fig. 6. Summary diagram of the developmental stages during avicularia formation. Developmental events observed in this study are also shown. Abbreviations: *ad* adductor muscle; *c* cilia; *h* hook; *m* mandible; *p* peduncle; *pa* palate; *pc* peduncle cushion; *r* rostrum; *vp* vestigial polypide.

independence or autonomy (Beklemishev, 1969). Our observations, however, showed that some avicularia survived and functioned after the loss of associated autozooids, suggesting that avicularia also possess individuality to some extent, although the definition of "individuality" is ambiguous in colonial animals such as bryozoans (cf. Clarke, 2010, 2011). This also suggests that there still could be some nutritional connections to other autozooids, probably through the funiculus.

In the sense that, to form a colony, multiple phenotypes are differentiated in a single species among related or clonal individuals, the coloniality and the heterozooid differentiation are similar to the caste differentiation in social insects (Wilson, 1975), although, in social insects, colony members are not directly connected to each other and possess apparent "individuality", while bryozoan zooids are connected to one another. Also, in the caste differentiation in social insects, multiple types of individuals, i.e., castes, differentiate during their postembryonic development, depending on various extrinsic factors (Miura, 2019). Furthermore, the soldier caste is a distinctive caste specialized in colony defense, so this is also analogous to the avicularia in bryozoans. The developmental process of soldier differentiation in termites is well documented (Miura and Maekawa, 2020), so similar analyses can also be applied to the study of avicularia formation in bryozoans.

This study has documented the detailed process of avicularia formation in a bryozoan species, *B. californica*, that possesses adventitious avicularia. It is still unknown whether the process of avicularia formation in *B. californica* is shared by other bryozoans, including other avicularia types, such as vicarious avicularia, interzooidal avicularia and vibracula. Since the morphological features of other avicularia types are similar to each other (Carter et al., 2010), they have been suggested to share some developmental processes. Also, the number of avicularia acquisitions in the bryozoan evolution is still unclear, although it is suggested that vicarious avicularia evolutionarily derive from autozooids (Silén, 1938; Cheetham et al., 2006). Therefore, it should be important to study the developmental process of vicarious avicularia, although it is difficult to culture the species with vicarious avicularia (Schack et al., 2019). In future studies, extensive phylogenetic analyses together with the investigations of avicularia formation in various bryozoan lineages will reveal the evolutionary process leading to the acquisition of avicularia.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

HY, MH, HK and TM designed the whole study. HY, SU, KO, HK and TM collected the study materials from the field. HY reared animals and observed the developmental processes. HY, MN and SU performed the experimental procedures. HY, JS and TM analyzed the data on rearing conditions. All of the authors wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs200143)

Supplementary Figure S1. Distal tip of a colony branch.

Supplementary Figure S2. Process of formation of muscle tissue in peduncle cushion.

Supplementary Figure S3. Observation of a degenerating autozooid that did not produce an avicularium.

Supplementary Figure S4. Observation of an avicularium formed on an autozooid that disappeared during the process of avicularia formation.

Supplementary Figure S5. Developmental process of avicularia formation observed at 15°C.

Supplementary Figure S6. Developmental process of avicularia formation observed at 20°C.

Supplementary Figure S7. Developmental process of avicularia formation observed at 25°C.

Supplementary Movie S1. Developing avicularium at early St. 6.

Supplementary Movie S2. Developing avicularium at mid St. 6.

Supplementary Movie S3. Developing avicularium at late St. 6.

Supplementary Movie S4. Completed avicularium at St. 7.

Supplementary Movie S5. Completed avicularium at St. 7 whose parental autozooid was dead, but the avicularium barely moved.

REFERENCES

Banta WC (1973) Evolution of avicularia in cheilostome Bryozoa. In "Animal Colonies" Ed by RS Boardman, AH Cheetham, WA Oliver, Dowden, Hutchinson and Ross, Inc, Pennsylvania, pp 295–303

Beklemishev WN (1969) Principles of Comparative Anatomy of Invertebrates, Vol 1. Oliver and Boyd, Edinburgh

Bock PE, Gordon DP (2013) Phylum Bryozoa Ehrenberg, 1831. Zootaxa 3703: 67–74

Borg F (1926) Studies on recent cyclostomatous Bryozoa. Zool Bidr Uppsala 10: 181–507

Calvet L (1900) Contributions a l'Histoire Naturelle des Bryozoaires Ectoproctes Marins. Trav Inst Zool, Montpellier

Carle KJ, Ruppert EE (1983) Comparative ultrastructure of the bryozoan funiculus: A blood vessel homologue. J Zool Syst Evol Res 21: 181–193

Carter MC, Gordon DP, Gardner JPA (2010) Polymorphism and variation in modular animals: morphometric and density analyses of bryozoan avicularia. Mar Ecol Prog Ser 399: 117–130

Carter MC, Lidgard S, Gordon DP, Gardner JPA (2011) Functional innovation through vestigialisation in a modular marine invertebrate. Biol J Linn 104: 63–74

Cheetham AH, Sanner J, Taylor PD, Ostrovsky AN (2006) Morphological differentiation of avicularia and the proliferation of species in mid-Cretaceous *Wilbertopora* Cheetham, 1954 (Bryozoa: Cheilostomata). J Paleontol 80: 49–71

Clarke E (2010) The problem of biological individuality. Biol Theory 5: 312–325

Clarke E (2011) Plant individuality and multilevel selection theory. In "The Major Transitions Revisited" Ed by K Sterelny, B Calcott, MIT Press, Cambridge MA

Cook PL (1968) Polyzoa from West Africa. The Malacostega. Part 1. Bull British Mus (Nat Hist) Zool 16: 115–160

Cook PL, Chimonides PJ (1978) Observations on living colonies of *Selenaria* (Bryozoa, Cheilostomata) I. Cah Biol Mar 19: 147–158

Cook PL, Chimonides PJ (1985) Recent and fossil Lunulitidae

(Bryozoa, Cheilostomata) 5. *Selenaria alata* Tenison Woods and related species. J Nat Hist 19: 337–358

Cook PL, Chimonides PJ (1987) Recent and fossil Lunulitidae (Bryozoa, Cheilostomata) 7. *Selenaria maculata* (Busk) and allied species from Australasia. J Nat Hist 21: 933–966

Darwin C (1872) On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life, 6th ed, John Murray, London

Gordon DP (1999) Bryozoan diversity in New Zealand and Australia. In "The Other 99%: The Conservation and Biodiversity of Invertebrates" Ed by W Ponder, D Lunney, Royal Zoological Society of New South Wales, Mosman, pp 199–204

Huxley TH (1856) Notes on the reproductive organs of the cheilostome Polyzoa. Quart J Microsc Sci 4: 191–192

Hyman LH (1959) The Invertebrates. Vol 5. Smaller Coelomate Groups: Chaetognatha, Hemichordata, Pogonophora, Phoronida, Ectoprocta, Brachiopoda, Sipunculida. The Coelomate Bilateria, McGraw-Hill Book Company Inc, New York

Johnston G (1847) A History of the British Zoophytes. Vol 2. Van Voorst, London

Kaufmann KW (1968) The biological role of Bugula-type avicularia (Bryozoa). In "Proceedings of the First International Conference on Bryozoa" Ed by E Annoscia, Atti Soc Ital Sci Nat 108: 54–60

Kaufmann KW (1971) The form and functions of the avicularia of Bugula (Phylum Ectoprocta). Postilla (Peabody Museum of Natural History, Yale University) 151: 1–26

Lidgard S, Carter MC, Dick MH, Gordon DP, Ostrovsky AN (2012) Division of labor and recurrent evolution of polymorphisms in a group of colonial animals. Evol Ecol 26: 233–257

Lutaenko KA, Furota T, Nakayama S, Shin K, Xu J (2013) Atlas of Marine Invasive Species in the NOWPAP Region. NOWPAP DINRAC, Beijing

Lutaud G (1961) Contribution á l'étude du bourgeonnement et de la croissance des colonies chez *Membranipora membranacea* (Linné) Bryozoaire Chilostome. Ann Soc R Zool Belg 91: 157–300

Matsuda K, Gotoh H, Tajika Y, Sushida T, Aonuma H, Niimi T, et al. (2017) Complex furrows in a 2D epithelial sheet code the 3D structure of a beetle horn. Sci Rep 7: 13939

Mawatari S (1971) Polymorphisms in Bryozoa. Proc Jpn Soc Syst Zool 43–44: 8–15

McKinney FK, Jackson JBC (1989) Bryozoan Evolution, Allen and Unwin, London

Miura T (2019) Juvenile hormone as a physiological regulator mediating phenotypic plasticity in pancrustaceans. Dev Growth Differ 61: 85–96

Miura T, Maekawa K (2020) The making of the defensive caste: Physiology, development and evolution of the soldier differentiation in termites. Evol Dev 22: e12335

Mukai H, Terakado K, Reed C (1997) Bryozoa. In "Microscopic Anatomy of Invertebrates" Ed by FW Harrison, RM Woollacott, Wiley, New York, pp 45–206

Nitsche H (1869) Beiträge zur Kenntniss der Bryozen. 1. Beobachtungen über die Entwicklungsgeschichte einiger chilostomen Bryozoen. 2. Ueber die Anatomie von *Pedicellina echinate* SARS. Z Wiss Zool 20: 1–36

Nitsche H (1871) Beiträge zur Kenntnis der Bryozoen 3. Über die Anatomie und Entwicklungsgeschichte von Flustra membranacea 4. Über die Morphologie der Bryozoen. Z Wiss Zool 21: 416–498, plates 25–27

Nordmann A (1840) Recherches microscopiques sur la Cellularia avicularia. In: Voyage dans la Russie méridionale et la Crimée, par la Hongrie, la Valachie et la Moldavie, exécuté en 1837 sous la direction de M Anatole de Demidoff, Vol III. Ernest Bourdin, Paris, pp 679–707

Reed CG (1991) Bryozoa. In "Echinoderms and lophophorates" Ed by AC Giese, JS Pearse, VB Pearse, Boxwood Press, Pacific Grove, pp 85–245

Robertson A (1905) Non-incrusting Cheilostomatous Bryozoa of the west coast of North America. Univ Calif Publ Zool 2: 235–322, plates 4–16

Ryland JS (1970) Bryozoans. Hutchinson University Library, London

Ryland JS (1976) Physiology and ecology of marine bryozoans. Adv Mar Biol 14: 285–443

Schack CR, Gordon DP, Ryan KG (2019) Modularity is the mother of invention: a review of polymorphism in bryozoans. Biol Rev 94: 773–809

Schopf TJM (1973) Ergonomics of polymorphism: its relation to the colony as the unit of natural selection in species of the phylum Ectoprocta. In "Animal Colonies" Ed by RS Boardman, AH Cheetham, WA Oliver, Dowden, Hutchinson and Ross, Inc, Stroudsburg, pp 247–294

Schwaha T (2018) Morphology and ontogeny of *Lophopus crystallinus* lophophore support the epistome as ancestral character of phylactolaemate bryozoans. Zoomorphology 137: 355–366

Schwaha T, Wood TS (2011) Organogenesis during budding and lophophoral morphology of *Hislopia malayensis* Annandale, 1916 (Bryozoa, Ctenostomata). BMC Dev Biol 11: 23

Schwaha T, Handschuh S, Redl E, Walzl M (2011) Organogenesis in the budding process of the freshwater bryozoan *Cristatella mucedo* Cuvier, 1798 (Bryozoa, Phylactolaemata). J Morphol 272: 320–341

Silén L (1938) Zur Kenntnis des Polymorphismus der Bryozoen. Die Avicularien der Cheilostomata Anasca. Zool Bidr Uppsala 17: 149–366

Silén L (1977) Polymorphism. In: "Biology of Bryozoans" Ed by RM Woollacott, RL Zimmer, Academic Press, New York, pp 184–231

Soule JD (1954) Post larval development in relation to the classification of the Bryozoa Ctenostomata. Bull S Calif Acad Sci 53: 13–34

Wilson EO (1975) Sociobiology. Belknap, Cambridge, MA

Winston JE (1984) Why bryozoans have avicularia—a review of the evidence. Am Mus Novitates 2789: 1–26

Winston JE (1986) Victims of avicularia. Mar Ecol: 193–199

Winston JE (1991) Avicularian behaviour—a progress report. In "Bryozoa Living and Fossil" Ed by FP Bigey, J-L d' Hondt, Bulletin de la Société des Sciences Naturelles de l'Ouest de la France, Mémoire HS 1: 531–540

Zar JH (1984) Data transformations. In: Biostatistical Analysis, Prentice Hall, Englewood Cliffs, pp 236–243