Patterns of sucker development in cuttlefishes

Ryosuke Kimbara[1], Mayuko Nakamura[1], Kohei Oguchi[1,2], Hisanori Kohtsuka[1], and Toru Miura[1,*]

[1]Misaki Marine Biological Station, School of Science, The University of Tokyo, Miura, Kanagawa, 238-0225, Japan

[2]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, 305-8566, Japan

[*]Corresponding author: Toru Miura, miu@mmbs.s.u-tokyo.ac.jp

ABSTRACT

Background: Morphological novelties have been acquired through evolutionary processes in relation to the acquisition of new life-history strategies together with novel functions of bodyparts. Cephalopod molluscs such as octopuses, squids and cuttlefishes possess novel morphological characteristics such as their overall

bodyplans and numerous arms. Among those novel morphologies, in particular, suckers arranged along the oral side of each arm possess multiple functions, such as capturing prey and locomotion, so that the sucker morphology is diversified among species, depending on their ecological niche. However, the detailed developmental process of sucker formation has remained unclear, although it is known that new suckers are formed or added throughout their life-time, including during both embryonic and postembryonic development. In the present study, therefore, focusing on two cuttlefish species, *Sepia esculenta* and *S. lycidas*, in which the sucker morphology is relatively simple, morphological and histological observations were carried out during embryonic and postembryonic development to elucidate the developmental process of sucker formation.

Results: The observations in both species clearly showed that the newly formed suckers were added on the oral side of the most distal tip of each arm during embryonic and postembryonic development. On the oral side of the arm tip, the epithelial tissue became swollen to form a ridge along the proximal-distal axis (distal sucker ridge). Next to the distal ridge, there were small dome-shaped bulges that are presumed to be the sucker primordia. Toward the proximal direction, the primordia became functional suckers, in which the inner tissues

differentiated to form the complex sucker structures. During postembryonic development, on both sides of the distal sucker ridge, epithelial tissues extended to form a sheath, covering the ridge for protection of undifferentiated suckers.

Conclusions: The developmental process of sucker formation, in which sucker primordia are generated from a ridge structure (distal sucker ridge) on the oral side at the distal-most arm tip, was shared in both cuttlefish species.

(313 words)

Keywords: Cephalopod, Cuttlefish, Novelty, Sucker, Embryogenesis, Postembryonic development, Sucker primordium, Distal sucker ridge

Background

Generally, in evolution, the acquisition of novel characteristics is known to play crucial roles in adaptation to new environments [1]. Among bilaterians,

51 the bodyplans of lophotrochozoans are the most diversified [2]. Especially,
52 molluscs exhibit diverse and unique characteristics, including their bodyplans [3,
53 4], and cephalopods (class Cephalopoda) are the most distinctive group in
54 molluscs [3, 5]. They show highly developed brains [6], ink sacs [7], and
55 chromatophores [8]. Extant cephalopod molluscs (class Cephalopoda) are
56 composed of 2 subclasses, Nautiloidea and Coleoidea, and Coleoidea is classified
57 into 2 superorders, i.e., Decapodiformes (squids and cuttlefishes) and
58 Octopodiformes [5, 9]. Numerous arms and suckers on arms are also important
59 novelties in cephalopods (**Fig. 1A**), although the basal cephalopod lineages, i.e.,
60 Nautiloidea, lack suckers [10]. Since cephalopod arms are used not only for
61 locomotion but also for prey capture and copulation, suckers should significantly
62 contribute to those functions [11].

63 Especially in Decapodiformes, the sucker morphology is diversified
64 among species [12]. Basically, an adult sucker is composed of an attachment,
65 face (infundibulum), a chamber for producing suction (acetabulum), and a
66 peduncle by which the acetabulum is attached to the arm surface, and there is a
67 proteinaceous ring with teeth on the rim of the infundibulum (sucker ring teeth)
68 (**Fig. 1B**) [13, 14]. In some species, furthermore, suckers are modified into

69 hooks, losing the sucking functions [12]. Thus, the sucker morphology is deeply
70 related to life-history strategies and the adaptive radiation in cephalopods [11,
71 15, 16].

72 There have so far been several studies on the development of arms in
73 Decapodiformes [17, 18]. It was reported that, in Decapodiformes, the number
74 of suckers increases as arms are elongated during postembryonic development
75 [10]. However, there are few studies focusing on the developmental process of
76 sucker formation throughout these animals' lifetime. In the embryonic
77 development of *Euprymna scolopes* (Sepiolida), small papillae were observed on
78 the oral side of the arm tip, although the process of sucker development was not
79 described in detail [17]. In some squid species belonging to Myopsida and
80 Oegopsida, the processes of embryonic development were described (*Illex*
81 *illecebrosus*: [19]; *Sepioteuthis lessoniana*: [20]; *Dosidicus gigas*: [21];
82 *Todarodes pacificus*: [22]), but the descriptions did not focus on sucker formation.
83 Thus, the pattern of sucker formation during embryonic and postembryonic
84 development has so far been poorly understood.

85 In this study, therefore, to get insights into the evolutionary process and
86 to understand the developmental mechanism of cephalopod suckers, the process

87 of sucker formation during development was investigated, focusing on 2 cuttlefish
88 species belonging to the order Sepiida, i.e., *Sepia esculenta* and *S*. *lycidas* (**Fig.**
89 **1C, D**). In Sepiida, which is thought to be an early-branched group among
90 Decapodiformes (although there are several hypotheses about this [23, 24]), the
91 sucker morphology is relatively simple without specialized structures such as
92 hooks [12] and is thought to show an ancestral state with some species diversity.
93 Sepiida cuttlefishes are relatively easy to rear after hatching because they are
94 benthic, while other groups of squids are pelagic and difficult to maintain in the
95 laboratory [25]. Furthermore, these 2 Sepiida species lay many large eggs that
96 are suitable for embryonic observations.

97 In the focal species, the embryonic stages can be identified based on
98 morphological features, according to a previous study in *Sepiella japonica*
99 [26] (**Fig. 1E**). In this study, the body axes of embryos and arms were defined
100 as shown in **Fig. 1F, G**. For postembryonic development, mantle length was
101 utilized as a criterion of growth because the growth speed could be variable
102 among individuals, so the time period elapsed during development is not suitable
103 as a growth index [27, 28]. Among the four pairs of arms and the pair of tentacles,
104 the second arms were focused on in this study, since they are the simplest arms,

while the others exhibit specialized morphologies like swimming arms or tentacles.

Since it was predicted that suckers were developed from undifferentiated tissues during development, observations on the external morphology and internal histological structures of cuttlefish arms during embryonic and postembryonic development were carried out to elucidate the pattern of sucker formation. Generally, in Coleoidea (octopuses, squids and cuttlefishes), small suckers are located at the distal tips of arms, indicating that the new suckers are formed at the distal tips. In this study, therefore, the distal arm tips were focused on.

Results

Increase of suckers throughout development in S. esculenta

Firstly, the number of suckers on the second arm were counted in embryos, juveniles and adults, to elucidate the increase pattern of suckers. No suckers were observed on arms in *S. esculenta* embryos of early developmental stages (St. 24-25), during which arms start to elongate. The number of suckers

(including primordial suckers) on the second arm of *S*. *esculenta* was counted in embryos just before hatching (St. 37-39: 47 suckers/arm) and sexually mature adults (181 suckers/arm), and the results showed that there were many more suckers in adults than in embryos (**Fig. 2**).

In addition, observation of the arm morphology at each developmental stage revealed that, at the arm tip, many small epithelial bulges (i.e., sucker primordia) were aligned next to the arm suckers on the oral side (**Fig. 3A-E**). No sucker primordia were observed until St. 24-25. The mean number of suckers (including sucker primordia) per second arm was 7 at St. 28-29, 24 at St. 31-33, 33 at St. 34-36, and 47 at St. 37-39, and the mean number of suckers of sexually mature adults was 181 (**Fig. 2B**)

Sucker formation during embryogenesis in S. esculenta

To investigate the patterns of sucker formation based on observations on the external morphologies and internal structures, the nucleus and cytoskeleton (F-actin) were stained and observed using a confocal laser scanning microscope (CLSM). The observation results showed that, in St-25 embryos at the early stage

of arm elongation, there were no structures of sucker primordia (**Fig. 3A**). At St. 26-27, epithelial tissues on the oral side of arm tips were observed to be swollen (**Fig. 3B**). At St. 28-29, on the oral side of the arm tip, the epithelial tissue formed a ridge along the proximal-distal axis (distal sucker ridge). On the more proximal area of the oral side, next to the distal sucker ridge, multiple dome-shaped bulges were located aligned in one or two rows in the proximal-distal direction, and the number of such bulges was increased in the more proximal part of the arm (**Fig. 3C**). At the middle to late embryonic stages (after St. 32), it was found that the distal sucker ridge was located at the tip of the oral side of the arm, and many small bulges (sucker primordia) were aligned on the proximal side of the distal sucker ridge (**Fig. 3D, E**).

From the distal to the proximal part of the arm, the number of suckers per row increased up to four. To examine the pattern of sucker-row formation in detail, observations of optical sections obtained from the basal part of each sucker primordium were carried out and revealed that two primordia were pinched off from one another, forming a gourd shape (**Fig. 3F, G, Additional file 1: Fig. S1**). Two pairs of gourd-shaped units were arranged alternately, forming 4 rows of suckers. Each unit of sucker primordium in the 4 rows was

157 clearly shown by the F-actin localization revealed by staining with phalloidin, and
158 consisted of 2 cell components: a single layer of epithelial cells and the inside
159 cells, which possessed relatively large nuclei (**Additional file 2: Fig. S2**).

160 At the stage just before hatching (St. 39), the gradual succession of stages
161 of the processes of sucker formation were observed along the distal-proximal
162 axis of the arm; immature sucker primordia were at the more distal part while
163 larger and well-developed suckers were at the more proximal part (**Fig. 3H**).
164 While dome-shaped sucker primordia were observed at the distal tip, sucker
165 primordia at more proximal positions showed constricted shapes, but without any
166 detailed structures (**Fig. 3H-J**). At the most proximal part of an arm, furthermore,
167 the structure of sucker primordia resembled the functional suckers in adults, with
168 an attachment surface, a cup-shaped structure, and a stalk connecting to the
169 arm, that corresponded, respectively, to an infundibulum, an acetabulum and a
170 peduncle (**Fig. 3K**). In the cup-shaped structure, a ring-shaped accumulation of
171 F-actin was observed along the cup edge (**Fig. 3E**).

172 The inner structures of arm tips were observed histologically, utilizing
173 paraffin sections. At St. 24-25, neither distal ridge nor dome-shaped bulges were
174 observed on the arms. However, the arrangement of epithelial cells was different

between the oral and aboral sides (**Fig. 3L**). On the aboral side, the epithelial cells were arranged in orderly alignment, while the arrangement of epithelial cells on the oral side appeared random and disordered (**Fig. 3L**). At St. 28-29, the epithelium at the tip of the oral side was single-layered and cells under the epithelium looked randomly arranged without any clear structures (**Fig. 3M**). The size of sucker primordia (i.e., dome-shaped bulges) gradually increased from distal to proximal parts. At St. 37-38, although the distal part with undifferentiated primordia was similar to those at the earlier stages (**Fig. 3N**), in the proximal part of the arm, the base of a sucker primordium was constricted, forming a cup-shaped structure, which was not observed at earlier stages. At St. 39, the distal arm tip was similar to that in the earlier stages; a distal ridge with a single epithelial layer, from which sucker primordia (i.e., dome-shaped bulges) were differentiated, and multiple sucker primordia were observed (**Fig. 3O-Q**). In the proximal part of the arm, the attachment surface and the cup-shaped structure (i.e., infundibulum and acetabulum) became more obvious, and nerve-like tissues were observed in the stalk of the cup structure, i.e., the peduncle (**Fig. 3O, R**).

Sucker formation during embryogenesis in S. lycidas

The sucker formation in *S*. *lycidas* was also observed to investigate whether the process seen in *S*. *esculenta* was shared among species. These observations showed that also in *S*. *lycidas*, a distal sucker ridge was formed on the oral side of the arm tip along the proximal-distal axis, and a cluster of multiple dome-shaped bulges (i.e., sucker primordia) was observed in the more proximal part, adjacent to the distal sucker ridge (**Fig. 4A-D**; St. 30-39). The number of primordial rows was only one at the distal arm tip, while it increased to two to four at more proximal positions. CLSM observations revealed that, as seen in *S*. *esculenta,* 2 bulges were present in one unit with a gourd shape, and 4-sucker rows were formed by zig-zag alignment of these gourd-shaped units (**Fig. 4E, F**). At St. 39 (**Fig. 4C, D**), the size of suckers was much larger in the proximal part than in the distal part of the arm, and an attachment surface (infundibulum), a cup-shaped structure (acetabulum) and a primordial stalk (peduncle) were clearly differentiated in the proximal part, whereas dome-shaped undifferentiated sucker primordia were still observed in the distal part (**Fig. 4G-I**).

Histological observations in *S*. *lycidas* also showed that, at the arm tip, the most distal ridge with a single epithelial layer was observed on the oral side,

211 from which dome-shaped sucker primordia were differentiated from the proximal
212 end (**Fig. 4J, K**; St. 33-39). At St. 39, in the distal part, dome-shaped sucker
213 primordia, in which the inner cell mass was undifferentiated, were similar to those
214 seen at the earlier stages (**Fig. 4L, M**). In the proximal parts, each sucker
215 primordium consisted of a cup-shaped structure and a stalk, which were
216 presumed to develop into the adult sucker components (infundibulum,
217 acetabulum and peduncle) (**Fig. 4N**).

218

219 *Sucker formation during postembryonic development in S. lycidas*

220 The external morphologies of arms and suckers in juveniles of *S. lycidas*,
221 observed by CLSM, showed that small dome-shaped bulges, i.e., sucker
222 primordia, were seen only in the distal part of the arm, and larger suckers in the
223 proximal arm part seemed to have functional structures like those seen in adult
224 cuttlefishes (**Fig. 5A-F**). Histological observations showed that the sucker size
225 was larger in the proximal part than that in the distal part. Most suckers, except
226 for sucker primordia in the distal tip, had distinct adult sucker structures, such
227 as infundibulum, acetabulum and peduncle (**Fig. 5G-I**). As in embryos, at the

most distal ridge a single epithelial layer was observed on the oral side of an arm tip, in which no clear tissue structures were seen (**Fig. 5G, H**).

Furthermore, SEM observations of the external morphology revealed that the epithelial tissues were expanded from the side of the arm's tip, covering the undifferentiated area at the distal end of the tip. This sheath-like structure was observed throughout postembryonic development (**Fig. 5J, Additional file 3: Fig. S3**). The distal ridge and dome-shaped sucker primordia were completely covered by the epithelial sheath. The epithelial sheath cover was also confirmed by observations on live specimens, to exclude the possibility that the epithelia had shrunk due to fixation for SEM observations (**Fig. 5K, Additional file 3: Fig. S3**).

Discussion

This study revealed morphogenetic processes of suckers on the second arm during embryonic and postembryonic development that are shared between 2 cuttlefish species, *S*. *esculenta* and *S*. *lycidas* (**Fig. 6**). As seen in other Coleoidea (octopuses, squids and cuttlefishes), the focal cuttlefishes possess numerous

245 small suckers that are located at the distal tips of arms. This clearly suggests
246 that the suckers are newly formed at the distal tips. The pattern of the increase
247 of the number of suckers in a second arm was firstly investigated in this study,
248 and the results indicated that that the suckers are newly added during embryonic
249 and postembryonic development (none at St. 25, about 50 suckers/arm at
250 hatching, about 200 suckers/arm in mature cuttlefish; **Fig. 2B**).

251 We found that during embryogenesis, at the oral side of the most distal
252 tip of the arm, a long narrow ridge (distal sucker ridge), followed by dome-shaped
253 bulges (sucker primordia) without clear inner structures, were observed, while at
254 the more proximal part, primordia that possessed differentiated sucker structures
255 were observed, as also seen in adult cuttlefishes. This strongly suggests that
256 undifferentiated suckers are newly formed in the distal part of an arm and they
257 develop to have functional sucker structures as they become located in a
258 relatively more proximal part, in association with the arm elongation. The distal
259 sucker ridge at the arm tip was constricted at its proximal side, adjacent to a
260 mass of dome-shaped bulges, suggesting that new sucker primordia were
261 produced and pinched off from the distal ridge. These small bulges (i.e., sucker
262 primordia) lined up in the distal part of the arms were also reported in *S*.

263 *officinalis* [18]. Therefore, it is suggested that, at least in the genus *Sepia*, the
264 sucker formation pattern in which suckers are formed at the most distal arm tip
265 during the arm elongation, is shared among species.

266 Adjacent to the proximal end of the distal sucker ridge, there were gourd-
267 shaped bulges that were visualized by actin localization (**Fig. 3F, G, 4E, F**). This
268 suggested that the gourd-shaped bulge is separated from the distal ridge, and
269 that it divides into two dome-shaped bulges that become two sucker primordia.
270 Pairs of sucker primordia derived from two gourd-shaped bulges were aligned in
271 a staggered array to form 4-sucker rows in the proximal part. This arrangement
272 of sucker row formation seems consistent with the fact that, in Coleoidea, the
273 number of suckers per row in adults is 2^n in many species [10].

274 The process of sucker formation during postembryonic development
275 showed a similar pattern to that observed during embryogenesis (**Fig. 5A-I**). In
276 the postembryonic development, however, sucker primordia on the arm tip were
277 covered by epithelial sheaths that extended from both sides of the arm tip (**Fig.**
278 **5J, K, Additional file 3: Fig. S3**), probably for protecting the undifferentiated,
279 vulnerable sucker primordia, since cuttlefish juveniles after hatching are exposed
280 to the external environment. This epithelial expansion was not seen during

281 embryonic development, and thus the expansion might start in response to some
282 stimuli from external environment.

283 In this study, the second arms of cuttlefishes were mainly studied.
284 However, since the number of suckers and/or the arrangement can be largely
285 different among arms and tentacles [12, 15], the differences of sucker
286 development among these body parts should also be compared to better
287 understand the fundamental process of sucker formation in future studies.

288 The sucker morphologies and the number of sucker rows are diversified
289 among cephalopod species [12, 29]. However, the ancestral state of suckers
290 cannot be inferred, since all the extant Coleoidea species (octopuses, squids and
291 cuttlefishes) possess well-developed suckers on their arms [30], while the extant
292 Nautilidea species (nautliuses) completely lack suckers [10], and there are no
293 intermediate cephalopod species. A few studies have been performed on the
294 sucker development in squids and cuttlefishes (e.g., [17]). The sucker formation
295 during embryogenesis in octopus (Octopodiformes) has also been studied, and in
296 octopus, remarkably fewer suckers are formed before hatching than in
297 cuttlefishes: 3 suckers in *Argonauta argo* and 8 suckers in *Eledone cirrosa*
298 develop in a row [31, 32]. Thus, the patterns of sucker formation in octopus seem

299 to be different from those in squids and cuttlefishes.

300 Furthermore, it could be assumed that the diversification of sucker
301 morphologies might contribute to the adaptive radiation of cephalopods, in which
302 benthic or pelagic lifestyles and diverse habitats have been acquired [33]. In that
303 sense, nautliuses (Nautilidea) attract special attention since they have many
304 arms without any suckers. In Nautilidea, the number of species is small and the
305 habitat is restricted to warm-water areas [34], supporting the idea that sucker
306 acquisition contributes to adaptive radiation. Thus, comparisons among species
307 across the cephalopod lineage will be required to understand the evolutionary
308 patterns of sucker formation and functions in Cephalopoda.

309

310 **Conclusions**

311 In this study, to elucidate the pattern of sucker formation, morphological
312 and histological observations were carried out during embryonic and
313 postembryonic development in *S*. *esculenta* and *S*. *lycidas*, focusing on the distal
314 arm tips, at which suckers were shown to be newly formed. The observations
315 showed that suckers are newly formed in the distal arm tips and functional sucker

316 structures are differentiated as they become located in a relatively proximal
317 region due to the arm elongation. Although in this study the sucker formation
318 pattern was revealed in *Sepia*, the sucker morphologies are diversified among
319 cephalopod species and the ancestral state of suckers cannot be inferred, since
320 there are no cephalopod species with intermediate sucker morphologies.
321 Therefore, comparisons among species across the cephalopod lineage will be
322 necessary to reveal the evolutionary patterns of sucker formation in Cephalopoda.

323

324 **Methods**

325 *Animals*

326 Sexually mature adults of *S. esculenta* and *S. lycidas* were captured in Sagami
327 bay by nets set off the coast of Okusu fishing port in Yokosuka city, Japan, in
328 April and May in 2019. Collected cuttlefishes were maintained in aquaria (1,240
329 × 760 ×550 mm, 3 individuals per aquarium) with circulating sea water at 14-
330 16°C for about a month (**Additional file 4: Fig. S4A**) and fed frozen Antarctic
331 krill. These aquaria were placed in a room under constant light condition even
332 during the night, to avoid sudden light on/off due to human activities, which may

cause stress to cuttlefishes. Cylindrical polyethylene nets were used as spawning beds, on which fertilized eggs were laid (**Additional file 4: Fig. S4A**). Fertilized eggs were collected and transferred into another aquarium, in which the water temperature was gradually raised to 21°C because higher temperature is known to promote cuttlefish embryogenesis [11].

After hatching, juveniles were maintained in a plastic container (715 × 410 × 200 mm) with an aeration apparatus and running sea water under constant light condition (**Additional file 4: Fig. S4B**). The sea water temperature rose up to 28°C. These juveniles were reared for 3 months at longest. Juvenile cuttlefishes were fed small mysids (Mysidae gen. sp.), shrimps (e.g., *Heptacarpus futilirostris, Palaemon serrifer and Lysmata vittata*) and crabs (e.g., *Gaetice depressus*), captured from coastal areas around Misaki Marine Biological Station. Embryos and juveniles were observed using a stereomicroscope (SZX16; Olympus, Tokyo, Japan), equipped with a digital camera (DP50; Olympus, Tokyo, Japan).

Fluorescent staining of nuclei and cytoskeletons

Fixation and fluorescent staining were performed according to previous studies (e.g., [35]). Briefly, embryos at each developmental stage and post-hatch juveniles with mantle length of 10-40 mm were fixed in 4% paraformaldehyde (PFA) in filtered sea water (FSW) for 1 to 3 hours after they were anesthetized with 7% $MgCl_2$. The fixed samples were then preserved in 0.3% Triton-X 100 in 1× phosphate-buffered saline (PBT) at 4°C until use. For post-hatch juveniles, the arm epithelium that covers suckers was removed under a stereoscopic microscope before the preparation, since it would be an obstacle for CLSM observations. The fixed samples were washed in PBT for 15 min at least three times before staining. The nuclei (DNA) and cytoskeletons (F-actin) were respectively stained with 4',6-diamidino-2-phenylindole (DAPI) (2 μg/ mL; Sigma, St Louis, MO, USA) and rhodamine-phalloidin (1:40; Invitrogen, Paisley, UK), for 1 hour at room temperature, and stained samples were then washed for 15 min in PBT at least three times. Stained samples (n = 4-5) were observed under a CLSM (FV3000; Olympus, Tokyo, Japan).

Histological analysis

To histologically observe the inner structures of suckers, paraffin sections were made according to the method described in previous studies (e.g., [36]). Embryos of *S*. *esculenta* and *S*. *lycidas* in each developmental stage and post-hatch juveniles of *S*. *lycidas* with mantle length of 10-40 mm were fixed in Bouin's solution (saturated aqueous picric acid solution/ formalin/ acetic acid = 15:5:1) or 4% PFA in FSW for longer than 8 hours after they were anesthetized with 7% $MgCl_2$. Then, fixed samples were preserved in 70% EtOH until use. Samples were dehydrated in increasing concentrations of ethanol, then transferred into xylene and finally embedded into paraffin. Serial sections (5-7 μm thick) of sagittal planes were prepared with a microtome (Spencer Lens Co., Buffalo, USA) and stained with hematoxylin and eosin. Tissues on slides were observed using an optical microscope (BX51; Olympus, Tokyo, Japan) and photographs were taken using a digital camera (DP74; Olympus, Tokyo, Japan) attached to the microscope.

Observations of external morphology

Scanning electron microscopy was carried out to investigate the external

morphology of the arm tip. Juveniles with mantle length of 20 mm, 30 mm or 40 mm were fixed in Bouin's solution for more than 8 hours after they were anesthetized with 7% $MgCl_2$, and then preserved in 70% EtOH until use. Then, the arms of fixed specimens were dehydrated in increasing concentrations of ethanol and transferred into hexamethyldisilazane for 1 hour, and then into *t*-butanol. After that, the fixed samples were freeze-dried using a Freeze Dryer ES-2030 (Hitachi Global, Tokyo, Japan), and coated with silver ions with an Ion Sputter E-1010 (Hitachi Global, Tokyo, Japan). Coated samples were observed with a JSM-5510LV scanning electron microscope (JEOL Ltd., Tokyo, Japan). Additionally, in order to exclude the possibility of shrinkage by the fixation process, the arms of live specimens were also observed with a stereomicroscope (SZX16; Olympus, Tokyo, Japan). Photographs were taken with a digital camera (DP50; Olympus, Tokyo, Japan) attached to the stereomicroscope.

Abbreviations

ac: acetabulum; in: infundibulum; p: peduncle; srt: sucker ring teeth; n: nerve; ML: mantle length.

Acknowledgements

We would like to express our gratitude to the staff of Yokohama Hakkeijima Sea Paradise, especially Sou Abe, for their assistance in collection of experimental animals. Thanks are also due to Michiyo Kawabata for helping us to rear the focal cuttlefishes.

Funding

This work was supported by a Grant-in-Aid for Scientific Research A (No. 18H04006) to TM from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RK and TM designed the study. RK, MN and KO carried out experiments and analyzed the data. HK was engaged in collection and maintenance of the focal animals. RK, MN, KO, HK and TM wrote the paper.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Although all experiments were carried out in Japan, where no ethics approval is required for the maintenance and handling of invertebrate species, rearing and fixation was performed with particular attention to replacement, reduction, and refinement of procedures in order to minimize animal suffering.

References

1. Erwin DH. Novelty and innovation in the history of life. Curr Biol. 2015;*25*(19):R930-R940.

2. Passamaneck Y, Halanych KM. Lophotrochozoan phylogeny assessed with LSU and SSU data: evidence of lophophorate polyphyly. Mol Phylogenet Evol. 2006;40(1):20-28.

3. Wollesen T, Monje SVR, Todt C, Degnan BM, Wanninger A. Ancestral role of Pax2/5/8 in molluscan brain and multimodal sensory system development. BMC Evol Biol. 2015;15(1):231.

4. Brusca RC, Moore W, Schuster M. Invertebrates. 3rd ed. Massachusetts: Sinauer Associates; 2016.

5. Kröger B, Vinther J, Fuchs D. Cephalopod origin and evolution: A congruent picture emerging from fossils, development and molecules. BioEssays. 2011;33(8):602–613.

6. Wollesen T, Loesel R, Wanninger A. Pygmy squids and giant brains: Mapping the complex cephalopod CNS by phalloidin staining of vibratome sections and whole-mount preparations. J Neurosci Methods. 2009;179(1):63–67.

7. Derby CD. Escape by inking and secreting: marine molluscs avoid predators through a rich array of chemicals and mechanisms. Biol Bull. 2007;213(3):274–289.

8. Hanlon RT, Messenger JB. Adaptive Coloration in Young Cuttlefish (*Sepia Officinalis* L.): The Morphology and Development of Body Patterns and Their Relation to Behaviour. Philos Trans R Soc B. 1988;320(1200):437–487.

9. Bather FA. (1888). Professor Blake and shell-growth in Cephalopoda. Ann Mag Nat Hist. 1888;1(6):421–427.

10. Naef A. Die Cephalopoden (Embryologie). Fauna Flora Golfo Napoli 1928; 35(2):1–357.

11. Boletzky S. From head to foot - and back again: Brachial crown development in the Coleoidea (Mollusca, Cephalopoda). Acta Univ Carol Geol. 2006;49(1–4):33–42.

464 12. Okutani T. Cuttlefishes and squids of the world. New ed. Hadano: Tokai
465 University Press; 2015.

466 13. Miserez A, Weaver JC, Pedersen PB, Schneeberk T, Hanlon RT, Kisailus D,
467 Birkedal H. (2009). Microstructural and biochemical characterization of the
468 nanoporous sucker rings from *Dosidicus gigas*. Adv Mater. 2009;21(4):401-
469 406.

470 14. Hou J, Wright E, Bonser RHC, Jeronimidis G. Development of Biomimetic
471 Squid-Inspired Suckers. J Bionic Eng. 2012;9:484–493.

472 15. Nixon M, Dilly PN. Sucker surfaces and prey capture. Symp Zool Soc Lond.
473 1977;38:447–511.

474 16. Nishiguchi MK, Mapes RH. Cephalopoda. In: Ponder WF, Lindberg DR,
475 editors. Phylogeny and evolution of the Mollusca. Berkeley: University of
476 California Press; 2008. p. 163-199.

477 17. Nödl M-T, Kerbl A, Walzl MG, Müller GB, Gert De Couet H. The cephalopod
478 arm crown: appendage formation and differentiation in the Hawaiian bobtail
479 squid *Euprymna scolopes*. Front Zool. 2016;13(1):44.

480 18. Tarazona OA, Lopez DH, Slota LA, Cohn MJ. Evolution of limb development
481 in cephalopod mollusks. eLife. 2019;8:e43828.

482 19. O'Dor RK, Balch N, Foy EA, Hirtle RWM, Johnston DA, Amaratunga T.
483 Embryonic Development of the Squid, *Illex illecebrosus*, and Effect of
484 Temperature on Development Rates. J Northwest Atl Fish Sci. 1982;3:41–
485 45.

486 20. Shigeno S, Tsuchiya K, Segawa S. Conserved topological patterns and
487 heterochronies in loliginid cephalopods: Comparative developmental
488 morphology of the oval squid *Sepioteuthis lessoniana*. Invertebr Reprod Dev.
489 2001;39(3):161–174.

490 21. Staaf DJ, Zeidberg LD, Gilly WF. Effects of temperature on embryonic
491 development of the Humboldt squid *Dosidicus gigas*. Mar Ecol Prog Ser.
492 2011;441:165–175.

493 22. Watanabe K, Sakurai Y, Segawa S, Okutani T. Development of the
494 ommastrephid squid *Todarodes pacificus*, from fertilized egg to
495 rhynchoteuthion paralarva. Am Malacol Bull. 1996;13(1/2):73–88.

496 23. Strugnell JM, Hall NE, Vecchione M, Fuchs D, Allcock AL. Whole
497 mitochondrial genome of the Ram's Horn Squid shines light on the
498 phylogenetic position of the monotypic order Spirulida (Haeckel, 1896). Mol
499 Phylogenet Evol. 2017;109:296-301.

500 24. Tanner AR, Fuchs D, Winkelmann IE, Gilbert MTP, Pankey MS, Ribeiro ÂM,
501 et al. Molecular clocks indicate turnover and diversification of modern
502 coleoid cephalopods during the Mesozoic Marine Revolution. Proc R Soc B.
503 2017;284(1850):20162818.

504 25. Iglesias J, Fuentes L, Villanueva R. Cephalopod culture. Berlin: Springer
505 Science & Business Media; 2014.

506 26. Yamamoto M. Normal Stages in the Development of the cuttlefish, *Sepiella*
507 *japonica* SASAKI. Zool Mag. 1982;91(2):146–157

508 27. Koueta N, Boucaud-Camou E. Food intake and growth in reared early
509 juvenile cuttlefish *Sepia officinalis* L. (Mollusca Cephalopoda). J Exp Mar
510 Biol Ecol. 1999;240(1): 93–109.

28. Martínez P, Moltschaniwskyj NA. Description of growth in the tropical cuttlefish *Sepia elliptica* using muscle tissue. J Mar Biol Assoc U K. 1999;79(2):317–321.

29. Roper CFE, Sweeney MJ, Nauen CE. FAO species catalogue, vol. 3, Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. FAO Fish Synop. 1984;3(125).

30. Young RE, Vecchione M. Analysis of morphology to determine primary sister-taxon relationships within coleoid cephalopods. Am Malacol Bull. 1996;12:91-112.

31. Mangold K, von Boletzky S, Frösch D. Reproductive biology and embryonic development of *Eledone cirrosa* (Cephalopoda: Octopoda). Mar Biol. 1971;8(2):109–117.

32. Fioroni P. Zur Epidermis-und Saugnapfentwicklung bei Octopoden, ein entwicklungsgeschichtlicher Vergleich. Rev Suisse Zool. 1982;89:355–374.

33. Young JZ. Brain, behaviour and evolution of cephalopods. In Symp Zool Soc Lond. 1977;38:377-434.

34. Hanlon RT, Messenger JB. Cephalopod behavior. Cambridge: Cambridge University Press; 1996.

35. Oguchi K, Shimoji H, Hayashi Y, Miura T. Reproductive organ development along the caste differentiation pathways in the dampwood termite *Hodotermopsis sjostedti*. Insect Soc 2016;63:519–529

36. Cornette R, Matsumoto T, Miura T. Histological analysis of fat body development and molting events during soldier differentiation in the damp-wood termite, *Hodotermopsis sjostedti* (Isoptera, Termopsidae). Zool Sci. 2007;24:1066–1074.

Figure legends

Fig. 1.

The general morphology, embryonic development, and the definition of axes in cuttlefishes. (A) A schematic image of the cuttlefish body plan. (B) The general structure of the sucker of Decapodiformes. (C) The golden cuttlefish *Sepia*

esculenta. (D) The kisslip cuttlefish *S*. *lycidas*. (E) A schematic image of developmental stages during embryogenesis, based on Yamamoto (1982). (F) The definition of the body axis of an embryo used in this study. (G) The definition of the axis of an arm. Abbreviations: ac, acetabulum; in, infundibulum; p, peduncle; srt, sucker ring teeth.

Fig. 2.

The number increase of suckers (or sucker primordia) during the growth in *S*. *esculenta*. (A) Schematic images of cuttlefish development, together with the corresponding sucker number in *S*. *esculenta*. (B) The transition of sucker number during embryonic and postembryonic development. Number under each column indicates the sample size. Error bars indicate standard deviation.

Fig. 3.

The process of sucker formation on the second arm of *S. esculenta* during embryonic development. Arms are oriented with distal to the right. (A-E) Confocal stacks of arms from oral view. Cyan: DAPI; red: phalloidin. Arrows: a long narrow

561 ridge (distal sucker ridge) on the arm tip. Arrowheads: the proximal constriction
562 of the distal sucker ridge. (A) St. 24-25. (B) St. 26-27. (C) St. 28-29. (D) St. 33-
563 36. (E) St. 39. (F, G) Optical sections of the base of the bulges at St. 39, stained
564 with phalloidin (F), or DAPI/phalloidin (G). White dotted frames indicate the
565 gourd-shaped actin localization. (H) A sagittal optical section at St. 39. (I-K)
566 Higher magnification of white boxed regions in H. White dotted frames indicate
567 the constricted sucker primordium (J) and the sucker primordium with
568 differentiated structures (K). (L-R) Histological sections in sagittal planes stained
569 with hematoxylin and eosin. Arms are oriented with oral to the bottom. (L) St.
570 24-25. (J) St. 28-29. (N) St. 37-38. (O) St. 39. (P-R) Higher magnification of the
571 bulges at St. 39; distal (P), middle (Q) and proximal (R) parts. Brackets indicate
572 the region where epithelial cells composed one layer. Scale bars: 50 μm.
573 Abbreviations: ac, acetabulum; in, infundibulum; n: nerve; p, peduncle.

574

575 **Fig. 4.**

576 The process of sucker formation of the second arm in *S. lycidas* during embryonic
577 development. Arms are oriented with distal to the right. (A-C) Confocal stacks of

arms from oral view. Arrows indicate distal sucker ridges on the arm tips. Arrowheads indicate the proximal constriction of the distal sucker ridges. (A) St. 30-32. (B) St. 33-36. (C) St. 39. (D) A sagittal optical section at St. 39. (E, F) Optical sections of the base of sucker primordia (dome-shaped bulges) at St. 33-36. White dotted frames indicate the gourd-shaped actin localization. (G-I) the magnification of sagittal section; distal (G), middle (H), and proximal (I) parts. White dotted frames indicate the constricted sucker primordium (H) and the sucker primordium with differentiated structures (I). (J-N) Histological sections in sagittal planes, stained with hematoxylin and eosin at St. 33-36 (J) and St. 39 (K). (L-N) Magnified images focusing on the primordial area at St. 39; distal (L), middle (M), and proximal (N) parts. Brackets indicate the regions where epithelial tissues are composed of one cell layer. Arrowheads indicate proximal constriction of distal sucker ridges. Scale bars: 50 μm. Abbreviations: ac, acetabulum; in, infundibulum; p, peduncle.

Fig. 5.

The postembryonic processes of sucker formation of the second arm in *S. lycidas*.

595 Arms are oriented with distal to the right. (A-F) Confocal stacks of arms from oral
596 view. (A) Second arm of an individual with ML (mantle length) 10 mm. (B) Higher
597 magnification of the white boxed region in A. (C) an individual of ML 30 mm. (D-
598 F) Higher magnification of the white boxed regions in C. (G-I) Histological
599 sections in sagittal planes of an individual with ML 10 mm, stained with
600 hematoxylin and eosin; distal (G), middle (H) and proximal (I) parts. (J, K) The
601 epithelium covering the arm tip in an individual with ML 30 mm; SEM observation
602 (J) and live specimen (K). Scale bars indicate 500 μm (A, C), 200 μm (D-F, J, K),
603 and 50 μm (B, G-I). Abbreviations: ac, acetabulum; in, infundibulum; p, peduncle.

604

605 **Fig. 6.**

606 A schematic diagram of sucker formation during embryonic and postembryonic
607 development. The second arm in *Sepia* is focused on. Abbreviations: ac,
608 acetabulum; in, infundibulum; p, peduncle.

609

610 **Additional files**

611 **Additional file 1:**

Figure S1. Optical sections (horizontal) at the base of sucker primordia in *S*. *esculenta* and *S*. *lycidas*. Arms are oriented with distal to right. (A-D) *S*. *esculenta* at St. 33-36 (A, B) and at St. 38-39 (C, D). (E-H) *S*. *lycidas* at St. 33-36 (E, F) and St. 38-39 (G, H). White frames indicate the gourd-shaped actin localization. Scale bars indicate 50 μm. (PDF)

Additional file 2:

Figure S2. Optical sections of the second arm in *S. esculenta* (St. 33-36) in the horizontal planes. Sections are obtained from the planes in which each primordial section area is largest. Scale bars indicate 50 μm. (PDF)

Additional file 3:

Figure S3. Epithelia covering the arm tip in *S*. *lycidas*. Arms are oriented with distal to the right. (A, C) SEM images. (B, D) Live specimens. Individuals with mantle length of 20 mm (A, B) and 40 mm (C, D) were used. Scale bars indicate 100 μm. (PDF)

Additional file 4:

Figure S4. The rearing system of cuttlefishes. A: adults. Arrow indicates a spawning bed. B: juveniles after hatching. (PDF)