Patterns of sucker development in cuttlefishes

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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,

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

Especially in Decapodiformes, the sucker morphology is diversified among species [12]. Basically, an adult sucker is composed of an attachment, face (infundibulum), a chamber for producing suction (acetabulum), and a peduncle by which the acetabulum is attached to the arm surface, and there is a proteinaceous ring with teeth on the rim of the infundibulum (sucker ring teeth) (**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

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

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

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

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

115

116 **Results**

117 *Increase of suckers throughout development in S. esculenta*

118 Firstly, the number of suckers on the second arm were counted in embryos,
119 juveniles and adults, to elucidate the increase pattern of suckers. No suckers
120 were observed on arms in *S. esculenta* embryos of early developmental stages
121 (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

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

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

The inner structures of arm tips were observed histologically, utilizing paraffin sections. At St. 24-25, neither distal ridge nor dome-shaped bulges were 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**).

193 *Sucker formation during embryogenesis in S. lycidas*

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

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

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

Sucker formation during postembryonic development in S. lycidas

The external morphologies of arms and suckers in juveniles of *S. lycidas*, observed by CLSM, showed that small dome-shaped bulges, i.e., sucker primordia, were seen only in the distal part of the arm, and larger suckers in the proximal arm part seemed to have functional structures like those seen in adult cuttlefishes (**Fig. 5A-F**). Histological observations showed that the sucker size was larger in the proximal part than that in the distal part. Most suckers, except for sucker primordia in the distal tip, had distinct adult sucker structures, such 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 (nautiliuses) 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, nautiliuses (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

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

Methods

Animals

Sexually mature adults of *S. esculenta* and *S. lycidas* were captured in Sagami bay by nets set off the coast of Okusu fishing port in Yokosuka city, Japan, in April and May in 2019. Collected cuttlefishes were maintained in aquaria (1,240 × 760 × 550 mm, 3 individuals per aquarium) with circulating sea water at 14–16°C for about a month (**Additional file 4: Fig. S4A**) and fed frozen Antarctic krill. These aquaria were placed in a room under constant light condition even 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% MgCl₂. 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% MgCl₂. 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% MgCl₂, 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.

401

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412

413 **Availability of data and materials**

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

416

417 **Authors' contributions**

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

421

422 **Competing interests**

423 The authors declare that they have no competing interests.

424

425 **Consent for publication**

426 Not applicable.

427

428 **Ethics approval and consent to participate**

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

433

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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*

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

549

550 **Fig. 2.**

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

556

557 **Fig. 3.**

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

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

Fig. 4.

The process of sucker formation of the second arm in *S. lycidas* during embryonic 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 μ m. Abbreviations: ac, acetabulum; in, infundibulum; p, peduncle.

Fig. 5.

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

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

Fig. 6.

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

Additional files

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 μ m. (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 μ m. (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 μ m. (PDF)

629 **Additional file 4:**

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