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PERSPECTIVE

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Embryonic development of the camouflaging dwarf cuttlefish, *Sepia bandensis*

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Abstract

Background: The dwarf cuttlefish *Sepia bandensis*, a camouflaging cephalopod from the Indo-Pacific, is a promising new model organism for neuroscience, developmental biology, and evolutionary studies. Cuttlefish dynamically camouflage to their surroundings by altering the color, pattern, and texture of their skin. The skin's "pixels" (chromatophores) are controlled by motor neurons projecting from the brain. Thus, camouflage is a visible representation of neural activity. In addition to camouflage, the dwarf cuttlefish uses dynamic skin patterns for social communication. Despite more than 500 million years of evolutionary separation, cuttlefish and vertebrates converged to form limbs, camera-type eyes and a closed circulatory system. Moreover, cuttlefish have a striking ability to regenerate their limbs. Interrogation of these unique biological features will benefit from the development of a new set of tools. Dwarf cuttlefish reach sexual maturity in 4 months, they lay dozens of eggs over their 9-month lifespan, and the embryos develop to hatching in 1 month.

Results: Here, we describe methods to culture dwarf cuttlefish embryos in vitro and define 25 stages of cuttlefish development.

Conclusion: This staging series serves as a foundation for future technologies that can be used to address a myriad of developmental, neurobiological, and evolutionary questions.

KEYWORDS

behavior, camouflage, cephalopod, cuttlefish, embryogenesis, gene editing, neuroscience, new model organism, staging series

1 | INTRODUCTION

The cephalopods are a group of marine mollusks that have fascinated scientists and philosophers since the time of Aristotle.[1] Although ancient cephalopods had an external shell, the coleoid (soft-bodied) cephalopods (cuttlefish, octopus, and squid) internalized or lost this shell over evolutionary time, exchanging physical protection for a faster and more diverse existence.[2] The coleoid cephalopods evolved neural control of skin patterns, the largest brains of the invertebrates,[3] and flexible motor control, enabling innovative strategies to avoid predation and exploit new ecologic niches.

Within the coleoid cephalopods, cuttlefish are characterized by the presence of eight arms (one of which is sexually dimorphic), two feeding tentacles and a cuttlebone, a calcium carbonate structure used for buoyancy (Figure 1C). Cuttlefish have a wide ecologic range, with species distributed across Asia, Europe, Africa and Australasia.[4] The European (or common) cuttlefish, *Sepia officinalis*, has been studied to elucidate its dynamic camouflage behavior.[5] However, this species is not

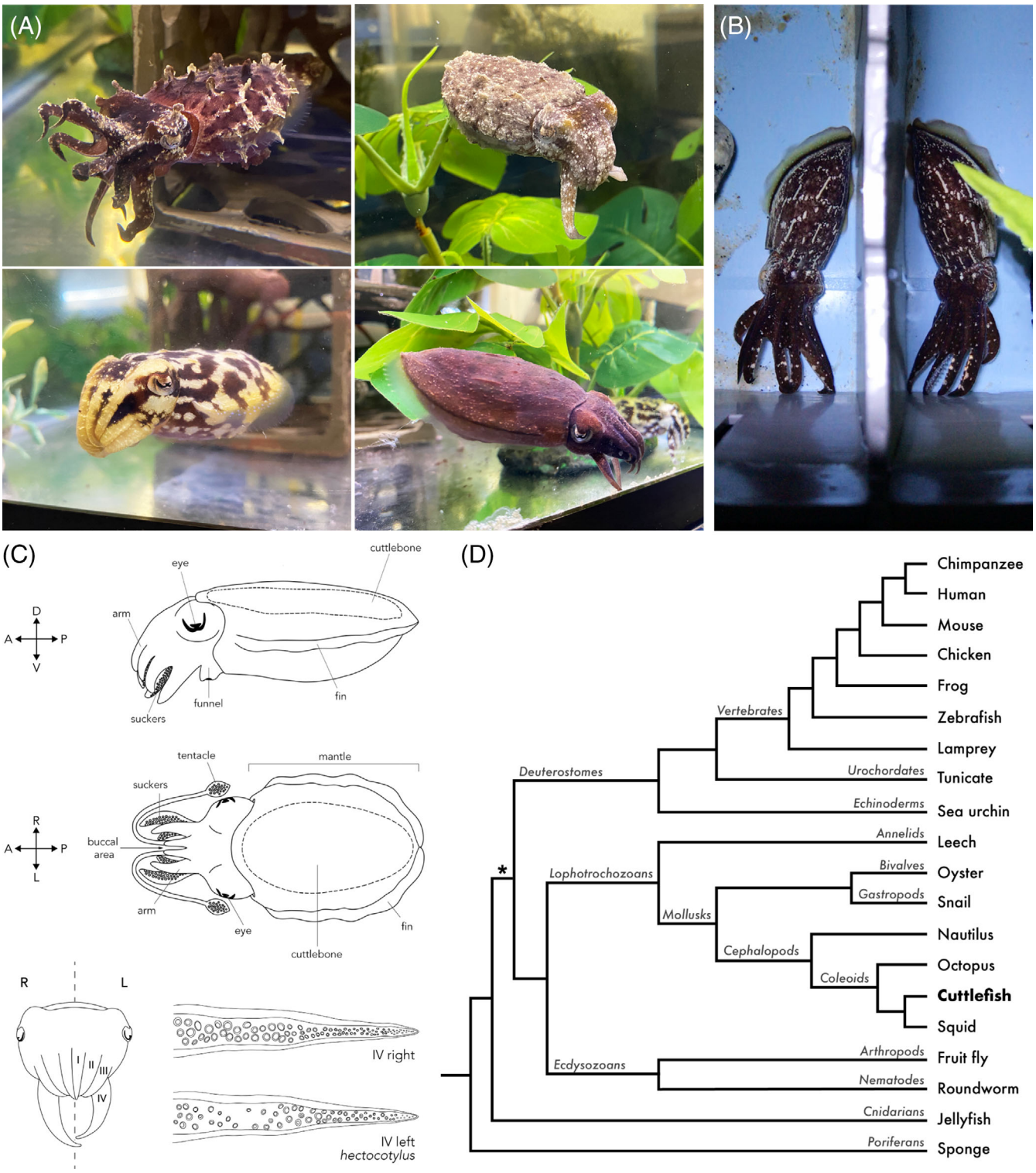

FIGURE 1 Dwarf cuttlefish create complex skin patterns for camouflage and social communication. A, Dwarf cuttlefish can generate a large array of body patterns using variations in color, the spatial frequency of patterns and three-dimensional texture. Cuttlefish skin displays can be associated with camouflage or social behaviors. B, Male dwarf cuttlefish produce a stereotyped aggression pattern when fighting other males or pursuing females. C, The major anatomical features of the dwarf cuttlefish. The feeding tentacles are normally hidden in the head, and are ejected to catch prey. Male dwarf cuttlefish possess a specialized arm with fewer suckers, the hectocotylus, which is used to place sperm in the female's buccal area during mating. It is the fourth left arm. D, Phylogenetic tree of animals. Cuttlefish are coleoid cephalopods and are members of the mollusk phylum. The last common ancestor of cephalopods and vertebrates lived over 500 million years ago (*). A anterior; D, dorsal; L, left; P, posterior; R, right; V, ventral

optimal for genetic and developmental studies: as a consequence of its cold-water habitat, embryos take 2 to 3 months to develop to hatching, and adults take 9 or more months to reach sexual maturity.[6] Dwarf cuttlefish, *Sepia bandensis*, are native to the Indo-Pacific, where tropical water temperatures enable embryos to hatch after 1 month of development, and animals can reach sexual maturity in 4 months. The species is small, allowing animals to be kept at high density in breeding tanks. Moreover, females can lay dozens of eggs during their lifetime (Table 1). Dwarf cuttlefish exhibit a broad and interesting behavioral repertoire, including skin camouflage, social communication by skin displays, and dynamic waves on their skin of unknown function (Figure 1A,B and Video S1).[7]

The establishment of the dwarf cuttlefish as a cephalopod model system to interrogate these interesting behaviors will require embryo culturing protocols and genetic tools. In this perspective, we review our current knowledge of cuttlefish development and neurobiology, and the genomic and neurobiological tools that will be needed to allow investigation of this organism. We then present methods to culture dwarf cuttlefish embryos in vitro and define 25 stages of development. This staging series serves as a foundation for future studies of development, and the generation of new genetic approaches to manipulate the genome.

2 | CEPHALOPOD DEVELOPMENT

Organisms that have served as developmental models are largely members of the ecdysozoans (eg, *Drosophila* and *C. elegans*) or the deuterostomes (eg, mouse and zebrafish) (Figure 1D). The third major group of animals, the lophotrochozoans, has been under-represented in developmental biology despite the fact that it encompasses nearly half of all animal phyla. Lophotrochozoan development is often characterized by holoblastic spiral cleavage and a larval embryonic stage. Although cephalopods are members of this group, they differ from many lophotrochozoans, undergoing meroblastic cleavage and direct development (no larval stage). The stages of embryogenesis have been described for a number of cephalopod species, including *Sepia officinalis*,[8,9] *Sepia pharaonis*,[10] *Euprymna scolopes*,[11] *Doryteuthis pealeii*,[12] *Todarodes pacificus*,[13] and *Octopus vulgaris*.[14] However, relatively little is known about the cellular and molecular basis of cephalopod embryogenesis.

Within the lophotrochozoans, cephalopods are members of the mollusk phylum (Figure 1D). Many mollusk body plans feature a shell, ventral foot and mantle.[15] During the course of evolution, the coleoid cephalopods lost or internalized their external shells and may have converted a ventral foot into prehensile limbs and a funnel.[16] Was the formation of new structures achieved through recruitment of existing gene networks? Gene expression analysis in the Hawaiian bobtail squid, *Euprymna scolopes*, has revealed the recruitment of the *Hox* gene family during limb patterning—a departure from its highly conserved role in bilaterian axial patterning. This suggests that lophotrochozoans have recruited ancient signaling pathways for conserved[17] as well as novel patterning tasks.[15]

In addition to their unusual embryogenesis and body plan, the coleoid cephalopods evolved a number of anatomical novelties that are similar to vertebrates, including a closed circulatory system and camera-type eyes. Was this dramatic convergence in anatomy governed by shared patterning mechanisms? In *Sepia officinalis* and *Sepia bandensis*, the three axes of limb patterning are controlled by the same signaling pathways used in arthropods and vertebrates.[18] Moreover, in the camera-type eye of the squid *Doryteuthis pealeii*, Notch signaling is important for photoreceptor differentiation, suggesting shared components of cephalopod and vertebrate eye development.[19] Genetic analysis in these organisms may expand our knowledge of the gene regulatory networks controlling the development of these tissues.

One of the most striking features of cuttlefish is their neurally-controlled skin camouflage, which they are capable of using as soon as they hatch from the egg. Cuttlefish skin is covered in thousands of pigment-filled saccules called chromatophores that are controlled by motor neurons projecting from the brain. When a motor neuron activates a chromatophore, the saccule is expanded by its surrounding muscles, creating a yellow, orange or brown pixel. Long-term tracking of *Sepia officinalis* chromatophores has revealed a dynamic developmental process in which chromatophores are born yellow, but after a few days transition to orange, and finally dark brown.[20] The asynchronous and continual emergence of new chromatophores throughout the animal's life ensures the presence of chromatophores of every color. This presents an interesting conundrum: the brain must dictate the activation of an appropriate pattern of chromatophores in order to camouflage effectively, but the chromatophores themselves are changing color over time. It is possible that the brain updates the identity of the motor neurons that innervate each chromatophore with each color transition. Alternatively, each chromatophore may be innervated by different motor neurons when it transitions to a different color.[20] How the brain retains knowledge of the changing color of a chromatophore is an interesting problem for further study.

TABLE 1 Practical considerations for raising dwarf cuttlefish in the lab

Dwarf cuttlefish feature	Suitability as a model organism
Small size	Dwarf cuttlefish reach a maximum size of ~10 cm so a full breeding colony can be kept in a relatively small facility.
Animals tolerate group housing	Multiple adult cuttlefish (or younger animals) can be co-housed, provided the tank is large enough (max. 5 adults per 10 gal), and there are sufficient hiding places (see Methods). By contrast, octopus species are often asocial and cannibalistic.
Animals reproduce in the lab	Provided dwarf cuttlefish are fed well and the water chemistry is properly maintained, males and females will spontaneously mate in the lab.
Dwarf cuttlefish can be raised in artificial saltwater or in natural seawater	Dwarf cuttlefish can be raised in artificial saltwater or in natural flowing seawater. Artificial saltwater is more expensive but provides consistent levels of essential compounds and trace elements. Natural seawater, by contrast, can vary in composition by location and season.
Sexual maturity at ~4 months	Male and female cuttlefish begin showing sexually dimorphic body patterns at 2 to 3 months. After ~4 months, animals begin mating and females lay fertilized eggs. Thus, dwarf cuttlefish take only ~33% longer to reach sexual maturity than zebrafish, a popular vertebrate model organism.
Multi-month reproductive period	The females of many cephalopod species undergo a single period of reproduction and then undergo accelerated aging and death. Female dwarf cuttlefish, by contrast, are reproductive for 1 to 2 months. During this period each female lays ~5 to 7 clutches of ~5 to 30 fertilized eggs, totaling <200 eggs.
Embryos develop without maternal care	Whereas some octopus species carry out dedicated maternal care for new embryos, female dwarf cuttlefish tie their embryos to coral and then leave them to develop. Thus, embryos can be removed from breeding tanks and placed in nursery tanks to hatch, without the need for elaborate care.
First cell division takes 8 hours	The first cell division of dwarf cuttlefish embryos takes ~8 hours, which provides a relatively large window in which to collect embryos and inject them. This compensates for the unpredictability of when female cuttlefish choose to lay eggs.
Commercially available	Dwarf cuttlefish embryos are available from commercial suppliers, and can be sent overnight to many locations. Thus, aquarium population sizes can be grown and maintained relatively easily.
Inbreeding is possible	Dwarf cuttlefish animals have been inbred for 4 generations (Connor Gibbons, personal communication), although the generational limit and long-terms effects of inbreeding have not yet been determined.
Rearing requires great expertise and time	Cuttlefish are exceptionally difficult animals to raise because they are voracious feeders and also extremely sensitive to water quality. They must be fed live shrimp at least three times per day, but this results in a large build-up of waste matter, which produces high levels of ammonia. Thus, systems must have very effective mechanical, chemical and biological filtration, which can take months to establish before the system is ready for animals. As a general rule, a 500-gal system with <200 cuttlefish of different life stages requires 2–5 hours of maintenance per day, 7 days a week.
Cuttlefish care is expensive	Juvenile and adult cuttlefish require multiple meals of live grass shrimp every day, while babies require live mysid shrimp for their nutrition (see Methods). Unless a lab is situated within driving distance of natural grass and mysid shrimp populations, the food source must be sent overnight by fishing companies, which can cost many thousands of dollars per year. For instance, to feed a facility of ~100 animals (from hatchlings to adults) a shipment of mysid and grass shrimp costs ~$1000/week (of which ~1/3 of the price is the cost of overnight shipping). The development of efficient mysid and grass shrimp culturing methods would greatly increase the practicability of raising cuttlefish species in inland labs. Dwarf cuttlefish can be trained to eat frozen food, therefore a cuttlefish facility could be maintained at much lower costs using a frozen diet. However, this feeding method is only suitable for a relatively small population—training animals, hand-feeding them, and removing waste can be very time consuming.

3 | CUTTLEFISH CAMOUFLAGE

Cuttlefish are masters of camouflage. They can change the color and pattern of their skin using chromatophores, they can reflect light using iridophores[21] and they can alter the texture of their skin using sub-cutaneous muscles (papillae) that are under neuronal control.[22] Thus, different combinations and colors of chromatophores, iridophores and papillae can create elaborate body patterns and textures that resemble features of the animal's natural environment.[23]

Cuttlefish present an interesting neurobiological system to ask how the physical properties of the visual world are represented by patterns of neural activity in the brain, and how this representation is transformed into an approximation of the visual world on the skin. Behavioral studies have provided initial insight into a cuttlefish's ability to resemble the environment. The body patterns generated by *Sepia officinalis* have been divided into three major classes: uniform, mottled and "disruptive" (a pattern comprising large patches of pigment).[24] Cuttlefish often generate body patterns that approximate the spatial frequency of the visual stimulus,[25] suggesting that the cuttlefish brain may extract the elementary features of visual scenes at different spatial frequencies, a property of primate visual processing.[26] Cuttlefish are not able to produce all skin patterns; each species has evolved a distinct repertoire of body patterns, thought to be generated by the summation of smaller pattern units.[27] Recently, powerful data processing and analytical methods were employed to track the expansion state of tens of thousands of chromatophores in the skin of a *Sepia officinalis* juvenile over multiple weeks. The application of statistical methods to the data defined skin pattern units and their underlying hierarchical control. This study provided important insight into the organization of motor unit control of cuttlefish color change.[20] The application of these sophisticated methods to a camouflaging animal should reveal a relationship between the visual input and patterned output on the skin.

The cuttlefish's skin pattern is a visible representation of neural activity. Understanding how the representation of the visual world is transformed in the cuttlefish brain will require the recording and manipulation of neural activity during camouflage. The development of electrophysiological methods or two-photon calcium imaging will allow an analysis of patterns of neural activity, but optical imaging awaits the development of transgenic animals. The development of genetic tools in the dwarf cuttlefish is possible due to its short generation time and prolific breeding. Transgenic tools that permit neural recordings and manipulations would also permit the study of stereotyped skin patterns that accompany social communication. Many species of cuttlefish, for example, display a dark striped skin pattern during aggressive interactions (Figure 1B). Others, including the dwarf cuttlefish, create dynamic pigmentation waves across their skin[7] that may be reflective of internal state (Video S1). Neural recordings coupled with behavioral analysis may permit an association between internal state and its representation in the brain and on the skin.

4 | CUTTLEFISH COGNITION

Cephalopods are cognitively advanced invertebrates. Experiments have suggested that cuttlefish are capable of multiple flexible behaviors that may be associated with complex cognitive mechanisms. Cuttlefish, for example, have demonstrated a quantitative ability: when presented with different quantities of shrimp, juvenile *Sepia pharaonis* cuttlefish consistently selected the larger quantity. If the animals were satiated, they selected the smaller of two meals, indicating that they can flexibly respond to foraging options dependent on their internal state.[28]

Behavioral experiments have also suggested that cuttlefish use episodic-like memory, a recollection of what, where and when an event occurred, a process typically associated with the more complex cognition of mammals and birds. *Sepia officinalis* trained to associate different locations ("where") with different prey items ("what") at different times ("when") successfully retrieved the correct prey item, demonstrating behavioral features of episodic-like memory.[29] Although the neural underpinnings of this form of memory are not known, lesion experiments in cuttlefish have demonstrated that the vertical lobe, a large region on the dorsal side of the cuttlefish brain, is required for memory.[30] Furthermore, slice physiology experiments have demonstrated long-term potentiation in the vertical lobe, a cellular mechanism of synaptic plasticity.[31]

Cuttlefish have evolved complex reproductive strategies to permit successful competitive mating. Australian giant cuttlefish, *Sepia apama*, use flexible strategies during mating, a behavior normally seen in cognitively advanced vertebrates.[32] Although large male cuttlefish are consistently more successful in male-male contests,[33] smaller males have been observed employing deceptive strategies, in which they impersonate female cuttlefish by expressing female body patterns and hiding their sexually dimorphic arms.[34] Male mourning cuttlefish (*Sepia plangon*) have been observed extending this behavior to include the simultaneous presentation of two body patterns: a male pattern facing a female, and a female pattern facing a male. This strategy was not observed when more than one male or female were present,[35] indicating the use of complex tactical deception.

How cuttlefish store and recall memories, employ navigation in learning, and use decision-making to select appropriate foraging and mating strategies remain unknown. Uncovering the neural mechanisms governing these behaviors may reveal novel mechanisms for cognitive functions.

5 | CEPHALOPOD AGING

Most coleoid cephalopod species live for only a few months to about 3 years. Most die shortly after mating or egg-laying, following an accelerated period of senescence.[36] In many species, animals perform no maternal or paternal care, yet rapidly develop hallmarks of aging after mating, including the loss of eyesight, reduction in long-term memory, and degeneration of the nervous system.[37] Although dwarf cuttlefish lay multiple clutches of eggs during adulthood, they also develop hallmarks of aging during their last 2 months of life. What triggers this accelerated aging and does this process share any similarities with aging in the vertebrate brain?

6 | CEPHALOPOD REGENERATION

Cephalopods are also capable of impressive feats of regeneration. Complete regeneration of the limb is achieved in a few weeks or months, depending on the species, location of the damage, water temperature, and age of the organism.[38,39] Interestingly, late in limb regeneration, chromatophores form on the skin,[39] raising questions about how they are innervated to permit appropriate color change. In *Octopus vulgaris*, if the motor neuron fibers that project from the brain to the chromatophores (pallial nerve) are crushed or cut, control of chromatophores is lost, resulting in uniform white skin. However, over the following weeks, the chromatophores regain their ability to expand, and camouflage is restored.[40] This implies that the nerve fibers form the correct connections to the chromatophores.[40] This task of appropriate axon targeting could be facilitated by protocadherins, a family of genes that is unusually large in cephalopods.[41]

7 | GENOMIC AND GENOME EDITING TOOLS

Recent advances in gene editing and nucleic acid sequencing technologies have created opportunities to establish more unusual creatures, notably the dwarf cuttlefish, as model organisms. RNA-Seq experiments can offer the first glimpse into the molecular mechanisms of cellular and organismal change over the course of development, regeneration or aging. Furthermore, single-cell RNA-Seq studies can uncover the cellular subtypes in the brain, or reveal the cellular changes underlying disease. The investigation of such transcriptomes requires the assembly of high-quality genomes. Long-read DNA sequencing technologies now permit the generation of chromosome-level, whole-genome assemblies in organisms for which there is no existing sequence. The ability to inbreed the dwarf cuttlefish, diminishing heterozygosity, has allowed us to initiate whole-genome sequencing (in collaboration with Benedict Paten and Paolo Carnevali). This sequence will join a growing list of cephalopod genomes, including *Octopus bimaculoides*,[41] *Octopus minor*,[42] *Euprymna scolopes*,[43] and *Architeuthis dux*,[44] which have provided insights into the evolutionary history of this unique group of mollusks.

Transgenesis methods have been successful in unusual model organisms, including sea anemones,[45] killifish,[46] hydra,[47] and in another member of the mollusk phylum, the slipper snail.[48] This raises the possibility of similar targeting success in cephalopods. CRISPR/Cas9 editing technologies now permit the manipulation of specific genes and the introduction of new genes into a large variety of organisms across evolution.[49] CRISPR/Cas9 mutagenesis was recently demonstrated in embryos of *Doryteuthis pealeii*, a wild-caught pelagic squid found seasonally in the American Northeast.[50] This species was not bred beyond the first generation, but we can anticipate future transmissible genetic modifications in other cephalopod models.

We have described several interesting features of cuttlefish biology that may serve as a model for studies of development, camouflage, cognition, regeneration and aging. The breadth of interesting biological phenomena, including striking cognitive and behavioral abilities, emphasize the need to develop cuttlefish tools to uncover molecular, cellular and circuit-level mechanisms. The development of genetic manipulation methods in the dwarf cuttlefish will require the ability to grow the embryos in vitro and obtain knowledge of dwarf cuttlefish development. We have determined methods to culture dwarf cuttlefish embryos in vitro under conditions suitable for microinjection, and here define 25 stages of development.

8 | RESULTS

8.1 | Dwarf cuttlefish embryo culturing

During mating, male and female cuttlefish embrace in a head-to-head posture (Figure 2A). During the embrace, the male retrieves a bundle of spermatophores from his

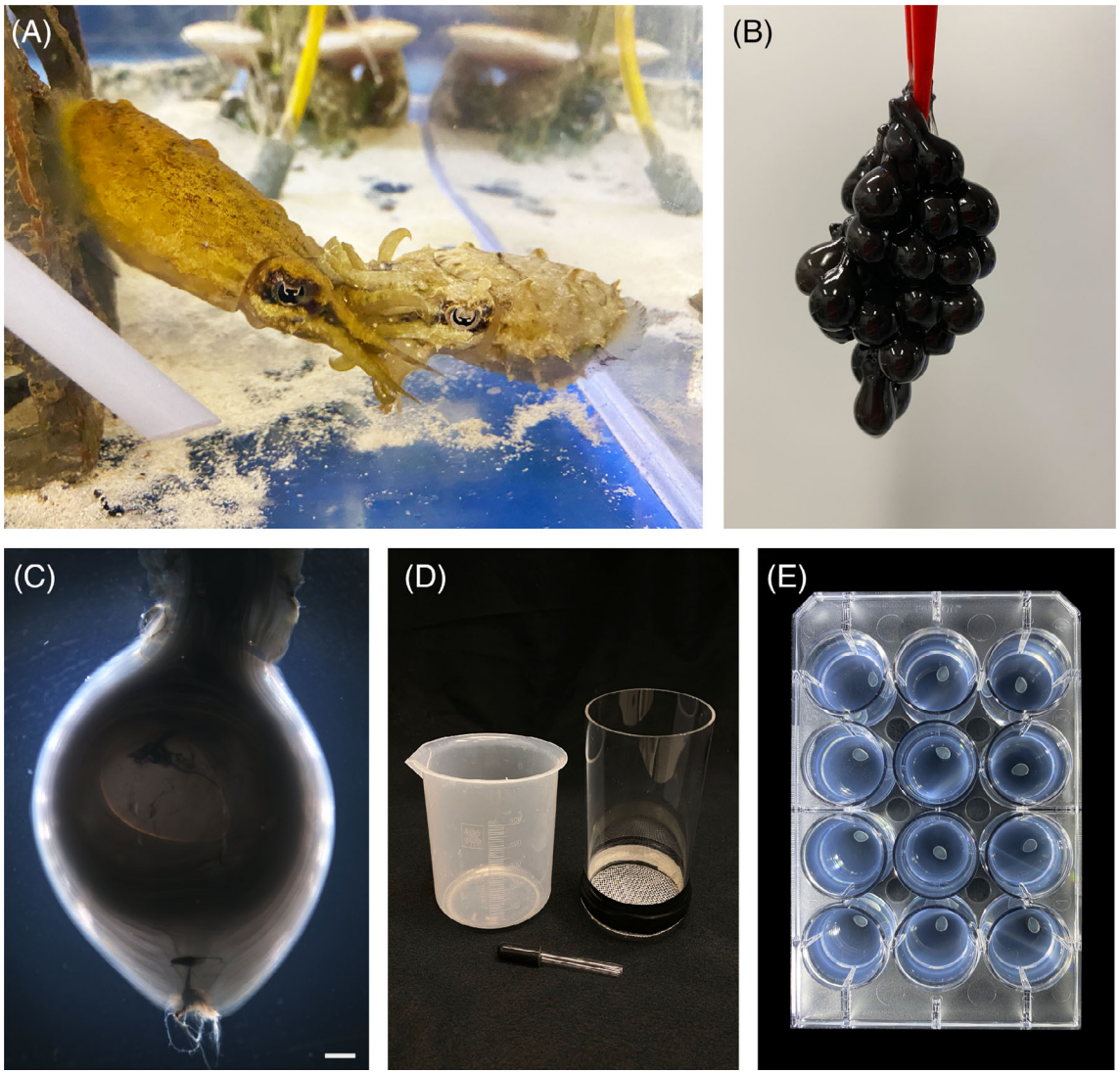

FIGURE 2 Dwarf cuttlefish mate in the lab and lay jelly-coated embryos. A, Male and female cuttlefish mate in a head-to-head posture. After mating, the female can store the sperm for weeks. B, Dwarf cuttlefish lay clutches of ~5 to 30 embryos over a period of a few hours. During spawning, the female releases an egg, fertilizes it using stored sperm, wraps it in black jelly and then attaches it to coral to develop. C, Removal of the outer, opaque layer of jelly reveals the many remaining jelly layers that surround each cuttlefish embryo. Scale bar, 1 mm. D, Dwarf cuttlefish embryos can be raised in vitro without their jelly. First, the embryos must be rinsed in alternating solutions of bleach and filtered artificial sea water (FASW) (see Methods), which can be performed efficiently using custom-made mesh baskets. After removing the jelly using forceps, embryos should be handled using a flame-polished glass pipette to avoid rupturing the yolk. E, Dwarf cuttlefish embryos can be raised without their jelly on agarose-coated petri dishes at 28°C

funnel using a hectocotylus (specialized arm with fewer suckers, Figure 1C), places it in the female's buccal area and grinds the spermatophores to release sperm.[51] Consistent with other cephalopod species,[51,52] we have observed female dwarf cuttlefish laying fertilized eggs after long periods of isolation, indicating that they can store sperm for weeks (unpublished observation). When a female dwarf cuttlefish is ready to lay eggs, she fertilizes each egg individually in her buccal area, wraps the fertilized egg in layers of inky jelly, and ties the egg mass to coral to develop (Figure 2B).

Embryonic manipulations that require microinjection are not feasible in the presence of embryonic jelly because it is opaque and impenetrable to microinjection needles (Figure 2C). We therefore modified methods[18] to raise cuttlefish embryos without their jelly. Similar to a previous study, we found that incubation of cuttlefish embryo clutches in bleach prior to jelly removal was essential for survival.[18] However, in contrast to previous work, a solution of filtered artificial sea water (FASW) supplemented with antibiotics and antimycotics was sufficient to support development of the embryos, without

the need for cell culture medium (see Methods). To streamline embryo bleaching and washing steps, we constructed a basket that allowed embryos to be easily transferred between beakers of solution (Figure 2D). After removing the embryos' jelly layers using forceps, we moved de-jellied embryos to agarose-coated 12-well plates (Figure 2E) using a flame-polished glass pipette (Figure 2D) (see Methods).

Existing cephalopod embryo culturing protocols feature daily or near-daily water changes.[50,53] In contrast to these species, dwarf cuttlefish development was most successful when the FASW was not replaced. Large water changes or physical manipulations (eg, rolling the embryos using forceps) inhibited development by introducing contamination or breaking the embryo's delicate chorion (Stage 17 onwards). In total, of 194 embryos raised under these conditions, 48 (25%) survived to hatching.

8.2 | Dwarf cuttlefish embryonic development

The determination of methods to raise dwarf cuttlefish embryos to hatching without their surrounding opaque jelly allowed us to observe embryonic development in detail and create an embryonic staging system. We based the early stages of the month-long embryonic development on a *Sepia officinalis* staging series,[8] and we defined later stages using a series of clear anatomical landmarks that can be easily identified without physically manipulating the embryo and inhibiting development. This led us to a 25-stage system of dwarf cuttlefish development, which contrasts with the 30-stage systems of *Sepia officinalis*[8,9] and *Sepia pharaonis*[10] (see Table 2). Dwarf cuttlefish development can be divided into three major periods: cleavage, gastrulation and organogenesis, similar to other cephalopod staging systems.[8-11,13,14]

TABLE 2 Comparison of cuttlefish embryonic staging systems

Major event	*Sepia bandensis* (this study)	*Sepia officinalis*[9]	*Sepia pharaonis*[10]
1-cell	**Stage 1**; 6 hpf	Stage 1	Stage 1
2-cell	**Stage 2**; 8 hpf	Stage 2	Stage 2
4-cell	**Stage 3**; 10 hpf	Stage 3	Stage 3
8-cell	**Stage 4**; 12 hpf	Stage 4	Stage 4
16-cell	**Stage 5**; 14 hpf	Stage 5	Stage 5
32-cell	**Stage 6**; 16 hpf	Stage 6	Stage 6
64-cell	**Stage 7**; 18 hpf	Stage 7	Stage 7
128-cell	**Stage 8**; 20 hpf	Stage 8	Stage 8
Blastodisc	**Stage 9**; 1 dpf	Stage 9	Stage 10
Layered blastodisc	**Stage 10**; 2 dpf	Stage 10	Stage 11
Blastodisc expansion	**Stage 11**; 3 dpf	Stage 11	Stage 12
10% epiboly	**Stage 12**; 4 dpf	Stage 12	Stage 12-13
30% epiboly	**Stage 13**; 5 dpf	Stage 13	Stage 13
50% epiboly	**Stage 14**; 6 dpf	Stage 14	Stage 14
75% epiboly	**Stage 15**; 7 dpf		Stage 15
Mantle primordium	**Stage 16**; 8 dpf		Stage 16
Arm primordia	**Stage 17**; 9 dpf	Stage 18	Stage 17
Elevated mantle	**Stage 18**; 10 dpf	Stage 21-22	Stage 19–20
First eye pigmentation	**Stage 19**; 13 dpf	Stage 24	Stage 22
Cuttlebone formation	**Stage 20**; 15 dpf	Stage 25	Stage 23
Ink sac formation	**Stage 21**; 17 dpf	Stage 26	Stage 25
3 cuttlebone layers	**Stage 22**; 19 dpf		Stage 26
Chromatophore formation	**Stage 23**; 3 wpf	Stage 25	Stage 24
Chromatophore innervation/expansion	**Stage 24**; 3.5 wpf	Stage 27	Stage 28
Hatching	**Stage 25**; 4 wpf	Stage 30	Stage 30

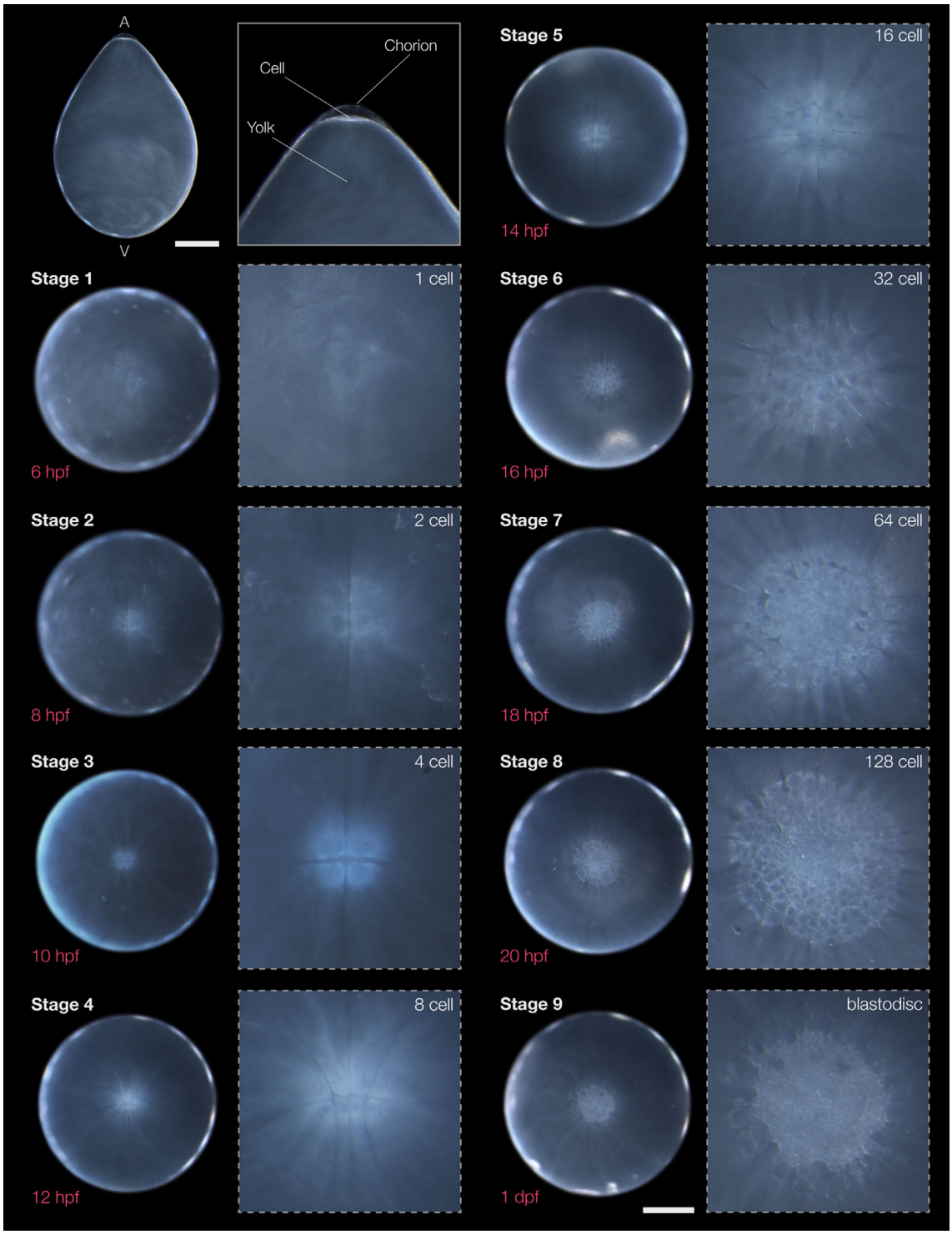

FIGURE 3 Cleavage. The single cell of the 1-cell-stage cuttlefish embryo sits at the animal pole of a large yolk cell. The entire embryo is surrounded by a clear chorion, which is only visible immediately above the cell (top left, lateral view). During the first phase of development the cells divide every 2 hours at 28°C. The first cell division is bilateral and occurs 8 hours post-fertilization (hpf) (Stage 2). The second cell division plane forms at an oblique angle to the first, creating the first embryonic asymmetry (Stage 3), which becomes more evident at Stage 4, when the embryo forms 6 larger cells and 2 smaller cells. At Stage 5, the cells have divided into two populations: blastocones, located at the periphery of the blastodisc, which will give rise to the yolk syncytium, and blastomeres, located in the center of the blastodisc, which will give rise to the embryo proper. From Stage 6 to Stage 8, the cells in the blastodisc continue to double every 2 hours. The blastodisc at 1 day post-fertilization (dpf) (Stage 9) comprises a single layer of cells. Sometimes gaps form within the blastodisc. Stages 1 to 9 are shown from an animal pole view, imaged with darkfield optics (left column, whole embryo; right column, high magnification view of the blastodisc). A, animal; V, vegetal; hpf, hours post-fertilization; dpf, days post-fertilization. Scale bars, 1 mm

8.3 | Cleavage

The cuttlefish embryo is telolecithal—a small, flat cell sits at one pole of a large yolk (~5 mm in length, Stage 1, Figure 3). The entire embryo is enveloped by a clear chorion that is only visible immediately above the cell where it separates from the yolk (Figure 3). In the first phase of embryonic development, the cell divides its volume every 2 hours in the absence of any net growth. Early cell divisions are meroblastic—they undergo incomplete cleavage—and remain connected by cytoplasm.[9] The first cell division occurs 8 hours post-fertilization and produces a bilaterally symmetric embryo (Stage 2, Figure 3). When the second cell division occurs 2 hours later, an oblique cleavage plane creates the first asymmetry (Stage 3, Figure 3), a feature observed in cuttlefish and squid species,[8-12,54] but not octopods.[14] At the 8-cell stage (Stage 4) the cellular asymmetries have become more pronounced, and by the 16-cell stage (Stage 5), the cells form two discrete populations. The blastocones, which are meroblastic, lie at the periphery of the blastodisc and are continuous with the yolk. They will contribute to the yolk syncytium.[55] The blastomeres, which have undergone complete cleavage, lie in the center of the blastodisc and will give rise to the embryo proper (Figure 3). This separation of blastocones and blastomeres is found during cuttlefish, squid and octopus embryogenesis.[8-11,13,14,54] The blastocones and blastomeres continue to divide every 2 hours (Stages 6-8), and at 24 hours post-fertilization a blastodisc of hundreds of cells sits on top of the yolk (Stage 9, Figure 3), bearing notable resemblance to the zebrafish blastula 3 hours post-fertilization.[56]

8.4 | Gastrulation

After the blastodisc is formed, the process of gastrulation begins, whereby the germ layers (endoderm, mesoderm and ectoderm) form and large-scale cellular rearrangements occur. First, the cells of the blastodisc rearrange to form a multilayered disc of cells surrounded by a single-layered marginal zone (Stage 10, Figure 4). The disc begins to expand, bearing some spatial and behavioral resemblance to the mesendodermal cells of the zebrafish embryo[57] (Stage 11, Figure 4). Over the next 5 days, the marginal zone leads a migration of cells over the entire yolk in a process called epiboly (Stages 12-15, Figure 4), a feature shared by coleoid cephalopods.[8-14,54] By 7 days post-fertilization, the majority of the yolk is enveloped by cells, and the cells that make up the

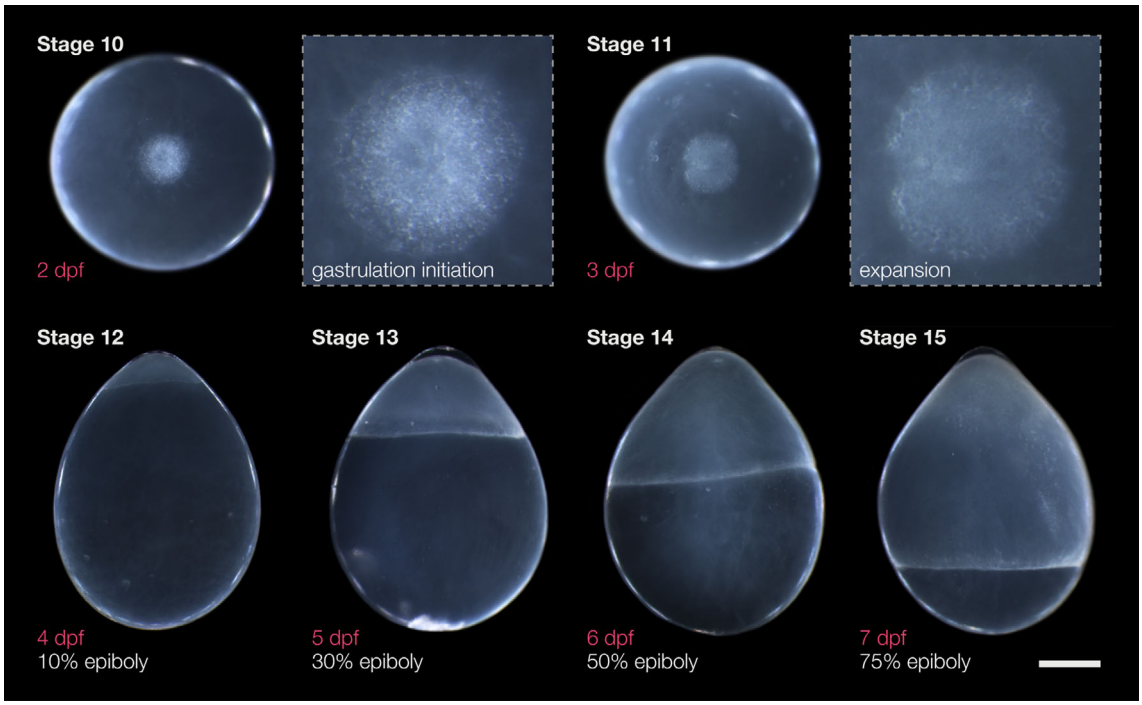

FIGURE 4 Gastrulation. In the second phase of development, the germ layers are formed, and an advancing layer of cells envelopes the yolk. First, the blastodisc rearranges to form a multilayered disc of cells (Stage 10), then the disc expands (Stage 11) and migrates over the yolk in a process called epiboly (Stages 12-15). This is the last stage during which the chorion is tightly attached to the surface of the cuttlefish embryo. Top row, animal pole views (including high magnification views of the blastodisc area); bottom row, lateral views of the embryo. dpf, days post-fertilization. Scale bar, 1 mm

embryo proper have begun the process of differentiation and reorganization[9] (Stage 15, Figure 4). This is the last stage during which the chorion is tightly attached to the surface of the cuttlefish embryo. In contrast to the 5 days of cuttlefish gastrulation, mouse gastrulation is completed in 2 days[58] and zebrafish gastrulation takes just 5 hours.[56]

8.5 | Organogenesis

When the yolk has been completely enveloped by cells, the migrating cell front zippers together at the vegetal pole of the yolk and the mantle primordium forms as a circular thickening of tissue at the opposite end of the embryo. By this stage, the chorion has begun to expand, and the embryo has rotated within the chorion so that the animal pole of the embryo no longer aligns with the pointed end of the chorion (Stage 16, Figure 5). Over the next day, the arm and eye primordia become visible, the mantle primordium thickens, and the chorion continues to expand (Stage 17, Figure 5). The mantle then elevates, and the envelope of cells around the yolk begins to contract, resulting in peristaltic waves across the yolk that permit the passage of blood and nutrients between the yolk and embryo[9] (Stage 18, Figure 5). Over the next 2 weeks, the embryo undergoes a dramatic period of growth, coincident with the formation of multiple organs and the absorption of yolk. A number of key morphological changes can be observed, including generation of the first eye pigmentation (Stage 19); formation of the first cuttlebone layer—a calcium carbonate structure used for buoyancy (Stage 20); formation of the ink sac, which will release ink to evade predators (Stage 21); conversion of the eye pigmentation from orange to dark brown (Stage 22); generation of the first chromatophores and papillae, which will later permit color and texture change, respectively (Stage 23); an increase in chromatophore and papillae number coupled with innervation of the chromatophores, allowing them to expand (Stage 24); and finally, the almost-complete absorption of yolk, which normally coincides with hatching from the jelly capsule (Stage 25, Figure 5). In total, dwarf cuttlefish embryonic development takes around 1 month.

8.6 | Post-hatching

After hatching, dwarf cuttlefish are benthic, remaining at the bottom of the tank by day. At night they are pelagic, ascending into the water column. Some cephalopod species, such as *Octopus vulgaris* and *Euprymna scolopes*, hatch as paralarva and must undergo an additional transformation into their juvenile form.[14,59] By contrast, cuttlefish species hatch as miniature versions of their adult form. Within a week of hatching, dwarf cuttlefish embryos begin feeding on mysid shrimp before transitioning to grass shrimp at around 1 month of age (see Methods). After 2 to 3 months of continual growth, male dwarf cuttlefish begin displaying sexually dimorphic behaviors (Figure 1B), reaching sexual maturity at around 4 months. Following a period of mating and egg-laying from 4 to 6 months of age, dwarf cuttlefish begin showing hallmarks of aging, including a reduction in eyesight and movement, resulting in a failure to catch prey. After a lifetime of continuous growth, dwarf cuttlefish die at around 9 months of age.

9 | DISCUSSION

This study describes an embryonic time course for the dwarf cuttlefish, *Sepia bandensis* (Figure 6), which we have divided into 25 easily-observable stages. The observation of development was facilitated by the determination of methods to raise dwarf cuttlefish embryos in vitro without their opaque jelly, providing future opportunities for microinjection and genetic manipulation. Although we were able to raise embryos to hatching (48/193; 25%), improvements to the culturing protocol that increase survival would be a valuable area for future study.

This staging series serves multiple functions. First, it permits the alignment of embryos from independent experiments by morphology rather than time. This is important because dwarf cuttlefish embryos, even within the same clutch, can exhibit variability in developmental timing. Second, it permits comparisons between embryos of different species that may share morphological features during development but exhibit heterochrony. Third, the staging series permits the health and progression of embryos to be monitored after microinjection of mRNAs, CRISPR/Cas9 components or transgenic DNA constructs. Finally, it permits detailed comparisons between normal embryos and those exhibiting abnormal developmental phenotypes caused by experimental manipulations or natural variation (eg, failed fertilization).

This staging series reveals that the development of the dwarf cuttlefish, *Sepia bandensis*, bears striking resemblance to *Sepia officinalis* and *Sepia pharaonis* embryogenesis (Table 2), with the exception of the larger size of *Sepia pharaonis* hatchlings[10] and slower embryogenesis of *Sepia officinalis*,[6] likely due to its temperate environment. Moreover, the major features of development, including the cellular arrangements and morphogenetic movements, appear to be shared across the coleoid cephalopods.[8-14,54] The notable exception is the process of reversion, which has only been observed in

FIGURE 5 Organogenesis. In the final, and longest phase of development, the embryo forms organs and undergoes substantial growth. First, the migrating cell front zippers together at the vegetal pole of the yolk and the mantle primordium forms as a circular thickening of tissue at the opposite end of the embryo. By this stage the chorion has begun to expand, and the entire embryo has rotated 90° within the chorion so that the animal pole of the embryo no longer aligns with the pointed end of the chorion (Stage 16). Next, the arm and eye primordia form (Stage 17). As the chorion continues to expand, the mantle elevates and the yolk envelope begins making periodic contractions (Stage 18). Over the next 2 weeks, the eyes develop their first pigmentation (Stage 19), the eye pigmentation darkens to orange (Stage 20), the ink sac becomes visible on the ventral side (Stage 21), the eye pigmentation darkens to brown (Stage 22), chromatophores and papillae form (Stage 23), the chromatophores become innervated and begin to expand (Stage 24), and the embryo completes its absorption of yolk, which is normally coincident with hatching from the jelly capsule (Stage 25). Under normal conditions, dwarf cuttlefish hatch at around 4 weeks post-fertilization (wpf) at 28°C. Note that the chorion begins expanding at Stage 17, and is not shown from Stage 19 onwards. Embryos are shown from both the dorsal and ventral views for Stages 21 to 24 as embryos usually lie dorsal-side-down in the petri dish. The developing layers of the cuttlebone are indicated in the figure. cb, cuttlebone; ch, chorion; chr, chromatophore; eye, eye primordium; fu, funnel; ink, ink sac; lim, limb primordium; ma, mantle; pg, pigment; pp, papillae; suc, sucker; te, tentacle; yo, yolk. Scale bar, 1 mm

octopods: whereas the blastodisc of developing squid and cuttlefish embryos generally forms and remains at (or near) the animal pole of the embryo, the octopus blastodisc forms at the animal pole and then migrates to the opposite end of the embryo before continuing development.[14,60]

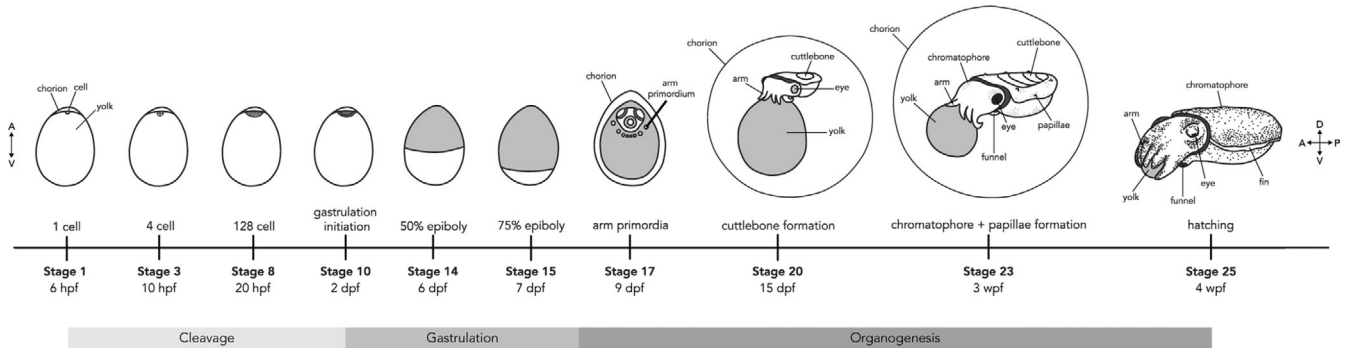

FIGURE 6 Overview of dwarf cuttlefish development. Dwarf cuttlefish development can be divided into three stages: cleavage, gastrulation and organogenesis. During cleavage, the embryo forms at the animal pole of the embryo and undergoes cell division every 2 hours (A, animal; V, vegetal). During gastrulation, the germ layers are formed and the embryo undergoes large cellular rearrangements. Finally, during organogenesis, the embryo undergoes substantial growth, it develops organs, the chorion expands, the embryo absorbs the yolk, and the embryo develops color-changing skin (A, anterior; D, dorsal; P, posterior; V, ventral). hpf, dpf, wpf: hours, days, weeks post-fertilization

The ability to observe early cuttlefish embryogenesis in vitro provides the opportunity to address interesting questions about transcription and cell fate. Do maternal determinants control the early cell divisions of the cuttlefish embryo? How and when does the embryo initiate zygotic transcription? Are the cells of the early embryo pluripotent or is their lineage specified? How are the axes specified in cuttlefish? Additionally, the study of cuttlefish embryogenesis may reveal how cuttlefish germ cells are specified. Does the embryo inherit maternally-provided germplasm, as found in zebrafish, or are primordial germ cells induced later in development, as found in mice? Do the embryonic chromatophores persist to adulthood, or are they continually replenished?

Scientific research is both driven and limited by the existence of experimental tools. This staging series represents a useful addition to the dwarf cuttlefish toolkit. The assembly of the genome and transcriptome, a brain atlas, methods for CRISPR/Cas9 mutagenesis and transgenesis, an embryonic fate map, and an RNA-seq cell atlas will add to this technologic armamentarium. Powerful computational and analytical techniques have recently been developed for tracking the movement and control of chromatophores in *Sepia officinalis*.[20] The application of similar methods to the dwarf cuttlefish would complement the development of molecular techniques, providing a powerful system to analyze the neural basis of camouflage behavior.

There is a tradition of conducting biological research on a small group of classic model organisms. These systems offer unparalleled resources for uncovering biological mechanisms, but discovery is limited by the biological phenomena exhibited by these model systems. The development of modern sequencing and gene editing tools allows us to expand the repertoire of model organisms to encompass a greater diversity of biologically fascinating organisms. This study describes the first dwarf cuttlefish tool in an expanding experimental toolkit that may provide novel insights in neuroscience, development, evolution and beyond.

10 | EXPERIMENTAL PROCEDURES

10.1 | Ethical use of animals

The use of cephalopods in laboratory research is currently not regulated in the USA, while in the European Union it is regulated by law.[61] However, Columbia University has established strict policies for the ethical use of cephalopods including operational oversight from the Institutional Animal Care and Use Committee (IACUC). All of the cuttlefish used in this study followed these strict ethical policies.

10.2 | Cuttlefish husbandry

Dwarf cuttlefish were raised in recirculating 250-gal systems of 32 parts per thousand (ppt) artificial seawater (ASW, Crystal Sea Marinemix) designed and built by 8 Arm Assistance (www.8armassistance.com). To establish the cuttlefish colony, cuttlefish embryos were ordered from an aquarium supplier (Quality Marine) and suspended in floating boxes made of 3/4″ plastic mesh in 5.5 or 10-gal tanks until hatching. Hatchling cuttlefish were fed with live mysid shrimp (*Mysidopsis bahia*, Aquatic Indicators) gut-loaded with freshly hatched brine shrimp (*Artemia sp*, E-Z Egg) twice per day. Juvenile

(>1 month) and adult cuttlefish were fed with live grass shrimp (*Palaemonetes pugio*, Aquatic Indicators) 3 times per day, with meal sizes of up to 9 grass shrimp per cuttlefish per day. Cuttlefish were co-housed in groups of <100 (hatchlings), <30 (juveniles) and <5 (adults) with a mix of sexes. Behavior was carefully monitored and animals were examined for beak marks (a permanent scarring of the skin resulting from fighting). When necessary, highly aggressive males were removed from group tanks and housed alone to avoid conflict. To maintain appropriate water conditions, aquarium water parameters were maintained at: ammonia <0.1 ppm (ppm), nitrite <0.1 ppm, nitrate <20 ppm and pH 8.1 to 8.4. Water changes, UV lights, pumps, and water quality parameters were monitored and controlled using a Neptune Apex system.

10.3 | Embryo collection

Male and female cuttlefish were co-housed in groups of 2 to 5 in 5.5- or 10-gal tanks with enrichment (rocks, artificial corals and plants). Breeding tanks were checked twice per day for embryos. Newly-laid embryos were removed from the tank using plastic tweezers and placed in a plastic beaker of ASW.

10.4 | Cuttlefish in vitro embryo culturing

Cuttlefish embryos were transferred to a handmade mesh basket (comprising plastic mesh taped to an acrylic cylinder) that was designed to fit within a 500 mL plastic beaker so the embryos could be easily transferred between solutions (Figure 2D). Embryos were rinsed in 150 mL of 0.22 μm-filtered ASW (FASW) and then incubated in 150 mL of a 1:20 dilution of bleach (sodium hypochlorite, 8.25%) in FASW for 5 seconds.[18] This process was repeated once, and then the embryos were washed vigorously in (a) 300 mL of sodium thiosulfate (4 tsp of sodium thiosulfate per 300 mL beaker of FASW); (b) 300 mL of fresh FASW; (c) a third beaker of 300 mL FASW for a final rinse. After bleaching, the color of the jelly turned from black to dark brown, and the jelly surface was slightly ruptured. The embryos were transferred to a deep petri dish (90 mm diameter × 25 mm deep; 8609-0625, USA Scientific) containing fresh FASW. To make the embryo incubation solution, one 1.5 mL tube of 100× antibiotic antimycotic solution (A5955, Sigma) was thawed and added to 150 mL of FASW to make a 1× solution. Embryos were transferred one-by-one to a new petri dish containing the 1× embryo incubation solution and the jelly layers were carefully torn away using forceps under a dissecting microscope. A wide-mouth glass dropper (14-955-501, Fisher Scientific) was used to move de-jellied embryos between dishes after the dropper's narrow opening was cut off using a ceramic tile (CTS, Sutter) and the sharp edge was melted using a bunsen burner (Figure 2D). De-jellied embryos were moved to a 12-well cell culture plate (353225, Falcon) pre-coated in a thin layer of 1% agarose and filled with 1× embryo incubation solution (Figure 2E). The embryos were incubated at 28°C on a 12-hour light/dark cycle. To minimize the risk of infection, embryos were not manipulated unless it was entirely necessary, and the instruments were disinfected with ethanol first. After Stage 16, the chorion of dwarf cuttlefish embryos starts expanding, and becomes increasingly easy to rupture. Embryos were not transferred or manipulated after Stage 17 otherwise the chorion ruptured and the embryos rarely survived. Embryo survival was highest when the same incubation solution was maintained for the entire 1-month period except for careful additions of a small volume of deionized water (<100 μL) every 3 to 5 days to supplement evaporation. Of the 193 embryos grown under these conditions, 95% (184/193) survived until gastrulation, 51% survived until early organogenesis (99/193), 42% survived until late organogenesis (81/193) and 25% survived until hatching (48/193).

10.5 | Embryo imaging

To capture images for the staging series, embryos at the desired stage were moved from their 12-well plate to a 90 mm agarose-coated petri dish (8609-0625, USA Scientific). A small (2 mm) well was cut in the middle of the agarose, and the embryo was placed in the well to prevent it from rolling. Embryos were imaged using a Zeiss SteREO Discovery.V20 microscope with an Axiocam 712 color. Brightness, contrast and color balance were adjusted uniformly to images using ImageJ.

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AUTHOR CONTRIBUTIONS

Tessa Montague: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review & editing. **Isabelle Rieth:** Methodology; visualization; writing-review & editing. **Richard Axel:** Funding acquisition; supervision; writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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