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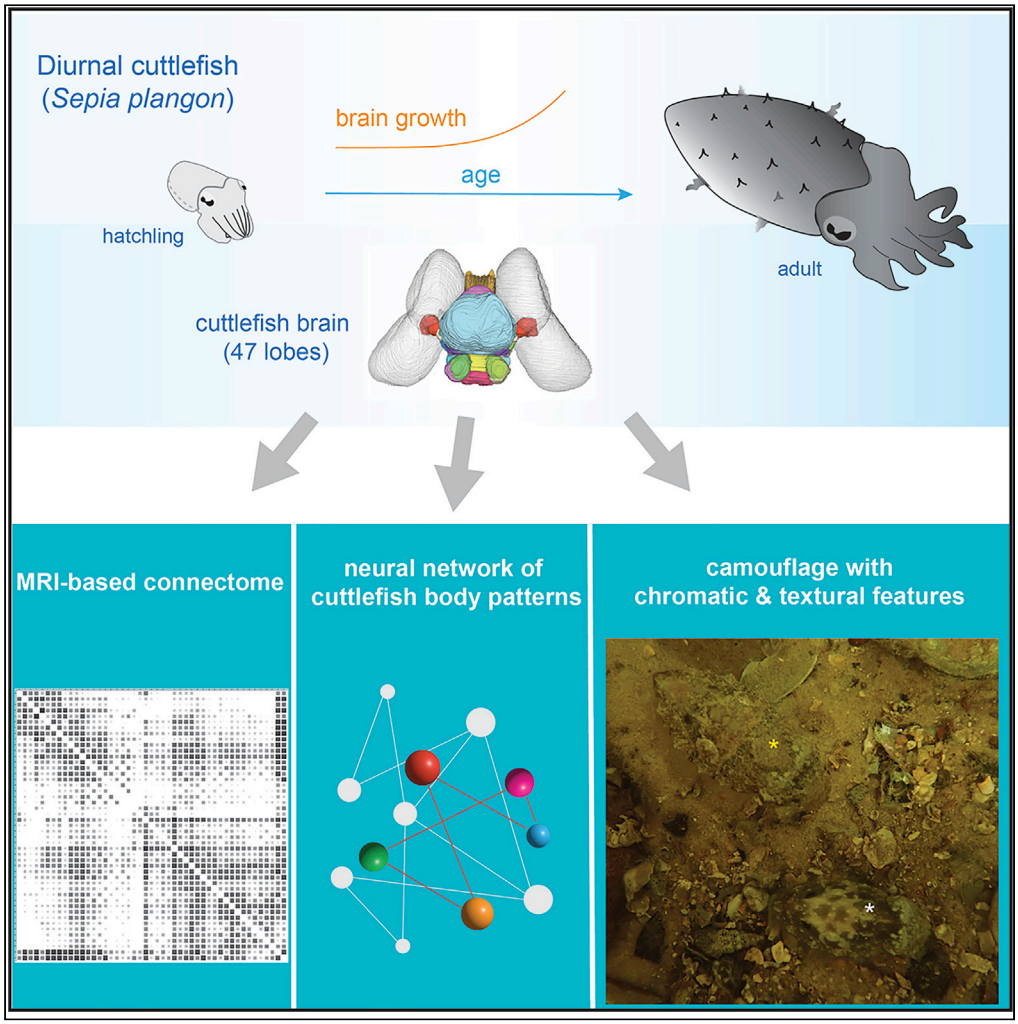

Wen-Sung Chung, Alejandra López-Galán, Nyoman D. Kurniawan, N. Justin Marshall

w.chung1@uq.edu.au

Highlights

The diurnal cuttlefish has the enlarged and complex visual and learning brain lobes

Identification of neural networks associated with camouflage and chemosenses

Evolutionary history and ecology requirement lead to cuttlefish brain heterogeneity

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The brain structure and the neural network features of the diurnal cuttlefish *Sepia plangon*

Wen-Sung Chung,[1,3,*] Alejandra López-Galán,[1] Nyoman D. Kurniawan,[2] and N. Justin Marshall[1]

SUMMARY

Cuttlefish are known for their rapid changes of appearance enabling camouflage and con-specific communication for mating or agonistic display. However, interpretation of their sophisticated behaviors and responsible brain areas is based on the better-studied squid brain atlas. Here we present the first detailed description of the neuroanatomical features of a tropical and diurnal cuttlefish, *Sepia plangon*, coupled with observations on ontogenetic changes in its visual and learning centers using a suite of MRI-based techniques and histology. We then make comparisons to a loliginid squid, treating it as a 'baseline', and also to other cuttlefish species to help construct a connectivity map of the cuttlefish brain. Differences in brain anatomy and the previously unknown neural connections associated with camouflage, motor control and chemosensory function are described. These findings link brain heterogeneity to ecological niches and lifestyle, feeding hypotheses and evolutionary history, and provide a timely, new technology update to older literature.

INTRODUCTION

Coleoid cephalopods (cuttlefish, squid and octopus) exhibit diverse adaptations in body form, brain layout, life modes and behavioral repertoires.[1–3] Octopus and squid (excluded *Argonauta* (pelagic octopus, also known as paper nautilus) and *Spirula* (ram horn squid)), do not use gas-filled floatation structures for buoyancy allowing these two groups to inhabit a broad range of ocean depths (0–6000 m).[4–6] By contrast, cuttlefish possess an internal chambered cuttlebone that actively adjusts the ratio between air and liquid to gain buoyancy.[7] Cuttlefish can therefore hover in the water column, usually close to the benthos, but the risk of cuttlebone implosion because of increased pressure with depth limits their habitat to above 400 m.[8] Of interest, for unknown reasons, cuttlefish also have a limited geographic distribution (high diversity in the Indo-Pacific but absence in the Americas and polar regions).[3,4,8,9]

Living close to or on the ocean floor, both cuttlefish and octopus have become masters of camouflage, blending with the benthos by control of body pattern, intensity and texture in astonishingly accurate imitation of their surroundings. This disappearing feat is used in both prey ambush and threat avoidance.[1,10–13] In fact, cuttlefishes spend most of their time in camouflage mode but may also rapidly switch body patterning to emphasize their presence, produce startle threats, attract mates or indeed cheat rival males.[1,14–19] The ability to alter their visual appearance is driven by neurally controlled chromatophore (colors) and muscular hydrostat (papillae) systems coordinated by circuits from a set of brain lobes; the optic lobe (OPL), the lateral basal lobe (lB) and the chromatophore lobe (Ch)).[12,20] Despite some recent attempts to prove otherwise,[21] several previous studies have shown cuttlefish to be colorblind,[10,17,22,23] their camouflage achieved through intensity, pattern and textural match alone on a baseline palette of ocean floor colors.

Over the past two decades, a growing number of studies have focused on the behavioral neurobiology of the cuttlefish and their remarkably rapid and cognitively complex reactions to novel challenges. For instance, cuttlefish can utilize spatial learning to solve maze tasks based on visual cues (e.g., landmark and e-vector of polarization light).[24,25] Object recognition in cuttlefish (e.g. visual equivalence, amodal completion and visual interpolation for contour completion) appears to use strategies close to those used in vertebrates.[26,27] The recent push toward comparisons of advanced cognitive behaviors (e.g.

[1]Queensland Brain Institute, The University of Queensland, St Lucia, QLD 4072, Australia

[2]Centre for Advanced Imaging, The University of Queensland, St Lucia, QLD 4072, Australia

[3]Lead contact

*Correspondence: w.chung1@uq.edu.au

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Table 1. List of ecological, behavioral and neuroanatomical features of cuttlefish and squid used in this study

Species	Habitat (depth)	Life mode	OPL shape
Sepia plangon[a]	Reef[c] (1–83 m)[4,19]	D[4]	C[a]
Sepia apama[a]	Sea grass/kelp bed/reef[c] (1–100 m)[4,72]	D[73]	C[a]
Metasepia tullbergi[a]	Reef[c] (1–83 m)[3,4]	D[4]	C[a]
Metasepia pfefferi[a]	Reef[c] (1–83 m)[4]	D[4]	C[a]
Sepia latimanus[a]	Reef[c] (1–30 m)[3,4,74]	D[3,4]	C[a]
Sepia officinalis	Predominantly in soft substrates; also found in the reefs of Mediterranean Sea[b] (1–200 m)[4,75]	N[4,7] Ca[1,29,40,41] in captivity	B[37,44,62]
Sepia pharaonis	Reef/soft substrate[c] (5–130 m)[3,4]	N[3,4]	B[32]
Sepiella japonica	Soft substrates[c] (1–50 m)[3,4]	N[76]	B[33]
Sepioteuthis lessoniana (squid)	Reef[c] (1–100 m)[3,4]	Ca[3,66,77]	B[63,78]

B- bean-shaped; C- croissant-shaped; Ca-cathemeral; D-diurnal; N- nocturnal.
[a]Indicates morphological features reported from the current study.
[b]As Eastern Atlantic and Mediterranean Sea.
[c]As Western Indo-Pacific Ocean.

number sense, episodic-like memory, self-control), has postulated that the ability of the cuttlefish in solving complex tasks and cognitive reactions approaches that of young humans.[28–30]

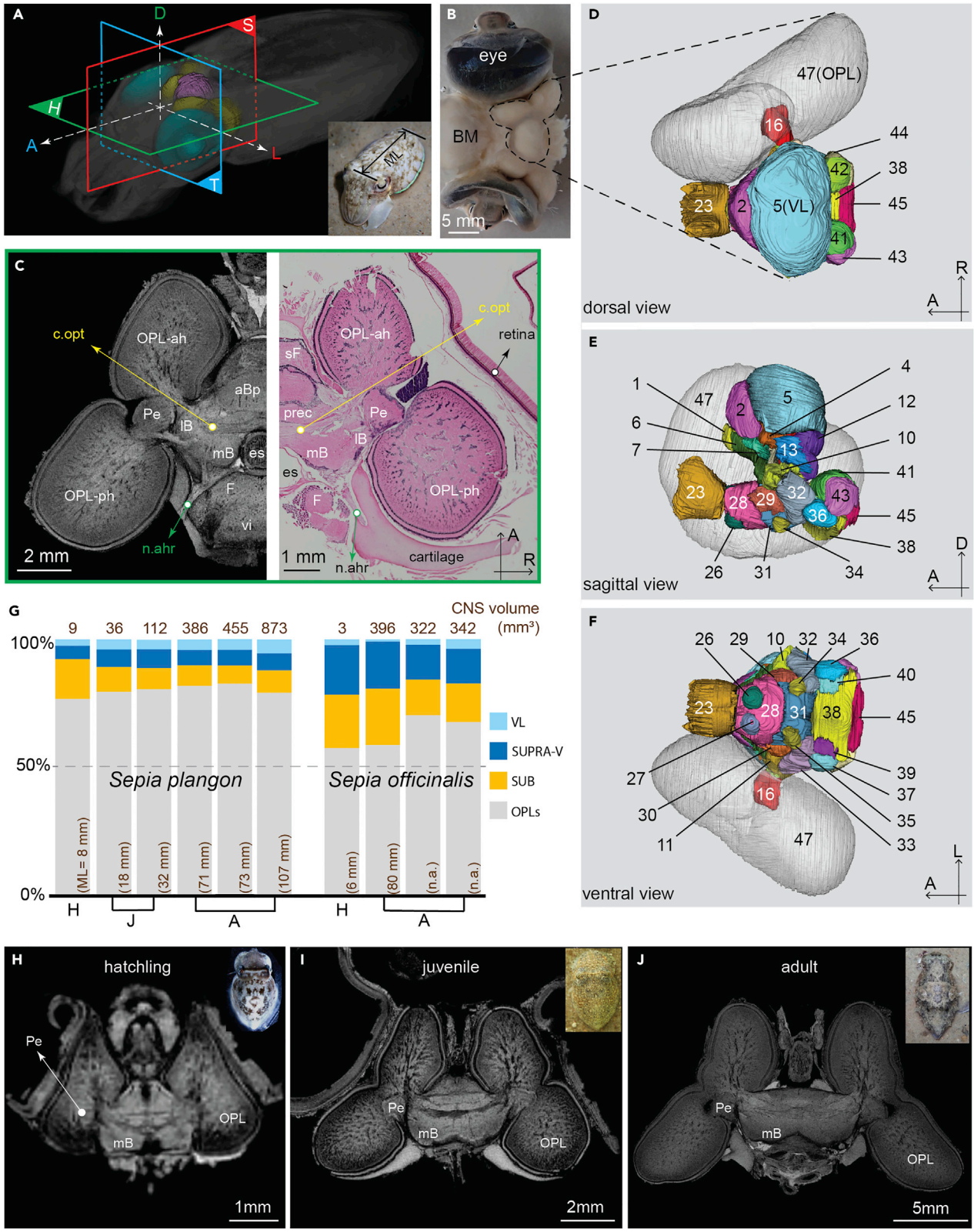
Although some of what we know of their biology and brain structure has been obtained from several Indo-Pacific cuttlefish species,[1,3,31–35] our current knowledge of the neurobiology and behavioral repertoire of cuttlefish is predominantly derived from the European common cuttlefish, *Sepia officinalis*.[7,10,12,13,22,29,34,36–43] Despite this historically long interest in the behavioral neurobiology of cuttlefish and some progress in descriptions of its central nervous system (CNS)[35–38,42–51] knowledge of its brain layout and underlying circuits is scant compared to octopuses[52–55] and loliginid squids.[56–63] In fact, an oversimplified assumption where little or no variation is postulated between squid and cuttlefish brains has been broadly applied for decades.[42,47,64]

Understanding the gross anatomy and circuit diagrams of any nervous system is the necessary first step toward understanding how evolution has shaped both brain structures and behaviors in cephalopods.[2,47,55,63,65–68] In this context we asked two questions here: (1) Whether the neuroanatomy of *S. officinalis* is representative of most cuttlefish, where there is data to form comparisons? (2) Whether the cuttlefish brain has specific adaptations in response to their habits and habitats? In order to describe the cuttlefish brain structure and extended neuroanatomical features described here, initially we use the previous publications of *S. officinalis*[37,62,69] in comparison with our description of the *Sepia plangon* brain. We then extend comparisons to a loliginid squid *Sepioteuthis lessoniana*,[63] treating this species as a phylogenetic 'baseline', and include comparison to the brain areas for ten other cuttlefish species from the three genera (*Sepia, Metasepia* and *Sepiella*) where data exist[12,32,33,35,37,43,47,56–63,70,71] (Tables 1 and S3). Observations on the relative enlargement of brain lobes, and brain folding are included in this extended comparison of species, relative to ecology and lifestyle as well as phylogenies mostly based on existing morphological but including some molecular data.[4,32,33,35,37,43,62]

RESULTS

Gross neuroanatomy of *S. plangon*

Dissection, contrast-enhanced 16.4 T magnetic resonance images (16.4T MRI) and resulting 3D reconstruction show that the brain of *S. plangon* is located just under the anterior projection of the cuttlebone (Figure 1 and Video S1). Supraesophageal (SUPRA, integrating sensory inputs, learning and memory) and subesophageal masses (SUB, coordinating locomotion and coloration) are encased by the cranial cartilage whereas the two optic lobes (OPLs, visual center) are partially covered by the orbital cartilage (Figure 1A). In gross anatomical terms this diurnal cuttlefish possesses a brain layout superficially similar to the largely nocturnal *S. officinalis* (histology[2,37,44,62] and MRI[43]) and shares a similar lobe arrangement such as the compact SUB where the short brachio-pedal connective link the brachial lobe (anterior SUB) to pedal


A
D
S
H
A
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T
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B
eye
BM
5 mm
D
47(OPL)
16
44
42
38
23
2
5(VL)
45
41
43
R
A
dorsal view
C
c.opt
OPL-ah
aBp
Pe
lB
mB
es
OPL-ph
F
vi
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2 mm
sF
prec
retina
cartilage
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1
47
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4
12
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13
10
41
23
28
29
32
43
36
45
38
26
31
34
D
A
sagittal view
G
CNS volume
(mm³)
9
36
112
386
455
873
3
396
322
342
100%
50%
0%
Sepia plangon
Sepia officinalis
VL
SUPRA-V
SUB
OPLs
(ML= 8 mm)
(18 mm)
(32 mm)
(71 mm)
(73 mm)
(107 mm)
(6 mm)
(80 mm)
(n.a.)
(n.a.)
H
J
A
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16
37
30
35
11
47
33
L
A
ventral view
H
hatchling
Pe
OPL
mB
1mm
I
juvenile
Pe
mB
OPL
2mm
J
adult
Pe
mB
OPL
5mm

Figure 1. The features of central nervous system (CNS) of the cuttlefish, *Sepia plangon*

(A) Live juvenile, *S. plangon*. ML - mantle length (18 mm) and 3D MRI rendering of an entire cuttlefish and the underlying CNS (including optic lobes (yellow) and central complex (pink)) and eyes (blue). H- horizontal; S- sagittal; T-transverse plane. A - anterior; P - posterior; D - dorsal; L – left; R - right lateral side. (See also Video S1).

(B) Isolated brain-eyes preparation (dorsal view). BM-buccal mass; OPL - optic lobe; VL-vertical lobe.

(C) Comparisons of horizontal sections between magnetic resonance histology (left) (ML = 107 mm) (isotropic resolution 30 μm) and conventional histology (right) (ML = 39 mm) (slice stained with hematoxylin and eosin (15 μm thickness)). es-esophagus; anterior posterior basal (aBp); optic connective (c.opt); anterior head retractor nerve (n.ahr); superior frontal (sF); lateral basal (lB); median basal (mB); precommisural (prec); peduncle (Pe); fin (F); visceral (vi); anterior horn of optic lobe (OPL-ah); posterior horn of optic lobe (OPL-ph). (See also Figure S1).

(D–F) 3D MRI rendering of lobe organization, including 47 lobes (15 of which are bilateral) (See also Tables S1 and S2): (1) inferior frontal lobe (iF); (2) superior frontal (sF); (3) posterior frontal (pF); (4) subvertical (sV); (5) vertical (VL); (6) anterior anterior basal (aBa); (7) anterior posterior basal (aBp); (8) precommissural (pr); (9) dorsal basal (dB); (10-11) interior basal (iB); (12) median basal (mB); (13-14) lateral basal (lB); (15-16) peduncle (Pe); (17-18) olfactory (of); (19-20) dorsolateral (D); (21) inferior buccal (iBu); (22) superior buccal (sBu); (23) brachial (Br); (24-25) anterior dorsal chromatophore (adC); (26-27) anterior ventral chromatophore (avC); (28) anterior pedal (aP); (29-30) lateral pedal (lP); (31) posterior pedal (pP); (32-33) dorsal magnocellular (dM); (34-35) ventral magnocellular (vM); (36-37) posterior magnocellular (pM); (38) palliovisceral (Pv); (39-40) lateral ventral palliovisceral (lvP); (41-42) fin (F); (43-44) posterior chromatophore (pC); (45) visceral (vi); (46-47) optic (OPL). (See also Tables S1 and S2).

(G) CNS volume and percentage of the four brain regions in *S. plangon* and *Sepia officinalis*. Color-coded symbols represent the brain regions as: VL, vertical lobe; OPLs, optic lobes; SUPRA-V, supraesophageal mass excluding VL; SUB, subesophageal mass. H, hatchling; J, juvenile; A, adult. Detailed volumetric data can be found in Table S2. The volumetric data of *S. officinalis* were based on the published literature.[2,62,69,79,80]

(H–J) Horizontal MRI slices (approximately mid-way through CNS) show neuroanatomical features in three ontogenetic stages. (H) ML = 8 mm. (I) ML = 32 mm. (J) ML = 107 mm.

See also Table S2, Videos S1, S2, and S3.

lobe complex (middle SUB), and the paired fin and posterior chromatophore lobes are located at the dorsal region of posterior SUB. In total 32 lobes were identified (15 of which are bilateral) and the unique features of *S. plangon* are described as follows with a comparison between adult and juvenile *S. plangon* brains to demonstrate the ontogenetic development of various brain features (Figures 1G–1J).

Notable neuroanatomical features of *S. plangon*

Several previously unknown neuroanatomical features, obvious at a gross anatomical level, were identified in *S. plangon*, including distinct enlargement of the OPL and VL, and morphological folding of the OPL into a croissant-shape (Figures 1, S1, and S2, and Videos S1, S2, and S3).

Croissant-shaped optic lobe

All MRI-examined *S. plangon* specimens (1 hatchling, 2 juveniles and 3 adults) possess distinct enlarged OPLs shaped like a croissant (Figure 1 and Videos S1, S2, and S3). The volume of the hatchling's OPLs is 7.11 mm^3 and rapidly increases up to 691 mm^3 at the adult stage (Figure 1G). The percentage of OPLs relative to total CNS volume in all stages we examined is approximately 80% (79.8 $\pm$ 1.8% (mean $\pm$ SD), n = 6) which is significantly larger than the moderately-enlarged and bean-shaped OPLs (65.8 $\pm$ 5.1%, n = 4) of *S. officinalis* (t-test, $p < 0.007$) (Figures 1G, Tables 1 and S3).

The croissant-shaped OPL is present over a broad range of body sizes (young juvenile - adult) (Figures 1H–1J and S1), and has a gyrification index (GI), that is a folding complexity measure used most often for mammalian cortex,[81] of between 1.01 $\pm$ 0.003 (mean $\pm$ SD) (2 juveniles) and 1.04 $\pm$ 0.014 (3 adults). Detailed morphological features are as follows:

(i) OPL horns. The dorsal 1/3 of the OPL is divided into two parts, forming two blunt horns that are closely opposed near the central line of the OPL (Figure S1, Video S3). With the cuttlefish resting on or just above the substrate, the anterior horn receives input from the posterior visual scene via the posterior vertical slit of its w-shaped pupil. The posterior horn is opposite to this and receives forward-directed visual input, vital for the ballistic tentacular strike during prey capture.

(ii) OPL sulcal folding. A second modification (again one found recently in the diurnal octopus[55]) is a curved-shaped sulcus at the lateral side receiving input from the medial retinal region looking out of the central crescent-shaped area of the pupil (Video S3).

Vertical lobe

Both hatchling and early juvenile possess a dome-shaped vertical lobe (VL, learning and memory center) with well-developed paired tracts linked to the superior frontal lobe (sF) and inferior frontal lobe (iF)

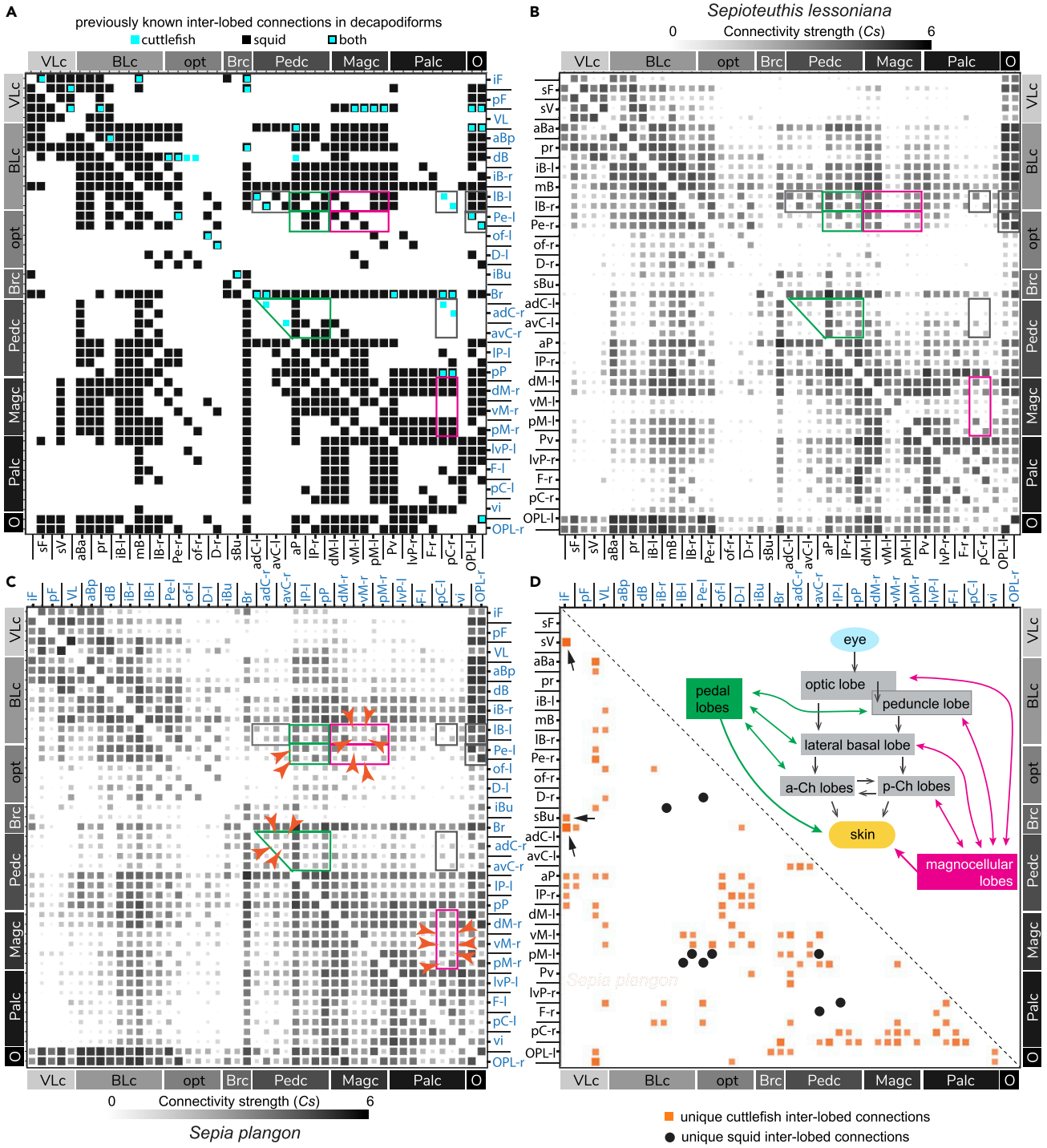


Figure 2. Comparisons of MRI-based connectivity matrices between the cuttlefish and the squid brains

(A) This matrix summarizes all described inter-lobed neural connections of cuttlefish (*Sepia officinalis*) (n = 45, blue squares)[2,37,47,61] and loliginid squids (n = 388, black squares) based on a suite of silver impregnation and cobalt filling results initiated by J.Z. Young and his colleagues[37,47,57–61] and our recent publication.[63] VLc, vertical lobe complex; BLc, basal lobe complex; opt, optic track complex; Brc, brachial lobe complex; Pedc, pedal lobe complex; Magc, magnocellular lobe complex; Palc, Palliovisceral lobe complex; O, optic lobes (See also Tables S1 and S2). Five gray boxes indicate the principle lobes and circuits governing the cephalopod chromatophore system initially described by Boycott[37] (cuttlefish), Young[58] (squid) and Dubas et al.[70] (squid). Three pink boxes represent additional inter-lobed network (squid) to expand the complexity of this system suggested by Novicki et al.[82] Additional set of camouflage-related tracts derived from the green and pink regions is suggested using tractography, see explanation below.

Figure 2. *Continued*

(B) An averaged probabilistic tractography connectivity matrix of the squid, *Sepioteuthis lessoniana* (5 juveniles).[63] The squid tractography reveals the 24 described squid coloration circuits (10 in gray boxes (100% positive rate) and 14 in pink boxes (78% positive rate missing out 2 sets of contralateral tracts (Pe-ventral M and posterior Ch – posterior M). Tractography derived from the lobes within the green highlighted regions shows strong degree of projection to their endpoints in chromatophore lobes.[63]

(C) An averaged probabilistic tractography connectivity matrix of the cuttlefish, *Sepia plangon* (3 adults). Aside from a similar connectivity pattern between cuttlefish and squid (A and B) (gray, pink and green regions), 18 inter-lobed connections are highlighted (orange arrow heads) due to their strong *Cs* values than in the squid and suggested as additional pathways for the cuttlefish camouflage.

(D) Using subtraction of the *Cs* values between the cuttlefish and squid matrices (B and C), unique inter-lobed connections ($\Delta Cs \geq 1.5$) are highlighted, cuttlefish (orange squares) and squid (black circles). Remarkably, three arrows indicate the cuttlefish chemosensory circuits linked to the inferior frontal lobe (iF), including iF-subvertical lobe (sV), iF-brachial lobe (Br) and iF-superior buccal lobe (sBu). Three color-coded highlighted regions reveal numerous previously-unknown connections related to the chromatophore lobes, suggesting a further complex circuit coordinating cuttlefish body patterning (the gray circuits were initially suggested by Boycott[37] and the pink and green circuits are proposed based on the tractography).

See also Figure S2.

(Figures 1D, 1E, and 2, Videos S2 and S3). Volumetric estimates of the VL reveal rapid growth during ontogeny from the hatchling (ca. 2.4% of CNS volume) toward 3.7% in juveniles and over 5% in adults ready for spawning (Figure 1G, Table S3). The expansion of VL during the post hatchling stage is faster than other brain regions of SUPRA in *S. plangon* (the percentage of VL relative to total SUPRA volume, from hatchling (31%) to juvenile (34.8 $\pm$ 1.7%) and adult (41.3 $\pm$ 2.6%) (Figure 1G), confirming the disproportionately ontogenetic growth of the cuttlefish VL.

Tractography and connectome

Using the same imaging procedure and the selection criteria established for the squid MRI-based connectome,[63] the averaged brain-wide connectome of *S. plangon* (3 adults) allows recovery of all known major inter-lobed tracts (n = 45) described in *S. officinalis*[2,37,45,47,56,60,61,83] and those (n = 388) in the loliginid squid[47,56–61,63,82] (Figure 2A). In addition, 181 blank spots (*Cs* = 0) in the averaged connectivity matrix from tractography are well-matched with the blanks from previous histology in *S. officinalis*,[2,37,45,47,56,60] demonstrating that our current procedure effectively eliminates false positives (Figure 2).

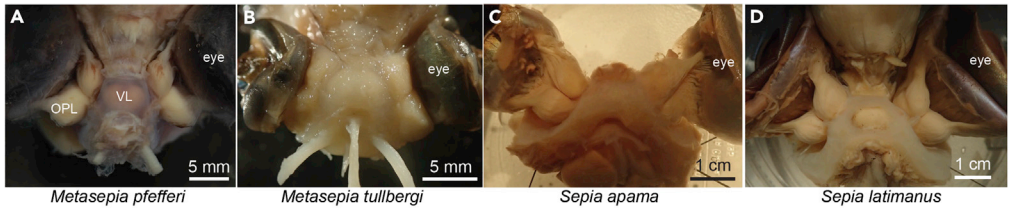
Despite a high degree of similarity between the connectomes of the squid, *S. lessoniana*,[63] and the cuttlefish, *S. plangon*, the connectivity strength of inter-lobed tractography (*Cs*, the logarithm of numbers of streamlines intersecting a pair of lobes) reveals the species-dependent patterns (e.g. *Cs* = 0.48–5.76 in *S. plangon* versus 0.48–5.1 in *S. lessoniana*). The highest *Cs* in *S. plangon* refers to the tractography between the VL and the subvertical lobe (sV) in contrast to it of *S. lessoniana* referred to the paired connectives linked with the brachial lobe (Br) and the anterior pedal lobe (aP) (Figures 2B, 2C, and 2S). Furthermore, a few remarkably strong inter-lobed connections can be identified as tracks unique to *S. plangon* but possibly present in other cuttlefish, including the network between the inferior frontal complex (iFLx, including inferior and posterior frontal lobes) and Br (chemosensory related circuits) (*Cs* = 2.0–3.8) and those related to the chromatophore (Ch), magnocellular and pedal lobes (coloration and locomotor circuits) (*Cs* = 1.6–2.8) (see the highlights in Figure 3D). In the squid connectome, similar tracts are either absent or with a much lower *Cs* value (Figures 2B–2D).

Chemosensory-related network

A previously unknown circuit between iF (chemosensory center) and sV (*Cs* = 2.8 $\pm$ 1.4) was identified in *S. plangon* (Figure 2D). In addition, although the percentage of brachial lobe (Br) relative to total CNS volume shows no difference between *S. plangon* (1.3 $\pm$ 0.3%, n = 3) and *S. lessoniana* (1.3 $\pm$ 0.5%, n = 5) (t-test, p = 0.79), the paired cerebral-brachial connectives between iF and Br are significantly stronger in cuttlefish (*Cs* = 3.6 $\pm$ 0.5) than in the squid (*Cs* = 1.8 $\pm$ 1.2) (t-test, p = 0.009) (Figures 2B–2D).

Vision-related network

The network between sV and OPLs in cuttlefish possess significantly stronger connectivity than squid (*Cs* = 4.1 $\pm$ 1.1 vs 2.6 $\pm$ 1.2, t-test, $p < 0.04$) (Figures 2B–2D). One additional set of neural components to coordinate the complex cuttlefish body patterns (a combination of chromatic and structural components) is revealed here (Figure 2D). The interweaving connections amongst OPLs and the connections related to coloration and locomotor control (lateral basal (lB), peduncle (Pe), chromatophore (Ch), magnocellular (M) and pedal (P) lobes) in the cuttlefish have strong connectivity (*Cs* = 2.9–5.2) where most have higher *Cs* values (ΔCs = 0.95 $\pm$ 0.52) than the counterparts in squid (Figures 2B–2D). Here we describe



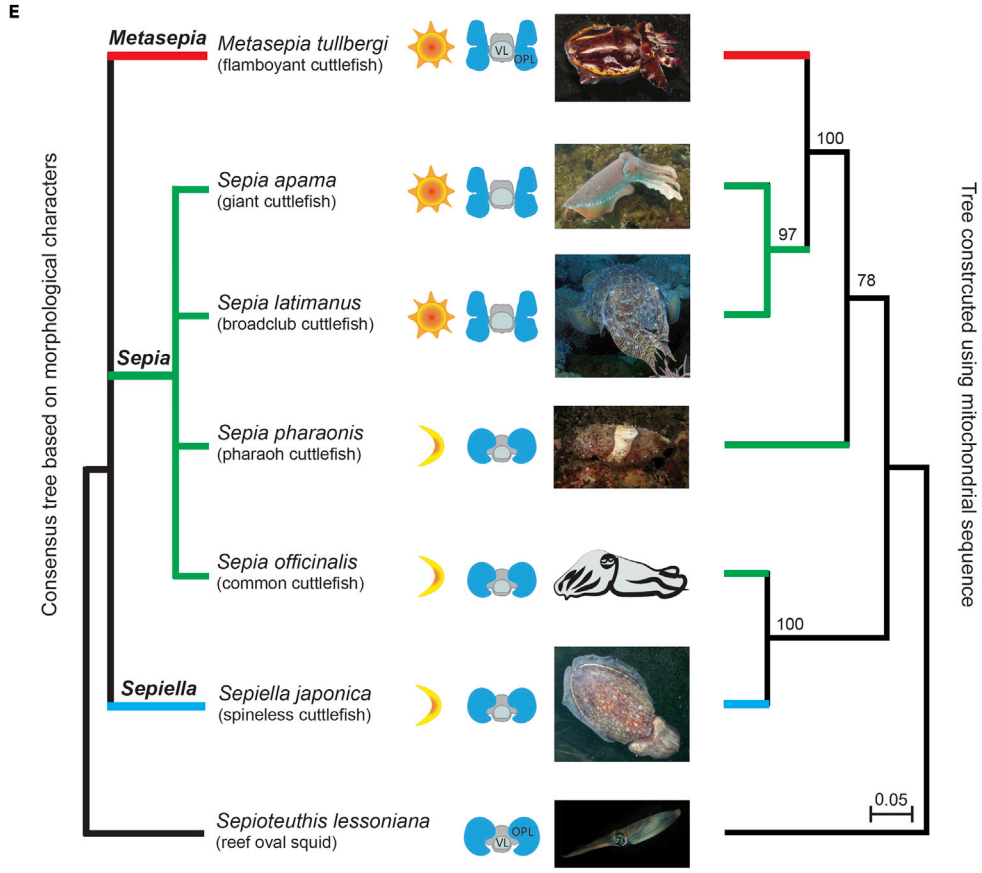


Figure 3. Neuroanatomical features of the optic lobe of the selected decapodiform cephalopods and correlations between phylogenetics and life modes

A similar feature to the croissant-shaped OPL described in *S. plangon* is identified in another four diurnal cuttlefish species, including.

(A) *Metasepia pfefferi* (adult, female, ML = 110 mm).

(B) *Metasepia tullbergi* (subadult, male, ML = 48 mm).

(C) *Sepia apama* (adult, male, ML = 203 mm).

(D) *Sepia latimanus* (adult, female, ML = 238 mm).

(E) Two possible phylogenetic relationships compared to brain morphologies. Using the squid as the outgroup, correlations between phylogenetics and neuroanatomical features are revealed in selected cuttlefish (6 species from 3 genera). Left: A consensus tree based on conventional morphological features (mainly cuttlebone morphology combined with tentacular, and reproductive characters) which are used to define the current three valid genera as *Sepia* (green bar), *Sepiella* (blue) and *Metasepia* (red).[3,4,9,84,85] Right: A molecular phylogenetical tree constructed using published entire mitochondrial DNA sequences of the selected species (STAR Methods). Given the disproportionately short sequence data of *S. plangon* and *M. pfefferi*, both of which possess croissant-shaped OPLs, both species were excluded. The bootstrap values are shown in front of the branch node. Notably, this molecular tree does not match the consensus tree (Left). For example, the two diurnal *Sepia* species, *S. apama* and *S. latimanus*, were grouped with *Metasepia*, whereas a nocturnal *S. officinalis* is grouped with *Sepiella*. Uncertainty around cuttlefish phylogeny (molecule versus morphology) has been noted repeatedly by previous studies.[86,87] Middle: Two types of OPLs are present in the selected cuttlefish. The neuroanatomical features (bean versus croissant OPL) and corresponding habit and habitats were based on the current

Figure 3. *Continued*
study and the published literature[3,4,7,63,73,76] (See also Tables 1 and S4). Two diurnal *Metasepia* species have the croissant-shaped OPL (A and B), whereas nocturnal *Sepiella* contains the bean-shaped OPL in common with *S. officinalis*. In contrast, two forms of OPL can be found in the members of *Sepia*, indicating that the two OPL forms might have been evolved multiple times within this largest cuttlefish group during its rapid diversification. The neuroanatomical features identified in this study could be included along with a growing number of behavioral, ecological and genomic data to refine cuttlefish phylogenetic analyses. The sun indicates diurnally active species and the moon as nocturnally active species, alongside diagramatic representations of brain regions: optic lobe (OPL) (dark blue); vertical lobe (VL) (light blue); central complex excluded VL (gray).
See also Tables S3 and S4.

18 additional inter-lobed connections potentially associated with cuttlefish camouflage because of their endpoints in chromatophore lobes and skin control areas, including 4 tracts amongst anterior Ch (*Cs* = 1.4–3.1), 6 tracts between posterior Ch and M (*Cs* = 2.3–3.8), 4 tracts linked to lB (lB-ventral M) (*Cs* = 2.4–2.9) and 4 tracts linked to Pe (Pe-anterior P, Pe-posterior-P, a pair of Pe-ventral M) (*Cs* = 2.5–2.9) (Figure 2C). Given these unique neuroanatomical features and known function of these six brain regions,[37] these previously-unknown cuttlefish circuits are likely involved in control of color and texture patterns alongside the two previously known circuits (OPL-lB-Ch and OPL-Pe-lB-Ch).[37,58,70,82]

Shapes of the cuttlefish optic lobes related to their life modes

Based on anatomical evidence alone, the croissant-shaped OPLs are identified here in another four diurnal Indo-Pacific cuttlefish species, including three from tropical waters (two flamboyant cuttlefish, *Metasepia pfefferi* and *Metasepia tullbergi*, and the broadclub cuttlefish, *Sepia latimanus*) and one in temperate waters (Australian giant cuttlefish, *Sepia apama*) (Figure 3 and Table 1). The impact of several variables (life modes and light conditions) on the modification of OPL are included in this extended comparison with the two types of phylogenetic relationship: (1) a consensus phylogeny based on morphological features alone (including cuttlebone, tentacular and hectocotylus characters).[4,9,84] (2) the molecular phylogeny constructed with the available full mitochondrial sequences (Figures 3E, Tables 1, S4 and STAR Methods).

Notably, the mitochondria-based phylogenetic relationship amongst the selected six cuttlefish species is mismatched with the well-known correlation of the three valid genera of cuttlefish (Figure 3E). Particularly, some species from the genus, *Sepia*, were associated closely with the genera, *Metasepia* and *Sepiella*, whereas these two genera contain the distinct morphological features unparalleled to those of *Sepia* (Figure 3E). Therefore, unlike the well-matched phylogenetic results (molecule and morphology) found in octopuses,[55] Pagel's λ and a phylogenetic generalized least squares (PGLS) analyses suggested that no clear link can be yet drawn between the molecular phylogeny and the OPL morphological changes (bean versus croissant shape) in cuttlefish (Pagel's $\lambda < 0.00001$ for all 7 species; test of $\lambda = 0$, $p = 1$) (Figure 3 and Table S4). This may be the result of a small dataset as well as the long standing unsolved controversial phylogenetical status of sepiids (Figure 3E), particularly the genus *Sepia* which currently represents over 92% of known cuttlefish species (115 species).[4,9]

Despite no attempt to clarify uncertainty of cuttlefish phylogeny in the current study, the qualitative analysis (neuroanatomical features related to the consensus phylogeny) combined with the quantitative analysis (PGLS, ambient light, $p = 0.003$ (mitochondria-based data)) may suggest that those diurnal dwellers' OPLs are potentially driven by the detailed requirements of their life modes (diurnal versus nocturnal). This type of analysis is generally subjective, often weighted by a pre-desired outcome and despite its intent, may ignore time frames of rapid evolutionary radiations, particularly within *Sepia*.[9,86] This finding also provides avenues for further research to refine analyses with a combination of molecular, morphological, ecological and behavioral aspects (see detailed discussion below).

DISCUSSION

In common with their major competitors, the fish, coastal cephalopods are successful and voracious vision dominant predators that live over a broad range of ecological niches. In contrast to our knowledge of fish neuroanatomical adaptations related to sensory perception, foraging modes and habitats,[88–91] establishing links between behavioral features and neuroanatomical modifications remains in its infancy for the cephalopods.[55,69,92] Using MRI-based techniques and conventional histology, we have started the first detailed comparison of neuroanatomical features and corresponding MRI-based connectomes between cuttlefish and squid (Figures 2 and S2). This work focuses on the diurnal cuttlefish and unfolds that

heterogeneity of neuroanatomical features indeed exist amongst cuttlefish. With our previous studies on squid and octopus[55,63,66] as well as numerous pioneering work on these brainy invertebrates,[2,12,32,37,38,45–47,50,65,69,93] this study is able to draw parallels and differences related to habitat and ecology (Figures 2,3 and S2).

Heterogeneity of cuttlefish brain structure linked to their life modes

For its body size, *S. plangon* possesses an enlarged brain compared to the other coastal species which are often active in dim conditions (Figure 1G and Table S3). For instance, the hatchling of *S. plangon* (ML = 6–9 mm) has significantly enlarged OPLs and VL, compared to those of *S. officinalis* (ML = 6–10 mm) (80 vs 60% and 2.4 vs 0.3% respectively)[62,94] (Figure 1G). With the black eggs of *S. officinalis*[34] and *Sepiella japonica*[3] which result in poor visibility of the outside scene, the translucent eggs of the diurnal cuttlefish species[3,72] allow embryos receive amounts of surrounding visual cues and react accordingly with flashing chromatophores. This early vision-related capability, presumably prepares these species for the post-hatching environment such as camouflage among coral, rubble or shallow coastal environments (e.g., seagrass meadow).[19] On the other hand, nocturnal *S. officinalis* and *S. japonica* often burry in sand to conceal the body outline during daytime and are more active hunting and interacting at night[4,7] and therefore do not need the OPL size or complexity seen in the diurnal species (Figure 3E).

The broadclub cuttlefish, *S. latimanus*, is a large diurnal reef-dwelling species also with a very large hatchling (ML = 11–15 mm) and in common with *S. plangon* already shows a visible croissant-shaped OPLs (ca. 82% of CNS volume).[74,95] The continuous and rapid expansion of the brain of these cuttlefish during their life span (Figures 1G,3D, and 3E), particularly the croissant-shaped OPL and large VL, may also reflect the role of vision and the complex decisions around learning and memory including foraging and threat avoidance in well-lit environments that contain both abundant food and visual predators.[42,72,93]

With the observation of morphological disparity amongst cuttlebones (102 species from 3 genera) across 17 biogeographical areas, Neige [9] proposed that a rapid diversification of cuttlefish from Japan to Europe occurred from an ancestor in the Indo-Pacific regions. However, this hypothesis has not been thoroughly tested yet with other factors such as genomic, behavioral or ecological data. To address the currently partially satisfied phylogenetic relationship of cuttlefish with refined analyses needs to include more morphological characters, both hard and soft structure, combined with a growing number of behavioral, ecological and genomic data.[1,3,9,84,86,87] The heterogeneity of cuttlefish brain structure found in this study provides additional characters at neuroanatomical and ecological aspects which could further test phylogenetical and evolutionary hypotheses and explain the observed patterns of these coastal water creatures.

Additional circuits for cuttlefish camouflage

Both chromatic and hydrostatic (papillae) systems are regularly used in the formation of cuttlefish body patterns for camouflage and courtship display.[1,3,12,13,16,19] For instance, *S. plangon* uses 34 chromatic components combined with 3 textural and 14 postural components for dynamic courtship displays (11 patterns used by female; 18 by male).[19] In contrast, squid mainly rely on chromatic components such as *S. lessoniana* assembling 27 chromatic components during reproductive interactions (7 patterns by female; 12 by male).[77] The complexity of camouflage tricks cuttlefish and squid demonstrate is also substantial, but again the former outstripping the latter. Cuttlefish camouflage contains a combination of cryptic coloration, skin texture and arm posture to conceal itself into the 3D characters of the surrounding scene[1,3,12,96] (Video S4). By contrast, the squid mainly relies on color changes on body surface to mimic the 2D background by manipulating colors to match with substrate while reaching close to floor and switching to countershading while hovering in water column.[3,18,97] Exactly how the cuttlefish nervous system dispatches signals via the additional 18 pathways identified here to govern skin patterns (Figure 2C) remains for future research.[11,13,18,98,99]

Neuroanatomical adaptations of coleoid cephalopods to different sensory modes

The proportion of neural processing investment in chemoreception and vision amongst cuttlefish, squid and octopus is variable.[2,55,63,66,69] All three groups possess large camera-like eyes and put considerable investment into the OPL processing of vision[66,67,69,78,100] (but see ecological differences discussed in Chung and Marshall[66] and Chung, et al.[55]). In addition, MRI-based connectomes provide new insights which confirm a high degree of similarity in the inter-lobed network between squid and cuttlefish CNS

(Figure 2). Vision-related tractography in particular (Figures 2B and 2C) highlights that cuttlefish and squid have adopted similar principles in design in response to visually-coordinated activities, including those regional networks such as OPL-SUPRA (e.g. mediating eye movements and coloration) and OPL-SUB (e.g. locomotion maneuver of arms, funnel and fins).[37,38,47,58,63]

The close to bottom dweller cuttlefish, *S. plangon*, and the water column dweller squid, *S. lessoniana*, possess relatively small chemosensory regions (iFLx), approximately 0.3–0.5% of CNS volume (Figure S2) compared to those of the entirely benthic octopuses (4–6%).[55,63,69] While comparing the volume ratio between the two sensory brain regions, vision (OPLs) vs chemoreception (iFLx), a further distinct difference was identified, such that the relative value reaches over 100-fold in cuttlefish (e.g. *S. officinalis*[73]; *S. plangon*(235)), >200 in loliginid squids (e.g. *S. lessoniana*(220); *Loligo forbesi* (305)) compared to a very low value around 10 in most benthic and nocturnal octopuses (e.g., *Octopus vulgaris* and *Hapalochlaena fasciata*).[55,63,69] Furthermore, a comparison of the *Cs* value (Br-iF) between *S. plangon* (3.77) and *S. lessoniana* (0.61) confirms a previous qualitative description indicating that strong cerebrobrachial connectives exist in cuttlefish, *S. officinalis*, whereas fewer stained neurons are seen in squid, *Loligo vulgaris*.[47] The increasing complexity of neural interconnection in cuttlefish chemosensory center indicates that they may favor chemosensory cues in daily tasks and more so than squid.

In the behavioral context, bait coated with amino acids, quinine or cephalopod ink, may be accepted or rejected by touching the bait using arms/tentacles in the cuttlefish, *Sepia esculenta*.[31] A similar bait handling behavior has been found in *S. plangon* and *S. latimanus* during the bait feeding in captivity. By contrast, using the same method rarely triggered feeding acceptance by squid that appear to need movement cues to trigger bait capture (personal observation). It is worth noting that octopus arms can rely on chemotactile sense alone for voracious foraging behavior within crevices that are inaccessible by vision.[101,102] This indicates that cuttlefish possess good contact chemosensory capabilities, somewhere between octopus and squid, an ability helpful in prey preference and tune foraging strategies.[1,3,31,66]

The features of sensory systems and underlying brain adaptations described here mirror ecology and habitat,[17,55,66,78,100] suggesting that the water column dwellers rely more on vision, whereas the more benthic groups favor a weighted combination of vision and chemoreception.

Limitations of study

This study reveals the first mesoscale MRI-based neural connectivity in the diurnal cuttlefish brain and extends comparisons to other cephalopod species for which the neuroanatomical data exist. However, it is possible that some limitations could affect the results presented here. To date, neither the morphology- nor the molecular approach is sufficient to pin down any characters to a common evolutionary origin, leaving contradictory results for cuttlefish phylogenetic relationships.[84,85,87] Remarkably, the main incongruence is the genus, *Sepia*, the biggest group of cuttlefish, where some members of this species complex were included in three different genera noted by multiple genomic studies.[86,87,103] As a result, the current PGLS test was thus not yet consolidated allowing the correlation between molecular phylogenetic signals and the shape of OPL. Future work needs to include more species and a combination of morphological (now including brain structures), molecular, behavioral, ecological and geographic data to generate a phylogenetic estimate.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Sepioteuthis lessoniana	Wild (Moreton Bay, QLD, Australia)	N/A
Sepia plangon	Wild (Moreton Bay, QLD, Australia)	N/A
Sepia latimanus	Wild (Lizard Island, QLD, Australia)	N/A
Sepia apama	Wild (Whyalla, SA, Australia)	N/A
Metasepia pfeffefferi	Wild (Moreton Bay, QLD, Australia)	N/A
Metasepia tullbergi	Wild (Kuei-Hou, Taiwan)	N/A
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde	Electron Microscopy Science	Cat# 15170
Magnesium chloride	Chem-Supply	Cat# MA029-500G
Magnevist	Bayer	Cat# NDC 50419-188-82
Fomblin	Solvay	Cat# LVOF066K
Deposited data		
MRI and histology	This study	https://doi.org/10.48610/0f9fcbe
Software and algorithms		
Paravision 6	Preclinical MRI software, Bruker Biospin	RRID:SCR_001964
MRtrix3	version 3.0.2, open-source software Tournier et al.[104]	RRID:SCR_006971
ITK-SNAP	version 3.8.0, open-source software Yushkevich et al.[105]	RRID:SCR_002010
Fiji	NIH, version 1.53c, open-source software Schindelin et al.[106]	RRID:SCR_002285
ANTs	Advanced normalization tools	RRID:SCR_004757
MEGA X	version 10.2.5, open-source software Kumar et al.[107]	RRID:SCR_000667
Rstudio	Version 1.4.1103, open-source software	RRID:SCR_000432
Helicon Focus Pro	version 7.6.4, Helicon Soft Ltd. Ukraine	RRID:SCR_014462
Adobe Illustrator	Adobe	RRID:SCR_010279
Other		
Complete mitochondrial genome of *Sepioteuthis lessoniana*	Akasaki et al.[108]	GenBank: NC_007894
Complete mitochondrial genome of *Metasepia tullbergi*	Lee et al.[103]	GenBank: MT974497.1
Complete mitochondrial genome of *Sepiella japonica*	GenBank	GenBank: NC_017749.1
Complete mitochondrial genome of *Sepia apama*	Kawashima et al.[109]	GenBank: NC_022466.1
Complete mitochondrial genome of *Sepia latimanus*	Kawashima et al.[109]	GenBank: NC_022467.1
Complete mitochondrial genome of *Sepia officinalis*	Akasaki et al.[108]	GenBank: NC_007895.1
Complete mitochondrial genome of *Sepia pharaonis*	Wang et al.[110]	GenBank: NC_021146.1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Wen-Sung Chung (w.chung1@uq.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper analyses existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- Data have been deposited at the University of Queensland's institutional repository, UQ eSpace, and are publicly available. DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The specimen collections (between 2017 and 2021) were conducted under a Great Barrier Reef Marine Park Permit (G17/38160.1), a Moreton Bay Marine Part Permit (QS2013/CVL625) and Queensland General Fisheries Permit (180731 & 202976). The mourning cuttlefish, *S. plangon* (n = 44, including 25 hatchlings; 11 juveniles; 4 adult males; 4 adult females), the flamboyant cuttlefish, *M. pfefferi* (n = 1, adult female), and oval squid, *S. lessoniana* (n = 5, juveniles), were collected using a seine net (water depth 1-3m) close to Moreton Bay Research Station, Stradbroke Island, QLD, Australia. Two broad club cuttlefish, *Sepia latimanus* (1 adult male and 1 adult female), were collected in 2019 using Scuba in Lizard Island, QLD, Australia. All experimental animals were held and handle according to the guidelines for the EU Directive 2010/63/EU for cephalopod welfare in order to minimize the suffering and distress of the animals. The experimental protocols and MRI procedures were performed underUniversity of Queensland Animal Ethics permit numbers: QBI/236/13/ARC/US AIRFORCE and QBI/304/16.

Additional two cuttlefish species used for the brain morphological examination were collected by local fishmen, including two Australian giant cuttlefish, *Sepia apama* (1 adult male and 1 adult female), from Whyalla, SA, Australia in 2009 and two *M. tullbergi* (1 subadult male and 1 subadult female) from Kuei-Hou, Taiwan in 2021.

METHOD DETAILS

Sample fixation

All animals were anesthetized in cool seawater (15°C) mixed with 2% $MgCl_2$ (Chem-Supply, Australia) and sacrificed by an overdose of $MgCl_2$. The specimens were then soaked into 10% neutral formalin or 4% paraformaldehyde (PFA) (EM grade, Electron Microscopy Sciences, Hatfield, USA) for at least 48 h and then transferred to 0.1% PFA-PBS fixative for storage at 4°C until further dissection for gross anatomy examination and histology.

Three adults of *S. plangon* (ML = 71–107 mm) and five juveniles of *S. lessoniana* (ML = 40–113 mm) for MR imaging were fixed using the transcardial perfusion protocol developed by Chung, et al.[63] In brief, the animals were anesthetized in cool seawater (15°C) mixed with 2% $MgCl_2$ and sacrificed by an overdose of $MgCl_2$prior to fixation. The transcardial perfusion protocol is using 4% PFA mixed with 0.1 M PBS with the rate of perfusion set to 2.5 mL per minute. The perfusion proceeded until 0.2 mL fixative per gram of specimen was used. Subsequently the muscle, skin and connective tissues around the brain were removed and the specimen was soaked in 4% PFA-PBS fixative for overnight to reduce morphological deformation of the brain.

Image stacking of the isolated brain-eyes

The isolated brain and eyes were imaged with the focus stacking method using a digital camera (Canon 5D4 camera with Canon MPE 65 mm Macro lens, Canon, Japan) mounted on the electronically-controlled focusing rack (Castel-Micro focusing rack, Novoflex, Germany). A sequence of close-up images was captured from the dorsal end of brain to the ventral end using 0.1 mm step for small samples or 0.25 mm step for large samples. Focus stacking (20–80 images) was processed using the software Helicon Focus Pro (version 7.6.4, Helicon Soft Ltd. Ukraine), rendering an image with a greater depth of field.

Conventional histology and imaging

The isolated cuttlefish brain and eyes were processed with a standard protocol of wax histology. In brief, the samples were dehydrated in increasing concentrations of ethanol, delipidised in xylene and paraffinized in a histology processor. Sections (10–15 μm thickness) were prepared using the microtome (Leica RM2235, Germany) and stained with Haematoxyline and Eosin. The selected horizontal sections were imaged using a digital camera (Canon 5D4 camera with Canon MPE 65 mm 1-5X Macro lens at 3X magnification, Canon, Japan) mounted on the electronically-controlled focusing rack (Castel-Micro focusing rack, Novoflex, Germany). Additional processing of images for brightness adjustment was performed using Fiji (version 1.53c, open-source software, https://imagej.net/).[106]

MRI procedure

Intact brain and eyeballs were isolated and repeatedly rinsed with 0.1 M PBS to minimise fixative residue. The isolated brain and eyes were then soaked into 0.1 M PBS containing magnetic resonance imaging (MRI) contrast agent, 0.2% ionic Gd-DTPA (Magnevist) (Bayer, Leverkusen, Germany), for 24–48 h to enhance image contrast prior to MR imaging.[55,63] Five contrast-enhanced cuttlefish brains and one intact cuttlefish juvenile were imaged following the protocol developed by Chung, et al.[63] The contrast-enhanced specimen was placed into a fomblin-filled (Fomblin oil, Y06/6 grade, Solvay, USA) container to prevent dehydration and then placed in a vacuum chamber for 3 min to remove air bubbles trapped inside esophagus or brain lobes. The container was then placed in a custom-built 20 mm diameter surface acoustic wave coil or 10 mm diameter quadrature coil (M2M Imaging, Brisbane, Australia). Both high resolution MR structural images and high angular resolution diffusion images (HARDI) were acquired using a 16.4 T (700 MHz) vertical wide-bore microimaging system (interfaced to an AVANCE I spectrometer running imaging software Paravision 6.0.1 (Bruker BioSpin, Karlsruhe, Germany) in the Center for Advanced Imaging at the University of Queensland. Imaging was performed at a room temperature (22°C) using a circulating water-cooling system.

Three dimensional (3D) high resolution structural images were acquired using fast low angle shot (FLASH) with the following parameters based on Chung and Marshall[66]: echo time (TE)/repetition time (TR) = 12/40 ms, average = 4, flip angle (FA) = 30°, field of view (FOV) = 7.5 × 6.4 × 6 mm to 21 × 13 × 13 mm for different individuals, 30 μm isotropic resolution. Total acquisition time for one brain was 1 h (hatchling) to 8.3 h (the largest brain).

After FLASH imaging, 3D high angular resolution diffusion-weighted imaging (HARDI) was acquired with the following parameters: TR = 300 ms, TE = 22 ms, 30 direction diffusion encoding with b-value = 3000 s/mm^2, two b0 images acquired without diffusion weighting and 80 μm isotropic resolution with 1.5 partial Fourier acceleration acquisition in the phase dimensions.[63] Total acquisition time for one brain was 16.5–35.5 h.

Estimates of lobe volume

Identification of the cuttlefish brain lobes was based on the published anatomical studies of cuttlefish and loliginid squids as an initial aid in determining the boundaries between tissue. 47 lobes previously defined by[37,57–61,63] were identified from the MRI data. The parcellation of the selected lobes and brains was then manually segmented using MRtrix3 (version 3.0.2, open-source software, http://www.mrtrix.org/)[104] and then estimates of volume of the selected lobes and an entire brain were calculated using ITK-SNAP (version 3.8.0, open-source software, http://www.itksnap.org/).[105] Considering variations of volume estimates of cephalopod brain which are strongly affected by the size and age of the individuals, the volumes of the lobes were expressed as percentages of the total CNS volume to circumvent this issue as suggested in previous studies.[55,63,69]

Construction of structural neural connectivity matrix

Our previous work demonstrated that the high resolution HARDI combined with conservative selection criteria enabled to accurately reveal the major neural tracts in the squid brain and octopus optic nerve tracts.[55,63] Adapting the same procedure to construct the brain-wide tractography of cuttlefish brain, the 47 lobes, regions of interest (ROIs) were used to construct tractography. Probabilistic fiber tracking was then performed using second order integration over the fiber orientation distribution (FOD) algorithm and the tracts were generated independently for each ROI (10 streamlines per voxel) with an optimized FOD amplitude cut-off value of 0.175 to generate biologically realistic tractography in cephalopod neural tissue at mesoscale. The brain-wide cuttlefish neural connectivity matrix where the connections and the corresponding connectivity strength (*Cs*) were mapped to the relevant cuttlefish brain lobes for each individual. The averaged pairwise *Cs* were also calculated and plotted in the matrices for further analysis with the previously-published squid matrix.[63]

Contour-based measurement of gyrification index (GI)

The degree of folding of the optic lobe was measured using the contour-based method.[55] We measured the GI by comparing the lengths of complete and outer contours of the selected brain lobes in a serial horizontal MR slices for the OPLs along with the dorsoventral axis using Fiji (version 1.53c, open-source software, https://imagej.net/).[106] The mean GI of the defined entire lobe is the ratio between the sum of the total outer contour and the sum of the superficially exposed surface contours.

Phylogenetic analyses

In order to understand whether the phylogenetic relationship or the life mode affect the modification of cuttlefish brain, the phylogenetic generalised least squires (PGLS) test was used to investigate the impact of several predictor variables (life modes, light conditions, and visual tasks) on the modification of neuroanatomical structure while controlling for potential phylogenetic signals in the responses.[111] Determination of the selected cuttlefish phylogenetic relationships was based on the published complete mtDNA sequence which were available from GenBank. Alignments of sequence were constructed using the multiple sequence alignment (MUSCLE) method with MEGA X (molecular evolutionary genetics analysis program version 10.2.5).[107] *S. lessoniana* was used as the outgroup. The phylogenetic tree of these selected species was generated by the Maximum-Likelihood method and the bootstrap confidence values (1000 replicates) were calculated with MEGA X.[107]

QUANTIFICATION AND STATISTICAL ANALYSIS

The phylogenetic signal was estimated with Pagel's λ using the package the CAPER v1.0.1 as implemented in the RStudio v1.4.1103. The relationship between the changes of brain anatomy and environmental characters (Table S3) was determined using the phylogenetic generalised least squares (PGLS) method with the CAPER package in RStudio.

The t-tests were used to determine if the diurnal cuttlefish has significantly enlarged brain lobes to those of the nocturnal species and the higher connectivity strength of the selected inter-lobed connectivity than those of the squid. All statistical analyses were performed with the RStudio, with significance set at $p < 0.05$.