


RESEARCH ARTICLE

Morphological changes of the optic lobe from late embryonic to adult stages in oval squids *Sepioteuthis lessoniana*

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Funding information

Ministry of Science and Technology of Taiwan (to CCC), Grant/Award Number: NSC-102-2628-B-007-001-MY3

Abstract

The optic lobe is the largest brain area within the central nervous system of cephalopods and it plays important roles in the processing of visual information, the regulation of body patterning, and locomotive behavior. The oval squid *Sepioteuthis lessoniana* has relatively large optic lobes that are responsible for visual communication via dynamic body patterning. It has been observed that the visual behaviors of oval squids change as the animals mature, yet little is known about how the structure of the optic lobes changes during development. The aim of the present study was to characterize the ontogenetic changes in neural organization of the optic lobes of *S. lessoniana* from late embryonic stage to adulthood. Magnetic resonance imaging and micro-CT scans were acquired to reconstruct the 3D-structure of the optic lobes and examine the external morphology at different developmental stages. In addition, optic lobe slices with nuclear staining were used to reveal changes in the internal morphology throughout development. As oval squids mature, the proportion of the brain making up the optic lobes increases continuously, and the optic lobes appear to have a prominent dent on the ventrolateral side. Inside the optic lobe, the cortex and the medulla expand steadily from the late embryonic stage to adulthood, but the cell islands in the tangential zone of the optic lobe decrease continuously in parallel. Interestingly, the size of the nuclei of cells within the medulla of the optic lobe increases throughout development. These findings suggest that the optic lobe undergoes continuous external morphological change and internal neural reorganization throughout the oval squid's development. These morphological changes in the optic lobe are likely to be responsible for changes in the visuomotor behavior of oval squids from hatching to adulthood.

KEYWORDS

cell islands, cephalopods, neural connections, ontogenetic development, visuomotor control

1 | INTRODUCTION

Cephalopods are known for their sophisticated behavior including camouflage, learning, memory, and cognitive functioning (Darmaillacq, Dickel, & Mather, 2014; Hanlon & Messenger, 1996). These behaviors are controlled by the most complex central nervous system (CNS) found in invertebrates (Hochner, 2008). The CNS of cephalopods mainly consists of brain tissues around the esophagus and includes the supraesophageal mass, the subesophageal mass, the periesophageal

lobes and a pair of optic lobes (Nixon & Young, 2003). These optic lobes are the largest brain areas in coleoid cephalopods (Boycott, 1961; Young, 1962, 1974) and they function as visual processing centers and upstream control centers for body patterning, locomotion, and learnt behaviors (Boycott, 1961; Chichery & Chanelet, 1976, 1978; Chung & Marshall, 2017). The optic lobe is located immediately behind the eye ball and receives visual signals from the retina directly (Boycott, 1961; Young, 1962, 1974). Across the chiasmata, the optic nerves project onto the outer surface of the optic lobe, namely the cortex or the deep

retina (Cajal, 1917), to allow visual information processing. Beneath the cortex is the medulla of the optic lobe and this area can be further divided into two major zones, the outer radial column zone and the center tangential zone (Young, 1974). The radial column zone is composed of numerous columnar structures of stacked cell somata and neural fibers (Young, 1974). In contrast, the tangential zone is less organized and has many clustered cell soma regions (also called the "cell islands") that are surrounded by neuropils (Young, 1974). The optic lobe then sends the output signals to other brain lobes including downstream motor centers for further information processing and the control of visual behavior (Young, 1974).

It has been well-documented that the various visual behaviors of cephalopods are affected by the early visual experiences during their embryonic and larval stages (Darmaillacq, Chichery, Shashar, & Dickel, 2006; Dickel, Boal, & Budelmann, 2000; Guibe & Dickel, 2011; Lee, Yan, & Chiao, 2010; Lee, Yan, & Chiao, 2012; Poirier, Chichery, & Dickel, 2005; Romagny, Darmaillacq, Guibe, Bellanger, & Dickel, 2012). In addition, the complexity and diversity of body patterning, which is used for visual communication, increases continuously from the juvenile stage to adulthood in cephalopods (Byrne, Griebel, Wood, & Mather, 2003; Jantzen & Havenhand, 2003; Moynihan & Rodaniche, 1982; Suzuki, Kimura, Ogawa, Hotta, & Oka, 2011; Yang et al., 1986). These behavioral changes and the associated plasticity throughout development are highly dependent on neural reorganization within the optic lobes (Liu, Liu, Su, & Chiao, 2017). Previous studies have described general features of optic lobe development in cephalopods (Marquis, 1989; Meister, 1972; Wentworth & Muntz, 1992). Specifically, it has been shown that both the volume and the proportion of the optic lobe in sepiolid squids keep increasing throughout the animal's embryonic stages (Kerbl et al., 2013). The neuropils within the optic lobe of the oegopsid, loliginid, and pygmy squids appear earlier in the cortex and tangential zone compared to the radial column zone (Shigeno, Kidokoro, Goto, Tsuchiya, & Segawa, 2001; Shigeno, Kidokoro, Tsuchiya, Segawa, & Yamamoto, 2001a; Yamamoto, Shimazaki, & Shigeno, 2003). Furthermore, the proportion of neuropils in the tangential zone shows a significant increase from embryos to juveniles in the oegopsid and loliginid squids (Kobayashi, Takayama, & Ikeda, 2013; Shigeno, Kidokoro, Tsuchiya, Segawa, & Yamamoto, 2001b; Shigeno, Tsuchiya, & Segawa, 2001). Despite these detailed morphological studies on various developing cephalopods, an ontogenetic characterization of the optic lobe from the late embryonic to juvenile and adult stages has not been conducted systematically.

The oval squid *Sepioteuthis lessoniana* displays dynamic body patterns during visual communication and these are particularly associated with reproductive behavior (Lin, Tsai, & Chiao, 2017). The visual system of *S. lessoniana* is particularly interesting. For example, it has been reported that there is a special retinal bump structure in the eye of *S. lessoniana* that has been correlated with the bobbing behavior used to estimate the distance of a small object (Chung & Marshall, 2014). In addition, the size of the retinal bump and the amount of bobbing behavior decrease as oval squids develop, which suggests that this structural alteration is associated with functional adaptation. In the present study, the optic lobes of *S. lessoniana* from late embryonic to

adult stage were characterized using magnetic resonance imaging (MRI) and micro-computerized tomography (micro-CT). The detailed internal structure of the optic lobes was also examined by histology and confocal microscopy. By systematically quantifying the morphological changes within the optic lobes throughout development, the present study provides the first structural evidence in the oval squid of brain changes from embryo to adulthood that seem to bring about behavioral adaptation.

2 | MATERIALS AND METHODS

2.1 | Animals

Five different developmental stages of *Sepioteuthis lessoniana*, Férussac, 1831 (embryo, hatchling, juvenile, subadult and adult) were examined as part of the present study. Embryos (stage 26) (Shigeno, Tsuchiya, & Segawa, 2001) and hatchlings were reared from eggs collected off the coast of Keelung, Taiwan. Juvenile (mantle length 3–5 cm), subadult (mantle length 8–15 cm), and adult (mantle length 20–30 cm) squids were captured in the same area. The specimens were transported to the National Tsing Hua University and kept in an aquarium with circulating sea water (24°C) before experimentation. For the whole brain MRI-scan (described below), a juvenile (mantle length 3 cm) and a subadult (mantle length 11.3 cm) were collected near the Moreton Bay Research Station, Stradbroke Island, Queensland, Australia. The maintenance and experimental protocols were covered by animal ethics permit (QBI/223/10/ARC/US AIRFORCE (NF)).

2.2 | Micro-CT and MRI

A hatchling was subjected to micro-CT at the National Synchrotron Radiation Research Center, Hsinchu, Taiwan. Before scanning, the animal was anesthetized using 3% MgCl₂ added to sea water (Mooney, Lee, & Hanlon, 2010), and then fixed with 10% formalin in sea water for one day. Next, the individual was incubated with 3% elemental iodine in 100% methanol for two days. A serial dehydration and embedding protocol (10%, 30%, 50%, 70%, 95%, absolute ethanol, followed by 2:1, 1:1, 1:2 mixtures of absolute ethanol and polypropylene, finally 100% polypropylene) was then applied. The scanning protocol follows the one described in a previous study (Tsai et al., 2014). The sample in the polypropylene tip was immobilized and mounted on a sample holder. The photon energy was 4–39 keV with maximum intensity of about 7 keV. The synchrotron source beam current was kept constant at 300 mA by topping up. A CdWO₄ scintillator placed 4–10 cm from the specimen converted X-rays into visible light. The projected images were captured with an optical lens and a CCD camera (RT3, SPOT IMAGING™, 1,600 × 1,200 pixels). The specimen was rotated from 0° to 180°, taking projected images every 0.3° with an exposure time of 300 ms. The projection images were computer reconstructed to obtain cross-sectional slices of 1,600 × 1,600 pixels.

One juvenile and one subadult squid were subjected to whole brain MRI using the protocol developed by Chung and Marshall (2017) at the Center of Advanced Imaging, University of Queensland, Australia. These

two squids were anesthetized in cold seawater mixed with 2% MgCl_2 preserved in neutral formalin in the field, and then transported back to the laboratory. The individuals were soaked into 0.1 mol L^{-1} PBS that contained the MRI contrast agent ionic Gd-DTPA (1% Magnevist, Bayer, Leverkusen, Germany), overnight before imaging. Each contrast-enhanced specimen was placed into the fomblin-filled (Fomblin oil, Y06/6 grade, Solvay) container to prevent dehydration, and then subjected to vacuum for 15 min to remove air bubbles trapped inside the tissue. The container was then placed in a custom-built surface acoustic wave coil (10 mm diameter). Imaging was performed using a 700MHz wide-bore microimaging system (Bruker Biospin, Karlsruhe, Germany) that consisted of a 16.4 T vertical bore magnet interfaced to an AVANCE II spectrometer. All scans were performed overnight (12–18 hr) using a T_2^* -weighted 3D-Flash sequence (TR/TE = 50 ms/14 ms, average = 8), which resulted in a voxel resolution of 30 μm .

The optic lobes from three squids, namely one juvenile (mantle length 4 cm), one subadult (mantle length 10 cm), and one adult (mantle length 23 cm), were subjected to the MRI scanning (9.4T, Bruker Bio-Spec 94/20 USR) to obtain their 3D-structures at the Kaohsiung Chang Gung Memorial Hospital, Taiwan. These three squids were anesthetized using sea water mixed with 3% MgCl_2 , preserved in 10% formalin, and then maintained in 70% ethanol. Before scanning, each individual was embedded in agar containing ferric ions to reduce background noise. The MRI-scanning system consisted of a self-shielded magnet with a 20-cm clear bore and a BGA-12S gradient insert (12-cm inner diameter). This setup offered a maximal gradient strength of 675 mT m^{-1} and a minimum slew rate of 4,673 T $\text{m}^{-1} \text{s}^{-1}$. The optic lobe was imaged at high resolution using the TurboRARE-3D-torun sequence (TR/TE = 2,000 ms/35 ms, NEX = 1, for the juvenile and subadult; TR/TE = 3,000 ms/35 ms, NEX = 1, for the adult).

2.3 | Histology and image acquisition

All the samples were anesthetized and fixed following the same protocol as in micro-CT and MRI-scans. The optic lobes were carefully dissected from the squid, except for the optic lobes of the embryonic and hatchling squids, which were sliced in situ using the head of the squid since the size of these specimens was too small to allow proper dissection. After fixation for three days, the specimens were transferred from 10% formalin to 70% ethanol. They were then incubated with a mixture of OCT (tissue freezing medium) and 30% sucrose solution for cryo-protection. Next, they were embedded in OCT, which was followed immediately by cryosectioning. Sagittal section slices (30 μm) were cut using a cryostat (CM3050S, Leica) and the middle slices were collected for further processing. The slices containing sections of the optic lobe were rinsed with PBS (phosphate-buffered saline) and then mounted in glycerol in the presence of the nuclear dye DAPI (4',6-diamidino-2-phenylindole) to visualize the nuclei. For the immunostaining image, the optic lobe slices were incubated with 10% normal donkey serum, 0.5% Triton X-100, and 0.1% sodium azide in PBS for 1 hr at room temperature. After blocking, the slices were incubated with the primary antibody against acetyl α -tubulin (dilution 1:200; T7451, Sigma) for 1 day at 4°C to label neural fibers (Klagges et al., 1996;

Shigeno & Yamamoto, 2002). After extensive rinsing with PBS, the secondary antibody, donkey anti-mouse IgG conjugated with DyLight fluorophore 488 (dilution 1:250; Jackson), was applied overnight at 4°C to visualize the immunoreactivity. The histological images of the optic lobes (six embryos, six hatchlings, seven juveniles, eight subadults, and five adults) were acquired using an upright fluorescent microscope (Axioskop 2 mot plus, Zeiss) with either a 5X (A-Plan, NA 0.12, Zeiss) or a 10X (Plan-Neofluor, NA 0.3; Zeiss) objective lens depending on the sample size. The high resolution DAPI images of the optic lobe slices were acquired using a confocal microscope (LSM 510, Zeiss) with a 40X objective lens (Plan-NEOFLUAR, NA 0.75, Zeiss).

2.4 | Image analysis

All images acquired from micro-CT and MRI were processed with Avizo software (FEI Company) for data analysis and 3D-reconstruction. The volume ratio between the pair of optic lobe and the total brain (including the optic lobes themselves) was determined from the whole brain images of the micro-CT and MRI. In addition, based on the 3D-reconstructions of the micro-CT and MRI data, the extent of the concaveness on the lateral side of the optic lobe was calculated using a custom-written MATLAB program (The Math-Works). The concave estimation involved finding the depth of the concave and divided this by the presumed width of the optic lobe without the dent.

Quantification of the neural organization from the histological images of the optic lobe slices was carried out using ImageJ (National Institutes of Health). The thicknesses of the cortex and of the radial column zone were calculated by averaging ten measurements from each sample. The areas of the optic lobe, medulla, tangential zone, and cell islands in these slices were also determined in a similar way. The density of the nuclei in the tangential zone was determined by dividing the area of the nuclei (the DAPI signal) by the total area of the tangential zone. The average size of the nuclei was determined by measuring the areas of randomly selected individual nuclei ($N = 30$) within the cortex, the radial column zone, and the tangential zone separately. Note that only the middle section of the optic lobe was examined in the present study, thus these measurements are the representation of the central optic lobe. For statistical analysis, one-way ANOVA and the post hoc Tukey's test were used to determine whether there was a significant difference between data sets (SigmaPlot, NA).

3 | RESULTS

3.1 | The optic lobe of *Sepioteuthis lessoniana* has a prominent dent on the ventrolateral side at late developmental stages

The high resolution whole brain MRI-images of oval squids show the relationship between the eye and optic lobe, as well as various other connections between different brain areas (Figure 1a–c; supporting information Movie S1). It is apparent that the pair of optic lobes on both sides of the squid head make up a large part of the space within

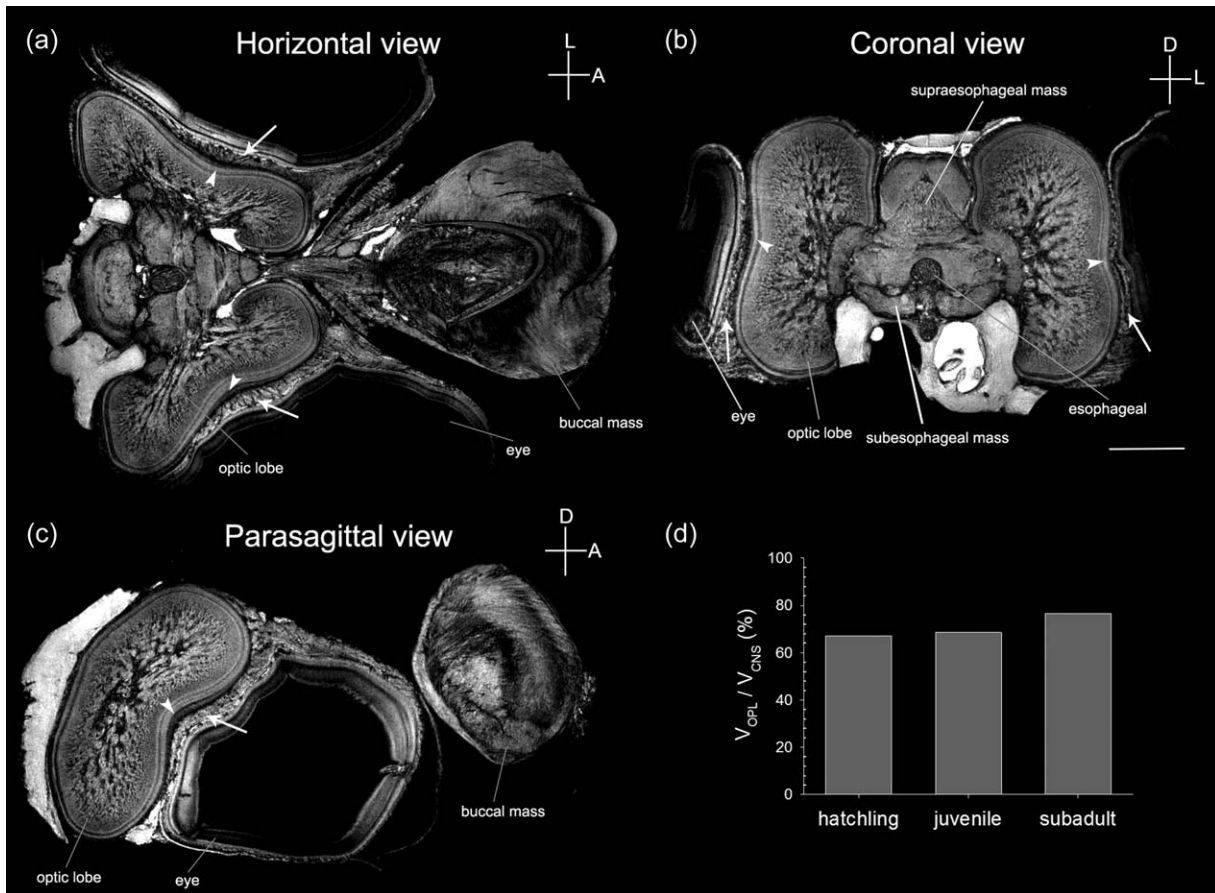


FIGURE 1 *Sepioteuthis lessoniana*, the optic lobes are the largest brain area in the head, and the proportion associated with the optic lobes increases throughout late development. (a) The horizontal section, (b) the coronal section, and (c) the parasagittal section of the whole brain MRI images of a subadult oval squid (mantle length 11.3 cm). A, anterior; L, left; D, dorsal; Arrowhead, the location of dent (or concave) on the optic lobe; Arrow, optic nerves which fill the space between the optic lobe and eye ball; Scale bar, 3 mm. (d) The optic lobes of hatchling and juvenile (mantle length 3 cm) oval squids occupy two-thirds of the total volume of the CNS, whereas those of subadult oval squid occupy up to three quarters of the total volume. The hatchling information was obtained from the micro-CT images, and the juvenile and subadult data were measured based on the MRI images

the squid brain. It was estimated from the micro-CT and MRI-scans that the optic lobes of the hatchling and juvenile oval squids made up two-thirds of the total brain volume, whereas those of the subadult made up as much as three quarters of the whole brain space (Figure 1d). This observation supports that the large volume of the optic lobes may be important in the oval squid's functions.

One of the most prominent features in these whole brain MRI-images is that the optic lobe has a dent or concavity on the ventrolateral side (Figure 1a–c). Three-dimensional models of the optic lobes at different developmental stages were reconstructed from the micro-CT and MRI-scans (supporting information Movies S2–S5) and these confirm this observation (Figure 2a). Although the concave area on the optic lobe was not evident when the squid hatched, it became more pronounced in the juvenile, subadult, and adult oval squids (Figure 2b). This finding suggests that the optic lobe of oval squids may be pressed ventrolaterally by the eye ball as the animal's eye continues to grow post-hatching and this squeezes the optic lobe inward. Alternatively, the optic lobe might not be pressed by the eye, rather it simply grows around the eye.

3.2 | The cell islands in the tangential zone of the optic lobe decrease continuously throughout development

To characterize the development of cellular organization inside the optic lobe, histological sections of the optic lobes from late embryo to adulthood were examined (Figure 3a). It is apparent that the boundary between the cortex and medulla became distinct before hatching. However, the total area of cell islands in the tangential zone were found to decrease continuously from late embryo to adulthood, while in parallel the area of neuropils increased steadily. To quantify these morphological changes related to neural organization during the development of the optic lobe, several structural features were measured and compared (Figure 4). It is evident that both the cortex and radial column zone increased steadily throughout development, yet overall the thickness of the radial column zone exceeded that of the cortex soon after hatching (Figure 4a). To further examine the thickness of the cortex at different regions of the optic lobe throughout the development, the cortical thickness at the dorsal and ventral sides were

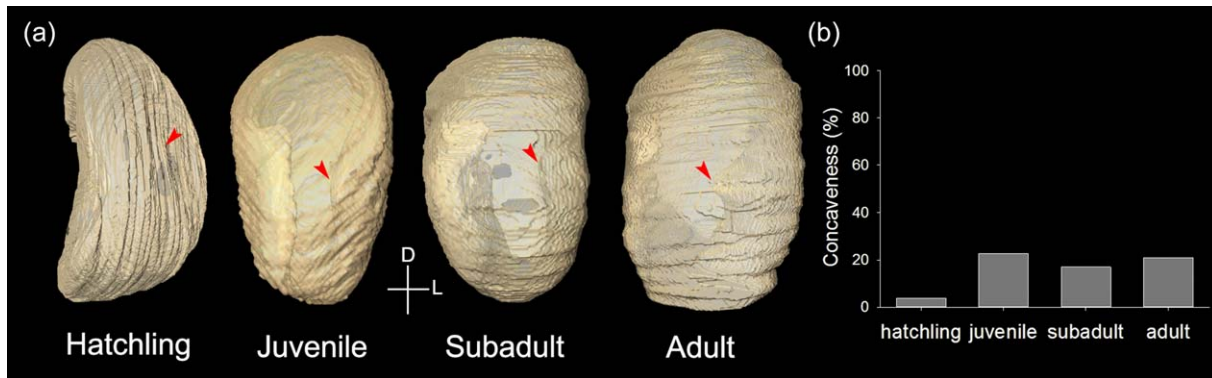


FIGURE 2 *Sepioteuthis lessoniana*, concavity on the lateral side of the optic lobe is evident in post-hatching oval squids. (a) The transparent 3D models of the left optic lobes at different developmental stages show the presence of a concave feature on the lateral side. The displayed sizes are not proportional to the original sizes. L, lateral; D, dorsal; Arrowheads, the location of the concave (dent) site. (b) The concave area on the optic lobe is not evident when the squid hatches, but become increasingly pronounced in the juvenile (mantle length 4 cm), subadult (mantle length 10 cm), and adult (mantle length 23 cm) oval squids

compared (supporting information Fig. S1). It is apparent that the ventral cortex was significantly thicker than the dorsal cortex at hatching, but the trend reversed when animals were subadults (supporting information Fig. S1). However, despite the size of the optic lobe, the medulla, and the tangential zone increased dramatically after hatching, the area of the cell islands increased only slightly during development (Figure 4b). When the proportions of the cortex, the radial column zone, and the tangential zone were compared at the various different developmental stages, it was noticeable that, from hatchling to adult, the percentage of the cortex decreased continuously, while at the same time the percentage of the tangential zone increased continuously (Figure 4c). Furthermore, the most dramatic morphological change was the fact that the density of the nuclei was found to decrease dramatically in the tangential zone throughout development, from about 80% in hatchlings to less than 20% in adults (Figure 4d). Besides these quantified morphological changes, we have also observed that cell somata inside the cell islands in the tangential zone were scattered and the cell islands showed hole-like structures when animals were in subadult and adult stages (Figure 3b–d). All of these observed changes in the neural organization of the optic lobe suggest that the tangential zone undergoes continuous reorganization from hatching to adulthood. Specifically, during the oval squid development, the proportion of neuropils, which are involved in neural connections, increased disproportionately, while at the same time, the cell islands decreased significantly.

3.3 | The mean size of the cell nuclei in the medulla of the central optic lobe increases throughout development

In addition to the general morphology changes within the optic lobe of developing oval squids, cell size seemed to change across the various developmental stages. By quantifying the size of cell nuclei via confocal images stained with DAPI, it was found that, from late embryos to adulthood, the mean size of the cell nuclei in the radial column zone and tangential zone increased significantly throughout development,

while at the same time the mean size of the cell nuclei in the cortex remained relatively constant (Figure 5). This unexpected finding suggests that, during the development of oval squids, the size of cell somata in the medulla of the central optic lobe increases as the brain grows larger.

4 | DISCUSSION

4.1 | Morphological changes of the optic lobe during development

In the present study, the proportion of the optic lobe relative to the whole brain in *S. lessoniana* shows an increase from the hatching to the subadult stage (Figure 1d). This finding suggests the importance of optic lobe function also increases as an oval squid mature. Furthermore, when the proportion of the optic lobe is measured in this species (76.55%, subadult *S. lessoniana*, ML = 11.3 cm), the result is similar to that measured in another species of the same genus (78.77%, *S. sepioidea*, ML = 15 cm) (Maddock & Young, 1987). These related findings support the idea that the size of the optic lobes in the genus of *Sepioteuthis* is relatively large when compared to other cephalopod genera or groups (Maddock & Young, 1987). The large optic lobe of *Sepioteuthis* thus may be a consequence of a high demand for visuomotor control of this squid's dynamic body patterning.

Unlike the retinal bump structure in the squid eye (Chung & Marshall, 2014, 2017), the dent or concavity on the lateral side of the optic lobe becomes evident only from the juvenile stage to adulthood (Figure 2). It is likely that the concavity of the optic lobe on the lateral side is a direct result of compression as the size of the eye ball increases during development. Another possibility is that the optic lobe may simply grow around the eye. Since the concave region is also the site corresponding to the optic nerve crossing or the chiasma (Figure 1), it may be a result of the physical constrain. Nevertheless, the shape change of the optic lobe after hatching may either have no functional significance in the oval squid or potentially keep the head in a streamline shape.

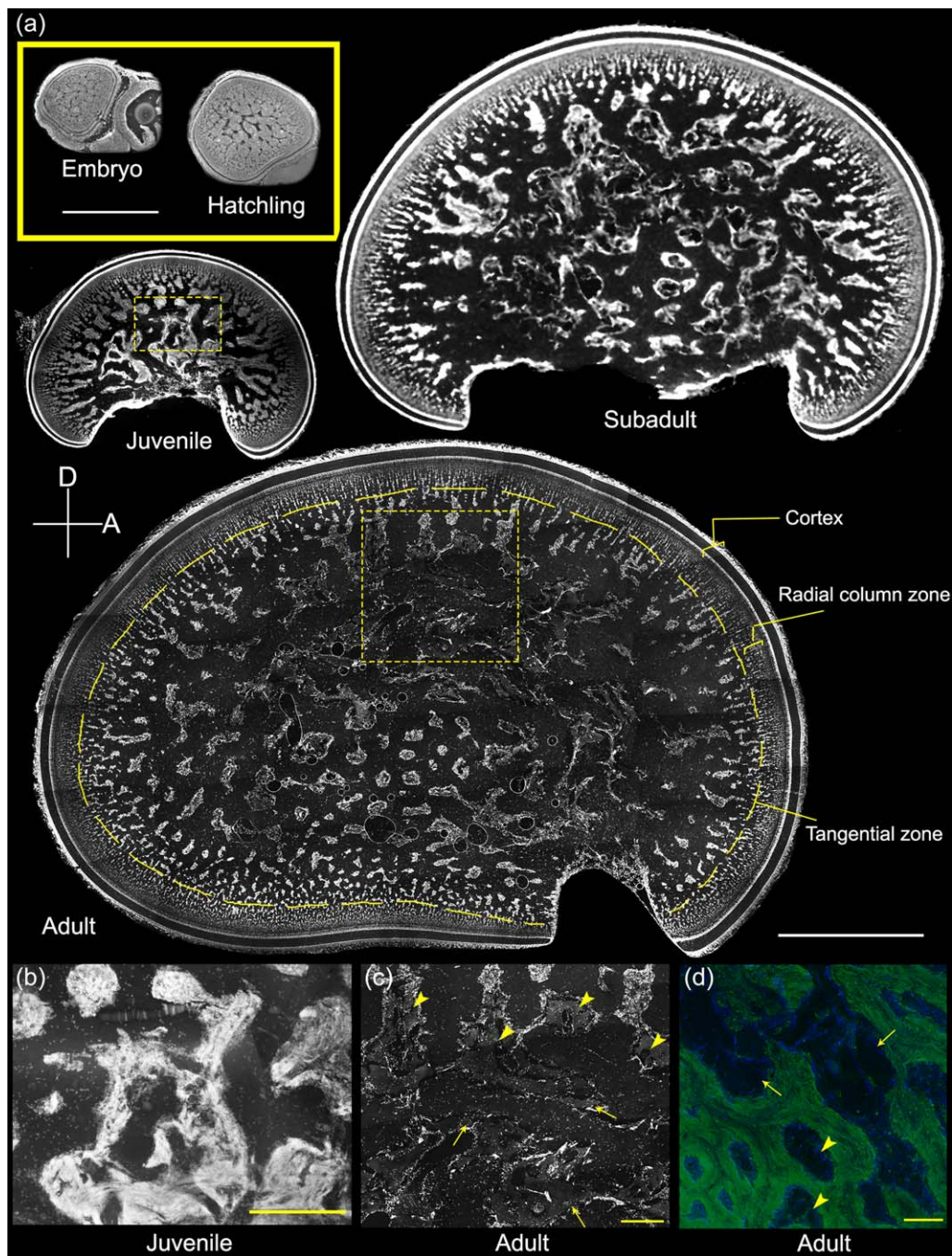


FIGURE 3 *Sepioteuthis lessoniana*, the morphology of the optic lobes continuously changes from embryo to adulthood. (a) Histological images of the middle sections of the optic lobes with nuclear staining (DAPI) were used to visualize the distribution of cell somata at different developmental stages of oval squids. The density of the nuclei within the tangential zone significantly decreases from late embryo to adulthood. A, anterior; D, dorsal. Scale bar, 3 mm; Inset scale bar, 1 mm. (b) The magnified image of the tangential zone of the optic lobe in the juvenile oval squid (dashed yellow box in panel a). Cell somata are densely packed in the cell islands. (c) The magnified image of the tangential zone of the optic lobe in the adult oval squid (dashed yellow box in panel a). There are several “holes” with only scattered cell somata inside the cell islands. Arrowheads, holes within the small cell islands in the peripheral region; Arrows, holes within the large cell islands in the central region. (d) The fluorescence image of the optic lobe section from another adult oval squid. Green, acetyl- α -tubulin immunostaining to label the neuropil region; Blue, DAPI staining to label the cell islands. Inside the cell islands, the holes are mostly empty without cell somata and neural fibers. Arrowheads, small holes in the cell islands; Arrows, large holes in the cell islands. Scale bars in panels b–d, 0.5 mm

A close examination of the internal structure changes in the optic lobe throughout development showed that the characteristics of the visual processing areas, namely the cortex and radial column zone

(Boycott, 1961; Young, 1974), establish earlier than the visuomotor area, namely the tangential zone (Boycott, 1961; Liu & Chiao, 2017; Sidel, 1982; Young, 1974). It has been known that there is a specific

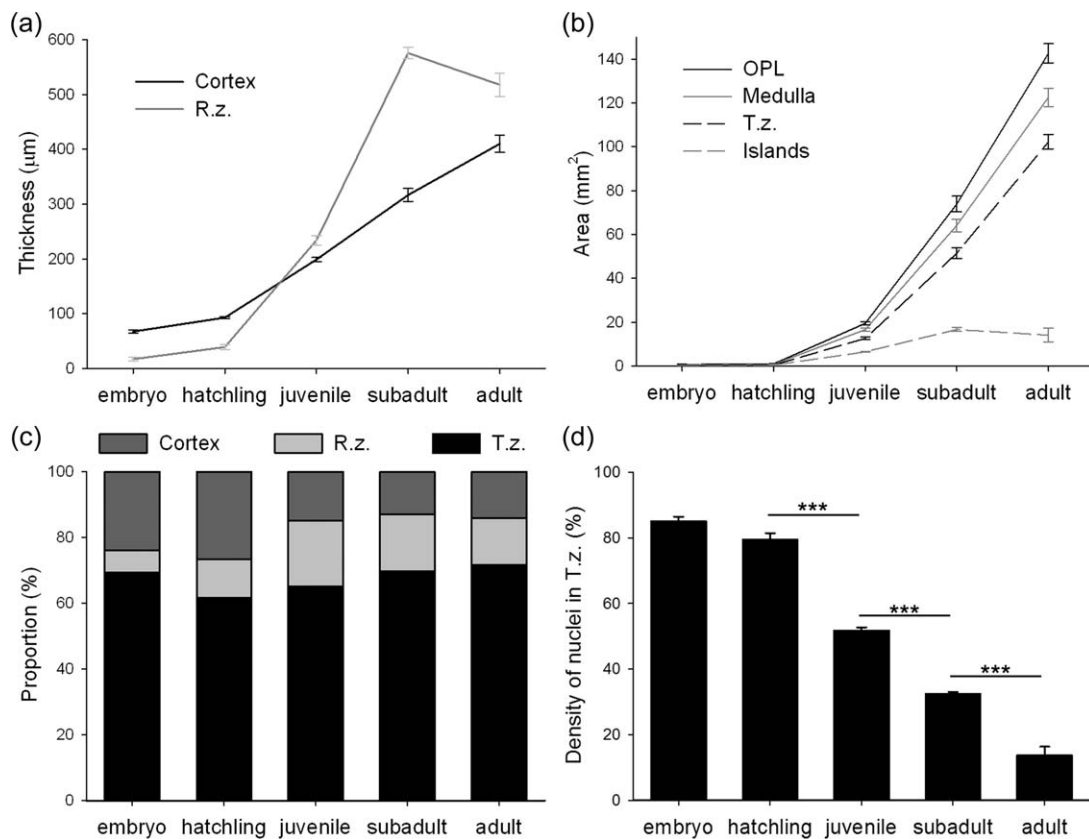


FIGURE 4 *Sepioteuthis lessoniana*, the proportion of cell islands in the tangential zone of the central optic lobe continuously decreases after hatching. (a) The thickness of the cortex and radial column zone shows an increasing trend from embryo to adulthood. (b) The size of the optic lobe increases significantly during development, but the cell islands do not expand proportionally. (c) The proportion associated with the tangential zone increases steadily after hatching at the expense of a proportional decrease in the cortex and radial column zone. (d) The density of nuclei in the tangential zone decreases significantly from hatchling to adult. R.z., radial column zone; T.z., tangential zone. ***, $p < .001$. Embryo ($N = 6$); hatchling ($N = 6$); juvenile ($N = 7$); subadult ($N = 8$); adult ($N = 5$)

dorsoventral representation of each retinal bundle within the optic lobe cortex of the octopus (Saidel, 1979). Along with our discovery, the ventral cortex is significantly thicker than the dorsal cortex when the squids are relatively young. However, the trend is reversed when the animals are relatively mature (supporting information Fig. S1). These findings suggest that the importance of visual input from different parts of the retina may vary depending on the developmental stage. Because the ventral cortex of the optic lobe receives visual input from the dorsal part of the retina which has a downward looking visual field (Young, 1962), the thicker cortex at this region may suggest that hatchlings rely more on visual information from below than from above for finding food. In contrast, when the oval squid grows up, the dorsal cortex of the optic lobe which corresponds to an upward looking visual field is relatively thicker and this may be important for the subadult squids to detect predators or find mates. Alternatively, this switch of the dominant cortex of the optic lobe during development may correlate with their visual tasks at different life stages (Chung & Marshall, 2014).

However, the number of neural fibers in the tangential zone, that is the neuropil, increase disproportionately faster than the cell somata in the same region, that are the cell islands (Figures 3 and 4). This finding supports the idea that the increased neural connections in the visuo-motor area are essential for coordinating the complex body patterning

and locomotive behaviors that occur later in life (Liu et al., 2017). It has been well-documented that the neurons in the tangential zone contain the higher-order visual neurons, the interneurons to the basal lobes that include visual processing reentry to the optic lobe, and the motor neurons to basal lobes, magnocellular lobes, and pedal lobes (Saidel, 1982; Young, 1962, 1974). In addition, the output of the tangential zone also includes the peduncle lobe, superior frontal lobe, and sub-vertical lobe for sensory and learning functions (Boycott, 1961; Messenger, 1979; Saidel, 1981; Shigeno & Ragsdale, 2015). In a recent developmental study, it has been shown that the visual sensory learning center such as the vertical lobe has the fastest growth rate among all visual-learning lobes from hatchling to juvenile stages in pygmy squids (Koizumi, Shigeno, Mizunami, & Tanaka, 2016). This result suggests that the sensory neurons and related areas running from the optic lobe to the vertical lobe and the superior frontal lobes might be the biggest growth area. Future studies aiming to characterizing the development of different neural connections in the tangential zone are needed to clarify the source of this disproportionately faster change of neuropils. Nevertheless, the increase of neuropils in the tangential zone during development is potentially correlated with the increased demand in integrating information from sensory, motor, and learning centers for visually guided behaviors.

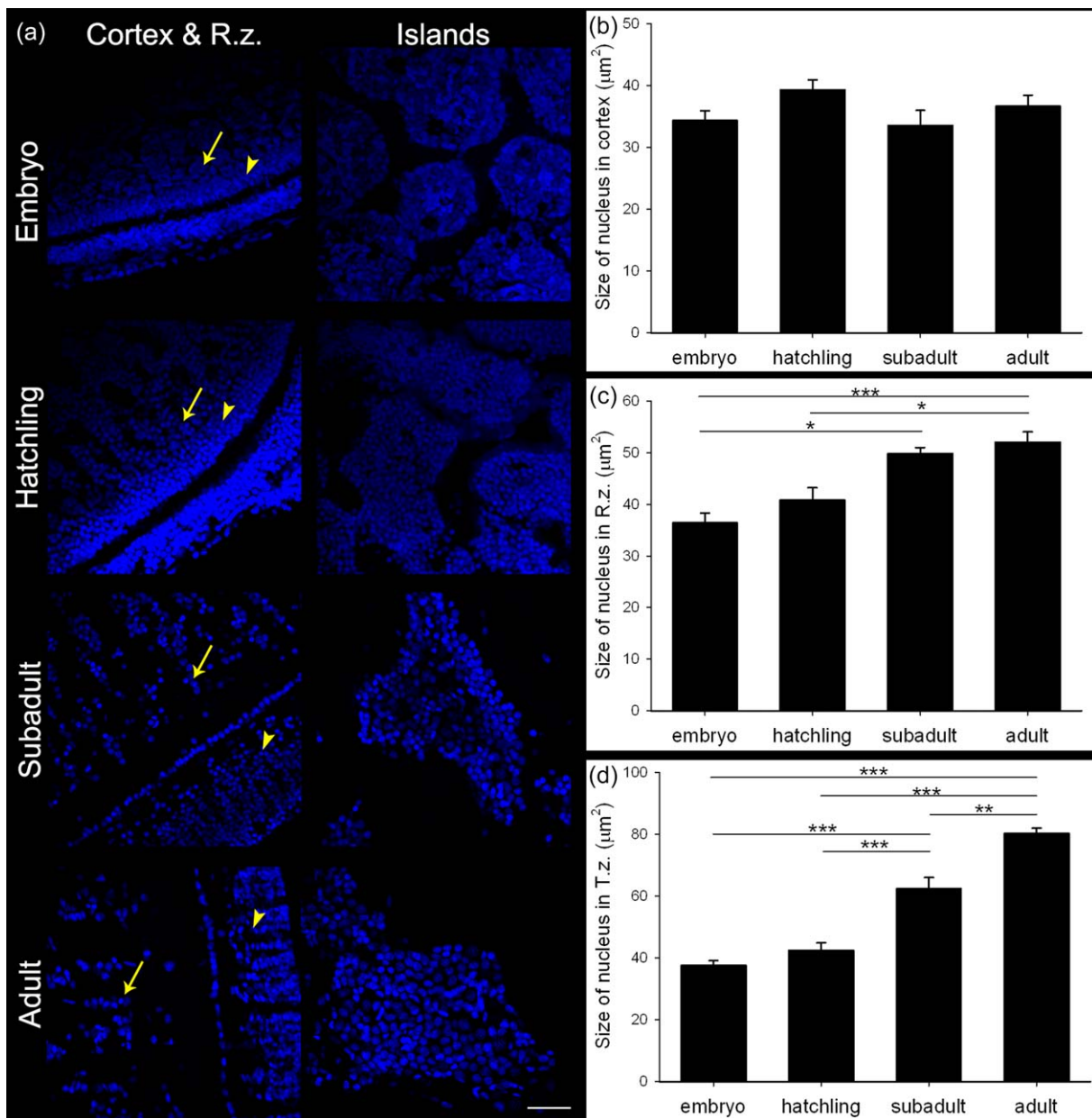


FIGURE 5 *Sepioteuthis lessoniana*, the size of the nuclei in the medulla of the central optic lobe increases significantly during the development. (a) Confocal images of optic lobes stained with DAPI to label the nuclei. The increase in the size of nuclei is more evident in the medulla than in the cortex. R.z., radial column zone. Arrowhead, the cortex; arrow, the radial column zone. (b) The size of nucleus in the cortex does not change from embryo to adulthood. (c) The size of nucleus in the radial column zone shows a steady trend toward an increase during development. (d) The nucleus size within the cell islands of the tangential zone increases significantly from hatchling to adult. Embryo ($N = 6$); hatchling ($N = 4$); subadult ($N = 2$); adult ($N = 4$)

Careful examination also revealed that not only the density of cell somata decreased significantly in subadult and adult stages but also the hole-like structures inside the cell islands also became prominent at these two stages (Figure 3b–d). From previous studies, it is likely that these hollow structures are the neurosecretory cells and thick vessels in the tangential zone, namely the subpedunculate tissues (Young, 1962, 1974), which reach into the central medulla of the optic lobe from the medial side. Although it is unknown how such neurosecretory cells contribute to the developmental change of the optic lobe, the fact that these hollow structures take up around 10% of the optic lobe area

in adult oval squids (supporting information Fig. S2) suggests that the subpedunculate tissues are more important in mature animals. However, this morphological feature does not occur in the optic lobe of adult cuttlefish *Sepia pharaonis* (as figures 3 and 5 in Liu et al., 2017) and this might reflect some species difference in the optic lobe organization.

Surprisingly, we also found that the mean sizes of the cell nucleus in the radial column zone and tangential zone increase during development (Figure 5). This suggests that the metabolic demand for neuronal function in the medulla increases from embryo to adulthood. In an

TABLE 1 A comparison of the optic lobe morphology between adult oval squids and cuttlefish

	<i>Sepioteuthis lessoniana</i> (Adult, N = 5)	<i>Sepia pharaonis</i> (Adult, N = 6)
Area of OPL (mm ²)	142.59 ± 4.40	80.74 ± 4.05
Area of medulla (mm ²)	122.59 ± 4.19	73.28 ± 3.66
Thickness of cortex (μm) [Area proportion (%)]	410.28 ± 15.92 (14.05 ± 0.59)	288.88 ± 9.28 (9.24 ± 0.53)
Thickness of r.z. (μm) [Area proportion (%)]	517.84 ± 21.10 (14.17 ± 0.75)	840.40 ± 30.69 (30.49 ± 0.25)
Area of t.z. (mm ²) [Area proportion (%)]	102.31 ± 3.30 (71.78 ± 1.15)	48.64 ± 2.28 (60.27 ± 0.40)
Nucleus size of cortex (μm ²)	36.64 ± 1.81	33.31 ± 0.70
Nucleus size of r.z. (μm ²)	52.07 ± 1.99	41.76 ± 1.54
Nucleus size of t.z. (μm ²)	80.32 ± 1.71	57.88 ± 3.33

^aMantel length: 23–30 cm in oval squids; 19.5–30.2 cm in cuttlefish.

^bOPL, optic lobe; r.z., radial column zone; t.z., tangential zone.

^cThe data of *Sepia pharaonis* is adapted from “Neural Organization of the Optic Lobe Changes Steadily from Late Embryonic Stage to Adulthood in Cuttlefish *Sepia pharaonis*,” by Y. C. Liu et al., 2017, *Frontiers in Physiology*, 8, p. 538.

earlier study, it has been shown that about 80–85% increase of total DNA content in the brain is attributable to the optic lobes during the growth of the octopus (Packard & Albergoni, 1970). This finding is consistent with our observation in the present study. Interestingly, an increase in the size of the cell soma size has also been reported in the song-control nuclei during the song development of zebra finches (Bottjer, Miesner, & Arnold, 1986). Thus, this finding supports the idea that the development of visuomotor behaviors requires a significant enhancement of cellular functioning in the oval squids.

4.2 | A comparison of the optic lobe development of oval squids and cuttlefish

In a separate study, the development of the optic lobe in cuttlefish *Sepia pharaonis* was characterized systematically (Liu et al., 2017). Because these two shallow water cephalopod species have quite different life styles (oval squids are pelagic and cuttlefish are benthic), a comparison of the optic lobe development of oval squids and cuttlefish is thus of great interest. In general, the head of squid is much narrower than that of cuttlefish, thus the brain lobes of oval squids are prominently more centralized than those of cuttlefish (Wild, Wollesen, Haszprunar, & Hess, 2015). As a consequence, the compression of the optic lobe by the eye ball is more significant in oval squids than in cuttlefish. This may explain why the optic lobe dent is present on the lateral side in oval squids, but is not present in cuttlefish. More importantly, when the size of the optic lobe from these two species of cephalopods were compared at different developmental stages, it is apparent that the optic lobes of the oval squid grow much faster than those of the cuttlefish (supporting information Fig. S3). This observation coincides with our present finding that the ratio of the optic lobe to the whole brain increases prominently from juvenile to subadult stages in the oval squid

(Figure 1d). As a result, the size of the optic lobe of the oval squids is nearly two folds larger than that of cuttlefish, when mature oval squids and cuttlefish with similar body size were compared (Table 1; Liu et al., 2017). This is also consistent with the results obtained during a previous optic lobe volumetric study that the optic lobe to CNS in loliginid species is generally larger than that in cuttlefish species (Maddock & Young, 1987). In addition, the same study also showed that the optic lobes of benthic octopuses which rely more on their chemical sense are significantly smaller than that of pelagic octopuses (Maddock & Young, 1987). This observation may suggest that the size of the optic lobe is related to the life style of these cephalopods.

Inside the optic lobe, the proportions of both the cortex and the tangential zone in oval squids are significantly larger than those of cuttlefish (Table 1; Liu et al., 2017). In addition, the mean size of the nuclei size in the medulla of the optic lobe is significantly larger in oval squids than in cuttlefish. Overall, the pelagic oval squids seem to have optic lobes with a thicker cortex, an enlarged tangential zone, and larger neural cells within the medulla. The results thus support the notion that pelagic cephalopods rely more on their visuomotor behaviors than their benthic counterparts (Frösch, 1971; Nixon & Young, 2003; Wollesen, Sukhsangchan, Seixas, Nabhitabhata, & Wanninger, 2012). However, both the pelagic and benthic cephalopods use astonishing camouflage and significant visual communication capabilities, both of which heavily depend on the functioning of their optic lobes, in addition to the other brain areas such as the posterior chromatophore lobes, anterior/lateral basal lobes, and peduncle lobes which are also involved in visuomotor coordination (Messenger, 1979; Nixon & Young, 2003; Shigeno & Yamamoto, 2002; Young, 1974, 1976, 1977). Thus, the exact relationship between structure and function within the optic lobes of squids and their relationship with life style requires further investigation in the future.

ACKNOWLEDGMENTS

We are grateful to Prof. Samuel H.H. Chan at the Kaohsiung Chang Gung Memorial Hospital for his generous support with the MRI scan. We also thank Dr. Hsiu-Ming Chang at the Brain Research Center, National Tsing Hua University, for helping with the AVIZO software and the provision of assistance with the 3D reconstruction of the optic lobe. We particularly thank Yueh-Chun Tsai for helping with the MATLAB programming that was used to quantify the optic lobe, and Chun-Yen Lin for collecting the oval squid eggs. Financial support came from the Ministry of Science and Technology of Taiwan (grant number: NSC-102-2628-B-007-001-MY3, to CCC).

AUTHOR CONTRIBUTIONS

Y.C.L. conceived, designed, carried out the work, and drafted the manuscript. W.S.C. and N.J.M. collected the whole brain MRI data and helped interpret the results. C.C.Y. and C.H.S. collected the optic lobe MRI data. S.T.H., F.L.C., and Y.H. collected the micro-CT data. T.H.L. helped plan experiments and was involved in clarifying data. C.C.C. coordinated the project, interpreted data, and revised the manuscript.

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How to cite this article: Liu Y-C, Chung W-S, Yu C-C, et al. Morphological changes of the optic lobe from late embryonic to adult stages in oval squids *Sepioteuthis lessoniana*. *Journal of Morphology*. 2018;279:75–85. <https://doi.org/10.1002/jmor.20755>