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Adaptation for Marine Environments by Locomotive Tunic Structure in Cuttlefish

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ABSTRACT.– Cephalopods have higher motility and more widely distributed in the ocean than other marine invertebrates. Especially members of the family Sepiidae have diverse habitats and locomotory modes. There are inner and outer tunics in the mantle, and the mantle muscle is sandwiched by inner and outer tunics. Their mantle is used for locomotion and respiration, and the tunics of the mantle supports its movements. However, the relationship between the tunic structure and habitats/locomotive modes of cuttlefish remains unclear. In this study, we made histological observations of the outer tunics of three species of Sepiidae with different locomotory modes and quantified the percentages of different collagen types (type I collagen: contributing tissue elasticity, type III collagen: contributing tissue flexibility). The structure and the collagen types of the outer tunic were related to the habitats and locomotory modes of Sepiidae. In *Metasepia tullbergi*, which walks on the sea bottom in shallow waters, the outer tunic has a reticular structure that mostly consists of type III collagen. This species' tunic is suitable for walking in shallower water. In *Sepia kobiensis*, which swims in deeper depth, the tunic has an oriented layer structure composed of type I collagen. This structure is suitable for swimming under higher water pressure. In *Sepia esculenta*, which swims at shallower depths, the tunic has a thick, reticular structure and is made of both type I and III collagen. This structure is suitable for swimming in shallower water. This study reveals Sepiidae have various locomotive tunic structure for adaptations for the diverse marine environment.

KEY WORDS: aquatic locomotion, cephalopod, collagen type, cuttlefish, mantle, outer tunic

INTRODUCTION

The ocean is a vast, aqueous environment with a three-dimensional structure. It contains extremely diverse environments, to which marine organisms have adapted through developing various locomotory modes (Amber and Christy 2016; Levinton and Levinton 1995). One of the most fundamental subjects in marine biology is clarifying the relationships between the habitats of marine organisms and their locomotory modes.

Cephalopods in the superfamily Decapodiformes, especially the cuttlefish (family Sepiidae), are interesting marine invertebrates in which to study this subject. Cuttlefish have exceptionally diverse means of motility (they walk as well as swim), and live in a wide range of habitats in the ocean (Yonge et al., 1998). Our goal in the present study was to clarify the relationship between the habitats and locomotory modes of cuttlefish from a functional morphology perspective.

The mantle is the most important organ for locomotion in cephalopod (Kurth, Thompson, and Kier 2014) They use their mantle to provide the jet propulsion that allows them to swim. This jet propulsion is a one of the unique locomotive feature of cephalopods, which allows them to move at high speeds (Milligan, Curtin, and Bone 1997; Nixon and Dilly 1977). In addition, *Metasepia* species raise ambulatory flaps from their mantle to walk on the seabed like a quadrupedal animal (Roper and Hochberg, 1998; Nixon and Young 2003). They also use their mantle as a water pump for respiration (Kier and Thompson 2003; Nixon and Young 2003; Packard and Trueman 1974). Therefore, even within the family Sepiidae, the use of the mantle differs among species, and its structure should therefore show differences among species that relate with differences in their habitats and locomotory modes.

The mantle consists of muscle, tunic, and epithelium tissues (Kangsanant, Vittayanont, and Tongraung 2008; Kier and Thompson 2003; Shadwick 2012). The tunic reinforces the mantle and increases the pressure in the mantle cavity to allow for swimming by strong jet propulsion (Ward and Wainwright 1972). The ambulatory flaps, which look like a pair of elongate, ventrally directed-fins (Roper and Hochberg, 1998) and are used for ambling on the bottom like quadruped (Nixon and Yonge, 2003), of *Metasepia* may also be formed from the tunic because of their positions. Therefore, it can be expected that differences in locomotory modes should correspond to differences in the structure of the tunic. However, no functional morphological study of the tunic has been done so far, although there have been some studies done on muscles (Packard 1974; Ward and Wainwright 1972).

The tunic consists of collagen fibers (Thompson and Kier 2001b). Collagen has many types, such as type I and type III collagen, where type I collagen contributes to tissue elasticity and type III collagen contributes to tissue flexibility, with the latter typically forming reticular fibers (Gosline and Shadwick 1983; Packard 1974; Packard and Trueman 1974). Type I collagen has been detected in *Sepia lycidas* (Nagai et al., 2001). Type III collagen has not been reported from cuttlefish, but it is expected that it will be detected in *Metasepia* species' ambulatory flaps because these are erected from the mantle (Roper and Hochberg1998; Nixon and Young 2003), and the erectile tissues of a hectocotylus in *Octopus bimaculoides* (instead of cuttlefish) were found to consist of reticular fibers (Kimura 1987), which are known to be made of type III collagen (Gosline and Shadwick 1983; Macgillivray et al. 1999; Packard and Trueman 1974)

In this study, we examined the structure of the tunic in cuttlefish, particularly with regard to the tunic structure of the mantle and the collagen types present therein. We compared the structures and collagen types of the tunic quantitatively and qualitatively among three species of Sepiidae; *Metasepia tullbergi* Appellöf, 1886, *Sepia kobiensis* Hoyle, 1885, and *Sepia esculenta* Hoyle, 1885, which have different habitats and locomotory modes.

MATERIALS AND METHODS

Specimens

We examined males of three species of Sepiidae, *Metasepia tullbergi*, *Sepia kobiensis*, and *Sepia esculenta. M. tullbergi* lives from shallow water to a depth of 100 m (House 1982), has limited swimming ability, and is characterized by walking using ambulatory flaps (Nixon and Young 2003). *S. kobiensis* is a species that swims in waters of 100 to 200 m depth. *S. esculenta* lives in shallower waters of 10-100 m depth and swims.

For each species, we examined three specimens (Table1). The specimens of *M. tullbergi* were wild-collected in Kumanonada, Mie prefecture, Japan, and transported to Tokyo, Japan. The animals were anesthetized, fixed in 10% formalin in seawater, and stored in 70% ethanol. The specimens of *S. kobinensis* and *S. esculenta* were specimens

TABLE 1. Specimens used in this study

Species	Mantle length (mm)	Body weight (g)	Museum number
Metasepia tullbergi	42.0	18.7	-
	44.4	22.1	-
	46.1	24.5	-
Sepia kobiensis	52.5	15.2	Mo. 75413
	56.3	15.4	Mo. 75413
	57.0	16.2	Mo. 75413
Sepia esculenta	50.1	17.5	Mo. 59003
	52.2	18.3	Mo. 58926
	55.3	21.7	Mo. 59003

belonging to the National Museum of Nature and Science, Tokyo, Japan, which had been fixed in 10% formalin in seawater and stored in 50% isopropanol.

Observations

A piece of the mantle of each specimen was cut parallel to the sagittal plane from the anterior end to the posterior end on the left ventral side. The pieces were dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin (melting point 56°C). Serial sections (10 µm thick) were cut parallel to the sagittal plane using a microtome (Yamato PR-50), put on slides, and stained with picrosirius red (Ito and Sakao 1983; Kimura 1987). The sections were observed using a bright-field microscope (Nikon ECULIPS Ts2).

The collagen types present in the outer tunics were identified using a polarized light microscope (NiconLV100POL), in which type I collagen appeared orange after staining, while type III collagen appeared green. The areas of each of the collagen types observed on the slides were measured from photographs taken by a microscope-mounted camera. The area of each collagen type was quantified by the photographs using Photoshop CS5. The collagen type percentages were then estimated by dividing the summed areas of each collagen type to the total areas of the outer tunic samples. The collagen type percentages in the different species were compared using multiple *t*-tests with the Holm method.

RESULTS

Morphological observations

Under light microscopy, collagen fibers at the lateral side of the outer tunic of *M. tullbergi* were densely entangled, and those at the center were sparsely reticulated and were separated by many gaps (Fig. 1A). In the tunic of *S. kobiensis*, all collagen fibers were aligned in the same direction to form layers. In the tunic of *S. esculenta*, the collagen fibers on the lateral side of the outer tunic were thicker and densely entangled; those on the inner side were thinner and somewhat layered and had a lower density.

Under polarized light, the peripheral part of the tunic of *M. tullbergi* consisted of type I collagen, and the center consisted of type III collagen. Most of the outer tunic of *S. kobiensis* consisted of type I collagen (Fig. 1B). Most of the outer tunic of *S. kobiensis* consisted of type I collagen. In *S. esculenta*, the outer part of the outer tunic consisted of type I collagen, while the inner part mainly consisted of type III collagen, and the few layers present consisted of type I collagen.

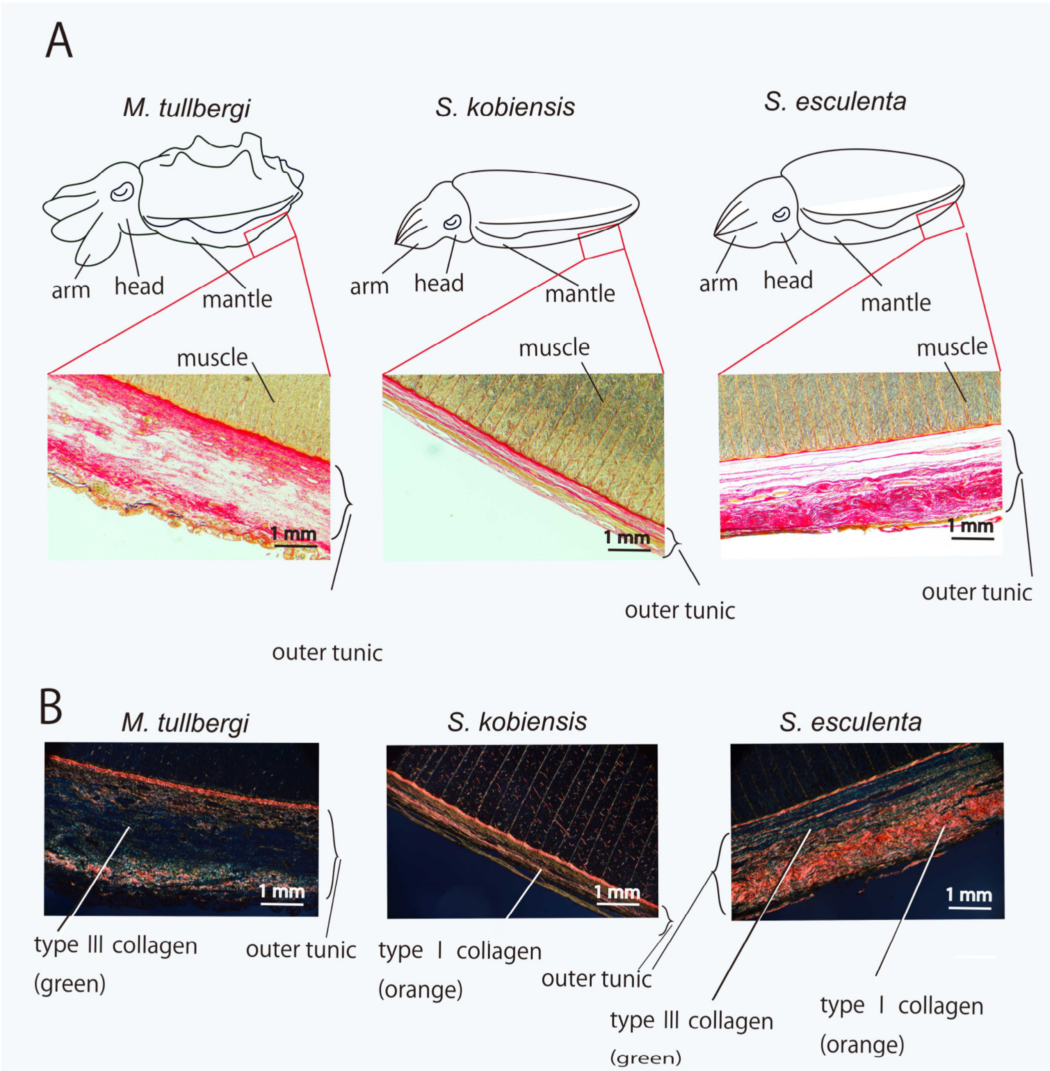

FIGURE 1. A: The part of outer tunics taken under light microscopy. B: The part of outer tunics taken under polarized microscopy.

The ratios of collagen types

Fig. 2 shows the percent content of each of the different collagen types present in the samples; the values shown are the averages of all the specimens. The percent content of type I and type III collagen in the *M. tullbergi* tunic were 27.4 and 72.6%, respectively, while those in *S. kobiensis* were 90.5 and 10.1%, respectively, and those in *S. esculenta* were 54.0 and 46.7%, respectively. These percentages were significantly different among species ($p < 0.01$).

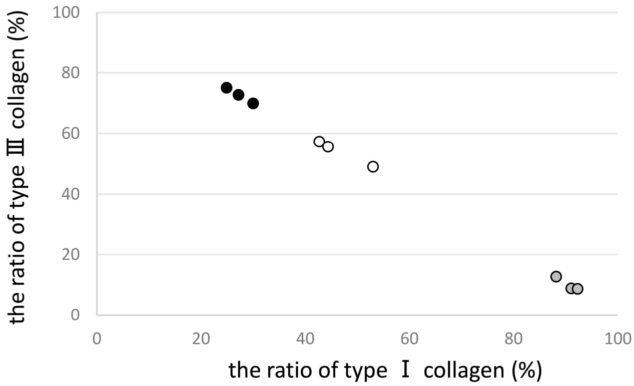

FIGURE 2. The ratios of collagen types. black dot: *Metasepia tullbergi*, white dot: *Sepia esculenta*, gray dot: *Sepia kobiensis*.

DISCUSSION

Among the three species of Sepiidae examined, much morphological diversity was found in their outer tunics. In short, the outer tunic of *M. tullbergi* seems to make this species' mantle more suitable for walking by reticular structure of type III collagen. *S. kobiensis* seems more suitable for aiding in swimming by layered structure of typeI collagen, and that of *S. esculenta* has properties intermediate between those of the two other species by both typeI and III collagen.

The outer tunic of *M. tullbergi* was found to mainly consist of type III collagen, with a reticular structure (Figs. 1, 2). *M. tullbergi* walks in shallow-water areas (Murakami et al. 2013; Nagai et al. 2001). For this type of locomotion, they erect their ambulatory flaps from their mantle and use them as the hind legs of a four-legged animal (Amber and Christy 2016; Nixon and Young 2003). Both the type III collagen and reticular structure of the tunic in this species contribute to the greater flexibility of the mantle. Type III collagen is known to make fibers more flexible than type I collagen (Gosline and Shadwick 1983; Packard and Trueman 1974), and the reticular structure of its fibers may allow them to change their shape more easily than the layered structure of type I fibers (cf. the erectile tissue of *Octopus*, which also has a reticular structure (Kimura 1987). Flexibility of the mantle may be needed to walk when there is poor footing to allow it to change its shape adaptively to the scaffold since they live on both shallow sandy bottoms and on the sea bottom in reef areas (Kimura 1987; Nagai et al. 2001).

The outer tunic of *S. kobiensis* almost entirely consists of type I collagen, with a layered structure (Figs. 1, 2). They swim at depths of 100 to 200 m. When decapodiforms swim, they use their mantle for both jet propulsion and respiration (Gosline et al. 1983). Type I collagen, with its layered structure, is suitable for aiding in such locomotion. Type I collagen is known to make fibers more elastic than type III (Gosline and Shadwick 1983; Macgillivray et al. 1999; Packard and Trueman 1974), A layered structure will also increase the strength and elasticity of the mantle; since the fiber direction shows a certain regularity, the connective tissue becomes very strong (Okutani 2015).

The outer tunic of *S. esculenta* consists of both type I and type III collagen. The outer side of the outer tunic is formed from densely entangled, thick fibers of type I collagen, and the inner side of the outer tunic is composed of a reticular structure of type III collagen and small amounts of layered type I collagen (Figs. 1, 2). This juvenile of the species moves by crawling or stabilizes itself on the sea bottom for most of the day (Ramasamy et al. 2012). The structure of the inner side of their outer tunic may also act as a shock absorber to guard the soft internal organs from impacts. In addition, the small amount of the layered structure of type I collagen present in inner side of their outer tunic may be used in jet propulsion during occasional swimming bouts.

In this study, we clarified the environmental adaptations of the structure of the outer tunic that occur in the Sepiidae. In addition to clarifying ecological adaptation strategies in cuttlefish, this study is a major step forward in understanding the environmental adaptations of cephalopods from the point of view of morphology, as well as their application to the field of biotechnology for underwater movement.

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