Studies on topographical distribution of rhabdom in the retina to estimate the visual function of three species of Teuthida(ツツイカ目3種の網膜上の感桿分布から推定した視機能に関する研究)

Master's Thesis

STUDIES ON TOPOGRAPHICAL DISTRIBUTION OF RHABDOM IN THE RETINA TO ESTIMATE THE VISUAL FUNCTION OF THREE SPECIES OF *Teuthida*

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Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

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WU YUNPENG

STUDIES ON TOPOGRAPHICAL DISTRIBUTION OF RHABDOMIN THE RETINA TO ESTIMATE THE VISUAL FUNCTION OF THREE SPECIES OF *Teuthida*

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修士学位論文内容要旨
Abstract

専　攻 Major	Marine life science	氏　名 Name	Wu Yunpeng
論文題目 Title	Studies on topographical distribution of rhabdom in the retina to estimate the visual function of three species of *Teuthida* (ツツイカ目 3 種の網膜上の感桿分布から推定した視機能に関する研究)		

[Objective] Previous studies of retinal histology of fish have established that the distribution of visual cell density often reflects both their habitats and feeding behaviors, while there are few studies concerned about the visual function related to the visual cell distribution of squid. The Japanese common squid *Todarodes pacificus*, Spear squid *Heterololigo bleekeri* and Firefly squid *Watasenia scintillans* are all important commercial squids in Japan with quite landing amounts every year and their habitats are different. However, the distributions of visual cell in relation to the habitat of these three species have not been fully examined or even unknown. In this study, the arrays of rhabdoms among three species of *Teuthida* were compared, the densities of visual cells were analyzed, the distributions of the rhabdoms were examined and the topographic maps of visual cell density were drew. Besides the visual axes in the horizontal and in the vertical plane were estimated, the visual acuities were calculated and comparisons of visual functions among the 3 species of *Teuthida* were also made in this study.

[Materials and Methods] The *Todarodes pacificus*, *Heterololigo bleekeri* and *Watasenia scintillans* were sampled in Wakasa bay Fukui prefecture in Jun 2015, Izu Shizuoka prefecture in Jan 2016 and in Toyama Bay during April 2016 in Toyama prefectures respectively. After fixed in the Bouin's fluid, the retina of each sample was divided into 37, 57 or 81 pieces according to the size of specimen, then made into the paraffin sections. The distributions of rhabdoms across the retina for each piece were observed using a light microscope OLYMPUS BX41N, the pictures were taken by the OLYMPUS E-330 camera. Calculated the visual cell density per square millimeter, tested and analyzed all the data by software of IBM SPSS Statistics 22.0. Then built iso-density contour maps of the visual cell densities by using the software of Golden Software Surfer 11. Visual axes were estimated from the visual cell density contour maps and visual acuities were estimated from the visual cell densities and the focal distance of the lenses.

[Results] The rhabdom arrays of *Todarodes pacificus* and *Watasenia scintillans* were similar, showed an orthogonal mosaic-like pattern array while the rhabdom array of *Heterololigo bleekeri* appeared to be in disorder, the adjacent two rhabdoms connected together tightly. Visual cell density differed in different parts of one retina and differs among different species. Visual cell densities were all higher in the posterior retina in all 3 species. For the *Todarodes pacificus*, which had a higher cell density than the other 2 species, the highest density region was ventral to the midline of the body, while the region of highest density was dorsal to the midline of the body in *Heterololigo bleekeri* and *Watasenia scintillans*. Visual axes showed difference for different species and suited for their habitat and feeding behaviour respectively. The visual axis of *Todarodes pacificus* was estimated to be upwards and forwards which might be advantageous in detecting prey items of above. While the downward and forward directed visual axes in *Heterololigo bleekeri* and *Watasenia scintillans* might to be advantageous in detecting items of its underside. The distributions of visual acuity were almost the same as the distributions of visual cell, the visual acuities were all higher in the posterior retina in all 3 species. In the *Todarodes pacificus* and *Heterololigo bleekeri*, the visual acuities were estimated to be 0.70, 0.74 which were higher than ordinary fish species, this may enhance their visual abilities to recognize distant objects and to promote a higher resolution. While there were no significant difference in visual acuities (around 0.48) among different specimen in *Watasenia scintillans*.

Chapter1 Introduction

Teuthida (also known as *Teuthoidea*), an order of the class *Cephalopod* (subclass *Coleoidea*, superorder *Decapodiformes*) are commonly known as squids. Two other orders of decapodiforme Cephalopods are also called squid, although they are taxonomically distinct from *Teuthida* and differ recognizably in their gross anatomical features. They are the bobtail squid of order *Sepiolida* and the ram's horn squid of the monotypic order *Spirulida*. The true squids arose in the early Mesozoic Period (Permian/Triassic) and have proliferated from the Jurassic to the Recent (Holocene). *Teuthida* is the largest *Cephalopod* order with around 300 species classified into 29 families (WoRMS, 2016). Two main groups, the Myopsida and the Oegopsida, are included in the *Teuthida*, and they occur in various marine habitats of the world (Roper and Clyde, 2014).

The *Teuthida*, like cuttlefish, characteristics include 10 circumoral (surrounding the mouth) appendages (eight arms and two tentacles); suckers with chitinous rings or hooks; a buccal membrane (lips); an internal, simple, rod or featherlike chitinous shell (pen or gladius); and eyes that are covered by a transparent membrane with a minute pore (myopsids) or eyes without any membrane, completely open to the sea. The *Teuthida* inhabit virtually all marine and estuarine habitats of the world, from surface waters to abyssal depths of 5000 m or more. *Teuthida* are strong swimmers and certain species can "fly" for short distances out of the water (Jabr, 2010) .They are foundation members of many ecosystems, as both prey and predator. Currently, in aggregate, more than 4.22 million metric tons (4.65 million tons) of squid are harvested annually in the worldwide squid fisheries (Roper and Clyde, 2014).

For most of the squids and fishes, the vision is one of the main sensory function and the studies on visual function have an important impact on improving efficiency of fishing gears in fishery production operation. Therefore many scholars carried out the relevant investigations and researches in terms of visual function of the squids and fishes from different aspects, by different methods and means (Ahlbert, 1976; Ali and Anctil, 1977; Budelmann, 1995; Jeanne, 2008; Miyazaki et al., 2008).

1.1 Studies on the eyeball construction of *Teuthida*(*Cephalopod*)

Cephalopods, as active marine predators, possess sensory organs specialized for use in aquatic conditions (Budelmann, 1995). The eye of *Cephalopod* is probably the most sophisticated eye of all invertebrates and is as complex as the vertebrate eye, though the two are not homologous. For their body size, *Cephalopod* eyes are relatively large (Nilsson, 2004). They have a camera-type eye which consists of an iris, a circular lens, vitreous cavity (eye gel), pigment cells, and photoreceptor cells that translate light from the light-sensitive retina into nerve signals which travel along the optic nerve to the brain (Jeanne, 2008). Unlike the vertebrate camera eye, the *Cephalopod*s' form as invaginations of the body surface (rather than outgrowths of the brain), and consequently they lack a cornea. Octopuses are the only *Cephalopods* with a completely protected "closed" cornea. That means that the eyes of squids and sepioids (cuttlefish, etc.) are in direct contact with sea water.

The pupil in *Cephalopods* is unique in that its morphology is different in octopuses, cuttlefish, and squid. Octopuses have a slit-shaped rectangular pupil. In cuttlefish it is W-shaped, and in squid it is round. It can narrow and widen in different brightness but resolves images poorly, so probably is useful only to detect light (Budelmann, 1995).

Most *Cephalopods* possess complex extraocular muscle systems that allow for very fine

control over the gross positioning of the eyes, which means unlike the vertebrate eye, a *Cephalopod* eye is focused through movement, much like the lens of a camera or telescope, rather than changing shape as the lens in the human eye does. The eye is approximately spherical, as is the lens, which is fully internal (Yamamoto, 1985).

1.2 Studies related to the retina of *Teuthida*(*Cephalopod*)

The *Cephalopod* retina, which develops from an ectodermal invagination in the head region, is in some respects remarkably different in construction from the vertebrate retina (Yamamoto et al., 1965). As revealed by previous researchers, the visual cell in *Cephalopod* retina is comprised of both a proximal and a distal segment. The nucleus of the cell is situated in the former, and rhabdomeres are carried by the latter on its two opposite lateral surfaces. Four rhabdomeres from four different cells combine to form a prismatic rhabdom, the functional unit of the *Cephalopod* retina. Rhabdoms are oriented in both vertical and horizontal planes, allowing the organism to perceive polarized light (Young, 1962).

From the infranuclear portions of the proximal segments long axonal fibers arise and dendritic collaterals spread into the plexiform or deepest layer of the retina. The optic nerve is composed of these axonal fibers originating directly from the visual cells (Schultze, 1869; Young, 1962). Other neuronal elements, such as bipolar or ganglion cells, are not present in the retina of the *Cephalopod*. Because of such structural simplicity and the superficial array of the rhabdoms, the *Cephalopod* retina has been one of the most favorable materials used not only for studying the comparative physiology of the retina but also for the investigation of the photoreceptive mechanism in primary sensory cells (Hagins et al.,1962; TASAKI et al.,1963; Tasaki et al.,1963).

Subsequent studies on the retinal ultrastructure in the *Cephalopod* by Wolken (1958),

Moody and Robertson (1960), and Moody and Parriss (1961) have demonstrated that the rhabdomeres consist of closely packed hexagonal arrays of tubules, 300 to 1000 Å in diameter, arranged approximately perpendicular to the light path(Yamamoto et al., 1965). The visual pigment in molluscs is proposed to be a rhodopsin(Wald and Hubbard，1957)，Hara & Hara then discussed the new photosensitive pigment found in the retina of the squid(Hara and Hara,1965).

1.3 Studies related to the visual functions of *Teuthida(Cephalopod)*

Similar to fish, *Cephalopod* species have a well-developed camera-type eye, but unlike the fish which has a complete study system on the visual function, the studies related to the visual functions of *Cephalopod* species are still deficient especially for *Teuthida* species.

Cephalopods have a remarkably high level of visual acuity, similar to that of vertebrates. Packard (1969) used the optomotor response and gave a result of visual acuity of Octopus *vulgaris* between 27' and 49' for very small animals (less than 3 g), and of 27' or better for slightly larger animals (12-22 g). Muntz & Gwyther (1988) found that the minimum separable visual acuity of Octopus *Pallidus* and Octopus *Australis* is less than 9.7'. Studies also show that cuttlefish have far superior visual acuity than fish. Watanuki et al. (2000) found that the visual acuity of cuttlefish *Sepia esculenta* is 0.36 when estimated from the bait recognition distance and the size of bait during feeding, and 0.89 when determined from the visual cell density at the visual equator and the focal length of the lens. Makimo and Miyazaki (2010) examined the visual acuities of 5 different species of decapodiformes and gave values of 9.5, 19.1, 19.2, 27.1 and 40.7 cycles/degree for *Euprymna morsei, Sepioteuthis lessoniana, Todarodes pacificus, Eucleoteuthis luminosa* and *Thysanoteuthis rhombus* respectively.

Flores et al. (1978) indicated that Japanese common squid *Todarodes pacificus* could not distinguish colors of jigs. Without color vision, therefore, all colored objects would appear as plain grey with difference of brightness due to the brightness contrast of the object against the surrounding background. But there exist one exception, The deep-sea firefly squid, *Watasenia scintillans*, has three visual pigments: The major one (A1 pigment) is based on retinal and has λ max = 484 nm, the second one (A2 pigment) is based on 3-dehydroretinal and has λ max = 500 nm, and the third one (A4 pigment) is based on 4-hydroxyretinal and has λ max = 470 nm (Matsui et al., 1988). Studied by HPLC analysis of the retinals, Seidou et al (1990) found that A1 pigment was not located in the specific region of the ventral retina receiving the down-welling light which contains very long photoreceptor cells, forming two strata. A2 and A4 pigment were found exclusively in the proximal pinkish stratum and in the distal yellowish stratum. Based on these findings, they surmised that the role of these pigments in the retina may be involved spectral discrimination (Seidou et al., 1990; Michinomae et al., 1994; INADA, 1996).

Suzuki et al. (1985) formed that the migration of retinal pigment of the squid *Todarodes pacificus* changed at approximately 1.0lx. Siriraksophon et al (1995) found the contrast threshold of *Todarodes pacificus* is far better than fishes' and justified that squid has an excellent visual sense. Besides researches also show that *Cephalopod* have a great sensitivity to the orientation of polarization of incoming light (Shashar et al.,1996; Shashar et al., 2000; Gronin et al., 2003) and widely divergent visual axes combined with highly mobile eyes (Schaeffel et al., 1999).

1.4 Objectives of studies

Studies of retinal histology of fish have established that the position of an area of high cell density reflects both the habitats and feeding behaviors. For example, in fish vision, cone photoreceptor array and/or topography of retinal ganglion cells (RGCs) across the whole retina often reflect species-specific feeding behavior and/or habitat (Beaudet, Flamarique, & Hawryshyn, 1997; Browman, Gordon, Evans, & O'Brien, 1990; S. Collin & Pettigrew, 1987; Shaun Patrick Collin & Pettigrew, 1988; Shaun P Collin & Pettigrew, 1989; Williamson & Keast, 1988). Collin & Pettigrew (1988) compared the iso-density contour maps of RGCs in 10 teleost species from different reef habitats. The fishes, whether living in enclosed environments or open water, shared the greatest density of RGCs in the temporal retina; in species from open water, dense RGCs also extended horizontally across the retinal meridian. Fishes from enclosed habitats are ambush hunters, whereas open-water fishes must keep watch for predators. The temporal peak in RGCs in both fish groups is presumed to allow binocular vision while feeding in front of the animal; the strong horizontal streak in open-water fishes may enhance their ability to detect predators elsewhere in the visual environment.

Similarities of visual ability and function between *cephalopods* and fish suggest that squid retinas and eyes may mirror the diversity documented among fishes. In these investigations of retinal histology and visual functions, however, attention has been focused exclusively on the fish, and only few descriptions have been presented on the rhabdom distribution and visual function of *cephalopods*.

Young (1963) measured rhabdom and other cells across the retina in the cuttlefish, *Sepia officinalis,* and the nearshore squid, *Loligo pealeii*, demonstrating a clear difference in the array of these retinal cells. A well-marked posterior fovea, with the longest rhabdoms, found

only in *Sepia officinalis*, was suggested to relate to the direction of the most frequent visual stimulation in their coastal life. Its absence in *L. pealeii* was related to the squid's habit of swimming in shallow and well illuminated water as shoals, looking backwards and forwards equally (Young, 1963). Makimo and Miyazaki (2010) investigated the density of retinal-cell nuclei across the whole retina in two coastal and three oceanic squid species, suggesting that for the coastal species, the region of highest density was dorsal to the midline of the body, meaning that the visual axis was likely directed downwards and forwards while for the oceanic species, the highest density region was ventral to the midline, meaning that the visual axis was directed upwards and forwards.

As we mentioned above, there exist more than 300 species of *Teuthida* in the world, while retinal variation has been noted among species (Young, 1962, 1963), few studies have related these differences to habitat.

The Japanese common squid *Todarodes pacificus*, spear squid *Heterololigo bleekeri* and firefly squid *Watasenia scintillans* are all commercial squids in Japan with quite a landing amount every year. All the 3 species have a well-developed camera-type eye with a remarkably high level of visual acuity, which is similar to that of vertebrates with widely divergent visual axes combined with highly mobile eyes. Previous studies showed that the visual cell distribution often reflect species-specific characters. However, the distribution of visual cell in relation to the habitat of these three species have not been fully examined. Few studies mentioned the differences among them. In this study, we investigated the topographical distribution of rhabdom in the retina of these 3 species of *Teuthida* by histological techniques, documented any differences in area or position of the region of highest cell density. Analyzed the density of rhabdom in the retina, tried to estimate the visual functions and made comparisons among them.

Chapter 2 compared the arrays of rhabdoms, analyzed the density of visual cells, introduced the distribution of the rhabdoms and produced the topographic maps of visual cell density of the 3 species of *Teuthida*.

Chapter 3 estimated the visual axes in the horizontal and in the vertical plane based on the data which we got in chapter 2, and introduced the visual acuity estimated by from the visual cell density and the focal length of the lens of the 3 species of *Teuthida*.

Chapter 4 made a comprehensive discussion about the comparisons on the visual axes and visual acuities related to their habitats and feeding behaviors.

In appendix part, it is focused on the problems that affecting the quality of the retina paraffin section, and introduced some improvements on the paraffin section preparation method according to problems existing in the paraffin section preparation.

Chapter2 Comparisons on distribution and density of visual cells of 3 species of *Teuthida*

2.1 Materials and methods

2.1.1 Organism materials

8 eyeballs from 4 individuals of *Todarodes pacificus*, 5 eyeballs from 3 individuals of *Heterololigo bleekeri* (one eyeball was used for testing the shrinkage rate of retina) and 6 eyeballs from 3 individuals of *Watasenia scintillans* were examined (Table 2.1).

The *Todarodes pacificus* specimens were jigged in Wakasa Bay, Fukui prefecture at night in Jun 2015. The *Heterololigo bleekeri* specimens were sampled and Izu, Shizuoka prefecture in daytime during Jan 2016. The *Watasenia scintillans* specimens were obtained in Toyama Bay, Toyama prefectures.

In order to ensure the accuracy of the experiments, the living samples are necessary, we chose the individuals in vivo as the samples. After measuring mantle length and weight, each sample of *Todarodes pacificus and Heterololigo bleekeri* was decapitated immediately and the head fixed in Bouin's fluid, as the size of *Watasenia scintillans* is small, the whole individuals were kept in the sample bottles with Bouin's fluid.

2.1.2 Retinal histology

After fixed in the Bouin's fluid for more than two days, each eyeball was removed from the head, after making an incision in the anterior side of the iris to mark the direction of the eyeball. After removal of the iris, lens, sclera and optic nerve, each retina was flattened by making peripheral incisions. As shown in the figure 2.1, the retinas were then divided into four pieces, in the figure D represents dorsal, V represents ventral, A represents anterior and

P means posterior. And the retinas then further sampled into 37-81 pieces according to the size of each specimen (Table 2.1).

The coordinates of these retinal pieces were calculated and recorded for restoring the distribution of the visual cells in the retina. Retinal pieces were dehydrated in an ethanol series and embedded in paraffin. Serial sections were cut tangentially at a thickness of 4 mm and stretched onto glass slides. These sections were then dewaxed with xylene, dehydrated in an ethanol series and stained with eosin. After clearing with xylene, sections were sealed by sealing mountant. Using a light microscope (OLYMPUS BX41N), we observed the layer of the outer segment of serial sections, found the rhabdom (the functional unit of the cephalopod retina) mosaic structures (fig. 2.2), then took the pictures by the OLYMPUS E-330 camera.

2.1.3 Data processing and analysis

Since the pictures of rhabdoms have been taken, we adjusted the contrast to make the rhabdoms to be identified more clearly and marked a square with length of 50μm by using software of Adobe Photoshop CS 6. We counted all the recognizable rhabdoms in this 50 × 50μm square and converted counts to cells/square millimeter. As has been mentioned, four rhabdomeres from four different cells combine to form a prismatic rhabdom, which can be calculated there exist two visual cells when one rhabdom exists in the picture. And taking the shrinkage rate (about 0.19) which emerged in the preparation of retina paraffin section into account, the final calculation formula of visual cell density of one square millimeter can be calculated:

$$C = 800A \times (1-0.19)$$

A is the number of rhabdoms in one 50 × 50μm square.

All the data was tested and analyzed by software of IBM SPSS Statistics 22.0. The densities of the 37 – 81 points sampled in each retina were used to build iso-density contour maps of the visual cell for each species by using the software of Golden Software Surfer 11.

2.2 Results

2.2.1 Comparison of the arrays of rhabdoms

Histological studies on retina of 3 species of *Teuthida* showed, the arrays of rhabdoms among *Todarodes pacificus*, *Heterololigo bleekeri* and *Watasenia scintillans* were different.

For all the three species under optical microscopy, as stated before, the structure of the rhabdom could be observed that four rhabdomeres from four different cells combine to form a prismatic rhabdom. In figure 2.2, we saw that for the rhabdom arrays of *Todarodes pacificus* and *Watasenia scintillans* were similar, all were arranged neatly and regularly and both horizontal and vertical were orderly, showing the shape of the regular square. Each rhabdom was independent from others, and the gap between the rhabdom and rhabdom was uniform, four adjacent individuals showing an orthogonal array, form a right angle cross. While for *Heterololigo bleekeri, Unlike Todarodes pacificus* and *Watasenia scintillans,* the array appeared to be in disorder, the adjacent two individuals were tightly connected together, and in the same direction, but differed from the other individuals, besides the gap between individuals was also not uniform.

The rhabdom arrays of different areas in retina for each species were also compared, it was found that only the size and density of rhabdoms differed in different areas, but the arrays did not change for different area.

2.2.2 Comparisons on density of visual cells

By using SPSS 22 statistical analysis software, One-Way ANOVA analyses of visual cell density (the number of cells per square millimeter) of each retina of each species and analyses of visual cell density among 3 species of *Teuthida* were conducted (Table 2.2-2.6).

For two retinas from the same individuals of same species, there was no significant difference ($P>0.05$) in the species of *Todarodes pacificus* and *Watasenia scintillans* (Table 2.2, 2.4), and there was also no significant difference ($P>0.05$) for the two retinas of *Heterololigo bleekeri* H1 (Table 2.3). For all the retinas of *Watasenia scintillans*, there was no significant difference ($P>0.05$) (Table 2.4), while there existed significant difference ($P<0.05$) among different individuals of *Todarodes pacificus* and *Watasenia scintillans* (Table 2.2, 2.4).

The results of multiple comparisons showed that there were significant differences between the 3 species of *Teuthida* ($P<0.05$) (Table 2.6). In addition, the means and std. deviation of each species were also analyzed in the tables.

2.2.3 Comparisons on visual cell density distribution

One-Way ANOVA analyses for visual cell density of the four areas (D-P, V-P, V-A, D-A) were conducted again, the results shown that, there were significant differences in the distributions of the different area which could be proved by iso-contour maps of visual cell density.

Through the maps of distribution of visual cell density, it could be proved that topographical distributions of visual cell across the whole retina differed among the species considered. The mean cell density of *Todarodes pacificus* was higher than *Heterololigo*

bleekeri and *Watasenia scintillans*. While the cell density of *Heterololigo bleekeri* was higher than *Watasenia scintillans*.

All cell densities were higher in the posterior retina in all species, for the *Todarodes pacificus*, the highest density region was ventral to the midline, while the region of highest density was dorsal to the midline of the body in Heterololigo bleekeri and *Watasenia scintillans*(Figure 2.3-2.5).

The highest density region changed outward with the growth of mental length (ML) in *Todarodes pacificus* (Figure 2.3(A, B, C)) and the density peaked at the periphery of the retina, with cells being sparse near its center in *Watasenia scintillans* (Figure 2.5).

2.3 Discussion

According to the previous studies, many day active fish with an acute vision have their visual cells arranged in very regular, mosaic-like patterns. This has been known for a long time and described by many authors (ENGSTRÖM, 1963; Saibil & Hewat, 1987; Stell, 1972; Wagner, 1975), some of whom pointed to the apparent similarity to the array of receptor cells in the insect eye. The highly organized orientation of the visual cells were taken as a prerequisite for the functional capacity of the eyes.

However, as for *Cephalopods*, there are few related studies in this area. In 2011, Marshall and Cronin suggested that rhabdomeres, the receptors in *Cephalopod* retinas, generally show orthogonal microtubule array, indicating sensitivity to e-vectors at 90° to each other. While microvilli could be arranged at any angle, this 90° array forms the basis of many polarisation-sensitive systems which animals utilise with navigation, sexual signalling and detecting water (Marshall & Cronin, 2011).

As stated before, while most *Cephalopods* have only one visual pigment, *Watasenia*

scintillans has three. They also have a double-layered retina with regular orthogonal mosaic arrays of rhabdomeres. These adaptations may have evolved to enable *Watasenia scintillans* to distinguish between ambient light and bioluminescence, and to help them decode the patterns of light created by other members of the species.

Although the real significance of the mosaic of receptor cells remained a matter of speculation, we can also surmised that *Todarodes pacificus* and *Watasenia scintillans* may have a better polarisation-sensitive systems than *Heterololigo bleekeri,* for the more developed mosaic array of rhabdoms. But this suspicion should be further verified by experiments, surveys in future.

The results shown that the maximum densities of 3 species of *Teuthida* are $1419\times10^2/mm^2$, $1069\times10^2/mm^2$, $849\times10^2/mm^2$ respectively, and this is generally consistent with the conclusions of previous studies(Makino & Miyazaki, 2010). Besides, Young (1963) reported cell density in the posterior central retina of the cuttlefish, *Sepia officinalis*, and the nearshore squid, *Loligo pealeii*, to be 104,000 and 51,000 cells/mm^2, respectively. These results are comparable to our results. But for *Todarodes pacificus,* it is found that the highest density region changed outward with the growth of ML, this conclusion is similar with the finding of Tominaga. The density of those 3 species will be analyzed and discuss particularly in chapter 3.

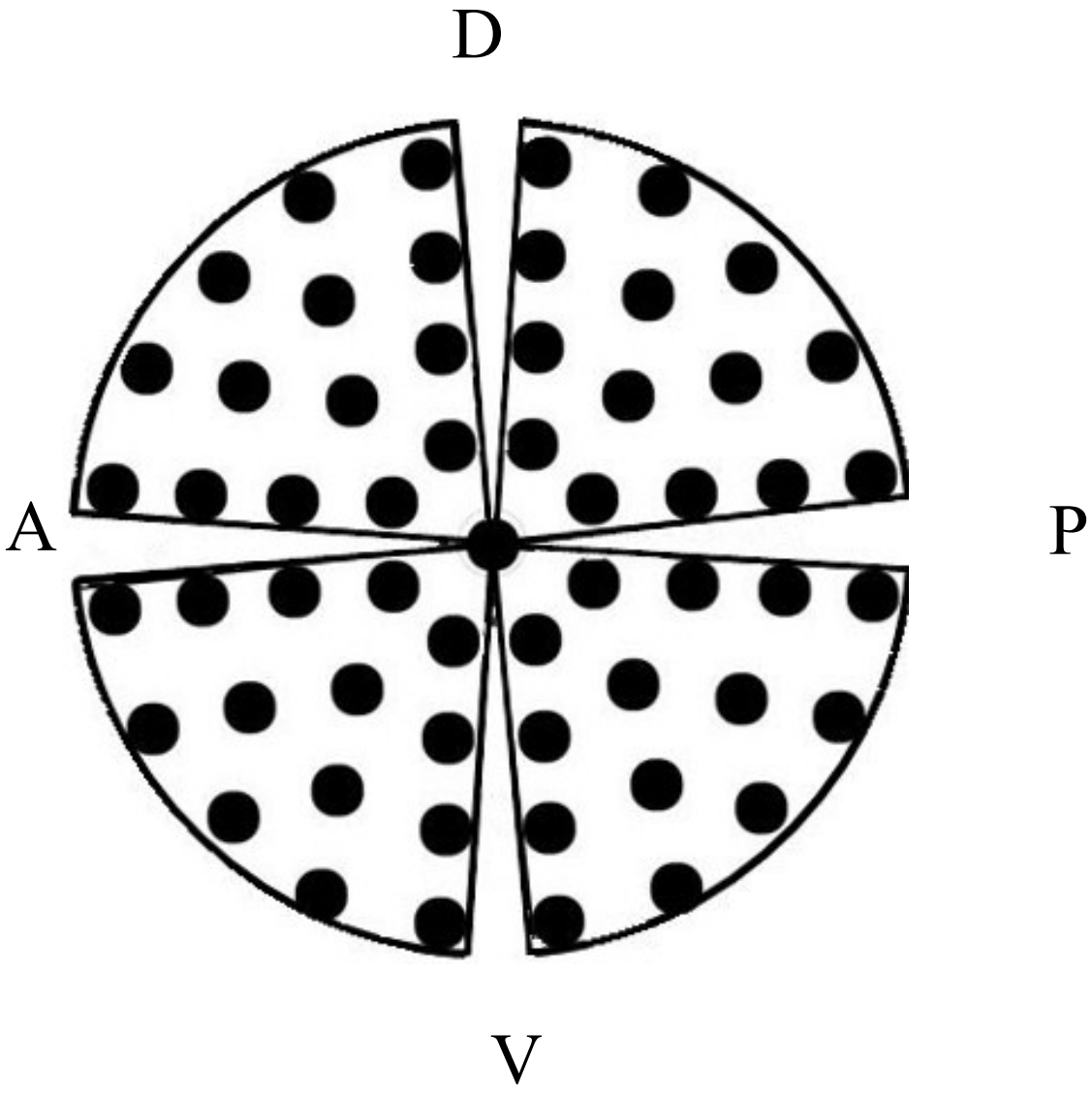


Figure. 2.1 Map showing the sampling sites on the retina

A., Anterior; D., Dorsal; V.,Ventral; P., Posterior

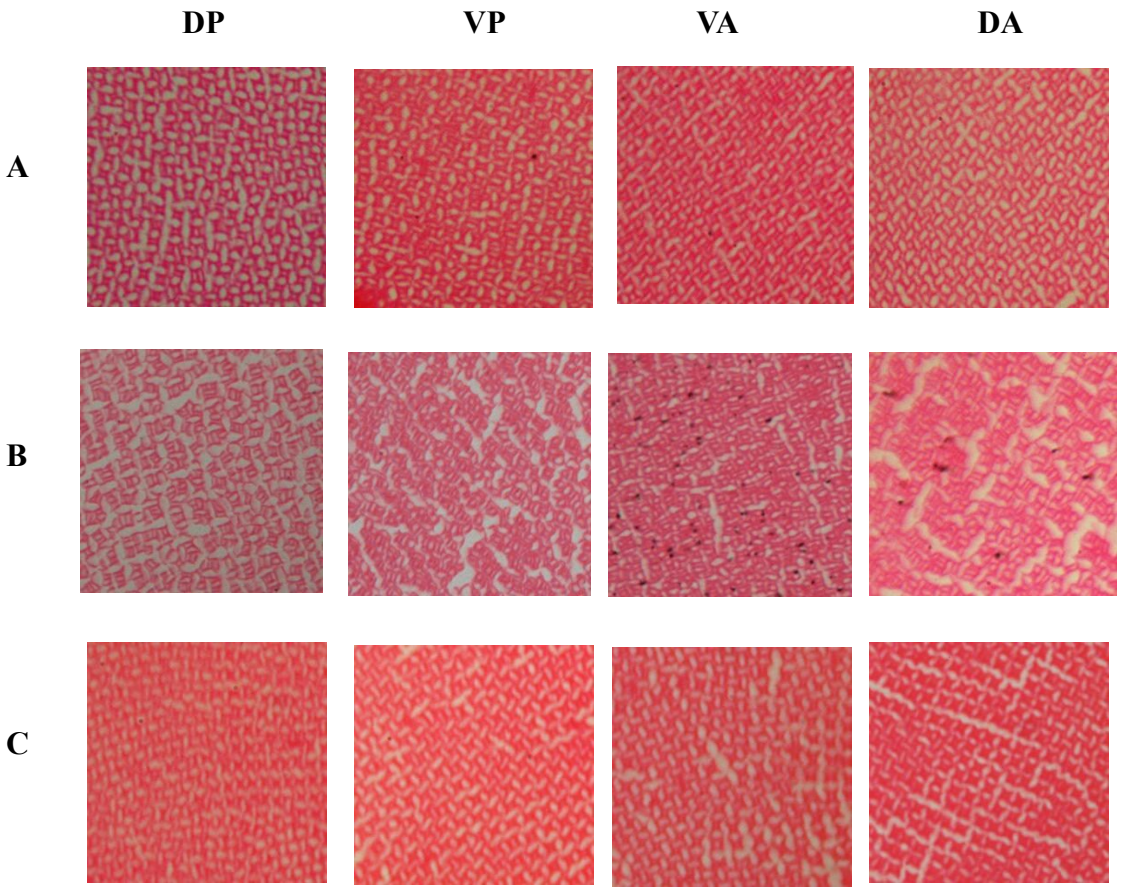
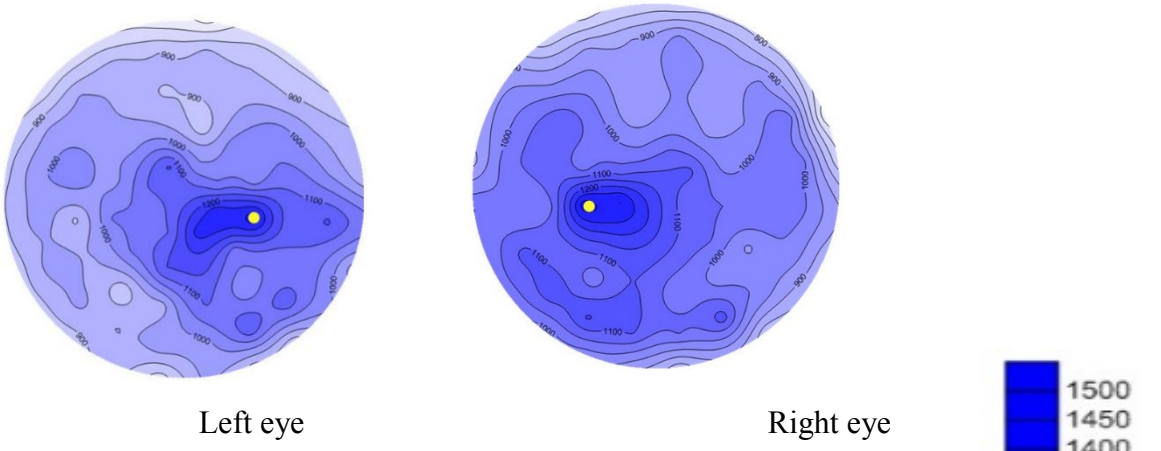


Figure 2.2 Rhabdoms of different parts in retina under optical microscope

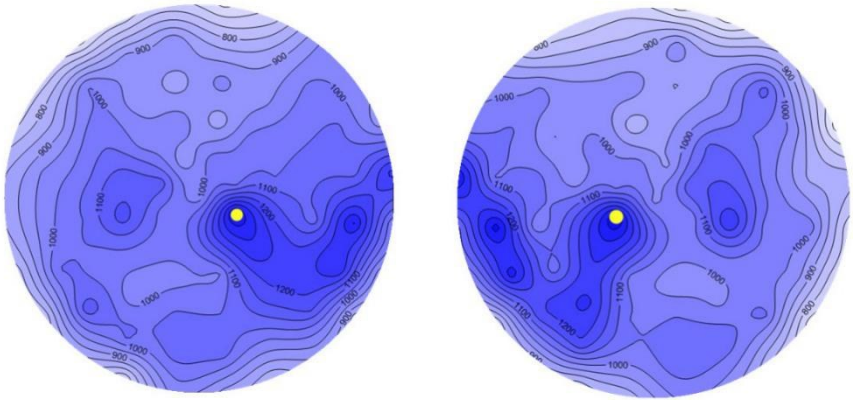
(A) *Todarodes pacificus,* (B) *Heterololigo bleekeri,* (C) *Watasenia scintillans*

(Magnification ×600 time)



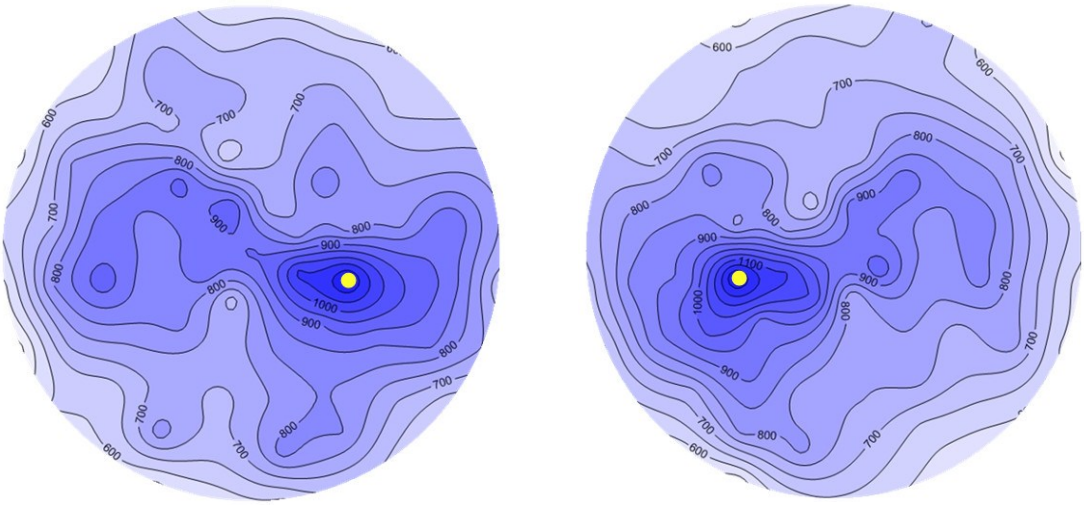
Left eye Right eye

Figure.2.3 (A) Distribution of visual cell density (T1)



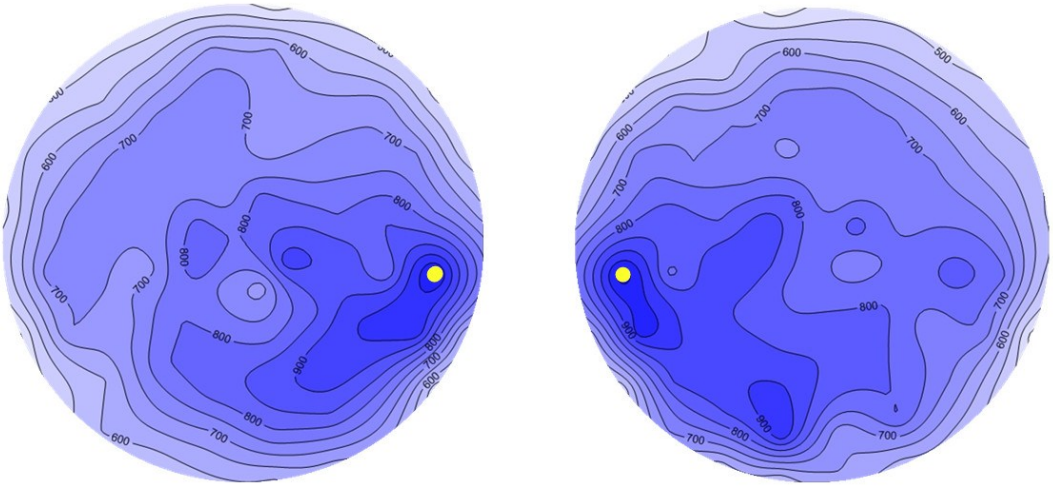
Left eye Right eye

Figure.2.3 (B) Distribution of visual cell density (T2)



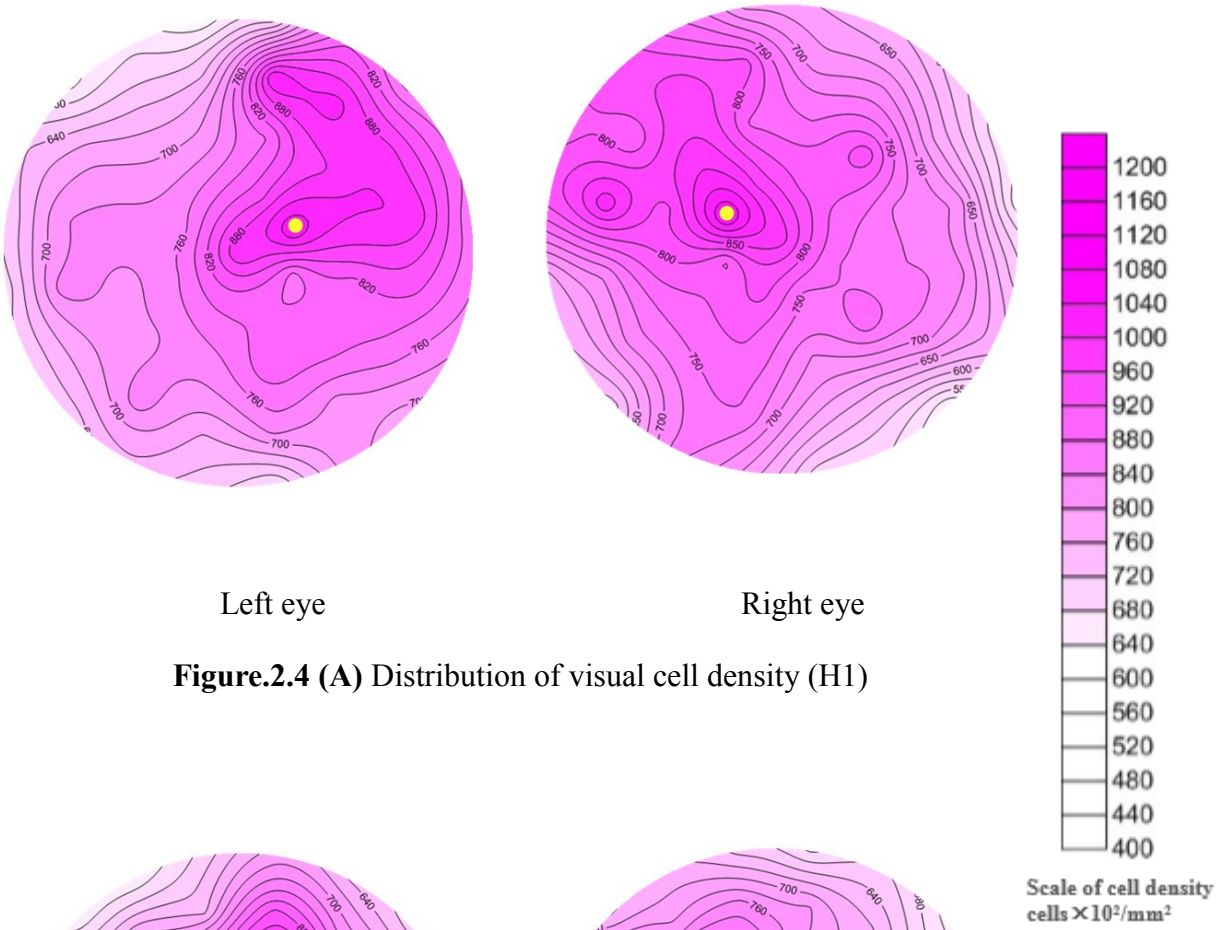
Left eye Right eye

Figure.2.3 (C) Distribution of visual cell density (T3)



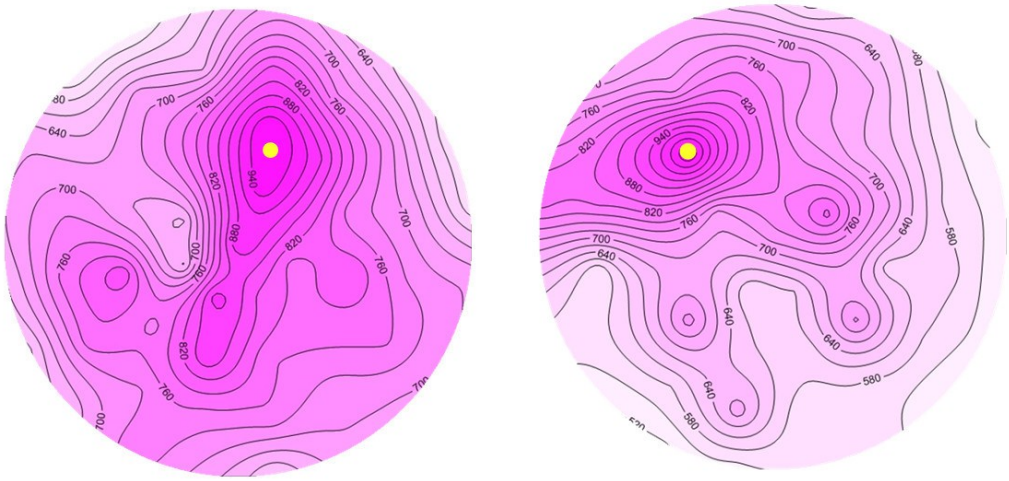
Left eye Right eye

Figure.2.3 (D) Distribution of visual cell density (T4)



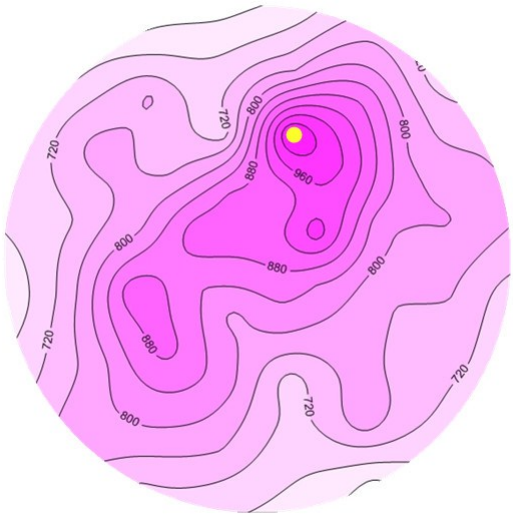
Left eye Right eye

Figure.2.4 (A) Distribution of visual cell density (H1)



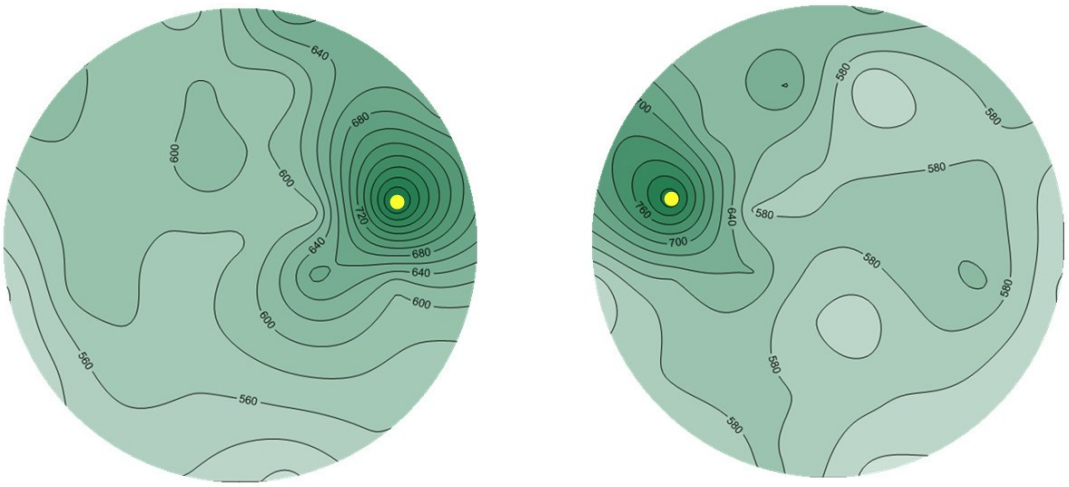
Left eye Right eye

Figure.2.4 (B) Distribution of visual cell density (H2)



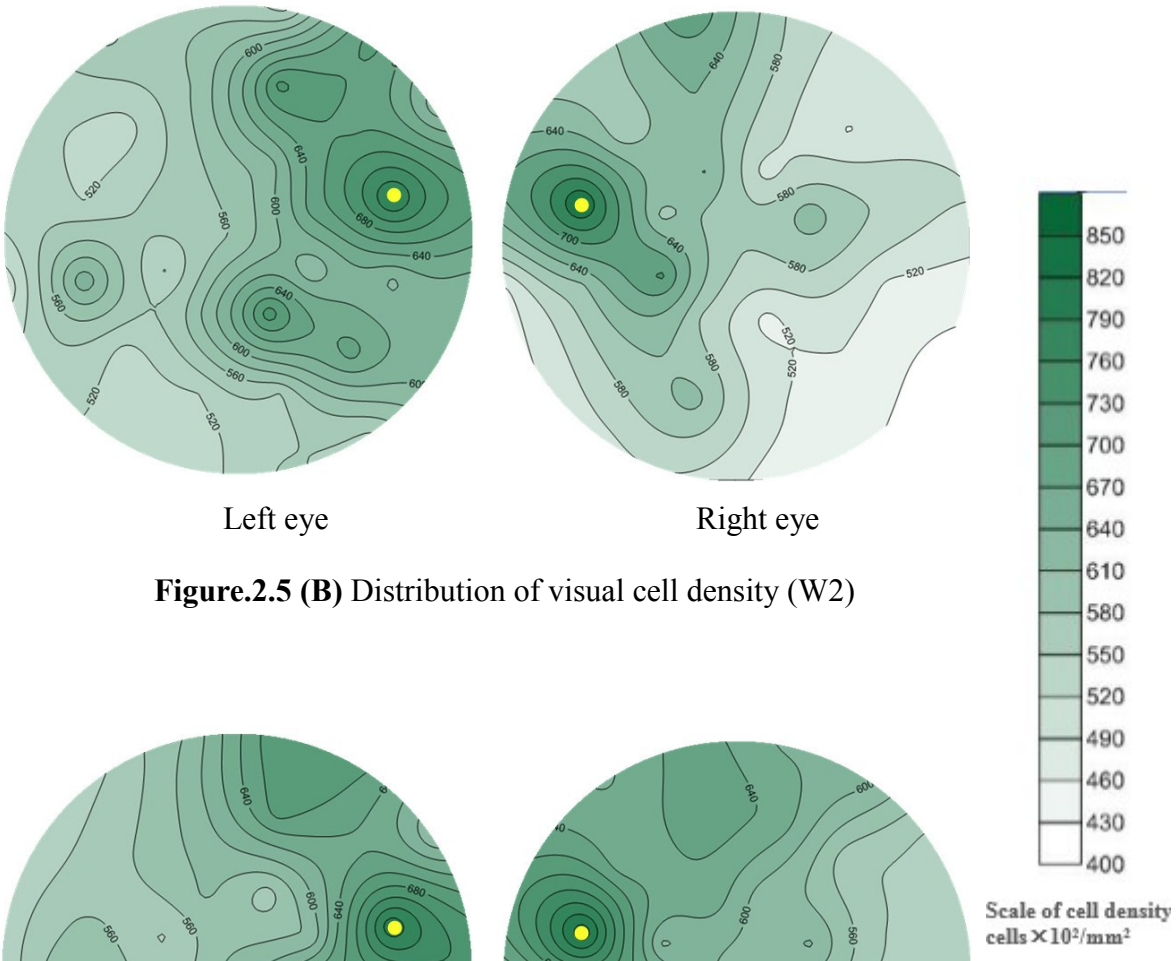
Left eye

Figure.2.4 (C) Distribution of visual cell density (H3)



Left eye Right eye

Figure.2.5 (A) Distribution of visual cell density (W1)



Left eye Right eye

Figure.2.5 (B) Distribution of visual cell density (W2)

Left eye Right eye

Figure.2.5 (C) Distribution of visual cell density (W3)

Table 2.1 Summary of the mantle length, weight and lens diameter of 3 species of *Teuthida*

Species	Number	Mantle length(mm)	Weight(g)	Lens diameter(mm)
Todarodes pacificus	T1	150	65.0	5.23
	T2	163	70	5.60
	T3	177	90.4	7.31
	T4	209	210.0	8.06
Heterololigo bleekeri	H1	168	78.5	7.26
	H2	175	91.5	7.31
	H3	207	124.5	7.47
Watasenia scintillans	W1	39	9.3	4.59
	W2	40.3	9.6	4.59
	W3	42.8	10.0	4.64

Table 2.2 Summary of the density of each retina of 4 specimens of *Todarodes pacificus*

Specimen (retina)	No. of retina pieces	Density of visual cells(cells×10^2/mm^2)			
		Mean	Std. Deviation	Minimum	Maximum
T1L	81	978.0	121.0	719	1341
T1R	81	984.0	129.4	642	1335
T2L	81	983.4	161.4	622	1419
T2R	79	998.6	160.4	667	1413
T3L	81	732.5	130.6	473	1166
T3R	81	746.6	143.2	480	1270
T4L	81	690.5	143.1	408	1069
T4R	81	697.7	140.3	408	1037
Total	646	850.0	195.7	408	1419

Table 2.3 Summary of the density of each retina of 3 specimens of *Heterololigo bleekeri*

Specimen (retina)	No. of retina pieces	Density of visual cells(cells×10^2/mm^2)			
		Mean	Std. Deviation	Minimum	Maximum
H1L	56	737.7	106.3	518	972
H1R	57	719.3	99.6	492	959
H2L	56	723.6	104.1	518	978
H2R	55	660.6	120.7	512	1069
H3L	56	766.7	91.2	622	1050

Table 2.4 Summary of the density of each retina of 3 specimens of *Watasenia scintillans*

specimen (retina)	No. of retina pieces	Density of visual cells(cells×10^2/mm^2)			
		Mean	Std. Deviation	Minimum	Maximum
W1L	36	598.2	59.7	512	836
W1R	37	591.4	65.5	505	849
W2L	36	577.8	63.7	480	745
W2R	36	576.4	69.3	480	797
W3L	37	587.0	61.9	505	758
W4R	36	585.2	58.6	492	778

Table 2.5 Summary of the density of 3 species of *Teuthida*

		Density of visual cells(cells×10^2/mm^2)			
Species	No. of retina pieces	Mean	Std. Deviation	Minimum	Maximum
Todarodes pacificus	646	851.0	195.7	408	1419
Heterololigo bleekeri	280	721.8	109.6	492	1069
Watasenia scintillans	218	586.0	62.9	480	849

Table 2.6 Multiple comparisons of density of 3 species of *Teuthida*

(I) Species		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Todarodes pacificus	*Heterololigo bleekeri*	252.6*	13.2	.0	226.7	278.5
	Watasenia scintillans	386.2*	15.1	.0	356.4	416.0
Heterololigo bleekeri	*Todarodes pacificus*	-252.6*	13.2	.0	-278.5	-226.7
	Watasenia scintillans	133.6*	16.1	.0	101.9	165.4
Watasenia scintillans	*Todarodes pacificus*	-386.2*	15.1	.0	-416.0	-356.4
	Heterololigo bleekeri	-133.6*	16.1	.0	-165.4	-101.9

*. The mean difference is significant at the 0.05 level.

Chapter3 Visual axis and visual acuity of 3 species of *Teuthida*

3.1 Materials and methods

3.1.1 Organism materials

The data of retinas from those 3 species of *Teuthida* which examined in chapter 2 were used in this chapter (Table 2.1). The highest density regions were used for estimating the visual axes, and highest densities of visual cells were used for calculating visual acuities of the 3 species of *Teuthida.*

3.1.2 A method of estimating the visual axis

In teleosts, the visual axis is generally defined as the direction linking the center of the lens and the retinal positions with the highest cell density (T Tamura, 1957). By using this theory, many studies estimated the visual axis of fish, and discussed about it(Tamotsu Tamura & Wisby, 1963). Applying this definition to our work, we estimated the visual axes from the vertical plane and from horizontal plane of the three species respectively.

The visual axes the vertical plane (observed from the lateral side) were traced from figures of distribution of visual cell density, by linking the center of the retina and the retinal positions with the highest cell density (figure 3.1). While for visual axes in horizontal plane (observed from the dorsal side), the highest density positions in the dorsal view of the eyecups were also estimated based on figures of distribution of visual cell density, by determining the distance of the highest-density position from the posterior edge relative to the equator line (figure 3.2).

3.1.3 Methods of calculating the visual acuity

Taking the special retina structure of retina nature of *Cephalopods* into consideration, and reference to other researches related to the visual acuity of other species, for example, in 1986 Mass and Supin tried to take a topographic study on the density of ganglion cells in the retina and calculated the visual acuity of the species of dolphins-the porpoise *Phocoena phocoena L(Mass, Supin, & Severtsov, 1986).*We decided to utilize the simple relationship between Matthiessen's ratio and nodal distance, which widely applied in fish visual optics to calculate the visual acuities of the 3 species of *Teuthida*. The formulas are as follows:

Matthiessen's ratio states that the distance from lens center to retina (posterior nodal distance,

PND) is 2.55 times the lens radius. Therefore, for a lens diameter of 5.60 mm (T2 in *Todarodes pacificus*, Table 2.1)

$$PND = 2.55 \times 2.80 \text{ (radius)}$$
$$PND = 7.14 \text{ mm}$$

The angle (α) (radian) subtending 1 mm on the retina can be calculated

$$\tan\alpha = 1\text{mm} / PND$$
$$\alpha = \arctan(1 / 7.14)$$
$$\alpha \approx 8.02\ ^\circ \text{(in degree)}$$

Or $\alpha \approx 0.140$ (in radians)

Within the highest density area of left retina of H2, there are 1.419×10^5 cells/mm^2 or 377 cells/mm.

The min spatial resolution may then be calculated by obtaining the number of cells subtended by one degree of visual arc, i.e.

$$\text{Cells per degree} = \sqrt{D} / \alpha$$
$$= 377 \text{ cells} / 8.02 \text{ degrees}$$
$$\approx 47.00$$
$$\text{Cycles per degree} = 1/2 \text{ cells per degree}$$
$$\approx 23.47 \text{ cycles per degree}$$

Or

$$\text{Visual acuity} = 1 / \text{min.of arc} = \frac{1}{\frac{1}{\sqrt{D}} \times \frac{180}{\pi} \times 60}$$

$$\approx 0.78$$

So, the maximum visual acuity of specimen T2 of *Todarodes pacificus* is about 0.78 or 23.47 cycles per degree.

As we mentioned above there are few researchers studied about the visual acuity of *Teuthida* (*decapodiformes*), in order to compare the results with the visual acuity of other species calculated by Tamura's methods. The visual acuities were also calculated by using Tamura's formula:

$$\alpha = \frac{1}{F} \left(\frac{0.1(1+S) \times 2}{\sqrt{n}} \right) \text{ (Angle in radians)}$$

Where F is the focal distance of the lens and it is same with PND above, S is the degree of shrinkage which is around 0.1, and n is the maximum number of visual cells per 0.1mm square in retina.

$$\text{min.of arc (minute)} = \frac{1}{\alpha \times \frac{180}{\pi} \times 60}$$

Take H2's data into this formula, we got that the maximum visual acuity of specimen T2 of *Todarodes pacificus* by Tamura's methods is about 0.36.

Same as the tissue of retina, there also exists shrinkage for the lens caused in the progress of fixing the eyeball. Therefore the shrinkage rate should be taken into consideration, here we consulted to Tominaga's shrinkage rate (about 6.3％), and applied it into our studies. Besides the minimum visual acuity of each specimen, visual acuity of each area in the retina was also calculated and compared here.

3.2 Results

3.2.1 Visual axis in the vertical plane

The visual axis the vertical plane (observed from the lateral side) were traced from figures of distribution of visual cell density of each retina. By using the Adobe Photoshop CC software, the visual axes were linked from the retinal positions with the highest cell density to the center of the retina, the angles (α) between the visual axes and vertical planes were also measured at the same time.

We estimated the visual axes of *Todarodes pacificus* to be upward and forward (Figure 3.1A), In *Heterololigo bleekeri* and *Watasenia scintillans* , the visual axes were estimated to be directed downwards and forwards when observed from the lateral side (Figure. 3.2A, 3.3A). However, according to the research of Mäthger et al in 2003, squids almost always hold a slightly downward posture while swimming or hovering(Mäthger, 2003), We therefore correspondingly depressed the visual axes of all the 3 species.

The visual axis angles in the vertical planes were measured and summarized in table 3.1, from the table, we can see that, the visual axis angles differed among different species; the visual axis angle of *Heterololigo bleekeri* was the highest among 3 species. The visual angle changed obviously with the difference of ML in *Todarodes pacificus* and *Heterololigo bleekeri* and there also existed significant difference of visual angles of same species in *Todarodes pacificus* and *Heterololigo bleekeri,* while there was no significant difference in *Watasenia scintillans*.

3.2.2 Visual axis in the horizontal plane

In the horizontal plane, the visual axes and visual axis angles were also presumed and measured by using the Adobe Photoshop CC software , although the angles of visual axis angles differed among species, the visual axes of all 3 species could also presumed to be directed laterally (Figure. 3.1B-3.3B). Based on the data we got (Table 3.1), the average value of visual axis angles of *Heterololigo bleekeri* was 77.8°, which was higher than the other two species. The species of *Watasenia scintillans* had the lowest angles of visual axes around 56.5 °, and there existed a widest range of visual axis angles in the horizontal plane in *Todarodes pacificus*, which were from 39.4° to 81.2°.

3.2.3 Visual acuity

By referring the lens shrinkage rate of the latest study (Tominaga, 2014) the lens diameters were returned to the original values, the visual cell densities were calculated in chapter 2, used the calculating methods which Mass & Supin and Tamura introduced in their papers, we got the visual acuities of all the 3 species of *Teuthida* (Table 3.2, 3.3).

Results showed that the average visual acuities of three species were 0.70, 0.74 and 0.42. According to the calculating formula, higher visual acuity relates to larger lens radius and higher retinal cell density. Although the lens radius and higher retinal cell density differed among different species, the highest visual acuities of *Todarodes pacificus* and *Heterololigo bleekeri* were similar in our experiments, which are 0.86 and 0.88. Similar to the visual axis angle, the *Todarodes pacificus* also had a widest range the visual acuity, besides the visual acuities increased with the growth of ML, so did in the species of the *Heterololigo bleekeri* (Table 3.2).

Although body sizes were much smaller than the other two species in *Watasenia scintillans*, the lens radius were still a relatively large proportion of the body size. But for the reason of low visual cell density, visual acuity of *Watasenia scintillans* turned to be smaller than *Todarodes pacificus* and *Heterololigo bleekeri*, which is about 0.42. And for different individuals of *Watasenia scintillans*, the visual acuities were differed ＜3% .

3.3 Discussion

Previous studies show that visual axis may suits for preying baits, for the *Todarodes pacificus* which is an oceanic species with an upper frontal visual axis, it is assumed that it might be advantageous in detecting prey items of above side. For the coastal species, which always hood a downward and forward directed visual axis, it is also proved that it is easily to detect baits of its downward side, and *Heterololigo bleekeri* happens to be a coastal species. While for *Watasenia scintillans*, although they live in the deep sea, this species come to sea surface to search for food at night, the downward and forward visual axis should enhance to forage items which come from under side.

The visual axes in the horizontal plane were directed laterally and forwards in all three species. It is also on prey items assumed that this might be advantageous in detecting items from the forward and lateral sides.

For the visual axis angle of *Todarodes pacificus*, even though there were only 4 individuals examined in our experiments, it could also be generally inferred the visual axis angle in the vertical plane decreased with the growth of mental length, and this is consistent with Tominaga's results. We assured that for the smaller individuals, the larger visual axis angle in the vertical plane might be advantageous in detecting predators and baits, while the dangers from predators decrease with the increase of body size, then the lower visual axis angle focus more on preying and other behaviors.

By using Mass & Supin, visual acuities were got out of quite different values, for example the maximum visual acuity of *Todarodes pacificus* is 0.98 (or 29.38 Cycles per degree) by Mass & Supin's way while 0.44 calculated in Tamura method. Here the acuities were only discussed among the three species and compared with the values calculated with the same methods by others. Average visual acuities of three species were 0.70 (25.55 cycles per degree), 0.74 (25.83 cycles per degree) and 0.42 (14.44 cycles per degree), compared previous species (*Sepioteuthis lessoniana, Uroteuthis edulis, Todarodes pacificus, Eucleoteuthis Luminosa, Thysanoteuthis rhombus* and *Euprymna morsei*) calculated by the same method (Makino, 2007), we confirmed that *Todarodes pacificus* and *Heterololigo bleekeri* had a good visual acuity which might be advantageous to their habitats.

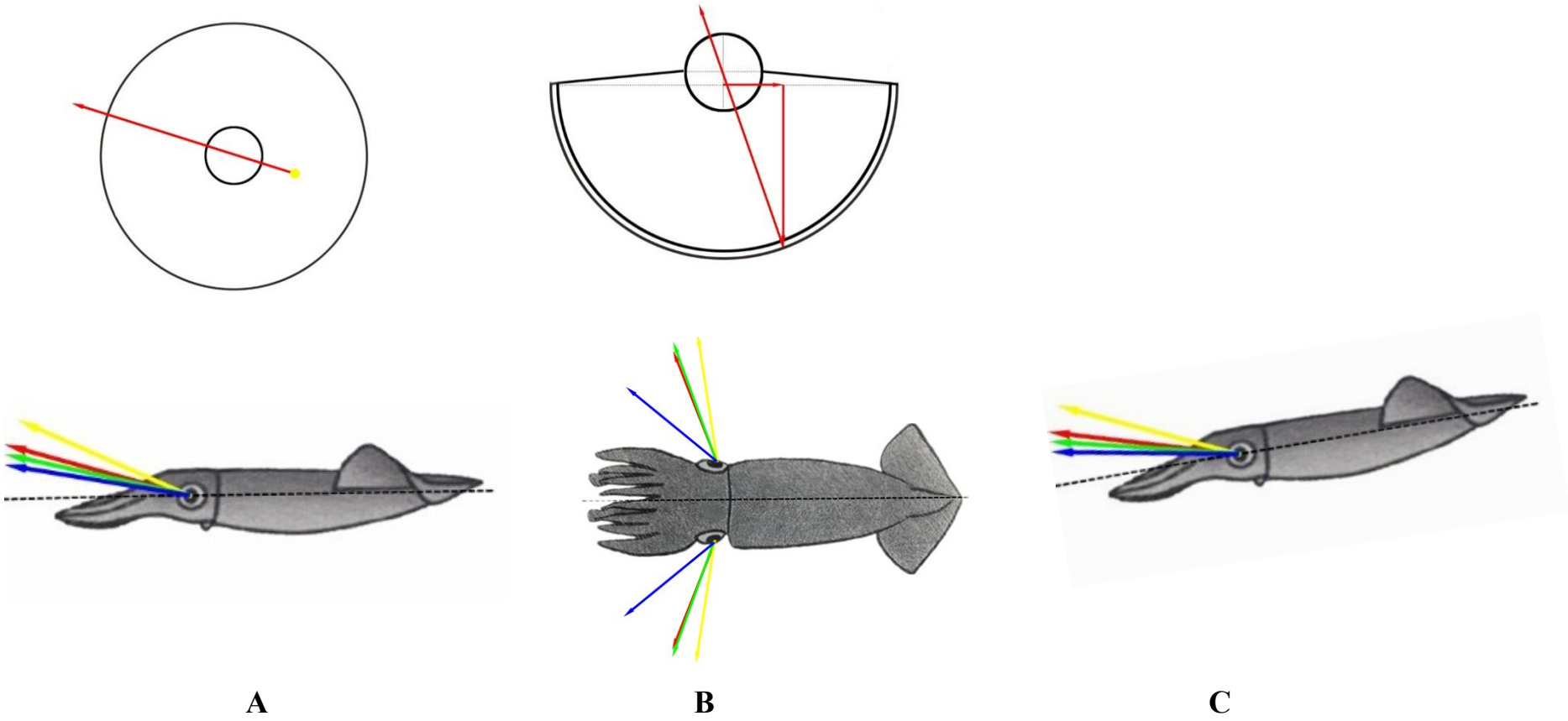
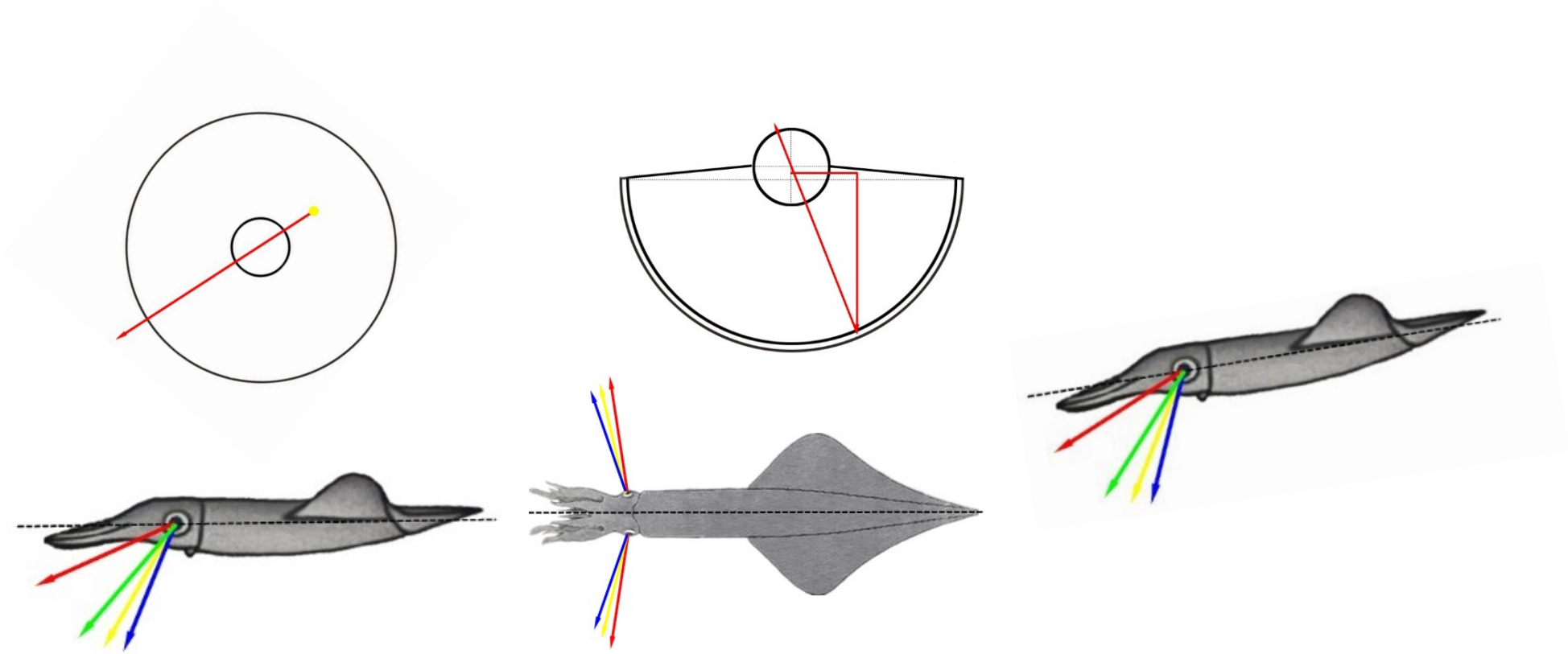


Figure .3.1 Schematic diagrams indicating visual axes of *Todarodes pacificus*

A: Visual axis in the vertical plane; B: Visual axis in the horizontal plane

C: visual axes in usual swimming or hovering posture

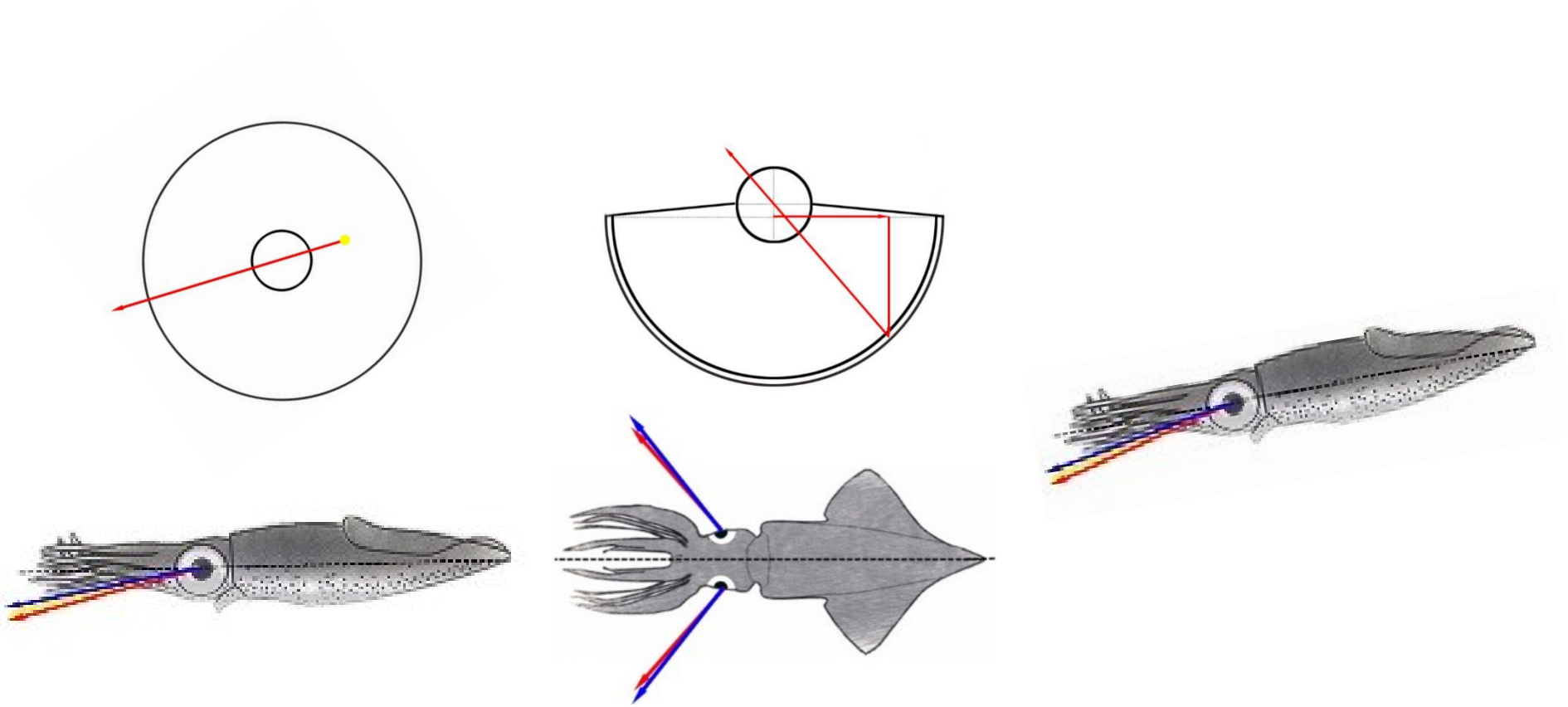


A **B** **C**

Figure. 3.2 Schematic diagrams indicating visual axes of *Heterololigo bleekeri*

A: Visual axis in the vertical plane; B: Visual axis in the horizontal plane

C: visual axes in usual swimming or hovering posture



A **B** **C**

Figure. 3.3 Schematic diagrams indicating visual axes of *Watasenia scintillans*

A: Visual axis in the vertical plane; B: Visual axis in the horizontal plane

C: visual axes in usual swimming or hovering posture

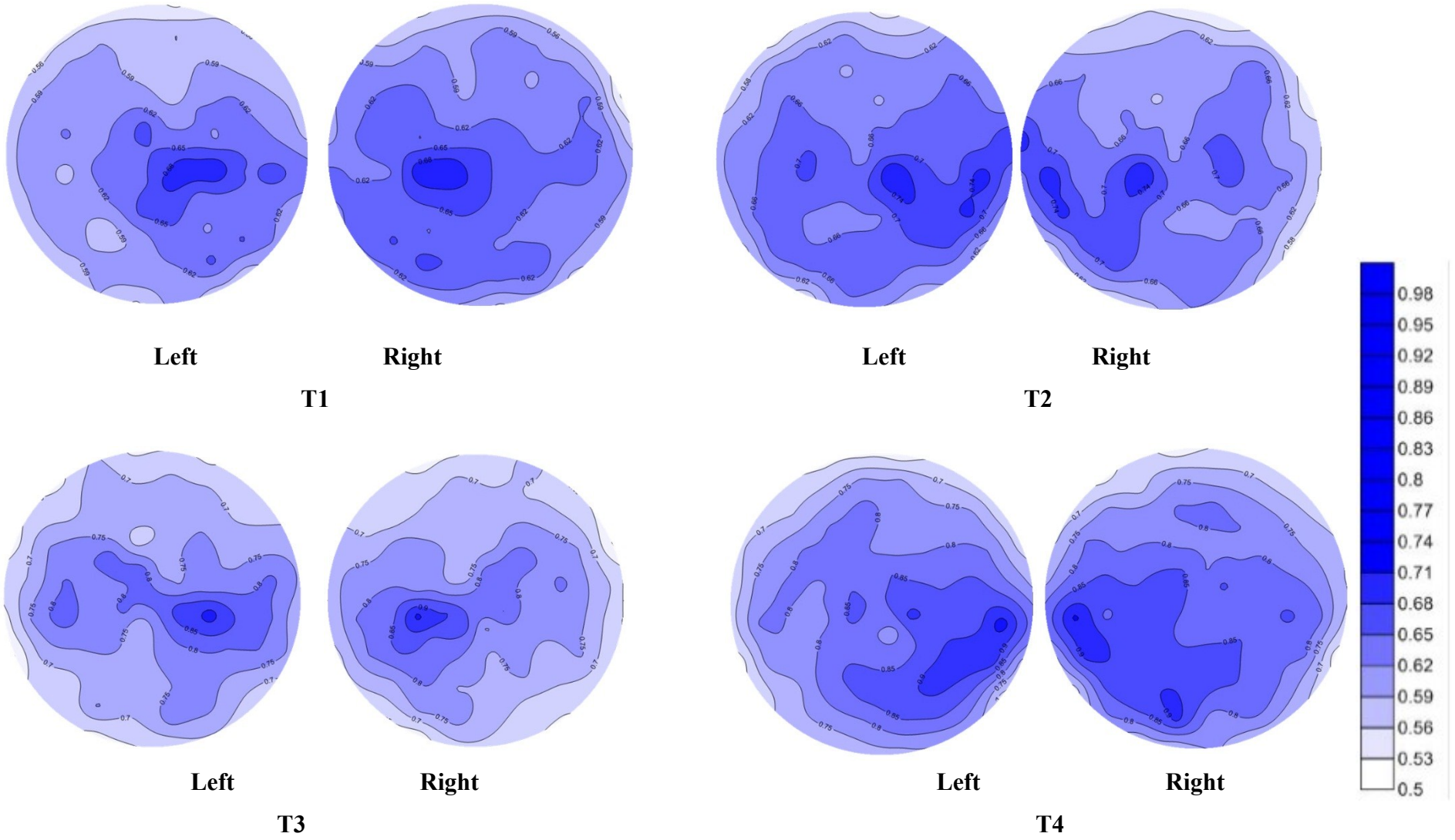


Figure. 3.4 Topographic distribution of visual acuity of *Todarodes pacificus*

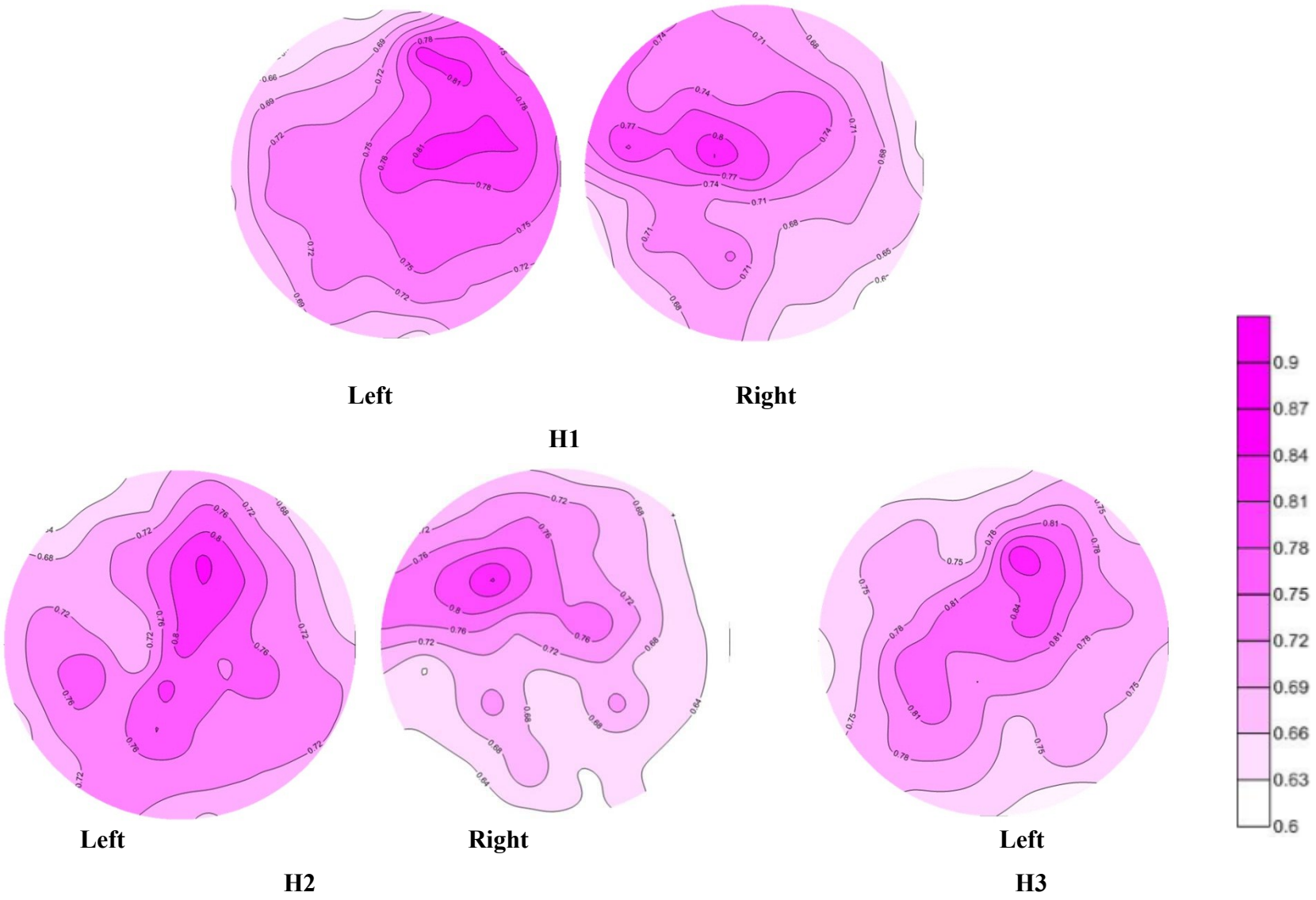


Figure. 3.5 Topographic distribution of visual acuity of *Heterololigo bleekeri*

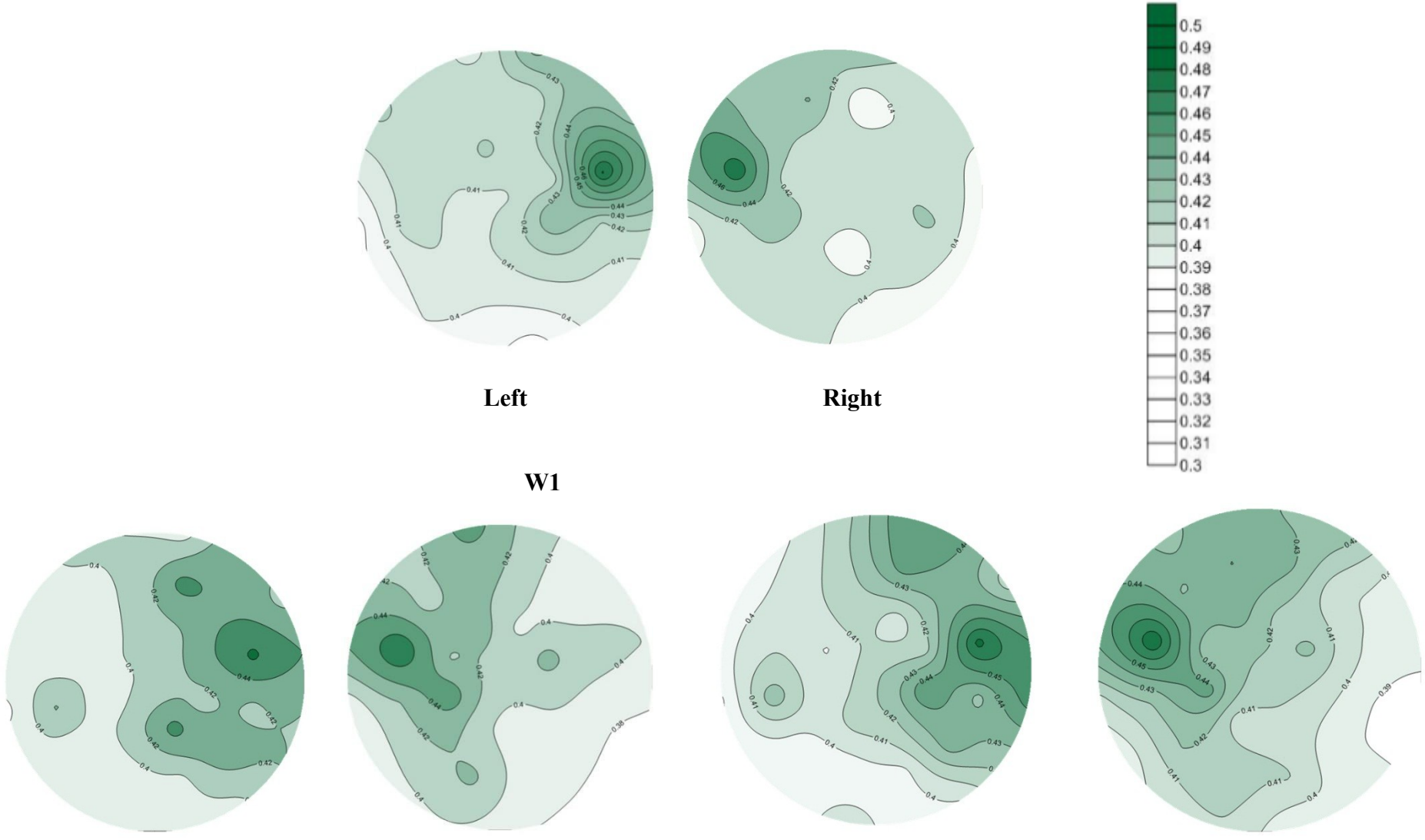


Figure. 3.6 Topographic distribution of visual acuity of *Watasenia scintillans*

Table 3.1 Summary of visual angles in vertical plane and horizontal plane of 3 species of *Teuthida*

Species	Number	Mantle length(mm)	lens diameter(mm)	Visual angle（°）			
				Left α	Right α	Left β	Right β
Todarodes pacificus	T1	150	5.23	15.4	15.4	68.2	68.2
	T2	163	5.60	23.5	23.5	81.2	81.2
	T3	177	7.31	12.9	12.9	68.8	68.8
	T4	209	8.06	9.2	9.2	39.4	39.4
Heterololigo bleekeri	H1	168	7.26	24.2	24.2	81.1	81.1
	H2	175	7.31	60.3	49.3	81	70.7
	H3	207	7.47	67.9	67.9	76.5	76.5
Watasenia scintillans	W1	39	4.59	15.2	15.3	49.2	49.2
	W2	40.3	4.59	14.9	14.9	51	51
	W3	42.8	4.64	13.6	13.6	49.2	49.2

Table 3.2 Summary of visual acuities of 3 species of *Teuthida*

			Visual acuity(M&S)		
Species	Number	Mantle length(mm)	Ave	Min	Max
	T1	150	0.61	0.49	0.71
	T2	163	0.65	0.52	0.78
	T3	177	0.74	0.59	0.97
Todarodes pacificus	T4	209	0.79	0.60	0.98
	H1	168	0.72	0.60	0.84
	H2	175	0.73	0.61	0.89
Heterololigo bleekeri	H3	207	0.77	0.69	0.90
	W1	39	0.42	0.38	0.50
	W2	40.3	0.41	0.37	0.48
Watasenia scintillans	W3	42.8	0.42	0.38	0.48

Table 3.3 Summary of maximum visual acuities of 3 species of *Teuthida*

Species	Number	Mantle length(mm)	Highest density of visual cells(cells×10^2/mm^2)		Maximum visual acuity(M&S)		Maximum visual acuity(Tamura)	
			Left eye	Right eye	Left eye	Right eye	Left eye	Right eye
Todarodes pacificus	T1	150	1341	1335	0.71	0.71	0.32	0.32
	T2	163	1419	1413	0.78	0.78	0.36	0.35
	T3	177	1166	1270	0.93	0.97	0.42	0.44
	T4	209	1069	1037	0.98	0.96	0.44	0.44
Heterololigo bleekeri	H1	168	972	959	0.84	0.83	0.38	0.38
	H2	175	978	1069	0.85	0.89	0.39	0.40
	H3	207	1050	N.D	0.9	N.D	0.41	N.D
Watasenia scintillans	W1	39	836	849	0.49	0.5	0.22	0.23
	W2	40.3	745	797	0.46	0.48	0.21	0.22
	W3	42.8	758	778	0.47	0.48	0.22	0.22

Chapter4 Comprehensive discussion

Studies on fish showed that the visual cell distribution often reflect species-specific characters such as the feeding behaviour or habitat. Similarities of retina structure and visual function between *Teuthida* and fish suggest that *Teuthida* retinas and eyes may mirror the diversity documented among fishes. Here we tried to discuss from visual axes and visual acuities estimated from the visual cell distribution of the three species and analyze the visual functions of them.

According to previous studies, the oceanic squids mostly feed on mesopelagic organisms including small fishes, cephalopods and crustaceans (Ivanovic & Brunetti, 1994; Mouat, Collins, & Pompert, 2001; Packard, 1972; Watanabe, Kubodera, Ichii, & Kawahara, 2004). As one kind of oceanic squids *Todarodes pacificus*, the large individuals feed predominantly on crustaceans (*Parathemisto japonica*) while smaller individuals feed on mesopelagic fish (*Maurolicus muelleri japonica*)(Okiyama, 1965). Besides they will also consume other types of squid, even resort to cannibalism of the smaller if food sources are short, for example, when trapped in nets together. In those considered here, this upper frontal visual axis might be advantageous in detecting prey items in the open water, silhouetted by light from above.

The species of *Heterololigo bleekeri* is a native species along the coast of Asia, it feed on benthic crustaceans in the juvenile stage, and then mainly feed on fish after reaching prematurity (Natsukari & Tashiro, 1991). As the *Heterololigo bleekeri* stays near on the sea bottom, its downward and forward directed visual axis may enhance its ability to forage for prey items of the sea bottom and detect the obstacles on the sea bottom. Besides many benthic on the sea bottom feed on the small fish or squid approaching from above, the downward and forward visual axis in the vertical plane might be advantageous in detecting predators on the sea bottom.

The *Watasenia scintillans* lives deep in the open ocean around Japan and is bioluminescent. Each tentacle of it has a photophore organ, which produces light. It uses its ability to sense and to produce light for counter illumination camouflage: it matches the brightness and color of its underside to the light coming from the surface, making it difficult for predators to detect it from its below.

The *Watasenia scintillans* spends the day at depths of several hundred meters, they migrate up to the surface to search for food when night falls. They are active predators by flashing the lights on and off, they can attract small fish and then pounce on them with their powerful tentacles (Sasaki, 1914). Because they prey from the surface at night, the downward and forward visual axis in the vertical plane should be advantageous in detecting small fish below themselves.

The visual axes in the horizontal plane were directed laterally and forwards in all three species. Although their monocular vision extends to almost 180° laterally, accuracy of attack on prey items may be significantly lower using monocular rather than binocular vision (Messenger, 1977). The lateral position of the eyes in squid may appear to limit binocular overlap, but well-developed extra-ocular muscles give the eyes high mobility and the lenses can move in any direction perpendicular to the papillary axis (Schaeffel, Murphy, & Howland, 1999). All three species detect the baits from front field and capture them with their powerful tentacles, therefore their binocular front vision suit for judging of distance and enables prey capture in the frontal(Budelmann & Young, 1993; Messenger, 1977).

Besides squid always clustered together in groups, for example the *Watasenia scintillans* can be seen gathering by the millions, and sometimes by the billions in Toyama Bay in Japan during the spawning season. The lateral visual axes in the horizontal plane may pertain to the ability to detect the conspecifics.

For the visual acuity studies of fish, a number of different methods have been employed to investigate visual acuity. Brunner and Yamanouchi studied the visual acuity of *Phoxinus laevis* and *Microcanthus strigatus*, respectively, by observing behavioural responses to different sized gratings(Brunner, 1935; Yamanouchi, 1956). Tamura studied the resolving power of the lens, dioptric accommodation and retinal cone density of 27 species of fish in a comparative study of form perception (Tamura, 1957). In 1999, Schaeffel et al attempted to calculate the anatomical resolving power of an adult cuttlefish using the average inter rhabdom spacing (millimeter) and the posterior nodal distance(Schaeffel et al., 1999).

But so far there is no clear conclusion for calculating the acuity of *Cephalopods*. So it is necessary to create a suitable calculation method of acuity for *Cephalopods*. As we mentioned before, Collin & Pettigrew (1988) examined in twelve species of reef teleosts and estimates of the spatial resolution of neurons within the ganglion cell layer calculated using Matthiessen's ratio. Upper limits of between 4 and 20 cycles per degree were found among these species, compared with these results, *Todarodes pacificus* and *Heterololigo bleekeri* have better visual acuities than ordinary species of fish.

Among the three species we examined, *Todarodes pacificus* had the highest visual acuity, besides the visual acuity also increased with the increase of body size. We consider that this oceanic species may therefore recognize distant objects. And as the body grows, more food is needed, this is just in line with the increase of visual acuity. For the *Heterololigo bleekeri*, it also had a high visual acuity almost same as the *Todarodes pacificus*, take the feeding habits and living environment into consideration, the high visual acuity may be suitable for detecting baits near the offshore.

It can be inferred from the formula, higher visual acuity relates to larger lens radius and higher retinal cell density. Although the lens radius of *Watasenia scintillans* was relatively large, due to its

slow visual cell density, the visual acuity proved lower than the other two species. To detect accurately a visual target or a point source like bioluminescence, the retina needs not only fine photoreceptor distribution, but the lens must also be able to provide high resolution. Although the lens resolution of *Watasenia scintillans* is unknown, the eye of this species may have specializations to detect bioluminescence by conspecifics and the three kinds of visual pigments, double-layered retina with regular orthogonal mosaic arrays of rhabdoms could also prove that.

In our study, new ways and methods were utilized for analyze and process the data, in the procedures returning the distribution of visual cell, the Golden Software of Surfer 11 was used to make the contour maps. This software has dozens of data algorithms, which can reduce the errors appeared in processing progress, therefore the visual cell distribution can be reproduced accurately. In addition, in the process of calculating the visual axis angle, the software of Adobe Photoshop was used to make the results become more accurate. This can serve as a reference for the further studies in future. We think for different disciplines, different research direction, research methods and means are common in a certain extent, through a combination of different methods or techniques, we can solve the problems encountered in many researches more quickly and accurately.

Appendix

Improvements of retina paraffin section preparation methods

The paraffin section is the main experimental method of histology and developmental biology. It is also an important method to observe the pathological changes in pathology. It has made a great contribution to scientific research and clinical diagnosis. This technology is applied the basic principle that animal (or plant) tissue can be well combined with paraffin. After specimen collecting, fixation, dehydrating, clearing, dipping wax, embedding, sectioning, baking sections, dewaxing, Hematoxylin-eosin staining, dehydration, clearing, the animal tissue can be made into transparent sections. By observing under a microscope or an electron microscope, it can accurately reflect the structure and morphology of the body; It can be used to determine quantitatively how much the sample size of the organizational structure, the number and amount of substance contained; At the same time, changes in the tissue can also be observed under different experimental conditions, thereby inform the activities of cells, cell differentiation and the relationship between different tissue cells (Lu and Wang, 2001; Yang, 2006).

The preparation process of the paraffin section is not complicated, but the factors affecting the quality of the section are various. In order to make the high quality section, every preparation step is very important. The eyeball structure of the squid is similar to the structure of the human eye, and it is a complex structure. Sclera is a kind of dense connective tissue, retina is soft and easy to fall off, lens is hard, and the vitreous is like jelly. Because of the fine structure, it is difficult to make paraffin section than the general tissue. For the reasons that the retina of the squid is very thin and soft, the retina is easy to get sagged, tattered or deform when making the paraffin sections. After long-term research and practice, we summed up the improvements of the paraffin section method in the main experimental steps, in this chapter, we will focus on the problems that affecting the quality of the retina paraffin section, and introduce some improvements about the paraffin section preparation method according to problems existing in the paraffin section preparation.

Fixation In order to maintain morphology, structure and composition of histocyte in the tissues, to keep the original shape and structure without deformation, the tissues (or organs) of animals (or plants) should be fixed by fixation liquid. Fixation liquid should be chosen according to the

characteristics of the tissues or organ.

Generally, the fixation liquid has the function of fixation and preservation, and is divided into the simple fixation liquid and the mixed fixation liquid. As the common simple fixation liquid, alcohol, formalin, acetic acid, picric acid, chromic acid, potassium dichromate, mercuric chloride and other simple fixation liquid, has the advantages of simplicity and convenience. The disadvantage is not easy to achieve the requirements of the ideal fixed. The mixed fixation liquid is made by mixing different kinds of liquid medicine according to a certain proportion, which can make the advantages and disadvantages of each other complement, and become a perfect fixation liquid. Although the preparation methods are more complex, the effect is better. Acetic acid - alcohol mixture, formalin acetic acid alcohol solution, Bouin's solution are widely used as the common mixed fixation liquid.

In our experiments, we often use the Bouin's solution as the fixation liquid to fix the eyeballs of squids. Formulated with picric acid saturated aqueous solution 75%, formalin (40% aqueous formaldehyde) 25% and glacial acetic acid 5% in de-ionized water ,the Bouin's solution has strong penetrating effect, it can fix the tissue evenly without significantly contracted, and it has good dyeing effect for the cell nucleus but for the cytoplasm the dyeing effect is poor. It can be used for fixing most organs and tissues, but it cannot prolong the shelf life of the tissue, so it cannot be used as a preservation solution. When fixing the eyeballs in the Bouin's solution, pay attention to the position of eyeballs in the liquid, to ensure that it can be completely submerged and fully fixed. For the firefly squid, due to its small size, it can be immersed in fixation liquid a whole unit, in order to facilitate the operation of the experiment (Han et al., 1994).

In addition, Eyeball Fixation Liquid is also a good choice for fixing the eyeballs, as a special fixation liquid for the eyeball, it could not only to get the eyeball fixed but also could be used as a preservative fluid for the longtime storage of the eyeball. The formula as followed, 40 ml acetone, 1.5 ml of glacial acetic acid, Mercuric chloride 2 g, Formaldehyde 10 ml, 40 ml of distilled water.

Dehydration After fixation of the fixed retina still contain some water, the water should be removed to make the specimen be easy to be cleared and wax dipped. Ethanol is often used as the dehydrating agent, because the retinal tissue contains much water, the direct use of anhydrous ethanol dehydration method will lead the tissue shrank and harden, so we use the gradient concentration method. We choose the concentration gradient of 80% → 85% → 90%→95%→100% ethanol, the concentration should be accurate, 95% and before the dehydration time is 30min, 100% concentration is divided into two steps, respectively, dehydrated for 30 min. Not completely dehydrated and excessive harden are two problems which is easy to appear in this process, to control excessive harden problem, as long as adjust each step of dehydration time; if tissue not completely dehydrated, it would be hard for the xylene and liquid paraffin to penetrate into the tissue in the subsequent clearing and

dipping wax processes (Yang, 2006). In order to ensure the accuracy of the dehydrating agent concentration, we should often replace the new ethanol.

Clearing In order to make it easier for paraffin to penetrate into the tissue ,it is necessary to clear the dehydrated tissues after dehydration. The most commonly used clearing agent is xylene, but the clearing time required to be strictly controlled. Due to the strong contraction characteristic of xylene, it is easy to make the retina tissue become brittle, clearing too long will make the tissue become tough and easily broken, making it difficult to ensure the integrity of the organizational structure. But if the clearing time is too short, the paraffin wax is not easy to penetrate into the retina in the dipping wax procedures. For retinal tissue, it usually takes around 60 min to reach the appropriate level clearing, whole process is divided into three steps, each step 20 minutes. We can extend some time of the clearing or increase the indoor temperature by using the air conditioner when the temperature is low.

Dipping wax Dipping wax is a procedure which use embedding agent penetrate into the tissue and replace xylene penetrated last procedure, the process is usually carried out in a constant temperature oven. The temperature of the constant temperature oven should be set higher than the melting point of paraffin wax 2 ~ 3 degrees Celsius, it would cause the tissue pieces become hard brittle and shrank if the temperature is too high ,which could result in the low quality of the slice or even failure. If the temperature is too low, paraffin wax cannot completely melt and difficult to penetrate into the internal tissue, causing the separation between the tissue and the paraffin wax, the bubble in the wax, the crack and so on. Duration of wax dipping also need to be control, for different tissues and organs the dipping time is different. In our experiment, the wax dipping time of *Cephalopod* retina tissue is 2 hours, divided into four steps each step takes 30 minutes.

Embedding Dipping wax and embedding progresses should be completed one time continuously to prevent the temperature difference occurs during operation. Different hardness of embedding wax should be chosen according to the temperature, in summer we should choose wax of high hardness and high melting point, while for winter, wax of low melting point and low hardness should be used. Besides, it should be based on the texture of the tissue to choose the appropriate wax, for the soft tissue for example brain, it should use wax with low hardness to get the tissue embedded, while for tough tissue such as the muscle, fibrous tissue, and it should choose a high hardness wax. Embedded temperature should not be too high, so as not to burn the tissue, bring adverse effects on staining lately.

Sectioning Sectioning is the key step to the section of good quality, when slicing the slice force should be uniform, not be overweight or too fast, otherwise it is easily lead to the slice of uneven thickness, or even the destruction of the wax block. Surrounding area of the embedded wax block should be neatly trimmed and made into appropriate size before sectioning, microtome should be set

stably and not easily get shaken; slicing blade should be tighten up, tilted to the appropriate angle. To prevent the slice from coiling when sectioning, humidity indoor should be maintained at a high level (40% and above), humidifier can also be used when necessary. Although so, slice breaking problems still occur from time to time, which not only related to the procedure of sectioning operation but also related to the prior steps, such as the time of dehydration was not appropriate or temperature was too high when dipping wax. In addition, because the part which we want to make into slices is located in the rhabdom layer of retina, what we want is the cross section of the rhabdom, particular attention should be paid in the sectioning procedure.

H.E staining Staining is an important step in the paraffin section preparation, and the H. E staining is used, here what is needed to pay attention to is whether hematoxylin staining is used or not. For the retinal transverse section which is used for observing the density of rhabdoms, what we want is the transverse cut of the extracellular part of visual cells, and there exist no nuclei in this part, so only the eosin staining could be used, while for longitudinal section which is used for observing the structure of the retina, the complete H. E staining should be performed. The main problem appears at this stage is the staining contrast is not clear, this may be caused by excessive eosin staining, the overdue staining solution may also enable tissue stained poorly. In addition not completely dewaxing could also lead to the not obvious contrast in the cells. Therefore, attentions should be paid to the dye solution, concentration should be accurately formulated, PH value should be moderate, and should not be precipitated. Sections should be dewaxed completely before staining. Avoid sun exposure during staining process.

Sealing when sealing, firstly put one side of coverslip on the slide which already dropped some mountant on it, then slow down the whole coverslip, completely eliminate the air, to avoid the formation of bubbles. Sealing mountant should be appropriately use, excess load of mountant will make the mountant overflowed from the coverslip and slide, effect observation when it is clotted, while too little volume of mountant will lead a not completely sealed result. In order to prevent the emergence of pigment particles and the bubbles in the sections, the sections should be immediately sealed after removing out from the xylene. In addition, uneven thickness of slice, incomplete expansion of section are also factors which could cause the failure of sealing.

To sum up, in order to prepare a high quality paraffin section, we must deal with each experimental details and solve the problems timely and reasonable. High level slicing techniques require long-term exploration and improvement, and it has important significance for the experimental research work to master paraffin section preparation technology.

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