Doctoral Thesis

The visual function of the ‘rough’ eyes of the northeast-Asian wood white butterfly, *Leptidea amurensis*

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Contents

General introduction

Living systems respond to environments physiologically and/or behaviorally. These responses are initiated by sensing of external information. One of the most important sources of information for many animals is light. Animals perceive typically two types of light information; the spectral and the intensity properties. Sensing light in almost all animals is performed by the visual organ, the eye. Most variable visual organs are compound eyes, since diverse species (most arthropods) possess them (Land and Nilsson 2002).

Compound eyes are composed of identical optical units called ommatidia. An ommatidium typically consists of two major components a lens unit and a rhabdom. The lens unit collects and focuses incoming light, while the rhabdom absorbs and transduces the light energy into neural signals. The lens unit (dioptric apparatus) consists of the corneal facet and the crystalline cone. The rhabdom is formed with microvilli of several photoreceptor cells (Warrant and McIntyre 1993).

There are two major designs in compound eyes, apposition type and superposition type. A major difference between two types is the number of dioptric apparatus that guide light to a single rhabdom. In apposition eyes, a single rhabdom receives light from one dioptric apparatus of the same ommatidium: an ommatidium is optically isolated from others. On the other hand, in superposition eyes, light from more than 100 dioptric apparatuses in some cases is focused onto a single rhabdom. There is a clear zone that separates the dioptric apparatus and rhabdom in superposition eyes. In general, diurnal insects such as butterflies have apposition eyes, while nocturnal insects like most moths have superposition eyes (Warrant and Mcintyre 1993).

There are two types of apposition eye: the focal type and the afocal type. In focal type, crystalline cone has a homogeneous refractive index, while in afocal type, strong gradient of refractive index exists especially in the proximal region of the crystalline cone. Most apposition eyes are of the focal type. The afocal apposition eye has been found only in butterflies. In the eye of butterflies, the crystalline cone works as a powerful lens in a similar manner of refractive superposition eyes (Nilsson et al., 1984, 1988). This design has been thought to be functionally advanced with higher efficiency of trapping light and better directional properties (Hateren and Nilsson 1987).

Even within butterflies, a variety of compound eyes have been found especially in their spectral organization of photoreceptors. The family Pieridae contains four subfamilies, Pierinae, Colidinae, Dismorphiinae and Pseudopontiinae (Braby et al. 2006). The eyes of the small white, *Pieris rapae* (Pierinae) and the eastern pale clouded yellow butterfly, *Colias erate* (Colidinae) have been studied in detail and found to have species-specific and sex-specific spectral sensitivities of photoreceptors (Qiu and Arikawa 2003; Wakakuwa et al. 2004; Arikawa et al. 2005; Ogawa et al. 2012, 2013). Pseudopontiinae contains the only one species that inhabits only in Africa. Then, I focused on a species in the subfamily Dismorphiinae that also occurs in Japan for studying evolution of color vision in Pieridae. Most species (about 100) in Dismorphiinae are distributed in South America and other species belong to a genus *Leptidea* inhabit the Palaearctic region (Europe and Asia). There are no Dismorphiinae species in Africa, North America and Southeast Asia (Yoshimoto 2000).

In the course of studying the visual system of *Leptidea amurensis*, I found a quite unique structure: the surface of the eyes appears rough. Therefore I termed this eye the ‘rough eye’. After I observed this unique structure, I happened to know a paper

describing this structure half century ago (Yagi 1964). But since the paper only briefly reported the surface structure of the eye, I decided to explore the internal structure as well as the visual function of rough eye of this species. In chapter I, I describe the structure of the compound eye through a combination of anatomy, molecular biology and intracellular electrophysiology, with a particular focus on the evolution of butterfly eyes. In chapter II, I compare the eye structure and light response between male and female and I describe the optical properties of ommatidia in the rough eye. Finally I discuss the function and biological significance of the rough eye.

Chapter I

Rough eyes of the northeast-Asian wood white *Leptidea amurensis*

Abstract

The Northeast-Asian wood white *Leptidea amurensis* (Lepidoptera, Pieridae) belongs to Dismorphiinae, a subfamily of the family Pieridae. I here studied the structure of the compound eye in this species through a combination of anatomy, molecular biology and intracellular electrophysiology, with a particular focus on the evolution of butterfly eyes. I found that their eyes consist of three types of ommatidia, with a basic set of one short, one middle and one long wavelength-absorbing visual pigment. The spectral sensitivities of the photoreceptors are rather simple, and peak in the ultraviolet, blue and green wavelength regions. The ommatidia have neither perirhabdomal nor fluorescent pigments, which modulate photoreceptor spectral sensitivities in a number of other butterfly species. These features are primitive, but the eyes of *Leptidea* exhibit a unique feature: the rough or bumpy appearance of the ventral two-thirds of the eye. The roughness is due to the irregular distribution of facets of two distinct sizes. As this phenomenon exists only in males, it may represent a newly evolved sex-related feature.

Introduction

Color vision of insects has been a major topic in the field of behavioral neurobiology ever since Karl von Frisch first demonstrated it in honeybees (Frisch 1914). Honeybees' vision is trichromatic, based on ultraviolet (UV), blue (B) and green (G)-sensitive photoreceptors in their compound eyes. The spectral sensitivity of each photoreceptor is primarily determined by the absorption spectrum of the visual pigment it expresses. In the case of bees, opsins of short (S), middle (M) and long (L) wavelength-absorbing visual pigments are expressed in the UV, B and G receptors respectively (Wakakuwa et al. 2005; Spaethe and Briscoe 2005).

Ommatidia are the basic structural units of compound eyes. Each one contains several photoreceptor cells of different spectral sensitivities. The combination of photoreceptor sensitivities differs among ommatidia, making the eye a patchwork of spectrally heterogeneous units. The ommatidial heterogeneity in the Japanese yellow swallowtail, *Papilio xuthus* has been described in detail (Arikawa 2003). The eye of *Papilio* has six classes of spectral receptors (UV, violet (V), B, G, red (R) and broad-band (BB)), which appear in three fixed combinations in the ommatidia (Arikawa 2003). Since then, I have investigated the extent to which eye organization is common among insects in general and flower-visiting butterflies in particular. Accumulated evidence suggests that the existence of three types of ommatidia is a widely shared trait, but the spectral sensitivity of individual photoreceptors appears to be almost species-specific, and even sex-specific in some cases. For example, the female small white, *Pieris rapae* (subfamily Pierinae, family Pieridae) has UV, V, B, G, R and dark-red (dR) receptors, while the male has double-peaked blue (dB) instead of V (Arikawa et al. 2005).

The wide variety in spectral sensitivities of photoreceptors is of course partially explained by the variety of opsins they contain (Arikawa et al. 2005; Awata et al. 2009; Ogawa et al. 2012). In addition, the cellular organization of the ommatidia is known to play a crucial role

(Stavenga and Arikawa 2011). Generally, reddish pigment surrounding the rhabdom (the photoreceptive organelle of an ommatidium) makes photoreceptors expressing green-absorbing visual pigment red sensitive (Wakakuwa et al. 2004). The pigmentation is generally weak in the dorsal part of the eye (Ribi 1979; Arikawa et al. 2009), so the shift in sensitivity is minor in this region (Ogawa et al. 2013). The abovementioned sexual dimorphism in spectral sensitivity is attributed to a difference between sexes in the distribution of fluorescent pigment (Arikawa et al. 2005; Ogawa et al. 2013).

The variety and complexity of butterfly eyes is impressive, but of course, such a complex organization must have evolved from simpler ones. The family Pieridae consists of four subfamilies, Pierinae, Coliadinae, Dismorphiinae and Pseudopontiinae. Pierinae and Coliadinae are sister taxa, containing about 700 and 250 species, respectively. This lineage is sister to two other smaller sister taxa, Dismorphiinae and Pseudopontiinae (Braby et al. 2006). Dismorphiinae is a subfamily with a limited geographical distribution and a relatively small number of species, and therefore may be ancestral. I here selected the northeast-Asian wood white, *L. amurensis* (Dismorphiinae, Pieridae) to identify their eye characteristics. I describe the external and internal structures of the eye at the electron-microscopic level, identifying and localizing opsin mRNAs and characterizing the spectral and polarization sensitivities of single photoreceptors. I thus identified three spectrally heterogeneous types of ommatidia in the *Leptidea* compound eye. The external morphology of the *Leptidea* eye is rather distinctive, due to its rough appearance as briefly reported previously (Yagi 1964).

Materials and Methods

Animals

I used summer form northeast-Asian wood white, *Leptidea amurensis*, captured around Nashigahara, Yamanashi or Sakuho, Nagano, Japan. The butterflies were fed with sucrose solution and kept in the laboratory for no longer than a week. For opsin characterization and comparison, I also used two Pierinae species: *Anthocharis scolymus* were collected around the Sokendai campus, Kanagawa, Japan, and *Hebomoia glaucippe* were provided by Gunma Insect World.

Anatomy

For scanning electron microscopy, heads were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (CB, pH 7.4) for 2 hr at room temperature. After a brief wash with CB, the heads were postfixed in 2% osmium tetroxide in CB for 2 hr at room temperature, and then dehydrated with acetone. After being infiltrated with propylene oxide, the heads were dried, platinum-coated and observed with a scanning electron microscope (JSM-6490LV, JEOL, Tokyo Japan).

For transmission electron microscopy, isolated eyes were fixed as above. Following infiltration, eyes were embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (H7650, Hitachi, Tokyo Japan).

For light microscopy, the eyes were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in CB and embedded in Quetol 812 without being postfixed with osmium tetroxide. The tissues were then cut into 5 μm sections and observed with a light microscope (BX60, Olympus, Tokyo, Japan).

Molecular biology

The method of molecular biology was as described previously (Awata et al. 2009), which was briefly as follows. I carried out RT-PCR using poly-A RNA extracted from retinal homogenate as the template and degenerate primers based on sequences of lepidopteran opsins. The full-length cDNAs were obtained using the 5' and 3' RACE methods. Phylogenetic trees based on the nucleotide sequences were reconstructed using Bayesian inference (BI) and also the maximum likelihood (ML) methods. The reliabilities were based on 100,000 replicate analyses (for BI) or 1000 bootstrap replicates (for ML).

The opsin mRNAs were localized in the retina by *in situ* hybridization. Isolated eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), embedded into paraffin and sectioned at 8–10 μm thickness. The sections were treated with 10 μg/ml proteinase K in phosphate-buffered Saline for 5 min at 37°C, and acetylated with 0.25% acetic acid in 0.1 M triethanolamine for 10 min prior to hybridization. Antisense RNA probes were synthesized from linearized plasmid carrying partial sequences of identified opsin mRNAs by *in vitro* transcription using digoxigenin-UTP. The probes were heat-treated and diluted at final concentration of 0.5 μg/ml in a hybridization solution. The heat-treated probe was applied to the sections at 55°C overnight. The hybridized probes were detected and immunohistochemically visualized using anti-digoxigenin.

Electrophysiology

A butterfly was mounted on a plastic stage set in a Faraday cage. A silver wire inserted in the head served as the reference electrode. A glass microelectrode filled with 3M KCl (resistance = ca. 100MΩ) was inserted into the retina through a small hole made in the cornea.

Monochromatic stimuli were delivered by a 500 W xenon arc lamp through a series of interference filters. The light was focused on the tip of an optical fiber that was attached to the perimeter device, where it provided a point light source (subtending 1° at the eye). The quantum flux of each monochromatic light was adjusted to a standard number of photons using an optical wedge.

After penetrating a photoreceptor, the spectral type of the impaled photoreceptor was determined using a series of monochromatic flashes of 30 msec duration. The response-stimulus intensity (V-log I) function was recorded over a range of 4 log units at the cell's peak wavelength (λ_{max}). The photoreceptor was subjected to further analyses only if the maximal response amplitude, V_{max}, exceeded 20 mV. I then recorded responses to a series of polarized light flashes at the receptor's λ_{max} at an intensity that elicited about 50% of the V_{max}. The *e*-vector orientation of the light stimulus was adjusted by rotating a polarization filter attached to the exit of the optical fiber. The *e*-vector orientation was initially set parallel to the dorso-ventral axis, which was defined as 0°. Both spectral and polarization responses were converted into sensitivity values. The *e*-vector orientation at which the polarization sensitivity curve peaks (ϕ_{peak}), and the polarization sensitivity ratio (P_S = maximal sensitivity / minimal sensitivity) were determined from a sinusoidal curve fitted to the data using the least-squares method.

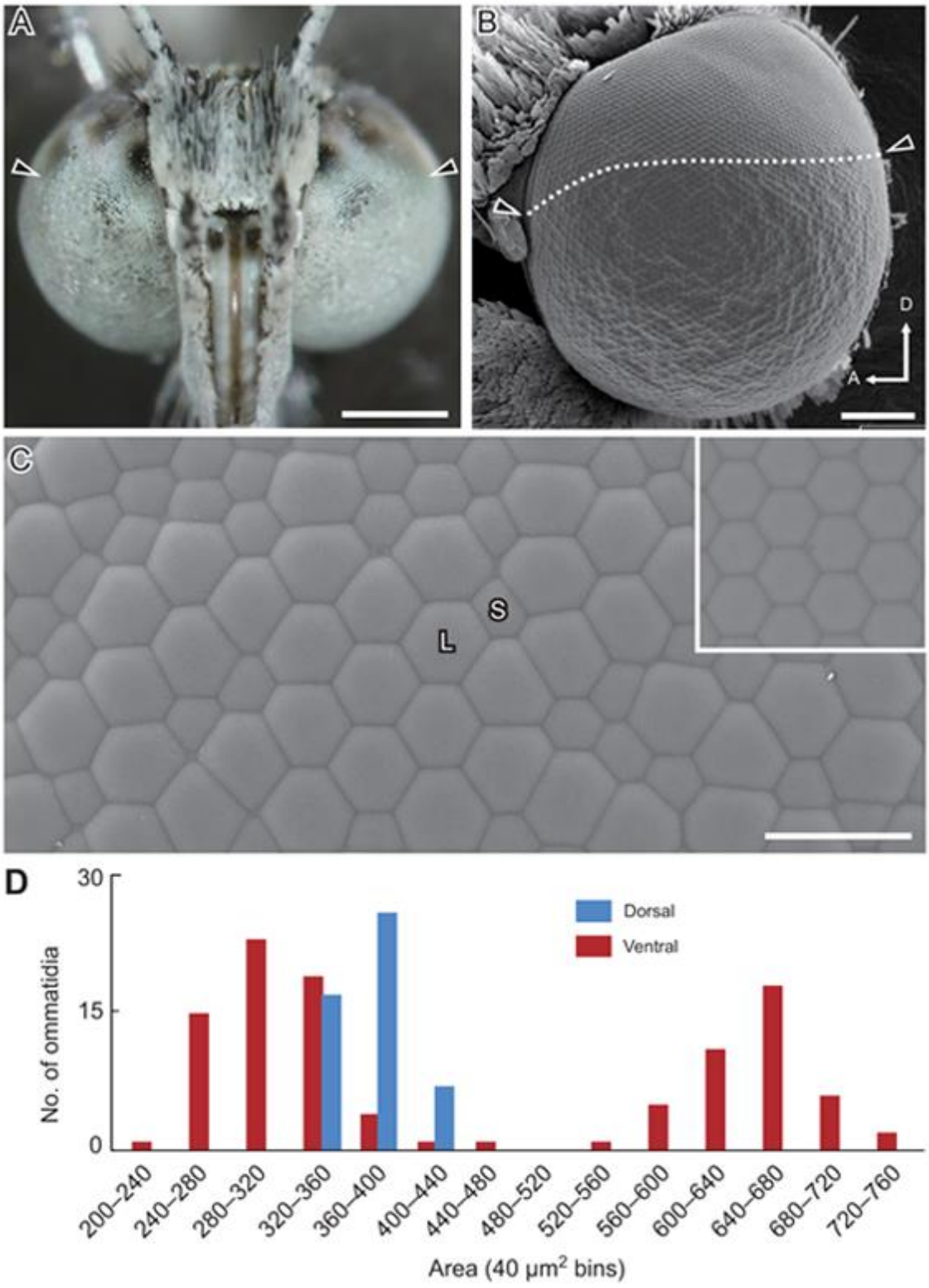
I marked some photoreceptors by injecting Lucifer yellow by applying 2 nA hyperpolarizing DC current for about 5 min after recording. The eyes were directly observed with a fluorescence microscope (BX60, Olympus, Tokyo Japan) under BV excitation to identify the ommatidium containing the dye-filled photoreceptor.

Results

Rough eyes

The eye of *Leptidea* is clearly divided into the dorsal and ventral regions (Fig. 1A and B, arrowheads). The surface of the ventral region is rough (Fig. 1C), the roughness being due to the variable sizes of the corneal facets (Fig. 1C). The dorsal region is smooth where the size of ommatidia appears uniform (Fig. 1C inset). I measured the areas of 106 facets in the ventral region of an individual as well as 50 dorsal facets of the same individual. Figure 1D shows histograms of the facet areas in the ventral and dorsal regions. The ventral histogram exhibits two peaks, one at 280-320 μm^2 and another at 640-680 μm^2, while the dorsal histogram has a single peak at 360-400 μm^2. Thus the ventral facets, unlike the dorsal, comprise two rather distinct groups: large and small.

Fig. 1. The eye of male *Leptidea amurensis*. (A) Frontal view. Arrowheads indicate the border between the dorsal and ventral regions. (B) A low-magnification SEM picture of a left eye. The dorsal region is smooth, while the ventral region appears rough. Dotted line indicates the border of regions. D, dorsal; A, anterior. (C) A highmagnification SEM picture of the ventral region, showing large (L) and small (S) facets. Inset shows a part of the dorsal region at the same magnification. (D) Histogram (40 μm^2 bins) of facet areas of the dorsal (blue) and the ventral (red) ommatidia. Scale bars, (A) 500 μm, (B)200 μm, (C) 50 μm.


A
B
D
A
C
S
L
D
30
15
0
No. of ommatidia
Dorsal
Ventral
200–240
240–280
280–320
320–360
360–400
400–440
440–480
480–520
520–560
560–600
600–640
640–680
680–720
720–760
Area (40 μm² bins)

Rhabdom ultrastructure

An ommatidium has a rhabdom (Fig. 2A) that consists of the rhabdomeres of nine photoreceptor cells, R1-9 (Fig. 2D). I could distinguish three types of ommatidia according to the fine structure of the rhabdom.

Figure 2 shows serial transverse sections of rhabdoms from three types of ommatidia at three different depths. The top of the pictures corresponds to the dorsal side (see Fig. 2A). I measured the areas of rhabdomeres at 20 μm intervals from the top to the bottom of the rhabdom (Fig. 3A, B, C). Figure 3D shows a diagram of the ommatidia with large and small facets (Fig. 3D). (The correspondence between ommatidial types and facet sizes is covered in the next section.)

Type I ommatidia have the largest rhabdoms, with R1-8 contributing along its entire length, while R9 adds a few microvilli at the base (Fig. 2J, 2K, 3A). As shown in Fig. 2A, D and G, R1 of type I contains curved microvilli in two orientations, while the microvilli of R2 are straight and parallel to the dorso-ventral (vertical) axis. The type I rhabdom is triangular at a depth of 170 μm (Fig. 2D), which is due to the different shapes of the R1 and R2 rhabdomeres: R1's rhabdomere is round, while R2's is rather rectangular. Further proximally, the rhabdom is elongated horizontally (210 μm, Fig. 2G) and then vertically (270 μm, Fig. 2J). The R1 photoreceptor's microvilli end at a depth of 250 μm (Fig. 2J and 3A). The microvilli of R3-8 are shorter and appear to be oriented either horizontally (R3 and R4) or diagonally (R5-8). The structures of R1 and R2 may be interchanged (see Fig. 4).

Type II ommatidia have smaller rhabdoms (Fig. 3B). The microvilli of both the R1 and R2 are straight and parallel to the vertical axis (Fig. 2B, E). The microvilli of R3 and R4 are aligned horizontally, and those of R5-8 are aligned diagonally (Fig. 2H).

The size of the rhabdom in type III ommatidia (Fig. 3C) is almost identical to that of

type II. The microvilli of R1 and R2 curve into two directions, indicating their reduced polarization sensitivity. The microvilli of R3-8 are aligned either horizontally (R3 and R4) or diagonally (R5-8) (Fig. 3I).

Note that the rhabdoms are not surrounded by perirhabdomal pigment at any depth (Fig. 2). These pigments are commonly found in Pierids (Qiu et al. 2002; Ribi 1978; Arikawa et al. 2009) and Papilionids (Awata et al. 2010; Arikawa 2003), so this feature is peculiar to *Leptidea*. In addition, I found no tracheal tapetum in *Leptidea*, which is also exceptional among Pierids.

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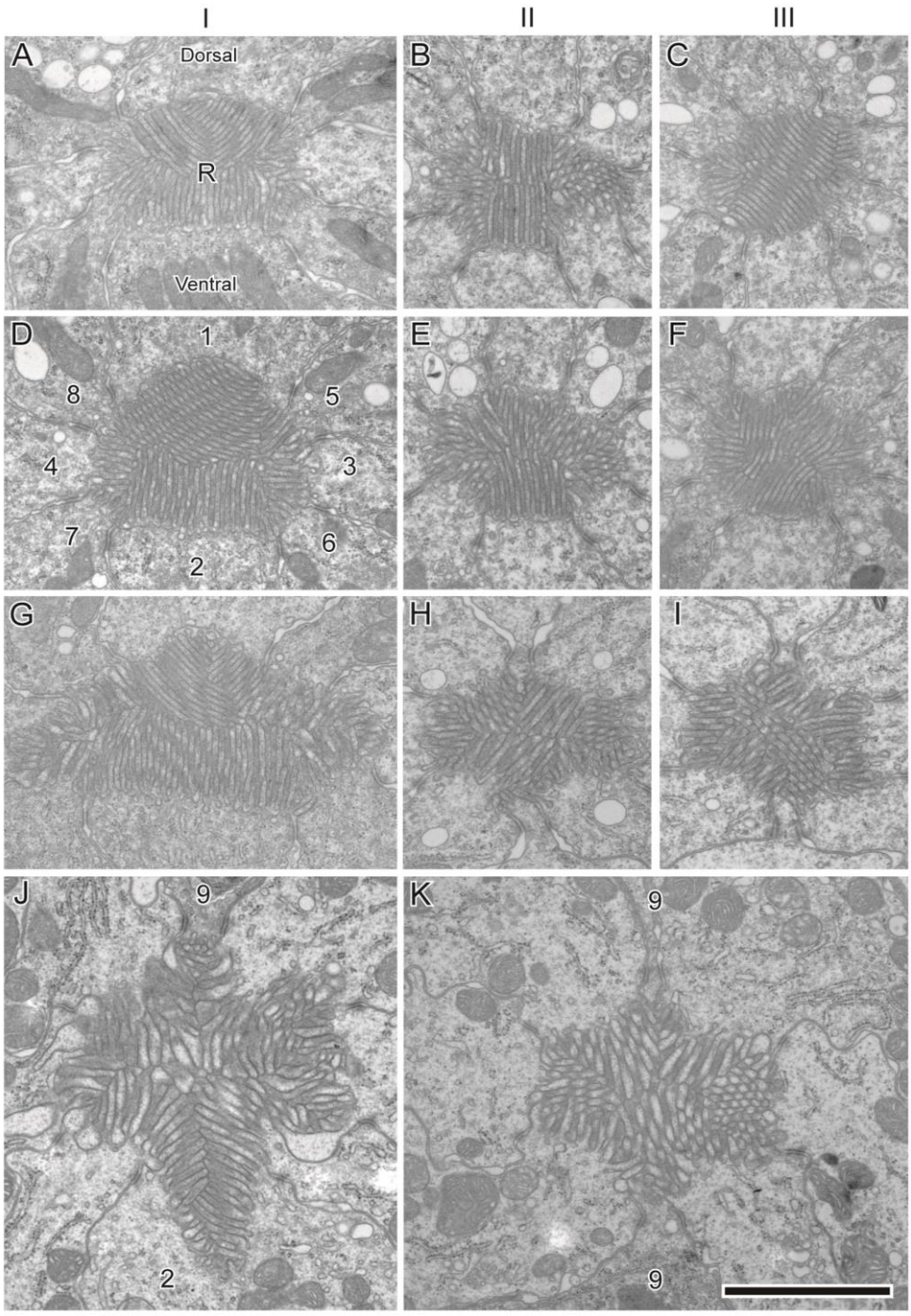

Fig. 2. Transmission electron micrographs of transverse sections of the rhabdom of three types of ommatidia (I, II and III) at four depths; 130 (A–C), 170 (D–F), 210 (G–I) and 270 μm (J, K) from the corneal surface. At 270 μm, type II and III ommatidia could not be distinguished (K). Scale bar, 2 μm

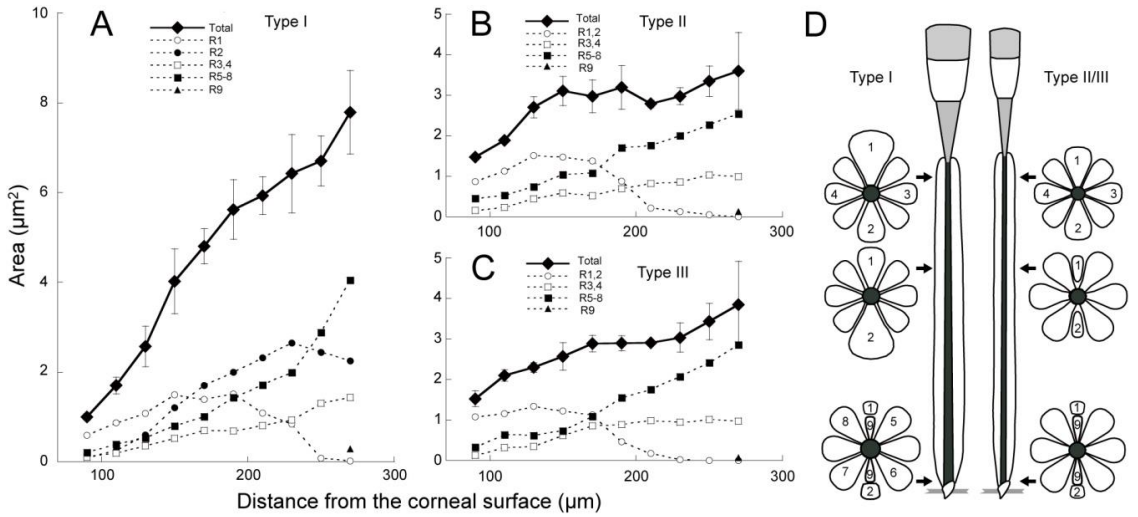

Fig. 3. Rhabdom (diamonds) and rhabdomere areas of photoreceptors R1–9 in type I (A), II (B) and III (C) ommatidia. For R3 and 4 and R5–8, combined areas are plotted. (D) Schematic diagram of a large (left) and small (right) ommatidia with transverse views at three depths. 1–9, photoreceptors R1–9.

Three opsins and localization

I identified three cDNAs encoding opsins in the eye of *Leptidea*. Based on phylogenetic analysis with other insect opsins (Fig. 4A), I identified these as a UV- (*Leptidea amurensis* UV, LaUV), a blue- (LaB) and a long wavelength-absorbing type (LaL). The arborizations agree with the phylogeny of Pieridae (Braby et al. 2006). The most conspicuous feature is that the *Leptidea* eyes express only one B opsin, while all other pierids studied to date (Pierinae and Coliadinae) have at least two opsins in the B clade. To confirm this, I analyzed *Anthocharis scolymus* and *Hebomoia glaucippe* (Pierinae), and found both to express two opsins (B and V) in the B clade. I thoroughly searched for additional *Leptidea* opsins in the B clade using degenerate primers based on the V opsins of other species, but found none.

I localized the mRNAs in the eye by *in situ* hybridization. Figure 4B-E shows four serial sections taken from an eye. Figure 4B is a section through the crystalline cone layer. The large and small cones correspond to the ommatidia with large and small facets, respectively. Figures 4C, D and E are sections labeled with probes specific to mRNAs of LaUV, LaB and LaL opsins, respectively. The labeling patterns of these probes are mutually exclusive; no photoreceptors appeared to be coexpressing mRNAs of two or more opsins.

The LaUV and LaB probes label R1 and R2 in a complementary manner, revealing three expression patterns (Fig. 4C, D). The ommatidia with large crystalline cones express one of each of the LaB and LaUV mRNAs in R1 and R2. This pattern most likely corresponds to the type I ommatidia where the ultrastructure of R1 and R2 markedly differs (Fig. 2). Types II and III have both R1 and R2 labeled with the same probe, either LaUV or LaB. The LaL probe labeled R3-8 in all ommatidia (Fig. 4).

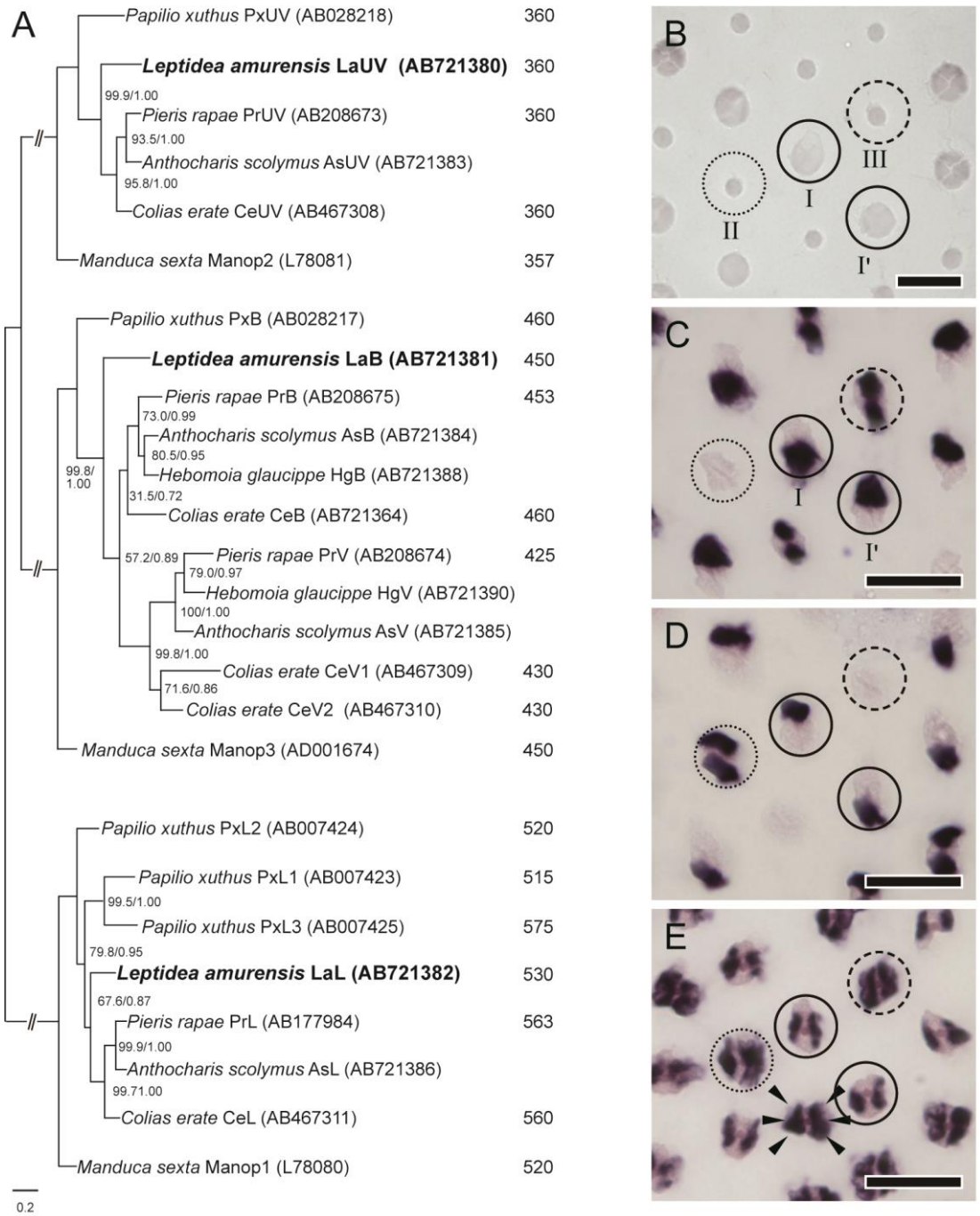

Fig. 4. *Leptidea amurensis* opsins. (A) Phylogeny of lepidopteran opsins, as determined by Bayesian inference analyses based on sequences of 1149 (UV), 768 (B) or 1149 (L) nucleotides. Maximum likelihood (ML) analyses produced a similar tree (not shown). The numbers at the nodes indicate the ML bootstrap values and Bayesian posterior probabilities. With the species and opsin names, the accession numbers and

the peak wavelength of absorption are indicated wherever available. (B–E) *In situ* hybridization of three opsin mRNAs in consecutive sections of *L. amurensis*. Solid, dotted and broken circles indicate type I, II and III ommatidia, respectively. (B) Section through the crystalline cone. (C) LaUV. Compositions of R1 and R2 in type I may be exchanged (I′). (D) LaB. (E) LaL. Arrowheads indicate six labeled photoreceptors. Scale bars, 20 μm.

Photoreceptor sensitivities

Intracellular recording revealed four distinct spectral sensitivity profiles, which I term ultraviolet (UV), broad-blue (bB), sharp-blue (sB) and green (G) sensitivity classes (Fig. 5, upper panels). I also measured the polarization sensitivity at the cell' s λ_{max} (Fig. 5, lower panels).

The λ_{max} of UV receptors is 360 nm (Fig. 5A). The average spectral sensitivity profile of UV receptors (n=13) reasonably matches with the absorption spectrum of a visual pigment peaking at 360 nm (R360) predicted using the Govardovski template (Govardovskii et al. 2000). The UV receptors are insensitive to polarization angle.

I found 9 bB receptors (Fig. 5B). The average spectral sensitivity peaks at 440 nm and matches well with the absorption spectrum of R450. The ϕ_{peak} is 10° in this receptor class (Fig. 5B), and the average P_s value is 2.43. I successfully labeled one bB receptor with Lucifer yellow, and localized it to an ommatidium with a small facet (Fig. 5B, inset).

I found 12 sB receptors (Fig. 5C). The average spectral sensitivity matches the template of R450 on the long wavelength side, but not in the UV region. The polarization sensitivity is indistinguishable from that of bB receptors: ϕ_{peak} = 0°, P_s = 2.54.

The average spectral sensitivity of G receptors matches with the predicted profile of R530. The ϕ_{peak} is variable: ϕ_{peak} = 50° (n=9), 95° (10) or 130° (7). The P_s value is about 1.55 in all cases.

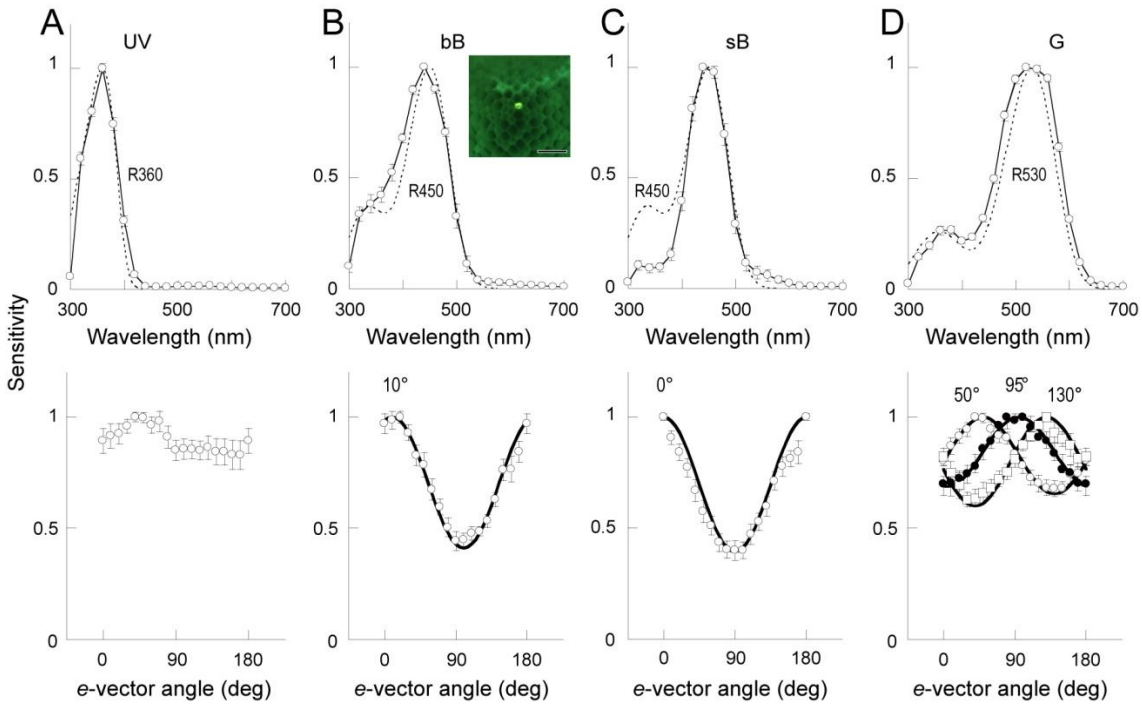

Fig. 5. Spectral (upper panels) and polarization (lower panels) sensitivities of UV (A), bB (B), sB (C) and G (D) receptors. Dotted lines in upper panels indicate absorption spectra of visual pigment predicted from the Govardovskii template (Govardovskii et al. 2000). Solid lines in lower panels of B–D are best-fit sinusoidal curves with the ϕpeak angle values. The inset picture in B is a fluorescent image showing a small ommatidium containing a Lucifer-Yellowinjected bB receptor.

Discussion

Eye roughness and spectral heterogeneity of the ommatidia

The most conspicuous feature of the eye of *Leptidea* is its rough appearance. The roughness is confined to the ventral two-thirds of the eye, which consists of irregularly distributed large and small facets. The existence of facets of different sizes in *Leptidea* was reported about a half century ago (Yagi 1964), but their internal structure has not been previously studied.

Although the surface of the eye appears rough, the internal structure is regular: the ommatidia are hexagonally arranged as seen in transverse sections (Fig. 4). Accumulated evidence suggests that insect compound eyes typically consist of three spectrally heterogeneous types of ommatidia (Arikawa and Stavenga 1997; Wakakuwa et al. 2007; Wakakuwa et al. 2005; Arikawa 2003; Spaethe and Briscoe 2005; White et al. 2003; Briscoe et al. 2003; Sison-Mangus et al. 2006). As expected, the ommatidia of *Leptidea* could be divided into three types as well. I found that the ommatidial heterogeneity is related to the facet sizes: large facets correspond to type I ommatidia, while small facets correspond to type II and III ommatidia. By combining TEM observation (Fig. 2), *in situ* hybridization (Fig. 4) and electrophysiology (Fig. 5), I have deduced the spectral properties of each type of ommatidium (Table 1).

Table 1. Three types of ommatidia in the ventral eye region of *Leptidea amurensis*

Type	Cornea	Ratio	Photoreceptor properties Microvilli / Spectral sensitivity / Opsin mRNA			
			R1	R2	R3 & 4	R5-8
I	Large	50%	Curved ultraviolet (UV) LaUV	Vertical sharp-blue (sB) LaB	Horizontal Green (G) LaL	Diagonal
II	Small	25%	Vertical broad-blue (bB) LaB		Horizontal Green (G) LaL	Diagonal
III	Small	25%	Curved Ultraviolet (UV) LaUV		Horizontal Green (G) LaL	Diagonal

All photoreceptors express exactly one of three opsin mRNAs, LaUV, LaB or LaL; I found no photoreceptors coexpressing two or more opsin mRNAs. R3-8 express the LaL mRNA in all ommatidia (Fig. 4E), and must be G sensitive. R1 and R2 express either the LaUV or LaB mRNA, so the short wavelength (UV, bB and sB) receptors can be assigned to R1 and/or R2 in certain combinations. I could not obtain any information about the basal R9 photoreceptors.

A notable feature of UV receptors is their negligible polarization sensitivity. Polarization sensitivity is reduced when the rhabdomeral microvilli are not aligned (Horvath and Varju 2004). Therefore, it is reasonable to assume that the UV receptors are R1 (or R2) of type I ommatidia (Fig. 2A), and both R1 and R2 of type III ommatidia (Fig. 2C), whose microvilli are curved and arranged in two different orientations.

Both types of blue receptors (bB and sB) have higher P_s values with ϕ_{peak} around 0°, indicating that the microvilli of these receptors are straight and vertically aligned. Clearly, they correspond to R2 (or R1) of type I and R1 and R2 of type II ommatidia, which are labeled with the LaB probe. Note that the spectral sensitivity of the bB receptor matches well with the predicted spectrum of a visual pigment peaking at 450 nm. The sB receptors contain the same visual pigment, but exhibit reduced sensitivity in the UV wavelength region. This phenomenon can most likely be attributed to the lateral filtering effect (Snyder et al. 1973). In the rhabdoms where UV receptors and blue receptors are colocalized, absorption by the UV receptor reduces the proportion of UV light that is absorbed by the blue receptors. Therefore, I conclude that sB receptors are localized in type I ommatidia, while the bB receptors are found in type II ommatidia (Fig. 4). Furthermore, I successfully stained a bB receptor with Lucifer yellow, and found it to be located in an ommatidium with a small facet (Fig. 5B, inset).

Evolutionary view

According to extensive phylogenetic analyses (Braby et al. 2006; Braby and Trueman 2006), Dismorphiinae may be an ancestral subfamily of the family Pieridae. Supporting this view, I have also found some primitive features in the compound eye of *Leptidea*.

The first of these features is the set of visual pigment opsins. The *Leptidea* eyes express three opsins, one in each of the short (S), middle (M) and long wavelength-absorbing (L) clades (Fig. 4A). This is the basic set of insect opsins; it is also found in bees, in a similar expression pattern (Wakakuwa et al. 2005). However, this scheme is unusually simple for a butterfly. Species of the subfamilies Pierinae and Coliadinae have duplicated M opsins (Fig. 4A); in *Pieris rapae* (Pierinae), for example, two M opsins, a 450 nm-absorbing PrB and a 420 nm-absorbing PrV were found (Arikawa et al. 2005).

About these *Pieris* M opsins, it has been proposed that the amino acids at the positions of 116 and 177 are crucial for spectral tuning based on results of site-directed mutations (Wakakuwa et al. 2010). PrB has serine at 116 (Ser116) and phenylalanine at 177 (Phe177), while PrV has alanine at 116 (Ala116) and tyrosine at 177 (Tyr177). Substituting Ser116 to Ala in PrB resulted in a 13 nm short-wavelength shift, and Phe177 to Tyr resulted in a 4 nm short-wavelength shift. The combination of S116 and F177 is retained in all lepidopteran B opsins including the *Leptidea* LaB. On the other hand, the amino acids of these sites in V opsins are variable (Wakakuwa et al. 2010), indicating that B opsins are ancestral to V opsins. The opsin phylogeny (Fig. 4A) also indicates that the duplication happened after the common ancestor of the lineages of Pierinae and Coliadinae diverged from those of Dismorphiinae and Pseudopontinae. In Papilionidae, L opsins are duplicated or triplicated in all species studied so far (*Papilio xuthus*, *Papilio glaucus*, *Parnassius glacialis*) (Awata et al. 2010; Briscoe 2000; Arikawa 2003).

Secondly, the *Leptidea* eye exhibits neither fluorescence nor perirhabdomal screening

pigments, both are crucial to fine-tune photoreceptor spectral sensitivities. Again in *Pieris rapae*, fluorescent pigment is concentrated in the distal tip of the rhabdom only in males. The pigment absorbs violet (420 nm) light, and thus changes violet receptors into double-peaked blue receptors in males (Arikawa et al. 2005). Similarly functioning fluorescent pigment is also found in *Colias* (Ogawa et al. 2012). Reddish screening pigments surround the distal tier of the rhabdom in several species (Ribi 1978; Arikawa and Stavenga 1997; Arikawa et al. 2009). These pigments absorb the boundary wave of light that propagates outside the rhabdom, and thus act as spectral filters. In *Pieris* and *Colias*, for example, this filtering effect turns proximal photoreceptors expressing green-absorbing visual pigment into "red" receptors (Wakakuwa et al. 2004; Ogawa et al. 2013). The spectral-tuning functions of these pigments appear to be evolutionary elaborations enhancing the animals' spectral discrimination ability. Lacking such pigments, *Leptidea* eyes seem quite primitive.

The third feature is the untiered nature of rhabdoms. The rhabdoms of Pierid and Papilionid species are clearly tiered with four distal, four proximal and one basal photoreceptor. The tiering strongly modifies the spectral sensitivity of proximal photoreceptors together with the perirhabdomal and fluorescent pigments (Stavenga and Arikawa 2011). Another possible reason for the evolution of the tiered rhabdom is the establishment of a channel for motion vision, which the R3 and R4 green receptor system may represent, at least in *Papilio* and *Pieris* (Matsushita et al. 2012). However, the rhabdoms of *Leptidea* exhibit little tiering (Fig. 3 and 4), in common with many other insects including Nymphalid butterflies (Matsushita et al. 2012; Kolb 1985; Gordon 1977), suggesting that the organization is ancestral.

Perspectives

Compound eyes composed with facets of different sizes are not rare. However, most of them are

systematically organized: the visual field in which the animal is most interested is covered by larger facets. The dorsal eye region of blowflies (Hateren et al. 1989) and dragonflies are such examples (Labhart and Nilsson 1995). For mantis shrimps, such a region corresponds to the "mid-band", which consists of six rows of large facets specialized for color and polarization vision (Marshall et al. 1991). One exception is a thrips, *Caliothrips phaseoli*, whose rudimentary compound eyes are random mixtures of large and small facets (Mazza et al. 2010).

Although most of the features of the *Leptidea* eyes appear to be primitive, the roughness due to the random array of large and small facets is unique among large and visual arthropods and may be evolutionarily novel. The roughness is found in the ventral region of male eyes only. This sexual dimorphism implies that it is related to some aspects of sexual behavior. Whether and how the eye roughness is biologically functional is an interesting issue to be addressed.

A hypothesis is related to sensitivity of lightness. The absolute sensitivity of an ommatidium is proportional to the diameters of the facet lens and the rhabdom (Snyder 1979). In addition, the rhabdom is larger in type I ommatidia (with large facets), suggesting that the acceptance angle may be wider there. If the photoreceptor gain is uniform, which does in fact appear to be the case in our preliminary measurements, then type I ommatidia would be more sensitive than the others. The male eye would then be a mixture of high and low sensitivity units, expanding the dynamic range of the entire eye. This may be beneficial for males of this open grassland species to find potential mates concealed in bushes.

Chapter II

Sexual dimorphism and its function of the eye of the butterfly, *Leptidea amurensis*

Abstract

The eyes of the male Northeast Asian wood white, *Leptidea amurensis*, appear “rough” due to irregular arrangement of facets of different shapes and sizes, while the eyes of females are not. To clarify the functional significance of the sexual dimorphism and its function, I compared the structure, optical and physiological properties in their eyes. Here I found that their eyes consist of three types of ommatidia: one type has large lens and rhabdom, and those in other types are small. These differences are clear in males, but less prominent in females. Moreover, the facets are hexagonal in females as in most other insects, but they are irregular in shape in males. I recorded the light intensity dependency of electroretinogram to elucidate the overall sensitivity, and found that the dynamic range of the intensity-response (*V-log I*) function is significantly wider in males than in females. Based on the anatomy of the ommatidia, I calculated the sensitivities of ommatidia: large ommatidia appear to be 1.4 times more sensitive than small ommatidia in males. The expanded dynamic range in males is probably attributed to the variability in sensitivity among ommatidia, and is probably beneficial for males to search for females in shaded patches in sunny grasslands.

Introduction

Structure and function of compound eyes have been studied more than a hundred years. The structural unit of compound eyes is an ommatidium. Each ommatidium has a lens system, the dioptric apparatus and a photoreceptive site, the rhabdom, which is formed by photoreceptors. Each ommatidium is recognizable from outside by a facetal lens. Facetal lenses of insects usually have similar size and hexagonal in shape, packed in a hexagonal lattice (Land 1997). There are two basic designs in compound eyes, superposition eyes and apposition eyes. While a rhabdom of superposition eyes receives light through many facets, a rhabdom of apposition eyes receives light entered in a single facet. Compound eyes of most diurnal insects including butterflies are of the apposition type. In eyes of butterflies, both cornea and crystalline cone act as lens and produce two-lens telescope-like optics system (Nilsson et al. 1984; 1988).

In recent years, ommatidial heterogeneity has been found in many insects including butterflies (Arikawa 2003; Qiu et al. 2002; Wakakuwa et al. 2005). There are three types of ommatidia in most insects. In the researches so far conducted, one of the main subjects addressed was heterogeneity of spectral sensitivity in ommatidia. An ommatidial type is generally defined by combinations of spectral receptors in an ommatidium.

I previously reported ommatidial heterogeneity of the eyes of *Leptidea amurensis* in males. There is a unique feature in the sizes of ommatidia. Three types of ommatidia were there and could be divided into large (typeI) and small ommatidia (typeII/III). Large ommatidia (LO) and small ommatidia (SO) are randomly distributed in the ventral two thirds of their eye. Due to LO and SO, the appearance of the eyes of males is rough. However, the eyes of females are not rough (Uchiyama *et al*. 2013).

Sexual dimorphism of compound eyes in Lepidoptera has been found in size (Yagi and Koyama 1963; Rutowski 2000), in spectral photoreceptor class (Arikawa et al. 2005; Ogawa et

al. 2012, 2013) etc. Requirements of visual function for performing sexual behavior may differ between males and females (Rutowski 1991, Hornstein et al. 2000), so I have assumed that the sexual dimorphism in eye structures is likely related to sexual behavior in *Leptidea amurensis*.

In order to address the question, I here performed anatomical as well as physiological experiments asking the following three specific questions. How is the sexual dimorphism in their eyes in detail? What are the optical and physiological properties of LO and SO? What is the possible function of the roughness in their eyes?

Materials and Methods

Animals

I used summer form northeast-Asian wood white, *Leptidea amurensis*, captured around Nashigahara, Yamanashi and Sakuho, Nagano, Japan. I also reared the butterflies from eggs that were laid on the host plants, *Vicia amoena* Fisch in the field or laboratory. The adult butterflies fed with sucrose solution and were kept in laboratory no longer than a week.

To compare properties of the eye, all the following experiments were carried out in the antero-lateral region of the ventral two-thirds of the eye.

Anatomy

For scanning electron microscopy, entire heads were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1mol/l sodium cacodylate buffer (CB, pH 7.4) for 2h at room temperature. After a brief wash with CB, the eyes were postfixed in 2% osmium tetroxide in CB for 2h at room temperature. Fixed heads were dehydrated with a graded series of acetone. After being infiltrated with propylene oxide, the heads were air-dried, platinum-coated and observed in a scanning electron microscope (JSM-6490LV, JEOL, Tokyo Japan).

For transmission electron microscopy, isolated eyes were fixed as above. Following dehydration with a graded series of acetone and infiltration with propylene oxide, eyes were embedded in Quetol 812. Ultrathin sections, cut with a diamond knife, were stained with uranyl acetate and lead citrate, and observed in a transmission electron microscope (H7650, Hitachi, Tokyo Japan).

For light microscopy, the eyes fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in CB were embedded in Quetol 812 without being postfixed with osmium tetroxide. The tissues were then cut into 5μm sections and observed with a light microscope

(BX60, Olympus, Tokyo, Japan).

Electrophysiology

Intracellular recordings

I measured the angular sensitivity of individual photoreceptors by intracellular electrophysiology. Monochromatic lights were delivered by a 500 W Xenon arc lamp via a series of interference filters ranging from 300 to 740 nm. The light was focused on the tip of an optical fiber, the other end of which was attached to a Cardan-arm perimeter device in a Faraday cage, where it provided a point light source (1° in diameter). The quantum flux of each monochromatic light was measured using a radiometer (Model-470D, Sanso, Tokyo, Japan) and adjusted to a standard number of photons using an optical wedge. For each experiment, a butterfly was mounted on a stage with its dorsal side up. A silver wire inserted into the head served as the reference electrode. A glass microelectrode filled with 3M KCl with a resistance of about 100 MΩ was inserted into the retina through a hole made in the cornea. Membrane potentials were recorded through a preamplifier (MEZ-7200; Nihon Kohden, Tokyo, Japan) connected to a computer via an AD converter (MP-150, BIOPAC Systems, USA).

After penetrating a photoreceptor, the optical fiber was adjusted so as to yield maximal responses. First, the spectral type of the impaled photoreceptor was determined using a series of monochromatic flashes of 30 ms duration. The response-stimulus intensity (V-log I) function was recorded over a range of 4 log unit at the cell's peak wavelength (λ_{max}) in nearly dark-adapted conditions. The photoreceptor was subjected to further analyses only if the maximal response amplitude, V_{max}, exceeded 20 mV. I then recorded angular responses of the cell, using flashes moving by steps of 0.2° through the center of a receptive field from the dorsal to ventral of the animal and vice versa at the receptor's λ_{max} at an intensity that elicits about 50 % of the V_{max}. These

responses were converted to sensitivity values.

Electroretinography

To determine the response to light intensity and spectral sensitivity of the eye as a whole, I recorded electroretinogram (ERG). Monochromatic stimuli and white light stimuli were provided by a 500 W Xenon arc lamp. The light beam was focused on the tip of an optical fiber whose other end was attached to a device in a Faraday cage. A butterfly was fixed on a plastic stage with its dorsal or ventral side up and then mounted in front of the exit pupil of the optical fiber.

To record the ERG from the ventral eye region, I covered the dorsal half of the eye with silver paint. A silver ring (approximately 900μm in diameter) was attached to the corneal surface with electric conductive paste. Another chlorinated silver wire was inserted into the head served as the indifferent electrode.

ERGs were recorded through a preamplifier (MEZ-7200; Nihon Kohden, Tokyo, Japan) connected to a computer via an AD converter (MP-150, BIOPAC Systems, USA). After 5 minutes dark adaptation, ERGs were measured in response to a series of white light flashes of varying intensities of duration 300 ms, spaced 20 s apart and to a series of equi-quantal monochromatic flashes of duration 300 ms, spaced 10 s apart.

The response-stimulus intensity (V-log I) function was recorded over a 4 log unit intensity range using white light. The V-log I data were fitted to the Naka-Rushton function, $V/V_{max} = I^n/(I^n + K^n)$, where I is the stimulus intensity, V is the response amplitude, V_{max} is the maximum response amplitude, K is the stimulus intensity eliciting 50 % of V_{max}, and n is the exponent.

Results

Anatomy of sexually dimorphic eyes

I observed morphological differences of the eyes of both sexes with scanning electron microscope (Fig. 6). While the eyes of males appear rough (Fig. 6A), female eyes look smoother (Fig. 6D). As described in the previous chapter, the eyes of males consist of facets of irregular shape (Fig. 6B). However, the eyes of females appeared to have hexagonal facets, whose size variation is much less than in male eyes (Fig. 6E). I analysed the distribution of the facet surface area in an eye of a female and compared it with the case in a male. The histogram of the female facet areas has a single peak, while it has two peaks in the male individual. The average size of female facets is slightly larger than male's smaller facets (Fig. 6F).

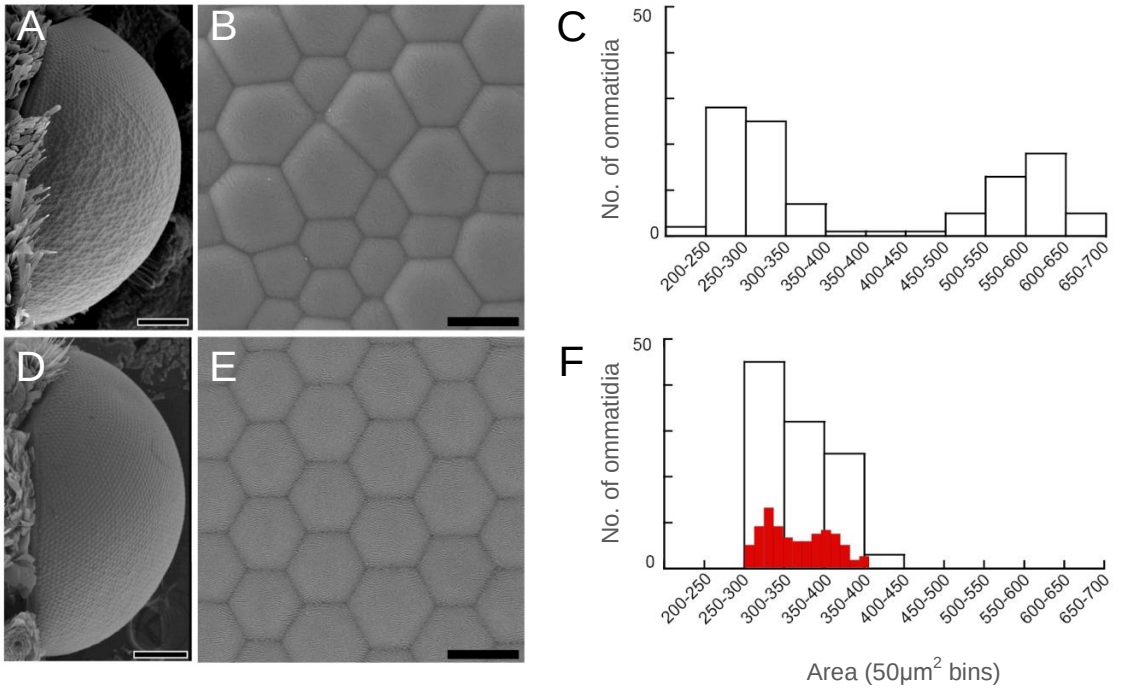

Fig. 6. Comparison of the eyes of a male (upper) and a female (lower) *Leptidea amurensis*. (A, D) A Low-magnification SEM picture of a left eye. (B, E) A high-magnification SEM picture of the ventral region. (C, F) Histogram of facet areas of the ventral ommatidia (white; 50 μm^2 bins, red; 12μm^2 bins). Scale bars, (A,D) 200μm, (B,E) 20μm.

I also compared crystalline cone sizes. There are two crystalline cone types of different sizes in both sexes, but the size difference is much smaller in females (Fig. 7B). I identified three types of ommatidia according to the rhabdom ultrastructure in females as well (Fig. 7C, for males see Fig. 2). The rhabdom structures in three types of ommatidia are similar in both sexes: for example, the variation in the microvillar orientation in three types is comparable to those in males. A conspicuous sexual difference is found in the total volume of the rhabdom. I estimated the volume of the rhabdom by reconstructing the rhabdom from a series of electron micrographs of rhabdom transverse sections. It turned out that the rhabdom volume is about half in females (Fig. 8).

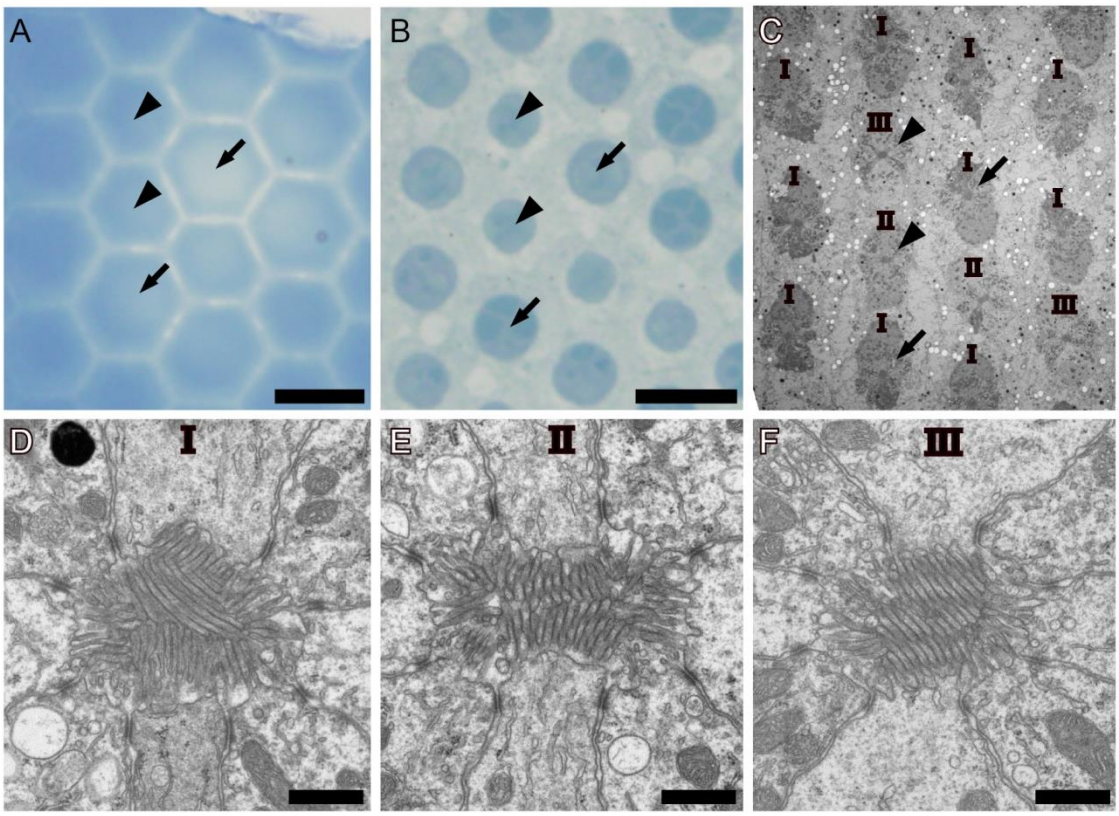

Fig. 7. Light micrographs and electron micrograph of consecutive transverse sections of the cornea (A), crystalline cone (B), photoreceptor (C) in a female. Arrowheads pointed small ommatidia and arrows pointed large ommatidia. Three types of rhabdom in a female eye at the depth of ca. 180 μm from the corneal surface (D:I, E:II, F:III). Scale bars, (A,B) 20μm, (D,E,F) 1μm.

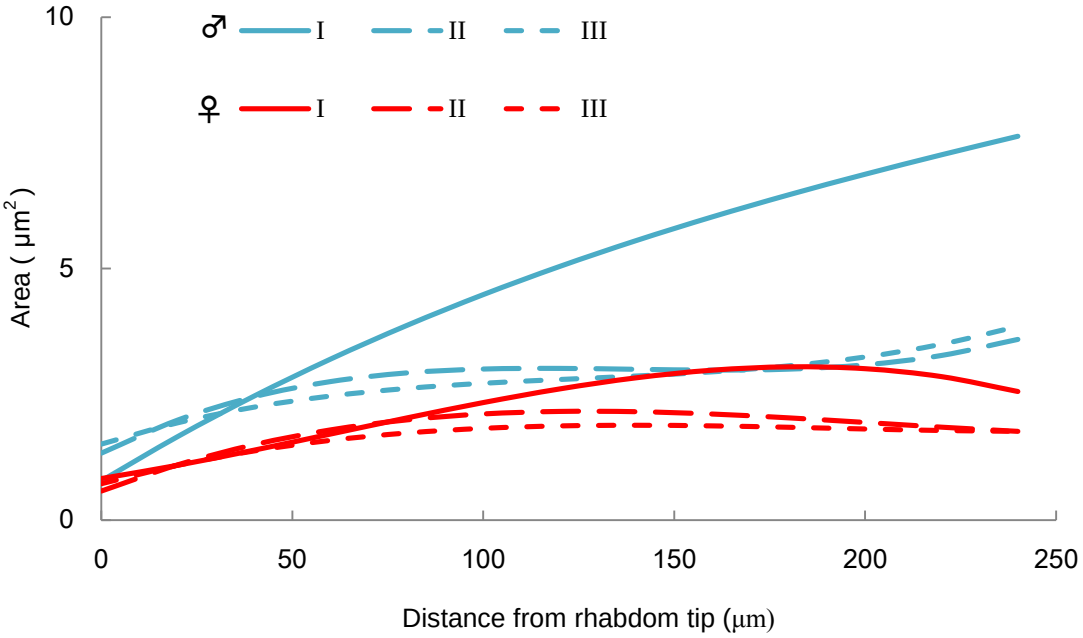

Fig. 8. The fitting curves of the rhabdom in three types of ommatidia in a male and a female. The estimated total volumes of the rhabdom are 1143.2 um^3 (type I in male), 679.2 um^3 (type II in male), 661.4 um^3 (type III in male), 552.3 um^3 (type I in female), 438.9 um^3 (type II/III in female) and 398.8 um^3 (type II/III in female).

Spectral sensitivities of the eyes as a whole

I measured the spectral sensitivities of ERG in males and females (Fig. 9). The spectral sensitivity of male eyes was the most sensitive at ultraviolet (360nm). In contrast, there are two peaks, one in UV (360nm) and the other in blue-green (500nm) region in females.

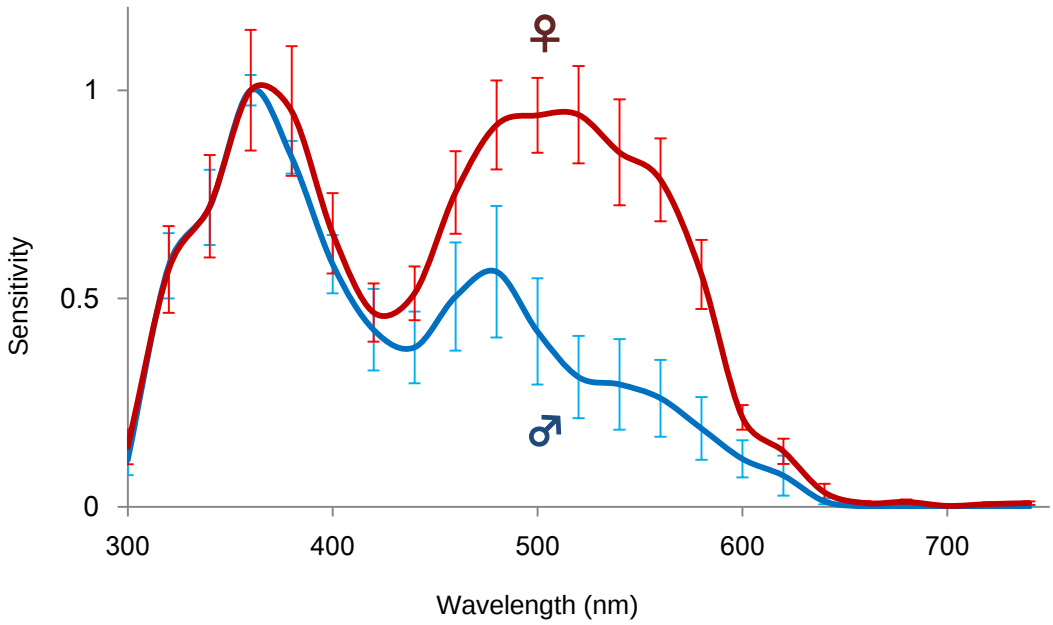

Fig. 9. Spectral sensitivities of ventral region of eyes in both sexes determined by ERG. Males (blue, N=10) and females (red, N=5).

Dynamic range of the eyes

V-log *I* curves determined by the ERG recording in ventral region of eyes in both sexes showed sexual dimorphism (Fig. 10). Parameters of *V*-log *I* curves were shown on Table 2. V_{max} of male was larger than that of female (Male: 5.17 ± 1.27, Female: 3.30 ± 1.03). The exponential slope, *n*, was steeper in females (*n*=0.68 ± 0.13) than that in males (*n*=0.57 ± 0.08). Males exhibited higher log K than in females (Male: 2.20 ± 0.65, Female: 1.75 ± 0.60).

I compared the dynamic ranges (light intensity range that elicits responses from 10% to 90% of maximal response) in the ERG-determined *V*-log *I* curves in both sexes. I thus found that the dynamic range was significantly wider in males (3.35 log units) than in females (2.80 log units) (Welch's t-test, P < 0.01) (Fig. 10).

Table 2. Comparison of parameters between male and female

	Male	Female	t-test
	63(N=8)	67(N=8)	
n	0.57 ± 0.08	0.68 ± 0.13	P < 0.01
V_{max}	5.17 ± 1.27	3.30 ± 1.03	P < 0.01
log*K*	2.20 ± 0.65	1.75 ± 0.60	P < 0.01

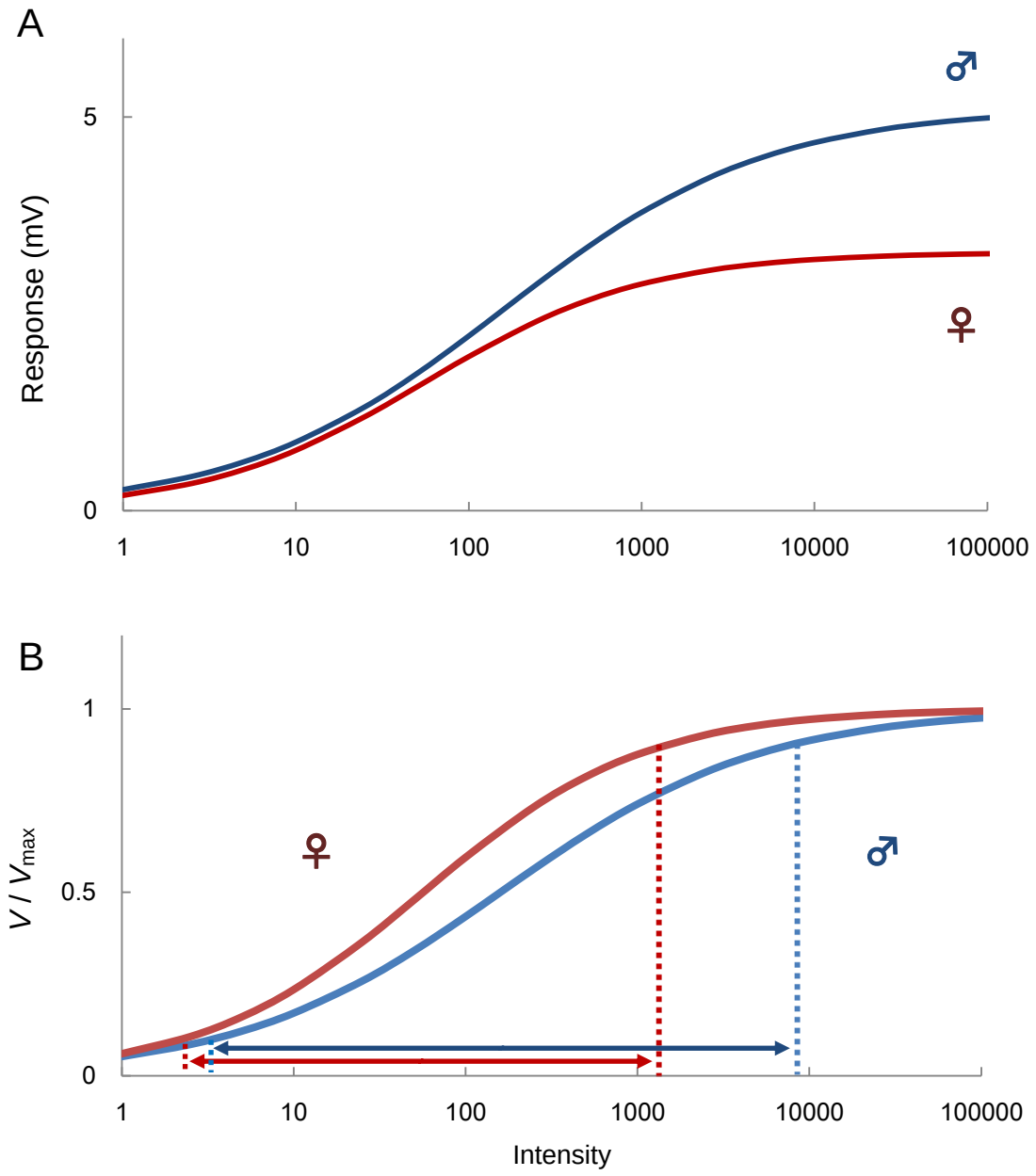

Fig. 10. *V-log I* curves from the eyes of males (blue) and females (red) determined by ERG. (B) Normalized curves to compare the dynamic range of the eyes. Arrows indicated the width of the dynamic range (10% - 90%).

Photoreceptor angular sensitivities in large and small ommatidia in males

To investigate optical properties of large and small ommatidia in the eye of males, I recorded spectral and angular sensitivities in single photoreceptors. The angular sensitivity of a photoreceptor represents the acceptance angle of the ommatidium in which the photoreceptor is located. There are two classes of blue receptors in males; one is in LO and another is in SO. Two classes of blue receptors can be distinguished based on their spectral sensitivities. The acceptance angle of ommatidia ($\Delta\rho$) is represented full width at half maximal. The distribution of angular sensitivity (AS) has two peaks which peaked around 1.75, 2.5 respectively (Fig 11A). Contrary to the size of lenses, small ommatidia has larger acceptance angle than large ones (Fig 11B). The $\Delta\rho$ of sB in LO (type I) is 1.65 ± 0.16 (N=6), bB in SO (type II) is 2.32 ± 0.37 (N=6).

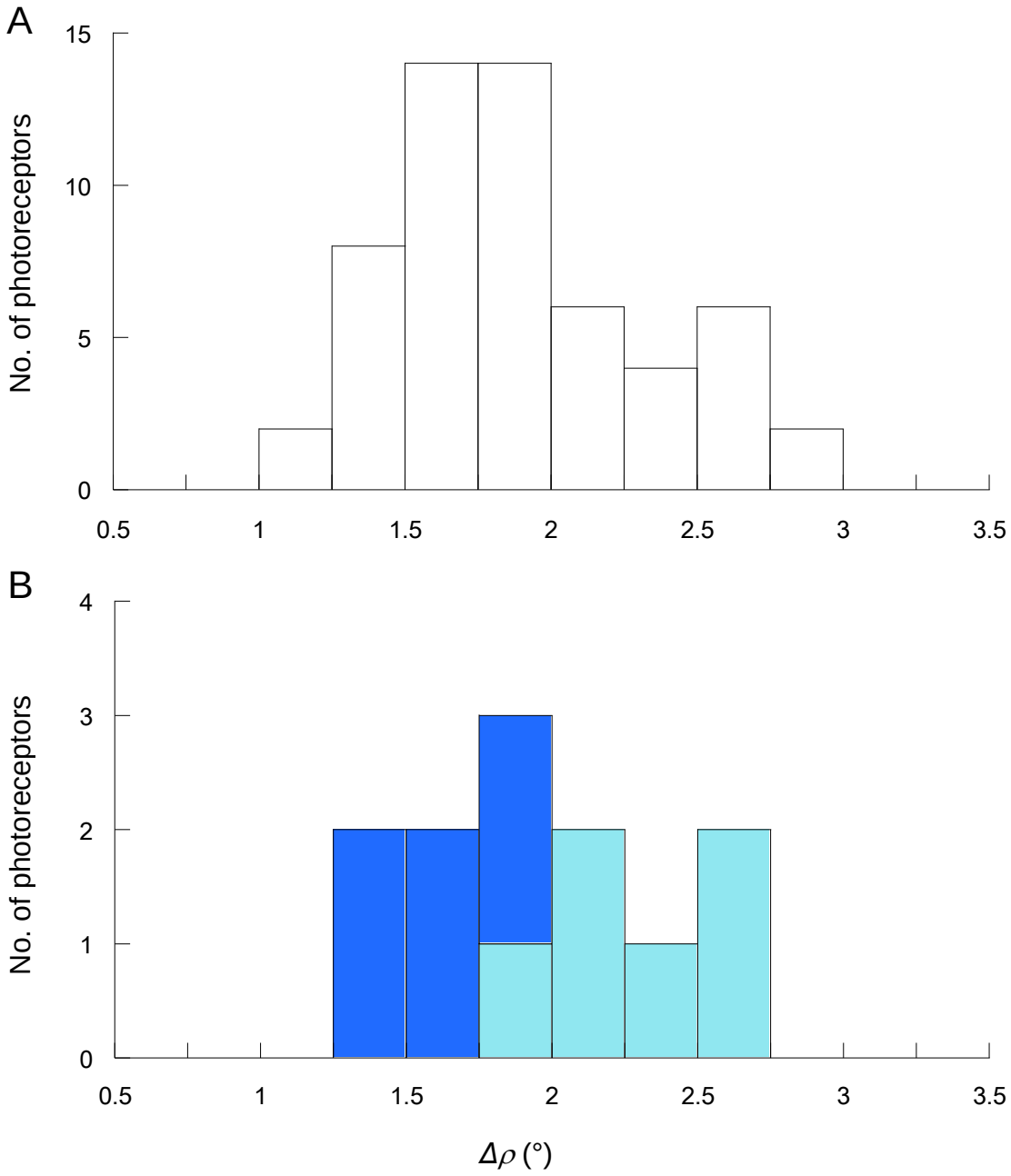

Fig. 11. Distribution of $\Delta\rho$ (bin width 0.25) for all of the recorded cells (A), and for blue cells, sB in LO (blue columns, N=6), bB in SO (light blue columns, N=6) (B).

The *V*-log *I* curves of large and small ommatidia in male

V-log *I* curves with monochromatic light from the both blue photoreceptors were obtained by intracellular recording (Fig 12). Comparing *V*-log *I* curve between two blue receptors, the maximum response (V_{max}) was significantly larger in sB (LO) (35.76 ± 7.6 mV) than in bB (SO) (26.68 ± 2.45 mV) (Welch's t-test, $P < 0.01$). Other factors, *K* (light intensity that evokes a response $V_{max}/2$) and *n* (exponential slope of the *V*-log *I* curve) are not significantly different between sB and bB (Table 3).

Table 3. Comparing average value of parameters between sB and bB

	V_{max}	*n*	Log *K*
sB(N=9)	35.76 ± 7.6	0.87 ± 0.08	2.41 ± 0.56
bB(N=8)	26.68 ± 2.45	0.89 ± 0.11	2.56 ± 0.32
t-test	P < 0.01	-	-

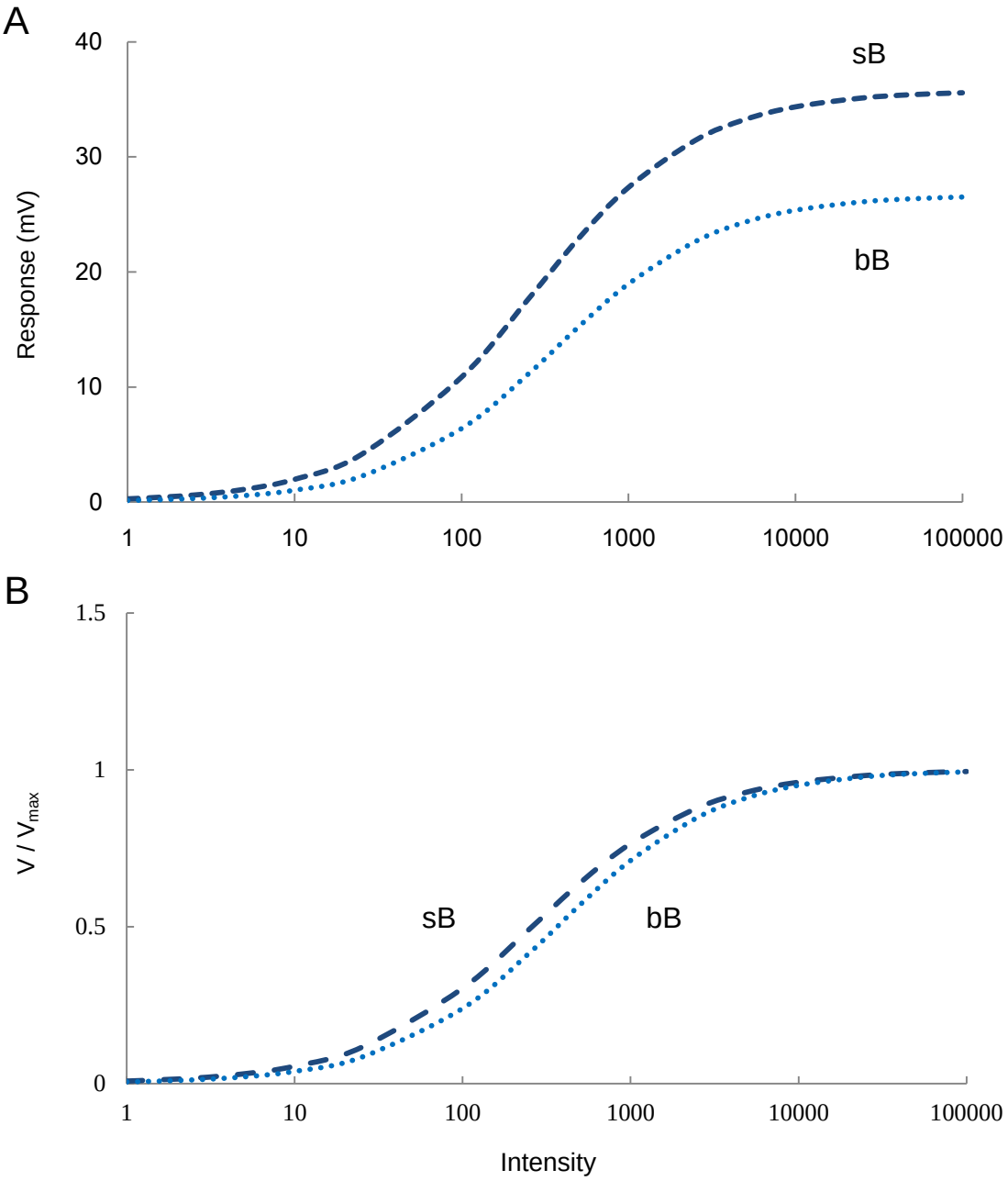

Fig. 12. *V*-log *I* curves recorded from blue receptors in male by intracellular recordings. Solid line is sB in LO, dotted line is bB in SO (A). Normalized *V*-log *I* curves (B)

Calculated sensitivity of ommatidia

The sensitivities of the ommatidia were calculated by the following equation (Frederiksen and Warrant 2008):

$$S = \left(\frac{\pi}{4}\right)^2 D^2 \Delta\rho^2 \frac{kl}{(2.3 + kl)} \quad (1)$$

where the parameters are the facet diameter, *D*, the acceptance angle of ommatidia, $\Delta\rho$, the absorbance coefficient, k, and the rhabdom length, *l*. The value of each parameters used in the calculation were listed in Table 4.

The calculated sensitivity of LO are 1.4 times larger than SO in male. I was not able to compare the sensitivities of male and female because I did not have the data of acceptance angle in females. However, because the size variation of female ommatidia is minor, I assumed their acceptance angle does not vary much among three ommatidial types.

Table 4. Parameters used to calculate the optical sensitivity

Symbol	Parameter	Unit	Male		Female	
			I	II / III	I	II / III
D	Facet diameter[a]	μm	31.6	21.8	24.9	22.5
$\Delta\rho$	Acceptance angle[b]	rad	0.029	0.040	0.040[c]	0.040[c]
k	Absorbance coefficient[d]	μm^{-1}	0.0115[e]	0.0067	0.0054[f]	0.0041[g]
L	Rhabdom length[h]	μm	260	260	260	260
S	Optical sensitivity	$\mu m^2 sr$	0.290	0.207	0.198*	0.193*

[a] D was calculated by the area of each size of ommtidia.

[b] $\Delta\rho$ (Fig.11) was converted from the degree to radians.

[c] I used $\Delta\rho$ of type II/III in male.

[d] I used k measured by Bruno et al (Bruno et al 1977) in type II/III of male as a standard.

[e] k in type I of male was derived by the ratio of rabdom volume: 0.0067 x 1.73

[f] k in type I of female was derived by the ratio of rabdom volume: 0.0067 x 0.80

[g] k in type II/III of female was derived by the ratio of rabdom volume: 0.0067 x 0.61

[h] Rhabdom length were almost same in each type of ommatidium.

* For comparison in female

Discussion

Sexual dimorphism in eye

Through comparing the eye structure of both sexes, I found that females also have three types of ommatidia: one type has large lens and rhabdom, and those in other two types are small. However, the difference in size between the large (type I) and the small ommatidia (type II/III) in females are smaller than in males. I found that the irregular shape of facets is a specific feature of male eyes.

The spectral sensitivities determined by ERG recording can be explained the variable combination of three absorption peaks of their visual pigments, LaUV (360nm), LaB (450) and LaG (530). This suggests they share identical set of opsins.

Dynamic range

The dynamic range of eyes is wider in males than in females. The rough eye of male that has two sized ommatidia and irregular shapes of facets probably relate to the difference.

Similar architecture has been developed in the sensor technology for a high dynamic range (HDR) imaging. HDR in imaging sensor such as digital cameras has been studied and developed, since human eyes have a remarkable dynamic range (Skorka and Joseph 2011). There are a few ways to improve dynamic range of imaging sensors (Kavusi and Gamal 2004). A novel scheme to obtain high dynamic range image is using multiple light detecting pixel that differ in size and therefore sensitivities (Nayar and Mitunaga 2000). In addition, HDR for motion imaging without suffering from a loss in spatial resolution was achieved by non-regular optical attenuation of individual pixels (Schöberl et al. 2013). An ommatidium acts like a pixel because it is furnished with a single light-sensing structure, the rhabdom, and is optically isolated from other ommatidia. The rough eye, which contains ommatidia of two different sizes,

is similar to these devises. If the ommatidia of different sizes have different sensitivities, the dynamic range will be expanded in the rough eye.

Sensitivity difference between large and small ommatidia

The differences in optical and physiological properties of LO and SO in males were found in acceptance angles, *V*-log *I* curves and estimated optical sensitivities.

Acceptance angle of photoreceptors approximated by $\Delta\rho = d/f$ where d is rhabdom diameter and f is focal length (Land and Nilsson 2002, Stavenga 2003). In apposition eye, increasing rhabdom diameter improves sensitivity by widening the acceptance angle. Although diameter of rhabdom is larger in LO than in SO over almost the entire depth, the rhabdom tip diameter is smaller in LO than in SO (Uchiyama et al. 2013). However, in eyes of butterflies, the angular sensitivity of ommatidia is not principally determined by rhabdom diameter (Land and Nilsson, 2002, Frederiksen and Warrant, 2008). The focal length is difficult to measure optically due to complex lens systems in eyes of butterflies. Radius of curvature of corneas in males are larger in LO (31.3 ± 7.0μm) than in SO (15.3 ± 6.3μm). Crystalline cones in SO emerged and ended more distally about 5μm than in LO (data not shown). These data indicated longer focal length in LO. This appears to be the cause of the observed difference in angular sensitivities between LO and SO.

V-log *I* curves and estimated optical sensitivities suggests that the LO are more sensitive to light than SO. The volume of rhabdom in LO are larger than in SO. That may be related to the V_{max} (saturated response value) in *V*-log *I* curves.

In the focal apposition eyes of two species of cockroaches, irregular facets that differ sizes and shapes have been reported (*Periplaneta Americana*, Butler 1973a; *Gromphadorhina portentosa*, Mishra and Meyer-Rochow 2008). In addition to the variation in sizes and shapes of

the lenses, the rhabdom of *Periplaneta americana* have variable sizes and shapes distributed randomly (Butler 1971, 1973a, b). The acceptance angle and sensitivity vary between photoreceptors due to the variability of these structures in *Periplaneta americana* (Butler and Horidge 1973a,b; Heimonen et al. 2006). This eye structure was supposed to be a functional adaptation to dim environment (Butler 1971, 1973a, b; Butler and Horidge 1973a,b Heimonen et al. 2006). The LO and SO of male *Leptidea amurensis* have irregular facet sizes and shapes, which probably extended the dynamic range of the eye. Although the LO have smaller acceptance angle than SO in average, the acceptance angle of LO and SO varies within each sized ommatidia most likely because of their irregular shapes.

Biological significance of the rough eye

Species in the genus *Leptidea* (including *L. sinapis*) have rough eyes (Yagi 1964). European species of *Leptidea, L. sinapis* and *L. reali* have been quite extensively studied ecologically, behaviorally and evolutionary (Friberg et al. 2013, Dincã et al. 2013). Males of both species spend their time flying to search females. Flying males approach whatever white and highly reflective objects approximately the sizes of females in herbage layers (Wikland 1977, Friberg et al. 2008). In both species, males cannot distinguish specific females, while females have the ability to discriminate their mates in courtship rituals (perhaps by chemical signals) (Freese and Fiedler 2002, Friberg et al. 2008).

Males of *L. amurensis* appear to use vision to detect white and highly reflective objects for searching females. Actually, I have repeatedly observed such courtship rituals (shown in Wikland 1977) in *L. amurensis* in their habitat. For the males, detecting females is one of the most important tasks.

Their habitat is localized in open and sunny places among forests and probably

restricted by the distribution of their larval food plants. Environmental conditions where *L. amurensis* is active were investigated in Nagano, Japan (Nakao et al. 2000). It has been shown that the adults fly in open grass fields more frequenly than in the forest from around 10:00 to 14:00. They prefer flying in conditions with the high photon flux density, the high ultraviolet intensity and high temperature (Nakao et al. 2000). These suggest *L. amurensis* are active in bright conditions and probably most of them are males searching for females. Searching for a female, which is hiding among bushes under bright light (the ratio of the highest and lowest light intensity should be quite large), wider dynamic range of eyes is certainly beneficial for males. Enhanced contrast sensitivity must be particularly useful in the dimmer and shady habitat for improved female detection. Moreover, flying frequently in and out between bright and shady environments most likely requires fast adaptation and extended dynamic range

General discussion and conclusion

In this thesis, I described the morphological and physiological basis of the eye of the northeast-Asian wood white *Leptidea amurensis*. I first studied the structure of the compound eye of males through a combination of anatomy, molecular biology and intracellular electrophysiology, with a particular focus on the evolution of butterfly eyes. Then, I explored the function of the eyes through comparing the properties of eye between sexes.

Comparing to other butterflies, number of types of photoreceptor is minimum in *L. amurensis*. The eye of males has only a basic set of spectral photoreceptors; ultraviolet, blue and green receptors, and does not have violet and red photoreceptors as in *Papilio*, *Pieris* and *Colias*. In addition, the eyes of males do not have duplicated blue opsins, which is a shared property among all Pierid butterflies so far studied (Uchiyama et al. 2013). More over, the eyes *Leptidea* do not contain peri-rhabdomeral and fluorescent pigments, which are found in not all but many butterflies. These pigments act as spectral filters, shifting the sensitivity peaks of photoreceptors to long wavelength direction, thus creating the eye spectrally richer (Arikawa et al. 2005, 2009; Ogawa et al. 2012,2013; Ribi 1978; Wakakuwa et al. 2004). Judging from these points, I have concluded that the eyes of *L. amurensis* have ancestral features.

One of the unique features of male eyes is the random distribution of the ommatidia of two different sizes with irregular facet shape. This feature is quite unique. Eyes with ommatidia of two different sizes are found in a thrips, *Caliothrips phaseoli* (Mazza et al. 2010). However, the function of the structure has not been studied in thrips. Irregular shapes of facets have been found in cockroaches, *Periplaneta americana* (Butler 1973a) and *Gromphadorhina portentosa* (Mishra and Meyer-Rochow 2008), and the

acceptance angle and sensitivity vary between photoreceptors due to irregular shapes of facets and to this eye structure might make dynamic range wider (Heimonen et al. 2006). The LO and SO in male of *L. amurensis* also have both irregular shapes of facets respectively. Thus *L. amurensis* have both features.

Design similar to *Leptidea* eye has been developed in today's imaging technology. The HDR video camera is achieved by non-regular optical attenuation of individual pixels and capable of light capturing a high dynamic range video without losing in spatial resolution (Schöberl et al. 2013).

Natural luminance varies in the intensity range of 8 log units if we compare those under sunlight and starlight (Land and Nilsson 2002), and typically luminance difference is at least 1.5- 2 log units (light source not included) in day condition (Nilsson 2009). Hence, the dynamic range of compound eye is generally wider in crepuscular and nocturnal animals. For example, in comparison of 46 species of Lepidoptera including butterflies and moths, the eyes of nocturnal moths have the broadest dynamic range (Eguchi and Horikoshi 1984). Most nocturnal insects have superposition eye types. *L. amurensis* is diurnal and active in bright condition. The diurnal apposition eye is designed for vision in bright light and therefore viewing dim condition is hard. Working range of the pupil corresponds to the habitat luminance of butterflies, and the pupil is working to maximize acuity over a wide range of luminance in woodland and crepuscular butterflies except grassland species such as *Pieris rapae* (Jonson et al. 1998). *L. amurensis* probably adapt to habitat luminance not only by the pupil mechanism but also by the characteristic roughness in the eye. The rough eye of *L. amurensis* seems to be another solution of expanding dynamic range for adapting various light environments for grasslands around woodlands. The male of *L. amurensis* probably have to fly both in sunny and shaded

places when searching potential mates, while newly emerged virgin females stay around the food plant in shaded places and wait to be found by males. The sexual dimorphisms in the visual system probably have evolved under the pressure to achieve better in the mating behavior in such a complicated light environment.

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