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Spatial distribution of opsin-encoding mRNAs in the tiered larval retinas of the sunburst diving beetle *Thermonectus marmoratus* (Coleoptera: Dytiscidae)

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SUMMARY

Larvae of the sunburst diving beetle, *Thermonectus marmoratus,* have a cluster of six stemmata (E1–6) and one eye patch on each side of the head. Each eye has two retinas: a distal retina that is closer to the lens, and a proximal retina that lies directly underneath. The distal retinas of E1 and E2 are made of a dorsal and a ventral stack of at least twelve photoreceptor layers. Could this arrangement be used to compensate for lens chromatic aberration, with shorter wavelengths detected by the distal layers and longer wavelengths by the proximal layers? To answer this question we molecularly identified opsins and their expression patterns in these eyes. We found three opsin-encoding genes. The distal retinas of all six eyes express long-wavelength opsin (TmLW) mRNA, whereas the proximal retinas express ultraviolet opsin (TmUV I) mRNA. In the proximal retinas of E1 and E2, the TmUV I mRNA is expressed only in the dorsal stack. A second ultraviolet opsin mRNA (TmUV II), is expressed in the proximal retinas of E1 and E2 (both stacks). The finding that longer-wavelength opsins are expressed distally to shorter-wavelength opsins makes it unlikely that this retinal arrangement is used to compensate for lens chromatic aberration. In addition, we also described opsin expression patterns in the medial retina of E1 and in the non-tiered retina of the lensless eye patch. To our knowledge, this is also the first report of multiple UV opsins being expressed in the same stemma.

Key words: stemmata, eye, *in situ* hybridization, visual pigments, visual system, insects.

INTRODUCTION

Adult insects are generally characterized by compound eyes, and the vast majority of the research in the vision field has been focused on understanding the form, function and evolution of these eyes. However, certain insects are also characterized by stemmata, which are the eyes of holometabolous insect larvae. Stemmata frequently are not multifaceted like compound eyes, but vary greatly from simple photosensitive organs (such as in many dipterans) to sophisticated camera-type eyes, such as those found in the tiger beetle *Cicindela chinensis* (Gilbert, 1994; Land and Nilsson, 2002; Toh and Mizutani, 1987; Toh and Mizutani, 1994; Toh and Okamura, 2007). Despite this diversity, careful morphological studies (Paulus, 1979) as well as more recent molecular work (Buschbeck and Friedrich, 2008; Liu and Friedrich, 2004) suggest that stemmata most probably evolved from the most anterior portion of the compound eyes of their hemimetabolous ancestors.

The stemmata of the sunburst diving beetle, *Termonectus marmoratus*, are also highly sophisticated. Some of the *T. marmoratus* stemmata are particularly interesting because their anatomy suggests a highly unusual functional organization (Mandapaka et al., 2006). In this paper, we investigate the presence and spatial distribution of opsins as an important step towards shedding light on the evolution and function of these peculiar eyes. Previous anatomical studies of *T. marmoratus* larvae revealed that this holometabolous insect contains six stemmata on each side of its head, E1–6 (Fig. 1A,B). Each of these stemmata has at least two distinct, tiered regions of retinular cells that constitute a distal and proximal retina. In addition, *T. marmoratus* has an untiered eye patch that lacks a lens, but has a retina that is situated directly beneath the cuticle (Fig. 1A). Behavioral experiments have revealed that E1 and E2 are forward-pointing principal eyes that are used to scan potential prey prior to capture (Buschbeck et al., 2007). E3–6 are secondary eyes that probably detect prey outside the visual field of the forward-pointing principal eyes. E1 and E2 point upward at an angle of about 35 deg. from the horizontal body plane and share many anatomical characteristics (Fig. 1C,D). Both are tubular in design and have a cylindrical crystalline cone-like structure underlying a large lens. The distal retina lies directly below this cone. Viewed sagittally, the rhabdomeric portion of the distal retina has a triangular shape, and is formed by retinular cells that are oriented orthogonally to the light path (Fig. 1C,D, insets). This orientation is unusual for insect retinular cells that are in general aligned with the light axis. In *T. marmoratus*, there are at least twelve orthogonal cell layers stacked on top of each other on both the dorsal and ventral eye side, resulting in dorsal and ventral stacks, which together form the distal rhabdom (Fig. 1D, insets). Below the distal retina lies the proximal retina which is formed by two rows of axially oriented retinular cells (Fig. 1C,D). In addition, E1 has a third retina that contributes to rhabdoms that are situated along the medial side of the eye (previously described as the lateral retina; Fig. 1D) (Mandapaka et al., 2006). This medial retina is absent in E2, which is the most obvious anatomical difference between the two tubular eyes.

E3–6 exhibit the same orientation of their rhabdoms within their distal and proximal retinas as do E1 and E2, but these secondary eyes are more spherical than cylindrical. All secondary eyes have a similar arrangement of their retinas with distal rhabdoms situated near the middle of each eye and a cytoplasm that projects to the periphery on both sides of the rhabdom. Retinular cells of the proximal retinas, by contrast, are 'in axis' with the light path and

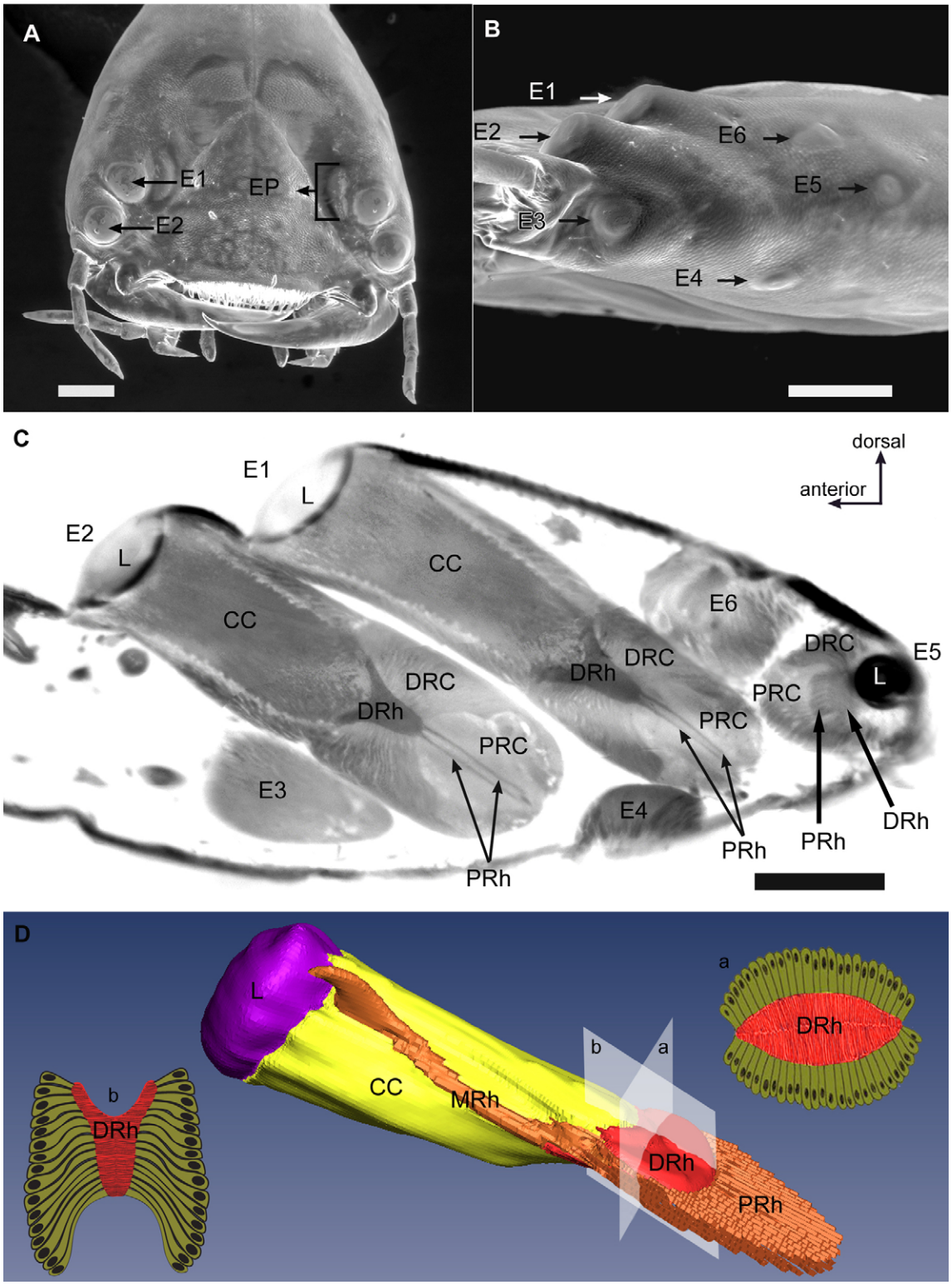

Fig. 1. First instar *T. marmoratus* larval eyes. (A) Frontodorsal view of a larva showing the principal eyes, E1 and E2, and the eye patch (EP). (B) Left lateral view of the head showing the secondary eyes, E3–6, as well as the two principal eyes. (C) An ethyl gallate-stained sagittal section showing the lenses (L), crystalline cone-like structures (CC), retinular cells of the distal (DRC) and proximal (PRC) retinas with their respective rhabdoms (distal, DRh; proximal, PRh). Different retinas (retinular cells and rhabdoms) are labeled on the principal eyes (E1 and E2) and on E5. Other secondary eyes (E3, E4, and E6) have an organization similar to that of E5, but are situated outside the plane of this section. In general, in all eyes (E1–6), distal retinular cells (DRC) stain darker with ethyl gallate than the proximal retinular cells (PRC). Rhabdoms also appear darker. (D) 3D reconstruction of E1 showing the lens (L), crystalline cone-like structure (CC), medial retina rhabdom (MRh), distal retina rhabdom (DRh), and the proximal retina rhabdom (PRh). Insets a and b are schematic drawings of respective sections through the distal retina (as indicated in the figure by sectional planes a and b), illustrating the orthogonal orientation of the dorsal and ventral retinular cells of the distal retina. The insets illustrate the rhabdoms (red) as well as cell bodies (green) which are not shown in the 3-D reconstruction. Scale bars, A and B, 200 μm; C, 100 μm.

lie along the back of the eyes (Fig. 1A, see E5). These eyes are also distributed around the animal's head, each eye oriented at a different angle (Mandapaka et al., 2006).

All in all, *T. marmoratus* has at least 26 retinas, and because of its bizarre organization, this visual system differs strongly from any other known visual systems (Mandapaka et al., 2006). How can such a complicated visual system function? There are a few organizational similarities to the eyes of jumping spiders (Salticidae). In these animals, the secondary eyes are fixed and act primarily as motion detectors while their principal, anteromedian (AM) eyes are used for target tracking (Land and Nilsson, 2002). Interestingly, the AM eyes of jumping spiders have a four-tiered retina, which has been hypothesized to be used to compensate for lens chromatic aberration (Blest et al., 1981; Land, 1969). Lens chromatic aberration results in shorter wavelengths being refracted more than longer ones. Hence, short wavelengths are in sharp focus closer to the lens than longer wavelengths. By having a tiered retina with short(er)-wavelength photoreceptors in layers closer to the lens and long(er)–wavelength receptors in the layers behind, one can compensate for lens chromatic aberration by focusing different wavelengths onto photoreceptor layers that are maximally sensitive to them. Consistent with this model, in the jumping spider *Plexippus* sp., the retinal layers farthest from the lens express long-wavelength sensitive (LW, or green) opsin receptors, whereas layers closer to the lens express ultraviolet-sensitive (UV) opsin receptors with spatial separations that approximately match the chromatic aberration of the lens (Blest et al., 1981). Considering that the unusual visual system of *T. marmoratus* larvae is also characterized by tiered retinas, and that the distal retinas of the principal eyes consist of many layers, the question arises as to whether specific photoreceptor subtypes could compensate for chromatic aberration in this species as well. Although this might not hold for the smaller secondary eyes, it is expected that the relatively long focal lengths of the tubular principal eyes lead to significant chromatic spread. For instance, for *Musca domestica* lenses, which have much smaller focal lengths of 50–80 μm, the focal plane for ultraviolet light (331 nm) lies about 4 μm in front of the focal plane for green light (582 nm) (McIntyre and Kirschfeld, 1982). Based on the several hundred micrometer separation between the lenses and retinas of the *T. marmoratus* E1 and E2, we expect that the lens chromatic aberration in these eyes is substantially larger than the one for *Musca domestica* lenses, separating light sufficiently for different wavelengths to be simultaneously focused at least within the different layers of the distal retinas. Therefore it is important to understand the spatial expression patterns of opsins in this visual system.

We have used molecular techniques to clone, identify and investigate the spatial distribution of opsin transcripts from the first instar larvae of *T. marmoratus*. We describe three independent opsin-encoding genes from these animals that account for all of the previously identified retinas of these larvae. Based on gene sequence and phylogenetic analyses, two opsins are UV sensitive, whereas one is LW sensitive. No blue (B)-sensitive opsin was identified, similar to recent studies in the *Tribolium* beetle lineage (Jackowska et al., 2007). These findings are consistent with an evolutionary loss of B-sensitive opsin in this lineage. The expression patterns of the different opsin genes indicate LW-sensitive distal retinas and UV-sensitive proximal retinas in all eyes. This expression pattern does not support the possibility of a chromatic aberration compensation mechanism, neither within the deep distal retinas of principal eyes nor between the distal and proximal retinas (since it is contrary to what would be expected for chromatic aberration compensation). To our knowledge, this is the first report of two UV-sensitive opsins expressed in the same stemma in a holometabolous insect.

MATERIALS AND METHODS

Animals

Adult and larval *T. marmoratus* Gray 1832 specimens, reared in our lab throughout the year, were offspring of the beetles provided by the Insectarium of the Cincinnati Zoo and Botanical Gardens, or from beetles collected in August 2004 near Tucson, AZ, USA. Adults were kept in fresh water aquariums at room temperature (RT) and fed daily with freshly killed crickets. After hatching, *T. marmoratus* larvae were separated from adults and fed with thawed brine shrimp and live mosquito larvae before they were used in experiments. Although second and third instar larvae appear to have a similar general eye organization (data not shown), all experiments were performed on first instar larvae only.

mRNA extraction, cDNA synthesis, PCR, and cloning

Larval heads were severed from intact animals in phosphate-buffered saline (PBS; pH 7.4) and kept on dry ice until at least ten heads were collected. Total RNA was extracted from larval heads using TRI Reagent® (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions and resuspended in 20 μl of H_2O. Single-stranded complementary DNA (cDNA) was synthesized from 1 μl of the extracted total RNA through reverse transcription (AffinityScript Reverse Transcriptase, Stratagene, La Jolla, CA, USA) with oligo(dT) primers. Consensus-degenerate hybrid oligonucleotide primers (Rose et al., 1998) that correspond to highly conserved amino acid sequences in LW-, UV- and B-wavelength-sensitive invertebrate opsins were designed using the online CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html; Table 1). All primers were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA). PCR reactions were performed using Taq DNA polymerase with premix, TAK_R001AM (Takara, Otsu, Shiga, Japan). Plant RNA Isolation Aid (Applied Biosystems) was used at a dilution of 1:10 per reaction to prevent PCR inhibition by polysaccharides present

Table 1. Degenerate (upper case) and specific (lower case) primers used in cloning *T. marmoratus* opsins

Primers	Sequence 5′>3′	Size
LWFw I	GAACCTGGCCTTCTCCGAYTTYNKNAT	27
LWRv I	ACGTTCATCTTCTTGGCCTGYTCNYKCAT	29
LWFw II	TTCGACCGGTACAACGTGATHGTNMANGG	29
LWRv II	CAGGTAGGGGGTCCAGGCVAWRAACCA	27
BlFw	CATGTTCATCATCAACCTGGCNATHTTYGA	30
BlRv	CGAAGGAGCAGGTGGACARRWANCCYTC	28
UVFw I	GAGCTGATGCACATCCCCSARCAYTGGYT	29
UVRv I	CGAAGGTGTCGGTCAGGTAGTCRAANSWRCA	31
UVFw II	TGGTCAAGACCCCCATCTTYAYGYAYAA	28
UVRv II	CAGATGGTGATGGCGGCYTTNGCDATNC	28
3′ RACE LW	tgatgaccatctcgctgtggttct	24
5′ RACE LW	aggtctcgtagtagcagttgacg	23
3′ RACE UV I	cgaagcaaccagaatcaacagacc	24
5′ RACE UV I	tagctccgatacctgataaagagc	24
3′ RACE UV II	tattctgcctgctatggaagtct	23
5′ RACE UV II	acctcggttgaatgagttgtaga	23

Degenerate primers were named according to the spectral class and their orientation, i.e. LWFw – long wavelength, forward; LWRv – long wavelength, reverse (see Fig. 2). The 3′ RACE primers shown are all forward and the 5′ RACE are all reverse.

from the head cuticle. A typical 25 µl reaction consisted of 2.5 µl PCR 10× buffer, 2.5 µl 25 mmol l^{-1} $MgCl_2$, 4 µl 10 mmol l^{-1} dNTPs, 1 µl 100 µmol l^{-1} forward primer, 1 µl 100 µmol l^{-1} reverse primer, 0.25 µl Taq DNA polymerase, 0.5 µl cDNA, 2.5 µl Plant RNA Isolation Aid and 10.75 µl H_2O. Samples were denatured at 94°C for 2 min prior to 35 cycles of PCR [94°C, 1 min; 48-55°C (depending on primer pair), 1 min; 72°C, 1 min], and finished with a 5 min 72°C extension. PCR products of appropriate length were gel purified with the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), subcloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA), and bidirectionally sequenced. 3′ RACE PCR reactions were performed using freshly isolated cDNA from *T. marmoratus* larvae synthesized with an adapted oligo(dT) primer (5′-GAC TCG AGT CGA CAT CGA TTT TTT TTT-3′) and subsequently amplified with opsin-specific forward primers (Table 1) and a universal reverse primer (from the adapted oligo(dT) primer: 5′-GAC TCG AGT CGA CAT CG-3′). 5′ RACE PCR reactions were performed using FirstChoice® RLM-RACE kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. 5′ RACE reaction forward primers were provided in the kit and opsin-specific reverse primers were synthesized (Table 1).

Sequencing and phylogenetic analysis

Each PCR product was sequenced from at least three independent clones. Full-length opsin cDNA sequences were assembled, translated and aligned with 46 selected arthropod opsin proteins (Table 2) using ClustalW implemented in MEGA 4.0 (Tamura et al., 2007). A phylogenetic tree was constructed using the neighbor-joining algorithm (Saitou and Nei, 1987) with Poisson correction (Zuckercandl and Pauling, 1965) and evaluated with 1000 bootstrap replicates (Felsenstein, 1985) as implemented in MEGA 4.0. Positions

Table 2. Forty-six opsin sequences used in this study for phylogenetic analysis

Opsin class	Species	Taxon	GenBank accession no.	λ_{max} (nm)	Reference
LW	*Apis mellifera*	Insecta	U26026	540	Townson et al., 1998
	Camponotus abdominalis	Insecta	U32502	510	Popp et al., 1996
	Drosophila melanogaster Rh6	Insecta	Z86118	508	Salcedo et al., 1999
	Limulus polyphemus (lateral eye)	Chelicerata	L03781	520	Smith et al., 1993
	L. polyphemus (ocelli)	Chelicerata	L03782	530	Smith et al., 1993
	Manduca sexta	Insecta	L78080	520	White et al., 1983
	Megoura viciae	Insecta	AF189714	LW predicted	Gao et al., 2000
	Neogonodactylus oerstedii Rh1	Crustacea	DQ646869	489	Cronin and Marshall, 1989
	N. oerstedii Rh2	Crustacea	DQ646870	528	Cronin and Marshall, 1989
	N. oerstedii Rh3	Crustacea	DQ646871	522	Cronin and Marshall, 1989
	Papilio glaucus Rh1	Insecta	AF077189	LW predicted	Briscoe, 2000
	P. glaucus Rh2	Insecta	AF077190		Briscoe, 2000
	P. glaucus Rh3	Insecta	AF067080		Briscoe, 2000
	P. glaucus Rh4	Insecta	AF077193		Briscoe, 2000
	Papilio xuthus Rh1	Insecta	AB007423	520	Arikawa et al., 1987
	P. xuthus Rh2	Insecta	AB007424	520	Arikawa et al., 1987
	P. xuthus Rh3	Insecta	AB007425	575	Arikawa et al., 1987
	Pieris rapae	Insecta	AB177984	540	Ichikawa and Tateda, 1982
	Procambarus clarkii	Insecta	S53494	533	Zeiger and Goldsmith, 1994
	Schistocerca gregaria	Insecta	X80072	520	Gartner and Towner, 1995
	Sphodromantis sp	Insecta	X71665	515	Rossel, 1979
	Tribolium castaneum	Insecta	XM_968054	LW predicted	Jackowska et al., 2007
	Vanessa cardui	Insecta	AF385333	530	Briscoe et al., 2003
Blue-green	*Calliphora vicina* Rh1	Insecta	M58334	490	Paul et al., 1986
	D. melanogaster Rh1	Insecta	K02315	478	Feiler et al., 1988
	D. melanogaster Rh2	Insecta	M12896	420	Feiler et al., 1988
	Hemigrapsus sanguineus Rh1	Crustacea	D50583	480	Sakamoto et al., 1996
	H. sanguineus Rh2	Crustacea	D50584	480	Sakamoto et al., 1996
Blue	*A. mellifera*	Insecta	AF004168	439	Townson et al., 1998
	D. melanogaster Rh5	Insecta	U67905	437	Salcedo et al., 1999
	M. sexta	Insecta	AD001674	450	White et al., 1983
	P. glaucus Rh6	Insecta	AF077192	Blue predicted	Briscoe, 2000
	P. xuthus Rh4	Insecta	AB028217	460	Arikawa et al., 1987
	S. gregaria	Insecta	X80072	430	Gartner and Towner, 1995
	V. cardui	Insecta	AF414075	470	Briscoe et al., 2003
UV	*A. mellifera*	Insecta	AF004169	353	Townson et al., 1998
	C. abdominalis	Insecta	AF042788	360	Smith et al., 1997
	Cataglyphis bombycinus	Insecta	AF042787	360	Smith et al., 1997
	D. melanogaster Rh3	Insecta	M17718	345	Feiler et al., 1992
	D. melanogaster Rh4	Insecta	AH001040	375	Feiler et al., 1992
	M. sexta	Insecta	L78081	357	White et al., 1983
	M. viciae	Insecta	AF189715	UV predicted	Gao et al., 2000
	P. glaucus Rh5	Insecta	AF077191	UV predicted	Briscoe, 2000
	P. xuthus Rh5	Insecta	AB028218	360	Arikawa et al., 1987
	T. castaneum	Insecta	XM_965251	UV predicted	Jackowska et al., 2007
	V. cardui	Insecta	AF414074	360	Briscoe et al., 2003

For opsins for which λ_{max} is not given, predictions were made based on molecular analysis. Adapted from Porter et al. (Porter et al., 2007).

containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (Pairwise deletion option).

Histology, environmental scanning electron microscopy (ESEM)

Histological sections were prepared using a protocol by Strausfeld and Seyan (Strausfeld and Seyan, 1985) with some minor modifications. Larvae were anesthetized by chilling, decapitated, and fixed 1–2 h at RT in 4% paraformaldehyde solution (Electron Microscopy Sciences, Fort Washington, PA, USA) in Sorensen's phosphate buffer, pH 7.4 (Electron Microscopy Sciences). After two 10 min washes in Sorensen's buffer, heads were transferred into 1% osmium tetroxide (OsO_4) solution (Electron Microscopy Sciences) in distilled water for 1 h on ice followed by 1 h at 20°C. Tissue was washed twice in distilled water and stained with saturated ethyl gallate for 1 h at 0°C and 1 h at 20°C. Finally, heads were dehydrated, embedded in Ultra-Low Viscosity Embedding Medium (Polysciences, Warrington, PA, USA) and serially sectioned at 8 µm. For SEM, whole animals were dried, mounted, and viewed with an ESEM XL30 (FEI Company, Hillsboro, OR, USA) microscope.

Single fluorescence in *situ* hybridization

Larval heads were fixed in 4% phosphate-buffered (PBS, pH 7.4) paraformaldehyde with 3.6% sucrose for ~1 h at RT, washed three times for 10 min in PBS, embedded in Neg-50 freezing medium (Thermo Scientific Inc., Waltham, MA, USA) and fast-frozen in liquid nitrogen. After 30 min equilibration at –20°C, 10 um sections were made using a Tissue-Tek cryostat (Ames Company, Elkhard, IN, USA). *In situ* preparations with fluorescent staining were performed using digoxigenin-labeled RNA. Antisense and sense riboprobes were generated using the SP6/T7 Transcription Kit with DIG RNA labeling mix (Roche Applied Sciences, Indianapolis, IN, USA). The riboprobes were synthesized either solely from the 3′ untranslated region (3′ UTR), or from the 3′ end of the coding sequence and the 3′ UTR (see Fig. 2). Tissue sections were prehybridized in ~100 µl per slide of hybridization buffer (0.3 mol l^{-1} NaCl, 2.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris, 50% formamide, 10% dextran sulphate, 2 mg ml^{-1} yeast total RNA, 1× Denhardt's medium) [adapted from Sakamoto et al. (Sakamoto et al., 1996)] for ~30 min at 55–60°C in a humidified chamber, and then hybridized in ~200 µl per slide of hybridization buffer with 0.5 µg ml^{-1} of labeled riboprobes for ~16 h at 55–60°C. Parafilm coverslips were removed from slides by soaking in 5× SSC (standard saline citrate) at 55–60°C, and sections were washed three times for 20 min in 0.1× SSC at 55–60°C. Sections were then washed in 1× PBS with 0.1% Triton X-100 (PBX) for 10 min at RT, blocked with 10% normal sheep serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBX

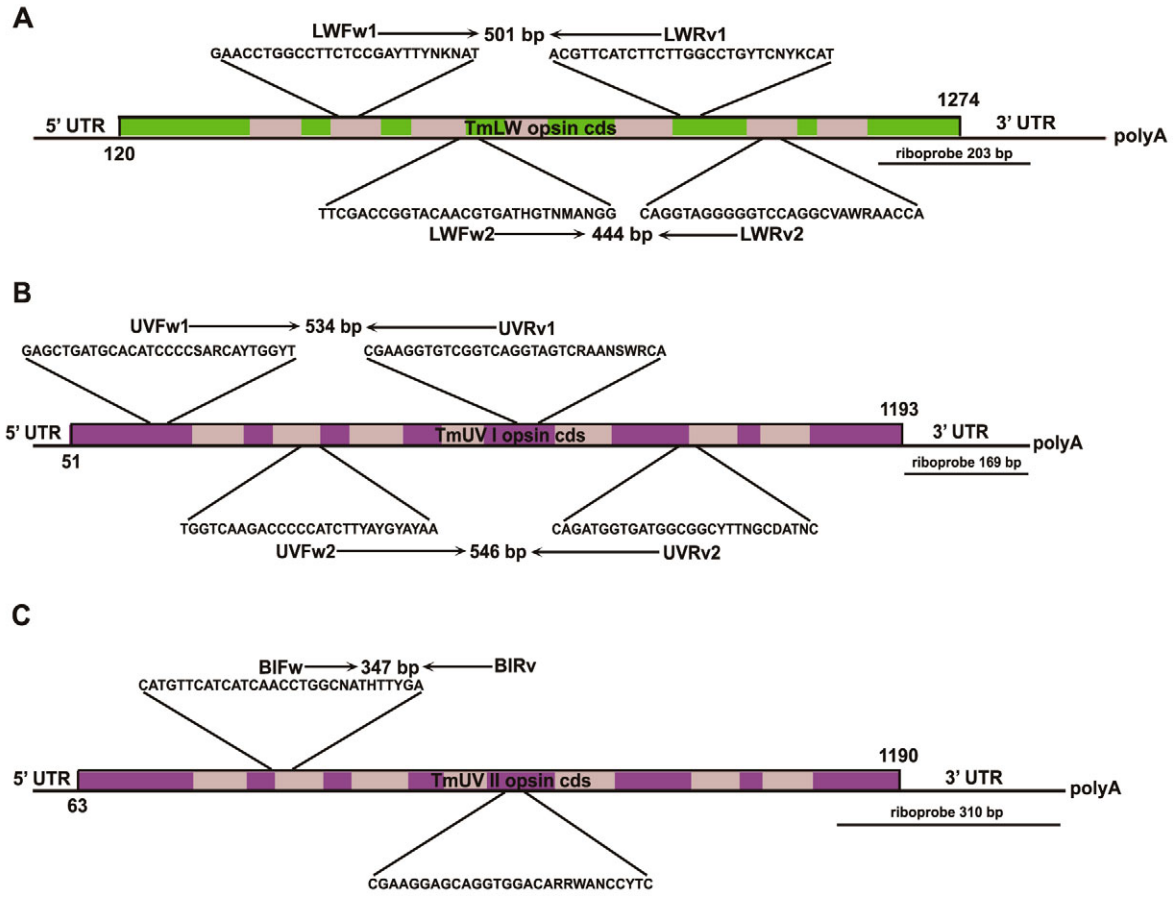

Fig. 2. Schematic illustration of the three cloned opsin cDNAs in *T. marmoratus* first instar larvae. (A) TmLW cDNA. (B) TmUV I cDNA. (C) TmUV II cDNA. Coding regions (cds) are shown in green in A, and violet in B and C. Gray regions are positions of the predicted transmembrane segments. Numbers at both ends of each cds indicate the positions of start and stop codons. Nucleotide sequences are of degenerate primers that were used to amplify the internal opsin fragments. Primer positions along with lengths of amplified fragments are also indicated, as well as the size and position of the riboprobes that were used in *in situ* hybridizations.

for 1 h at RT, and incubated at 4°C overnight with sheep anti-digoxigenin-AP (Roche Applied Sciences, Indianapolis, IN, USA) diluted 1:500 (1.5 i.u. ml^{-1}) in blocking solution. Sections were then washed three times for 30 min in PBX at RT. Alkaline phosphatase activity was developed using Fast Red Tablets (Roche Applied Sciences, Indianapolis, IN, USA) according to manufacturer's instructions. Sections were washed again for 5 min in PBX at RT, rinsed with distilled water and mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Fluorescence images were taken using an Olympus BX51 microscope (with filter set for Texas Red) equipped with an Olympus 60806 digital camera (Olympus America Inc., Center Valley, PA, USA), and adjusted for brightness and contrast with Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA).

Double chromogenic *in situ* hybridization

The procedure used for double *in situ* hybridization was identical to the one given above for single fluorescence *in situ* hybridization, except for the following:

Double *in situ* hybridizations were performed using digoxigenin- and fluorescein (Fluorescein RNA labeling mix; Roche Applied Sciences, Indianapolis, IN, USA)-labeled RNA probes simultaneously, with 1 μg ml^{-1} of labeled riboprobes (0.5 μg ml^{-1} per probe) in the hybridization buffer. After the blocking step, sections were incubated at 4°C overnight simultaneously with sheep anti-digoxigenin-AP and anti-fluorescein-POD (Roche Applied Sciences) diluted 1:500 (1.5 i.u. ml^{-1}) and 1:100 (1.5 units ml^{-1}), respectively in blocking solution. Since fluorescence was not used for double *in situ* hybridizations, the detection method was also different. Fluorescein-labeled riboprobes were identified first by detecting horseradish peroxidase activity using a DAB Substrate Kit (Vector Laboratories, Burlingame, CA, USA), followed with a 5 min wash in PBX at RT. Alkaline phosphatase activity was developed second, using 1-Step NBT/BCIP plus Suppressor (Thermo Scientific Inc., Waltham, MA, USA). Images were taken with the same equipment used for fluorescence *in situs*, but without fluorescence filters.

RESULTS

Opsin sequences and phylogenetic analysis

Using degenerate PCR primers corresponding to highly conserved amino acid sequences from LW-, B-, and UV-sensitive invertebrate opsins (Table 1), we performed RT-PCR from RNA isolated from first instar *T. marmoratus* larvae. Primers LWFw I + LWRv I and LWFw II + LWRv II amplified 501 bp and 444 bp fragments, respectively, of an identical long-wavelength opsin, which we call TmLW (Fig. 2A; GenBank accession no.: EU921225). UVFw I + UVRv I and UVFw II + UVRv II amplified 534 bp and 546 bp fragments, respectively, of a single UV-sensitive opsin encoding gene, called TmUV I (Fig. 2B, EU921226). BlFw + BlRv, made against conserved sequences in insect B-sensitive opsins, amplified a 347 bp fragment of a third opsin under low stringency PCR conditions, which by BLAST homology, corresponds to a second UV-wavelength-sensitive opsin, TmUV II (Fig. 2C, EU921227). Corresponding fragments of TmLW and TmUV I were also amplified under these conditions.

Full-length cDNAs for these three opsins were acquired using 5′ and 3′ RACE PCR, and their full-length deduced amino acid sequences were assembled (Fig. 3). TmLW cDNA encodes a 384 amino acid opsin, TmUV I cDNA encodes a 380 aa opsin, and TmUV II cDNA encodes a 375 aa opsin. As shown by the alignment in Fig. 3, TmLW, TmUV I and TmUV II are 31.70% identical (red residues/asterisks), and the two UV opsins are 75.65% identical. Consistent with other arthropod opsins, and from hydropathicity analysis, all three *T. marmoratus* opsin cDNAs are predicted to code for seven transmembrane segments. In addition, each opsin contains conserved amino acid residues commonly associated with insect opsins, including: (1) a Lys in the seventh transmembrane segment (Lys333) that is the site of attachment for the Shiff's base linkage to the retinal chromophore (black arrow) (Gartner and Towner, 1995; Townson et al., 1998; Wang et al., 1980), (2) two conserved Cys residues predicted to form a stabilizing disulfide linkage (Gartner and Towner, 1995; Townson et al., 1998) at positions 134 and 212, (3) an Asn residue at the N-terminus (TmUV I and TmUV II, Asn9; TmLW, Asn5) that represents a potential glycosylation site (Gartner and Towner, 1995; Townson et al., 1998), and (4) two helix–loop conserved sequences (DRY, QAKKMNV) potential G-protein binding sites (Gartner and Towner, 1995; Townson et al., 1998).

To confirm Blast predictions of the spectral class of the cloned opsin cDNAs, and to examine their relationship with other known arthropod opsins, a phylogenetic tree was generated based on these and 46 other known opsin amino-acid sequences. As shown in Fig. 4, opsins that share similar spectral characteristics are more related to each other than opsins from different spectral classes. In our analysis, both TmUV I and TmUV II are nested within the UV branch of the tree, most closely related to the UV opsin of another beetle, *Tribolium castaneum*. Likewise, the long-wavelength opsin (TmLW) is grouped together with other LW opsins, again with *Tribolium castaneum* LW opsin as its closest neighbor. We also noted that, as in *Tribolium castaneum* (Jackowska et al., 2007), we were unable to amplify a B-sensitive opsin from *T. marmoratus* larvae, even under low-stringency PCR conditions, suggesting that the B family of insect opsin has commonly been lost from the coleopteran phylogenetic branch (see Discussion).

Spatial expression of TmLW, TmUV I and TmUV II mRNAs

We next located expression of TmLW, TmUV I and TmUV II mRNAs in the first instar larvae visual system using *in situ* hybridization. Sense probes did not produce any apparent staining (data not shown). As summarized in Table 3, the mRNAs corresponding to the three opsin genes isolated by PCR were found in all known retinas in the head of the first instar larvae. Specific expression patterns are described below.

Opsin mRNA expression in the distal and proximal retinas of eyes 1 and 2

As described earlier and shown in Fig. 5A, E1 and E2 are anatomically similar, having a characteristic tubular design with narrow distal and proximal retinas, each made of a dorsal and ventral rhabdom. E1 and E2 also exhibit very similar opsin expression patterns (see Figs 5 and 6), so in the text below, we will refer to the distal and proximal retinas from either E1 or E2 without distinction. As shown by fluorescent *in situ* hybridization, TmLW mRNA is expressed highly and specifically in the distal retinular cells (DRC) of the *T. marmoratus* principal eyes (Fig. 5B), whereas the TmUV II mRNA is restricted to the proximal retinular cells (PRC; Fig. 5C). This expression pattern was confirmed with non-fluorescent chromogenic double in *situ* hybridizations for TmLW and TmUV II mRNAs (Fig. 5D–F). Higher magnification images (Fig. 5E,F) illustrate that the nuclei of the retinular cell bodies appear at the periphery as spherical regions of weaker staining for both TmLW (Fig. 5E) and Tm UVII (Fig. 5F) mRNAs. As expected, the rhabdomeric regions of each retina (DRh and PRh) do not stain with any of the opsin probes, leaving translucent regions in the center.

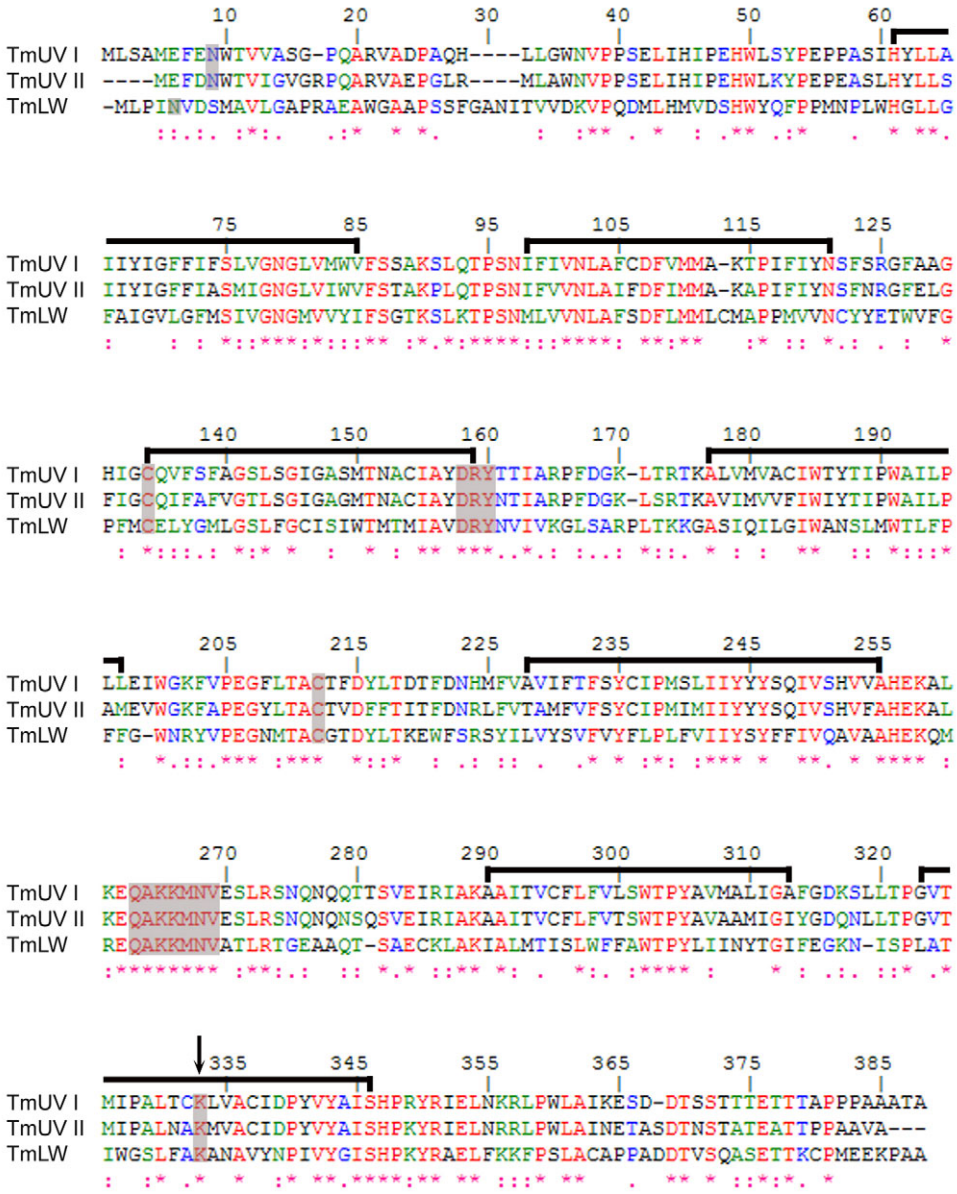

Fig. 3. Amino-acid alignment in ClustalW of the three opsins cloned as cDNA from the first instar larval heads of *T. marmoratus*. Brackets indicate transmembrane regions predicted from hydropathicity analysis and the alignment with other opsins. Identical residues are in red (*), strongly similar ones are in green (:), weakly similar ones are in blue (.). The arrow indicates the site of the chromophore Schiff-base linkage. Conserved amino-acid residues are in gray (for more details see text). The sequences have been deposited in GenBank under accession numbers EU921225 (TmLW), EU921226 (TmUV I), EU921227 (TmUV II).

We also stained E1 and E2 for TmUV I mRNA, and found that, like TmUV II, it is expressed in the proximal retinas of the principal eyes. However, TmUV I mRNA expression is restricted to the dorsal stack of photoreceptors, whereas TmUV II mRNA is present in both the dorsal and ventral stacks (Fig. 6). This is apparent in sagittal (Fig. 6A) as well as in cross sections (Fig. 6B,D) of E1 and E2. Although both UV opsins are present in the dorsal proximal retina, TmUV I mRNA expression appears weaker than TmUV II mRNA expression (compare Fig. 5C with Fig. 6A, and Fig. 6B with D). This is possibly because the probe used for detecting TmUV I mRNA is approximately half the length of the probe for TmUV II mRNA or because TmUV I mRNA is present in fewer cells or at lower levels than TmUV II mRNA. Future protein localization studies should help to clarify this issue.

Opsin mRNA expression in the E1 medial retina

In addition to the distal and proximal retinas in both E1 and E2, E1 also has a third, narrow retina that extends along the median border of the eye tube (Fig. 1D). This was originally described as the lateral retina, but since the retina itself is situated along the medial side of the eye, we now refer to this retina as the medial retina. As shown in Fig. 7, all three opsin mRNAs are expressed in the E1 medial retina. Fig. 7B–E illustrate cross sections at the approximate location of E1, which is indicated in Fig. 7A. As shown with fluorescent staining in Fig. 7B, TmLW mRNA is expressed in the most dorsal and ventral portions of the medial retina. TmUV II mRNA, however, shows a somewhat different staining pattern, being restricted to central parts of the medial retina (Fig. 7C). This is more obvious in Fig. 7D, which shows that TmUV II mRNA is limited to the central portion of the medial retina with TmLW mRNA surrounding it from the dorsolateral and ventrolateral sides. TmUV I mRNA is also expressed in the medial retina and similarly to the proximal retina, its expression level appears lower than that of TmUV II mRNA (Fig. 7E). However, in general, their expression patterns overlap. Another interesting finding from these studies is that the medial retina is contiguous with the proximal retina (Fig. 1D), which is most clearly visible on sagittal sections (Fig. 7F). This shows that a band of TmUV II-expressing cells from the medial retina is present on

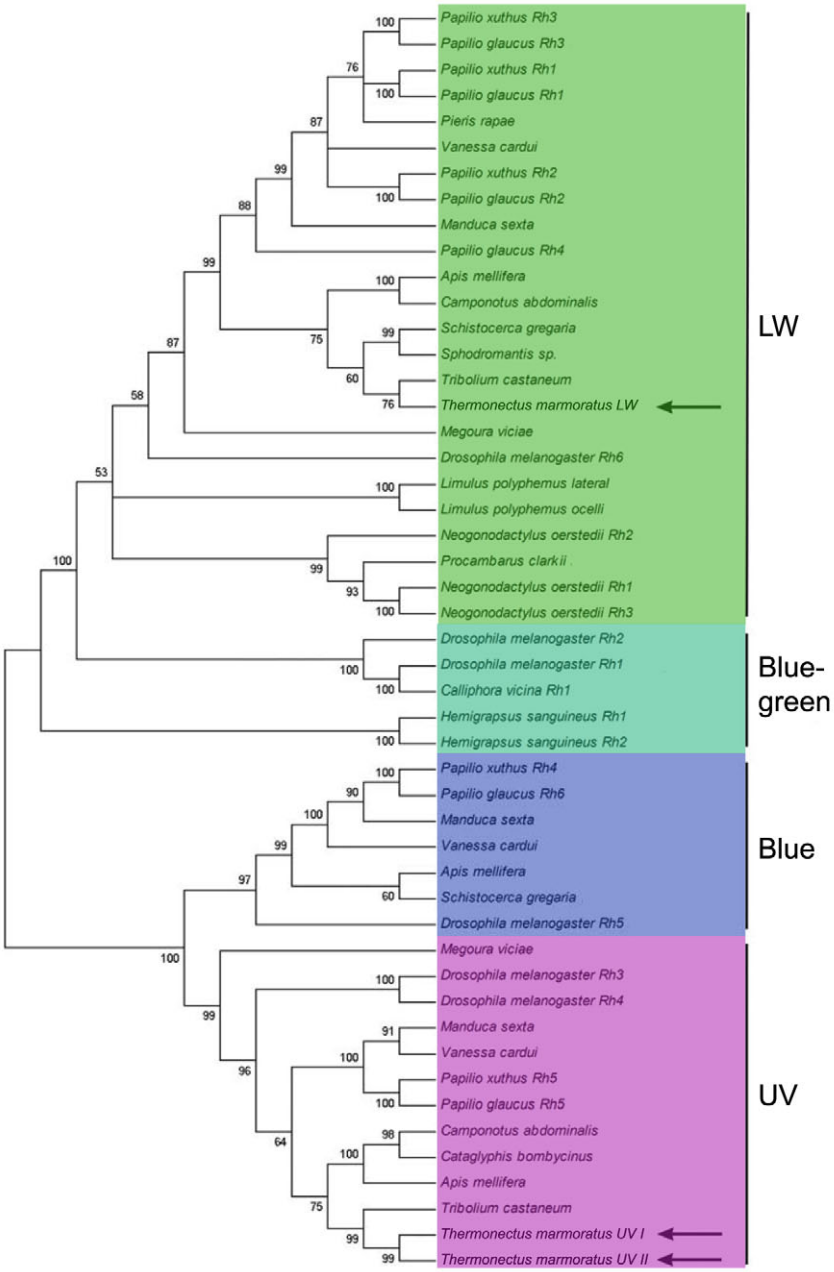

Fig. 4. Phylogenetic reconstruction of *T. marmoratus* larval opsins and 46 other known arthropod opsins. For accession numbers and spectral absorbencies, see Table 2. The numbers appearing above nodes represent bootstrap percentages of 1000 replications. Spectral clades are delineated. Arrows indicate positions of the *T. marmoratus* larval opsins.

the surface of the LW-expressing distal retina. It is also apparent that the peripheral TmLW-expressing portion of the medial retina arises from the distal retina (Fig. 7F, black arrow). This has also been confirmed with fluorescence *in situ* hybridization (data not shown).

Together, these studies reveal that TmUV I and TmUV II mRNAs are coexpressed in the proximal retinas of E1 and E2, as well as in the central region of the E1 medial retina. TmLW mRNA, by contrast, is highly expressed in the dorsal retinas of E1 and E2, and in the periphery of the E1 medial retina. Thus, LW and UV-sensitive opsins are distinctly separated into different regions of each eye.

Opsin mRNA expression in eyes 3–6

All four secondary eyes are characterized by two retinas, distal and proximal, which in terms of their general organization, are comparable with the distal and proximal retinas of the principal eyes. The general organization of these eyes is illustrated in an ethyl gallate-stained section in Fig. 1A (see E5). As shown in Fig. 8, all four secondary eyes exhibit a segregation of opsins similar to the two principal eyes: TmLW mRNA is expressed in the distal retinas and TmUV I mRNA is expressed in the proximal retinas. By contrast, TmUV II mRNA is not detected anywhere within eyes 3–6 (data not shown).

TmLW mRNA is found in the retinular cells of the distal retina that are situated on both sides of the distal rhabdom lying beneath the lens (Fig. 8A,C,E,G). Fast Red staining also shows strong non-specific staining of the lens that is also present in our control preparations (data not shown). As shown in Fig. 8B,D,F and H, TmUV I mRNA is expressed in the proximal retinas of secondary eyes, generally in the back of the eyes where the proximal retinular cells are situated. It is absent from the rhabdomeric region (situated underneath the lens), as well as from the eye's periphery where the

Table 3. Spatial expression of the three cloned opsins in the *T. marmoratus* larval visual system

Eye	Retina	TmLW	TmUV I	TmUV II
1	Distal	+	–	–
	Proximal	–	+	+
	Medial	+	+	+
2	Distal	+	–	–
	Proximal	–	+	+
3	Distal	+	–	–
	Proximal	–	+	–
4	Distal	+	–	–
	Proximal	–	+	–
5	Distal	+	-	–
	Proximal	-	+	–
6	Distal	+	–	–
	Proximal	–	+	–
Eye patch		+	+	–

distal TmLW-expressing cells are located. In E3 (Fig. 8B) the TmUV I mRNA staining pattern separates into two relatively narrow bands (see Fig. 8B, inset).

Opsin mRNA expression in the eye patch

In addition to E1–6 on the dorsal surface of each side of the head, a non-lens-containing eye patch is present. Fig. 9 illustrates that TmLW and TmUV I are expressed in the eye patch of *T. marmoratus* but, as in the secondary eyes, TmUV II mRNA is not expressed in this retina (data not shown). Consistent with previous transmission electron microscopy studies showing that the rhabdomeric region of the eye patch is characterized by rhabdoms that wrap around cytoplasmic regions of the retinular cells (Mandapaka et al., 2006), the rhabdomeric region of the eye patch shows some opsin mRNA staining (Fig. 9). One significant difference between the eye patch and the secondary eyes, however, is that TmUV I-expressing cells are interspersed at more or less regular intervals with TmLW-expressing cells (Fig. 9C). This is also visible in Fig. 9B, where the unstained regions in between the TmUV I-positive cells presumably represent cells that express TmLW mRNA. The alternating stained and unstained regions/cells can also be seen in Fig. 9A, although to a lesser extent than in Fig. 9B. Thus, in contrast to all other eyes of these larvae, different opsin mRNAs are expressed in neighboring cells in the eye patch, rather than being physically separated into different retinal tiers.

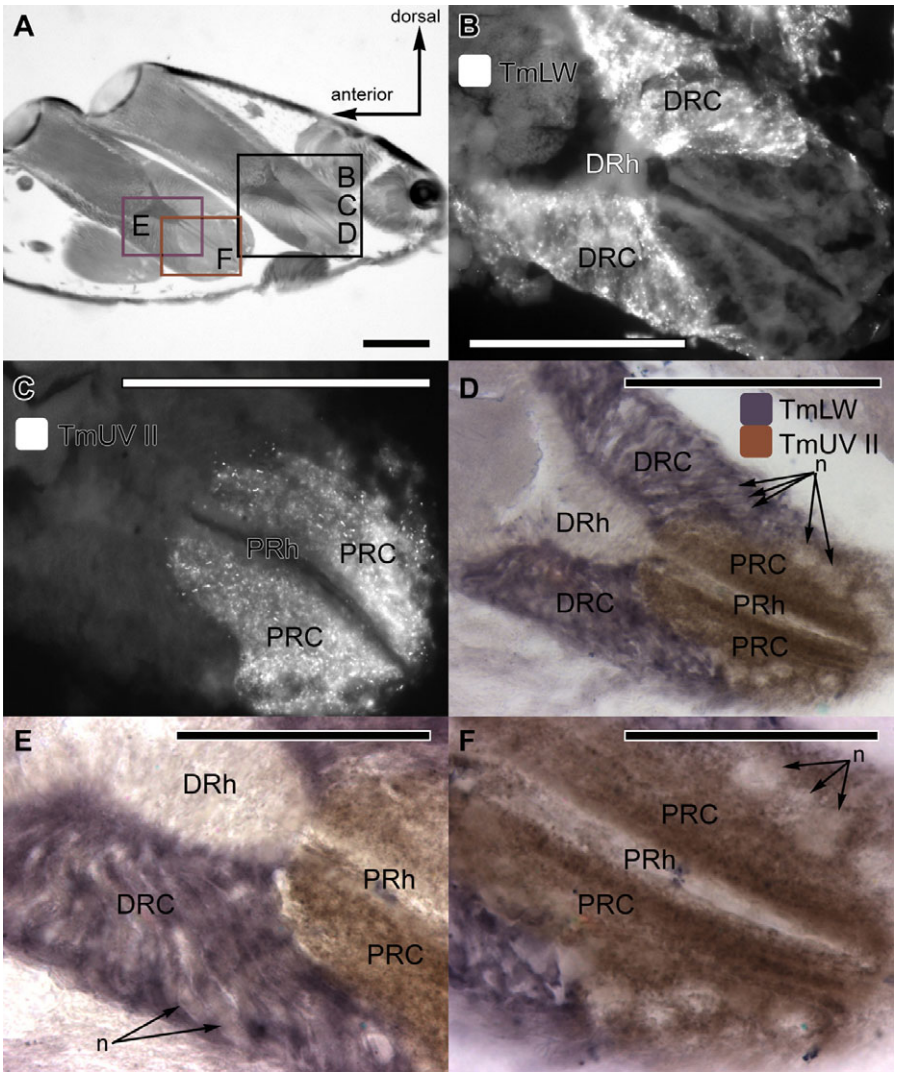

Fig. 5. Distribution of opsin mRNAs in the principal eyes as examined by *in situ* hybridization; all images are of sagittal sections. (A) An overview histological section showing the positions of the *in situ* images. (B) E1, fluorescent staining of TmLW mRNA illustrating its expression in the distal retina. (C) E1, fluorescent staining of TmUV II mRNA illustrating its expression in the proximal retina. (D) E1, double chromogenic staining of TmLW (purple) TmUV II (brown) mRNAs. (E) E2, double chromogenic staining of TmLW (purple) and TmUV II (brown) mRNAs illustrating primarily the distal retina at larger magnification. (F) E2, double staining for TmLW (purple) and TmUV II (brown) mRNAs illustrating primarily the proximal retina at larger magnification. DRC, retinular cells of the distal retina; PRC, retinular cells of the proximal retina; DRh, distal rhabdom; PRh, proximal rhabdom n, nuclei. Scale bars: A, 100 μm; B-D, 100 μm; E,F, 50 μm.

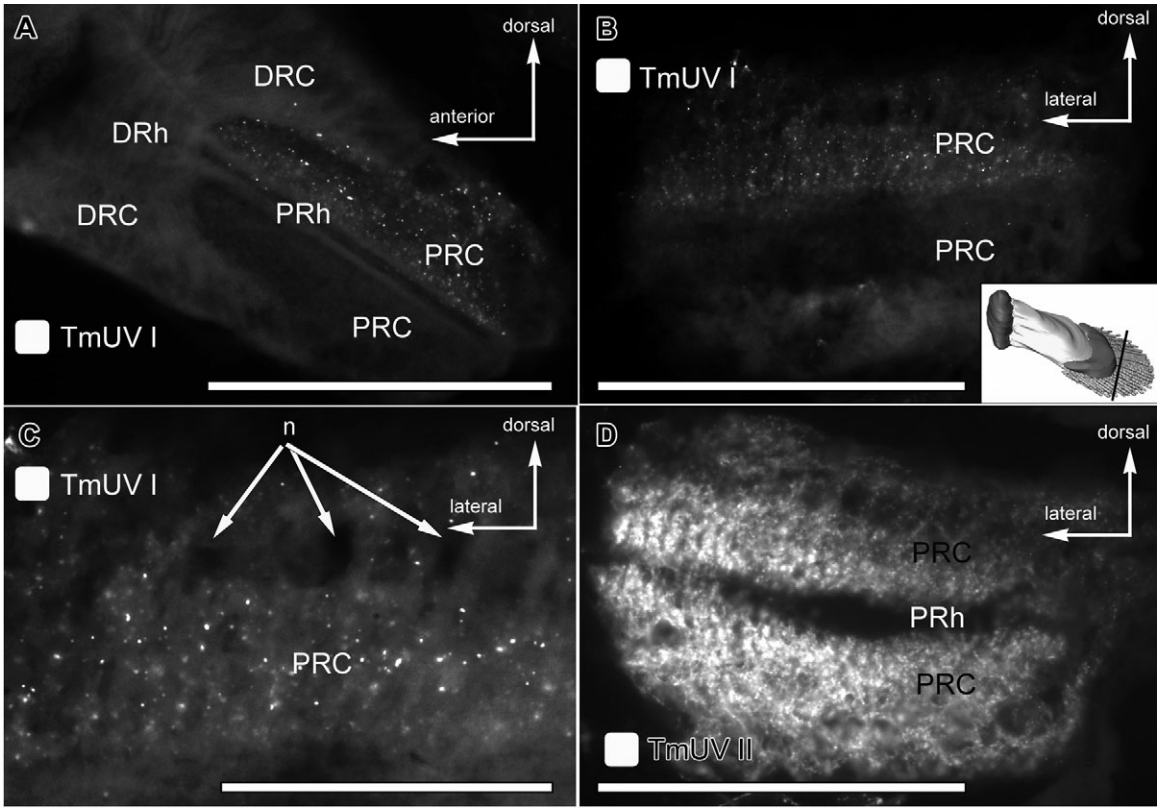

Fig. 6. Distribution of TmUV I and TmUV II mRNAs in the proximal retina of the principal eyes as examined by *in situ* hybridization. A, sagittal section; B-D, cross sections through the proximal retina (see inset in B). (A) E1, fluorescent staining of TmUV I mRNA illustrating its expression in the dorsal stack of the proximal retina. (B) E2, fluorescent staining of TmUV I mRNA in a cross section through the proximal retina as indicated in the inset. Note that the expression is only present in the dorsal stack. (C) E2, fluorescent staining of TmUV I mRNA in a cross section through the dorsal stack of the proximal retina at a higher magnification. (D) E2, fluorescent staining of TmUV II mRNA in a cross section through the proximal retina. Note the stronger hybridization signal of TmUV II mRNA compared with that of TmUV I mRNA in B and C. DRC, retinular cells of the distal retina; PRC, retinular cells of the proximal retina; DRh, distal rhabdom; PRh, proximal rhabdom; n, nuclei. Scale bars: A,B,D, 100 μm; C, 50 μm.

DISCUSSION

Distribution of opsin mRNAs in the larval eyes of *Thermonectus marmoratus*

T. marmoratus larvae are characterized by six stemmata and an eye patch. We cloned full-length coding sequences for three distinct opsins using mRNA from these larval heads: one long-wavelength (TmLW) and two UV-wavelength (TmUV I and TmUV II). Together, these three opsins account for all regions of the larval head for which photoreceptive cells have previously been identified based on histology (Mandapaka et al., 2006), as illustrated schematically in Fig. 10. Interestingly, in all six stemmata, LW *versus* UV opsin mRNA expression is clearly separated, closely following morphological distinctions between different retinas. Specifically, the distal retina in all eyes exclusively expresses the TmLW mRNA, whereas the proximal retina of all eyes expresses the UV TmUV I mRNA. Moreover, we find evidence for the presence of a second UV opsin mRNA (TmUV II) that is uniquely expressed in the scanning principal eyes, E1 and E2, which are used during prey approach (Buschbeck et al., 2007). To our knowledge, this is the first report of two UV-sensitive opsins expressed in the same stemma in a holometabolous insect. Furthermore, this distribution is in contrast to that in other arthropods such as jumping spiders and many crustaceans and insects (Kelber, 2006) in which short wavelength-sensitive receptors are distal to long wavelength-sensitive receptors. In fact, to our knowledge, this is the first example of a tiered system with the UV-sensitive cells in the proximal portion. Finally, we find that in the eye spot the patterns of expression of LW and UV I opsins are not spatially separate in clearly distinct regions of the retina, but rather, they are regularly spaced with neighboring cells frequently expressing different mRNAs.

Comparison of *T. marmoratus* opsin subclasses with those of other invertebrates

Although insect photoreceptors can be grouped into four distinct spectral classes with peak sensitivities in ultra-violet (UV), blue (B), blue-green (middle-wavelength, MW) and green (LW), most insects possess only three distinct classes: UV, B and LW (Briscoe and Chittka, 2001). However, we failed to identify a B-sensitive opsin in the *T. marmoratus* larval visual system. There are several possible explanations for this lack of a B opsin. First, it is possible that the loss of the B opsin occurred during the evolution of a lineage to which *T. marmoratus* belongs. This is supported by the fact that the adult form of the red flour beetle, *Tribolium castaneum*, also appears to lack the B opsin but expresses one for UV and one for LW (Jackowska et al., 2007). Consistent with this explanation, ERG spectral sensitivity measurements in the adult eye of another beetle, *Tenebrio molitor*, peak only in the UV and green ranges (Yinon, 1970). Therefore, it is possible that the B opsin was lost prior to the evolution of these three species of Coleoptera.

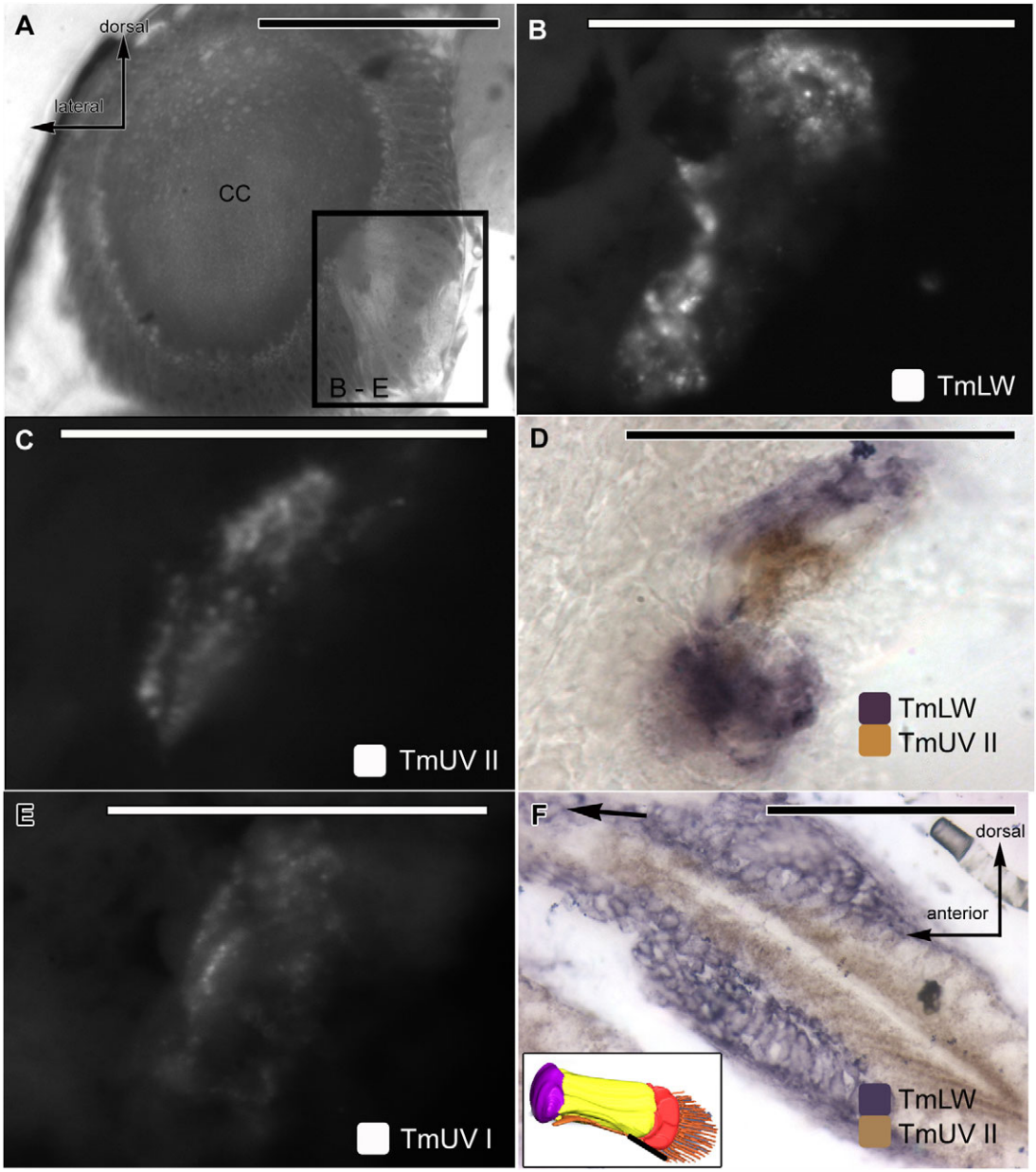

Fig. 7. Distribution of opsin mRNAs in the medial retina of E1 as examined by *in situ* hybridization. A-E are cross sections through E1; F is a sagittal section (see inset). (A) An overview histological cross section through E1 showing the position of the medial retina as seen in B–E. (B) Fluorescent staining of TmLW mRNA showing its expression in the dorsal and ventral parts of the medial retina. Note the absence of signal in the central part of the medial retina. (C) Fluorescent staining of TmUV II mRNA showing its expression in the more central parts of the medial retina. (D) Double chromogenic staining of TmLW (purple) and TmUV II (brown) mRNAs. Note how the purple staining (TmLW mRNA) surrounds the brown staining (TmUV II mRNA) from the dorsolateral and ventrolateral sides. (E) Fluorescent staining of TmUV I mRNA. Note that the hybridization signal covers a similar region as the expression pattern of TmUV II mRNA (see C). (F) Double chromogenic staining for TmLW (purple) and TmUV II (brown) mRNAs in a sagittal section, as indicated in the inset. Note the band of UV-expressing tissue (brown) extending along the margin of the distal retina. It appears that the UV-sensitive part of the medial retina is anatomically connected with the UV-sensitive proximal retina. Furthermore, the LW-sensitive part of the medial retina may be an extension of the distal retina (black arrow). CC, crystalline cone like structure. Scale bars: A,F, 100 μm; B–E, 50 μm.

An alternative explanation for only identifying two classes of opsins is that only the larval stemmata, but not adult *T. marmoratus* compound eyes, lack the B-sensitive opsin. It is not unusual that larval stemmata express fewer receptor classes than adult compound eyes. For instance, photoreceptors in the Bolwig's organ in *Drosophila* larvae only express two of the six opsin-encoding genes present in the *Drosophila* genome: B-sensitive Rh5 and LW-sensitive Rh6 (Malpel et al., 2002), whereas the adult compound eye expresses five of the six opsins which fall into all three clades (UV, B and LW). Indeed, it has been recently proposed that UV-expressing cells in the Bolwig's organ were lost during the evolution of the *Drosophila* larval eyes (Friedrich, 2008). Similarly, in *T. marmoratus* stemmata, B-opsin-expressing photoreceptors might have been lost during the evolution of the larval eyes, or B-sensitive cells switched to UV expression. However, it is worth noting that some holometabolous insects,

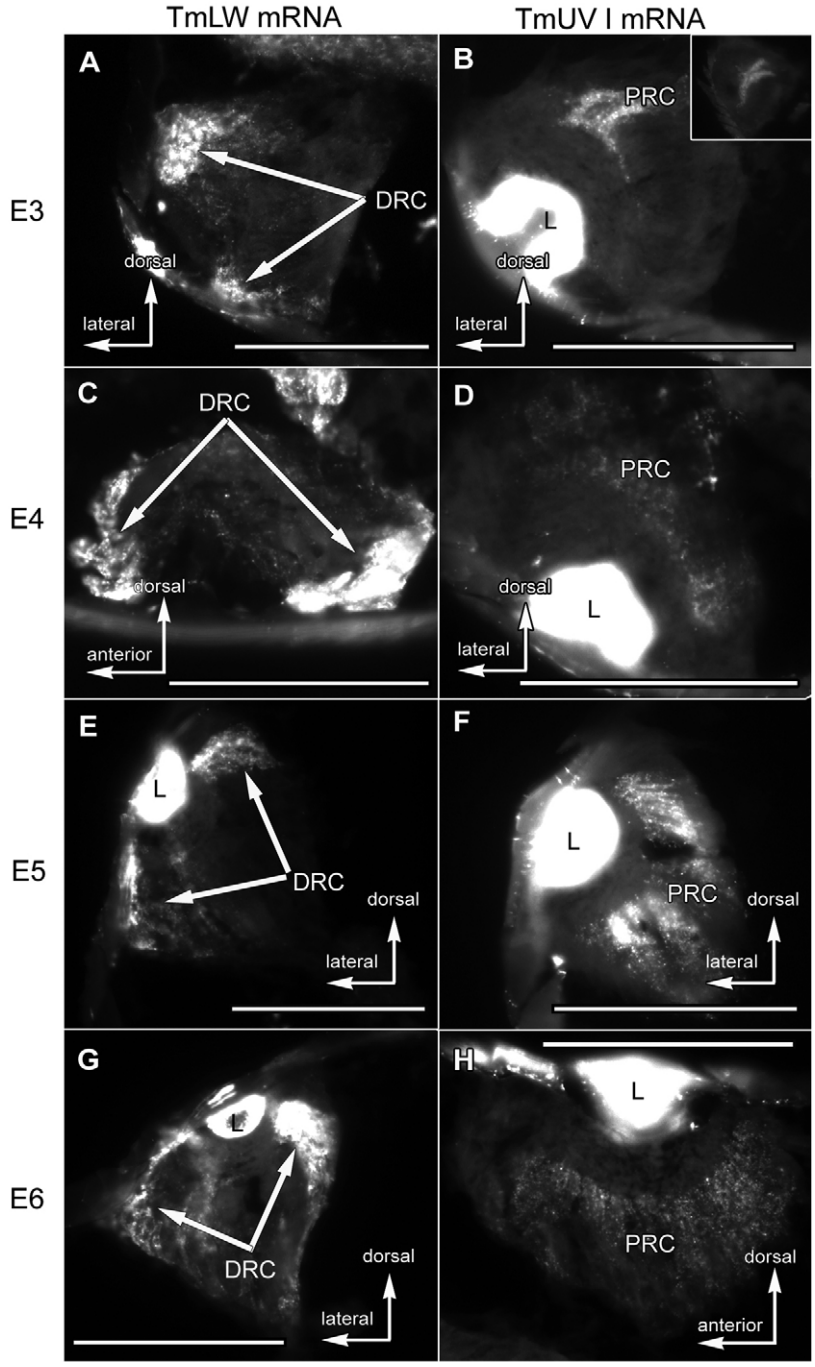

Fig. 8. Distribution of TmLW and TmUV I mRNAs in the secondary eyes as examined by fluorescence *in situ* hybridization. Staining of TmLW mRNA is illustrated in the left panels and TmUV I mRNA in the right panels. Each row is a different eye, as indicated on the left side of the figure. The terms 'sagittal' and 'cross' refer to the sectional plane of the head. All eyes are shown in cross sections, except for E4 in C (sagittal) and E6 in H, (sagittal). E3–5 have comparable arrangements of their retinas with distal retinular cells (DRC) projecting to the periphery on both sides of the rhabdoms, and proximal retinular cells (PRC) extending to the back of the eyes (see Fig. 1, E5). (A, C, E and G) TmLW mRNA is present in the lateral eye region, which is associated with the distal retina. (B, D, F and H) TmUV II mRNA is present in the back of the eyes where the proximal retinular cells are located. In E3 the signal is localized in a relatively small area covering two narrow bands. This is most apparent in the inset in B, which is a section through E3 without the lens. L, lenses. Scale bars: A–H, 100 μm.

such as *Papilio* and several other lepidopteran species, express all three spectral clades in their larval stemmata (Gilbert, 1994). We do not currently know which opsins are expressed in the compound eye of adult *T. marmoratus*, but additional physiological and molecular studies involving both larval and adult specimens of a variety of holometabolous insects are likely to shed light on some of these questions.

Presence of two UV-sensitive opsins in *T. marmoratus*

To our knowledge, this is the first report of the expression of two related but similar opsins within the UV subclass being expressed in the same stemma in a holometabolous insect. Our phylogenetic analysis suggests that the two UV opsins are more closely related to each other than to any of the other UV opsins (including that of *Tribolium castaneum*), raising the possibility of a relatively recent gene duplication. Interestingly, besides being expressed in the medial retina of E1, TmUV I is present in all *T. marmoratus* proximal retinas and the eye patch, whereas TmUV II is only expressed in the proximal retinas of the primary E1 and E2 eyes. In addition, within the E1 and E2 proximal retinas, the distributions of TmUV I and TmUV II are not identical (Fig. 6). TmUV I mRNA appears to be weakly expressed only in the dorsal stack of the proximal retinas whereas TmUV II mRNA is strongly expressed in both stacks of the proximal retinas. Since the retinular cells in this region are densely packed, it is currently not possible to ascertain whether some cells of the dorsal stack of the proximal retinas of the principal eyes

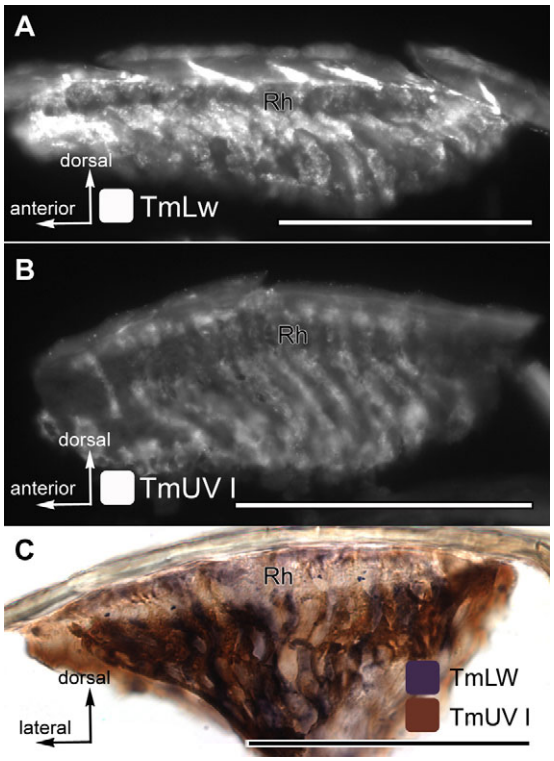

Fig. 9. Distribution of TmLW and TmUV I mRNAs in the eye patch retina as examined by *in situ* hybridization. (A) Sagittal head section illustrating the fluorescent hybridization signal for TmLW mRNA in the eye patch retina. (B) Sagittal head section illustrating the fluorescent hybridization signal for TmUV I mRNA in the eye patch retina. (C) Frontal (cross) head section illustrating chromogenic double staining for TmLW (purple) and TmUV I (brown) mRNA in the eye patch retina. Rh, rhabdom. Scale bars: A,B, 100 μm; C, 50 μm.

violate the 'one receptor' rule in sensory neurons (Mazzoni et al., 2004; Mombaerts, 2004). Coexpression of LW and UV opsins has been revealed for another beetle, the flower beetle, *Tribolium castaneum*, although in compound eyes (Jackowska et al., 2007). Also, Mazzoni et al. (Mazzoni et al., 2008) showed that a small subset of R7 photoreceptors in the *Drosophila* compound eye coexpresses both UV opsins, Rh3 and Rh4. Opsin coexpression has also been reported in several butterfly species (Arikawa et al., 2003; Awata et al., 2009; Sison-Mangus et al., 2006). However, the differences in expression patterns we observe for TmUV I and TmUV II suggest the possibility that different sized or shaped photoreceptor subpopulations are present in these retinas, which are specialized for different functions. Unfortunately, our *in situ* preparations do not provide sufficient information to identify the individual cell populations and ascertain their opsin expression. It is also important to note that rhabdoms in this area are characterized by multiple narrow cells (Mandapaka et al., 2006), which could account for this seemingly overlapping expression.

The other retina that expresses both UV mRNAs is in the medial retina of E1 (Fig. 7). Here too, we cannot rule out the possibility that these two UV opsins are coexpressed in the same cells. However, the similarities we find between the proximal and medial retina may relate to their tight anatomical connection. In fact, based on opsin expression results, it appears that the central, UV-sensitive part of the medial retina represents an extension of the proximal retina of E1. This is consistent with ultrastructural data, in which

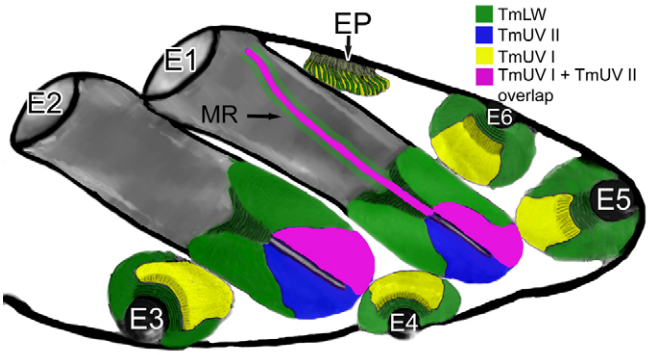

Fig. 10. Schematic illustration of the spectral sensitivity of the entire *T. marmoratus* first instar larva visual system based on opsin expression patterns. Rhabdoms are shown as stripped regions. EP, eye patch; MR, medial retina.

rhabdoms of the central part of the medial retina and of the proximal retinas of E1 and E2, appear to be very similar (Mandapaka et al., 2006).

Functional implications

Although the overall functional organization of *T. marmoratus* larval eyes is still largely unclear, certain functions are indicated by the distribution of the opsins. First, as mentioned in the Introduction, one reason for animals having multiple, sequentially arranged retinas (or multiple layers of a single retina) is to compensate for lens chromatic aberration (Blest et al., 1981; Land, 1969). However, for this mechanism to work, photoreceptor cells closer to the lens must be sensitive to more strongly refracted, shorter wavelengths while deeper photoreceptor layers would be excited by less strongly refracted, longer wavelengths. This is not the case for *T. marmoratus* in which all the layers of the distal retinas of the principal eyes express the same LW opsin. The implication of this finding is that the sharpest image that is sampled by the photoreceptors of this retina is influenced primarily by the distance of a viewed object, with minimal contribution of chromatic aberration. Moreover, our data suggest that the sequence of the two retinas in *T. marmoratus* larval eyes follows a pattern contrary to what would be expected from lens chromatic aberration, with long wavelength receptors being closer to the lens than short wavelength receptors. Therefore it is unlikely that this arrangement compensates for lens chromatic aberration. Instead, our data suggest that any lens with a single focal plane would be unable to sharply focus any object at a given distance onto both retinas.

A second functional implication of the opsin distribution is that the distal retina may filter out UV light that would otherwise excite the proximal retina. Since LW opsins typically have a β peak (Stavenga et al., 1993), it is unclear how much of the UV light remains to excite the proximate retina. Typically, invertebrate rhabdomeric photoreceptors absorb ~1% of the incident light per 1 μm of their length (Land and Nilsson, 2002). Although it is unclear how well this value fits for the highly unusual orthogonally oriented retinular cells found in the *T. marmoratus* stemmata, it is expected that at least half of the UV light should pass the ~50 μm thick distal retina. It also remains unclear whether sufficient light is absorbed by individual layers of the distal retina for photoreceptors to function independently. Several photoreceptors may have to be pooled in order to absorb sufficient light to function. However, it is noteworthy that these larvae live in shallow water in the

southwestern United States, where light levels are frequently very high.

Many of the functional aspects rest upon just which wavelengths are absorbed by each of the three opsins, which, at this point, remains unclear. Nevertheless the unusual sequence of LW-sensitive photoreceptors followed by UV-sensitive photoreceptors further supports the idea that the eyes of these highly efficient, visually guided predators function in a novel, but as yet undescribed, way.

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REFERENCES

Arikawa, K., Inokuma, K. and Eguchi, E. (1987). Pentachromatic visual system in a butterfly. *Naturwissenschaften* **74**, 297-298.

Arikawa, K., Mizuno, S., Kinoshita, M. and Stavenga, D. G. (2003). Coexpression of two visual pigments in a photoreceptor causes an abnormally broad spectral sensitivity in the eye of the butterfly *Papilio xuthus*. *J. Neurosci.* **23**, 4527-4532.

Awata, H., Wakakuwa, M. and Arikawa, K. (2009). Evolution of color vision in pierid butterflies: blue opsin duplication, ommatidial heterogeneity and eye regionalization in *Colias erate*. *J. Comp. Physiol. A* **195**, 401-408.

Blest, A. D., Hardie, R. C., McIntyre, P. and Williams, D. S. (1981). The spectral sensitivities of identified receptors and the function of retinal tiering in the principal eyes of a jumping spider. *J. Comp. Physiol. A* **145**, 227-239.

Briscoe, A. D. (2000). Six opsins from the butterfly *Papilio glaucus*: molecular phylogenetic evidence for paralogous origins of red-sensitive visual pigments in insects. *J. Mol. Evol.* **51**, 110-121.

Briscoe, A. D. and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol.* **46**, 471-510.

Briscoe, A. D., Bernard, G. D., Szeto, A. S., Nagy, L. M. and White, R. H. (2003). Not all butterfly eyes are created equal: rhodopsin absorption spectra, molecular identification, and localization of ultraviolet-, blue-, and green-sensitive rhodopsin-encoding mRNAs in the retina of *Vanessa cardui*. *J. Comp. Neurol.* **458**, 334-349.

Buschbeck, E. and Friedrich, M. (2008). Evolution of insect eyes: tales of ancient heritage, deconstruction, reconstruction, remodeling, and recycling. *Evolution: Education and Outreach* **1**, 448-462.

Buschbeck, E., Sbita, S. and Morgan, R. (2007). Scanning behavior by larvae of the predacious diving beetle, *Thermonectus marmoratus* (Coleoptera: Dytiscidae) enlarges visual field prior to prey capture. *J. Comp. Physiol. A* **193**, 973-982.

Cronin, T. W. and Marshall, N. J. (1989). Multiple spectral classes of photoreceptors in the retinas of gonodactyloid stomatopod crustaceans. *J. Comp. Physiol. A* **166**, 261-275.

Feiler, R., Harris, W. A., Kirschfeld, K., Wehrhahn, C. and Zuker, C. S. (1988). Targeted misexpression of a *Drosophila* opsin gene leads to altered visual function. *Nature* **333**, 737-741.

Feiler, R., Bjornson, R., Kirschfeld, K., Mismer, D., Rubin, G. M., Smith, D. P., Socolich, M. and Zuker, C. S. (1992). Ectopic expression of ultraviolet-rhodopsins in the blue photoreceptor cells of Drosophila: visual physiology and photochemistry of transgenic animals. *J. Neurosci.* **12**, 3862-3868.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

Friedrich, M. (2008). Opsins and cell fate in the Drosophila Bolwig organ: tricky lessons in homology inference. *BioEssays* **30**, 980-993.

Gao, N., Foster, R. G. and Hardie, J. (2000). Two opsin genes from the vetch aphid, *Megoura viciae*. *Insect Mol. Biol.* **9**, 197-202.

Gartner, W. and Towner, P. (1995). Invertebrate visual pigments. *Photochem. Photobiol.* **62**, 1-16.

Gilbert, C. (1994). Form and function of stemmata in larvae of holometabolous insects. *Annu. Rev. Entomol.* **39**, 323-349.

Ichikawa, T. and Tateda, H. (1982). Distribution of color receptors in the larval eyes of four species of lepidoptera. *J. Comp. Physiol. A* **149**, 317-324.

Jackowska, M., Bao, R., Liu, Z., McDonald, E., Cook, T. and Friedrich, M. (2007). Genomic and gene regulatory signatures of cryptozoic adaptation: Loss of blue sensitive photoreceptors through expansion of long wavelength-opsin expression in the red flour beetle *Tribolium castaneum*. *Front. Zool.* **4**, 24.

Kelber, A. (2006). Invertebrate colour vision. In *Invertebrate vision* (ed. E. Warrant and D.-E. Nilsson), pp. 250-290. New York: Cambridge University Press.

Land, M. F. (1969). Structure of the retinae of the principal eyes of jumping spiders (Salticidae: Dendryphantinae) in relation to visual optics. *J. Exp. Biol.* **51**, 443-470.

Land, M. F. and Nilsson, D.-E. (2002). *Animal eyes*. New York: Oxford University Press.

Liu, Z. and Friedrich, M. (2004). The Tribolium homologue of glass and the evolution of insect larval eyes. *Dev. Biol.* **269**, 36-54.

Malpel, S., Klarsfeld, A. and Rouyer, F. (2002). Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. *Development* **129**, 1443-1453.

Mandapaka, K., Buschbeck, E. and Morgan, R. (2006). Twenty-eight retinas but only twelve eyes: an anatomical analysis of the larval visual system of the diving beetle *Thermonectus marmoratus* (Coleoptera: Dytiscidae). *J. Comp. Neurol.* **497**, 166-181.

Mazzoni, E. O., Desplan, C. and Celik, A. (2004). 'One receptor' rules in sensory neurons. *Dev. Neurosci.* **26**, 388-395.

Mazzoni, E. O., Celik, A., Wernet, M. F., Vasiliauskas, D., Johnston, R. J., Cook, T. A., Pichaud, F. and Desplan, C. (2008). Iroquois complex genes induce co-expression of rhodopsins in Drosophila. *PLoS Biol.* **6**, e97.

McIntyre, P. and Kirschfeld, K. (1982). Chromatic aberration of a dipteran corneal lens. *J. Comp. Physiol. A* **146**, 493-500.

Mombaerts, P. (2004). Odorant receptor gene choice in olfactory sensory neurons: the one receptor-one neuron hypothesis revisited. *Curr. Opin. Neurobiol.* **14**, 31-36.

Paul, R., Steiner, A. and Gemperlein, R. (1986). Spectral sensitivity of *Calliphora erythrocephala* and other insect species studied with Fourier Interferometric Stimulation (FIS). *J. Comp. Physiol. A* **158**, 669-680.

Paulus, H. F. (1979). Eye structure and the monophyly of the Arthropoda. In *Arthropod phylogeny* (ed. A. P. Gupta), pp. 299-384. New York: Van Nostrad Reinhold.

Popp, M. P., Grisshammer, R., Hargrave, P. A. and Smith, W. C. (1996). Ant opsins: sequences from the Saharan silver ant and the carpenter ant. *Invert. Neurosci.* **1**, 323-329.

Porter, M. L., Cronin, T. W., McClellan, D. A. and Crandall, K. A. (2007). Molecular characterization of crustacean visual pigments and the evolution of pancrustacean opsins. *Mol. Biol. Evol.* **24**, 253-268.

Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M. and Henikoff, S. (1998). Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res.* **26**, 1628-1635.

Rossel, S. (1979). Regional differences in photoreceptor performance in the eye of the praying mantis. *J. Comp. Physiol. A* **131**, 95-112.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425.

Sakamoto, K., Hisatomi, O., Tokunaga, F. and Eguchi, E. (1996). Two opsins from the compound eye of the crab *Hemigrapsus sanguineus*. *J. Exp. Biol.* **199**, 441-450.

Salcedo, E., Huber, A., Henrich, S., Chadwell, L. V., Chou, W.-H., Paulsen, R. and Britt, S. G. (1999). Blue- and green-absorbing visual pigments of Drosophila: ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. *J. Neurosci.* **19**, 10716-10726.

Sison-Mangus, M. P., Bernard, G. D., Lampel, J. and Briscoe, A. D. (2006). Beauty in the eye of the beholder: the two blue opsins of lycaenid butterflies and the opsin gene-driven evolution of sexually dimorphic eyes. *J. Exp. Biol.* **209**, 3079-3090.

Smith, W., Ayers, D., Popp, M. and Hargrave, P. (1997). Short wavelength-sensitive opsins from the Saharan silver and carpenter ants. *Invert. Neurosci.* **3**, 49-56.

Smith, W. C., Price, D. A., Greenberg, R. M. and Battelle, B. A. (1993). Opsins from the lateral eyes and ocelli of the horseshoe crab, *Limulus polyphemus*. *Proc. Natl. Acad. Sci. USA* **90**, 6150-6154.

Stavenga, D. G., Smits, R. P. and Hoenders, B. J. (1993). Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vision Res.* **33**, 1011-1017.

Strausfeld, N. J. and Seyan, H. S. (1985). Convergence of visual, haltere, and prosternal inputs at neck motor neurons of *Calliphora erythrocephala*. *Cell Tissue Res.* **240**, 601-615.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596-1599.

Toh, Y. and Mizutani, A. (1987). Visual-system of the tiger beetle (*Cicindela chinensis*) larva.1. structure. *Zool. Sci.* **4**, 974-974.

Toh, Y. and Mizutani, A. (1994). Structure of the visual system of the larva of the tiger beetle (*Cicindela chinensis*). *Cell Tissue Res.* **278**, 125-134.

Toh, Y. and Okamura, J. Y. (2007). Morphological and optical properties of the corneal lens and retinal structure in the posterior large sternma of the tiger beetle larva. *Vision Res.* **47**, 1756-1768.

Townson, S. M., Chang, B. S. W., Salcedo, E., Chadwell, L. V., Pierce, N. E. and Britt, S. G. (1998). Honeybee blue- and ultraviolet-sensitive opsins: cloning, heterologous expression in Drosophila, and physiological characterization. *J. Neurosci.* **18**, 2412-2422.

Wang, J. K., McDowell, J. H. and Hargrave, P. A. (1980). Site of attachment of 11-cis-retinal in bovine rhodopsin. *Biochemistry* **19**, 5111-5117.

White, R. H., Brown, P. K., Hurley, A. K. and Bennett, R. R. (1983). Rhodopsins, retinula cell ultrastructure, and receptor potentials in the developing pupal eye of the moth *Manduca sexta*. *J. Comp. Physiol. A* **150**, 153-163.

Yinon, U. (1970). The visual mechanisms of *Tenebrio molitor*: some aspects of the spectral response. *J. Exp. Biol.* **53**, 221-229.

Zeiger, J. and Goldsmith, T. H. (1994). Behavior of crayfish rhodopsin and metarhodopsin in digitonin: the 510 and 562 nm "visual pigments" are artifacts. *Vision Res.* **34**, 2679-2688.

Zuckercandl, E. and Pauling, L. (1965). Evolutionary divergence and convergence in proteins. In *Evolving Genes and Proteins* (ed. V. Bryson and H. J. Vogel), pp. 97-165. New York: Academic Press.