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SHORT COMMUNICATION

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Adult stemmata of the butterfly *Vanessa cardui* express UV and green opsin mRNAs

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Abstract Adult stemmata are distinctive insect photoreceptors located on the posterior surfaces of the optic lobes. They originate as larval eyes that migrate inward during metamorphosis. We used a combination of light microscopy and in situ hybridization to examine their anatomical organization in the butterfly *Vanessa cardui* and to test for the presence of visual pigments, the light sensitive components of the visual transduction pathway. The bilateral cluster of six internal stemmata is located near the ventral edge of the lamina. They retain the dark screening pigment and overlying crystalline cones of the larval stemmata. We found two opsin mRNAs expressed in the stemmata that are also expressed, respectively, in UV-sensitive and green-sensitive photoreceptor cells in the compound eye. A third mRNA that is expressed in blue-sensitive photoreceptor cells of the compound eye was not expressed in the stemmata. Our results reinforce the idea that the adult stemmata are not merely developmental remnants of larval eyes, but remain functional, possibly as components of the circadian input channel.

Keywords Extraretinal opsins · Photoreceptor · Visual pigment · Vanessa cardui (Insecta)

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Introduction

The larval photoreceptor organs of holometabolous insects, known as stemmata, lose their attachment to the head capsule at metamorphosis and migrate inward along the developing optic nerve of the adult (e.g., Norlander and Edwards 1969; Gilbert 1994). They end up on the posterior surface of the adult optic lobe, readily distinguished by their dark screening pigment. Such adult derivatives of larval stemmata have been reported for lepidoptera (Ichikawa 1991), beetles (Schulz et al. 1984), ants (Felisberti and Ventura 1996), flies (Seifert et al. 1987), caddisflies (Hagberg 1986), and other insects (Fleissner and Fleissner 2003; Gilbert 1994). Originally thought to be degenerate remnants in adults, accumulating morphological and physiological evidence indicates that the stemmata remain functional in adults (Ichikawa 1991; Gilbert 1994).

The larval photoreceptor in *Drosophila* and other muscomorphan flies is Bolwig's organ (Strange 1961), the homologue of the stemmata of other insects (Melzer Von and Paulus 1989). Like stemmata, Bolwig's photoreceptors are retained in adults at the posterior margin of the compound eye as the Hofbauer–Buchner eyelet (Yasuyama and Meinertzhagen 1999). The green-sensitive RH6 *Drosophila* rhodopsin (Salcedo et al. 1999; Yasuyama and Meinertzhagen 1999) and blue-absorbing RH5 rhodopsin (Salcedo et al. 1999; Malpel et al. 2002) were found to be expressed in eyelet cells, whereas known UV-sensitive rhodopsins (RH3 and RH4) were not detected. Little is known about the function of adult stemmata or their photopigments in other insects.

Vanessa cardui has joined the swallowtail butterfly, *Papilio xuthus* (Kitamoto et al. 1998, 2000), and the sphingid moth, *Manduca sexta* (White et al. 2003), as a lepidopteran species in which visual pigments have been characterized (Briscoe et al. 2003). Three opsin-encoding mRNAs are expressed in the compound eyes of *Vanessa*. These encode a UV-sensitive rhodopsin with peak absorption at 360 nm, a blue-sensitive rhodopsin at 470 nm, and a green-sensitive rhodopsin at 530 nm. The fact that

compound eye-specific opsins are also expressed in the Hofbauer–Buchner eyelet of *Drosophila* makes it interesting to see if this pattern of opsin expression is conserved in other insects. To further our understanding of the photoreceptor systems in day-flying lepidopterans, we asked whether the opsins of the compound eye in *Vanessa* are also expressed in the adult stemmata.

Materials and methods

Animals

Vanessa cardui pupae were purchased commercially (Carolina Biological Supply Company) and allowed to eclose in a humid chamber. The adults were hand-fed a sucrose solution.

In situ hybridization

Riboprobes were made from three distinct opsin-encoding 3′RACE products isolated from a cDNA pool synthesized from compound eye mRNA as previously described (Briscoe et al. 2003). Approximately one third of the coding region and all of the 3′UTR was included in the synthesis reaction. The probes were 684 bp (UV opsin, AF414074), 873 bp (blue opsin, AF414075), and 678 bp (LW opsin, AF385333) in length. Briefly, M13 (−20) and M13 forward primers were used in a 50-µl PCR reaction with cloned plasmid DNA as a starting template. The PCR product was separated on a 1% agarose gel, and the appropriate band was cut out and purified using the GeneClean kit (Qbiogene). The purified DNA was used as the template for a digoxigenin-labeling reaction using either the SP6 or T7 RNA polymerase (DIG RNA labeling kit, Roche). Probe yield was quantified using a dot blot. The sections were incubated in hybridization buffer (0.3 mM NaCl, 2.5 mM ethylene-tetraacetic acid, 20 mM TRIS-Cl, pH 8.0, 50% formamide, 10% dextran sulfate, 200 µg/ml yeast tRNA, and ×1 Denhart's medium) for 30 min at 60°C. Then approximately 40 ng of probe diluted in hybridization buffer was added to each slide. The sections were coverslipped and incubated overnight at 60°C in a humid chamber. The sections were then washed with ×2, ×1, and ×0.1 standard saline citrate (SSC) and 0.1% Tween, for 10 min to increase accessibility of the probe. An alkaline-phosphatase conjugated anti-digoxigenin antibody, Fab fragment (1:1,000 in ×1 phosphate-buffered saline, PBS) was added to the slides and incubated 2 h at room temperature. Probe detection was via 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP), nitro blue tetrazolium (NBT), and 0.1% Tween 20 colorimetric detection in alkaline phosphatase developing solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM TRIS, pH 9.5).

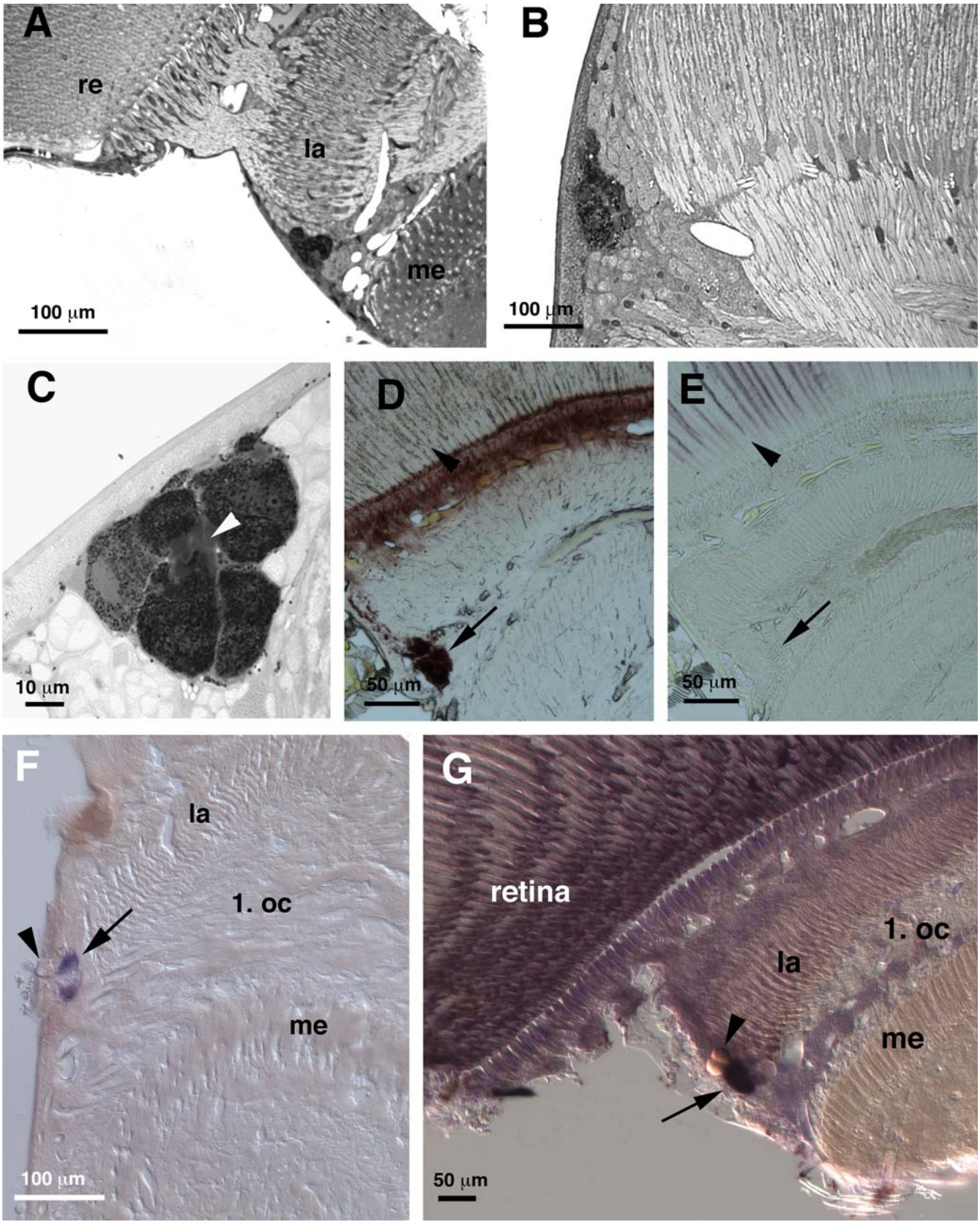
Fig. 1 Methylene-blue-stained light micrographs (**a–c**) of *Vanessa cardui* retina and optic lobe showing the location of the adult stemmata along the ventral margin of the lamina and (**d–g**) blue, UV, and green opsin mRNA expression as indicated by detection of digoxigenin-labeled riboprobes. **c** *Arrowhead* indicates location of rhabdomeres. **d** Frozen section of retina and optic lobe photographed prior to performing the in situ hybridization. *Arrow* indicates the presence of darkly pigmented stemmata. *Arrowhead* indicates same ommatidia as in **e**. **e** Same section as in **d**, following in situ hybridization using the blue opsin antisense riboprobe and colorimetric detection using BCIP, NBT, and alkaline phosphatase. *Arrow* indicates the location of the same stemmata shown in **d**, showing the lack of screening pigments due to washing and the lack of blue opsin mRNA expression. *Arrowhead* indicates the presence of blue opsin mRNA in the retinular cells of the compound eye. **f** *Arrow* indicates lobes of the stemmata that express UV opsin mRNA, suggesting that some but not all photoreceptor cells of the stemmata express the UV opsin transcript. *Arrowhead* indicates crystalline cones. **g** *Arrow* indicates strong green opsin mRNA expression in the stemmata. Dark staining of the photoreceptor cells of the retina is also evident, as is lighter staining of the lamina. Crystalline cone-like structures are also visible above the stemmata (*arrowhead*). *re* Retina, *la* lamina, *me* medulla, *1. oc* first optic chiasma.

Tissue preparation and light microscopy

For anatomy (Fig. 1a–c), opened head capsules were fixed in cacodylate-buffered glutaraldehyde solution. After aldehyde fixation for 1–2 h, the tissue was postfixed in 0.5% osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Spurr's resin (Polysciences, Warrington, PA, USA). One-micrometer sections were stained with methylene blue. Digital light micrographs were obtained with an Olympus BX60 microscope and Scionimage software, and processed in Adobe Photoshop with only magnification, contrast, and density adjusted. For riboprobe detection (Fig. 1d–g), *Vanessa* heads were fixed as in Briscoe et al. (2003). Cryostat sections (14–16 µm) were cut (Microm, HM 500 OMV) and photographed before and after in situ hybridization using an Axioskop two plus microscope connected to an AxioCam Hrc digital camera (Zeiss Goettingen, Germany).

Results and discussion

Bilateral groups of six stemmata can be seen on the larval head capsule. We found the six stemmata clustered near the ventral edge of the lamina, similar to those described in adult *P. xuthus* (Ichikawa 1991). The stemmatal cells contained a dense concentration of screening pigment granules surrounding rhabdoms. The stemmata were surmounted by the crystalline cone cells that were present in the larvae (Fig. 1a–d). In pierid and papilionid caterpillars, each larval stemma contains seven photoreceptor cells (Ichikawa and Tateda 1982). Although larval *Vanessa* has not yet been investigated, it is probably similar. Thus, in principle there could be a total of 42 photoreceptors in the extraocular organ. It would be interesting to investigate further whether all of these photoreceptor cells are retained in the adult stemmata of



Vanessa or whether (as in *Drosophila*) some are lost during the course of development.

Green opsin riboprobe hybridization similar in intensity to that observed in the compound eye was localized to the stemmatal cells (Fig. 1g). Similar intensity was also

observed between the retina and stemmata photoreceptors with the UV opsin riboprobe (data not shown). Blue opsin mRNA localized to the retinular cells of the compound eye, in contrast, was not detected in the stemmata (Fig. 1d, e). To our knowledge, this is the first report of specific opsin transcripts localized to extraretinal photoreceptor cells in a butterfly.

Our molecular results from *Vanessa* should be considered in light of the electrophysiological measurements of Ichikawa (1991) on the adult stemmata of another butterfly, *P. xuthus*. Whereas we found only UV- and green-sensitive rhodopsins, Ichikawa's spectral sensitivity measurements identified three cell types—green-sensitive, blue-sensitive, and UV-sensitive—the same three that are present in the larval stemmata of *Papilio* and several other lepidopteran species (Gilbert 1994). Thus it seems likely to us that a blue-sensitive rhodopsin differing in sequence from that of the compound eye is in fact expressed in the adult (and perhaps larval) stemmata of *Vanessa*. However, there are other possibilities: the larval stemmata of *Vanessa* may lack blue-sensitive cells; blue-sensitive cells present in the larva may degenerate at metamorphosis; or blue-sensitive larval cells may cease to express their blue-sensitive rhodopsin or switch to green or UV expression. Clearly these alternatives can only be resolved by more extensive morphological, electrophysiological, and molecular studies with *Vanessa*.

Our results from *Vanessa* are also consistent with opsin expression results from *Drosophila*. Several recent studies indicate that the cells of its stemmatal homologue, Bolwig's organ, and its adult derivative, the Hofbauer–Buchner eyelet, both express two rhodopsins: blue-sensitive RH5 and green-sensitive RH6 (Yasuyama and Meinertzhagen 1999; Malpel et al. 2002). We should note that an early report (Pollock and Benzer 1988) on opsin expression in Bolwig's organ is incorrect (Malpel et al. 2002; Pichaud and Desplan, personal communication). Thus it is likely the case that larval and adult stemmata in *Vanessa* (as in *Drosophila*) express the same opsins, and except for the uncertainty discussed above, they are opsins that are also expressed in the compound eye.

What might be the function of adult stemmata in *Vanessa* and other insects? Stemmata, while immediately useful to the larva for location of food (Harris et al. 1995) and avoidance of predators, may also be important for regulating life history traits such as the timing of pupation. It has also been suggested that in adults they play a role in entrainment of circadian rhythms (Ichikawa 1991; Fleissner 1982). In this regard, *Drosophila* is currently the most intensely investigated species. Eyelet photoreceptors have been shown to play a role in the entrainment of circadian activity rhythms in *Drosophila* under normal 12-h light / 12-h dark photoperiods in studies of mutant flies that lack them (Regier et al. 2003). Since long-wavelength receptors have been typically implicated in circadian systems (e.g., Felisberti et al. 1997), it will be interesting to see if all classes of adult stemmatal photoreceptors work in concert to track changes in the spectral content of ambient light.

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