

Review



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Insect opsins and evo-devo: what have we learned in 25 years?

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The visual pigments known as opsins are the primary molecular basis for colour vision in animals. Insects are among the most diverse of animal groups and their visual systems reflect a variety of life histories. The study of insect opsins in the fruit fly *Drosophila melanogaster* has led to major advances in the fields of neuroscience, development and evolution. In the last 25 years, research in *D. melanogaster* has improved our understanding of opsin genotype–phenotype relationships while comparative work in other insects has expanded our understanding of the evolution of insect eyes via gene duplication, coexpression and homologue switching. Even so, until recently, technology and sampling have limited our understanding of the fundamental mechanisms that evolution uses to shape the diversity of insect eyes. With the advent of genome editing and *in vitro* expression assays, the study of insect opsins is poised to reveal new frontiers in evolutionary biology, visual neuroscience, and animal behaviour.

This article is part of the theme issue 'Understanding colour vision: molecular, physiological, neuronal and behavioural studies in arthropods'.

1. Introduction

Opsins are the most widely used photopigments across animals. Present in basal lineages Ctenophora, Placozoa and Cnidaria, opsins have been widely studied to understand the evolution of eyes and vision [1–5]. In the past 25 years, research into the molecular evolution, expression and function of insect opsins has revealed extraordinary diversity of insect visual systems owing to gene duplication and loss, coexpression and spectral tuning of opsins [6–9]. Since von Frisch established that honeybees could see in colour over 100 years ago, the study of insect colour vision has provided fundamental insights into sensory physiology and animal behaviour [10].

The canonical role of opsins is that of light detection. Animal opsins are a monophyletic clade of seven transmembrane G-protein-coupled receptors [5]. Visual opsins bind a vitamin A-derived molecule called the chromophore (11-*cis*-retinal in vertebrates and 3-hydroxy-11-*cis*-retinal in the fruit fly *Drosophila melanogaster*) [11] (see box 1 for a glossary of terms). The chromophore binds to the opsin protein at a conserved lysine residue, forming a functional rhodopsin visual pigment molecule. Light activates rhodopsin through a chromophore conformational change from 11-*cis* to all-*trans*, which initiates the G-protein-mediated phototransduction cascade, leading to ion exchange and amplification of light information into a cellular signal [12]. Opsin protein sequences, opsin coexpression and filtering effects determine the wavelength of light to which photoreceptor cells respond. Opsins are particularly useful in the study of genotype–phenotype relationships because their sequence and expression are directly related to cell physiology and animal behaviour. The degree of overlap in spectral sensitivities allows animals to discriminate between wavelengths of light and underlies their colour vision (see also non-spectral colours [13]).

Box 1. Glossary of terms.

Bolwig organ	name for the larval eye in <i>D. melanogaster</i>
BRh, B opsin	blue-absorbing opsin homologue shared by insects
cell differentiation	in development, when a cell phenotypically specializes and expresses the functional proteins of its specific adult cell type, e.g. Rh3-expressing photoreceptor cell
cell specification	in development, the point at which a cell is committed to a particular fate, e.g. photoreceptor cell precursor
chromophore	vitamin A derived molecule that absorbs photons of light
ciliary opsin (c-opsin)	monophyletic clade of opsins historically associated with the outer segment (a modified cilium) of vertebrate photoreceptor cells, though now known to be found also in invertebrates
Cnidaria	animal group including jellyfish, anemones, and corals
Coleoptera	insect group including beetles
colour vision	the ability to discriminate between light stimuli of differing wavelengths
compound eye	the main visual organ of insects, made up of many repeating ommatidia
Ctenophora	early-branching animal group including comb jellies
Diptera	insect group including flies and mosquitoes
DRA	the dorsal-most row(s) of ommatidia in the compound eye, typically expressing more ultraviolet or B opsin, associated with polarized light detection and navigation
ERG	method of recording a sum of photoreceptor sensitivities from a region of the eye extracellularly
eyelet	photosensitive visual structure in the brain of adult insects
gene regulatory network	a group of genes that interact with each other, affecting downstream gene expression and phenotype
holometabolous insects	insects that undergo complete metamorphosis
homologue	gene found in multiple groups with a shared common ancestor, can be an orthologue or paralogue
homologue/paralogue switching	process by which one homologue/paralogue of a gene is swapped for another, achieving a similar function with a new gene
Hymenoptera	insect group including ants, bees and wasps
intracellular recording	a method of recording from individual neurons, the best way to measure individual photoreceptor cell physiology
lamina	distal-most layer of the insect optic lobe, associated with motion and contrast processing
Lepidoptera	insect group including moths and butterflies
LWRh, LW opsin	long-wavelength- or green-absorbing opsin homologue in insects
medulla	more proximal layer of the insect optic lobe, associated with colour vision processing
microspectrophotometry	a method measuring opsin absorbance spectra in the eye
morphogenetic furrow	a physical groove of differentiating cells that sweeps in a wave across the developing <i>D. melanogaster</i> eye
non-visual opsins	opsins that are not historically associated with vision, outside of rhabdomeric and ciliary opsin clades
non-spectral colour	a colour that is perceived by sampling from non-overlapping photoreceptors
ocellus	single chambered eye found on the dorsal head of most insects, with circadian and navigation-related functions
Odonata	insect group including dragonflies, mayflies and damselflies
ommatidium	a single unit of the compound eye containing photoreceptor cells, pigment cells and a focusing apparatus
opsin	visual pigment protein
orthologue	gene found in multiple animal groups related by inheritance from a single common ancestor, e.g. UVRh
Orthoptera	insect group including crickets and grasshoppers
paralogue	one of two or more genes in a species related by duplication within a single lineage, such as <i>LWRh1</i> and <i>LWRh2</i>
Pax6	transcription factor expressed early in development that determines eye tissue in many animals
photoreceptor cell	light-sensitive cell typically expressing opsin, typically sensory neural cell
phototaxis	an animal's movement toward or away from light
phototransduction	a G-protein-mediated signalling pathway by which light information is transduced into a biochemical signal in photoreceptor cells
Placozoa	early-branching group of small, free living, simple animals
polarized light	photons of light that travel in a wave along a single plane

precursor cell	cell that undergoes a limited number of divisions to form daughter cells that differentiate
retrotransposition	mechanism of gene duplication by which a transposable element inserts intronless mRNA encoding a gene back into the genome as DNA
rhabdomere	structure formed by photoreceptor cell microvilli through which light is guided and absorbed by expressed opsins
rhabdomeric opsin (r-opsin)	monophyletic clade of opsins historically associated with expression in the microvillar rhabdom of invertebrate photoreceptor cells, though now known to be found also in vertebrates
rhodopsin	functional visual pigment complex of opsin protein and vitamin A-derived chromophore
sexual dimorphism	distinct phenotype between males and females; can be gene expression, physiology, behaviour or morphology
spectral sensitivity	the degree of response of a photoreceptor cell over wavelengths of light
spectral tuning	process by which the sensitivity of a photoreceptor cell is shifted to detect new wavelengths of light, either evolutionarily or via physiological plasticity
stemmata	simple larval eyes with a limited number of opsins expressed
tetrachromacy	having four independent sensory channels for colour vision
trichromacy	having three independent sensory channels for colour vision (similar to normal human vision)
turbinate eye	specialized dorsal compound eye enlarged on vertical stalks and unique to mayflies, in addition to the usual compound eye
UVRh, UV opsin	ultraviolet-absorbing opsin homologue shared by insects

In addition to opsin biochemical function, understanding the developmental processes leading to when and where opsins are expressed is important to understanding colour vision. Linking Pax6 (Eyeless) to eyes in all animals was first recognized and functionally validated in *D. melanogaster*, and the genetic tractability of this organism led to many advances in how gene regulation is related to a complex organ like the eye [14]. Studies of photoreceptor cell development uniquely connect early development through terminal differentiation to mature cell and tissue phenotype. New research has revealed the complexity of the regulatory networks that specify photoreceptor cell fate and differentiation in *D. melanogaster*, and the relationship between these regulatory networks and terminal cell differentiation is perhaps one of the best understood of any complex trait in biology. We first review what we have learned in *D. melanogaster* and examine how this gene regulation has evolved in other insects. We relate this to how the evolution of colour vision has proceeded via opsin gene duplication and to changes in opsin gene expression. While descriptions of opsin expression patterns have expanded in other insects over the past 25 years, much work remains to characterize the developmental genetics behind opsin expression in other species. To conclude, we suggest future avenues of research in insect opsins as models for neuroscience, evolutionary biology and development.

2. What have we learned about opsin regulation from *Drosophila melanogaster*?

(a) Genetic regulation of specification and terminal differentiation of insect photoreceptor cells

Much of what we know about opsin-expressing photoreceptor cell development in insects comes from *D. melanogaster*. Over the past 25 years, work in *D. melanogaster* has shown that an intricate process involving both stochastic and deterministic mechanisms has led to the retinal mosaic in the compound

eye. Opsin regulation is intertwined with photoreceptor cell differentiation and has taught us about cell differentiation more broadly. While many reviews summarize eye development (such as early determination by the retinal determination network) in *D. melanogaster*, we focus on photoreceptor cell specification (when the cell becomes committed to a particular fate) and terminal differentiation (when the cell matures and expresses markers of its specific adult function), synthesizing previous information with the most recent work [15–18].

In *D. melanogaster*, like other insects, the compound eye is made up of many repeating unit-eyes called ommatidia. Each ommatidium is made up of eight photoreceptor cells labelled R1–R8. The outer R1–6 cells express the opsin Rh1 in every ommatidium in the retina and are broadband contrast and motion-sensing cells that project to the lamina, while R7/R8 are the ‘inner’ photoreceptors that project to the medulla, known to be involved in colour processing (figure 1a). The opsin-expressing microvilli of these two cells are stacked along the proximo-distal axis (R7 more distal) in the ommatidium, sampling from the same point in space. The typical R7 cell expresses either Rh3 or Rh4 (ultraviolet (UV)) opsin and induces R8 to express either Rh5 (blue) or Rh6 (long-wavelength or LW) opsin, respectively [20,21]. The ommatidia expressing Rh3 in R7 and Rh5 in R8 are known as pale ommatidia while ommatidia expressing Rh4 in R7 and Rh6 in R8 are known as yellow ommatidia. The pale and yellow types are stochastically found throughout the retina at a ratio of 35:65 in typical laboratory fly stocks [17,22]. In *D. melanogaster*, like in other insects [23], the dorsal-most row of ommatidia is known as the dorsal rim area (DRA) and expresses only Rh3 in both R7 and R8 cells. Insect DRA ommatidia are typically enriched with UV or blue-sensing cells and are involved in polarized light detection for navigation [24,25]. A specialized yellow ommatidial type in *D. melanogaster* is found only in the dorsal third of the eye and in addition to Rh4 also expresses Rh3 in the R7 cell [17] (figure 1a).

In *D. melanogaster*, photoreceptor cell specification begins first with the R8 cell. Atonal increases Senseless expression,

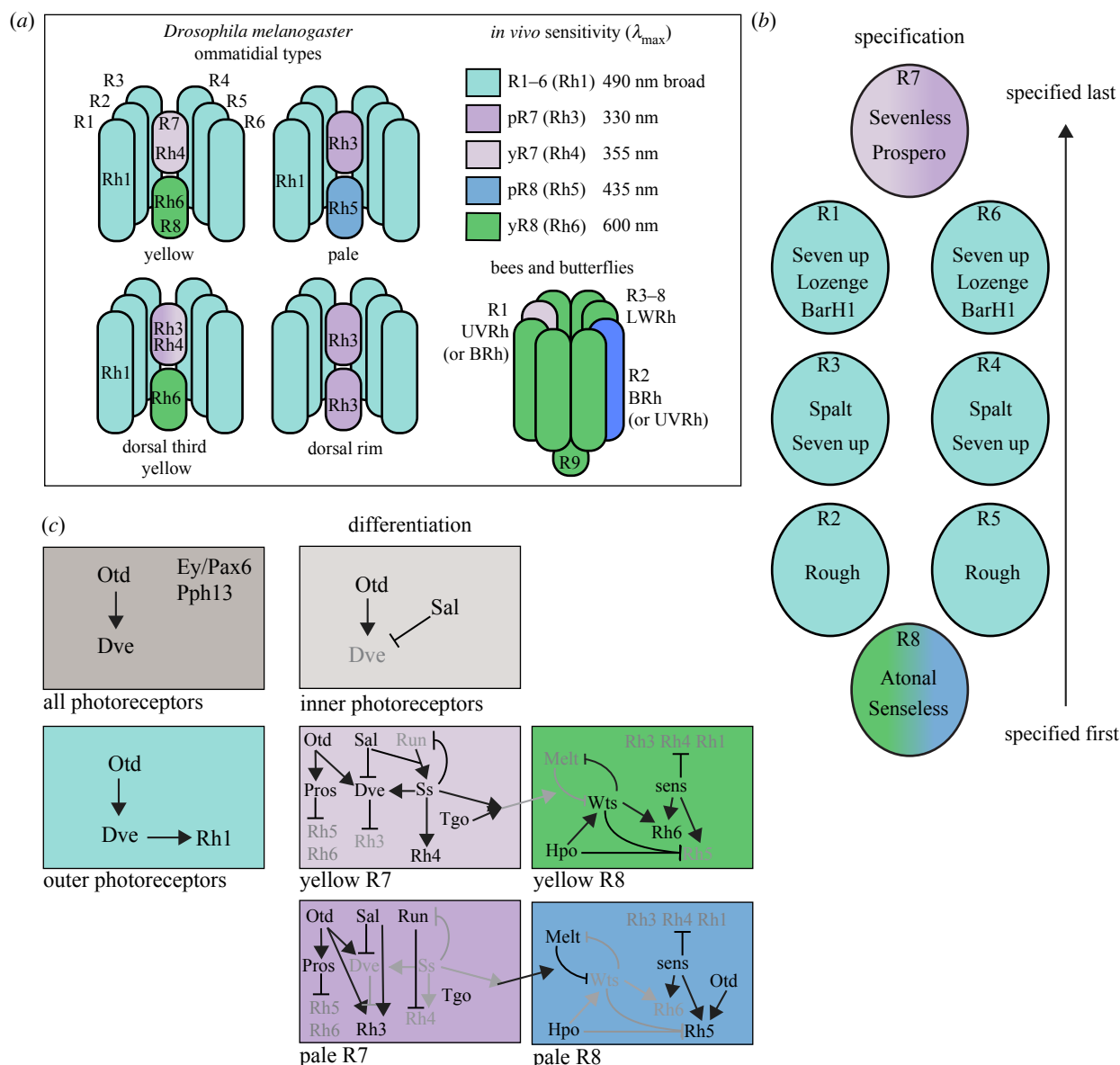


Figure 1. Insect photoreceptor cell arrangements, specification and regulation of differentiation. (a) Top, schematic representation of *D. melanogaster* ommatidial types. R1–R6 labels outer photoreceptor cells while R7 and R8 are inner photoreceptor cells involved in colour vision. Distinct ommatidia vary by opsin expression in inner photoreceptor cells. Right, corresponding maximum sensitivities ($\lambda_{\max}$) of photoreceptor cells measured *in vivo* incorporate pigment filtering and other photoreceptor cell dynamics (from [19]). Bottom, Hymenoptera and Lepidoptera have an additional photoreceptor cell in their ommatidia. The R1/R2 cells in butterflies and R1/R5 cells in bees are the 'inner' photoreceptors and contribute to colour vision, and both are R7-like in the *D. melanogaster* numbering scheme. The small R9 cell in bees and butterflies is equivalent to the R8 cell in *D. melanogaster*. The additional photoreceptors have allowed for three main ommatidial types with regard to UVRh opsin expression in the retinas of bees and butterflies as opposed to two in *D. melanogaster*. (b) The specification of photoreceptor cells in each ommatidium is stereotyped and proceeds via temporal differences in the expression of transcription factors. R8 is specified first, followed by R2/R5, then R3/R4, then R1/R6, with R7 specified last. Transcription factors known to be important for subtype specification are shown within each cell. (c) Once cells are specified an interconnected series of transcription factors are expressed to terminally differentiate each photoreceptor cell. These networks are characterized by a series of feed-forward bistable loops of regulation, mutual inhibition and a combination of stochastic and deterministic choices. Arrows are positive regulatory relationships while T's are negative regulatory relationships. Black shows active pathways while grey indicates pathways not activated in each subtype. Otd, Orthodenticle; Dve, Defective proneuroblast; Ey, Eyeless; Pph13, PvuII-PstI homology 13; Sal, Spalt complex; Pros, Prospero; Run, Run; Ss, Spineless; Tgo, Tango; Melt, Melted; Wts, Warts; Sens, Senseless; Hpo, Hippo pathway.

inhibiting Rough, leading to R8 cell fate (figure 1b). The cell-adhesion protein Hibris is also involved in signalling for proper R8 specification [26]. R2 and R5 are specified next, where Rough is highly expressed and represses Senseless leading to R2 and R5 cell fate. Rough suppresses Seven up in R2/R5, but Rough is not expressed and Seven up is upregulated in R3/R4 and R1/R6 which are next to be specified. R3/R4 cell fate requires the Spalt complex [27] while R1/R6 cell fate requires Lozenge which in turn upregulates BarH1 in R1/R6. Finally R7 is specified last, where the receptor

tyrosine kinase Sevenless is expressed and binds to Boss in the R8 cell. Epidermal growth factor receptor (EGFR) signalling from R1/R6 activates the Ras pathway which in turn upregulates Prospero in R7. Notch is also involved in blocking differentiation in R7 until Sevenless is upregulated highly enough to overcome this, in a distinct mechanism from EGFR signalling in R1–R6 [28] (figure 1b).

Next, photoreceptor cell differentiation involves a complex series of overlapping events that work in concert to ensure proper and robust opsin expression in each cell. Rh1

has been shown to be directly activated by Pax6/Eyeless [29]. Defective proventriculus (Dve) is also expressed in R1–R6 and activates Rh1. Differentiating R7 and R8 inner photoreceptors, both of which mediate colour vision (the former of which also contributes to motion vision [30]), involves the precise restriction of specific opsins and activation of others. Following specification, the Spalt complex is a marker for inner photoreceptor cell differentiation (R7/R8) [31] where it blocks Dve and thus Rh1 expression. R7 expresses Prospero, which inhibits Rh5 and Rh6 opsin expression [32]. R8 expresses Senseless which promotes expression of these opsins and blocks Rh3/Rh4 expression [33]. Then, a stochastic choice in each R7 results in Spineless expression in a subset of cells across the retina as differentiation proceeds [34,35]. Spineless leads to Rh4 expression and yellow ommatidial fate. Together with Tango, Spineless induces the R8 cell to express Rh6. In the absence of Spineless, Rh3 is expressed in R7 and Rh5 is expressed in R8 (figure 1c).

A variety of interdependent regulatory loops pattern the retina and dictate opsin expression. A bistable loop of the mutually inhibiting proteins Melted and Warts ensures robust expression of opsins in R8 cells. Whereas Melted promotes Rh5 and blocks Warts, Warts promotes Rh6 and blocks Melted [36]. Another feedforward loop involves Orthodenticle (Otd/Crx). In the absence of Dve, Otd is a permissive factor that leads to pale ommatidial fate by activating Rh3 and Rh5 expression in R7 and R8 cells. Spalt complex blocks Dve in pale ommatidia, but Dve expression in yellow ommatidia represses this permissive Otd, blocking Rh3 and Rh5 expression. In the dorsal third retina, lower Dve expression and Iroquois complex expression activate Rh3 coexpression with Rh4 in dorsal yellow ommatidia [16,37,38]. Recently, another feedback loop was discovered, showing that interaction with stereotyped upstream Runt expression contributes to the stochastic Spineless expression that determines pale or yellow ommatidial types [39] (figure 1c). Feedforward, feedback and bistable loops build redundancy and modularity into this system and have become hallmarks of robust developmental processes in other complex traits. The study of these genetic regulatory programmes has contributed to our modern view of development, and further study will continue to yield new insights that explain how complex traits develop.

The *cis* regulatory biochemical interactions of some of these genetic relationships have been elucidated. As mentioned above, Pax6/Eyeless binds directly to the *Rh1* promoter and activates expression. There is also evidence that Pax6 is a general activator for all *D. melanogaster* rhodopsins [29,40]. In R8 cells which are competent to express Rh5 throughout their lifetime, expression and functional activity of Rh6 directly inhibits Rh5 expression [41]. A conserved 11 bp palindromic motif is found in the proximal promoter of phototransduction genes expressed in all *D. melanogaster* photoreceptors but is modified in rhodopsins with restricted photoreceptor cell expression domains, such as Rh1 found only in R1–6 cells or Rh5 found only in R8 cells. Differences in rhodopsin expression are owing to specific base-pair mutations that break the palindrome, resulting in changes to this site's affinity for binding repressors and activators, PvuII-PstI homology 13 (Pph13), Dve and Otd [42]. Recent work swapping promoter domains from multiple rhodopsins has revealed a complex regulatory landscape in addition to

this palindromic sequence that delineates photoreceptor subset specific expression. Each rhodopsin-specific promoter landscape evolved by duplication from a simple single pan-photoreceptor rhodopsin followed by subsequent divergence for specificity of expression [43]. This highlights the importance of duplication as a mechanism for generating novel genetic material (reviewed below), and that by linking development and evolutionary biology we can more deeply understand how visual systems work [43].

3. Genome editing

Advances in CRISPR/Cas9 editing technology have allowed researchers to validate gene functions and discover genetic interactions. In terms of insect eye development, in *Drosophila*, CRISPR deletion of an *eyeless* enhancer site resulted in a small eye phenotype [44]. This enhancer was not only important for *eyeless* expression, but also for Decapentaplegic expression and proper formation of the morphogenetic furrow [44]. In terms of photoreceptor cell fate, CRISPR knockouts revealed the genes and interactions necessary for compound eye retinal mosaic choices [45]. While eye development genes are difficult to knock out owing to their deleterious effects, opsin and eye colour gene knockouts have revealed genes important for visually guided behaviour [46], phototaxis [47] and eye pigmentation [48–51].

4. Opsin regulation in other insects

In other insects, relatively little is known about development and opsin regulation, despite Hymenoptera and Lepidoptera being major visual ecology models [52–54]. One key theme to emerge in the last 25 years is the understanding of homology between the inner colour photoreceptor cells between *D. melanogaster*, Lepidoptera, and Hymenoptera. In butterflies and bees but not beetles, the equivalent of *D. melanogaster* inner photoreceptor R7 was independently 'duplicated' with two R7-like cells spanning the length of the ommatidium, while the R8 cell has been miniaturized and sits at the proximal-most part of the ommatidium. In Lepidoptera and Hymenoptera, this proximal cell is called R9. R1/R2 and R9 project to the medulla similar to R7/R8 in *D. melanogaster*, all presumably contributing to colour vision [55]. The difference is that R1/R2 (R7-like) in bees and butterflies alter their particular opsin expression with either blue- or UV-absorbing opsins, unlike in *D. melanogaster* where R7/R8 do this. The R9 cell (R8 homologue) so far has only been shown to express long-wavelength opsin (LWRh) in all ommatidial types although more sampling is required [18,56]. In most other insects, including crickets, locusts, dragonflies and beetles, no such cell type diversification occurred, and the arrangement of photoreceptors in ommatidia is similar to *D. melanogaster*. This suggests the additional R7-like cell evolved independently in bees and butterflies (figure 1a, bottom).

Until recently, the molecular logic, which led to three ommatidial types in the main retina of bees and butterflies and only two main types in *D. melanogaster*, was unknown. As mentioned above, in *D. melanogaster*, Spineless is expressed stochastically in a subset of R7 cells and directs the expression of particular opsins in both R7 and R8 yielding either pale or yellow ommatidia. In the swallowtail butterfly *Papilio xuthus*, Spineless is also expressed stochastically in a

subset of R1 and R2 cells, both of which are homologous to the *D. melanogaster* R7, with ON expression leading to blue opsin (BRh) expression and OFF yielding ultraviolet opsin (UVRh) expression [45]. As a result, there are two Spineless decisions within each ommatidium yielding three combinations of UV/UV UV/blue or blue/blue ommatidial types [45]. (This pattern is complicated in nymphalid butterflies with the recent discovery of the coexpression of BRh and LWRh opsins in a subset of R1/R2 cells in *Heliconius*, producing three additional ommatidial classes, and the existence of R1/R2 photoreceptors with both blue- and LW sensitivities across a variety of nymphalid species [57,58].) In *Heliconius*, which are rare among Lepidoptera and other insects for duplicating a UV opsin [8], yet more combinations of ommatidial types exist where a choice between BRh, UVRh1 or UVRh2 is made [59]. Counts of ommatidial types in multiple species within this genus have shown stereotyped percentages for these novel ommatidial types, suggesting there may be an additional stochastic yet well-controlled step similar to what we see with Spineless [60]. Other than this, there is no experimental evidence showing that genetic regulation in *D. melanogaster* opsin-expressing cells is conserved in either Hymenoptera or Lepidoptera.

Although little functional data exist, orthologous genes known to be involved in *D. melanogaster* eye development have been identified in the visual systems of many other insects and are thought to be involved in similar ways to *D. melanogaster* differentiation. Recent work has suggested at least some transcription factor orthologues (Pax2/5/8, Optix, Ocelliless and Araucan/Caupolican) involved in *D. melanogaster* photoreceptor differentiation are potentially regulating UV opsin expression in *Heliconius* butterflies [57]. In *Tribolium*, Pph13 and Otd are required for proper regulation of both rhabdomeric opsin transcription and rhabdomere morphogenesis [61]. The *Glass* gene is also required for proper photoreceptor cell differentiation and for Pph13 expression in both *D. melanogaster* and *Tribolium* [62]. Unlike *D. melanogaster* and *Tribolium*, in scarabaeid beetles, RNAi-mediated knockdown of Otd induces functional ectopic eyes [63]. These ectopic eyes are located in the medial dorsal head, where the ocelli (simple eyes) are found in other insects, but nearly all beetles have lost their ocelli [64], so this may be a reactivation of some vestigial pathway. So far only a handful of insect orders are represented within the opsin regulation literature, and the next years should bring functional studies in more diverse taxa.

5. Larval photoreceptors and ocelli

Holometabolous insects have evolved complete metamorphosis from ancestral insect lineages that do not have such an extreme transformation [65]. The larvae of holometabolous insects typically have simple eyes called stemmata with a limited number of photoreceptors and opsins compared to the adult compound eye. Other insects that do not exhibit complete metamorphosis have compound eyes in larval stages, suggesting stemmata are derived evolutionarily from compound eyes [66]. Although most stemmata are simple, some lineages have elaborated these structures considerably, especially among holometabolous larvae [66]. The molecular differences between larval eyes and adult structures are poorly studied in most insects, obscuring

conclusions about the homology of eyes within arthropods and across the animal tree of life.

Nearly all functional molecular data come from *D. melanogaster*. The larval eye, also known as the Bolwig organ, is made up of 12 photoreceptor cells that express the same opsins as R8 cells in adults (Rh5, four cells or Rh6, eight cells) [67]. Primary precursor cells are specified by Atonal [68], then these cells recruit secondary precursors via EGFR signalling [67]. The primary precursors differentiate into Rh5-expressing cells while the secondary precursors express Rh6. Despite a similarity to the 35:65 ratio seen in adult ommatidia, this process is deterministic and not driven by stochastic choices [68]. Spalt and Otd are required for Rh5 expression and repression of Seven up while Seven up blocks Spalt and thus Rh5 in Rh6-expressing cells [67,68].

During metamorphosis, the Rh6-expressing cells of the Bolwig organ die, and the Rh5 cells switch to Rh6 expression forming an adult extraretinal photosensitive structure known as the eyelet, which mediates circadian entrainment [69,70]. Senseless also acts to maintain Rh5 in larval photoreceptor cells, while it blocks apoptosis during metamorphosis and promotes Rh6 expression in the adult eyelet [71]. Furthermore, Pph13 (Hazy) is required for Rh5 and Rh6 expression in the larva but during metamorphosis blocks Senseless expression in Rh6-expressing cells, leading to apoptosis [71]. Not much is known about the stemmata of other insects; however in the adult butterfly, *Vanessa cardui*, eyelets exist and express both UVRh and LWRh [72], unlike in *D. melanogaster* where only the UV-absorbing Rh5 is expressed.

The adult forms of winged insects also contain medial ocelli, single chambered, optical structures on the dorsal side of the head [73]. These ocelli and compound eyes are thought to have been split from some ancestral eye prior to the rise of Arthropoda [74]. Subsequent duplication of opsins allowed for differential expression and thus separation of distinct visual tasks [75]. In *D. melanogaster*, Rh2 is expressed only in the ocelli, resulting in violet-sensitive photoreceptor cells [76]. It has recently been shown that Homothorax (Hth) is expressed in ocelli and represses Rh1 expression while promoting Rh2 in these photoreceptor cells [77]. Hth appears to cause a fate switch between Rh1 and Rh2 because Rh2 expression can only be induced by ectopic Hth in outer photoreceptor cells of the compound eye, which normally express Rh1. This observation further supports that Rh1 and Rh2 are the result of a gene duplication that allowed for neofunctionalization of light-mediated tasks [77]. Ocellus-specific duplicates of opsins are common in most insect orders: specific UV and LW opsins are found only in ocelli in the cricket *Gryllus bimaculatus* [78], in multiple dragonfly species [7], and in bees [79,80] (figure 2). Together this further supports that opsin gene duplication has coincided with the evolution of novel sensory structures and allowed for neofunctionalization of light-mediated tasks.

6. Opsin expression patterns in insect photoreceptor cells

In contrast with the dearth of genetic regulatory studies of opsins across insects, studies of opsin expression have expanded to include multiple insect orders and have contributed to shifts in thinking about receptor patterning and function. The number of opsins and their spatial patterns of

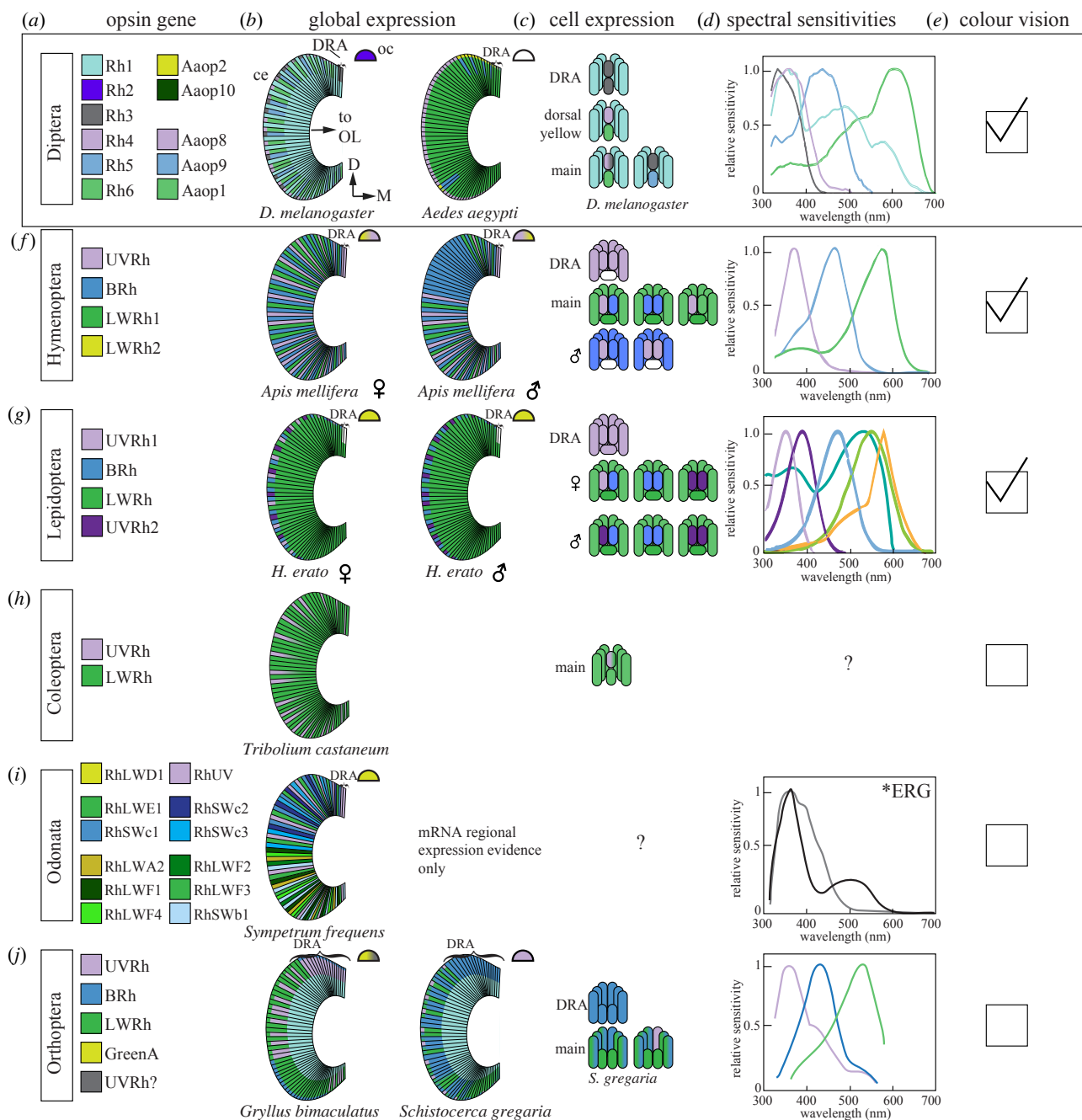


Figure 2. Holistic opsin sequence, expression pattern and visual system function is only known in a few insects. Cartoons of exemplar systems are shown from major insect orders. (a) Opsin sequences in example dipterans are coloured to match the general wavelength of absorption. Use of the same colour indicates orthologous sequences. Names are specific to literature in each species. (b) Broad opsin expression patterns in the compound eyes (ce) and ocelli (oc) are shown. Dorsal rim area (DRA) expression is distinct in the dorsal-most cell layers, if present. For all cartoons, colours correspond to sequences in the first column, gradient colours indicate co-expression, dorsal is up, medial is to the right. White, expression is unknown. Tiered retinal opsin expression is shown if known. Photoreceptor axons project to the optic lobe (OL) from the proximal side of the eye. (c) Expression in single cells is known for fewer insect examples. Opsin-expressing cells are arranged as ommatidial types present in the compound eye. (d) Single-cell resolution of photoreceptor spectral sensitivities is known in still fewer insect species. Relative sensitivity is shown over the visible light spectrum. (e) The presence of colour-discrimination behavioural data is indicated by a tick. Data in (e) and (d) are unknown in many insects. (f) In Hymenoptera, the honeybee *Apis mellifera* is best studied for colour vision behaviour, although other well-studied hymenopteran models (such as bumblebees) also exist. The honeybee eye is sexually dimorphic with respect to opsin expression. (g) Lepidoptera is one of the best-studied insect groups for colour vision. Molecular, physiological and behavioural examples exist in moths and butterflies. The *Heliconius erato* eye is sexually dimorphic and *Heliconius* is one of the few insect genera where colour vision has been studied from molecular genetics to behaviour. (h) Beetles have lost the BRh opsin, and the retina is dominated by LWRh and UVRh opsins. (i) Dragonflies are known to have greatly expanded their opsin repertoires based on transcriptomics but single-cell level expression data remain to be gathered. (j) Crickets and locusts are classic models for studying the DRA owing to its conspicuousness in these species. Single-cell expression and physiology has been worked out but few colour-specific behavioural studies exist. References: (a–e) [17,19,81,82], (f) [83,84], (g) [45,56,57,59,60,85–92], (h) [93], (i) [7] and (j) [78,94].

expression in the eye and/or their inferred expression based on physiological measurements has been reviewed across insects [6,17,85]. Regions, bands and spots of specialized ommatidia expressing unique combinations of opsins are

found throughout insects and can be sex-specific [17] (figure 2). The DRA is found in many insects, and evidence from *D. melanogaster* (above) suggests that distinct regulatory pathways lead to UV or blue opsins expressed in all or a

subset of photoreceptor cells of the ommatidia in the DRA. Dorsal/ventral expression and sex-specific regions of specialized ommatidia in bees and flies are used for locating prey or mates in flight. These patterns of opsin expression remain interesting from both developmental and behavioural perspectives and merit further study [17].

One way our understanding of sensory neurons has changed significantly is the 'one cell one receptor' rule, stating that in sensory cells, a single molecular receptor is expressed to the exclusion of all other possible receptors in any individual cell [95]. It was thought until recently that the expression of two opsins in a single photoreceptor cell would diminish colour vision capability by the generation of a broadband receptor. In contrast with this view, multiple insects have independently evolved opsin coexpression in single photoreceptor cells (figure 2) and new, though limited, evidence suggests coexpression contributes to colour vision in some instances [57,58,96]. In *D. melanogaster*, the dorsal yellow ommatidial type expresses both Rh3 and Rh4 in the R7 cell. The Rh1-expressing outer cells, though not coexpressed with another opsin, are modified broadband receptors which participate in colour vision [97]. In butterflies, a variety of different opsin coexpression patterns is common, with examples of coexpressing photoreceptor cells found in nearly all major families. In the lycaenid *Lycaena rubidus*, one *BRh* duplicate is coexpressed with *LWRh* opsin messenger RNA (mRNA) in the dorsal eye of females only in all R3-8 (outer) photoreceptor cells [98]. In Pieridae, *Colias erate* expresses three distinct opsin mRNAs in a single cell [99]. *Papilio xuthus* (Papilionidae) has been shown to coexpress two green-absorbing opsins. Both opsins participate in phototransduction, generating a double-peaked green cell [96], which is used in the butterfly's tetrachromatic colour vision system [86]. Recently, a LW+B opsin cell type has been found in *Heliconius* butterfly R1/R2 cells, and intracellular recordings have identified a blue-green broadband cell across Nymphalidae that could be this coexpressing cell type [57,58]. In the desert locust *Schistocerca gregaria*, most photoreceptor cells in the main retina coexpress blue and green opsin mRNAs [100]. While not all photoreceptor cells with opsin coexpression are involved in colour vision, some probably are. Future work including behavioural assays should focus on how photoreceptors with opsin coexpression contribute to colour vision.

(a) A note of caution: opsin messenger RNA does not always equal opsin protein

Most studies of opsin expression in non-*Drosophila* insects examine opsin mRNA expression using transcriptomics instead of opsin protein expression using immunohistochemistry. We, along with other researchers, have found that quantifiable opsin mRNA expression levels do not always correspond to opsin protein expression. For instance in the butterfly *Heliconius melpomene*, *UVRh2* is expressed at low levels in the eye and brain as assayed using RNA-Seq but no protein is detected [60,101]. A similar finding of opsin mRNA expression in the absence of opsin protein expression has been found in bats and in the swallowtail butterfly *Papilio xuthus* [102,103]. These findings suggest more mechanistic studies of post-transcriptional opsin regulation are needed to better understand the relationship between opsin genotype and phenotype.

7. Opsin duplication

Studies of opsins can tell us about general trends and fates of gene duplicates. Current knowledge indicates that duplications can occur by a variety of mechanisms even in closely related groups [104]. Similarly, selection can act on different sites to achieve almost identical outcomes and selective pressure weakens with additional duplication events [105,106]. The vast majority of recent findings in insect opsins have come from surveys of opsin genes or mRNAs together with phylogenetic studies. Opsin genes evolve by coding sequence variation, gene duplication and gene loss. The insect opsin gene family includes four non-visual opsin and three visual opsin clades, roughly corresponding to mRNAs encoding UV-, blue- and LW-absorbing photopigments (summarized in figure 3 and considered below). General trends are that *UVRh* is duplicated less often than *BRh* and *LWRh*; *BRh* has been lost in multiple insect orders and *LWRh* is most variable with multiple duplications in most insect orders. Overall *UVRh*, *BRh* and *LWRh* duplicates have resulted in diversity in insect visual system function including modifications of sensitivity to light via spectral tuning mutations and the evolution of sexual dimorphisms of the eye (figures 2 and 3).

(a) Long-wavelength opsins and tandem duplication

In insects, the most duplications and largest diversity in opsins are that of *LWRh*, a finding foreshadowed by the first publication describing butterfly opsins in 1998 [117]. While there are many hypotheses linking *LWRh* duplications to ecology, the significance of *LWRh* duplications and expansions across most insects remains unclear because few studies have examined the behavioural context in which a particular receptor is used (e.g. [88,118]). Most Hymenoptera have two *LWRh* opsins (one of which is expressed in ocelli) [119–121]. A comprehensive study in Lepidoptera found that opsin duplications are more common in diurnal species and that *LWRh* was duplicated more often than other opsins; specifically, *LWRh* has had approximately 10 duplication events, while six have occurred in *BRh* and three in *UVRh* [8]. In Diptera, the current *LWRh* complement in mosquitoes has been produced by an estimated 18 or 19 duplication events [109,122]. At the genetic level, signatures of gene evolution mechanisms include genes located in tandem owing to unequal crossing over or a lack of introns by retrotransposition [123]. In insects, we see *LWRh* evolution by both of these mechanisms and probably others that are not as easy to identify. In the water strider *Gerris buenoi* and in *Anopheles gambiae*, four and five *LWRh* genes, respectively, are located in tandem [110,122]. Some *LWRh* paralogues in moths and *Anopheles* are intronless and are proposed to have evolved by retrotransposition [109,111,124]. In mayflies, four *LWRh* genes are in genomic clusters that vary in size and have undergone rearrangement between species [112]. A limitation in investigating the molecular mechanisms by which *LWRh* genes are undergoing expansions is a lack of high-quality contiguous insect genomes. With chromosome-level genome assemblies, searches for other signatures of gene evolution via retrotransposition and other mechanisms become possible.

(b) Blue duplications, expansions and localized expression in butterflies, bees and Paleoptera

Independent duplications of *BRh* have been found in butterflies. *Pieris rapae* and *Lycaena rubidus* have two *BRh* that

	ptero	RGR	arth	Rh7	UV	Blue	LW	reference
Archaeognatha								
<i>Machilis hrabei</i>		1			1	1	2	[114]
Zygentoma								
<i>Tricholepidion gertschi</i>		0			0	0	3	[114]
Odonata								
<i>Indolestes peregrinus</i>	1	1	1	1	1	2	8	[7]
<i>Epiophlebia superstes</i>	1	1	1	1	1	7	11	[7]
<i>Anax parthenope</i>	1	1	0	1	1	8	21	[7]
<i>Sympetrum frequens</i>	1	1	1	1	1	5	10	[7]
Emphemeroptera								
<i>Ephemera danica</i>		0			1	3	5	[112]
<i>Cloeon dipterum</i>					4	2	4	[112]
Orthoptera								
<i>Schistocerca gregaria</i>		0			1	1	1	[100]
<i>Gryllus bimaculatus</i>		0			1	1	2	[78]
Hemiptera								
<i>Acyrtosiphon pisum</i>	1	0	1	2	2	0	1	[111]
<i>Apolygus lucorum</i>					1	0	2	[116]
<i>Nesidiocoris tenuis</i>					1	0	3	[116]
<i>Gerris buenoi</i>	1	0	1	1	1	0	4	[110]
Phthiraptera								
<i>Pediculus humanus</i>	0	0	0	1	1	0	1	[7]
Hymenoptera								
<i>Apis mellifera</i>	1	0	0	0	1	1	2	[111]
<i>Nasonia vitripennis</i>	0	0	0	0	1	1	2	[111]
<i>Ceratosolen solmsi</i>					1	1	2	[114]
<i>Bombus impatiens</i>					1	1	2	[147]
Coleoptera								
<i>Tribolium castaneum</i>	1	0	0	0	1	0	1	[7]
<i>Agrilus planipennis</i>					2	0	2	[113]
<i>Photinus pyralis</i>					1	0	1	[113]
<i>Brassicogethes aeneus</i>					3	0	2	[113]
Diptera								
<i>Drosophila melanogaster</i>	0	0	0	1	2	1	3	[111]
<i>Anopheles gambiae</i>	2	0	0	1	1	1	5	[109]
<i>Aedes aegypti</i>	1	0	0	1	1	1	5	[109]
<i>Culex quinquefasciatus</i>	1			1	1	1	7	[109]
Lepidoptera								
<i>Heliconius melpomene</i>	1	2		1	2	1	1	[89]
<i>Danaus plexippus</i>	1	2		1	1	1	1	[89]
<i>Papilio glaucus</i>					1	1	4	[115]
<i>Bombyx mori</i>	1	1		1	1	1	2	[89]
<i>Manduca sexta</i>	1	2		1	1	1	1	[89]

Figure 3. Opsin diversity in representative insects. The arthropod opsin complement includes canonical c-opsins, canonical r-opsins, retinal pigment epithelium G protein-coupled receptor (RGR) and neuropsin [5]. Within arthropods, it is estimated that there were anywhere between 1 and 3 UV/B/LW rhodopsin, 1 c-opsin, 1 arthropsin and 1–2 peropsin before the Chelicerate split [107,108]. The ancestral insect eye was probably trichromatic through the use of an ultraviolet (UVRh), blue (BRh) and long-wavelength (LWRh) rhodopsin [6]. Empty boxes represent missing data. The numbers in the boxes show number of gene copies present in that species. Figure made using data from [7,78,100,101,109–116].

underlie sexual dimorphism in their compound eyes [98,125]. In *Pieris rapae*, males have a double peak in blue sensitivity; in *Lycaena rubidus* females, BRh is coexpressed with LWRh in R3-8 cells while in males, BRh has entirely replaced LWRh in the R3-8 cells in the dorsal region of the compound eye [98,125]. In both of these cases, the sexually dimorphic eye is believed to be important for male behaviour and possibly for vision in

the blue spectrum. A related pattern of BRh opsin expression is that of the honeybee *Apis mellifera*. Honeybee drones have different ommatidia and photoreceptor morphology in their dorsal eye region compared to workers [126]; at late pupal stages, drones express a larger proportion of UVRh and BRh compared to LWRh mRNAs while worker bees predominantly express LWRh in their developing compound eye [127].

Paleoptera (damselflies and dragonflies) has multiple independent expansions in the number of *BRh* copies. Damselfly and dragonfly adults are diurnal, brightly coloured, territorial and possess large eyes. Larvae are aquatic. In 12 species surveyed, all species had 1 to 8 *BRh* genes [7]. Opsin regional expression in adult eyes, ocelli and larval eyes varies between species [7]. In Odonata, *BRh* transcripts are divided into three subgroups. Interestingly, *BRh* from group a are upregulated in larval eyes, *BRh* from group b are upregulated in the ventral adult eye, and *BRh* from group c (which has the most duplications) are expressed in the dorsal region of the adult eye [7]. Comparative opsin studies in mayflies have not been as in-depth. *Cloeon dipterum* has two *BRh* opsin genes which are located in tandem; one copy is also specific to male turbinate eyes [112]. Variation in Paleoptera visual systems is speculated to be related to differences in behaviour, light ecology and microhabitat.

(c) Polarized light detection and paralogue switching in Orthoptera

Although *BRh*, to our knowledge, is not duplicated in Orthoptera, it is probably functioning in polarized light detection. Insects use polarized light for orientation and navigation (see reviews [128,129]). The specialized DRA found in many insects often expresses only *UVRh* to detect polarized light [23,128,130]. However, in Orthoptera, *BRh* is expressed in the DRA and hypothesized to be important for polarized light detection [131,132]. In the cricket, *Gryllus bimaculatus* *BRh* and *UVRh* are expressed in the DRA while in the desert locust *Schistocerca gregaria*, only *BRh* is expressed in all photoreceptor cells of the DRA [78,100]. This is an example of paralogue switching where Orthoptera have swapped *UVRh* typically found in most insect DRAs for the *BRh* paralogue. Studies thus far have shown that both *UVRh* and *BRh* can be used for polarized light detection. While *LWRh* is not typically used in polarized light detection, butterflies furnish an exception [86,89,133–135].

(d) Ultraviolet opsin duplications

UVRh duplications are rarer among surveyed insects compared to *BRh* and *LWRh* duplications. The mayfly *Cloeon dipterum* has four copies of *UVRh*, and one of the *UVRh* genes is only expressed in male-specific turbinate eyes [112]. *UVRh* duplications in the pea aphid (*Acyrtosiphon pisum*) and in beetles (order Coleoptera) appear to have evolved to overcome the loss of *BRh* [113,136]. A survey of 175 moths and butterflies found only two independent UV duplications, in *Triodia sylvina* (Hepialidae) and *Chilo suppressalis* (Crambidae), in addition to that of *Heliconius*, described below [8]. Another lepidopteran with multiple *UVRh* copies is the armyworm *Mythimna separata*. *Mythimna separata* has three *UVRh* genes but only one copy is expressed at a level similar to other visual opsin genes while the other two copies are very lowly expressed [137]. In Lepidoptera where opsin evolution has been extensively studied, the first discovery of a *UVRh* duplication was made in the butterfly genus *Heliconius*, famous for Müllerian mimicry [90]. In one of the duplicates, *UVRh2*, some of the sites evolved under positive selection within *Heliconius* compared to *UVRh1*, and in other systems, these sites are associated with spectral tuning [90]. Physiological recordings indicate *UVRh1* encodes

a UV receptor, while *UVRh2* encodes a violet receptor. Following duplication of *UVRh*, a remarkable diversity in opsin expression patterns, including several forms of sexually dimorphic UV opsin expression, has evolved within the genus [60]. These opsin expression pattern differences, filtering effects, and spectral tuning correspond to a diversity of photoreceptor cell sensitivities [57,59,60]. Recent behavioural experiments show that the two UV opsins are used in UV colour vision in foraging *Heliconius erato* females, while *H. erato* males, which express only one UV opsin, are colour blind to the same UV discrimination task [89].

8. The role of opsins in colour vision: physiology and behaviour

Studies of opsin presence, absence and expression establish an experimental framework connecting molecular evolution to expression level changes, photoreceptor sensitivity, and colour visual behaviour. In *Heliconius* butterflies, UV opsin duplication and divergence [90] led to sexually dimorphic expression differences and photoreceptor cell sensitivities [59,60], which has resulted in differences in adult colour visual behaviour in the UV [89]. Transcriptomics and genomics have been used to identify *UVRh*, *BRh* and *LWRh* genes while electroretinograms, microspectrophotometry and intracellular recordings have been used to determine eye and photoreceptor cell spectral sensitivities [85]. However, in only a limited number of species and orders have opsins been connected molecularly to sensory neuron phenotype, and in even fewer to organismal behaviour (figure 2).

(a) *In vitro* expression systems to study insect visual pigment absorption spectra

A major contribution to the field of visual ecology is the refinement of an *in vitro* insect opsin-expressing system which permits the measurement of their absorption spectrum [9]. Vertebrate ciliary-type opsins are often expressed and purified in *in vitro* systems, and this has greatly aided in understanding the specific contribution of opsin absorbance to photoreceptor cell physiology, especially when *in vivo* electrophysiology is a challenge [138,139]. These systems also allow for targeted mutagenesis experiments to measure the effect on opsin absorbance of spectral tuning site changes [140]. Although insect rhabdomeric-opsins are ancestrally related to vertebrate opsins, *in vitro* expression systems have proven challenging [81,141] (but see [87,142]). The new expression system established by Liénard *et al.* [9] should greatly facilitate future research on insect opsin phenotypes.

(b) A need for behavioural studies

Behavioural studies are crucial for addressing the link between opsin genotype and phenotype yet continue to be relatively rare. Among hymenopterans, ants are believed to have UV, LW and possibly B photoreceptors. Yet, behavioural studies have found them to be dichromatic, where some are capable of discriminating between UV and green wavelengths of light, while others can discriminate UV from blue, and UV from green, but not between blue and green [143–145]. In Lepidoptera, behavioural studies have found that gene duplications and filtering pigments drive shifts in

butterfly sensitivities allowing them to discriminate in the UV, blue, green and red [86,88,89,133]. It is clear from available evidence that there is a direct relationship between opsin genes and phenotypes, but it is important to remember that much of what we know about insect opsins is based on limited sequence data, and that much work remains in order to build a more complete picture of opsin and eye evolution in insects.

9. Concluding remarks

Colour visual systems with three–four colour receptor channels should be sufficient to adequately sample over the entire visual spectrum in bright daylight. Nonetheless cycles of opsin duplications occur, leading to increased numbers of colour channels in the visual systems of insects. The number of channels subsequently gets pruned, which explains the high level of turnover we see among opsins throughout insect evolution [146]. Insects that make transitions between light environments, such as fireflies, diving beetles and some moths, have additional selective constraints shifting opsin repertoires in predictable ways [8,147,148]. However we still do not fully understand the selective

pressures for particular sensory arrangements and further physiological and behavioural studies are needed to better understand the role of selection and drift in the evolution and diversity of sensory systems. Lepidoptera and Hymenoptera are rich with examples of unique visual ecology and evolutionary transitions such as switches from nocturnal to diurnal flying, and these transitions change opsin repertoires [8,149]. With CRISPR/Cas9 genome editing becoming more accessible in these insect orders, and with refinements of *in vitro* opsin expression systems, investigating opsin spectral tuning, expression and gene regulation in these classical visual ecology models will be a fruitful avenue for future research.

Data accessibility. This article has no additional data.

Authors' contributions. K.J.M.: conceptualization, data curation, formal analysis, writing—original draft, writing—review and editing; A.D.B.: conceptualization, funding acquisition, writing—review and editing; A.M.-M.: conceptualization, formal analysis, writing—original draft, writing—review and editing.

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