Integrative and Comparative Biology

Integrative and Comparative Biology, volume 56, number 5, pp. 809–819
doi:10.1093/icb/icw093

Society for Integrative and Comparative Biology

SYMPOSIUM

Simple Eyes, Extraocular Photoreceptors and Opsins in the American Horseshoe Crab

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From the symposium "Extraocular, Nonvisual, and Simple Photoreceptors" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2016 at Portland, Oregon.

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Synopsis The eyes and photoreceptors of the American horseshoe crab *Limulus polyphemus* have been studied since the 1930s, and this work has been critical for understanding basic mechanisms of vision. One of the attractions of *Limulus* as a preparation for studies of vision is that it has three different types of eyes—a pair of later compound, image-forming eyes and two types of simple eyes, a pair of median ocelli, and three pair of larval eyes. Each eye type is tractable for experimentation. *Limulus* also has extraocular photoreceptors in its segmental ganglia and tail. The current contribution focuses on photoreceptors in *Limulus* larval eyes and ocelli and its extraocular photoreceptors with the goal of summarizing what is currently known and not known about their physiology and function and the opsins they express. The *Limulus* genome encodes a surprisingly large number of opsins (18), and studies of their expression pattern have raised new questions about the role of opsin co-expression, the functions of peropsins expressed outside of eyes, and the physiological relevance of opsins with apparently very low expression levels. Studies of opsin expression in *Limulus* lead one to wonder whether photoreceptors yet to be discovered might be present throughout its central nervous system.

Introduction

The visual system of the American horseshoe crab *Limulus polyphemus* Linnaeus 1758 has been studied extensively beginning in the 1930s (Hartline and Graham 1932). This work has produced an extensive literature describing the structure of its eyes and photoreceptors (e.g., Jones et al. 1971;Fahrenbach 1975; Calman and Chamberlain 1982; Herman 1991) and the physiology of its photoreceptors that has been critical for our understanding of basic mechanisms of vision including phototransduction (e.g., Brown et al. 1984; Payne et al. 1986), light and dark adaptation (e.g., Lisman et al. 2002), visual information processing (Hartline et al. 1956), and the effects of circadian rhythms on visual function (reviewed in Battelle 2013). *Limulus polyphemus*, hereafter referred to as *Limulus*, is a xiphosauran chelicerate, the sister group to arachnids (Regier et al. 2010; Edgecombe and Legg 2014); therefore, investigations of its visual system may also provide insights into visual functions of the (eu)chelicerate ancestor (Nilsson and Kelber 2007) and contribute to our understanding of the evolution of vision in arthropods.

Limulus, has three different types of eyes: a pair of image-forming lateral compound eyes, a pair of median ocelli, and three pair of larval eyes—lateral, median, and ventral. Median ocelli and larval eyes do not form images and are considered simple eyes. In addition, extraocular photoreceptors have been identified in its segmental ganglia and tail (Fig. 1). The compound eyes were an early focus of study, and their structure and function are understood in considerable detail (Smith and Baumann 1969; Fahrenbach 1975, Battelle 2006). The present contribution focuses on the simple eyes and extraocular photoreceptors with the goal of summarizing current knowledge of their structure and function, the impact of illuminating each on behavior and their visual pigments.

Advanced Access publication July 21, 2016

Structure and function

Larval eyes

All three pair of larval eyes develop in embryos before the lateral compound eyes and median ocelli, and they persist in adults. Each larval eye consists of a cluster of two types of photoreceptors—giant photoreceptors measuring about 150 μm long and 70 μm wide in adults and smaller photoreceptors about half the size of the giants (Calman and Chamberlain 1982; Herman 1991). The giant photoreceptors are sensitive to visible light with peak sensitivity at about 520–525 nm (Millecchia et al. 1966); the smaller photoreceptors are UV-VIS cells, meaning they show two peaks of photosensitivity, one in the UV at about 320 nm and a second in the visible range at about 520 nm (Battelle et al. 2014).

In adults, lateral larval eyes are located at the posterior edge of each lateral compound eye, and median larval eyes are beneath the carapace between the median ocelli. Ventral larval eyes consist of a pair of optic nerves that extend anteriorly from the brain and terminate in an end organ that is attached to a specialized region on the ventral cuticle. This specialized region is devoid of pigment and has a small lens-like structure over each end organ (Patten 1894 and personal observations) (Fig. 1B). Photoreceptors in adult ventral eyes cluster in the end organ and at the posterior ends of the optic nerves on the brain. They are also scattered along the lengths of the optic nerves. Since lateral and median larval eyes are beneath the carapace in adults, it is not clear whether in adults they receive significant light to respond to and influence behavior. However, photoreceptors in the ventral larval eyes clearly respond to light in adult animals. Light responses have been recorded from the ventral eye end organs of intact adults (Kass and Renninger 1988), and light-driven rhabdom shedding has been observed in adult ventral larval eye photoreceptors *in vivo* under conditions of natural illumination (Katti et al. 2010). In naturally behaving animals, ventral

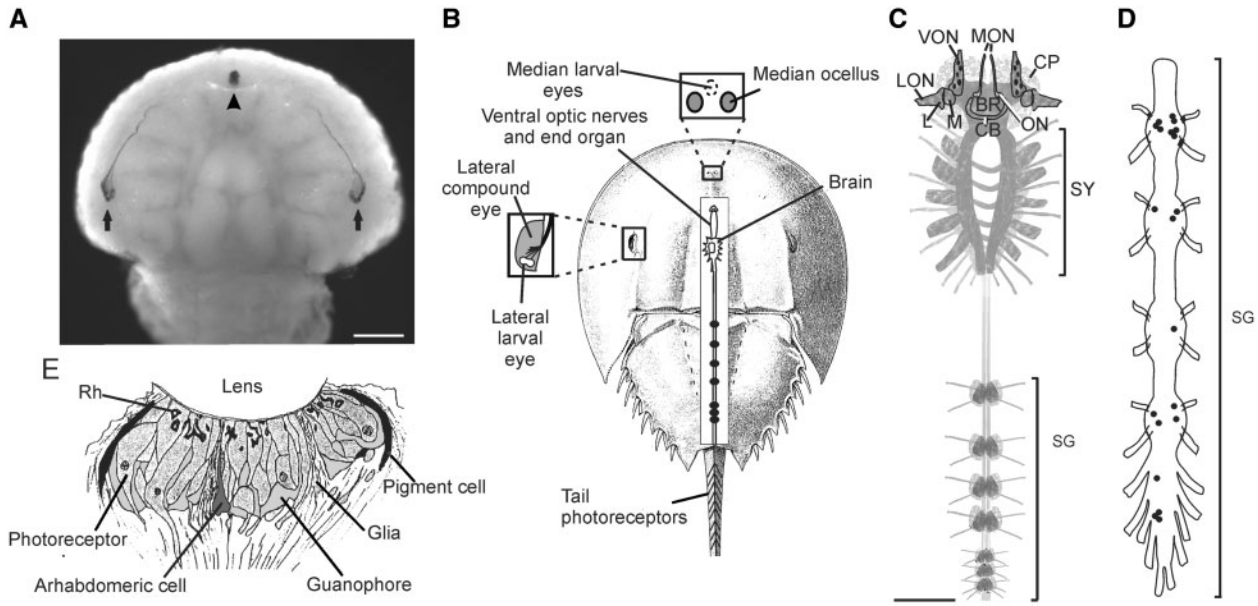

Fig. 1 Locations of *Limulus* eyes, CNS, and extraocular photoreceptors and the structure of it CNS and median ocelli. (**A**) *Limulus* embryo early following its fourth embryonic molt immunostained for visual arrestin showing well developed lateral larval eyes with photoreceptor axons extending toward the brain (arrows) and fused median larval eyes (arrow head). The lateral larval photoreceptors and their axons surround a region where the lateral compound eyes will later develop. Scale bar, 250 μm. Modified from Harzsch et al. (2006) with permission. (**B**) Schematic of a dorsal view of an adult *Limulus* showing the locations of the lateral compound eyes, median ocelli, and median and lateral larval eyes. The central cut-away shows the locations of the brain, ventral optic nerves and end organ, synganglion, and segmental ganglia. Presumed locations of photoreceptors along the length of the tail are indicated. (**C**) Schematic of a dorsal view of the central nervous system from a juvenile animal measuring 2–2.5 cm across the prosoma showing structures visible on the dorsal brain. BR, brain; CB, central body; CP corpora pedunculata; L, lamina; LON, lateral optic nerve; M, medulla; MON, median optic nerve; ON, ocellar neuropile; SG, segmental ganglia; SY, synganglion; VON, ventral optic nerve. Scale bar, 1 mm. (**D**) Schematic of a ventral view of segmental ganglia (SG) from an adult showing locations of 20 photoreceptive neurons. Redrawn from Mori et al. (2004) with permission. (**E**) Schematic of a longitudinal section through a median ocellus showing clusters of photoreceptors, arhabdomeric cells, and guanophores separated by glial partitions. Rhabdomeres are near the base of the lens. Rh, rhabdom. Modified from Battelle et al. (2014) after Jones et al. (1971).

larval eyes can be exposed to light whenever the animal moves on its walking legs, swims—it swims upside down—or becomes inverted on the beach while spawning. Illuminating the ventral larval eyes of adults can also phase-shift the animal's circadian clock (Horne and Renninger 1988). The latter finding provides clear evidence that the ventral larval eyes function in adults and can influence behavior.

The relative importance of larval eyes for light-driven behaviors in adults, compared with the lateral and median eyes, can be debated, however, larval eyes probably provide the major photic input to embryos and newly hatched larvae. During development, *Limulus* undergo four embryonic molts before they hatch as free-swimming larvae called trilobite larvae (Sekiguchi et al. 1982). Using photoreceptor-specific antibodies, photoreceptors in lateral eye ommatidia and median ocelli are first detected late during the trilobite larval stage (Harzsch et al. 2006). By contrast, photoreceptors in lateral and median larval eyes are detected soon after the first embryonic molt. Following the second embryonic molt, lateral larval eye photoreceptors and their axons partially surround an area where ommatidia of the compound eye appear later (Fig. 1A) (Harzsch et al. 2006; Blackburn et al. 2008).

It is not known how early in development larval photoreceptors begin to respond to light. Light responses from the lateral eye region were detected with electroretinogram recordings at the time of hatching (French 1980), which is before ommatidia of the lateral compound eye develop, but larval photoreceptors probably respond to light earlier. Arrestin protein is detected in larval photoreceptors soon after the third embryonic molt indicating that at least some proteins required for the photoresponse are expressed quite early (Harzsch et al. 2006). It is also not known how early in development photoreceptor axons from the lateral and median larval eyes reach the brain, but their projections to brain target areas (Fig. 2) are in place between the third and fourth embryonic molts (Blackburn et al. 2008 and N. L. Brown and BA. Battelle, unpublished data). Ventral larval eye development was not studied during very early embryonic stages, but ventral larval photoreceptors are detected on the anterior brain between the third and fourth embryonic molts (N. L. Brown and BA. Battelle, unpublished data). Each of the larval eyes is also innervated by efferent neurons that are driven by a central circadian clock. These efferents fire action potentials only during the subjective night. In response to this input, ventral larval eyes, and presumably also median and lateral larval eyes, show increased sensitivity to light at night (reviewed in Battelle 2002).

Larval photoreceptors respond to light stimuli with graded depolarizations, and only graded potentials are conducted by their axons (Millecchia and Mauro 1969). This raises the question of whether signals from larval photoreceptors reach the brain. Their graded potentials probably do reach the brain in embryo, larvae, and young juveniles when their optic nerves are short. Furthermore, larval photoreceptor axons have large diameters and low axial resistance. There is some evidence that graded potentials from larval photoreceptors reach the brain even in adults (Behrens and Fahy 1981).

The central questions of whether and how visible light influences the development or behavior of embryos have been addressed in only preliminary studies (French 1980), but these showed that the time to hatching is accelerated among animals reared in a 12:12 light-dark cycle compared with those reared in constant light or constant darkness, and that more animals hatched during the light phase of the 12:12 light-dark cycle compared with the dark phase. Thus, visible light may be one of many important environmental cues influencing *Limulus* embryonic development and hatching (Ehlinger and Tankersley 2003). When *Limulus* hatch and become free-swimming trilobite larvae, the larval eyes are still their only eyes. Because larval eyes are located on different

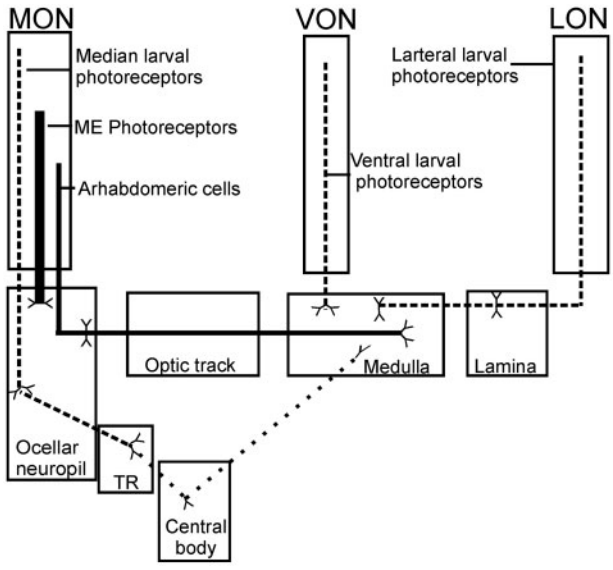

Fig. 2 Summary diagram of central projections from larval eyes and median ocelli. LON, lateral optic nerve; ME, median eye; MON, median optic nerve; TR, terminal region for axons of the median larval photoreceptors; VON, ventral optic nerve. Based on Chamberlain and Barlow (1980); Calman et al. (1991); Harzsch et al. (2006).

regions of the animal's body—dorsal-lateral, dorsal-anterior, and ventral (Fig. 1)—and each eye projects to a different region of the brain (Fig. 2), it seems reasonable to speculate that they provide directional information to the newly hatched animals.

Median ocelli

Based on the shape of the lens and organization of the underlying retina, median ocelli are thought not to form images but to provide directional information (Fahrenbach 1975). The large size of the lens aperture compared with that of the underlying pseudo-ommatidia (see below) suggests that the eye is particularly adapted for operating in low light levels, and electrophysiological and behavioral studies suggest median ocelli are particularly important for detecting UV light (Nolte and Brown 1972; Lall and Chapman 1973).

Each ocellus has a single, weakly converging lens below which lay elongated photoreceptors with convoluted rhabdomeres positioned close to the base of the lens (Fig. 1E). The retina consists of a number of loosely organized cell clusters, called pseudo-ommatidia containing UV light-sensitive and visible light-sensitive photoreceptors, quanophores—cells containing reflective crystals of guanine—and usually one arhabdomeric cell. These cell clusters are separated from one another by partitions of pigment cells and glia. Glial processes also penetrate photoreceptors and line the base of the retina (Jones et al. 1971; Fahrenbach 1975). Within each cell cluster the relative number of visible light- compared with UV light-sensitive photoreceptors vary widely, but electrophysiological recordings show that on average 70% of photoreceptors are maximally sensitive to UV light at 360 nm (Nolte and Brown 1972). Peak sensitivity of the visible light photoreceptors is 520–525 nm, like the photoreceptors in the larval eyes, but their overall spectral sensitivity is different from that recorded from larval photoreceptors (Nolte and Brown 1970) hinting that the visible light-sensitive opsins expressed in median eyes and larval eyes are different. No cells with dual sensitivity to UV and visible light were detected (Nolte and Brown 1970, 1972). Like photoreceptors in larval eyes, photoreceptors in median ocelli generate graded depolarizations when stimulated with light, and although the photoreceptors project axons to the brain (Fig. 2), arhabdomeric cells are thought principally responsible for transmitting visual information from the ocelli to the brain (Nolte and Brown 1972). Arhabdomeric cells are electrically coupled to photoreceptors and generate action potentials (spikes) when photoreceptors depolarize. Their axons project to the ocellar neuropil, through the optic track and to the medulla (Fig. 2). Interestingly, arhabdomeric cells are electrically coupled only to UV photoreceptors (Nolte and Brown 1972).

Illuminating median ocelli with UV light produces a positive phototactic response in adult animals whereas iIlluminating median ocelli with visible light produces no phototactic response (Lall and Chapman 1973). The significance of the behavioral response to UV light is not clear, but because UV light attenuates sharply with water depth, it is speculated that *Limulus* use their median ocelli as depth detectors to help guide them to shallow water and beaches for spawning (Lall and Chapman 1973). In addition, illluminating median ocelli with UV light at night increases the sensitivity of the lateral compound eye to light (Westerman and Barlow 1981; Herzog and Barlow 1991). The central circuitry underlying this phenomenon is not understood, but electrically stimulating afferents from median ocelli increases the activity of clock-driven efferent neurons innervating the lateral compound eyes (Kass and Barlow 1992), and this input is known to increase lateral eye sensitivity at night (Barlow et al. 1977; Barlow 1983). *Limulus* use their lateral compound eyes to find mates (Barlow et al. 1982). Therefore, UV light reflected from the moon at night and detected by the median ocelli may enhance nighttime spawning success.

Effects of visible light on the output of median ocelli are less well understood. Although no behavioral responses have been reported in response to illuminating median ocelli with visible light, when long wavelength light is applied during a prolonged UV stimulus, spike activity in arhabdomeric cells is suppressed (Nolte and Brown 1972). Thus, visible light may modulate the UV light-driven output from ocelli. In addition, illuminating median ocelli with visible light can phase-shift the animals' circadian clock (Horne and Renninger 1988).

Extraocular photoreceptors

Extraocular photoreceptors have been detected with electrophysiological approaches in each segmental ganglion and in the tail (Fig. 1B,D) (Hanna et al. 1988; Mori and Kuramoto 2004; Mori et al. 2004). In isolated preparations of ventral nerve cord, spontaneous activity of motor neurons projecting out segmental nerves is modulated by illuminating the ganglia (Mori and Kuramoto 2004). Intracellular recordings (Mori et al. 2004) suggest that photoreceptors represent less than 2% of cells in segmental

ganglia, that they are maximally sensitive to light at 425 nm or below, that they fire spontaneous action potentials in the dark, and that their rate of firing increases with light intensity. Filling these cells with dye typically revealed a roughly 100 μm diameter soma with a single axon projecting to the contralateral side of the nerve cord and then longitudinally in the nerve cord in one or both directions. Motor neurons projecting out segmental nerves innervate the heart, viscera, and gills; therefore, it is speculated that light impinging on photoreceptors in segmental ganglia modulates the activity of these organs (Mori et al. 2004).

Evidence for tail photoreceptors comes from studies showing that illuminating the tail with broad spectrum visible light can phase-shift animals' circadian clock (Hanna et al. 1988). These same studies showed that photosensitivity is apparently distributed along the length of the tail, but tail photoreceptors have not been identified, and their spectral tuning has not been characterized.

Opsin expression

The results of spectral recordings from characterized *Limulus* photoreceptors (Table 1) suggested that *Limulus* expressed a relatively limited number of opsins: 1. A UV light-sensitive opsin expressed in median ocelli and larval eyes (Nolte and Brown 1970; Battelle et al. 2014). 2. A long wavelength-sensitive (LWS) opsin expressed in both the lateral compound eye and larval eyes (Nolte and Brown 1970). 3. A LWS sensitive opsin expressed in median ocelli that is different from the opsin expressed in larval and lateral compound eyes (Nolte and Brown 1970). 4. A short wavelength-sensitive opsin expressed in segmental ganglia (Mori et al. 2004). 5. A visible light sensitive opsin expressed in the tail with unknown spectral sensitivity (Hanna et al. 1988). Therefore, it was a surprise to discover 18 opsin genes in the *Limulus* genome (Battelle et al. 2016) (Fig. 3B; Supplementary Table S1). All *Limulus* opsins have been characterized and their distributions in eyes, central nervous system (CNS), and tail have been examined (Table 1, Fig. 3).

Limulus opsins cluster among three of the four major opsin classes (Battelle et al. 2016). Fourteen are R-type opsins. These include: seven LWS opsins (LpOps1-4 and 6-8); an opsin (LpOps5) that clusters among median wavelength sensitive opsins in phylogenetic analyses but which has a maximum sensitivity indistinguishable from that of LWS LpOps1-4 (Battelle et al. 2014); a UV opsin (LpUVOps1), three UV-short wavelength sensitive (SWS) opsins (LpUVOps 2, LpOps 9, and 10), which cluster among UV7-type opsins and two arthopsins (LpArthOps1 and 2). In addition there are two C-type opsins (LpCOps1 and 2) and two RGR/Go-type or Group 4 opsins (peropsins LpPerOps1 and 2).

Table 1. Maximum spectral sensitivities of *Limulus* photoreceptors identified with electrophysiological recordings, the opsin they are known to express and other opsins expressed in the same tissue.

Photoreceptive tissue	Photoreceptor type	Maximum sensitivity in nm	Opsins confirmed[a] in identified photoreceptors	Other opsins expressed in the tissue detected by RT-PCR	References
Lateral eye	**Retinular cell**	**520–525**	**LpOps1-4, 5**	**LpOps9, PerOps1, UVOps1**	Battelle et al. (2014, 2015, 2016); Katti et al. (2010); Nolte and Brown (1970); and. Smith et al. (1993).
Larval eyes	Giant	**520–525**	**LpOps1-4, 5**	**LpOps9, UVOps2; ArthOps1; PerOps1**	Battelle et al. (2001); Battelle et al. (2014, 2015, 2016); Katti et al. (2010); and Millecchia et al. (1966)
	Smaller	**520–525 and 350**	**LpOps5, UVOps1**		
Median ocelli	**Visible light sensitive**	**520–525**	**LpOps7,8,9**	**LpOps1-4, 5, 9, PerOps1**	Battelle et al. (2014, 2015, 2016); Katti et al. (2010); Nolte and Brown (1970); and Smith et al. (1993).
	UV light sensitive	**350**	**LpUVOPs1**		
Segmental ganglia	**100 μm neurons**	**425 or below**	**None**	**LpOps1-4,5,9 UVOps2, Arthops1, PerOps1, COps1,2**	Battelle et al. (2016); Mori and Kuramoto (2004); and Mori et al. (2004);
Tail	**Unknown**	**Visible light sensitive Maximum Sensitivity Unknown**	**None**	**LpOps1-4,9,10, UVOps2, ArthOps1, PerOps1, COps1,2**	Battelle et al. (2016) and Hanna et al. (1988)

[a]Opsin expression confirmed by *in situ* hybridization or immunocytochemical assays.

LpOps1-4 genes are considered a set because the coding regions of their transcripts are 99% identical to one another, and their gene products cannot be distinguished from one another using *in situ* hybridization or immunocytochemical assays (Dalal et al. 2003). LpOps1-4 genes are located on the same scaffold and may have resulted from tandem duplication events (Battelle et al. 2016). Other opsins appear to be paralogous pairs (LpOps6 and 7; LpUVOps2 and LpOps9; LpArthops1 and 2; LpCOps1 and 2; LpPerOps1 and 2) (Battelle et al. 2016) and may be products of a whole-genome duplication (Nossa et al., 2014; Kenny et al., 2015).

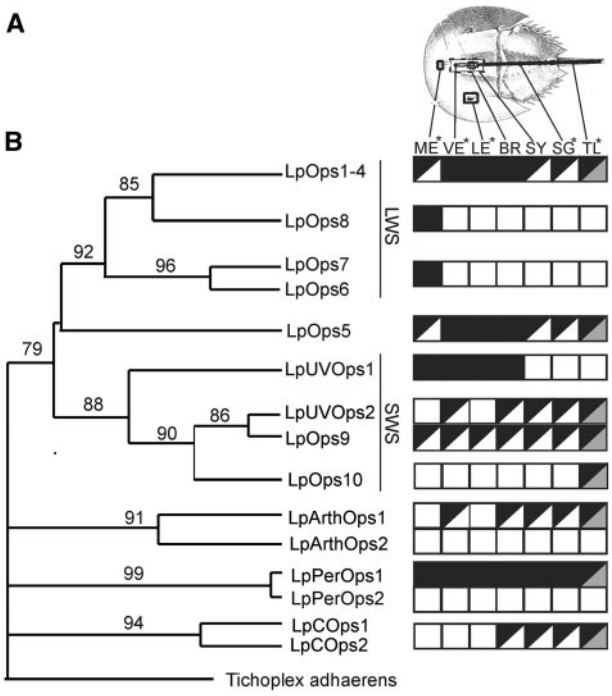

Fig. 3 *Limulus* opsins and their expression pattern. (**A**) Schematic of *Limulus* showing the tissues assayed for opsin expression. (**B**) Left. Phylogenetic tree of *Limulus* opsins constructed using a maximum likelihood analysis of amino acid sequences (Phylogen.fr platform: http://www.phylogeny.fr/simple_phylogeny.cgi) with *Trichoplex adhaerens* opsin (Accession number EDV21947) as the out group. Accession numbers for the *Limulus* opsins are provided in Supplementary Table S1. Numbers on branches are aLRT (approximate likelihood-ratio test) values for nodes supported by greater than 70%. *Limulus* opsins that cluster among other arthropod long wavelength sensitive (LWS) and UV-short wavelength-sensitive (UV-SWS) are indicated. (B) Right. Tissue distributions of opsins shown at the left. Tissues assayed are listed at the top. (*) Tissues in which photosensitive cells have been detected using electrophysiological techniques (see text). Solid boxes, opsin detected by RT-PCR and *in situ* hybridization/immunocytochemistry. Black/white boxes, opsin detected by RT-PCR but not by *in situ* hybridization/immunocytochemistry. Black/gray boxes, opsin detected by RT-PCR but not assayed with *in situ* hybridization/immunocytochemistry. Solid white box, transcript not detected by RT-PCR. A single set of boxes is shown for opsin paralogs with the same distribution. ME, median eye; VE, ventral eye; LE, lateral eye; BR brain; SY, synganglion; SG, segmental ganglia; TL, tail. Data from Battelle et al. (2016).

Of particular interest for the present discussion are the tissue and cellular distributions of these opsins and how their expression pattern might enhance our understanding of ocular and extraocular photoreception. In a series of studies, the tissue expression pattern of each opsin was assayed with RT-PCR, and their cellular distributions in eyes and CNS was examined with *in situ* hybridization assays. Immunocytochemistry was also applied to search for LpOps1-4, 5, 6, UVOps1, and PerOps1 (Smith et al., 1993; Katti et al.,2010; Battelle et al., 2014; 2015; 2016). The results of this work are summarized in Table 1 and Fig. 3. The cellular distribution of opsins in the tail could not be examined because of the difficulty of applying morphological techniques to the tail.

Tissue distribution

Transcripts encoding each of the opsins were detected by RT-PCR in the CNS dissected from young juveniles between their first and second juvenile molts, in adult eyes, in the CNS from adults and older juveniles measuring 2.5–3.5 cm across the prosoma, or in the tails from older juveniles, with one exception. LpPerOps2 transcripts were not detected in any tissues assayed. RT-PCR assays showed that the expression of some opsins is tissue specific. LpOps6, 7, and 8 were detected only in the median ocelli and LpOps10 only in the tail. Transcripts for other opsins were detected in multiple tissues. LpOps1-4, 5, 9, and PerOps1 were detected in all tissues assayed; LpUVOps2 and Arthops1 in all tissues except the lateral compound eyes and median ocelli; LpCOps1 and 2 throughout the CNS and in the tail but not in the eyes; LpUVOps1 in each eye type and the brain but not in other CNS tissues or the tail. LpArthOps2 was detected in the CNS of young juveniles but not in any tissues from older animals; thus, LpArthOps2 may be expressed only early in development (Battelle et al. 2016).

Cellular distribution

In situ hybridization and immunocytochemical assays further clarified opsin expression patterns but also raised new questions. These assays revealed that most photoreceptors in the simple eyes express more than one opsin. Furthermore, some opsin transcripts detected by RT-PCR in a tissue were not detected with *in situ* hybridization assays, and the

encoded proteins were not detected by immunocytochemistry. Where opsin transcripts are not detected by *in situ* hybridization the assumption is that their expression level is low.

*Simple eyes.*The cellular distribution of opsins in larval eyes was examined in adult ventral larval eyes, which are considered representative for all three pairs of larval eyes (Fahrenbach 1975; Harzsch et al. 2006; Battelle et al. 2014). In the ventral larval eyes, RT-PCR assays detected transcripts for 10 different opsins: Lp1-4, 5, 9, UVOps1 and 2, Arthrops1 and PerOps1 (Dalal et al. 2003; Katti et al. 2010; Battelle et al. 2015, 2016). *In situ* hybridization and immunocytochemical assays confirmed the expression of Lp1-4, 5, UVOps1 and PerOps1, and immunocytochemistry showed co-expression of LpOps1-4 and 5 in giant photoreceptors and LpOps5 and UVOps1 in the smaller photoreceptors. LpPerOps1 expression was confirmed by *in situ* hybridization and immunocytochemistry in glia surrounding photoreceptors (Battelle et al. 2001; Katti et al. 2010; Battelle et al. 2014, 2015). By contrast, transcripts encoding LpOps9, UVOps2, and ArthOp1 were not detected in the ventral eyes with *in situ* hybridization assays (Battelle et al. 2016).

In the median ocelli, RT-PCR assays detected transcripts for 11 different opsins: LpOps1-4, 5, 6, 7, 8, 9, UVOps1, and PerOps1 (Smith et al. 1993; Katti et al. 2010; Battelle et al. 2014, 2015, 2016). Combined *in situ* and immunocytochemical assays revealed that a majority of photoreceptors express LpUVOps1, the same UV opsin expressed in larval eyes, and that the remaining photoreceptors co-express the LWS opsins LpOps6, 7, and 8. No photoreceptors co-express LpUVOps1 and a visible light sensitive opsin (Battelle et al. 2015). These data are consistent with the electrophysiological studies described above showing that the majority of photoreceptors in the median ocelli are sensitive to UV light, that the visible light sensitive photoreceptors contain photopigment(s) different from that found in the larval eyes and that there are no photoreceptors with dual sensitivity to UV and visible light. LpPerOps1-expressing glia were identified surrounding the retina, in partitions between pseudo-ommatidia and between photoreceptors (Battelle et al. 2015). As was the case in the larval eyes, a number of opsin transcripts detected in the median ocelli by RT-PCR were not detected by *in situ* hybridization (LpOps1-4, 5, and 9). Furthermore, LpOps1-4 and 5 proteins were not detected in the median ocelli by immunocytochemistry using antibodies that clearly detected these opsins in ventral larval eyes (Battelle et al. 2014, 2016).

Central nervous system. All 10 opsin transcripts detected in the ventral larval eyes by RT-PCR were also detected in the brain. This was expected because some ventral photoreceptor cell bodies are located on the brain. Indeed, *in situ* hybridization assays of brain whole mounts confirmed the expression of LpOps1-4, 5, and UVOps1 transcripts in ventral photoreceptor cell bodies located on the brain (Battelle et al. 2016), and LpPerOps1 transcripts in cells associated with ventral photoreceptors (Fig. 4; Battelle et al. 2016). LpOps1-4 transcripts were also observed in axons of lateral eye photoreceptors where they enter the brain (Fig. 1C), but no cell bodies or processes elsewhere in the brain stained positively for LpOps1-4, 5, or UVOps1 transcripts, and only ventral photoreceptor cell bodies stained positively for LpOps1-4, 5, and UVOps1 proteins (Battelle et al. 2016). Thus, LpOps1-4, 5, and UVOps1 transcripts in the brain can be fully explained as originating from photoreceptors in the eyes. Since LpUVOps1 is not detected elsewhere in the CNS, this opsin may be eye specific.

Since LpOps9; LpUVOps2 and LpArthOp1 transcripts are also present in the ventral larval eyes, ventral photoreceptors may also be the source of these transcripts detected in the brain. But as in the ventral larval eyes themselves, transcripts for these opsins were not detected with *in situ* hybridization assays in ventral photoreceptors located on the brain, nor were they detected in cells elsewhere in the brain. LpCOps1 and 2 transcripts in the brain cannot be explained as originating from eyes, yet no LpCOps1-or 2-expessing neurons or process were detected in the brain (Battelle et al. 2016). The only opsin transcripts detected in the brain by *in situ* hybridization that have a cellular distribution which cannot be fully explained by an association with eye photoreceptors are those encoding LpPerOps1. In addition to being in cells surrounding ventral photoreceptor cell bodies, LPerOps1 transcripts were detected at the periphery of lateral optic nerves and in what appear to be processes in the central body (Fig. 4A).

In the synganglion and segmental ganglia, RT-PCR assays revealed transcripts encoding the same complement of 11 opsins (Fig. 4B); however, *in situ* hybridization and immunocytochemistry assays failed to confirm expression of any of these opsins in neurons. Only LpPerOps1 transcripts were detected with *in situ* hybridization. These were found associated with neuronal clusters between the large nerves projecting into the periphery, and with two or three bilateral neuronal clusters in each segmental ganglion (Fig. 4B,C). Immunocytochemistry confirmed that in

both tissues LpPerOps1 is expressed in glia surrounding neurons (Battelle et al. 2016). All opsin transcripts detected by RT-PCR in the synganglion and segmental ganglia were also detected in the tail. In addition, LpOps10 transcripts were detected in the tail. Thus, LpOps10 appears uniquely expressed in tail (Battelle et al. 2016).

Unanswered questions

What is the impact of simple eyes and extraocular photoreceptors on animal behavior?

As was discussed above, illuminating the ventral larval eyes in adult animals can influence the phase of the animal's circadian clock. Except for this effect on circadian rhythms, nearly nothing is known about the importance of larval eyes for the behavior of adults. Although larval eyes appear early in development, the influence of visible light on the behaviors of embryo and newly hatched larvae remains largely unexplored, and the relative importance of UV light has not been investigated. Photoreceptors in all larval eyes probably respond to light for at least several years post-hatching because juveniles remain only lightly pigmented for at least several years. But again, the functional relevance of illuminating larval eyes in older juveniles is completely unknown.

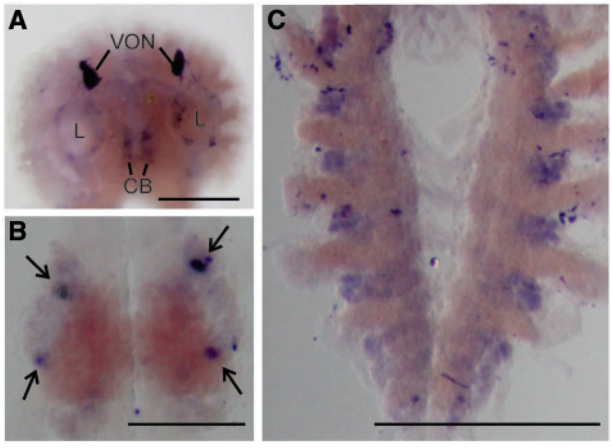

Fig. 4 Distribution of LpPerOps1 transcripts in CNS. LpPerOps1 transcripts were detected by *in situ* hybridization in brain, synganglion, and segmental ganglia of older juveniles. Representative whole-mount of the CNS of an older juvenile incubated with antisense probe targeting LpPerOps1 transcripts. (**A**) Brain, dorsal view. Transcripts were detected in the ventral optic nerve (VON), in cells surrounding the lateral optic nerve (L) and in the central body (CB). Scale bar, 0.5 mm. (**C**) Synganglion, ventral view. Transcript was associated with cell clusters located between large nerve roots projecting to the periphery. Scale bar, 0.5 mm. (**B**) Third segmental ganglion, ventral view. Transcript was typically associated with two, sometimes three, bilateral cell clusters (arrows) in each segmental ganglion. Scale bar, 0.25 mm.

Photoreceptors in ventral larval eyes clearly respond to light in adults, and since these are the only photoreceptors on the ventral surface they may provide important directional information, but this has not been tested.

Illuminating median ocelli with UV light produces two clearly documented functional responses: positive phototaxis during the day, and during the night, an increase in the sensitivity of the lateral compound eyes. Although projections from ocellar photoreceptors and arhabdomeric cells in the brain are known, the central circuits that give rise to these responses are not known. Furthermore, the role of visible light photoreceptors in median ocelli remains an enigma except for their ability to phase shift the animal's circadian clock (Hanna et al. 1988).

Little is known about the photoreceptors in segmental ganglia or their function. It is not yet known where their photosensitivity resides, dendrites or soma, how their activity influences motor neurons or what specific visceral functions (if any) are influenced by light. In adults, it is not clear whether light reaches segmental ganglia with sufficient intensity to stimulate these photoreceptors; they may have a greater role in the physiology of the nearly transparent juveniles. Although many opsin transcripts were detected in segmental ganglia, the opsin(s) responsible for the photosensitivity of these cells is not known. Since the cells are maximally sensitive to short wavelength light, LWS LpOps1-4 and LpOps5 are not candidates, but a number of other possibilities remain: LpOps9, Arthop1, and COps1 and 2.

Even less is known about photoreceptors in the tail. The tail is clearly photosensitive along its full length, and illuminating the tail can phase-shift the animal's circadian clock (Hanna et al. 1988), but neither the photoreceptors responsible nor their spectral sensitivity have been characterized. Also unknown is the nature of the central circuitry underlying the effects of illuminating the tail on the animal's central circadian clock, which is located in the brain (Kass and Barlow 1992). Any of the many opsins expressed in the tail may contribute to its photosensitivity, but LpOps10 is particularly interesting because its expression appears tail specific (Battelle et al. 2016).

What is the functional relevance of opsin co-expression? Of opsins that are expressed at very low levels? Of the broad expression of LpPerOps1?

Where co-expressed opsins have different spectral sensitivities, they presumably broaden the spectral sensitivity of the photoreceptor. For example, the

smaller photoreceptors in larval eyes described above express LWS LpOps5 and LpUVOps1, and they are sensitive to both visible and UV light. It is more difficult to explain the functional relevance of co-expression where the opsins have the same spectral sensitivity, as in the giant photoreceptors of larval eyes that co-express LpOps1-4 and 5 (Katti et al. 2010; Battelle et al. 2014). However, in these giant photoreceptors, the concentrations of LpOps1-4 and 5 proteins in rhabdomes are regulated differently. The concentration of LpOps1-4 in rhabdomes changes dramatically day-to-night whereas the concentration of LpOps5 remains relatively stable (Katti et al. 2010). A decrease in total opsin concentration in these rhabdomes probably reduces photoreceptor sensitivity, but it is not yet known whether a change in relative concentrations of LpOps1-4 and 5 impacts photoreceptor function. The visible light sensitive photoreceptors in median ocelli also express multiple LWS opsins (LpOps6, 7, and 8) (Battelle et al. 2015). The spectral sensitivities of the individual opsins are not yet known, nor is it known whether their relative levels change day-to-night.

A particularly puzzling finding is that transcripts encoding a number of opsins detected by RT-PCR in *Limulus* simple eyes and CNS were not detected by *in situ* hybridization, nor were the encoded proteins detected by immunocytochemistry (Table 1 and Fig. 3). In eyes, where some opsins are clearly highly expressed, it is tempting to consider those opsins expressed at apparently much lower levels as trace opsins with no functional significance. However, this may not be the case. *In situ* hybridization and immunocytochemical assays failed to confirm neuronal expression of any opsin in the CNS, yet photosensitive neurons are present in segmental ganglia. This may mean that even low levels of opsin expression can support a physiological response to light. If this is the case, finding multiple opsins expressed throughout the *Limulus* CNS could point to the interesting possibility that photosensitivity in the *Limulus* CNS is far more wide-spread than currently appreciated with functions yet to be discovered.

The expression pattern of LpPerOps1 raises new questions about its function. Peropsins were originally detected in eyes (Sun et al. 1997), and as in *Limulus*, most often in cells closely associated with photoreceptors (Battelle et al. 2015). The best current hypothesis regarding peropsin functions is that they are bistable photopigments and retinal photoisomerases (Nagata et al. 2010) that in invertebrates recycle chromophore released from rhodopsin internalized and degraded during rhabdom shedding (Wang et al. 2012). The presence of LpPerOps1 in cells within fiber tracks in the brain and surrounding many neurons in the synganglion and segmental ganglia suggests LpPerOps1 must have other functions as well.

Although photosensitivity in the American horseshoe crab has been studied for more than 80 years and much has been learned, clearly much remains to be discovered. A recently-generated high-quality genome assembly of *Limulus* (Battelle et al. 2016) should hasten these discoveries.

Funding

Work from the Battelle laboratory described in this review was funded by grants from the National Science Foundation.

Supplementary data

Supplementary data available at *ICB* online.

References

Barlow RB. 1983. Circadian-rhythms in the *Limulus* visual-system. J Neurosci 3:856–70.

Barlow RB, Bolanowski SJ, Brachman ML. 1977. Efferent optic-nerve fibers mediate circadian-rhythms in *Limulus* eye. Science 197:86–9.

Barlow RB Jr, Ireland LC, Kass L. 1982. Vision has a role in *Limulus* mating behavior. Nature 296:65–66.

Battelle BA. 2002. Circadian efferent input to *Limulus* eyes: anatomy, circuitry, and impact. Microsc Res Tech 58:345–55.

Battelle BA. 2006. The eyes of *Limulus polyphemus* (Xiphosura, Chelicerata) and their afferent and efferent projections. Arthropod Struct Dev 35:261–74.

Battelle BA. 2013. What the clock tells the eyes: Lessons from an ancient arthropod. Integr Comp Biol 53:144–53.

Battelle BA, Dabdoub A, Malone MA, Andrews AW, Cacciatore C, Calman BG, Smith WC, Payne R. 2001. Immunocytochemical localization of opsin, visual arrestin, myosin III, and calmodulin in *Limulus* lateral eye retinular cells and ventral photoreceptors. J Comp Neurol 435:211–25.

Battelle BA, Kempler KE, Harrison A, Dugger DR, Payne R. 2014. Opsin expression in *Limulus* eyes: a UV opsin is expressed in each eye type and co-expressed with a visible light-sensitive opsin in ventral larval eyes. J Exp Biol 217:3133–45.

Battelle BA, Kempler KE, Saraf SR, Marten CE, Dugger DR Jr, Speiser DI, Oakley TH. 2015. Opsins in *Limulus* eyes: characterization of three visible light-sensitive opsins unique to and co-expressed in median eye photoreceptors and a peropsin/RGR that is expressed in all eyes. J Exp Biol 218:466–79.

Battelle BA, Ryan JF, Kempler KE, Saraf SR, Marten CE, Warren WC, Minx P, Montague MJ, Green PJ, Schmidt SA, et al. 2016. Opsin repertoire and expression patterns in horseshoe crabs: evidence from the genome of *Limulus*

polyphemus (Arthropoda: Chelicerata). Genome Biol Evol 8:1571–89.

Behrens M, Fahy JL. 1981. Slow potentials in non-spiking optic nerve fibers in the peripheral visual system *Limulus.* J Comp Physiol 141:239–47.

Blackburn DC, Conley KW, Plachetzki DC, Kempler K, Battelle BA, Brown NL. 2008. Isolation and expression of Pax6 and atonal homologues in the American horseshoe crab, *Limulus polyphemus.* Dev Dyn 237:2209–19.

Brown JE, Rubin LJ, Ghalayin AJ, Tarver AP, Irvine RF, Berridge MJ, Anderson RE. 1984. *myo*-inositide polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. Nature 311:160–163.

Calman BG, Chamberlain S. 1982. Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. J Gen Physiol 80:839–62.

Calman BG, Lauerman MA, Andrews AW, Schmidt M, Battelle BA. 1991. Central projections of *Limulus* photoreceptor cells revealed by a photoreceptor-specific monoclonal antibody. J Comp Neurol 313:553–62.

Chamberlain SC, Barlow RB Jr. 1980. Neuroanatomy of the visual afferents in the horseshoe crab (*Limulus polyphemus*). J Comp Neurol 192:387–400.

Dalal JS, Jinks RN, Cacciatore C, Greenberg RM, Battelle BA. 2003. *Limulus* opsins: diurnal regulation of expression. Vis Neurosci 20:523–34.

Edgecombe GD, Legg DA. 2014. Origins and early evolution of arthropods. Palaeontology 57:457–68.

Ehlinger GS, Tankersley RA. 2003. Larval hatching in the horseshoe crab, *Limulus polyphemus*: facilitation by environmental cues. J Exp Mar Biol Ecol 292:199–212.

Fahrenbach WH. 1975. Visual system of horseshoe crab *Limulus polyphemus.* Int Rev Cytol 41:285–349.

French KA. 1980. The development of photoreception in Limulus polyphemus: morphology, electrophysiology and behavior. Boston: Boston University Graduate School. p. 1–168.

Hanna WJB, Horne JA, Renninger GH. 1988. Circadian photoreceptor organs in *Limulus*.2. The telson. J Comp Physiol A 162:133–40.

Hartline HK, Graham CH. 1932. Nerve impulses from single receptors in the eye. J Cell Comp Physiol 1:277–95.

Hartline HK, Wagner HG, Ratliff F. 1956. Inhibition in the eye of *Limulus.* J Gen Physiol 39:651–73.

Harzsch S, Vilpoux K, Blackburn DC, Platchetzki D, Brown NL, Melzer RR, Kempler KE, Battelle BA. 2006. Evolution of arthropod visual systems: development of the eyes and central visual pathways in the horseshoe crab *Limulus polyphemus* Linnaeus, 1758 (Chelicerata, Xiphosura). Dev Dyn 235:2641–55.

Herman KG. 1991. Two classes of *Limulus* ventral photoreceptors. J Comp Neurol 303:1–10.

Herzog ED, Barlow RB. 1991. Ultraviolet-light from the nighttime sky enhances retinal sensitivity of *Limulus.* Biol Bull 181:321–2.

Horne JA, Renninger GH. 1988. Circadian photoreceptor organs in *Limulus*.1. Ventral, median, and lateral eyes. J Comp Physiol A 162:127–32.

Jones C, Nolte J, Brown JE. 1971. Anatomy of median ocellus of *Limulus.* Z Zellforsch Mikrosk Anat 118:297–309.

Kass L, Barlow RB. 1992. A circadian clock in the *Limulus* brain transmits synchronous efferent signals to all eyes. Vis Neurosci 9:493–504.

Kass L, Renninger GH. 1988. Circadian change in function of *Limulus* ventral photoreceptors. Vis Neurosci 1:3–11.

Katti C, Kempler K, Porter ML, Legg A, Gonzalez R, Garcia-Rivera E, Dugger D, Battelle BA. 2010. Opsin co-expression in *Limulus* photoreceptors: differential regulation by light and a circadian clock. J Exp Biol 213:2589–601.

Kenny N, Chan K, Nong W, Qu Z, Maeso I, Yip H, Chan T, Kwan H, Holland P, Chu K, et al.. 2015. Ancestral whole-genome duplication in the marine chelicerate horseshoe crabs. Heredity 116:1–10.

Lall AB, Chapman RM. 1973. Phototaxis in *Limulus* under natural conditions—evidence for reception of near-ultraviolet light in median dorsal ocellus. J Exp Biol 58:213–24.

Lisman JE, Richard EA, Raghavachari S, Payne R. 2002. Simultaneous roles for Ca2+ in excitation and adaptation of *Limulus* ventral photoreceptors. Adv Exp Med Biol 514:507–38.

Millecchia R, Mauro A. 1969. The ventral photoreceptors of *Limulus* II. J Gen Physiol 54:310–30.

Millecchia R, Bradbury J, Mauro A. 1966. Simple photoreceptors in *Limulus polyphemus.* Science 154:1199–201.

Mori K, Kuramoto T. 2004. Photosensitivity of the central nervous system of *Limulus polyphemus.* Zool Sci 21:731–7.

Mori K, Saito T, Kuramoto T. 2004. Physiological and morphological identification of photosensitive neurons in the opisthosomal ganglia of *Limulus polyphemus.* Biol Bull 207:209–16.

Nagata T, Koyanagi M, Tsukamoto H, Terakita A. 2010. Identification and characterization of a protostome homologue of peropsin from a jumping spider. J Comp Physiol A 196:51–9.

Nilsson DE, Kelber A. 2007. A functional analysis of compound eye evolution. Arthropod Struct Dev 36:373–85.

Nolte J, Brown JE. 1970. Spectral sensitivities of single receptor cells in lateral, median, and ventral eyes of normal and white-eyed *Limulus.* J Gen Physiol 55:787–801.

Nolte J, Brown JE. 1972. Electrophysiological properties of cells in median ocellus of *Limulus.* J Gen Physiol 59:167–85.

Nossa C, Havlak P, Yue J-X, Lv J, Vincent K, Brockmann H, Putnam N. 2014. Joint assembly and genetic mapping of the Atlantic horseshoe crab genome reveals ancient whole-genome duplication. GigaScience 3:9.

Patten W. 1894. On the morphology and physiology of the brain and sense organs of *Limulus.* Q J Microsc Sci 3:1–96.

Payne R, Corson DW, Fein A, Berridge MJ. 1986. Excitation and adaptation of *Limulus* ventral photoreceptors by inositol 1,4,5 trisphosphate result from a rise in intracellular calcium. J Gen Physiol 88:127–42.

Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, Wetzer R, Martin JW, Cunningham CW. 2010. Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 463:1079–98.

Sekiguchi K, Yamamichi Y, Costlow JD. 1982. Horseshoe crab developmental studies I. Normal embryonic development of *Limulus polyphemus* compared with *Tachypleus tridentatus.* In: Bonaventura J, Bonaventura C, Tesh S, editors.

Physiology and biology of horseshoe crabs. New York: Alan R. Liss. p. 53–73.

Smith TG, Baumann F. 1969. The functional organization within the ommatidium of the lateral eye of *Limulus*. Prog Brain Res 31:313–49.

Smith WC, Price DA, Greenberg RM, Battelle BA. 1993. Opsins from the lateral eyes and ocelli of the horseshoe-crab, *Limulus-polyphemus*. Proc Natl Acad Sci USA 90:6150–4.

Sun H, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. 1997. Peropsin, a novel visual pigment-like protein located in the apical microvilli of the retinal pigment epithelium. Proc Natl Acad Sci USA 94:9893–8.

Wang XY, Wang T, Ni JFD, von Lintig J, Montell C. 2012. The *Drosophila* visual cycle and de novo chromophore synthesis depends on rdhB. J Neurosci 32:3485–91.

Westerman LA, Barlow RB. 1981. Ultraviolet responses of the *Limulus* median ocellus. Biol Bull 161:352–3.