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RESEARCH ARTICLE

The unusual eyes of *Xenos peckii* (Strepsiptera: Xenidae) have green- and UV-sensitive photoreceptors

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ABSTRACT

The highly specialized evolution of Strepsiptera has produced one of the most unusual eyes among mature insects, perhaps in line with their extremely complex and challenging life cycle. This relatively rare insect order is one of the few for which it has been unclear what spectral classes of photoreceptors any of its members may possess, an even more apt question given the nocturnal evolution of the group. To address this question, we performed electroretinograms on adult male *Xenos peckii*: we measured spectral responses to equi-quantal monochromatic light flashes of different wavelengths, and established *V*log*I* relationships to calculate spectral sensitivities. Based on opsin template fits, we found maximal spectral sensitivity (λ_{max}) in the green domain at 539 nm. Application of a green light to 'bleach' green receptors revealed that a UV peak was contributed to by an independent UV opsin with a λ_{max} of 346 nm. Transcriptomics and a phylogenetic analysis including 50 other opsin sequences further confirmed the presence of these two opsin classes. While these findings do not necessarily indicate that these unorthodox insects have color vision, they raise the possibility that UV vision plays an important role in the ability of *X. peckii* males to find the very cryptic strepsipteran females that are situated within their wasp hosts.

KEY WORDS: Invertebrate vision, Color vision, Spectral sensitivity

INTRODUCTION

Strepsiptera are a small, curious order of obligate endoparasitic insects whose complex life histories have raised many unanswered questions. These include several aspects of their visual physiology, which is the subject of this investigation.

Strepsiptera have diverged so strongly from other insect orders that they are best known for the extreme difficulty of placing them phylogenetically (Kristensen, 1981; Wiegmann et al., 2009). Recent research has resolved the issue quite satisfactorily, however: there is now very strong morphological and genomic evidence supporting Strepsiptera as the sister group to Coleoptera (Cook, 2014; Niehuis et al., 2012). *Xenos peckii* Kirby 1813 is a diurnal species of Strepsiptera that uses paper wasps as its host. As in most strepsipteran species, adult female *X. peckii* never leave their host. Instead, adult females are larviform, lacking wings, eyes and legs. Unlike any other known holometabolous insect, adult female Strepsiptera mature without pupating (Kathirithamby, 1989). Their neotenic bodies remain within their hosts, within which they give birth to live young. These triungulins are mobile and find new hosts by entering into wasp larvae of the same nest, or by riding uninfected wasps to other nests to enter larvae there (Hughes et al., 2003). Toward the end of summer, developing *X. peckii* breach the cuticle of the abdomen of their, by then, adult wasp hosts. Males pupate without exiting their hosts, and only later eclose, becoming airborne immediately. Mature, unmated females emit a sex pheromone (Cvačka et al., 2012; Tolasch et al., 2012), which attracts adult males (Fig. 1A) through olfaction, while females also protrude their cephalothorax out of the wasp, potentially providing an additional visual signal. Adult male *X. peckii* are about 4 mm long. They, like other male Strepsiptera, have a very well-developed flight apparatus (Pohl and Beutel, 2008), including halteres that are homologous with the forewings of other insects and are important for flight control (Pix et al., 1993). By means of their semicircular hindwings, they are able to fly immediately upon eclosing from the pupal case (Smith and Kathirithamby, 1984). Once airborne, with the assistance of their elaborate antennae and prominent eyes (Buschbeck et al., 1999, 2003), they search incessantly for a virgin female with which to mate. Males die within a few hours of eclosing, but females persist long enough for their offspring to mature. In the case of *X. peckii*, this includes overwintering, which they are able to induce even in unmated wasps by hormonal manipulation (Strambi and Girardie, 1973).

Strepsipteran eyes are remarkable. Unlike typical compound eyes, which consist of ommatidia that each collect information from a single point in space, the strepsipteran eye is constructed of a number of single-chamber eyes that are aggregated into a larger eye (Fig. 1B). A single-chamber eye differs from an ommatidium in that it has a retina large enough to contain spatial information. In *X. peckii*, each eyelet has a retina that consists of about 100 receptors (Buschbeck et al., 1999), onto which a small image is projected. Because of the characteristics of lenses, the image is inverted within each eyelet. However, in *X. peckii*, the original orientation of each image is restored via downstream wiring (Buschbeck et al., 2003), allowing the eye as a whole to produce a combined image of higher acuity (Maksimovic et al., 2007) than the 50 or so pixels that *X. peckii* would be able to represent if each of the eyelets only resolved a single point in space (as is typical for compound eye ommatidia).

While this extraordinary eye organization continues to inspire novel camera designs (Brückner et al., 2011; Druart et al., 2009; Keum et al., 2016), its evolution remains unclear. However, some insight can be gained from the fact that a large number of strepsipteran species appear to be nocturnal (Pohl and Beutel, 2008). Although rarely experimentally confirmed, the inability of adult male Strepsiptera to feed or drink (Pohl and Beutel, 2008), coupled with the relative frequency with which males (particularly those of basal clades) are caught in light traps (Kathirithamby, 1989; Khalaf, 1968; Shepard, 1979), the activity patterns of their hosts and the absence of

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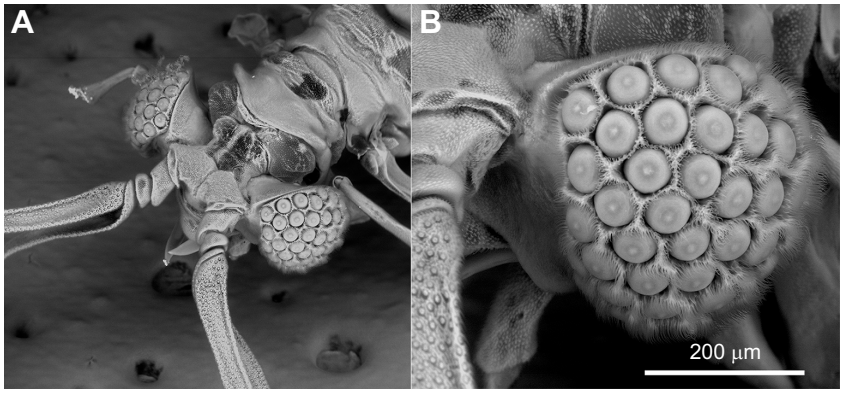

Fig. 1. Twisted-wing parasites such as *Xenos peckii*, are characterized by 'eyelets' of unusually large diameter, each of which contains its own extended retina. (A) An overview of an adult male *X. peckii* head, illustrating the presence of two large eyes. (B) A magnification of the left eye illustrates the shape and position of individual eyelet lenses, each of which is surrounded by dense setae ('hairs').

sightings of free-flying males, all support strepsipteran nocturnal ancestry. Furthermore, several attributes of the strepsipteran eye – even those of diurnal species – are reminiscent of nocturnal insects, raising the possibility that even though *X. peckii* is a diurnal species, their extraordinary eyes owe their existence to a nocturnal evolutionary history (Buschbeck et al., 2003).

As photons are limited at night, one might expect that a nocturnal lifestyle could lead to a reduction of photoreceptor classes, and there is evidence for such reduction, at least in mammals. Most mammals have dichromatic vision (Osorio and Vorobyev, 2008), but some nocturnal groups have become monochromatic (Kelber et al., 2003). When light is dim, available photons may be used to boost sensitivity rather than the ability to discriminate color. It is therefore plausible that the nocturnal ancestry of Strepsiptera led to the reduction or absence of color vision in this group. It is also notable that in insects known to have color vision, the color-mediating photoreceptors typically pass straight through the lamina, the first neuropil of the visual system, and terminate in the second layer, the medulla (Morante and Desplan, 2008). In contrast, photoreceptors that are associated with motion vision tend to terminate in the lamina (Heisenberg and Buchner, 1977). In *X. peckii*, all identified projections terminate in the lamina (Buschbeck et al., 2003), possibly indicating that color vision in this insect group is absent. However, more recent data have emerged indicating that apparently parallel visual pathways are not as clearly separated as has long been believed (Kelber and Henze, 2013). For example, in *Drosophila*, it has been demonstrated that the outer photoreceptors R1–R6 (which terminate in the lamina) can also mediate color vision (Schnaitmann et al., 2013). Despite severely reduced light levels at night, it has been noted that color vision in nocturnal insects is more common than historically believed (Kelber and Roth, 2006). For example, the hawk moth, *Deilephila elpenor*, can distinguish colors by dim starlight (Kelber et al., 2002). The majority of insects studied so far have three color channels, with photoreceptors specialized for absorbing light in the green, blue and UV ranges (Briscoe and Chittka, 2001; Kelber, 2006; Osorio and Vorobyev, 2008).

Taken together, the question of whether or not Strepsiptera have the visual machinery necessary to detect color arises. To address this question, we used extracellular recordings (electroretinograms, ERGs) of photoreceptor responses to equal-intensity but differently colored light flashes to investigate the spectral response properties of *X. peckii*, a diurnal strepsipteran, and among the best-known species in this order.

MATERIALS AND METHODS

The strepsipteran *X. peckii* is relatively difficult to find, but in mid-summer 2012 we came across a fertilized female Strepsiptera within its queen *Polistes fuscatus* host. This female subsequently produced triungulins that allowed us to raise a generation of Strepsiptera. To do so, the host wasp was kept separately in a cool and dark environment, and over a period of 10 days she was periodically handled to elicit the emergence of triungulins. These were then picked up with a soft brush and placed directly onto *Polistes fuscatus* larvae of colonies that were reared separately in small wooden nest boxes. To access wasp larvae, nest boxes were cooled to 4°C. This allowed triungulins to be placed onto the nests without interference from the adult wasps that tend their larvae. Adult wasps were fed honey–water and freshly killed crickets. Once stylopized wasps emerged, they were monitored closely, and separated from the nest as soon as *X. peckii* puparia became visible.

In our laboratory, eclosed adult male Strepsiptera were only fully healthy for 2–3 h at room temperature. Therefore, one of the biggest challenges was to secure them immediately after emergence. To do so, we moved stylopized wasps into a dark chamber and then every morning, or every other morning, we placed them in separate containers under bright light that triggered the emergence of mature males. When multiple adult male Strepsiptera eclosed in rapid succession, some of them were placed in a refrigerator at 4°C for up to 3 h to keep them viable until we could record from them.

ERGs

To record ERGs from the *X. peckii* eye, each insect was immobilized by mounting it on a cover-slip using dental wax. A cotton wick inserted into a glass capillary tube filled with a solution of NaCl (0.9% w/v NaCl) served as the measuring electrode and was placed on the surface of the eye. The reference electrode was another glass electrode, also filled with NaCl solution and placed into the abdomen of the Strepsiptera. All recordings were performed in a Faraday cage, on a TMC 66-501 vibration isolation table (Technical Manufacturing Corporation, Peabody, MA, USA) using standard electrophysiological equipment, including an A-M Systems Neuroprobe amplifier 1600 (A-M Systems, Inc., Sequim, WA, USA), Tektronix Oscilloscope 5111A (Tektronix, Inc., Beaverton, OR, USA) and an iWorx Data Aquisition System (HAI 118, iWorx Systems, Inc., Dover, NH, USA). Data were acquired at a sampling rate of 10,000 Hz and stored on a PC computer using iWorx LabScribe software (iWorx Systems, Inc.), and analyzed as outlined below using customized programs (available upon request) in MATLAB (The Mathworks, Inc., Natick, MA, USA).

The eye was then stimulated with equi-quantal monochromatic light pulses and the voltage responses of photoreceptors were recorded. These light flashes were obtained from a 150 W xenon arc lamp coupled to an Oriel Cornerstone 130 1/8 m 74,000 monochromator (Oriel Instruments, Stratford, CT, USA). The

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intensity of the stimulus was controlled with a Newport circular variable neutral density filter 50Q04AV.2 (Newport Corporation, Irvine, CA, USA) operated with a Newport Newstep Controller NSC200 (Newport Corporation). The filter was mounted onto a Newport NSR-12 motorized rotator stage (Newport Corporation) and placed in line with the output slit of the monochromator. A converging lens (f=10 cm) was used to focus the light from the monochromator onto the tip of an optic fiber, the other end of which was positioned a few millimeters from the strepsipteran eye. Prior to the experiment, the intensity of the light at the tip of the fiber was calibrated using an Ocean Optics USB2000+ spectrometer (Ocean Optics, Inc., Dunedin, FL, USA). Specific neutral density filter positions allowed for equi-quantal light stimulation at different wavelengths.

To assess the spectral response, the intensity of monochromatic light flashes was pre-calibrated to 6.5×10^{13} photons cm^{-2} s^{-1} for all wavelengths (stimulus intensities ranged from 6.0×10^{13} to 6.8×10^{13} photons cm^{-2} s^{-1}). A typical spectral response recording consisted of equi-quantal monochromatic light stimuli ranging from 300 to 640 nm in 20 nm steps. To verify the stability of the recording, this was followed by a set of simulations in the opposite direction (640–300 nm). For most animals, additional recordings were taken later in the experiments, and data were averaged over up to four measurements for each animal. For each of these measurements, at each wavelength, three consecutive flashes (each 300 ms long with a 1.7 s interval) were presented. A 10 s time interval between consecutive wavelengths allowed the eye to recover between light stimulations of different wavelengths. Immediately afterwards, responses to monochromatic light stimuli at 500 nm (near the putative peak) ranging from 4.8×10^{11} to 8.5×10^{14} photons cm^{-2} s^{-1} in 0.25 log steps were recorded to later generate the response–stimulus intensity (Vlog$I$) function.

As initial measurements showed a secondary peak around 350 nm, we performed additional measurements to determine whether UV sensitivity is independent of green sensitivity, or whether it merely reflects a typical beta peak of a green opsin (Stavenga et al., 1993). To do so, we re-measured the response to light pulses across the spectrum while using a green LED (525 nm; superbrightleds.com) to 'bleach' green receptors ('green-bleach'). As these measurements revealed a prominent peak in the UV range, the Vlog$I$ relationship was also established for the green-bleach paradigm for intensities of 380 nm light ranging from 4.78×10^{11} to 2.68×10^{14} photons cm^{-2} s^{-1}. Finally, a 380 nm UV LED (RL5-UV0315-380 from superbrightleds.com) was used to bleach out the majority of the response across the spectrum.

Analysis

Both the spectral response and Vlog$I$ results were analyzed using in-house MATLAB code. Briefly, data were first smoothed with the following function: (filter(ones(1,windowsize)/windowsize,1, data)), with windowsize=50. For each pulse, a baseline value was determined as the average of 100 points surrounding stimulus onset (see red points in Fig. 2B). The response was defined as the average of 100 points (equaling 10 ms) surrounding the minimum response that occurred during each stimulation (see magenta points in Fig. 2B).

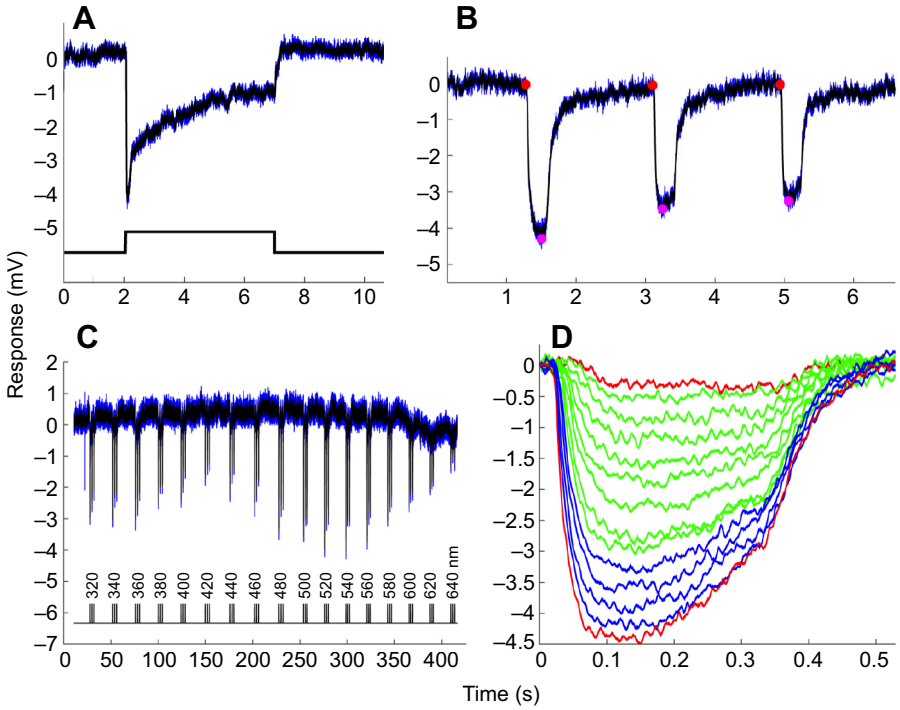

Fig. 2. Examples of electroretinogram (ERG) responses. (A) Response to a bright green light pulse near saturation. As in other insects, the response is characterized by a fast transient component, as well as an extended receptor potential. (B) Example of the three consecutive responses that were the basis of our spectral response measurements. In this example, green (520 nm) light pulses were administered at 6.5×10^{13} photons cm^{-2} s^{-1}. Red dots indicate the base values and magenta dots indicate response values that were identified by our in-house analysis program. (C) Example recording of the entire spectrum, from 320 to 640 nm wavelength. At each wavelength, three pulses were administered as indicated in B. The bottom trace illustrates stimuli (and wavelength values) and the top trace illustrates the recorded response (in blue, with smoothed data in black). (D) Superimposed responses to green light of all intensities that were presented in our Vlog$I$ measurements. The weakest and strongest responses are illustrated in red, and intermediate responses are in green and blue. The light intensity that was used for further analysis lies between the intensity that elicited the largest response that is plotted in green (4.8×10^{13} photons cm^{-2} s^{-1}), and the intensity that elicited the smallest response that is plotted in blue (8.5×10^{13} photons cm^{-2} s^{-1}), demonstrating that photoreceptors were not saturated in these measurements.

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To validate the stability of our recordings, multiple measurements were plotted on top of each other. As the analysis revealed that for each wavelength the first pulse was systematically larger than the other pulses, the analysis was performed for the first pulse of the three stimuli only, as well as averaged across the three pulses. Because comparison of these two analyses revealed no systematic difference in regards to the spectral findings (Fig. 3A,B), further analysis was completed by averaging the results of the three pulses.

To convert our spectral response measurements to spectral sensitivity curves, we used the hyperbolic Naka–Rushton (NR) function (Eqn 1), where V_{max} is the maximum response amplitude, I is the stimulus intensity, k is the stimulus intensity at $V_{max}/2$ and n is the slope of the function (Menzel et al., 1986; Naka and Rushton, 1966; Skorupski and Chittka, 2010):

$$\frac{V}{V_{max}} = \frac{I^n}{I^n + k^n}. \quad (1)$$

Our Vlog$I$ data were fitted to this function using the MATLAB curve-fitting tool *cftool* to obtain values for k, n and V_{max}. To establish the peak green sensitivity, the Vlog$I$ data for 500 nm were used. To establish the UV peak, the Vlog$I$ data were taken at 380 nm under green-bleach conditions. Each fit then was used to extrapolate the Vlog$I$ curves for all other wavelengths. The spectral sensitivity curve was then determined as the reciprocal of the photon count required to elicit equal response amplitudes at wavelengths ranging from 320 to 640 nm. Finally, these spectral sensitivity data were fitted (with *cftool*) to the Govardovskii (Govardovskii et al., 2000) and Stavenga (Stavenga et al., 1993) rhodopsin absorption templates to find the maximal sensitivity of the opsin in question.

Transcriptomics and phylogenetic analysis

The RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA) was utilized for RNA isolation of two intact animals. To assess the quality of RNA, extractions were subjected to spectrophotometic analysis utilizing a NanoDrop 1000 Spectrometer (Thermo Fisher Scientific, MA, USA) where the $A_{260/280}$ absorbance ratio yielded measurements of ~2.0 for RNA extracts, indicating that all RNA measurements were relatively pure. RNA-seq utilized the Illumina HiSeq 2500 (75 bp) with a Ribo-zero preparation at Cincinnati Children's Hospital Core Sequencing Facility (Cincinnati, OH, USA). The raw read FASTQ files were assembled utilizing SeqMan NGen default assembly parameters (DNASTAR. v. 12.0, Madison, WI, USA). The annotation of contigs was carried out using Blast2GO (BioBam, Valencia, Spain) with default parameters using the blastx database (Altschul et al., 1997). To contrast our mRNA sequences against other opsins, we utilized the blastx algorithm to predict the amino acid sequences of the opsins. Amino acid sequences of 50 known additional opsins from GenBank (Table S1) were aligned using the ClustalW algorithm (Saitou and Nei, 1987). This alignment was subjected to a neighbor-joining algorithm to perform a phylogenetic analysis as implemented in MEGA v. 6.06 (Tamura et al., 2007). Bootstrap values were derived from 1000 bootstrap replicates.

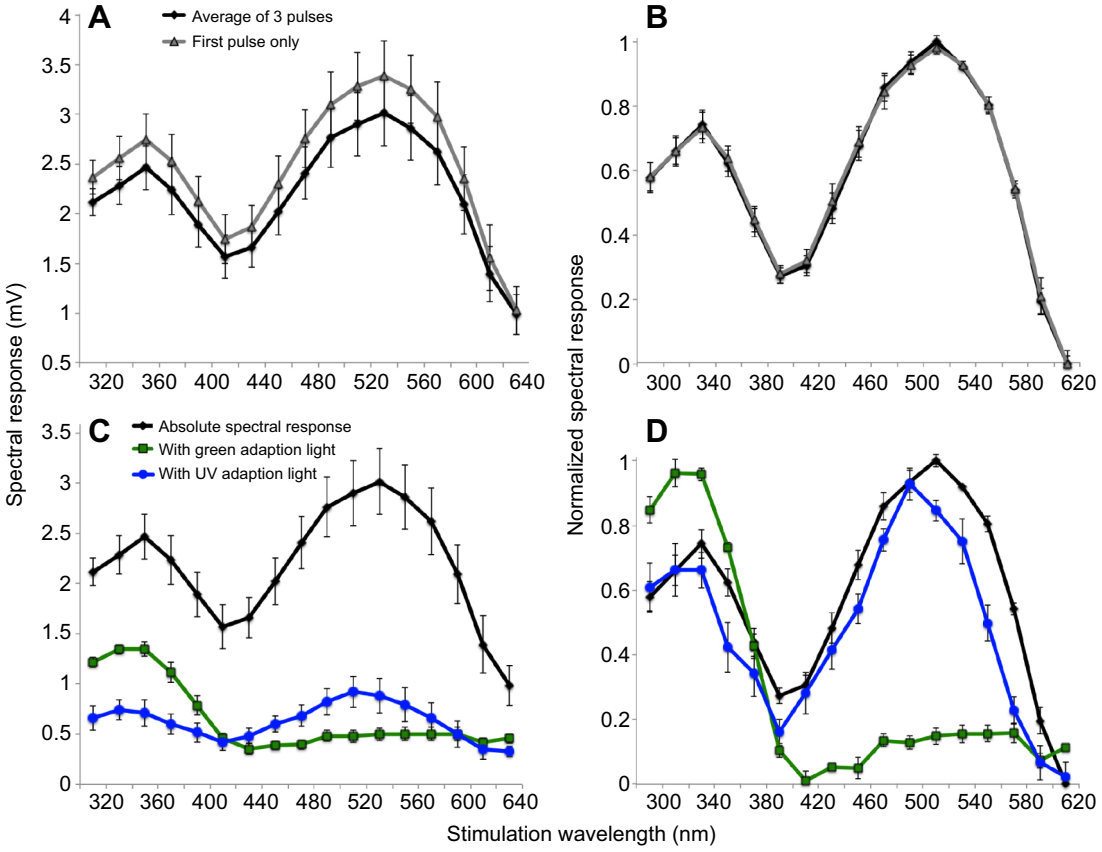

Fig. 3. Spectral response recordings. (A) Average response curves of our analysis based on all three pulses, and of the first pulse only. (B) These two types of analysis yield essentially identical results when normalized. (C) Under strong selective stimulation of green-sensitive receptors ('green-bleach'), a UV response remains. In contrast, a UV-bleach light greatly attenuates the entire response, indicating that the long wavelength (LW) opsin contains a beta peak. (D) Normalizing the data illustrates that the spectral characteristics of the response under the UV-bleach light are qualitatively similar to those of the non-attenuated response. All curves represent means±s.e.

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RESULTS

ERGs and spectral response measurements

Our initial recordings of longer light stimuli revealed that the wave shape of the strepsipteran ERG looks like that of typical insect photoreceptors (Fig. 2A). Near-saturation responses are characterized by a transient strong response, followed by an extended persistent activation. To measure the spectral response, three consecutive light pulses of equal wavelength and intensity were used, resulting in responses as illustrated in Fig. 2B. Fig. 2C illustrates the raw data for one recording from 320 to 640 nm, in which particularly strong responses are notable around 350 nm as well as around 540 nm. For further analysis (see below), and to ensure that our measurements were performed within the linear range of the receptor response, we also established the relationship between stimulus intensity and response at 500 nm. A set of response curves to each light intensity illustrates minor light intensity-related changes in the overall shape of the responses (Fig. 2D). Spectral response measurements were performed at a light intensity of ~6.5×10^{13} photons cm^{-2} s^{-1}. This intensity elicited responses that were in the upper mid-portion of the receptor's range.

Our initial analysis revealed that the second and third pulse of each stimulus consistently showed a slightly smaller response, presumably because receptors did not fully dark adapt between pulses. Independent analysis of only the first pulse, and of all three pulses, showed comparable results in regard to the spectral qualities of the data, with the main difference being that the three-pulse analysis led to slightly smaller response magnitudes than the first-pulse only analysis (Fig. 3A). However, normalization of the data led to essentially identical traces (Fig. 3B), demonstrating that these two analysis methods are comparable with respect to spectral response properties of the strepsipteran eye.

Because our initial analysis revealed the presence of a peak in the UV region, we performed further tests to establish whether this UV response simply represents the beta peak of a longer wavelength or whether it could be the manifestation of an independent UV opsin. Specifically, we used a green-bleach light (at 525 nm) to saturate the green receptor. The rationale of this experiment is that constant activation of the green opsin leads to a constant response (both its UV and green components) independent of additional stimulation of opsins that are outside the range of the 'bleach light'. Fig. 3C illustrates that under these conditions a UV response (though reduced in size) remained, whereas the green response was essentially absent, indicating that at least a portion of the initial UV response was independent of the green opsin. In contrast, UV-bleach light resulted in a strongly reduced response across the spectrum, indicating that all opsins that contributed to the initial response had a UV component; this response curve regained a comparable shape to the original measurements when normalized (Fig. 3D), suggesting that the UV-bleach attenuated the response approximately equally throughout the spectrum.

Establishment of spectral sensitivity maxima

To convert our spectral response measurements of the green peak to spectral sensitivity data to which opsin templates can be applied, we first established the photoreceptor response characteristic of a series of 500 nm light pulses of different intensities (4.8×10^{11} to 8.5×10^{14} photons cm^{-2} s^{-1}). Fig. 4A illustrates these measurements for each of the seven male Strepsiptera that were measured, as well as the NR function (Naka and Rushton, 1966) fit that was used to calculate the *V*log*I* response, and to convert the data to spectral sensitivity curves. Govardovskii (Govardovskii et al., 2000) and Stavenga (Stavenga et al., 1993) opsin templates were then applied to the green peak (situated between 440 and 620 nm) of each spectral response curve. The Govardovskii template resulted in maximal sensitivities (λ_{max}) between 533.7 nm and 545.9 nm with a mean (±s.e.) peak sensitivity of 538.7±1.7 nm. The Stavenga template resulted in nearly identical results, with a λ_{max} between 533.6 nm and 546 nm and a mean (±s.e.) peak sensitivity of 538.7±1.7 nm. Template fits to these mean sensitivity values, as well as the mean (±s.e.) measurements are illustrated in Fig. 4B. To establish the spectral sensitivity maxima of the UV opsin, we established the photoreceptor response characteristic of a series of 380 nm light pulses of different intensities (4.78×10^{11} to 2.68×10^{14} photons cm^{-2} s^{-1}), while applying the green-bleach light. Fig. 5A illustrates these measurements for each of the five male Strepsiptera that were successfully measured, as well as the NR function (Naka and Rushton, 1966) fit that was used to calculate the *V*log*I* response, and the conversion to spectral sensitivity curves. Two measurements were excluded based on electrical noise that confounded the analysis. The Govardovskii template resulted in λ_{max} values between 331.4 nm and 354 nm with a mean (±s.e.) sensitivity of 346.1±4.1 nm. Here too, the Stavenga template resulted in nearly identical results, with a λ_{max} between 331.3 nm and 353.8 nm and a mean (±s.e.) sensitivity of 345.9±4.1 nm. Template fits to these mean sensitivity values, as well as the mean (±s.e.) measurements are illustrated in Fig. 5B.

Transcriptomics and phylogenetic analysis of opsins

We used a molecular approach to independently investigate photoreceptor types that may be present in the strepsipteran eye.

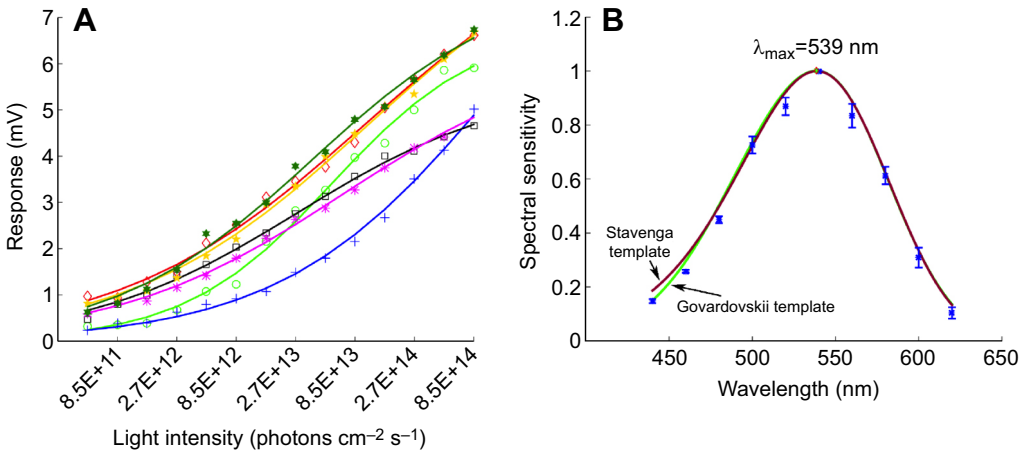

Fig. 4. The spectral sensitivity of the green-sensitive opsin. (A) The *V*log*I* relationship for 500 nm light stimuli was established in seven Strepsiptera (measurements for each individual are illustrated with their own color and symbol) and fitted with the Naka–Rushton (NR) function (see Eqn 1; plotted in respective colors). (B) Values obtained from the NR function were used to calculate spectral sensitivity curves to which Stavenga (brown) and Govardovskii (green) templates were applied. Both fits resulted in a λ_{max} of 539 nm.

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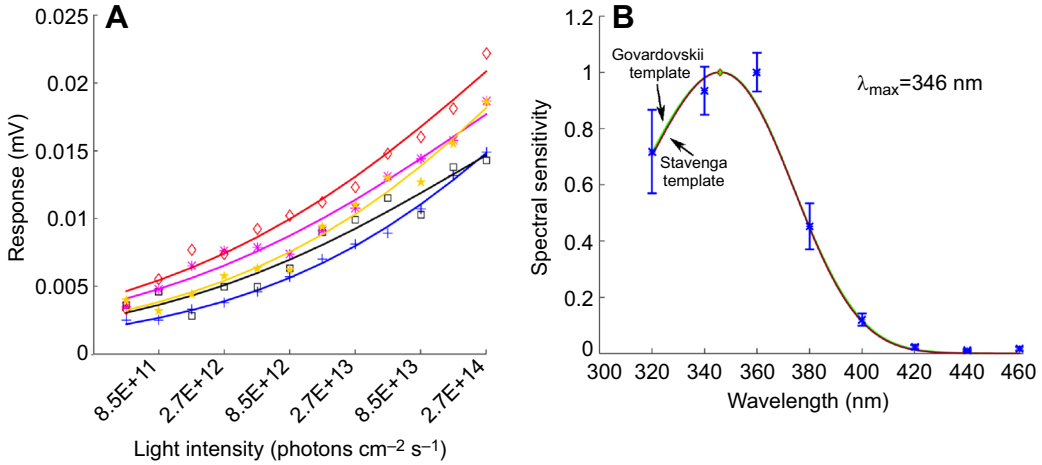

Fig. 5. The spectral sensitivity of the UV-sensitive opsin. (A) The *V*log*I* relationship for 380 nm light stimuli was established in five Strepsiptera (measurements for each individual are illustrated with their own color and symbol) during the application of a bleach light, and fitted with the NR function (see Eqn 1; fits are plotted in respective colors). (B) Values obtained from the NR function were used to calculate spectral sensitivity curves to which Stavenga (brown) and Govardovskii (green) templates were applied. Both fits resulted in a λ_{max} of 346 nm.

Specifically, we identified possible opsins from a transcriptome of male *X. peckii*. The 23,308,238 reads, 75 bp in length, from this project have been deposited in the NCBI Sequence Read Archive. Their *de novo* assembly aligned a total of 9854 contigs. One tool utilized to assess the assembly quality was the contig N50 which resulted in an average length of 1253 bp, which on average was represented 12 times. From the *de novo* assembly, a total of 6879 contigs were assigned an annotation, including two opsin proteins: one long-wavelength sensitive and one UV sensitive (see below for GenBank accession numbers). To determine the relative expression of each opsin, we mapped the raw reads back to a templated assembly of the two opsin sequences. The long-wavelength opsin had 8100 sequences that mapped back, whereas the UV opsin only had 630.

Our transcriptome did not resolve a blue-sensitive opsin, and there was no evidence for additional long-wavelength or UV opsin types, or any other opsin. We further investigated these categorizations, confirming the presence of a 7-transmembrane class 1 receptor, a sequence typical for opsins, and performed a phylogenetic analysis (Attwood and Findlay, 1994). As shown in Fig. 6, opsins that share similar spectral characteristics cluster more closely to each other than opsins from different spectral classes. Our phylogenetic tree resulted in monophyletic clades for all LW opsins and for all UV opsins. In our analysis, the long-wavelength opsin (*Xenos peckii* LW) is nested well within the LW opsins clade, and the UV opsin (*Xenos peckii* UV) is nested in the UV clade.

DISCUSSION

The ability to see and discriminate objects on the basis of their color is an important attribute for the ecology of many organisms. Most insects are thought to have trichromatic vision, the presumably ancestral form, while some (including multiple groups of butterflies) have even evolved tetrachromatic vision (Briscoe and Chittka, 2001; Eguchi et al., 1982) with the addition of a red channel (Bernard, 1979). The ability to differentiate objects based on their color can be important for many aspects of their lives, including the ability to efficiently locate food sources such as flowers, select oviposition sites and find mates. Finding a mate is the most important challenge in the life of an adult male Strepsiptera, which in the few hours of his eclosed life is only concerned with mating. In *X. peckii*, the larviform female is situated primarily within the abdomen of her wasp host. Although it recently has become clear that she actively participates in attracting a male (Hrabar et al., 2014), only a small and, for our eyes, rather cryptic portion of her body is exposed. Still, the male finds her often enough to propagate the species, and his unique strepsipteran eye type (Buschbeck et al., 1999, 2003) may play an important role in that. Given the unorthodox eye organization and the lack of data in regard to what spectral classes of photoreceptors might be present in them, it has been difficult to hypothesize whether color vision could be involved. In fact, presumably because they are difficult to find and work with, Strepsiptera are among the few holometabolous insect orders for which spectral sensitivity data had been wholly absent, even though their unconventional eyes make them particularly interesting.

In part, Strepsiptera have been understudied because they are relatively difficult to find, and their short adult lifespan imposes additional challenges for research projects that rely on live specimens. Because adult males are short-lived, all data need to be collected within a few hours of their emergence. In this study, we succeeded in lab-rearing a population, and in measuring spectral response characteristics of a representative set of adult male Strepsiptera. Our initial measurements showed maximal responses to green light, with a secondary response in the UV domain. Based on our calculated spectral sensitivity and fits to both the Govardovskii (Govardovskii et al., 2000) and Stavenga (Stavenga et al., 1993) opsin templates, the maximal spectral sensitivity is 539 nm, well in line with long-wavelength receptors of other insects. In fact, with typical λ_{max} values of ~530 nm, insects so far have remarkably consistent peak green sensitivities, despite a large variety of ecological backgrounds (Briscoe and Chittka, 2001). Our assessment of the green sensitivity peak is particularly robust, as all specimens were measured at least twice, and often four times, with comparable results. With fewer measurements (for one specimen we only had one measurement and for the remaining specimens we had two), and smaller signal-to-noise ratios, our UV opsin analysis is slightly less robust. In addition to extinguishing the green peak, the green-bleach also reduced the size of the UV peak, indicating that the green opsin has some sensitivity in the UV (as is typical for long-wavelength opsins). Nevertheless, our analysis showed robust results, with both applied opsin templates suggesting a λ_{max} of 346 nm. Like the green sensitivity, the strepsipteran UV sensitivity

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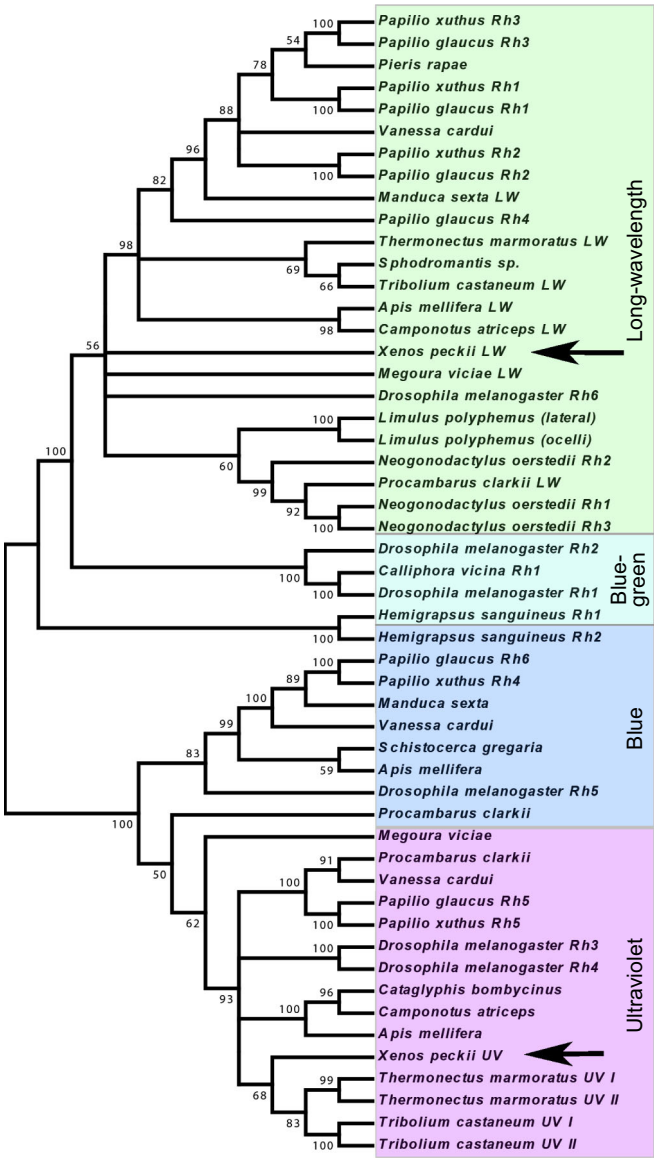

Fig. 6. Phylogenetic analysis of opsins. A neighbor-joining phylogenetic tree (with bootstrap values) was obtained from 50 amino acid sequences obtained through GenBank, as well as our own two *Xenos* sequences, illustrating that the *X. peckii* LW opsin is nested well within the long wavelength clade, and the *X. peckii* UV opsin is well nested within the UV clade.

lies well within the range of UV sensitivities of other insects, and is quite comparable to their typical value of around 350 nm (Briscoe and Chittka, 2001).

It is noteworthy that our initial measurement included stimulation at 300 nm that resulted in a surprisingly high sensitivity, as though Strepsiptera have a unique sensitivity to UVB in addition to UVA. However, for technical reasons the 300 nm stimulus of our setup was least precisely calibrated, so we therefore did not sufficiently trust those data to include them in this publication. Furthermore, no UVB opsin was implicated in our transcriptomic analysis. Nevertheless, it would be worthwhile to further investigate the spectral response characteristics of *X. peckii* in the very short-wavelength domain, especially in the light of recent findings that UVB sensitivity is important in some other arthropods. For example, it plays a role in communication in jumping spiders (Painting et al., 2016), and a UVB receptor of slightly longer wavelength than would be predicted for *X. peckii* has been identified in certain stomatopods (Kleinlogel and Marshall, 2009). It also has been suggested that a powerful cut-off filter could convert a UVA receptor into a UVB receptor in thrips (Mazza et al., 2010).

Although extracellular methods can never be completely conclusive, our data are most consistent with the absence of a blue receptor. Most telling here are our recordings with a 520 nm bleach light, with a relatively narrow spectrum (its width at half height was less than 50 nm). As blue receptors have a typical λ_{max} of ~440 nm, it is unlikely that the green-bleach light would have bleached out a blue opsin if it were present. However, under these conditions, our measurements show that *X. peckii* response curves are at their minimum at 440 nm (Fig. 3D), making it very unlikely that a blue-sensitive opsin in any way contributed to the measured response curves. Despite ancestral trichromacy, the absence of a blue-sensitive opsin has been noted in several insect orders, including representatives of Coleoptera, Hymenoptera, Neuroptera and Blattodea (Briscoe and Chittka, 2001). Particularly noteworthy is that in the strepsipteran sister group Coleoptera, the absence of a blue-sensitive opsin has been reported more often than its presence, including in the flour beetle *Tribolium castaneum* (Jackowska et al., 2007), and at least in the larval form of the diving beetle *Thermonectus marmoratus* (Maksimovic et al., 2009, 2011). In some fireflies, blue opsins also may be absent, or at least restricted to certain areas of their visual fields (Lall et al., 1982). This group of beetles is also known for variable green sensitivity in diurnal and nocturnal species, both through tuning of screening pigments and shifts in opsin sensitivity (Cronin et al., 2000). All in all, our findings raise the possibility that blue opsins are frequently absent within the entire coleopteran–strepsipteran clade.

In *X. peckii*, the condition of dichromacy is further supported through our transcriptomics analysis. Our initial BLAST results identified a long-wavelength- and a UV-sensitive opsin. The placement of these two opsin genes in our phylogenetic analysis of opsin genes confirmed that prediction, as in both cases strepsipteran opsins are well nested within opsins of the same spectral class. Though spectral characterization of Coleoptera has been limited to date, it is satisfying to note that the *X. peckii* UV opsin is positioned at the base of the beetle clade, which is in line with current phylogenetic theory that places Strepsiptera as sister group to Coleoptera (Niehuis et al., 2012).

Finally, we would like to emphasize that the presence of two distinct opsins does not mean that *X. peckii* has actual color vision. Color vision requires direct comparison of identical visual fields, the possibility of which largely depends on whether or not UV and green receptors project into the same eyelets. Backfills from portions of the eye showed that photoreceptors of respective eyelets terminated in the lamina (Buschbeck et al., 2003), as though these eyelets were characterized by only one receptor type. But UV and green receptors could be intermingled, leading to similar histological projections, or, alternatively, there could be specializations within the eyelet array, such as a dorsal rim area, which in other insects is converted to a UV-rich polarization sensor (Dacke et al., 2002; Labhart, 1980). Based on the number of reads that mapped to the two opsins, the green opsin appears to be more widely expressed than the UV opsin, but further molecular studies, such as expression analysis, are necessary to resolve this question. Based on the opsin sequence, such studies now can be executed when additional material becomes available. True color vision also depends on the presence of a neural substrate that can adequately process photoreceptor input. However, the presence of distinct UV and green opsins suggests that UV–green coloration could play a

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significant role in strepsipteran ecology, such as helping the male to find the female. Toward that end, it would be interesting to determine whether the *X. peckii* female, which is rather cryptic in the visual spectrum, selectively reflects UV. If so, this could help explain another aspect of the complex life cycle of these extraordinary insects.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.J. wrote portions of the manuscript and assisted in the data collection. S.P.N. collected the majority of the physiological data and A.S. worked on trancriptomics and bioinformatics. E.K.B. organized specimens, conceived and designed experiments, analyzed the physiology data and drafted and revised the manuscript.

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Data availability
Transcriptomic data were deposited in the NCBI Sequence Read Archive, and are available under SRP090411. Opsin sequences were submitted to NCBI GenBank and are available under the following accession numbers: BankIt1954825 long-wavelength, KX898496 and BankIt1954825 UV-wavelength, KX898497. ERG analysis software will be shared upon request.

Supplementary information
Supplementary information available online at
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Supplementary Table S1 with References

Table S1: 50 opsin sequences (with GenBank accession numbers) used for this study. For opsins for which λ_{max} is not given, predictions were made based on molecular analysis. Adapted from Porter et al. (Porter et al., 2007) and Maksimovic et al. (Maksimovic et al., 2011).

	Species	Taxon	GenBank #	λ_{max}	Reference
LONG WAVELENGTH	*Apis mellifera*	Insecta	U26026	540	Townson et al., 1998
	Thermonectus marmoratus	Insecta	EU921225.1	520-540	Maksimovic et al., 2011
	Camponotus abdominalis	Insecta	U32502	510	Popp et al., 1996
	Drosophila melanogaster Rh6	Insecta	Z86118	508	Salcedo et al., 1999
	Limulus polyphemus (lateral eye)	Chelicerata	L03781	520	Smith et al., 1993
	Limulus polyphemus (ocelli)	Chelicerata	L03782	530	Smith et al., 1993
	Manduca sexta	Insecta	L78080	520	White et al., 1983
	Megoura viciae	Insecta	AF189714	LW predicted	Gao et al., 2000
	Neogonodactylus oerstedii Rh1	Crustacea	DQ646869	489	Cronin and Marshall, 1989
	Neogonodactylus oerstedii Rh2	Crustacea	DQ646870	528	Cronin and Marshall, 1989
	Neogonodactylus oerstedii Rh3	Crustacea	DQ646871	522	Cronin and Marshall, 1989
	Papilio glaucus Rh1	Insecta	AF077189	LW predicted	Briscoe, 2000
	Papilio glaucus Rh2	Insecta	AF077190	LW predicted	Briscoe, 2000
	Papilio glaucus Rh3	Insecta	AF067080	LW predicted	Briscoe, 2000
	Papilio glaucus Rh4	Insecta	AF077193	LW predicted	Briscoe, 2000
	Papilio xuthus Rh1	Insecta	AB007423	520	Arikawa et al., 1987
	Papilio xuthus Rh2	Insecta	AB007424	520	Arikawa et al., 1987
	Papilio xuthus Rh3	Insecta	AB007425	575	Arikawa et al., 1987
	Pieris rapae	Insecta	AB177984	540	Ichikawa and Tateda, 1982
	Procambarus clarkii	Crustacea	KT304796.1	533	Zeiger and Goldsmith, 1994
	Schistocerca gregaria	Insecta	X80072	520	Gartner and Towner, 1995
	Sphodromantis sp	Insecta	X71665	515	Rossel, 1979
	Tribolium castaneum	Insecta	NM_001162519.1	LW predicted	Jackowska et al., 2007
	Vanessa cardui	Insecta	AF385333	530	Briscoe et al., 2003
BLUE-GREEN	*Calliphora vicina Rh1*	Insecta	M58334	490	Paul et al., 1986
	Drosophila melanogaster Rh1	Insecta	K02315	478	Feiler et al., 1988
	Drosophila melanogaster Rh2	Insecta	M12896	420	Feiler et al., 1988
	Hemigrapsus sanguineus Rh1	Crustacea	D50583	480	Sakamoto et al., 1996
	Hemigrapsus sanguineus Rh2	Crustacea	D50584	480	Sakamoto et al., 1996
BLUE	*Apis mellifera*	Insecta	AF004168	439	Townson et al., 1998
	Drosophila melanogaster Rh5	Insecta	U67905	437	Salcedo et al., 1999
	Manduca sexta	Insecta	AD001674	450	White et al., 1983
	Papilio glaucus Rh6	Insecta	AF077192	Blue predicted	Briscoe, 2000
	Papilio xuthus Rh4	Insecta	AB028217	460	Arikawa et al., 1987

	Schistocerca gregaria	Insecta	X80072	430	Gartner and Towner, 1995
	Vanessa cardui	Insecta	AF414075	470	Briscoe et al., 2003
ULTRA VIOLET	*Apis mellifera*	Insecta	AF004169	353	Townson et al., 1998
	Camponotus abdominalis	Insecta	AF042788	360	Smith et al., 1997
	Cataglyphis bombycinus	Insecta	AF042787	360	Smith et al., 1997
	Drosophila melanogaster Rh3	Insecta	M17718	345	Feiler et al., 1992
	Drosophila melanogaster Rh4	Insecta	AH001040	375	Feiler et al., 1992
	Manduca sexta	Insecta	L78081	357	White et al., 1983
	Megoura viciae	Insecta	AF189715	UV predicted	Gao et al., 2000
	Papilio glaucus Rh5	Insecta	AF077191	UV predicted	Briscoe, 2000
	Papilio xuthus Rh5	Insecta	AB028218	360	Arikawa et al., 1987
	Tribolium castaneum	Insecta	XM_965251	UV predicted	Jackowska et al., 2007
	Vanessa cardui	Insecta	AF414074	360	Briscoe et al., 2003
	Procambarus clarkii	Crustacea	KT304797.1	440	Kingston and Cronin, 2015
	Thermonectus marmoratus	Insecta	EU921226.1	UV predicted	Maksimovic et al., 2009
	Thermonectus marmoratus	Insecta	EU921227.1	374	Maksimovic et al., 2011

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