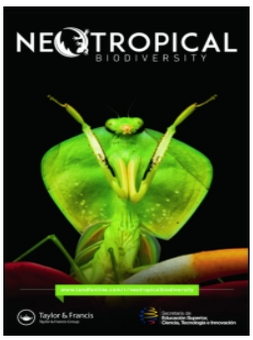

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Sub-functionalization of dorsal and ventral eyes in a whirligig beetle (Coleoptera: Gyrinidae)

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ABSTRACT

Compound eyes in nocturnal or fossorial insects generally express visible light opsins at higher levels than diurnal insects. In this study, we tested whether dorsal (above water) and ventral eyes (below water) of the diurnal four-eyed whirligig beetle *Gyretes sericeus* Laboulbène, 1853, resemble opsin expression and function of diurnal or nocturnal insect eyes respectively. By immunocytochemistry, we compared expression of green LW-opsin in dorsal and ventral rhabdoms of whirligig beetle ommatidia. Basal rhabdomeres (bR) showed comparable expression levels of LW-opsin in both dorsal and ventral ommatidia. In contrast, the inner proximal (R1p) and distal (R1d) bR showed a weak and narrow expression dorsally, whereas R1p and R1d showed a higher and expanded expression ventrally. To test whether dorso-ventral specialization of ommatidia results in functional differences in light response, we studied the behavior of beetles after selective eye-occlusion experiments. During phototaxis experiments, whirligig beetles typically showed a clear preference for light. Positive phototaxis was mainly disrupted after dorsal eye occlusion suggesting dorsal dominance of light sensing. Together, differences in opsin expression, structure, and function of whirligig beetle eyes suggest dorso-ventral sub-functionalizations resembling eye adaptations of diurnal and nocturnal insects. We discuss how dorsal and ventral eye specializations may have evolved in adephagan beetles.

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Apposition; *Gyretes*; rhodopsin; rhabdom; superposition

1. Introduction

The aquatic whirligig beetles (Gyrinidae) are opportunistic scavengers that feed during the day on randomly dispersed nutrient particles, usually dead insects at the water surface [1]. They form groups to optimize foraging and predator avoidance [2]. Thus, ecological and behavioral factors during the daytime activities require optimal color discrimination and effective image formation. In contrast, other sensory mechanisms may be used by whirligig beetles for swimming in dark environments or flying at night, as whirligig beetles are thought to disperse during the night by flying [3].

Whirligig beetles (Coleoptera:Gyrinidae) have an exceptional eye conformation that is adapted for life in an air/water interphase. These beetles contain a pair of ventral submerged eyes pointing the subaquatic environment and another pair of dorsal eyes oriented to the environment above the water surface [4]. While ventral eyes are submitted to low levels of light, dorsal eyes are exposed to large quantities of light [5]. When whirligig beetles are on alert, stimuli to dorsal eyes prevail for escape responses over stimuli to the opposite side (i.e. ventral eyes), suggesting there is a dominance of dorsal visual organs [6,7]. Moreover, perpetual differences in the input of light are likely to generate adaptive sub-functionalization in the anatomy and photoreception of dorsal and ventral eyes of whirligig beetles. Previous studies by Wachmann & Schröer (1975) already suggested that ventral eye anatomy of whirligig beetles showed resemblance to a superposition-like eye of nocturnal insects. Among other features, their analyses showed that ventral eyes contained larger ommatidia with a constriction on the first rhabdom, similar to a clear zone with unpigmented cells in nocturnal eyes, allowing a better absorbance of light. Thus, opposite eye adaptations to light sensitivity within the same individual [6] make the whirligig beetle a particularly interesting model for developmental and evolutionary studies.

Compound eyes of arthropods contain simple units of photoreception, also known as ommatidia [8–12], which rest on the basement membrane that connects the eye to the central nervous system [13]. Each ommatidium is composed of the cornea and crystalline cone that focus light stimulus to the photoreceptor cells below. Photoreceptor cells R1 to R7 and/or R8 absorb different wavelengths depending on the presence of different photo pigments (i.e. opsins), and activate the ganglion cells of the optic nerve to project signals to the brain [14]. Inside the photoreceptor cells, there are special molecular pigments, better known as opsins, which allow for color vision in these animals. Opsins are G-protein coupled receptors used and adapted for light perception that under certain

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conditions have shown a large plasticity of responses to environmental change [14–16]. In some nocturnal insects adapted for dark vision, green photopigments (Rh1, Rh2, or LW-opsin) together with some coexpression of UV sensitive opsins (Rh4, Rh3, or UV-opsin) are uniformly expressed in photoreceptor cells along the length of the omatidia. This pattern of expression was acquired at the expense of blue opsins. In contrast, diurnal insects show the standard pattern of opsin expression, including subjugated green and/or UV opsins in the central R7 and R8 basal rhabdom (bR), as well as in regions of the bR close to the basement membrane of the ommatidia [17].

In this study, we show that the anatomical differences observed between the two pairs of eyes in the whirligig beetle *Gyretes sericeus* also show functional differences in their response to light sensitivity in eye occlusion behavioral experiments. In addition, we assess the previously described resemblance of Gyrinidae ventral eyes to nocturnal insect eyes (i.e. a uniform expression of green LW-opsin), as well as a similar expression of LW-opsin of Gyrinidae dorsal eyes to the eyes of other diurnal insects (i.e. expression in rhabdom 7 & 8 and most proximal regions of the dorsal eye ommatidia). In summary, our work provides evidence for a modal subfunctionalization of dorsal and ventral eyes in whirligig beetles supported by differences in structure, expression, and function of the eyes. Finally, we discuss how these eye adaptations may reflect certain evolutionary trajectories in adephagan beetles based on current understanding of phylogenetic relationships on the group.

2. Materials and methods

2.1. *Animal collecting*

Insects were collected manually in two locations: (1) in La Almenara stream, located on the outskirts of Santa María, Boyacá, Colombia (4°51′27.0479″N, −73° 15′54.7823″O, 793 m.a.s.l.) and (2) in a lake located in the ecological park "Matarrendonda," on the road to Bogotá-Choachí, Cundinamarca, Colombia (4°33′ 42.0062″N, −74°00′02.8759″O, 3331 m.a.s.l.). Animals were transported to Universidad de Los Andes in a polystyrene chamber with ice and moist tissue paper to keep humidity and temperatures down (10°C) to ensure the survival of the specimens. In the laboratory, individuals were maintained in distilled water at room temperature (RT) 15 ± 2°C in a small aquarium (20.5 cm × 20.5 cm × 5.7 cm). The colony was maintained with distilled water and fed daily with frozen fruit flies (*Drosophila melanogaster*) and Tipulid mosquitoes. Individuals were indetified as *Gyretes sericeus* (Figure 1) following identification characters featured in Miller & Bergsten [18], and commentaries from G. Gustafson.

2.2. *Immunocytochemistry*

Fluorescent whole mount immunocytochemistry: beetle eyes were dissected and fixed in 4% Paraformaldehyde (PFA). After fixation samples were washed 3 times in Phosphate-buffered saline (PBS) solution and blocked with PBT/milk solution (1 × PBS + 0.1% Triton

Figure 1. Photograph of *G. sericeus*. Identification was based mainly on the antennal segments and the texture of the elytra. Specimens are available at the Museum of Zoology of 'Universidad de Los Andes', Bogotá, Colombia. Images by Yiselle Cano, used with permission. Scale bars = 50 mm.

X100 + 5% powdered milk, pH 7.2) for 1 h. Because a coleopteran specific antibody is not available, we used rhodopsin type 1 (4C5) Mouse mAb, an antibody against *Drosophila melanogaster* Rh1, which corresponds to a LW-opsin (Developmental Studies Hybridoma Bank, DSHB). Related LW-opsin sequences are conserved and have been found to be expressed in retinal photoreceptors of the closely related sunburst diving beetle *Thermonectus marmoratus* (Coleoptera: Dytiscidae), the more distantly related flour beetle *Tribolium castaneum*, as well as *Drosophila* species (Maksimovic et al. 2009). Compound eyes were incubated over night at 4°C with the rhodopsin type 1 (4C5) Mouse mAb 1:100 in PBT (1 × PBS + 0.1% Triton X100, pH 7.2). Samples were washed 3 × 10 min with PBT at RT, and incubated over night at 4° C with goat anti-mouse IgG AlexaFluor-488 conjugated secondary antibody (Invitrogen, A32723) 1:500 in PBT. The next day samples were washed 3 × 10 min again with PBT and 30 min with PBS. Finally, the retinas were mounted in two drops of autoclaved glycerol and pictures of the tissue were taken with a Nikon AZ100M fluorescent stereomicroscope. Ten specimens were analyzed in total.

Colorimetric immunohistochemistry: For histological preparations, individual heads were dissected with scissors and fixed in formaldehyde (4% in PBS) over night at 4°C. Tissues were dehydrated in ethanol series (30, 50, 70, 90, and 100%), followed by two xylol washes, and kept for 30 min in a 1:1 Xylol: Paraffin solution, and embedded in paraffin blocks. After this, eyes were embed in paraffin and sliced in cross sections of 6 µm using a Leitz 1300 large base sledge microtome. Cross sections on slides were then treated in xylol and rehydrated again in decreasing ethanol dilutions (as previously described) to stain with Eosin and Hematoxilin. Later on, the rhodopsin type 1 (4C5) Mouse mAb was incubated in 1:100 PBT over night at 4°C. After 3 × 10 min PBT washes, samples were incubated with anti-rabbit IgG-AP (Santa Cruz Biotechnology, TX, US, sc-2034) at 1:200, as a secondary antibody for 4 h. The next day samples were washed 3 × 10 min in PBT and twice in PBS. For the color reaction, we used NBT/BCIP (Promega, US) solution for 20 min. Samples were washed 3X with PBT and 1X in distilled water. Afterwards, the samples were dehydrated 1 with 30 min washes in the following ethanol dilutions: 30, 50, 70, 80 and 100%. Finally xylol was applied for another 30 min to be coverslipped with Permount mounting medium (Fisher Scientific). Histological sections were photographed with a ZEISS Axio Imager A2 microscope. In total 10 individuals were used successfully for sections.

2.3. *Behavioral tests*

We constructed a behavioral arena that consisted of a T-shaped maze to test for light preference of laboratory whirligig beetles (Figure 2(a)). To acclimatize all individuals to similar light stimulus and exclude any influence of light sensitivity before the experiments, we placed each individual in a separate dark pool for 5 min followed by a free-swimming period in the starting pool of the T-shaped arena for another brief period (1 min) in absence of light. Following the acclimatization period, the beetle was allowed to swim the maze to the decision point. Here, the animal had to decide between remaining in a bright chamber with a light bulb intensity around 1.7364e14 photons/cm^2.s and a light wavelength of 530 nm, remaining in the dark chamber covered with an aluminum hood on top of a black background. Preliminary behavioral tests were done throughout the day, from morning (8 AM) to the afternoon (5 PM), to discard possible circadian influences on the behavioral responses. Eye occlusion treatments to stimulate light input into dorsal or ventral eye pairs selectively, dry black nail polish was applied carefully

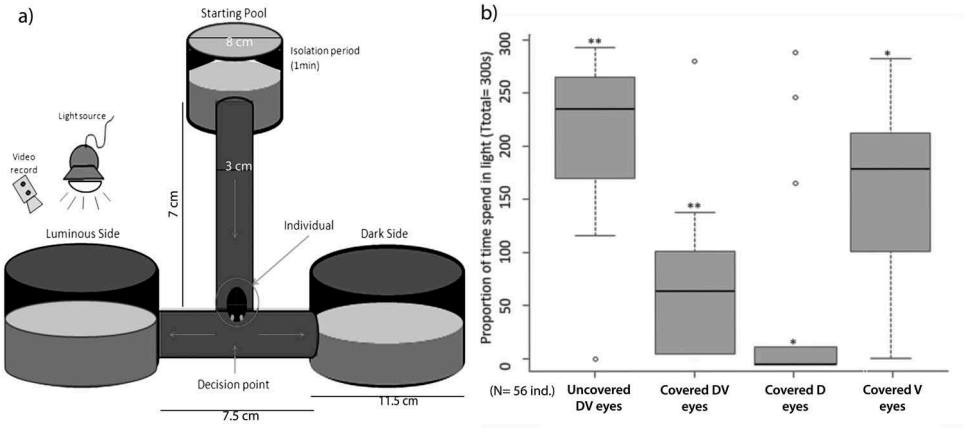

Figure 2. Experiment view and general results of behavioral tests. a) Assembly for the test where the beetle had to stay in the starting pool for an adaptation period and shortly after the time spend in the light was recorder. b) Light response behavior in beetles with dorsal eye occlusion is disrupted. (Control) No sensory alteration and normal eye conditions, (Covered) Dorsal and ventral eyes covered, (Dorsal) Ventral eyes covered, (Ventral) Dorsal eyes covered (n = 56, 14 individuals per treatment).

on the eyes with a fine brush. Black nail polish is insoluble in water and generates a permanent blockage of light. The beetles showed no obvious alteration on their ability to swim or move freely in the water. We recorded the time spent by the beetles on the bright chamber during a lapse of 5 min using a Panasonic Lumix DMC-FS42 video camera. Four treatments were used to evaluate light sensing functional responses of dorsal vs. ventral eye-pairs: (1) no sensory alteration in uncovered eye conditions (Control), (2) complete sensory alteration by carefully covering dorsal and ventral eyes with nail polish (Covered), (3) partial sensory alteration by covering ventral eyes only (i.e. dorsal visualization only), and (4) partial sensory alteration by covering dorsal eyes only (i.e. ventral visualization only). We used 14 individual replicates for each treatment.

2.4. *Statistical analysis*

We used a Shapiro–Wilk test to test normality of our data. Normality was rejected so we decided to use a Kruskal–Wallis test and Wilcoxon tests to assess differences on light attraction between the eye treatments as previously described (Table 1). All statistical tests and box plots were made with the R software version 3.0.2 [19].

3. Results

3.1. *Behavioral assays*

Systemic eye occlusion assays in whirligig beetles showed that the dorsal eyes mainly mediated photo-tactic responses. In all three eye-occlusion treatments, which included: (a) beetles with dorsal and ventral eyes covered, (b) beetles with ventral eyes covered, and (c) beetles with only dorsal eyes covered, the time spent under the light was significantly lower from the controls with no eye-occlusion (Figure 2(b)). Most categories showed significant differences to the controls and among treatments after a Wilcoxon test analysis under a significance of α = 0.05 (Table 2). When dorsal and ventral eyes were covered (Covered DV eyes) positive phototactic responses of the beetles were significantly lower than beetles with uncovered eyes (controls) (Figure 2 and Table 2). Beetles showed the lowest phtototactic response after dorsal eyes were covered (Covered D eyes) (Figure 2 and Table 2), although some outliers were found to respond to phototaxis probably due to some remaining light sensitivity of ventral eyes (blank circles). Both of these experiments show that the beetles prefer to use dorsal eyes over ventral eyes for phototaxis. Furthermore, when ventral eyes were covered and dorsal eyes uncovered, the light response was no different from the uncovered controls (Figure 2, Tables 1 and 2).

Table 1. Test for normality in treatments and detection of treatments differences, α = 0.05.

	p-values			
Tests	*Control*	*Covered*	*Dorsal*	*Ventral*
Shapiro–Wilk	0.02327	0.007686	0.1729	2.148 e −05
Kruskal–Wallis	0.0001492**			

** Statistic significance for differences in all treatments, X^2 = 20.2697, *df* = 3.

3.2. *Anatomical characterization of dorsal and ventral eyes*

Green opsin immunochemical reaction shows expression deeply located within dorsal ommatidia at relatively low levels, whereas it was expressed at relatively higher levels externally and throughout the ventral ommatidia. Figure 2 indicates possible differential expression patterns on ventral and dorsal eyes. Opsin expression in dorsal eyes is concentrated in photoreceptors which are closer to the basal membrane (Figure 3(c,d & d')), where it is been described is the location from the second to the eighth rhabdom [7]. In contrast, ventral eyes showed expression along the complete length of the ommatidia (Figure 3(f-i)), starting externally on the location of the first rhabdom, extending through the clear middle zone and up to the basal membrane of the eye, which comes into contact with the optic nerve. Thus, functional diversification of vision in whirligig beetles is supported by different expression patterns of opsins observed in dorsal and ventral eyes.

4. Discussion

4.1. *Sub-functionalization of dorsal and ventral eye morphologies*

Our observations of an inner opsin expression in the central bR of dorsal eyes (Figure 3(d')) correlates with the hypothesis that this conformation of the bR serves as a filter to the incoming light for better reception and sensory discrimination. Dorsal eyes of Gyrinidae expressed relatively higher levels of opsin in more basally located positions (i.e. internally), helping to form an apposition eye-structure. We believe whirligig beetles use this structural conformation to form high quality images together with other expressing opsins, and possibly allow the animal to have a better visual discrimination.

Table 2. Wilcoxon test for differences between treatments, α = 0.05.

	p-values			
Treatments	*Control*	*Covered*	*Dorsal*	*Ventral*
Control	–	+	+	+
Covered	0.0006104*	–	+	+
Dorsal	0.1769	0.01074*	–	+
Ventral	0.004166*	0.7598	0.01074*	–

*Tests that show significant difference comparing a pair of treatments

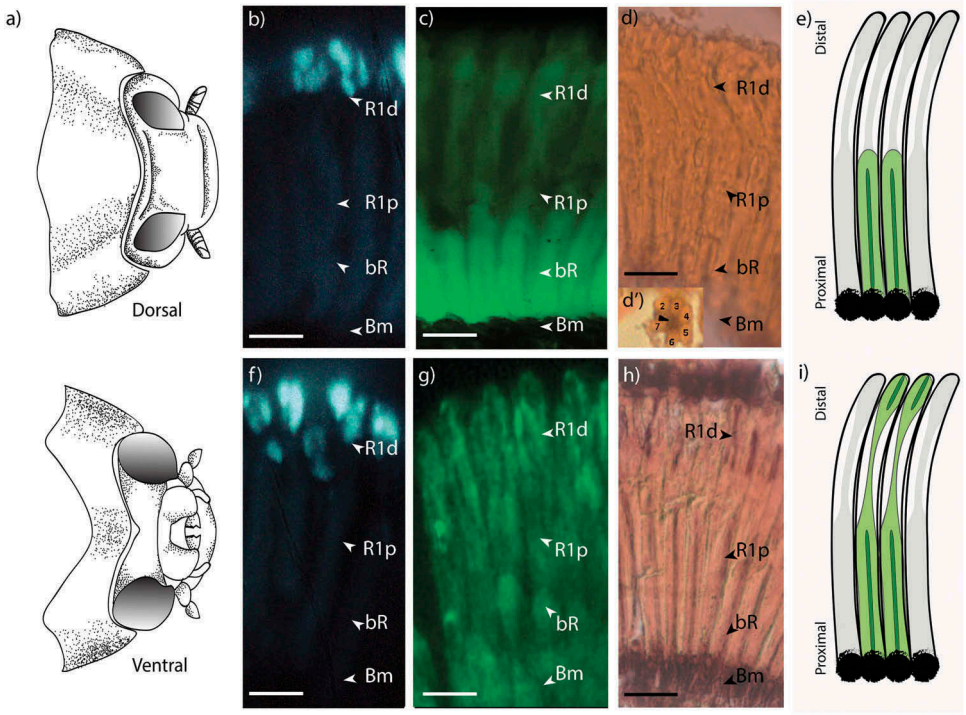

Figure 3. Expression patterning of the green opsin in both eye types, confirming apposition and superposition-like structure in dorsal and ventral eyes respectively. a) Dorsal, lateral and ventral view of beetle eyes. b) & f) DAPI staining delimitating cell nuclei. c) & g) Fluorescent immunostaining of the photopigment in dorsal and ventral rhabdomeres. d) & h) Eosin and AP antibody staining to the ommatidia, green opsin marked as purple. d') Basal view of the rhabdomeres agglomeration showing the photopigment expression on the eight rhabdom e) & i) Schemes illustrating the proposed distribution for green opsin in both dorsal and ventral eyes. R1d = Distal rhabdomere 1; R1p = Proximal rhabdomere 1; bR = Basal rhabdomere; Bm = Basal membrane. Scale bars = 20 µm.

A superposition-like conformation of bR in the ventral side shows consistency with previous descriptions made by Bott, Wachmann and Schröer [7,20]. The external region of ventral eyes directs a large quantity of light into a clear zone in the middle containing one rhabdom [21]. Transverse sections of the outer ommatidia of ventral eyes in whirligig beetles showed opsin expression in both the inner and the first outer bR (Figure 3(g, h)) suggests a more light-sensitive conformation. More distally located expression of green opsin in ventral eyes shortens the distance from the stimulus to the photoreceptors in the ommatidia increasing the speed of visual processing [22] and allowing the beetles to better sense light under low illumination conditions.

In addition to the green opsin expression pattern described above, ventral eyes showed similarities with superposition-like conformation, particularly by the first bR morphology. With the shape of an inversed drop, and a constriction in the median part to create a clear zone-like area, the first rhabdom is located close to the crystalline cone in the external part of the ommatidia. These features allow light to converge in a space between the crystalline cone and the bR (Figure 3(g, h) [7].). A greater sensitivity to light has tradeoffs for image acquisition and resolution. While shorter is the distance between the stimulus and light-reception, a more chromatically aberrated image is generated [21,23,24]. Animals can correct this problem by changing the form of the lens or displaying an asymmetric sensory pigments location in respect to the ommatidium or cornea [23,25,26] (e.g. external short wave pigments for increased light sensitivity) (Figure 3(i)).

Our results predict that ventral superposition-like eyes in Gyrinidae may have poor resolution of images although a higher light sensitivity. Specialized eyes of the closely related diving dyctiscid beetle larvae show green sensitive-opsin expression in the distal retina that is closer to the lens [21,27], resembling the general organization and expression pattern we observe in the ventral adult eyes of gyrinid beetles. Dyctiscid beetle larvae, also known as water tigers, have more predatory and aquatic life habits than the whirligig beetles (Gyrinidae). A light sensitive specialization of eyes in these two beetles may serve as adaptations to their fundamentally aquatic lifestyles, and less likely represent adaptations to their predatory feeding behaviors.

4.2. *Evolution of night/day vision in Gyrinidae beetles*

Aquatic adephagan beetles (Hydradephaga) have mostly nocturnal habits [28,29], except for the

whirligig beetles that are mostly diurnal and live in the water interphase [30,31]. According to two recent phylogenies discussing the position of Gyrinidae inside Hydradephaga [32,33], the evolution of apposition and superposition-like eyes in gyrinids may differ. In one recent phylogenetic study using ultraconserved elements (UCEs), the Gyrinidae are placed as sister group to the geadephagan beetles and the rest of hydroadephagan beetles [32]. A paraphyletic Hydradephaga means that either aquatic habits in this group of beetles evolved twice, or aquatic habits were lost once in the geadephagan beetles. However, another phylogenetic study using complete mitochondrial genome sequences published the same year supported a sister group relationship between a monophyletic Geadephaga and a monophyletic Hydradephaga, which included the Gyrinidae [33]. If the ancestral state of Adephaga (Geadephaga + Hydradephaga) resembles apposition eyes of most diurnal beetles with daylight perception, then the dorsal apposition-like eyes in the gyrinids would have gone through a loss in the Hydradephaga and regain of the character in the Gyrinidae. On the other hand, if the ancestral state of Adephaga resembles superposition eyes of nocturnal insects, then the dorsal apposition-like eyes of the Gyrinidae would have evolved independently.

The predaceus diving beetles (Dytiscidae), another well-studied family of the Hydradephaga clade, also presents a similar configuration of opsin distribution as occurs in the ventral eyes of Gyrinidae [21,24]. When compared to whirligig beetles (Gyrinidae), diving beetles (Dyctiscidae) are more active and generally prey on larger animals. However, these differences in microhabitats and behaviour did not result in any obvious or large differences in eye conformation among these two aquatic beetle groups. Therefore, our results show similarities among the ventral superposition-like eyes of whirligig beetles to the eyes of other aquatic beetles suggesting a single evolutionary acquisition of dark adaptations in these eyes.

The sub-functionalization of dorsoventral eyes in *G. sericeus* with a prevalent use of dorsal eyes for light responses raises questions about the role and functional implication of ventral eyes for the organism. A predominant use of dorsal eyes over ventral eyes has also been suggested before to serve for better spatial recognition and positioning, which resulted in effects on the overall neural organization of this beetle [34]. Future research in *G. sericeus* should focus on how dorsal and ventral stimuli are processed by the nervous system, and specifically how a sub-functionalization of photosensorial systems in the dorsal and ventral side of whirligig beetles is related to the ecological and evolutionary history of the beetles.

Adaptations of whirligig beetle eyes to different environmental conditions are evolutionarily plastic. Thus, our studies show that the evolution of photosensory systems in beetles is very dynamic and that the configuration of the retina, photoreceptors, and opsin expression in beetle eyes can easily adapt for optimal perception under different environmental conditions. In summary, we show that apposition and superposition conformations of eyes that are typically observed in beetles (and other insects) with diurnal or nocturnal habits resemble the apposition-like and superposition-like eyes observed in the aerial/dorsal or aquatic/ventral eyes of whirligig beetles.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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