## RESEARCH ARTICLE

# Establishment of correctly focused eyes may not require visual input in arthropods

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## ABSTRACT

For proper function, vertebrate and invertebrate visual systems must be able to achieve and maintain emmetropia, a state where distant objects are in focus on the retina. In vertebrates, this is accomplished through a combination of genetic control during early development and homeostatic visual input that fine-tunes the optics of the eye. While emmetropization has long been researched in vertebrates, it is largely unknown how emmetropia is established in arthropods. We used a micro-ophthalmoscope to directly measure how the lens projects images onto the retina in the eyes of small, live arthropods, allowing us to compare the refractive states of light-reared and dark-reared arthropods. First, we measured the image-forming larval eyes of diving beetles (*Thermonectus marmoratus*), which are known to grow rapidly and dramatically between larval instars. Then, we measured the image-forming principal anterior-median eyes of jumping spiders (*Phidippus audax*) after emergence from their egg cases. Finally, we measured individual ommatidia in the compound eyes of flesh flies (*Sarcophaga bullata*) that had developed and emerged under either light or dark conditions. Surprisingly, and in sharp contrast to vertebrates, our data for this diverse set of arthropods suggest that visual input is inconsequential in regard to achieving well-focused eyes. Although it remains unclear whether visual input that is received after the initial development further improves focusing, these results suggest that at least the initial coordination between the lens refractive power and eye size in arthropods may be more strongly predetermined by developmental factors than is typically the case in vertebrates.

KEY WORDS: Refractive error, Eye development, Invertebrate vision, Optics, Emmetropization

## INTRODUCTION

Eyes are among the most complex sensory organs (Land and Nilsson, 2012) and animals depend on an optimized visual system for survival (Cronin et al., 2014). As such, the visual systems of vertebrates and invertebrates alike must be able to achieve and maintain a state of correct focusing, which needs to be established during development and then maintained, even if the eye grows. A correctly focused eye is characterized by a precise match between the focal length of its optics and the distance between the lens and the retina. In emmetropic eyes, the images of objects at infinity fall directly onto the retina. If images are positioned in front of the retina, the eyes are near-sighted or myopic, and if they are positioned

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behind the retina, the eyes are far-sighted or hyperopic. Typically, such deviations are considered to be refractive errors, although some invertebrate eyes may have evolved to focus at distances other than infinity (Stowasser et al., 2017).

How eyes develop their optics correctly has been investigated intensively in vertebrates (Flitcroft, 2013; McBrien and Barnes, 1984; Troilo, 1992; Wallman and Winawer, 2004) and has become a particularly important question considering that a drastic increase in myopia has been found (Dolgin, 2015; Matsuda and Park, 2019). Many arthropods also have sophisticated image-forming eyes (Land and Nilsson, 2012) that require precise focusing and therefore must also have mechanisms that tightly control eye growth. However, the mechanisms that coordinate lens refraction and the spacing between the lens and retina during the development of arthropod eyes remain elusive.

Studies in vertebrates and in squid (Sivak and Sivak, 2019; Turnbull et al., 2015) have suggested that the coordination of eye growth towards emmetropia is accomplished through a combination of gene regulation during early development (passive regulation) and homeostatic feedback in the form of visual input from the environment (light) that fine-tunes the eye (active regulation). Vertebrates are typically born with eyes that are either myopic or hyperopic (Wallman and Winawer, 2004) and later grow to establish emmetropia (Schaeffel and Feldkaemper, 2015). Passive emmetropization, understood as the non-visual execution of a genetic plan, often continues postnatally and acts in combination with the physical constraints that are imposed on a growing eye (Mark, 1972), thus allowing eye growth to diminish refractive errors. Studies in numerous vertebrates also provide strong evidence that visual feedback has a role in controlling eye growth. For example, with adequate visual input, tree shrews were able to recover from visual manipulations (Siegwart and Norton, 2010). Other support derives from studies in fish, where chromatic defocus was compensated for (Kroger and Wagner, 1996), and from guinea pigs (McFadden et al., 2004), primates (Hung et al., 1995) and chicks (Schaeffel et al., 1988; Troilo and Wallman, 1991; Wahl et al., 2015, 2016), typically by monitoring compensation of growing eyes after imposing defocus through spectacle lenses. In the study of Troilo and Wallman (1991), a refractive error was induced in chicks via visual manipulations, including dark-rearing. The chicks were ultimately able to recover emmetropia by actively adjusting the growth of their vitreous chambers. Specifically, growth stopped in eyes that were recovering from myopia and continued in eyes that suffered from hyperopia. Since then, it has become clear that image contrast is a critical regulator (Wahl et al., 2015, 2016). To the best of our knowledge, the only study that addresses a similar mechanism in invertebrates was performed on squid (Turnbull et al., 2015), which found that squid were able to compensate for refractive errors introduced by raising them under abnormal lighting conditions (orange or blue light).

Correctly focused eyes are particularly important for arthropods with high-resolution camera-type eyes, including visually guided

predators such as some jumping spiders (Jakob et al., 2018; Land, 1969, 1972) and predatory holometabolous insect larvae (Buschbeck, 2014; Gilbert, 1994; Toh and Mizutani, 1987; Toh and Okamura, 2007). Correct focus is also important for arthropods with compound eyes (Warrant and McIntyre, 1993), such as flies, which rely on their visual system for fast flight control, for example during mating flights or predatory behavior (Wardill et al., 2017). In compound eyes, proper focusing promotes optimal light capture, and in flies the neural superposition organization relies on the ability to independently resolve multiple sampling points within each ommatidium (Nilsson, 1989). To gain a better understanding of how arthropods develop emmetropic eyes, we examined species with both fundamentally different eye designs and diverse phylogenetic backgrounds. Specifically, we investigated how the visual systems of the diving beetle larva *Thermonectus marmoratus*, the jumping spider *Phidippus audax* and the flesh fly *Sarcophaga bullata* develop with and without access to visual input.

The larvae of sunburst diving beetles (*T. marmoratus*) have sophisticated camera-type image-forming eyes with elaborate optics (Stowasser et al., 2010). To catch prey, these larvae perform a scanning behavior (Buschbeck et al., 2007) and their precise optics are likely important for assessing distance (Bland et al., 2014). *Thermonectus marmoratus* has three larval instars that differ dramatically both in body size and in eye size. At each molt, larvae shed not only their exoskeleton but also a small portion of the lens, which undergoes dramatic reformation to produce longer focal lengths for larger eyes (Werner and Buschbeck, 2015). Proper focusing of the eye is re-established approximately 8 h post-molt. As the dramatic changes at this developmental transition are already documented, they represent a particularly good point of investigation for this study. The precise organization of the principal eyes [anterior-median (AM) eyes] of jumping spiders into multiple layers is also known to be important for distance vision (Nagata et al., 2012). However, it is unclear how the optics changes between instars in *P. audax*; thus, we focused on their initial development. Upon emergence from their egg cases, jumping spiders rely on their visual systems to capture prey, so their eyes are expected to be fully functional at that time point. Finally, we assessed the refractive state of individual ommatidia of the compound eye of *S. bullata*, again focusing on their eye development immediately after emerging from a developmental stage.

To the best of our knowledge, this represents the first investigation into whether light fosters active regulation in developing arthropod eyes, possibly due to the technical challenge associated with precise measurements of such small visual systems. This challenge has been overcome using a micro-ophthalmoscope imaging technique that allows direct measurements of refractive errors in small, live arthropod photoreceptors based on photoreceptor autofluorescence (Stowasser et al., 2017). In combination with lens focal length measurements, we were able to quantify the position of images within the eye relative to its retina or specific retina layers.

## MATERIALS AND METHODS
### Animal husbandry
*Thermonectus marmoratus* Gray 1832 larvae were offspring of our beetle colony, which was originally collected in Arizona and obtained through Bugs of America (Portal, AZ, USA). The colony was maintained in freshwater aquaria at approximately 28°C. The larvae were reared individually and fed a daily diet of bloodworms and mosquitoes. All larvae were raised under a 14 h light:10 h dark cycle and fed daily until the day they were expected to molt into

their 3rd larval stage. At this point, the larvae were divided into control or dark-reared groups and deprived of food. The control larvae were kept under regular light:dark conditions, whereas the dark-reared larvae were deprived of light until measurements were taken. The measurements for all individuals were performed 1 day post-molt into the 3rd instar to ensure sufficient time for full reformation of their lenses (Werner and Buschbeck, 2015).

*Phidippus audax* (Hentz 1845) spiderlings were obtained from egg sacks that were laid after mating individuals from our lab population, which were originally wild caught in Cincinnati, OH, USA, purchased from Phids.net (West Palm Beach, FL, USA) or collected in the vicinity of Pittsburgh, PA, USA. The control and test groups each used spiderlings from two egg clutches. The spiders were kept in our animal husbandry room at 28°C under a 14 h light:10 h dark cycle. For the dark-treatment group, the egg clutches were deprived of light shortly after being laid, whereas the control egg clutches were kept under regular light:dark conditions. Upon emergence, the spiderlings were separated into individual vials with access to water but no food to control for any potential effect diet could have on their visual development, as the test animals were unable to hunt without light. All spiderlings remained in their respective light or dark conditions until measurements were performed, within 10 days of emergence, a time period in which no further molts were observed.

*Sarcophaga bullata* (Parker 1916) flies were obtained as pupae from Carolina Biological Supply Company (Burlington, NC, USA). The fly pupae were separated into three groups: a control group that was measured during the daytime, a control group that was measured during the night-time and a dark-reared group. The night-time control group was introduced to account for the possible effects of retinomotor movements that have previously been described in insect compound eyes (Walcott, 1971; Warrant and McIntyre, 1993). All flies were raised in incubators at 25°C with access to sugar and water. The daytime control flies were raised under a 14 h light:10 h dark cycle and, after emergence, measurements were taken during the flies' daytime hours. The night-time control flies were raised under a time-shifted light:dark cycle, with measurements taken during the flies' night-time hours. The dark-reared flies were deprived of light until measurements were taken. Measurements of male and female flies were performed within 10 days of emergence into adults from their pupal stage.

### Establishment of refractive errors
Our micro-ophthalmoscope allowed us to measure the refractive errors of arthropod photoreceptors based on the autofluorescence of their receptors, as described by Stowasser et al. (2017). In brief, the animal is mounted with the eyes of interest looking up vertically and positioned under an objective so that the lens of the eye of interest is in sharp focus for the camera. Thereafter, an accessory lens is added to allow imaging of the eye's retina by visualizing the red autofluorescence that can be generated using a typical Texas Red filter. The accessory lens can be moved along a rail, with the refractive state of the eye determining the accessory lens position that yields a focused retinal image. The direction that the lens is moved in reference to a zero position (indicating emmetropia) is informative in regard to the eye being myopic (near-sighted) or hyperopic (far-sighted). The principal eyes of *T. marmoratus* (E1 and E2) were imaged with a 20× water objective and were evaluated in regard to the best-focused images, which correspond to the second (longer) focus of the bifocal lens (Stowasser et al., 2010). The AM eyes of *P. audax* were imaged with a 10× UPlanFL objective and the individual ommatidia of *S. bullata*

were imaged with a 40× UPlanFL objective (Olympus, Center Valley, PA, USA). For each objective, we established a calibration curve for the ophthalmoscope that allowed us to determine the relationship between the accessory lens position and the corresponding in-focus object distance. After imaging, the eyes were either dissected or fixed for histology to determine the lens focal length, which is necessary to determine refractive errors quantitatively.

### Focal length measurements

Individual focal length measurements were taken to determine where an image was focused within the eye relative to the position of the retina for a specific animal. Focal length measurements are needed for quantitative evaluation of an animal's focus using the micro-ophthalmoscope. Two different techniques were applied to determine the focal length. Modified versions of the hanging-drop method were applied for the jumping spiders and beetle larvae, the lenses of which are sufficiently large for reliable measurements. For flesh flies, the hanging-drop method provides less reliable measurements; thus, we assessed the focal length through an image magnification method based on obtaining the absolute size of the ommatidial photoreceptor array through histology.

### Hanging-drop method

We followed a previously used hanging-drop method (modified after Homann, 1924) as described in detail by Stowasser et al. (2017). In brief, the focal length of the animal's lens was calculated from the image magnification (Land, 1969; Land and Nilsson, 2012). The dissected spider lenses were mounted upside down, floating on a drop of Ringer solution (O'shea and Adams, 1981), and the diving beetle larval lenses were submerged within a less concentrated Ringer solution (see Stowasser and Buschbeck, 2014; Stowasser et al., 2010). To determine the image magnification, we took images at 5 µm intervals through the lenses (using a 10× objective) of an object (USAF 1951 negative test target, Edmund Optics, Barrington, NJ, USA) that was placed at effective infinity for the animal's lens (distance of 4.3, 12.1 or 13.4 cm). First, the best-focused image was found by using a custom-written MATLAB program that evaluated the images based on their edge sharpness. Then, the focal length of the dissected lens was calculated based on the magnification of the best-focused image in comparison with the size of the object.

### Histological assessment

To obtain histological images of the photoreceptors, fly heads were fixed at 4°C in a solution of 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in Sørensen's phosphate buffer at pH 7.4 for up to 24 h. The tissue was then rinsed and fixed in a 1% osmium tetroxide ($OsO_4$) solution for 1 h on ice and then 1 h at room temperature. Next, the tissue was rinsed with water and dehydrated in a series of increasingly concentrated alcohol solutions and ultimately acetone. The tissue was then embedded in an ultra-low viscosity embedding medium (Polysciences, Inc., Warrington, PA, USA), sectioned at intervals of 8 µm, and mounted on slides. The absolute sizes of the photoreceptor arrays could then be determined by evaluating histological images against those of a micrometer scale using Adobe Photoshop CC 2018. The absolute sizes of the photoreceptor array images produced by the animals' eyes were determined from opthalmoscope images that were corrected for the opthalmoscope's image magnification. The eyes' focal lengths could then be calculated from the image magnification between the histological and opthalmoscope images.

## RESULTS

To investigate arthropod eye growth towards emmetropia, we assessed the refractive state of visually deprived and control animals using micro-ophthalmoscope measurements. For each individual, the refractive error (relative to emmetropia) was evaluated in terms of the object distance for which the animal's corresponding photoreceptor arrays would be in focus. This was calculated based on the range (and center point) of micro-ophthalmoscope accessory lens positions that resulted in the clearest focused images of the retina, in combination with a previously established objective calibration. Specifically, the calibration established the correlation between accessory positions and in-focus object distances. To better evaluate and illustrate the data, calculations were based on the reciprocal of the object distance, which puts the infinite focus of emmetropia into the center. Using the focal lengths measured independently for each individual in combination with the object distance, we were able to evaluate the refractive state of all dark-reared and control animals quantitatively in terms of where the image of an object was focused within the eye relative to the retina. This can also be thought of as the distance between the surface of the retina and the focal plane of the eye's lens.

### *Thermonectus marmoratus*

The proximal retina of the right eye 1 (E1) and eye 2 (E2; Fig. 1A,B) of 10 dark-reared and 10 control *T. marmoratus* larvae was imaged with the ophthalmoscope for each individual. The range and center point of the object distances at which the animal's retina was in focus were plotted for each individual. The object distances for E1 (Fig. 1C) and E2 (Fig. 1F) indicate that both were focused close to emmetropia, with the majority of individuals exhibiting slight myopia (near-sightedness). The differences between the dark-reared and control individuals were not significant for E1 ($P=0.193$) or E2 ($P=0.282$) based on a two-sample $t$-test assuming unequal variance. The variance of the ophthalmoscope measurements for the average of the range of focus was significantly lower for the test animals than for the controls ($F$-test for variance; $P=8.7\times10^{-5}$ for E1, $P=3.2\times10^{-5}$ for E2). The focal lengths for E1 ranged from ∼452.4 to 595.2 µm for the control animals (mean±s.d. 514 ±46.9 µm) and from ∼520.4 to 608.8 µm for the test animals (567 ±31.3 µm; Fig. 1D). For E2, the focal lengths ranged from ∼397.9 to 581.6 µm for the control animals (508.8±55.3 µm) and from ∼445.6 to 602.06 µm for the test animals (514.6±45.7 µm; Fig. 1G), with E2 having a shorter focal length than E1 on average. There was no significant difference in focal length between the control and test animals for E2 ($P=0.802$), but the difference was significant for E1 ($P=0.008$) based on a two-sample $t$-test assuming equal variance.

Subsequently, the object distances and focal lengths were used to determine where the image was focused relative to the proximal retina for each individual. For E1, the values for the control animals ranged from −20.8 to 19.4 µm (mean±s.d. 15.4±19 µm) and those for the test animals from −16.03 to 12.8 µm (6.3±12.8 µm; Fig. 1E). For E2, the values for the control animals ranged from −17.5 to 40.9 µm (14.9±19 µm) and those for the test animals from −15.6 to 25.2 µm (5.7±13.7 µm; Fig. 1H). The difference was not significant for either E1 ($P=0.182$) or E2 ($P=0.233$), nor was there a significant difference in regard to the variance. Nevertheless, it is noteworthy that the focus of the control larvae, on average, was slightly more myopic than that of the dark-reared larvae (Fig. 1C,F;E,H).

### *Phidippus audax*

The AM eyes of jumping spiders are known to be organized into multiple layers (Land, 1969), two of which are visible in the
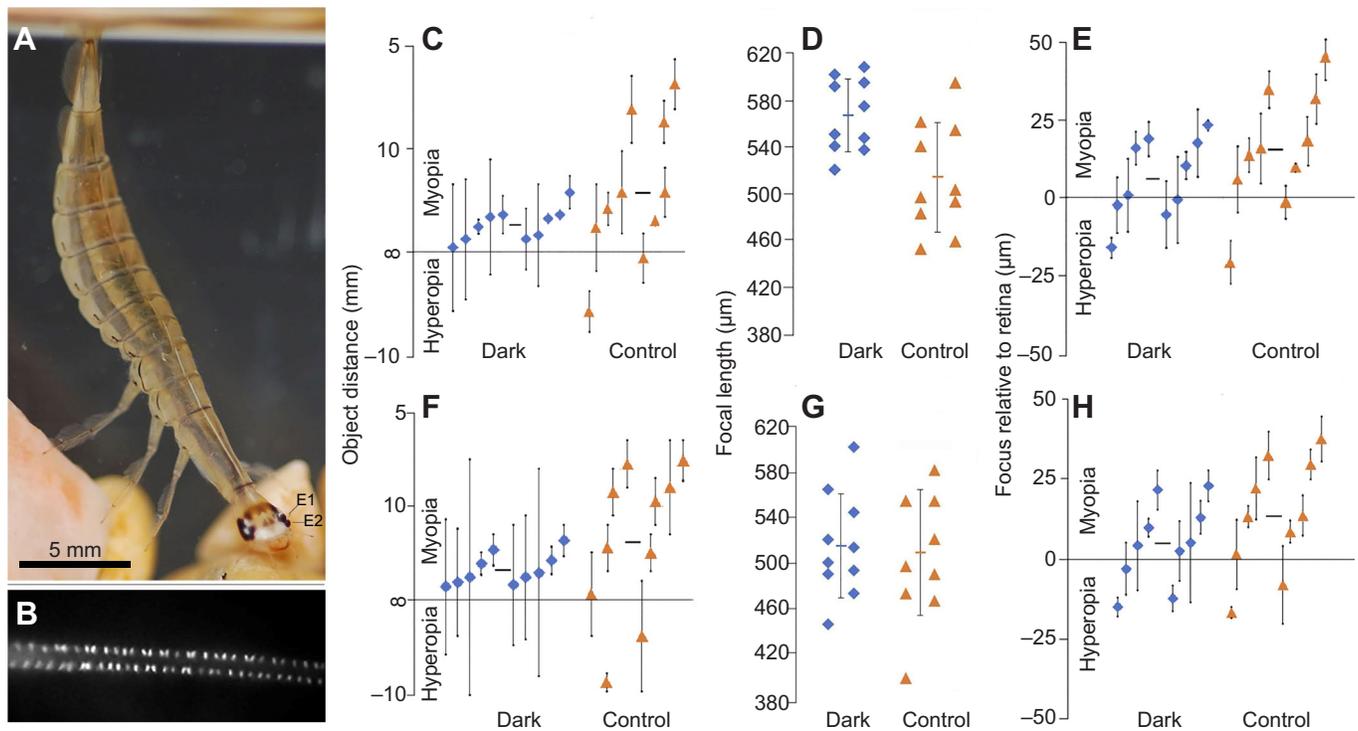
**Fig. 1. *Thermonectus marmoratus* optical analysis.** (A) External image of a *T. marmoratus* larva in water showing the principal front-facing eyes E1 and E2. (B) Ophthalmoscope image of the proximal retina showing two rows of rhabdoms. (C–H) Analysis of E1 (C–E) and E2 (F–H). (C,F) Micro-ophthalmoscope measurements showing object distances for the range (represented by bars) over which the E1 (C) and E2 (F) photoreceptors are optimally focused for each individual as well as their average (dark-reared individuals, *N*=10; control individuals, *N*=10). The overall averages are indicated by horizontal lines. (D,G) Measured focal lengths for dark-reared and control individuals from isolated lenses. (E,H) Range of distances (and averages) over which images are focused with respect to the surface of the retina based on the micro-ophthalmoscope measurements in combination with the focal length measurements for each individual.

peripheral region (Fig. 2A) and can be imaged individually with the micro-ophthalmoscope (Stowasser et al., 2017). These visible layers are layer 1, which exhibits a staircase organization, and layer 2. The central part of the retina was observed as a generally bright area (Fig. 2B) without individually discernible photoreceptors, possibly because of its high resolution or the presence of layers 3 and 4, which were not individually discernible using the ophthalmoscope. For each dark-reared spiderling (*N*=16) and control spiderling (*N*=9), the right AM eye was imaged. The object distances were plotted, showing the range of object distances and the center points at which the animals' retinas were in focus (Fig. 2C,F). The individual object distances for the dark-reared and control individuals did not show significant differences for layer 2 (*P*=0.681) or layer 1 (*P*=0.647) based on a two-sample *t*-test assuming equal variance. The average object distances for the dark-reared group were very similar to those of the controls for both layers (Fig. 2C,F). The focal lengths measured for the AM eyes of each individual ranged from ∼395 to 460.6 µm (mean±s.d. 418.6± 22 µm) for the control animals and from 377.7 to 463.7 µm (423.5±19.7 µm) for the test animals (Fig. 2D). There was no significant difference between the focal lengths of the dark-reared and control spiderlings (*P*=0.569).

Each individual was also evaluated to determine where the image of an object at infinity was focused relative to the retina based on each individual's focal length. For layer 2, the values ranged from −67.3 to −16.6 µm (mean±s.d. −38.1±15.8 µm) for the control animals and from −67.2 to 15.2 µm (−36.1±19.1 µm) for the test animals (Fig. 2E). For layer 1, the values ranged from −34 to 24.9 µm (−3.5±17.9 µm) for the control animals and from −48.54

to 50.5 µm (−0.76±21.9 µm) for the test animals (Fig. 2H). The difference between the test and control animals was not significant for layer 2 (*P*=0.797) or layer 1 (*P*=0.638). Note that the graphs of how the image is focused on the retina do not account for the possible effects of a negative lens that is formed by the surface between the proximal end of the lens tube and the retina layers. This lens, which is an important component of the telescopic function of adult spiders (Williams and McIntyre, 1980), could potentially shift the focus slightly deeper into the photoreceptor array. Taken together, our measurements of jumping spider AM eyes reveal no significant difference between the control and dark-reared animals. As has been previously noted for adult jumping spiders (Stowasser et al., 2017), layer 1 is focused very close to emmetropia. By contrast, layer 2 is hyperopic, with the image focused approximately 37 µm below its top surface.

### *Sarcophaga bullata*

To test whether light exposure influences the refractive state of insect compound eyes, we measured individual ommatidia of *S. bullata* flies that were forced to emerge in the dark (*N*=10) or were reared under normal conditions (daytime control; *N*=10). In addition, to account for possible circadian-related rhabdom shifts (Walcott, 1971; Warrant and McIntyre, 1993), an additional control group was measured during their night-time (night-time control; *N*=10). For each individual, we imaged a single ommatidium (Fig. 3B) on one side of the fly's head, which was ∼9 lens rows into the array from the frontal center border of the eye. A single ommatidium could be targeted by sufficiently closing the illuminating aperture around an individual lens (Fig. 3A, right).
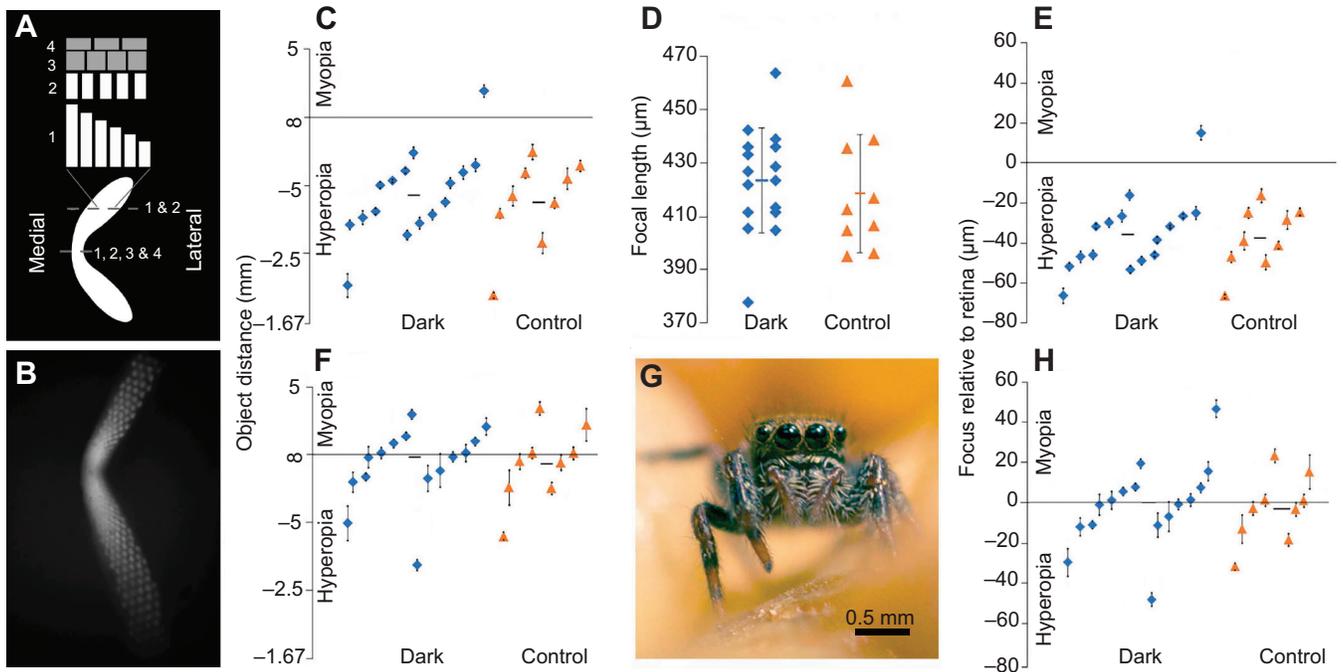
**Fig. 2. *Phidippus audax* optical analysis.** (A) Schematic diagram of the four layers of retina in the principal anterior-median (AM) eyes of jumping spiders (modified from Stowasser et al., 2017). (B) Ophthalmoscope image of the boomerang-shaped AM retina, focused on layer 1. (C) Micro-ophthalmoscope measurements showing the range of object distances, as well as the average, for which the photoreceptors of layer 2 are focused for each individual (dark-reared individuals, *N*=16; control individuals, *N*=9). The overall averages are indicated by horizontal lines. (D) Measured focal lengths as well as average and standard deviation for AM eye lenses. (E) Range of distances (and averages) at which images are focused with respect to the surface of layer 2 of the retina based on micro-ophthalmoscope measurements in combination with focal length measurements for each individual. The overall averages are indicated by horizontal lines. (F) Object distances for layer 1 (as in C for layer 2). (G) Image of a *P. audax* spiderling, illustrating the prominent principal AM eyes. (H) Range of distances (and averages) over which images are focused with respect to the surface of layer 1 of the retina (as in E for layer 2).

This selectivity was confirmed by the observation of one lens in the fly's lens array, in which the autofluorescence had bleached after the measurements (Fig. 3A, left). The *S. bullata* object distances were plotted as the range of object distances and the center point for which the retina of each individual was in focus (Fig. 3D). Based on a one-way ANOVA, there was a significant difference (*P*=0.033) between the groups. However, based on a *post hoc* Tukey–Kramer test, there was only a significant difference between the two control groups (daytime and night-time controls; *q*=4.178 with a critical value of 3.53). There was no significant difference between the daytime control and the dark-reared flies (*q*=1.98 with a critical value of 3.53) or the night-time control and the dark-reared flies (*q*=2.19 with a critical value 3.53). Fig. 3C illustrates a frontal cross-section through an *S. bullata* compound eye, which was used to determine the fly's focal length based on image magnification. The focal lengths were between 52 and 64.4 μm (mean±s.d. 59.7 ±4.6 μm) for the dark-reared individuals, between 57.2 and 70.3 μm (64.8±5 μm) for the daytime control individuals, and between 53.3 and 70 μm (62.6±6.6 μm) for the night-time control individuals (Fig. 3E). Based on a one-way ANOVA, there was no significant difference (*P*=0.2937) between the groups for focal length.

As the histological assessment was not successful for every individual, our calculations of where the image was focused relative to the retina had fewer samples (*N*=7 for dark-reared flies, *N*=6 for daytime control flies and *N*=9 for night-time control flies; Fig. 3F). The values ranged from −2.7 to 1.1 μm for the dark-reared individuals, from −3.5 to 0.27 μm for the daytime control individuals and from −2.3 to 2.4 μm for the night-time control individuals. Again, based on a one-way ANOVA, there was a significant difference

(*P*=0.0418), which a *post hoc* Tukey–Kramer test revealed to be due to differences between the daytime and night-time controls (*q*=3.87 with a critical value of 3.59). However, there was no significant difference between the daytime control and the dark-reared flies (*q*=2.41 with a critical value of 3.59) or the night-time control and the dark-reared flies (*q*=1.38 with a critical value 3.59). On average, the focus of the daytime control flies was slightly deeper into the retina (−2.07±1.38 μm; towards hyperopia) than that of the dark-reared flies (−0.66±1.5 μm) or the night-time control flies (0.07±1.5 μm), which were essentially emmetropic. Taken together, we observed no significant differences between the dark-reared flies and the control groups, as was the case for the beetle larvae and the spiderlings.

## DISCUSSION

To the best of our knowledge, this study is the first to directly address the question of how well arthropod eyes are focused after their initial development, and to test whether their refractive state is influenced by visual input. Specifically, we assessed the refractive state of dark-reared and control individuals of three phylogenetically distant arthropods that are characterized by optically diverse eye types. Unexpectedly, all three arthropods used for this study developed relatively well-focused eyes with comparable levels of focus regardless of whether they were deprived of visual feedback. These results suggest that visual input is not required at least for the initial refractive development of these arthropods.

Our investigation of *T. marmoratus* larvae showed that for both E1 and E2, the clearest images, on average, were focused close to emmetropia (with slight myopia) in relation to the proximal retina. This finding in regard to E1, as well as our focal length
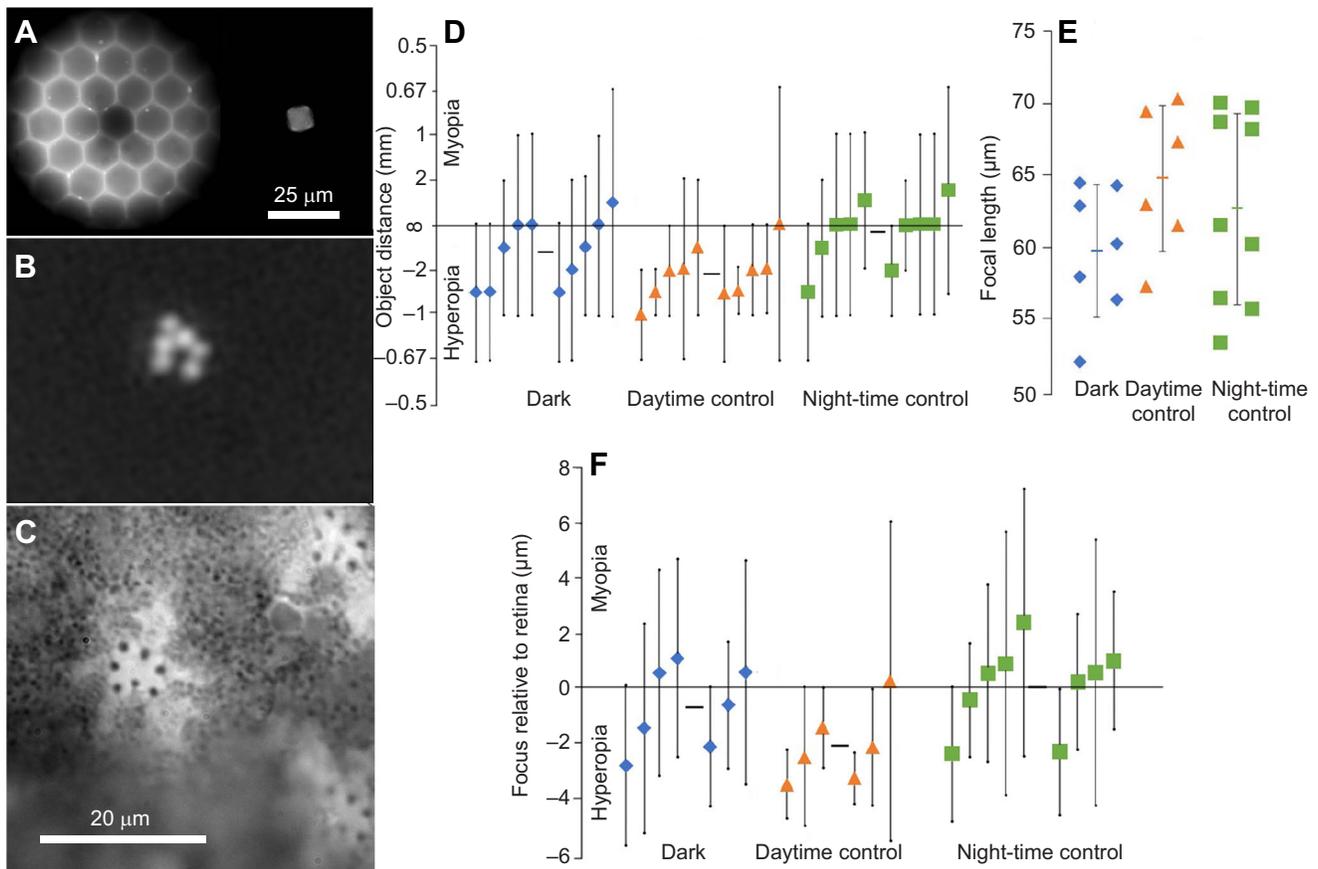
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**Fig. 3. *Sarcophaga bullata* optical analysis.** (A) Ophthalmoscope post-measurement image of the surface of an *S. bullata* compound eye lens array, illustrating bleaching of a single ommatidium (left) that was illuminated by a small square light beam (right). (B) Ophthalmoscope image showing the photoreceptors of a single ommatidium. (C) Histological section of *S. bullata* photoreceptors. (D) Micro-ophthalmoscope measurements showing the range of object distances, as well as the average, for which the photoreceptors are focused for each individual (dark-reared individuals, *N*=10; daytime control individuals, *N*=10; night-time control individuals, *N*=10). The overall averages are indicated by horizontal lines. (E) Measured focal lengths for each individual calculated from the image magnification between the histological and optical images (dark-reared individuals, *N*=7; daytime control individuals, *N*=6; night-time control individuals. *N*=9). (F) Range of distances (and averages) over which images are focused with respect to the surface of the retina. The overall averages are indicated by horizontal lines.

measurements, correspond well with the results of a previous study (Stowasser and Buschbeck, 2014) that used a less-direct method. However, the previous study suggested that E2 was more myopic than indicated by our current measurements. As the previous study (Stowasser and Buschbeck, 2014) was performed on slightly older individuals that were allowed to hunt for several days, further investigation is required to determine whether E2 becomes more near-sighted later in development. Although not statistically significant, the finding that the dark-reared larvae showed slightly less variation than the controls, and were on average focused slightly more towards emmetropia, is consistent with such a change. Thus, it is possible that while a passive regulatory mechanism is the more dominant process, visual feedback could start to initiate a small change in a few individuals. By contrast, the dark-reared larvae, which molted without any visual feedback, would rely fully on their developmental program, resulting in less variation. Regardless, this effect is minor and, overall, our data show that the focus of both principal larval eyes was comparable between individuals that molted in the dark and control animals.

The AM eyes of *P. audax* have a retina that is organized in layers. For these spiders, our experiments revealed no significant differences between the dark-reared and control individuals in regard to refractive errors, with similarities between control and test

animals in regard to not only the average values but also the variance. As expected, our data showed a clear difference in focus between layer 2 and layer 1, with layer 1 being focused close to emmetropia and layer 2 being hyperopic (far-sighted). These findings are consistent with previous results for *Metaphiddipus aeneolus* (Land, 1969) and what has been found, following the same methods, for late juvenile *P. audax* (Stowasser et al., 2017). The latter is interesting, as it suggests that young spiderlings are already focused in the same way as older spiders, and hence that the eyes of very young spiderlings are similar to those of older spiders in terms of their refractive states. Although the focus in regard to the layering seems to stay constant, it is noteworthy that the range of focus is larger, as expected for these spiderlings, which necessarily have a much shorter focal distance. Our data on how images would be focused on the retina based on the focal lengths of the overlying lenses also places the focus of layer 1 close to emmetropia, similar to the object distance data. These findings suggest that in these young spiderlings, the image shift from the negative lens that is formed by the retina's surface must be relatively minor. Among other optical features, it has recently been noted that the anterior-lateral eyes of *P. audax* spiderlings already have a full complement of photoreceptors (Goté et al., 2019).

In regard to how images are focused on the retina, both the beetle larvae and jumping spiders showed a much larger range of variation

than the flies. This difference is likely due to both the beetle larvae and jumping spiders having camera-type eyes with relatively large lenses, whereas the compound eyes of *S. bullata* flies are characterized by many much smaller lenses with shorter focal lengths and less variation in regards to where images are focused relative to the retina. However, our data also illustrate how the short focal length leads to an impressively large range of object distances that are well resolved (Fig. 3D). This large range indicates that precise focusing may be less critical for compound eyes than for camera-type eyes, at least in regards to maintaining suitable spatial resolution. It is conceivable that in these eyes the ability to maximally gather light more strongly affects proper focusing than the constraints for image resolution. As was the case for the beetle larvae and spiderlings, no significant difference in focus was observed between the dark-reared and both the daytime and night-time control animals of *S. bullata*. Interestingly, the daytime control flies were significantly more hyperopic than the night-time control flies. Although circadian and light-induced differences in the rhabdom position have been observed in other insects (Narendra et al., 2016; Walcott, 1971; Warrant and McIntyre, 1993), our results revealed an opposite effect to that expected based on the literature, which generally suggests that the rhabdomeres of light-adapted animals move away from the lens. Regardless, our findings clearly indicate that by the time the flies emerge, they already have precisely focused images on their retinae. This finding is in line with a study on praying mantises that were raised with monocular occluded eyes (Mathis et al., 1992), which found that their stereopsis did not require binocular visual input to develop, even though this is the case in vertebrates. The extraordinarily high level of precision, which must be achieved through a developmental program that does not rely on visual feedback, further demonstrates the value of insect compound eyes as a model system for highly precise organogenesis.

Taken together, our measurements show that all three arthropods have good optics from the start of active life (spiders and flies) or following a molt (diving beetle larvae). These findings suggest that the coordination between lens refraction and retina position is more strongly genetically predetermined in arthropods than in vertebrates and perhaps even cephalopods (Schaeffel et al., 1988; Troilo and Wallman, 1991). Although arthropods and cephalopods are both invertebrates, and species from both groups have camera-type eyes, the previous study in squid demonstrated that an active mechanism dominates the final refractive state (Turnbull et al., 2015), whereas our findings are consistent with the primary mechanism for arthropods being passive regulation. Many invertebrates require full function by the time they emerge. For example, *P. audax* spiderlings must be able to hunt as soon as they emerge from their egg cases. Similar to the larvae of *T. marmoratus*, the spiderlings undergo frequent molts but appear to have fully functional eyes after molting. Although the exact effect of molting on the eyes of *P. audax* is unknown, it has been established that they grow between instars (Goté et al., 2019). Thus, similar to *T. marmoratus* larvae (Werner and Buschbeck, 2015), it is likely that spiderlings have to re-establish correctly focused eyes with each molt. Many arthropods (including *T. marmoratus* larvae) undergo multiple molts within just a few days, which may have necessitated the evolution of an extremely effective developmental mechanism for eyes that are properly focused from the beginning. However, it is important to note that our study exclusively focused on determining whether visual input alters initial eye development. It remains unclear whether visual input could further improve the optics of these arthropod eyes at later times.

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