

RESEARCH ARTICLE

Multiple spectral channels in branchiopods. II. Role in light-dependent behavior and natural light environments

Nicolas Lessios^{1,2,*,\$}, Ronald L. Rutowski^{1,‡} and Jonathan H. Cohen^{3,‡}

ABSTRACT

Light is a primary environmental factor used by aquatic invertebrates for depth selection behavior. Many branchiopod crustaceans live in ephemeral aquatic habitats. All branchiopod crustaceans studied to date express four or more visual opsins in their compound eyes. We asked whether two branchiopods, *Triops longicaudatus* and *Streptocephalus mackini*, use multiple spectral channels to regulate their position in the water column. At the lowest intensities that elicited photonegative behavior, both species had broad spectral bandwidths, suggesting they use multiple spectral photoreceptor classes. Male *S. mackini* were more likely to maintain a vertical position 8.0–12.0 cm below the surface than females, independently of whether females were present. Male photopositive behavior at low intensity was restricted to a narrow bandwidth centered at 532 nm, suggesting a single photoreceptor class is used to maintain position above females. We compared ephemeral pools from two regions in Arizona and found that diffuse light attenuation coefficients were two orders of magnitude greater than the most heavily attenuating coastal waters. At less than 1 m of depth, pools were often dimmer than terrestrial habitats under starlight. Soil particle size distribution in each region affected spectral light environments, and behavioral responses of field-caught shrimp were adapted to the spectral properties of their region. The results suggest that branchiopods predominantly use luminance vision summed from multiple spectral photoreceptor classes for depth selection in dim, spectrally variable environments. The neuroanatomical basis for summation is described in a companion paper.

KEY WORDS: Pancrustacea, Behavior, Color vision, Electroretinography, Opsin

INTRODUCTION

In deep-sea aquatic habitats, attenuated ambient light is known to shape the spectral information received by the visual systems of its inhabitants (Frank et al., 2012; Lythgoe, 1984; Lythgoe and Partridge, 1989). Far less is known in comparison about the extent of light attenuation in shallow freshwater environments, or how the behaviors of temporary freshwater pool inhabitants might be configured for stereotyped behavioral responses when presented with light of differing wavelengths or intensities (Cohen and

Forward, 2009). Branchiopod crustaceans are frequent inhabitants of shallow freshwater habitats ranging from highly light-transmissive lakes to heavily occluded inland waters (Dumont and Negrea, 2002; Williams, 2005). Fossils from the Devonian suggest that ancestral marine branchiopods colonized temporary freshwater habitats, and that this transition is responsible for many of the shared derived characteristics of extant branchiopods (Dumont and Negrea, 2002; Gueriau et al., 2016; Olesen, 2007). Ephemeral pools are types of temporary freshwater habitats that have periods in which they are fully desiccated (Williams, 2005). Many species of branchiopods are found in such habitats, as there are typically no fish to predate them (Brendonck et al., 2008; Dumont and Negrea, 2002; Gueriau et al., 2016). As a result, branchiopod crustaceans are passive dispersers, and many species have resting eggs that can withstand complete desiccation (Brendonck, 1996).

Branchiopods have evolved characteristics such as resting eggs that are clearly adaptations for temporary freshwater habitats. But they also have optic lobe neuroanatomy that is simpler in comparison to other pancrustaceans, a group of arthropods that includes hexapods and crustaceans (Ma et al., 2012). The structure of the optic lobe of branchiopods could be adaptive because of the costs of maintaining unnecessary neuroanatomical structures. In other pancrustaceans, three nested visual neuropils function as a series of information processing centers (Wolff and Strausfeld, 2016). Color information in other pancrustaceans is thought to be processed in two of their neuropils, the medulla and the lobula (Dyer et al., 2011; Kleinlogel et al., 2003; Melnattur et al., 2014; Morante and Desplan, 2008; Paulk et al., 2008, 2009; Strausfeld, 2012). The structural properties of the visual neuropils that have been identified in branchiopods to date, which do not include a medulla or a lobula, therefore suggest a lesser ability to process color information. In a companion paper, we have clarified how the structure of the lamina, the first optic neuropil, is configured (Lessios et al., 2018). Here, we hypothesize that light attenuation and the spectral content available in ephemeral pools act as a selective pressure for light-dependent behavior that is determined by a simplified set of optic neuropils. We describe light-dependent behavior and attenuation in natural habitats of the anostracan *Streptocephalus mackini* Moore 1966 and the notostracan *Triops longicaudatus* (LeConte 1846).

The biotic and abiotic factors that determine light attenuation in aquatic environments can be complex. Therefore, *in situ* measurements of irradiance are often the most descriptive measurements of the overall light level and environmental variation experienced by an aquatic animal (Johnsen, 2012). Studies of spectral attenuation in freshwater habitats first measured downwelling irradiance in highly transmissive waters (Jerlov, 1968, 1976). Subsequent study of inland turbid bodies of water found that they strongly attenuate light (Kirk, 1981). More recently, heavy attenuation in turbid bodies of water has been determined by remote sensing of large bodies of water and also from direct measurements of highly attenuating estuarine habitats (Lawrenz et al., 2010; Simon and

¹School of Life Sciences, Arizona State University, Tempe, AZ 85287 USA.

²Department of Neuroscience, University of Arizona, 611 Gould-Simpson, Tucson, AZ 85721, USA. ³School of Marine Science and Policy, College of Earth, Ocean and Environment, University of Delaware, 700 Pilottown Road, Lewes, DE 19958, USA.

*Present address: Department of Neuroscience, University of Arizona, 611 Gould-Simpson, Tucson, AZ 85721, USA.

‡These authors contributed equally to this work

\$Author for correspondence (nlessios@email.arizona.edu)

id N.L., 0000-0002-1767-0153; J.H.C., 0000-0002-2032-7874

Shanmugam, 2016). Downwelling irradiance in desert ephemeral pools has not been assessed to date (Scholnick, 1994; Williams, 2005). The temporary waters inhabited by many branchiopod crustaceans are characterized by high levels of particulate matter and by suspended soil in the water column (Brendonck et al., 2008; Williams, 2005). The size of a given soil particle determines how long it remains in the water column and how it attenuates light (Partridge and Cummings, 1999). Therefore, light attenuation in temporary waters also varies regionally because of soil particle size distribution (percent sand/silt/clay) affecting the number of particles of a given size that are suspended in the water column. How long particles remain in the water column depends on the presence of wind or biological activity of wading birds or other swimming animals. Given the potential for heavy attenuation in temporary waters, we predicted that ephemeral pools inhabited by branchiopods would be dimmer and more spectrally variable than other bodies of freshwater that have been categorized, or than terrestrial habitats.

Despite having only two neuropils in their optic lobes, all branchiopods studied to date express four or more rhabdomeric opsins in their compound eyes (Kashiyama et al., 2009). In a companion paper (Lessios et al., 2018), we report that these opsins are most likely configured in four spectral photoreceptor classes in their compound eyes, which agrees with electroretinography of branchiopods studied to date, and the most recent rhabdomeric opsin phylogeny of pancrustacean compound eyes (Henze and Oakley, 2015; Smith and Macagno, 1990). In the first optic neuropil, the lamina, we identified lateral processes as neuroanatomical candidates for spatial summation in dim light conditions. Some pancrustaceans are thought to pool responses from multiple spectral photoreceptor classes in the lamina for visual behavior in dim light conditions (Menzel, 1974; Menzel and Greggers, 1985). However, it remains to be determined whether spatial summation takes place from both multiple ommatidia and spectral photoreceptor classes (Nilsson and Kelber, 2007). Given that there is evidence of broadband neurons in the lamina of other pancrustaceans (Kelber, 2006; Menzel, 1974; Menzel and Greggers, 1985; Yang and Osorio, 1996), broadband summation may take place in the lamina of branchiopods. Many pancrustaceans also possess long visual fibers that extend from the retina to the second optic neuropil. The long visual fibers of pancrustaceans can terminate in these deeper optic neuropils, and in some cases, lateral processes of the long visual fibers have been found in the lamina (Shimohigashi and Tominaga, 1999). Given the structure of the first optic neuropil we have reported for these two species, we predict that light-dependent behavior could involve summation from multiple spectral photoreceptor classes and would therefore have broad spectral bandwidth. Because anostracan branchiopods have been reported to have long visual fibers (Nässel et al., 1978), we predict wavelength-specific behavior of narrow bandwidth.

In the present study, we asked whether two species of branchiopods, *T. longicaudatus* and *S. mackini*, use multiple spectral channels for light-dependent behavior that uses luminance, rather than color vision, and if so, how this relates to their natural environment. We took *in situ* measurements of downwelling irradiance in temporary freshwater pools inhabited by *T. longicaudatus* and *S. mackini*. To better understand some of the factors affecting regional attenuation, we performed soil texture analysis on dry soil samples from two regions in Arizona to identify particle size distributions, and we analyzed total suspended solids from water samples taken in the field. We observed behavioral responses of populations from separate regions to calibrated light stimuli of narrow spectral bandwidth to ask whether visual performance is adapted for the spectral regime of each region. To ask

whether branchiopods use light to maintain position in the water column by actively swimming, we observed behavioral responses under broad-spectrum lighting conditions, as well as in the dark. It should be noted that although comparing horizontal and vertical behavior provides insight regarding stereotyped responses to light, it still does not approximate responses to a natural light field in ephemeral pools, which as of yet remains unknown. An overarching hypothesis supported by the present study is that the visual system must serve the behavioral requirements of the organism in question, and that sensory structures at the periphery of the nervous system, such as eyes, are more easily modified, and potentially more developmentally plastic, than centralized neural structures (Land and Nilsson, 2012). Our aim was to study behavioral responses of branchiopods with simple optic lobe neuropils that explain the presence of multiple spectral channels in branchiopod compound eyes.

MATERIALS AND METHODS

Study sites and light measurements in the field

We collected dry soil from the upper 2.0 cm of dry ephemeral pools located in the Sonoran Desert in central Arizona and near Willcox Playa in Southeastern Arizona (33.670°N, 111.464°W, and 32.221°N, 109.827°W). Resting eggs were contained in the soil samples. We collected soil throughout the catchment area of each dry pool (approximately 2000 m²), then mixed soil thoroughly within each sub-sample to ensure that eggs produced by different individuals were contained in all samples. *Streptocephalus mackini* lack a head-shield, so catching them in the field and transporting them without damage was not possible. We therefore only reared *S. mackini* from soil samples. We caught adult *T. longicaudatus* in a single pool in Central Arizona, as well as from a single pool in Southeastern Arizona (32.221°N, 109.827°W) using a dip-net that caused no damage. We reared shrimp to test their behavioral responses to light. Shrimp were reared under conditions with total suspended solid particulate matter similar to that measured during seasonal monsoonal wet periods in 2012 and 2013 (0.40±0.23 g l⁻¹ mean±s.d.).

We measured downwelling irradiance in the field at 1.0 cm depth intervals, with the shallowest measurement taken with the collector just below the water surface. We took measurements every 3 days from the time of an initial filling event in six ephemeral pool communities (100 m² or greater), between 10:00 and 14:00 h. These measurements were taken for 16 days total. We used a USB 2000+ spectrometer (Ocean Optics, Dunedin, FL, USA), 10 m cable, and a CR2-OR 0.635 cm diameter waterproof cosine corrector (StellarNet, Tampa, FL, USA) with a spectroradiometric calibration from Ocean Optics. At the time of initial filling of each pool, a GPS point was taken above the deepest point of each pool. Depth at these coordinates was measured on the day of each irradiance measurement. The mean (±s.d.) depth of these pools was 122±44 cm. Latitude and longitude coordinates, determined by GPS, are presented for each ephemeral pool in Table S1. Local attenuation coefficients were calculated using paired measurements from just below the surface and at 1 cm depth. These were averaged from three replicate measurements taken at each site, from a different spot in the pool, on each sampling day. Because of heavy attenuation at depths greater than 1 cm at some field sites leading to loss of signal at short wavelengths, measurements were compared at this depth from 350 to 700 nm.

Soil texture analysis

To determine whether ephemeral pool light environments are affected by attenuation that occurs because of soil suspended in the water column, we performed soil texture analysis (percent sand/silt/

clay) in the laboratory on dry soil samples using a hydrometer procedure according to methods used by McKenna and Sala (2016). We took hydrometer readings 40 s after we added processed soil (after all sand particles had settled) and after 7 h (after all silt particles settled). Clay particles can remain suspended in the water column for a week or more (Elliott et al., 1999). We asked whether suspended soil in the water column differed between two regions because of soil particle size distribution. To estimate total suspended solids in filled ephemeral pools, we performed vacuum filtration analysis on 10 ml water samples collected from the upper 10 cm of each pool every 3 days from the time of filling. These measurements were taken for 16 days total. We filtered total suspended solids using GF/F filters, and then dried and weighed them on a balance (Mettler Toledo XS105, Columbus, OH, USA) according to methods used by Deng et al. (2015). We used soil particle size distributions to estimate the suspended silt and clay found in water samples. We used a log transformation of total suspended solid estimates to establish normality (Shapiro–Wilk test) for statistical comparisons.

Behavioral experiments

Rearing

We reared shrimp in wading pools in a greenhouse facility under natural sunlight (Fig. S1). Wading pools were each filled with 80 liters of deionized water and then we mixed 1.5 liters of collected soil into the water. After 24 h, we added an additional 20 liters of deionized water and maintained a constant volume of 100 liters by adding deionized water as needed. We maintained a 25°C:15°C, 12 h:12 h light:dark diel cycle using a programmable thermostat and light timer. Under these conditions, shrimp developed brood pouches within 5–7 days after hydration of resting eggs, comparable to development rates observed in the field (N.L., personal observation). We did not add food supplement until we observed shrimp with brood pouches because most larval food is contained in the soil (Centeno et al., 1993). After observing adult shrimp, we mixed 10 ml of 0.025 g ml⁻¹ Brewer's yeast solution (Twinlab, Inc., Grand Rapids, MI, USA) into each wading pool once a week. In order to verify that the shrimp we were testing were the species we had identified using their morphology, we extracted DNA from a subset of individuals and amplified a cytochrome oxidase I (COI) mitochondrial segment (Folmer et al., 1994) from 20 individuals used in behavioral trials, verifying the species as *T. longicaudatus* and *S. mackini*. The tested populations of *T. longicaudatus* consist only of self-fertilizing hermaphrodites (Maeda-Martínez et al., 1997), verified using the presence of brood pouches in every individual. Sexes are separate in *S. mackini*. Individuals of each sex in the latter were identified using the morphology of second antennal appendages (Belk, 1975).

Horizontal distribution

We used a behavioral assay in a horizontal trough with light at one end to assess responsiveness to light under constant gravitational cues that could otherwise elicit a gravitaxis response, following testing procedures described in Cohen et al. (2010). We placed the testing trough 55 cm away from the light source, which consisted of a 500 W tungsten lamp housed in a slide projector (ViewLex, Inc., Holbrook, NY, USA) with a cooling fan. In control trials we only turned on the cooling fan. We limited infrared radiation from the light source using a hot mirror (Edmund Optics, Barrington, NJ, USA). We controlled wavelength using interference filters with a bandwidth at half maximum of 12.5 nm (Ditric Optics Inc., Marlborough, MA, USA), with center wavelengths ranging from

410 to 636 nm. We controlled irradiance using neutral density filters (Edmund Optics) and measured this using the spectrometer and equipment described earlier for field measurements. The minimum stimulus intensity centered at each wavelength in these experiments is shown in Fig. S1B.

Swimming behavior in response to narrow-bandwidth light in the horizontal plane was tested in a Plexiglas trough (40×10×10 cm) divided into five equal longitudinal sections, where the dividers could be removed and replaced in unison. Each trial consisted of filling the trough with 2.0 liters of aerated deionized water, placing a group of eight shrimp in the center compartment, and giving them 30 s to acclimate. We then turned on the light source and cooling fan, removed the dividers and allowed shrimp to move freely for 45 s. We then replaced the dividers and counted the numbers of animals in each chamber, with the number found in the two chambers nearer the light source scored as 'towards' and those found in the chambers further from the light source counted as 'away'. The group of shrimp was returned to the central section and allowed to acclimate for 5 min prior to testing at a different intensity. The lowest photon flux experienced by animals in the middle section of the trough was set to 10^{3.5} photons cm⁻² s⁻¹ at all wavelengths tested (Fig. S1B). Starting at this lowest intensity, we tested each group four times using two log unit intervals of increasing photon flux. After the final experimental condition at 10^{9.5} photons cm⁻² s⁻¹, we tested the same individuals once more under dark conditions to ensure no trail following occurred and that shrimp remained behaviorally responsive. We tested each group of shrimp at only one wavelength or under repeated control conditions. Testing took place between 09:00 and 16:00 h. We allowed shrimp in the experiments to dark-adapt for at least 1 h in aerated deionized water in the darkroom before testing. Between trials of increasing irradiance, we carefully removed shrimp and rinsed out the Plexiglas trough to eliminate the possibility of trail following. We calculated a response index (R_{ind}) for each trial to create a single response metric ranging from -1 to 1:

$$R_{\text{ind}} = \frac{n_{\text{towards}} - n_{\text{away}}}{n_{\text{total}}}, \quad (1)$$

where n_{towards} is the number of animals moving towards a calibrated light source, n_{away} is the number moving away (see below) and n_{total} is the total number of animals.

Vertical distribution

To assess behavior during active swimming of *S. mackini* and *T. longicaudatus* in the vertical plane in the presence of gravitational cues that could elicit a gravitaxis response, we marked a cylindrical glass testing column into five horizontal sections of 4 cm each (Fig. 2A, inset). We used this for testing in both a greenhouse setting under broad-spectrum light including UV (Fig. S2A), and for controls in a darkroom. Testing groups included 10 shrimp in each trial, with groups of *S. mackini* consisting of either 10 males, 10 females, or five males and five females. We placed the testing column in the center of a cardboard outer housing. The outer housing had walls that limited the view of the surroundings. The outer housing walls and the base of the column were painted flat black. A hole in the outer housing was used to position a video camera that maintained the entire column in its field. We placed a removable frame containing parchment paper above the apparatus during testing to serve as a broad-band diffuser of downwelling greenhouse light (Fig. S1).

We acclimated shrimp in deionized water for 3 h prior to testing active swimming. We tested shrimp in the greenhouse between

10:00 and 14:30 h. Six trials were carried out on each testing day. We alternated the testing order to avoid effects of time of testing. Between trials, we removed the water and cleaned the column. We carried out 12 replicates for each group composition of *S. mackini* and 10 replicates of *T. longicaudatus*. At the beginning of a trial, we pipetted a testing group gently into the center of the vertical column. During a day of testing, the solar orb passed symmetrically over the testing apparatus. We only tested on cloudless days. Downwelling light levels at the top of the testing column were measured at 14:00 h on a cloudless day.

We recorded video in the greenhouse light condition using a SDR-S7 digital video camera (Panasonic), through a 4 cm diameter opening in the outer cardboard housing. We recorded video in the dark using a C5900 H.264 wireless IP camera (Esy, Beijing, China) with a built-in infrared light source. We observed shrimp on the camera's viewscreen or a laptop screen during testing, recording the numbers of dead or non-responsive shrimp every 12 min. We later extracted individual frames using a VLC media player (VideoLAN, Paris, France) from the end of each 1-min interval of recording. We did not include the first minute interval in analyses as shrimp often displayed an initial flight reaction before resuming regular swimming. We assigned a random accession number to video files from each trial and scored each frame manually. The scorer was blind to the group composition of *S. mackini* as it was not possible to resolve sexual characteristics in the extracted frames. Based on preliminary results, we stopped experimental trials after 36 min of continuous video to ensure only active animals were being tested. Preliminary analysis indicated the 10th frame had the fewest individuals with a flight reaction, and the fewest number of unresponsive animals. Therefore, to avoid pseudoreplication from each trial, we compared the frame extracted at 10 min among replicates. A two-way chi-square test determined whether the frequencies of animals in each vertical section was dependent on group composition and whether greenhouse experiments differed from darkroom controls. To compare the numbers of animals in each section of the vertical column, we performed a sequential Bonferroni correction (Hochberg, 1988). We carried out a control experiment to compare sinking speeds of shrimp that were not swimming. We anesthetized shrimp in carbonated water for 3 s, after which we observed no gill-swimming. We then transferred them into the testing column and recorded the time to sink to the bottom to determine an average sinking rate.

RESULTS

Behavior in the horizontal

We quantified a behavioral response index of notostracan and anostracan branchiopods to narrow-bandwidth light in the horizontal plane under constant gravitational cues that could otherwise elicit a gravitaxis response (Fig. 1). *Triops longicaudatus* moved away from the light source at all tested wavelengths except 550 nm and at all intensities greater than $10^{3.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (mixed ANOVA: between-subjects factor $F_{8,18}=3.67$, $P=0.010$; within-subjects factor $F_{4,72}=15.99$, $P<0.0001$; interaction $F_{32,72}=0.702$, $P=0.865$; Fig. 1A,B). The lowest response intensity different from the control for *T. longicaudatus* was $10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$ ($P<0.01$; Fig. 1A, asterisks). Male *S. mackini* moved away from light at intensities above $10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (mixed ANOVA: between-subjects factor $F_{8,18}=5.611$, $P=0.001$; within-subjects factor $F_{4,72}=76.29$, $P<0.0001$; interaction $F_{32,72}=4.465$, $P<0.0001$; Fig. 1C, asterisks). Female *S. mackini* also moved away from light at intensities above $10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (mixed ANOVA: between-subjects factor $F_{4,72}=8.817$, $P<0.0001$; within-subjects factor $F_{4,72}=64.445$, $P<0.0001$; interaction $F_{32,72}=1.704$, $P=0.032$; Fig. 1D, asterisks).

At intensities of $10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$, *S. mackini* males moved towards a light source of peak emission of 532 nm (Fig. 1C, asterisks). Female *S. mackini* did not move towards light at any of the tested wavelengths and intensities (Fig. 1D). The minimum stimulus intensity that elicited a behavioral response for both species of branchiopods is $10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (Fig. 1). This falls below the intensity of dim starlight in a terrestrial habitat, which is $10^{6.0}$ photons $\text{cm}^{-2} \text{s}^{-1}$ (Kelber et al., 2002).

Behavior in the water column

To determine whether notostracan and anostracan branchiopods use light for depth selection in the presence of gravitational cues that could elicit a gravitaxis response, we assessed behavior under diffuse broad-spectrum light (Fig. S1). Groups of shrimp were tested in a vertical cylindrical column (inset Fig. 2A). *Streptocephalus mackini* tended to swim away from the surface in the greenhouse and were more likely to sink in the dark (Fig. 2B). We found the vertical distribution of *S. mackini* to be dependent on group composition (chi-square test of independence, $\chi^2=19.13$, $P=0.014$, $N=257$; Fig. 2A). Males were more likely to maintain a vertical position of 8.0–12.0 cm from the surface than females, independently of the presence of females (Fig. 2A, asterisks). We found that *T. longicaudatus* tended to maintain a vertical position between 16.0 and 20.0 cm away from the surface in light conditions, with more sporadic movements towards the surface under dark conditions (chi-square test of independence, $\chi^2=22.96$, $P=0.004$, $N=187$; Fig. 2B). To further assess whether shrimp actively maintained a vertical position, we measured the sinking rates of anesthetized shrimp (Fig. 2C). Sinking rates indicated that male *S. mackini* sink faster than females (one-way ANOVA followed by Tukey's *post hoc* test $F_{2,40}=24.20$, $P<0.01$), even though males tended to swim slightly above females in the greenhouse experiments (Fig. 2A). Together, these results suggest that both species of branchiopods use downwelling light to regulate their position in the water column when gravitational cues could also be used for gravitaxis.

Regional light environments and effects on behavior

To compare behavioral responses of two natural populations of branchiopods from two separate regions in Arizona, we carried out behavioral assays on field-caught *T. longicaudatus*. We found that *T. longicaudatus* from Central Arizona had a broader response spectrum than the population from Southeastern Arizona (Fig. 3). The latter population responded significantly to light at >470 nm (one-way ANOVA followed by Dunnett's *post hoc* test relative to control $F_{8,64}=5.419$, $P<0.001$; Fig. 3).

We took measurements of downwelling irradiance during seasonal monsoonal wet periods to describe the spectral properties of ephemeral pool light environments. We found that the spectral quality (λ_{P50} , the wavelength at which 50% of photon flux distribution occurs) of ephemeral pools varied considerably among two regions (Fig. 4A). From the time of initial filling until 16 days later, we found a standard deviation of 70.4 nm among all habitats of two regions, a standard deviation of 26.4 nm within Central Arizona habitats and a standard deviation of 57.4 nm within Southeastern Arizona. The λ_{P50} of the ephemeral pool habitat where eggs were collected after its desiccation for the behavioral response index assay was 534 ± 18.0 nm (mean \pm s.d.; Fig. 4A, dark gray). In order to model intensity loss with depth, irradiance measurements were used to calculate diffuse attenuation coefficients. At less than 1 m of depth, ephemeral pool habitats tended to reach light intensities lower than those of terrestrial habitats under dim starlight

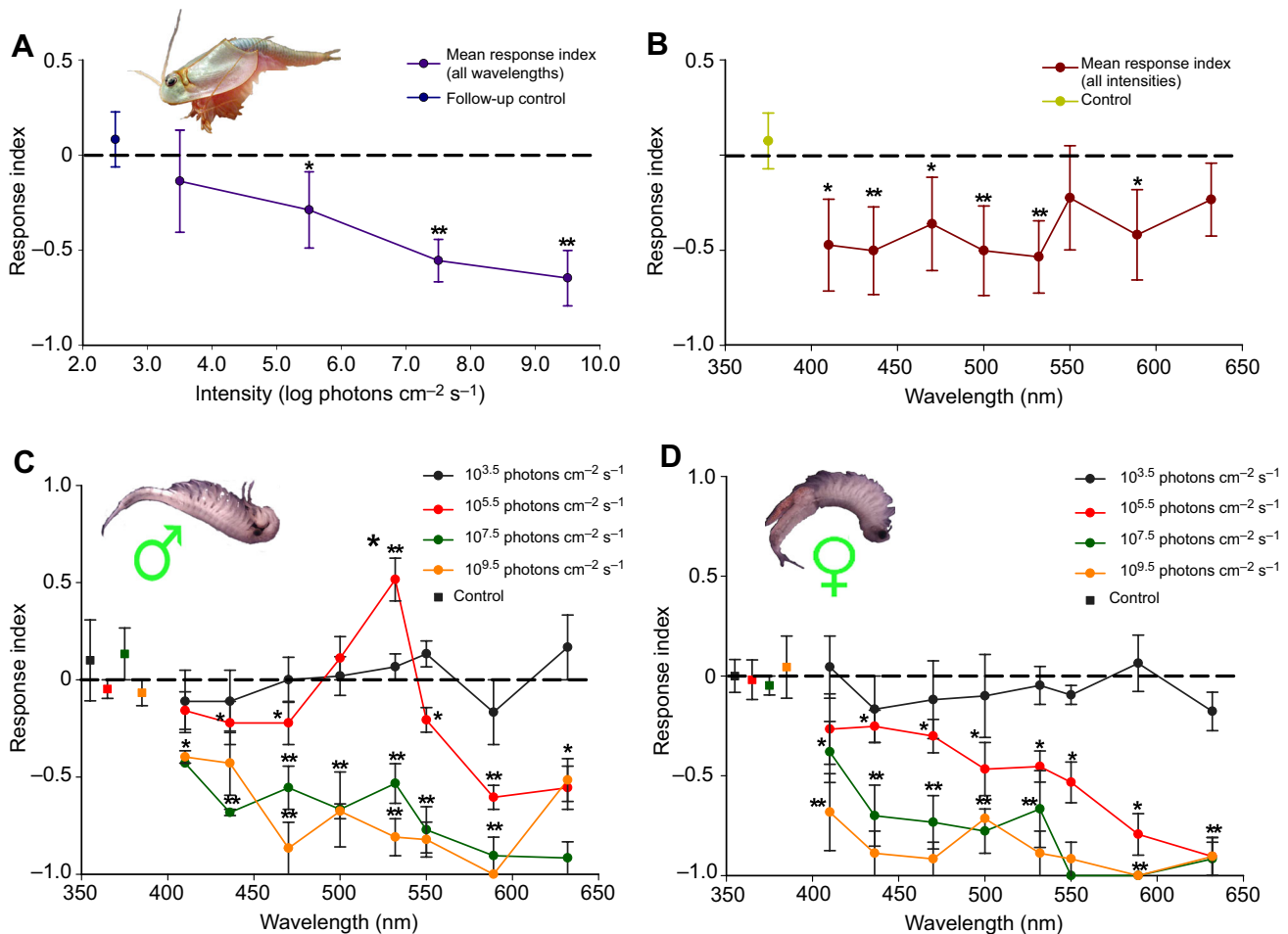


Fig. 1. Behavioral response index of dark-acclimated branchiopods. (A,B) *Triops longicaudatus*. As there was no significant interaction ($F_{32,72}=0.702$, $P=0.865$), data are presented as marginal means \pm s.e. across all tested intensities (A) and wavelengths (B). Main effects of wavelength (between-subjects factor) and intensity (within-subjects factor) were significant (mixed ANOVA, between-subjects factor $F_{8,18}=3.67$, $P=0.010$; within-subjects factor $F_{4,72}=15.99$, $P<0.0001$). (C) *Streptocephalus mackini* males. There was a significant effect of interaction ($F_{32,72}=4.465$, $P<0.0001$). Main effects of wavelength (between-subjects factor) and intensity (within-subjects factor) were significant (mixed ANOVA, between-subjects factor $F_{8,18}=5.611$, $P=0.001$, within-subjects factor $F_{4,72}=76.29$, $P<0.0001$). (D) *S. mackini* females. There was a significant effect of interaction ($F_{32,72}=1.704$, $P=0.032$). Main effects of wavelength (between-subjects factor) and intensity (within-subjects factor) were significant (mixed ANOVA, between-subjects factor $F_{4,72}=8.817$, $P<0.0001$, within-subjects factor $F_{4,72}=64.445$, $P<0.0001$). Post hoc test significance is indicated at $*P<0.05$ and at $**P<0.01$. Main effects with Bonferroni correction in A and B, and two-way Dunnett's test versus control in C and D ($n=24$ individuals for each wavelength/intensity).

(Fig. 4B). Diffuse spectral attenuation coefficients are presented in 25 nm bins in Fig. 4C for comparison to other aquatic environments (Jerlov, 1976). Note that the clearest waters transmit the most light at 480 nm, which is not the case for other highly attenuating waters such as coastal marine environments, or as we describe here, ephemeral pools (Fig. 4C). Light attenuation per centimeter in the water column of these pools is approximately two orders of magnitude higher than the most heavily attenuating coastal waters (Fig. 4C).

To determine whether soil particle size distribution could explain regional differences in light attenuation, we used hydrometers (Fig. 5A) to carry out soil texture analysis on dry soil samples from Central and Southeastern Arizona (Fig. 5B,C). Particle size distribution was dependent on the region (two-way ANOVA with region and particle size distribution as factors, interaction $F_{2,54}=180.22$, $P<0.001$; Fig. 5C). Soil texture analysis includes the largest particles (sand) that settle from a suspended solution in 40 s or less. It is unlikely that sand particles remain suspended in the water column throughout the life cycle of branchiopod populations in the field. Excluding sand, Central Arizona had a mean \pm s.d.

particle size distribution of $66.9 \pm 1.9\%$ silt and $33.1 \pm 1.9\%$ clay. Southeastern Arizona had $41.0 \pm 2.0\%$ silt and $59.0 \pm 2.3\%$ clay.

To determine total suspended solids of water samples taken in the field, we performed vacuum filtration of water samples. Southeastern Arizona had higher total suspended solids (mean \pm s.d. 1.734 ± 0.98 g l⁻¹) than Central Arizona (0.397 ± 0.190 g l⁻¹). We then used soil particle size distribution measurements from each region to estimate suspended soil by particle size. Southeastern Arizona had significantly more suspended clay than silt particles, whereas Central Arizona had more suspended silt than clay (two-way ANOVA on log-transformed suspended soil, with region and particle size distribution as factors, interaction $F_{1,32}=6.136$, $P=0.019$; Fig. 5D). Clay particles have smaller diameters (<0.002 mm) than silt particle diameters (<0.05 , >0.002 mm). Therefore, clay includes particles with radii that are roughly one-tenth the wavelength of UV and short wavelength light, which leads predominantly to scatter of shorter wavelength light than that caused by silt particles (Johnsen, 2012). Through scattering, clay contributes to higher attenuation of short wavelength light in Southeastern Arizona than in Central Arizona.

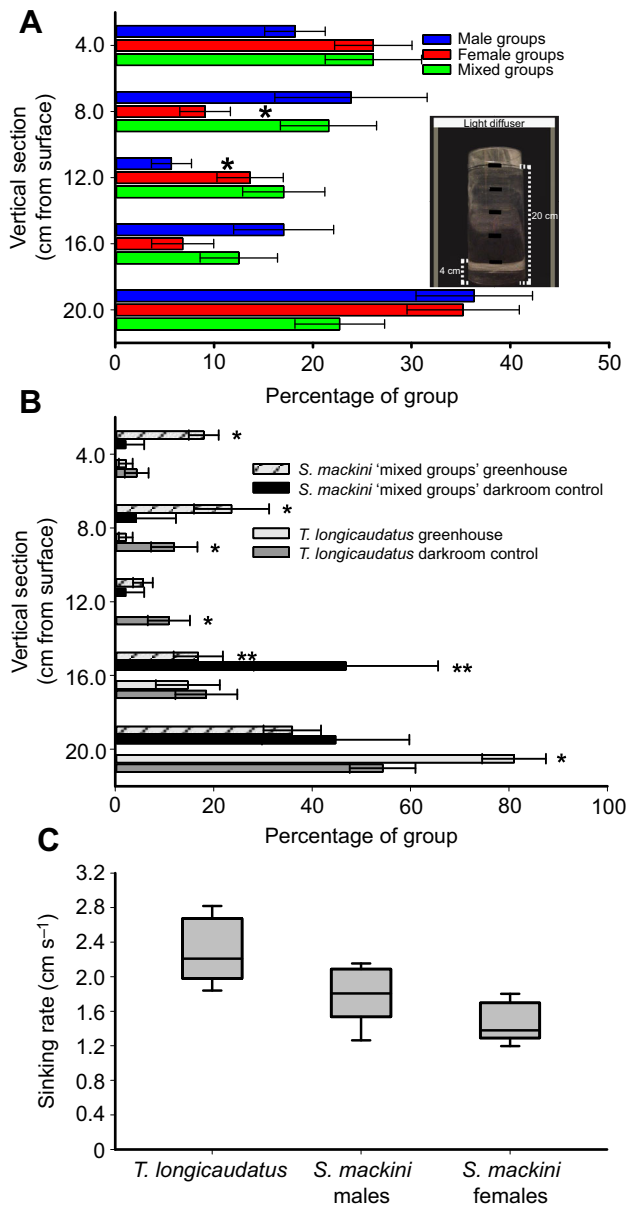


Fig. 2. Behavioral distribution in the water column. (A) *S. mackini* vertical distribution under diffuse, broad-spectrum light ($n=120$ individuals, in 12 trials of groups consisting of males, females and mixed groups). 'Mixed groups' contained five males and five females in each trial. Sequential Bonferroni-corrected test significance at each position is indicated at $*P<0.05$. (B) Comparisons between vertical distributions in greenhouse light environment and darkroom control tests for *T. longicaudatus* ($N=187$) and *S. mackini* ($N=205$). Error bars indicate s.e.m. Bonferroni-corrected significance at each position is indicated at $*P<0.05$ and at $**P<0.01$. (C) Mean sinking rates of anesthetized *T. longicaudatus* ($n=12$) and *S. mackini* males ($n=13$) and females ($n=15$). Whiskers indicate 95% confidence intervals.

The temporal variation of light and suspended soil for each region is presented in Fig. 6. The pools in this study filled from seasonal rains that took place in 2012 and 2013. Large single rains filled each pool (time since filling=0). λ_{P50} , total irradiance and total suspended solids varied irregularly over time. A clear peak in measured suspended solids and a minimum in total irradiance occurred 6 days after initial filling (Fig. 6C,B). Clay particles can remain suspended for weeks, silt for up to 8 h and sand particles for

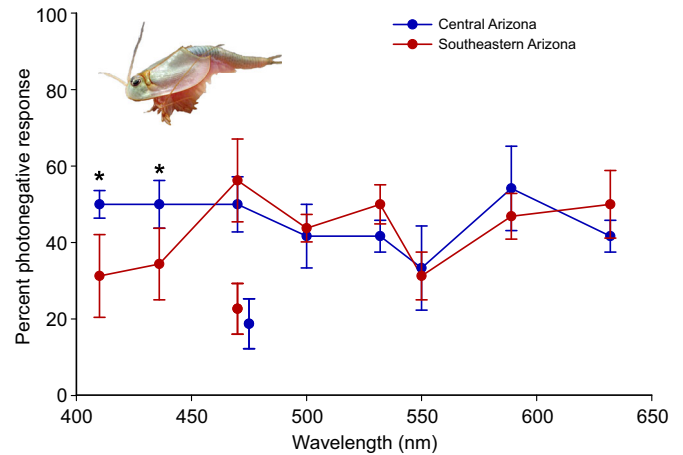


Fig. 3. Behavioral responses of field-caught animals from two regions. Responses were measured away from light when tested in a horizontal trough. Data are presented as means \pm s.e. Field-caught *T. longicaudatus* ($n=4$ trials) at threshold response intensities ($10^{5.5}$ photons $\text{cm}^{-2} \text{s}^{-1}$) are significant at <470 nm in Central Arizona (blue points, one-way ANOVA followed by Dunnett's *post hoc* test relative to control, $F_{8,64}=6.428$, $P<0.001$), but not in Southeastern Arizona (red points, one-way ANOVA followed by Dunnett's *post hoc* test relative to control, $F_{8,64}=5.419$, $P<0.001$). Asterisks indicate significance relative to control ($*P<0.05$).

a matter of seconds. Mixing from wind or other disturbances would result in an increase of suspended silt, and a temporary drop in total irradiance over this time frame. Our estimates of suspended clay versus silt would not detect a change over time within a region, as texture analysis was performed on dry soil samples to determine particle size distribution (Fig. 4B), and was used to estimate suspended soil (Figs 5D and 6D). The filled area of each pool used in this study was $>100 \text{ m}^2$. In smaller pools, it has been reported that the presence of notostracans contributes to suspended solids in the water column, but these experiments have not been carried out for pools $>100 \text{ m}^2$, and the density of notostracans could vary with total water volume. In mesocosm experiments, the presence of notostracans increases total suspended solids because of their swimming activity (Waterkeyn et al., 2011). We first observed adult branchiopods 5 to 7 days from initial filling in every pool, coinciding with the peak in suspended solids at 6 days from filling.

DISCUSSION

Light attenuation and behavioral depth regulation in temporary waters

Light is one of the primary environmental factors used by zooplankton for depth selection behavior (Cohen and Forward, 2009; De Meester et al., 1999). We show that two species of branchiopod crustaceans found in ephemeral pools, *T. longicaudatus* (Order Notostraca) and *S. mackini* (Order Anostraca), use light to regulate their depth in the water column. Early studies suggested that a suspension feeding branchiopod, *Daphnia magna* (Order Cladocera), might use spectral preferences, termed 'color dances', to regulate its vertical and horizontal position toward alga-rich water (Lubbock, 1888; Smith and Baylor, 1953). Recent studies have shown considerable regional variation in shallow freshwater spectral environments (Sabbah et al., 2011). All branchiopods are passive dispersers and are often found in hydrologically isolated, environmentally variable freshwater habitats (Brendonck et al., 2008; Williams, 2005), so adaptation to local environments would be expected. Because behavioral responses of *Daphnia* depend on wavelength, branchiopod responses may depend on regional light

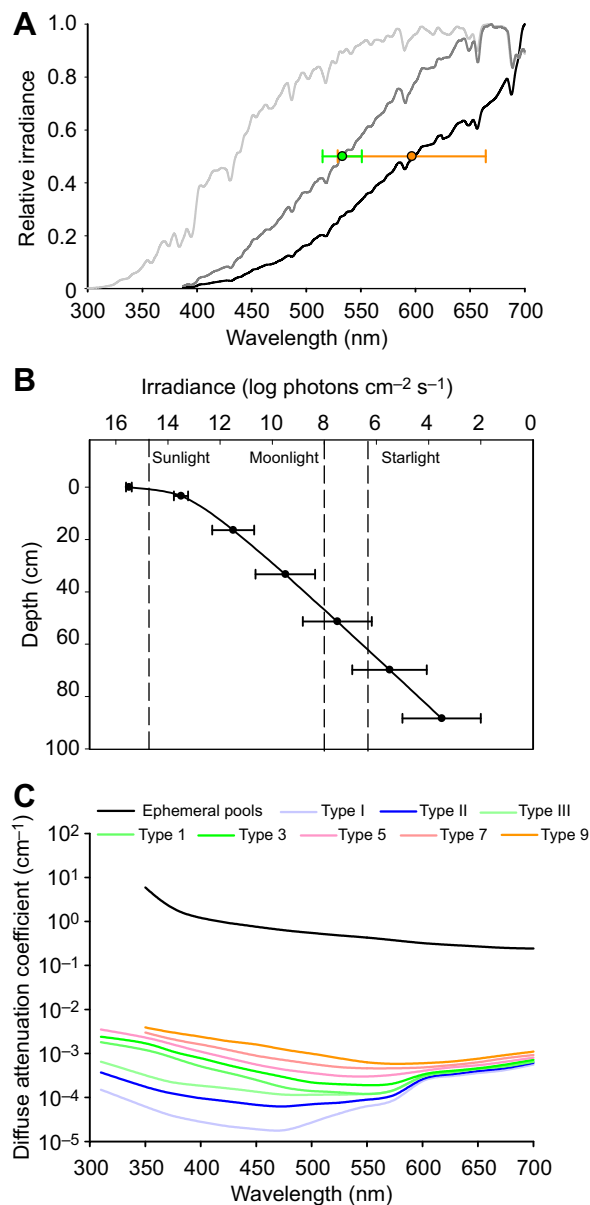


Fig. 4. Environmental light variation in shallow freshwater habitats.

(A) Relative averaged downwelling irradiance at 0 cm, just below the surface (light gray) and at 5 cm depth (dark gray) for the primary ephemeral pool for this study ($N=9$) and Lambert–Beer modeled irradiance at 5 cm depth (black) for six ephemeral pool habitats greater than 100 m² in Arizona, USA, measured between 10:00 and 14:00 h on cloudless days ($N=86$). The green and orange circles indicate the wavelength at which 50% of the photon flux distribution occurs ($\lambda_{P50} \pm s.d.$) at 5 cm (not shown for surface measurements). Irradiances were each standardized relative to peak photon flux of each measurement from 300 to 700 nm. (B) Comparison of intensity loss from 350 to 700 nm as a function of depth in all ephemeral freshwater pools ($N=6$) measured in this study in Arizona. Dashed reference lines are included for comparison to light levels of terrestrial habitats, and represent sunlight, moonlight (full moon) and starlight under clear conditions. Downwelling irradiances were modeled at $\lambda_{P50} \pm s.d.$ for each diffuse attenuation coefficient calculated from irradiances taken at 0 cm and 1 cm, using Lambert–Beer attenuation in order to provide the median intensity profile with depth shown here at two log unit intensity intervals. Error bars represent the relative error in modeled log intensity of $\lambda_{P50} \pm s.d.$ among both regions and over time. (C) Diffuse attenuation coefficients of downwelling irradiance, shown here for comparison to other bodies of water categorized by Jerlov (1976). The black line corresponds to median attenuation coefficients at 1.0 cm for ephemeral pools in Arizona ($N=86$), measured at 1 nm intervals, binned here to 25 nm for comparison with values reproduced from Jerlov (1976). Note that Jerlov (1976) use attenuation in the upper 10 m of the water column, only shown here for comparison to our values at 1 cm. Values used here are presented in Table S2. The bodies of water range from the least-attenuating (Jerlov Type I, light gray) to coastal and heavily attenuating (Jerlov Type 9, orange).

maintained at a much higher rate than would be expected given homogenization by gene conversion (Colbourne et al., 2011). In a companion paper (Lessios et al., 2018), we suggest that branchiopods have maintained four spectral classes of photoreceptors in their compound eyes, which is supported by the number of opsins expressed in anostracan and notostracan compound eyes (Kashiyama et al., 2009). Based on the number of spectral photoreceptor classes alone, it might be assumed that these species possess tetrachromatic vision. A 518-million-year-old stem mandibulate fossil had optic lobe neuropils that are associated with color vision in extant pancrustaceans (Ma et al., 2012). Branchiopod fossils that are 365 million years old have been found with evidence of resting eggs, suggesting that they inhabited ephemeral and most likely turbid bodies of water (Gueriau et al., 2016). Given that branchiopods have simplified optic lobes, we suggest that the opsins which are configured for four spectral photoreceptor classes have diversified throughout the evolutionary history of notostracans and anostracans for broad-bandwidth behavioral responses in temporary habitats.

Based on the spatial variation and regional effects of light environment on behavior (Fig. 3), we suggest that each population is adapted to the spectral conditions of its specific region. There are many compound eye parameters that could change spectral sensitivities of the compound eyes. Some studies have modeled visual pigment concentration, k , the absorption coefficient per micrometer of the photoreceptors, and have been able to identify differences in relative contribution of one or more photoreceptor classes based on habitat or regional light environment (Johnson et al., 2002; Lessios, 2017). However, empirical evidence is currently lacking of routine changes in visual pigment concentration in rhabdomeric photoreceptors among individuals (Fuse et al., 2014), and other photoreceptor parameters that have an effect on spectral sensitivity could change in development.

Multiple spectral classes predominantly for luminance-driven behavior

The broad spectral bandwidths we found for photonegative behavior at low intensity suggest that both *T. longicaudatus* and *S. mackini*

environment, rather than spectral preferences associated only with the transmission properties of algae (Stearns, 1975). We found that regional light environments inhabited by notostracan and anostracan branchiopods differed in the attenuation of short wavelength light, and that smaller suspended particles (clay) led to UV attenuation with depth. Our results from field-caught *T. longicaudatus* from separate regions support the prediction that behavioral responses are adapted to the spectrum of downwelling irradiance of a given region at depth.

The levels of suspended soil we have described in ephemeral desert pool habitats lead to rapid light attenuation with depth. The branchiopod species studied here inhabit spectrally variable light environments that are often dimmer than terrestrial habitats under starlight. Such patterns of regional variation in light environments have consequences for the expression of multiple opsin proteins, which are the primary genetic components of visual pigments expressed in photoreceptors (Bowmaker, 1999). A branchiopod selective history in variable environments has led to one of the highest numbers of duplicated opsins found in animal genomes sequenced to date. There is also evidence that recently duplicated paralogs are

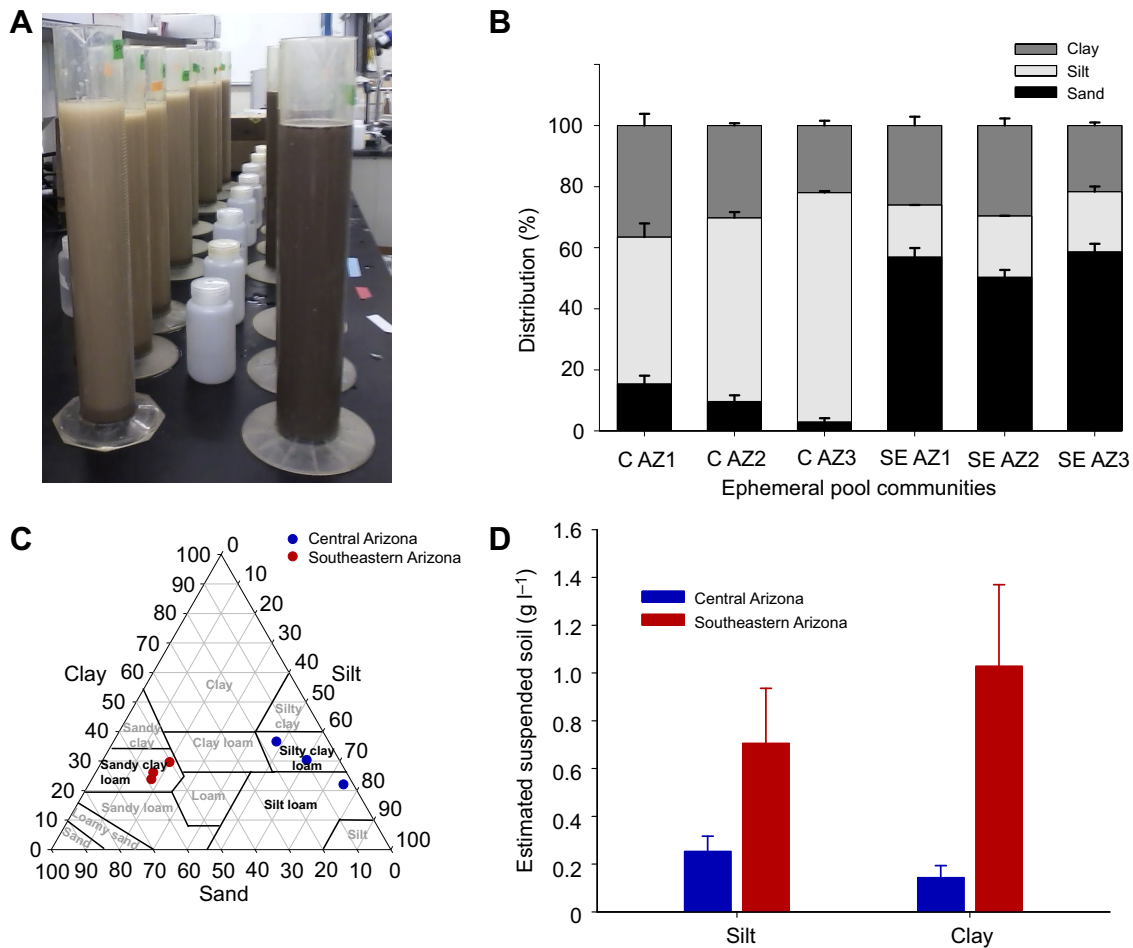


Fig. 5. Regional soil analysis. (A) Hydrometers used for soil analyses of each region, with Southeastern Arizona soil samples on the left, and Central Arizona soil samples on the right. (B) Soil texture triangle indicating particle size distributions of soil from each region, with classifications of soil categories reported by USDA (1993). (C) Soil texture analysis for each point shown in the soil texture triangle (means \pm s.d., $n=3$ soil samples from each pool). SE AZ, Southeastern Arizona; C AZ, Central Arizona. (D) Size distribution estimates of total suspended solids from water samples taken in the field ($N=88$). Only silt and clay are shown here as sand particles remain suspended for <40 s, meaning they are not likely to affect attenuation in the water column. Silt and clay particles remain suspended in solution for up to 8 h and for 8 h or longer, respectively.

generally employ multiple spectral photoreceptor classes for depth-selection behavior (Fig. 1). In *T. longicaudatus*, summation of outputs from multiple spectral classes could take place from broad receptive fields present in the lamina that we have identified in a companion paper (Lessios et al., 2018). The narrow bandwidth of photopositive responses we found for male *S. mackini* strongly supports the use, and the presence, of a single spectral photoreceptor class with an absorbance peak near 532 nm identified in a companion paper (Lessios et al., 2018). There are several possibilities regarding how a single receptor could provide input for narrow-bandwidth photopositive behavior. Long visual fibers have been reported in anostracans (Nässel et al., 1978). A long visual fiber that sums responses from several ommatidia in the proximal lamina could be configured to a single photoreceptor class to control wavelength-specific behavior. An alternative would be that short visual fibers from a single photoreceptor class make contact with other terminals in one of the main layers of the lamina and they are compared synaptically with other receptor classes. If there are synaptic comparisons of photoreceptor outputs, it could involve color vision for behavior. However, because male *S. mackini* photopositive responses to light centered at 532 nm were restricted to a narrow waveband, we refer to them as a wavelength-specific behavior

(Goldsmith, 1990; Kelber and Osorio, 2010). Crustaceans and insects that employ wavelength-specific behavior tend to use a subset of their photoreceptors for behavior (Cohen et al., 2010; Kolb and Scherer, 1982), but it remains unknown whether color opponency is involved for such wavelength-specific behavior (Kelber and Osorio, 2010). Furthermore, as we have found males of *S. mackini* to swim slightly above females, we suggest they could be using a single photoreceptor class to actively maintain a position at shallower depth than females. This is supported by some accounts that report vision is involved in anostracan mate choice (Belk and Martin, 1991).

We have found that ephemeral pool habitats, at less than 1 m of depth, are often dimmer than terrestrial habitats under starlight. In a companion article (Lessios et al., 2018), we found that branchiopod apposition compound eyes are likely to generate reliable signals at such intensities. There is some neuroanatomical evidence that pancrustaceans might incorporate summation from compound eye ommatidia in the lamina of their optic lobes for reliable vision at low intensities (Glantz, 2014; O'Carroll and Warrant, 2017; Warrant, 2017). Support for summation from multiple photoreceptor spectral classes for achromatic visual behavior at low intensity has so far been limited to behavior described in honeybees (Menzel and Greggers, 1985). We suggest that *T. longicaudatus* and *S. mackini*

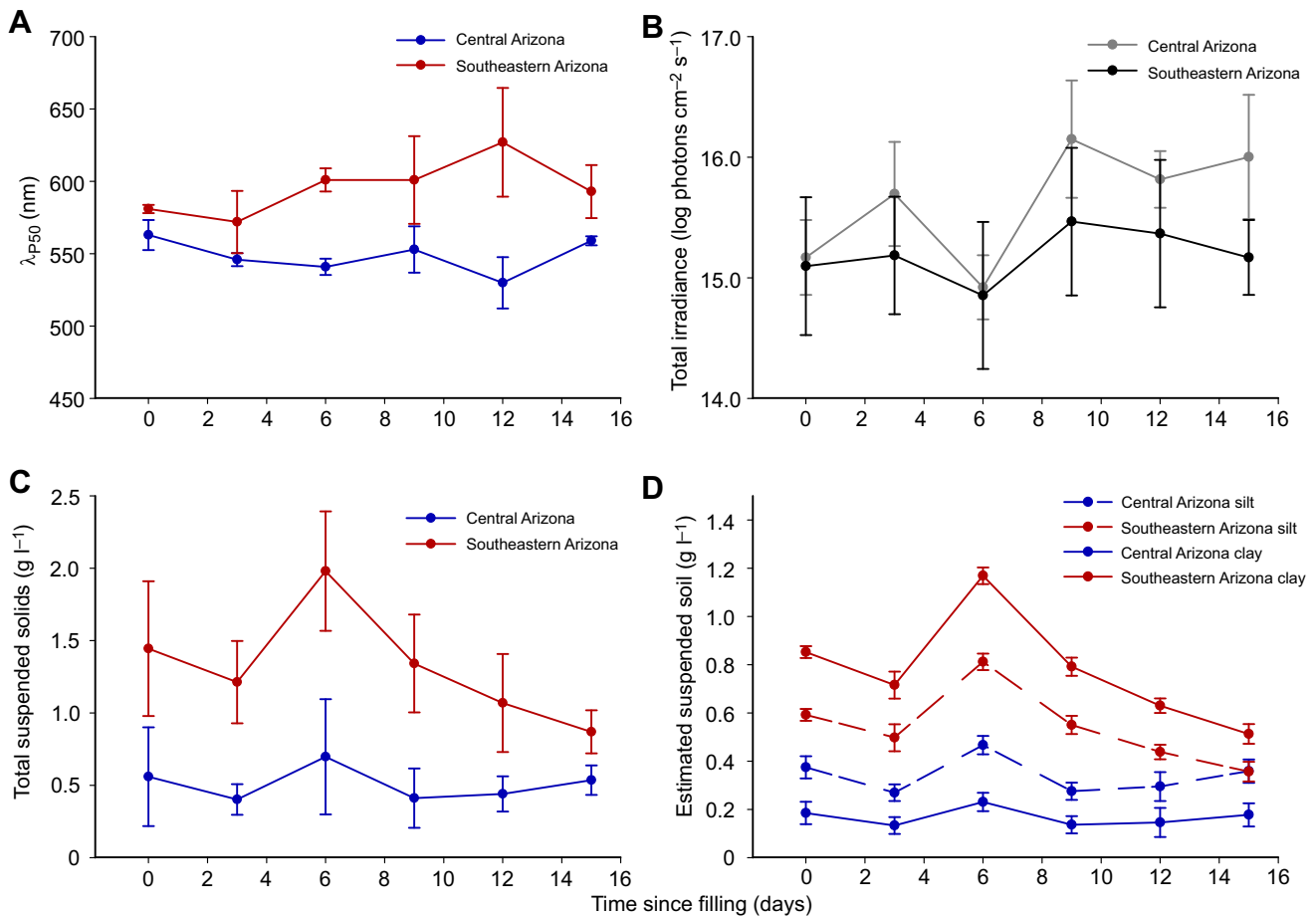


Fig. 6. Total downwelling irradiance, λ_{P50} , and total suspended solids over time starting at initial pool filling in Southeastern and Central Arizona. (A) λ_{P50} , the wavelength at which 50% of the photon flux distribution occurs at 5 cm depth. (B) Total downwelling irradiance at 5 cm depth (300–700 nm) from Central Arizona (gray) and Southeastern Arizona (black). (C) Total suspended solids taken from water samples collected in the upper 10 cm from each pool. (D) Suspended soil of silt (dashed lines) and clay (solid lines) estimated using total suspended solids weighted by particle size distribution.

are similarly employing summation for the behavior at low intensity described here. We infer that animals with simplified visual systems, such as branchiopods, are likely to have stereotyped light-dependent responses. If their habitats reach low intensities, their visual systems are likely to be configured to employ summation from multiple spectral classes in the peripheral nervous system. Research of light-dependent behavior that employs summation from multiple photoreceptor classes and wavelength-specific behavior is needed to fully understand the selective pressures that drive opsin diversification and maintenance. We suggest that future studies which make inferences about color vision should continue to use integrative approaches incorporating electrophysiology, neuroanatomy and behavior.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.L.; Methodology: N.L.; Formal analysis: N.L., J.H.C.; Resources: N.L., R.L.R.; Writing - original draft: N.L.; Writing - review & editing: N.L., R.L.R., J.H.C.; Supervision: R.L.R., J.H.C.; Funding acquisition: N.L.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.165878.supplemental>

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