ANATOMICAL COMPARISONS OF NEURAL SYSTEMS IN SIGHTED EPIGEAN AND TROGLOBITIC CRAYFISH SPECIES

Robin L. Cooper, Hao Li, Ling Yun Long, John L. Cole, and Hilary L. Hopper

(RLC, HL, YL, ILC) Thomas Hunt Morgan School of Biological Sciences, Center for Ecology, Evolution, and Behavior, University of Kentucky, Lexington, Kentucky 40506-0225, U.S.A.;
(HLH) Department of Geography, University of Kentucky, Lexington, Kentucky 40506-0225, U.S.A.
(corresponding author (RLC) e-mail: RLCOOP1@pop.uky.edu)

ABSTRACT

The activity of visual systems is known to affect development of the neural tissue associated with vision in both vertebrates and invertebrates. Three species of crayfish were compared for variations in the gross structures of the eye and of the underlying neural tissue of the optic system that were associated with environmental adaptation. The troglobitic crayfish Orconectes australis packardi and two epigean crayfish, Cambarus tenebrosus and Procambarus clarkii, were used. Cambarus tenebrosus raised in the cave are functionally blind although ommatidia develop, indicating that the primary sensory structures still develop without normal input. Troglobitic crayfish have lost the genomic ability to form a functional visual system. Electrophysiological records from neurons within the optic stalk of O. australis packardi showed no response to light. The neuronal ganglia within the eye stalk of C. tenebrosus are disorganized which could be the reason for the lack of a behavioral response related to sight. Second order neurons associated with olfaction arise in the central brain and send processes to lobula within the eye stalk via the protocerebral tract. Cross sections of this tract revealed that the troglobitic crayfish have more olfactory projection neurons and fewer large axon profiles than the other two crayfish, suggesting that O. australis packardi has more neural processing devoted to olfaction as an adaptation to cave life.

Neural activity is widely recognized as a contributing factor to neural development in a variety of animal species. The pioneering work of Wiesel and Hubel (1963) demonstrated how gross morphological alterations occur in synaptic organization in those parts of the brain related to vision. The present hypothesis is that the blind crayfish of an obligatory (troglobitic) cave species and the behaviorally blind crayfish of a surface (epigean) species found in caves both have reduced numbers of neuronal and retinal structures for the optic system as compared to species that make use of vision. The troglobitic crayfish Orconectes australis packardi Rhoades, the cave facultative species Cambarus tenebrosus Hay, and the surface crayfish Procambarus clarkii (Girard) were used in these studies. Even though C. tenebrosus still retains pigment in the eyes, the ability of the animal to use vision has not been previously addressed. Mellon (1977) has shown that members of the troglobitic crayfish species Procambarus erythrops Relyea and Sutton retain eye stalk musculature and reflexive eye movements such as protective eye withdrawal, geotactic eye stabilization, and proprioceptive nystagmus.

Crustaceans such as crayfish and lobsters are used to study the establishment and maintenance of social hierarchy by indexing the degree of dominance and submissiveness during paired interactions (Kravitz et al., 1980; Huber et al., 1997; Li et al., 1998). The crustacean social behaviors studied to date have evolved around visual posturing with a display of cheliped spread (meral spread) during the initial and maintenance phases of dominance behavior (Bruski and Dunham, 1987). The blind troglobitic and blind troglophilic crayfish do not show such obvious postural behaviors upon interactions in the pools of water within their natural cave settings. A plausible reason for the lack of display is that such behavior has no gain for blind opponents; nor are they able to see the result of such posturing. Reduced visual structures and behaviors save metabolic energy for other needs.

Because the cave crayfish have a reduced visual system, it seems likely that tactile and/or chemosensory systems would be enhanced. Detailed developmental studies trac-
ing olfactory afferent projections from the antennular nerve to the olfactory lobe in the central brain, and interneurons projecting from the olfactory lobe to lobula in the eye stalk, have recently been undertaken (Harzsch et al., 1997; Schmidt, 1997) although not in cave species. The olfactory projection neurons (OPN) have their cell bodies within cluster #10 of the central brain and send their axonal projections via the olfactory globular tract (OGT) to hemiellipsoid bodies in the eye stalk ganglion (Harzsch et al., 1997; Schmidt, 1997). The OGT forms a distinct subgroup of small axons within the protocerebral tract. The protocerebral tract is the nerve bundle of the eye stalk that contains the axons to and from the ganglia within the eye stalk and the central brain (Wiersma and Yamaguchi, 1966, 1967; Nunnemacher and Davis, 1968; Schmidt, 1997; Sandeman et al., 1998). The OPN bundle has been identified in cross sections of the protocerebral tract within the shore crab, *Carcinus maenas* (Linnaeus) (see Schmidt, 1997). The regional distribution is also observed in cross sections of all three species of crayfish used in this study. Because this olfactory path is of a distinct neuronal type and is isolated within the protocerebral tract (Schmidt and Ache, 1996; Schmidt, 1997), the OPN provides a good measure to determine if differences occur in the olfactory interneurons among the adult blind cave crayfish and the adult sighted crayfish. Simultaneously, examination can be made for other differences in visual neuron projections to the central brain. Additionally, it has been shown that new cells appear within cluster #10 throughout the life span of those crustaceans examined to date (Sandeman et al., 1998).

The purpose of this study was to examine the gross morphology of the crustacean eye among three species of crayfish, two of which show varying degrees of cave adaptation (or, at least, of evolutionary time in caves). In addition, the profile of neurons within the protocerebral tract and neural structures within the eye stalk were examined for indications that there has been a loss of visual information available for use by the blind animal. It was anticipated that this loss would be paired with an enhancement in the number of olfactory projecting neurons from the central brain to lobula within the eye stalk. This initial study lays the groundwork for future studies to address comparisons of those olfactory organs contained in the antennular flagella, and the number of aesthetasc sensilla of blind cave-adapted crayfish.

This work has been presented in abstract form (Cole et al., 1998).

**MATERIALS AND METHODS**

*Animals.*—Experiments were performed on three species of crayfish, *Procambarus clarkii* measuring 6–10 cm in body length were obtained from Achafalaya Biological Supply Co. (Raceland, Louisiana). *Orconectes australis packardi* and *Cambarus tenellus* were obtained by the authors from the Sloan's Valley Cave System in Pulaski County, Kentucky. A Kentucky Fish and Wildlife permit was obtained to collect these animals within this cave system and to use them for these experiments. Animals were housed in an aquatic facility within the laboratory in complete darkness and fed fish-food pellets.

*Anatomy.*—The crayfish specimens were photographed with a Pentax X2M camera with a 90-mm macro lens. The live animals were held at approximately 30° to 45° angles clockwise from the camera in order to allow the eye stalks to extend at their maximum distance from the carapace and to elicit and document a natural geotactic response of the eyestalk position. Measurements were made of structures from calibrated photographic prints.

One of the eye stalks from each animal was used for cross sections, and the other eye was used for longitudinal sections for light and transmission electron microscopy (TEM).

After the eye stalk was cut and held in place, the preparation was fixed with 2.5% vol. glutaraldehyde, 0.5% vol. formaldehyde dissolved in a buffer (0.1 M sodium cacodylate, 0.022% wt. calcium chloride, 4% wt. sucrose, and adjusted to pH of 7.4) for 1 h with 2 changes of solution. The tissue was subsequently processed for TEM (Jahromi and Atwood, 1974; Cooper, 1998).

In order to obtain axonal measurements, thin sections of the nerves were photographed at 1,500x and printed at 2.5x to form montages. Maximum and minimum diameters of the axons were measured. The square root of the product of the maximum and minimum diameters provided the mean diameter of the axon.

*Electrophysiology.*—In order to obtain retinograms, one tip of a silver wire was placed under the dorsal thoracic carapace and a second silver wire was placed just under the cuticular surface within the array of ommatidia. The signals were amplified by an extracellular P15 amplifier (Grass Instruments). The signals were recorded to VHS tape (Vetter, 400) as well as on-line to a Power Macintosh 9500 computer via a MacLab/4s interface (ADInstruments). All events were measured and calibrated with MacLab Chart software version 3.5.4. Calibration pulses were provided internally by the P15 amplifier. The computer acquisition rate was at 10KHz.

Illumination of the eyes was provided by a fiber-optic source from a Lumina (Chu Technical Corp.) in which a single optic fiber of either 0.5-mm or 1.0-mm diameter was passed over regions of the ommatidia or held directly over the eye to illuminate the entire eye.

*Behavior.*—Observational studies were made of all three species as to their ability to detect a hand passing 10 cm
overhead in the presence of dim red light, bright red light, and dim white light. Other conspecific partners were placed face-to-face out of water on moist sand just far enough apart so that their antennae did not touch. Observations were made to examine any postural position- ing that would indicate a response to the hand waving or to an opponent. In addition, rotation of the body was performed to examine oculomotor reflexes, and focused light was shone at the optic cap to determine if the eye-lowering reflex was present.

RESULTS

General External Eye Morphology

The cuticle forming the eye stalk was present in all three species. The stalk lengths were similar in P. clarkii and C. tenebrosus, but the stalk was grossly reduced in O. australis packardi. Figure 1 is a side view of each species showing the position of the eyes with a maximal eye stalk extension.

The eyes of P. clarkii and C. tenebrosus were faceted, although corneal size varied extensively between the two species (Fig. 1). This is clearly seen in the images obtained from a dissecting microscope. The cornea in O. australis packardi could not be observed with surface imaging or in cross sections taken from the eye-cap region (Fig. 2). Six crayfish of this species were examined under a dissecting microscope. The cornea appeared to be absent in this species. The most visual of the crayfish, P. clarkii, had a larger cap surface area and more ommatidia than C. tenebrosus. In order to resolve and count the ommatidia, photographic montages were made. The ommatidial dimensions were the same between the two species, but there were fewer of them in C. tenebrosus. Counts of the total ommatidia from one eye revealed 3,596 in P. clarkii and 1,859 in C. tenebrosus.

Ommatidial Structure

Longitudinal sections through the eye and the stalk showed large differences in the underlying neural anatomy of the visual system in the three species (Fig. 3). As similarly shown by Mellon (1977) for the troglobitic crayfish Procambarus erythrropus, the structured laminae were most distorted in O. australis packardi. Because O. australis packardi has even less of a cornea than P. erythrropus, it was not surprising that the structures were less defined. In C. tenebrosus there was better retention of neural structures in the eye stalk visual system than for O. australis packardi, although the most distinct lobula structures in an eye stalk were observed in P. clarkii.

Protocerebral Tract

Cross sections taken at the base of the eye stalk distal to the central brain allowed a direct count of the number of axons in each of the three species. Cross sections of the protocerebral tract in the three species as seen with a light microscope revealed the size differences of the nerve (Fig. 4). A representative cross section of the eye stalk from O. australis packardi is shown in Fig. 5A. At a higher magnification (Fig. 5B, C) of a region of the nerve bundle, a typical distribution in the size differences in axonal diameters was obvious. Montages of these high magnifications were made so that accurate measurements could be made of the axon diameters. In addition, size distributions of axon profiles were made for ease in comparative analysis among these three species and for use in future studies (Fig. 6A–C).

There are likely motor axons within the nerve bundles, because sections were made proximal to some of the muscle attachments within the eye stalk. The motor axons could be the larger axon profiles, because more distal sections past the muscular regions did not contain such large axons. Mellon et al. (1976) provided a detailed study of the eye motor axon anatomy and position for sighted crayfish. The present study does not include a systematic survey of axon profiles along the length of the eye stalk among the different species. Nonetheless it is of value to report that the subset of axons composed by the OPN were clearly delimited in all the cross sections as the smallest axon profiles and formed the wedge-shaped structure within the protocerebral tract. The electron micrograph of a cross section from the protocerebral tract of O. australis packardi (Fig. 5A) demonstrates the unique small axon diameters of the OPN as compared with the other axons within the protocerebral tract (Fig. 5B, C).

Electrophysiology

Electrical signals were elicited in the eye of P. clarkii by shining light over the eye for brief moments (horizontal line in Fig. 7A) or in a sweeping manner (angled horizontal lines) across the cornea of ommatidia array. The sweeping motions of the light are indicated as the solid lines in Fig. 7B, with the
Fig. 1. Lateral profiles of the eye stalk on the crayfish of the epigean sighted *Procambarus clarkii* (top), and two cave crayfish, *Cambarus tenebrosus* (middle) and *Orconectes australis packardi* (bottom). The animals were tilted about 30–45° while the pictures were taken. Note the geotactic response of the eye stalk position. The right panel are photographs of the most distal portion of the eye stalk. The top-to-bottom order is *P. clarkii*, *C. tenebrosus*, and *O. australis packardi*. The arrow in the bottom picture points to the distal part of the eye in this species which is difficult to see because of the lack of dark pigment. Scale bar for right panel is 2.5 mm; for left panel, scale bar is 286 μm.
Fig. 2. Comparisons of cross sections in the cap region in epigean and troglobitic crayfish. Shown is a cross section of the distal region to examine for ommatidial structures. The top-to-bottom order is Procambarus clarkii, Cambarus tenebrosus, and Orconectes australis packardi. Note that only ommatidia are observed from external and internal morphology in P. clarkii and C. tenebrosus. Scale bar is 1.38 mm for left panel and 0.138 mm for right panel.
Fig. 3. Longitudinal sections of the eye stalks in sighted and behavioral blind crayfish. The sighted *Procambarus clarkii* (A), and two cave crayfish, *Cambarus tenebrosus* (B), and *Oroconetes australis packardi* (C), are shown at the same scale (2.5 mm) for comparative purposes. The enlargement of *O. australis packardi* (D) demonstrates the lack of orderly arrangement of the optic neural structures and lack of projection to the eye cap (scale 1.27 mm). The various regions are identified and named accordingly as by Mellon (1977), Strausfeld and Nüssel (1981), and Kirk et al. (1982)—1. lamina ganglionaris; 2. medulla externa; 3. medulla interna; 4. terminal medulla.

Responses shown directly below the lines. The electrical response shows a relationship to the intensity of the light. As shown in Fig. 7C, illumination was given in short bursts with increasing intensity upon the next exposure, then the light was turned back to the intensity of the first exposure but this time the light was left over the eye for continuous illumination. This response adapted relatively quickly during the exposure time. When repeating similar experimental perturbations but with freshly obtained *C. tenebrosus* no sig-


Fig. 4. Cross section of protocerebral tract just prior to the central brain reveals the size differences among the species. In thick sections photographed with light microscopy, the differences among the three species can be seen—Procambarus clarkii (A), Cambarus tenebrosus (B), and Orconectes australis packardi (C). The olfactory projection tract (OPN) is outlined in white. Scale bar is shown in B and is the same for all at 23 μm.
3 d and the animals were adapted to dim room light, small responses could be observed to light stimuli in *C. tenebrosus* (Fig. 8C). These results indicate that in the eyes of *P. clarkii* electrical signals can be measured easily in response to light, but that in the eyes of *C. tenebrosus* the pigments bleach out readily upon exposure to light after the animals are removed from the cave.

**Behavior**

The behavioral studies mimicked the results obtained with the retinograms in that only *P. clarkii* responded to hand waving over its head in dim white light and outdoor lighting. The typical response was the raising of chelipeds in what is termed a “startle” response. This postural behavior was seen following the pairing of one animal with another in a face-to-face position. This species did not show any response to hand waving in the presence of dim red lighting. The other two species, *C. tenebrosus* and *O. australis packardi*, did not show any responses in any of the lighting paradigms or in the pairing experiments with a conspecific animal.

In the cave setting, *C. tenebrosus* and *O. australis packardi* have co-existed and have lived without light by use of non-visual behaviors. Even though there was some response to light by *C. tenebrosus*, no behavioral responses could be induced from hand waving over the eyes (e.g., inducing shadows) or from pairing conspecifics in dim white light. These behavioral tests were done on wet sand to avoid the animal’s use of chemical cues, and observations were made only when the animals were far enough apart that their antennae could not come in contact. Any time the cornea was touched in any of the three crayfish species, an eye stalk withdrawal occurred. This type of reflex is normally observed in crabs and crayfish (Burrows, 1967; Burrows and Horridge, 1968; Mellon, 1977).

**DISCUSSION**

The eye of the troglobitic cave crayfish *O. australis packardi* lacks facets, resulting in the absence of a cornea. This lack of visual receptors has led to an alteration of neural tissue within the eye stalk as compared to sighted crayfish. The developmental loss of visual receptors is perhaps due to an altered genome in *O. australis packardi*, because *C. tenebrosus* retains ommatidia and a cornea,
Fig. 6. The distribution of the axon dimensions found at the base of the protocerebral tract reveals differences among epigean and cave crayfish. The histograms show the frequency of occurrence in the variously sized axons. The bin widths used in the histograms were 0.3 mm for all three species, Procambarus clarkii (A), Cambarus tenebrosus (B), and Orconectes australis packardi (C) are all plotted with the same scale for comparative purposes. The left panels are enlarged to the right to highlight the lower occurring frequencies.
Fig. 7. Retinograms made in the distal region of the eye stalk of the epigean crayfish. The sighted *Procambarus clarkii* showed neural responses each time a light passed over the eye. (A) The light was turned on (bars) or off (no bar). (B) The fiber-optic light source swept past the eye at variable rates (relative rates as indicated by the slope of the bars). (C) The light was pulsed on or off and later in the trace the light remained on to test for accommodation. Calibrations shown in Fig. 8.
Fig. 8. The retinograms in the cave crayfish revealed differences from the epigean species, *Orconectes australis packardi* and *Cambarus tenebrosus* did not show responses when given the same stimuli as for the Procambarus clarkii. (A) The light was turned on (bars) or off (no bar), *Cambarus tenebrosus*. (B) Same as in A, but for *Orconectes australis packardi*. When the eye was touched by the optic light fiber, a reflex withdrawal presented a large artifact in the baseline. (C) After the recording wires were in place and the crayfish (*C. tenebrosus*) was left in dim white light (16 h) and dark (8 h) for 3 d, small responses could be observed upon illumination of the cornea. (D) P–15 calibrations for all traces in Figs. 6 and 7.
organisms lose functions that are not used. In the case of *O. australis packardi* even the juvenile stages appear to lack eye pigmentation, indicating the influence of heredity.

Morphological studies of crayfish fossils and the geological evidence suggest that the *Orconectes* of the Cumberland Plateau in Kentucky originated from *Procambarus* stock and probably entered the karst systems during the Miocene period (Rhoades, 1944, 1962; Hobbs and Barr, 1960; Hobbs et al., 1977; Barr, 1985). This long period may have provided sufficient time for the development of variations in the visual systems among isolated cave crayfish populations. It should be kept in mind, however, that following 69 generations of *Drosophila* raised in the dark to induce eye loss, the animals still possessed vision (Payne, 1911). It remains to be determined the evolutionary time scale needed for functional changes to occur.

The behavior of the cave crayfish within a cave or in the experimental laboratory tanks indicates that the animals depend on the use of antennae, which continuously sweep over the terrain in front of them when walking (Cole et al., 1998; Li and Cooper, 1999). This action may very well enhance chemosensory (i.e., olfaction) ability by causing water to flow over the aesthetasc sensilla on the antennules (Ache and Derby, 1985; Moore et al., 1991). It remains to be determined if cave crayfish have more neurons and neural space in the central brain dedicated to primary tactile and chemosensory sense from the antennae and antennules.

Investigations are under way to determine by electron microscopy the number of axons and their size profiles from cross sections made at the base of the antennae and antennules in the same species used for this study. Because it has already been determined that the OPN forms a distinct tract within the protocerebral tract for relaying olfactory information to the hemiellipsoid body within the eye stalk, and because it has now been shown that there are more axons within this bundle in the adult blind cave crayfish than in the larger adult sighted crayfish, there is some indication that there are more primary sensory neurons in the chemosensory antennules. Because it is known that these neurons increase with age of the animal, further detailed studies at the various stages of development among these animals would be useful for a deeper comparative study among these three
species. The fact that there are substantially more OPN in the cave crayfish is a good demonstration of the potential increased reliance on olfaction in the blind cave crayfish.

Because there are so little distinctive neural tissue and lobular structures within the eye stalk of *O. australis packardi*, it is hard to identify the particular structures with confidence. The reason that any neural tissue remains in the optic stalk of *O. australis packardi* is probably due to the many other functions the eye stalk carries out, such as hormonal secretion of the molt inhibiting hormone (Skinner, 1985; Fingerman, 1987, 1995) as well as other factors (see Barrera-Mera and Berdeja-Garcia, 1979; Aréchiga *et al.*, 1990).

The numbers of ommatidia per eye in *P. clarkii* and in *C. tenebrosus* are within the range reported for *Pacifastacus leniusculus* (~2,500) of the same range of body mass (Kirk *et al.*, 1982). It would be of interest to raise eggs in a lighted surface environment from a gravid *C. tenebrosus* caught in the cave to determine if use may have a developmental role on the numbers of ommatidia and eye-stalk neuronal structures. Pigment within the eye of crustaceans is known to migrate in response to light, which is thought to be regulation for light adaptation (Stavenga, 1979). It is unknown if pigment migration occurs in *C. tenebrosus* in the cave or if it would occur if specimens were exposed to a light:dark cycle. Such studies in pigment migration could be addressed by exposing *P. clarkii* to cave (absolute darkness) conditions during development. Possibly the lack of exposure to light may also induce *P. clarkii* to show a lack of diurnal cycles, also shown to be lacking in a troglobitic crayfish (Park *et al.*, 1941).

In spite of the small eye stalks in *P. erythrops* (see Mellon, 1977) and *O. australis packardi*, they both have oculomotor reflexes for protective eye withdrawal and geotactic eye stabilization. The large axons with mitochondria in the protocerebral tract are probably motor neurons that innervate the eye-stalk musculature. These motor neurons have been reported in *Procambarus erythrops*, by Mellon (1977). Possibly there are cuticular sensory structures in the eye stalk that might contribute to a percentage of the total number of axons observed in the cross sections.

Investigations of a variety of surface and cave crustaceans at various stages in development will provide insight as to the evolutionary process of vision loss with simultaneous enhancement of other sensory modalities, allowing cave animals better chances for survival. For example, Mellon (1977) reported that *P. erythrops* possesses a cornea, whereas it has been shown that *O. australis packardi* does not. Because there are numerous cave crustaceans with various degrees of structural loss in visual components, such a study is feasible for structural and functional correlations related to the evolutionary processes in well-defined ecological niches, such as in isolated caves (Poulsom, 1964). Crayfish in general do show a high degree of plasticity in their nervous system and muscles based on activity or use (Cooper *et al.*, 1998). Studies on both amblyopsid fishes and the crustacean *Gammarus minus* have provided indirect anatomical support for a trade-off between optic and olfactory systems in cave-dwelling forms (Poulsom, 1963; Voneida and Fish, 1984; Culver, 1987).

**Acknowledgements**

Illustrations were provided by the courtesy of Hye Won Cooper. Superb electron microscopy technical assistance was provided by Ms. Mary Gall Engle and Mr. Richard Watson (Univ. of Kentucky). Gratitude is given to Dr. Tom Barr (Tennessee) and to Dr. Horton Hobbs (Wittenberg Univ.) for help in identification of cave organisms. Thanks is given to Mr. Tom Crockett for access to the cave entrance on his property. Appreciation is expressed to Dr. H. B. Hartman for use of optic fibers. Thanks are also given to Hye Won Cooper for helping to count and measure axon profiles. Thanks is given to Dr. Hugo Arechiga (Mexico) for useful discussion on references to optic structure. Funding was provided by University of Kentucky Research and Graduate Studies Office (R.L.C.) and NSF grant IBN-9808631(R.L.C.). This work is in partial fulfilment of the Ph.D. requirements for Mr. Hao Li. This paper is dedicated to the late Mrs. Cathy Crockett, who was a nationally recognized ecologically minded caver.

**Literature Cited**


Rhoades, R. 1944. The crayfishes of Kentucky, with notes on variation, distribution and descriptions of species and subspecies.—American Midland Naturalist 31: 111–149.


Voneida, T. J., and S. E. Fish. 1984. CNS changes related to the reduction of visual input in a naturally blind fish Anophtichthys hubbsi.—American Zoologist 24: 775–782.


———, and T. Yamaguchi. 1966. The neuronal components of the optic nerve of the crayfish as studied by single-unit analysis.—Journal of Comparative Neurology 128: 333–358.


ACCEPTED: 19 September 2000.