

Communication using eye roll reflective signalling

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Body reflections in the ultraviolet (UV) are a common occurrence in nature. Despite the abundance of such signals and the presence of UV cones in the retinas of many vertebrates, the function of UV cones in the majority of taxa remains unclear. Here, we report on an unusual communication system in the razorback sucker, *Xyrauchen texanus*, that involves flash signals produced by quick eye rolls. Behavioural experiments and field observations indicate that this form of communication is used to signal territorial presence between males. The flash signal shows highest contrast in the UV region of the visual spectrum ($\lambda_{\max} \sim 380$ nm), corresponding to the maximum wavelength of absorption of the UV cone mechanism in suckers. Furthermore, these cones are restricted to the dorsal retina of the animal and the upwelling light background is such that their relative sensitivity would be enhanced by chromatic adaptation of the other cone mechanisms. Thus, the UV cones in the sucker have optimal characteristics (both in terms of absorbance and retinal topography) to constitute the main detectors of the flash signal. Our findings provide the first ecological evidence for restricted distribution of UV cones in the retina of a vertebrate.

Keywords: visual communication; ultraviolet cones; sucker; territoriality

1. INTRODUCTION

Light signals based on reflections from exposed body surfaces are a widely used means of animal communication. Such signals are known, or believed, to play a role in mate attraction (Endler 1987; Fleishman *et al.* 1993; Bennett *et al.* 1996; Hunt *et al.* 1998; Kodric-Brown & Johnson 2002; Cummings *et al.* 2003; Boulcott *et al.* 2005), dominance (Thompson & Moore 1991; Marchetti 1993; Baube 1997) or orientation within a group (e.g. as in the schooling behaviour of fishes; Rowe & Denton 1997). Of particular interest in recent decades is the observation that many of these signals comprise ultraviolet (UV) wavelengths (approx. 340–400 nm), which overlap the absorbance spectrum of UV cones in the retinas of many animals (Tovée 1995). With the exception of some non-raptor bird species (Bennett *et al.* 1996; Hunt *et al.* 1998) and one fish (Cummings *et al.* 2003), where UV cone-mediated sensitivity is known to play a role in sexual selection, the function of UV cones in other vertebrate taxa is unknown or disputed (see White *et al.* 2003, 2005; Kellie *et al.* 2004; Lukáts *et al.* 2005; Cheng *et al.* 2006). Furthermore, in species where UV cones have been mapped onto the retinal surface, these are often restricted to specific areas (e.g. the ventral retina of the mouse, Lukáts *et al.* 2005; the dorsal retina of juvenile salmonid fishes, Cheng & Novales Flamarique 2004; Cheng *et al.* 2006), the reason for which is also unclear.

Our studies on the razorback sucker, *Xyrauchen texanus*, have focused on the role that the UV cones are likely to play as part of a novel communication system

subserving territoriality. While filming the reproductive (spawning) behaviour of this species in the Colorado River, we discovered an unusual signalling system used by this fish that involves rolling out the eyes to produce two flashes of light. Intrigued by this behaviour, we proceeded to measure the characteristics of the signal and carried out behavioural experiments to figure out its purpose. We hypothesized that if the flashing served to attract mates, then females would swim closer to a flashing model than to a non-flashing model. Conversely, if the signal was territorial, then males would swim further away from a flashing model compared with a non-flashing model, as only males appear to hold territories in nature (Mueller 1989). Since the signal comprised UV wavelengths, we characterized the position of the UV cones in the retina to test whether their location was conducive with a role in its detection.

2. MATERIAL AND METHODS

(a) Animals

The fish studied was the razorback sucker, *X. texanus*, which is the largest catostomid in North America. The study used adult wild-stock fishes from the Colorado River kept in outdoor raceways at Willow Beach National Fish Hatchery (Arizona, USA). The weight and the total length \pm s.d. of the fish used in the experiments were: for females ($n=35$): 738 ± 251 g, 41.1 ± 4.93 cm; for males ($n=35$): 754 ± 166 g, 42.3 ± 3.2 cm. Underwater filming of reproductive behaviour was carried out by positioning a black and white-output camera at 1 m depth at various spawning locations along the Colorado River. All field observations and experimentation complied with institutional guidelines, which followed those of the Canadian Council for Animal Care.

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(b) Measurements of reflection signals

We used a USB-2000 spectroradiometer (Ocean Optics) equipped with a liquid light guide (600 μm diameter input, 0.22 NA) to measure the light reflected from the top of the eye of anaesthetized fish and from the surrounding head skin. To do this, the fish was anaesthetized by immersion in a solution containing 25 mg l⁻¹ buffered MS-222 and placed in a holder equipped with an attachment for the liquid light guide. The attachment could be displaced in the horizontal plane and the input end of the guide rotated in the vertical plane. All fish were placed in the same way, by aligning the pupil of the left eye with a horizontal marker on the holder. Similarly, the guide's input was consistently positioned 2 cm from the top of the fish's eye at an angle of approximately 20° from the vertical. For eye reflectance measurements, the eye was slightly rolled out with pincers to expose the top region responsible for the 'flash'. For head skin measurements, the guide was repositioned at the same distance and angle but with respect to the head skin next to the eye. These measurements were carried out at 1 m depth in the Colorado River, adjacent to the hatchery. The measurements used seven fish of similar size and took place between 12.45 and 13.30 h on a clear sunny day. The use of a precision holding apparatus, similar size fish and chosen time of day permitted us to minimize the differences due to field conditions, fish and probe positioning. Measurements of the light reflected from the river bottom (1–3 m depth) were also conducted from 10 cm below the surface using the positioning arm of the holding apparatus at the beginning and at the end of the fish reflectance measurements. The reflectance contrast between the eye roll signal and that from the skin on the head immediate to the eye, or that from the upwelling light reflected from the bottom of the river, was computed as the difference between both signals divided by the sum and expressed as a percentage.

(c) Quantum catch estimates

To obtain a measure of detection efficiency of the flash signal by each cone type in the razorback retina (maximally sensitive to either UV, blue, green or red), we calculated the ratio of quantum catch of the flash signal to that of the upwelling river light, the latter representing the relative adaptation state of the cone. Pigment absorbance spectra were generated using an eighth-order polynomial template (Palacios *et al.* 1996) from maximum wavelengths of absorption/sensitivity derived by microspectrophotometry and optic nerve recordings ($\lambda_{\text{max}} = 380$ nm (UV), 433 nm (blue), 544 nm (green) and 630 nm (red); Novales Flamarique & Hárosi 1997; Novales Flamarique & Hawryshyn 1998). These values were used to compute absorbance using the equation: $\text{absorbance} = 1 - 10^{-(\text{absorbance})(S)(l)}$, where S is the transverse specific density (approx. 0.0115 μm^{-1}) and l is the outer segment length (approx. 10 μm) of cones in adult sucker (Novales Flamarique & Hárosi 1997). For each cone type, the absorbance values were multiplied by either the average upwelling river light or the average eye flash signal. These values were then integrated across the spectrum to give the respective quantum catches (see Novales Flamarique & Hárosi 2000). The ratio of quantum catch of flash signal to that of upwelling light is a relative measure of efficiency of signal detection taking into account adaptation due to background lighting.

(d) Behavioural experiments

To figure out the purpose, if any, of the flash signal, we carried out behavioural experiments in which groups of five fishes were exposed to two razorback fish models, each located on either end of a 3600 l tank (length \times width \times height: 250 \times 100 \times 125 cm). The models were equipped with white light emitting diodes (LEDs; Philips Lumileds) for eyes whose emission (flash intensity, duration and frequency) was controlled remotely. The bottom two-thirds of each LED was painted opaque to mimic the upwelling flash produced by the fish eye roll. The bottom of the tank was covered with gravel and rocks from the Colorado River to simulate spawning habitat. The tank was filled with fresh river water every day and was illuminated by direct sunlight during the experiments. Five fish (males or females) were tested during each experiment to mimic the spawning aggregations that occur in nature. Fish behaviour was recorded with a wide-angle camera positioned 2 m above the tank.

Each experiment involved an acclimation period of 50 min with no model flashing, after which the behaviour was recorded for 16 min. One of the models was then made to flash for 16 min, once every 30 s for 0.5 s. The flashing of this model was then suppressed and the opposite model was made to flash in similar fashion for the next 16 min. Following 30 min of acclimation, another 16 min of recordings were made without models flashing. The distance of each fish from the flashing model was used to compute an average location for the five fish every minute. Distances were obtained similarly during the two non-flashing intervals of the experiment (in this case, half of the distances were taken with respect to each model). Seven such trials (replicates) were performed with female fish and seven with male fish; all fish in a given trial were naive. The data, which fitted the assumptions of normality and homogeneity of variance, were used to assess differences in distance as a function of diode state (flashing or not) using the general linear models (GLM) univariate option in SPSS (v. 15). The GLM was of the form: $\text{distance} = \beta - \text{intercept} + \beta_1 (\text{diode ON}) + \beta_2 (\text{diode OFF}) + \beta_3 (\text{model left}) + \beta_4 (\text{model right}) + \sum_{i=5-11} \beta_i (\text{trial})_i + \text{error}$. In other words, we tested for the effect of diode state on fish distance when controlling for the position of the flashing model (left or right) and trial number.

(e) Localization of UV cones

Since the UV opsin mRNA sequence of the razorback sucker is unknown, we used a halibut UV riboprobe to study the distribution of UV cones in the retina by *in situ* hybridization. Previous research has shown that halibut-derived UV riboprobes consistently label UV opsin mRNA in the single cones of a multitude of fishes resulting in expression patterns which are equal to those obtained with species-specific riboprobes (Raymond *et al.* 1995; Stenkamp *et al.* 1997; Forsell *et al.* 2001; Cheng *et al.* 2006). Halibut (*Hippoglossus hippoglossus*) ultraviolet-sensitive opsin partial cDNA (HUV) was generated by RT-PCR amplification from halibut total RNA isolated from homogenized eyes (RNAqueous-Midi; Ambion). HUV cDNA was synthesized (Ready-To-Go RT-PCR beads; Amersham Biosciences) using primers that were designed from a published UV opsin sequence for halibut (accession no. AF156264, our probe corresponds to bases 33–354 of this sequence; UV forward primer 5'-ACG TTT CTA ATG TGA GTC CC-3'; UV reverse primer 5'-AGG CTC CGA ATG GTT TAC AA-3'). Reverse transcription with UV reverse primer was carried out at

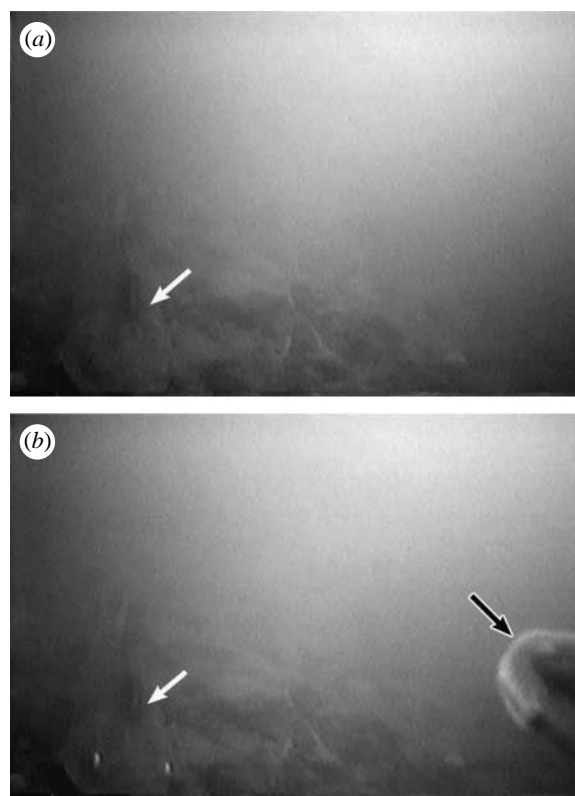


Figure 1. Eye roll behaviour of the razorback sucker. (a) A male sucker (white arrow) rests camouflaged on the river bed at 1 m depth. (b) Two flash signals (visible as white marks at the bottom left of the panel) produced by simultaneous eye rolls deter a roving male (black arrow) from approaching.

42°C for 15 min. Cycling parameters for the subsequent PCR were: 95°C × 5 min, 32 cycles of 95°C × 30 s, 55°C × 30 s and 72°C × 1 min, and 1 cycle of 72°C × 10 min. HUV cDNA was purified and cloned into TOPO TA cloning vector pCRII (Invitrogen) and sequenced by AmpliTaq Dye terminator cycle sequencing (UBC Sequencing Laboratory). The identity of the sequence was confirmed by comparing it to the nucleotide sequence databases using the BLASTN program. To make the cRNA probe, a PCR fragment containing the HUV insert and an RNA promoter amplified from the pCRII vector was used to generate digoxigenin (DIG)-labelled RNA sense and antisense riboprobes by *in vitro* transcription (DIG RNA labeling kit; Roche Diagnostics).

Retinas ($n=3$) used for this analysis were fixed in 4% paraformaldehyde in 0.06 M PBS overnight at 4°C, washed 3 × 1 h in 0.06 M PBS, cryoprotected in sucrose solution (30% sucrose, 0.1 M phosphate buffer in OCT medium) overnight at 4°C and cryo-embedded in 100% OCT medium (Cedar Lane Laboratories). Blocks were cut in 7–10 µm steps and the resulting cryosections were used for *in situ* hybridization with the riboprobe as per published studies (Cheng *et al.* 2006). Briefly, the procedure involved rehydrating the sections, permeabilization in 10 µg ml⁻¹ proteinase K (Sigma) for 7 min, followed by exposure to 0.1 M triethanolamine containing 0.25% acetic anhydride, dehydration and hybridization overnight at 50°C with 1–5 µg cRNA probe in hybridization solution containing 50% formamide, dextran sulphate and goat serum. Sections were then treated with RNase A (Sigma) and incubated with anti-DIG Fab fragments conjugated to alkaline phosphatase (1 : 2000; Roche Diagnostics) overnight at room temperature. The DIG-labelled probes

were visualized using 5-bromo-4-chloro-3-indolyl phosphate with 4-nitroblue tetrazolium chloride (NBT/BCIP; Roche Diagnostics). Sense probes were used as negative controls and did not hybridize in any of the retinas. Atlantic halibut retina was processed in parallel as a positive control. Digital images of sections were acquired with an E-600 Nikon microscope equipped with a DXM-100 digital camera and DIC optics.

3. RESULTS

Underwater video sequences acquired during the spawning season showed a peculiar behaviour exhibited by male razorback suckers. These fishes were barely visible on the river bottom (figure 1a), their presence only revealed by two sudden flashes of light (figure 1b) arising from quick (approx. 0.5 s) eye rolls that exposed the back of each eye to the downwelling light. This behaviour was observed when roving males came to an area occupied by other males resting on the bottom.

The flash signal produced by the eye roll was 4.1 times more intense than that reflected from the head skin next to the eye, and it was 4.4 times more intense than the light reflected from the river bottom (figure 2a). Furthermore, the reflectance contrast between the eye roll signal and that of the head skin or that from the upwelling river light showed maxima in the UV wavelengths (less than 400 nm; figure 2b). Flash signal contrast with the light reflected from the river bottom was also very high (above 80%) in the long (red) wavelengths (greater than 700 nm; figure 2b) because, as per the UV region of the spectrum (wavelengths less than 370 nm), there was no upwelling river light beyond approximately 720 nm (figure 2a).

As a consequence of the upwelling river light spectrum, quantum catch of this background light was highest for the green cone. When cone quantum catches were normalized by the latter, the ratios obtained were: 0.043 (UV), 0.24 (blue), 1 (green) and 0.88 (red). The ratios of flash signal quantum catch to that of upwelling river light for the various cone types were: 14 (UV), 6.5 (blue), 2.7 (green) and 3.67 (red). The UV cone had therefore the highest signal quantum catch over background adaptation.

When fish behaviour was recorded in the experimental tank, males swam closer to the non-flashing model (average distance ± s.d.: 114 ± 7.72 cm) than when no model flashed (136 ± 11.8 cm). The GLM statistic $\beta = -32.77$ ($\alpha < 0.001$) indicated that the fish were on average 32.77 cm further away from a model when it flashed versus when it was silent. In contrast, female distance from the models was not influenced by diode state (average distance ± s.d.: 118 ± 9.82 and 121 ± 9.67 cm for non-flashing and flashing intervals, respectively; $\beta = -0.089$, $\alpha = 0.98$).

The retina of the razorback sucker had large rods and single cones of different sizes (figure 3). This retina was structurally similar to that described for other suckers, where two unusual attributes (for a fish) have been documented: these are the presence of pseudo-double cones and the lack of cone mosaic structure at the ellipsoid level (Ali & Anctil 1976; Novales Flamarique & Hárosi 1997; Novales Flamarique & Hawryshyn 1998). Pseudo-double cones are absent in fresh retinal preparations demonstrating that there is no permanent association between double cone members (Novales Flamarique & Hárosi 1997).

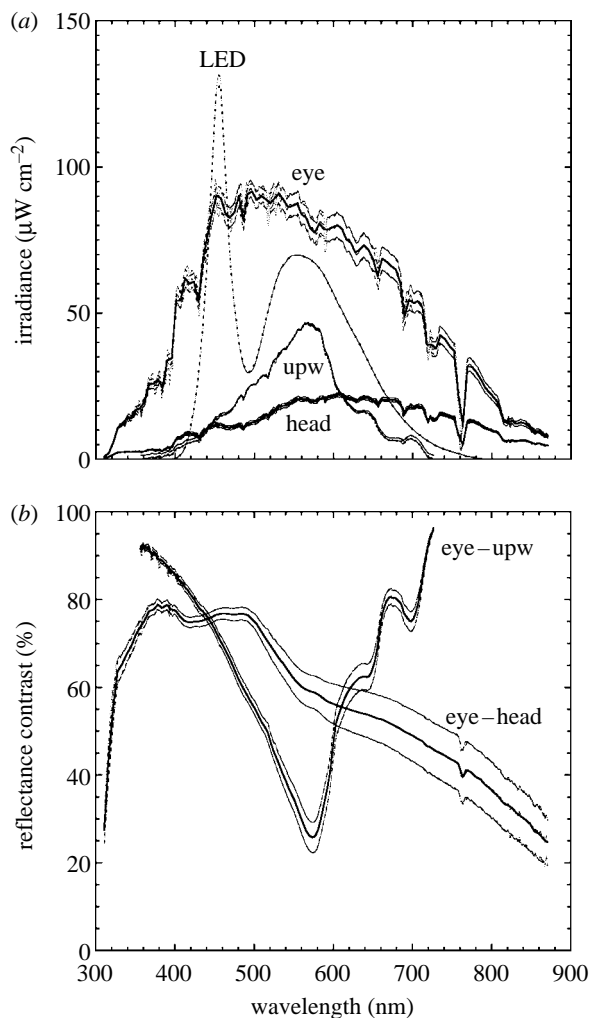


Figure 2. Intensity and reflectance contrast of the eye roll signal. (a) Intensity of the flash signal produced by the eye roll (eye), the skin on the head adjacent to the eye (head), the upwelling light from the bottom of the river (upw) at the start and at the end of fish reflectance measurements (1–3 m depth, both curves overlap) and the LED used in the models. Also shown are the 95% confidence intervals associated with the eye roll and head skin signals. (b) Reflectance contrast and associated 95% confidence limits when comparing the eye signal to that from the head skin (eye–head) and that from the eye signal with respect to the upwelling river light (eye–upw).

When *in situ* hybridization experiments were carried out on cryosections, the UV riboprobe labelled single cones in the dorsal but not in the ventral retina (figure 3). The distribution of UV cones was therefore restricted to the upper half of the retina of the adult razorback sucker.

4. DISCUSSION

The eye roll behaviour is a novel and effective means of communication between conspecifics. Our results suggest that this signalling system is used by male suckers to advertise their presence to other roving males. Underwater observations support this conclusion in that only males rest on the bottom, at established distances from each other, while female presence draws multiple males into the water column to form a spawning group (Mueller 1989). There is no apparent female choice based on signal quality (all signals are similar) as females are observed to roam

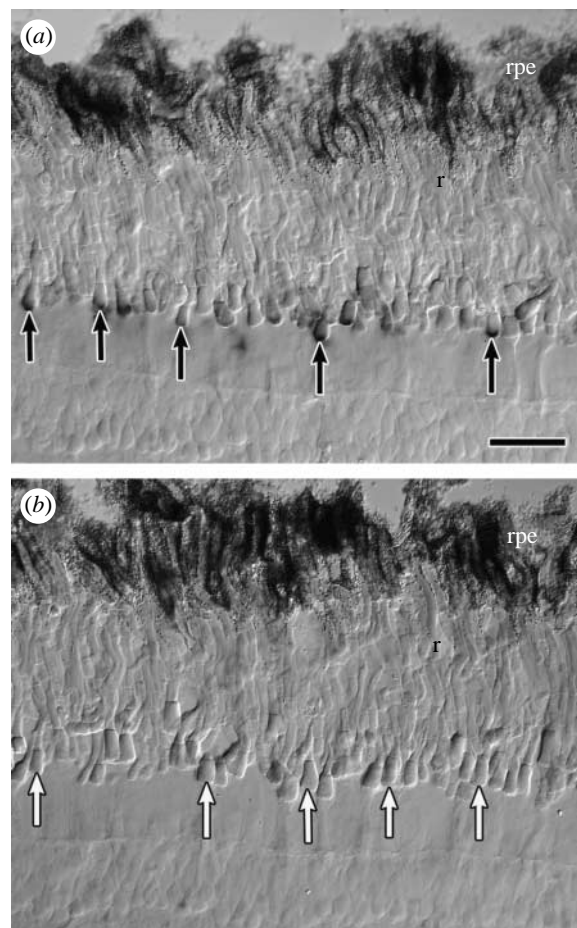


Figure 3. Photomicrographs of radial cryosections from the retina of the razorback sucker. (a) Dorsal retina showing single cones labelled with the UV riboprobe (black arrows). (b) Ventral retina showing lack of cone labelling with the UV riboprobe (white arrows). Abbreviations: rpe, retinal pigment epithelium; r, rod. Scale bar (in (a)), 40 μm , holds for both panels.

into male-established areas when no eye rolls are taking place. In addition, the eye roll signal is visible primarily from above (only the top of the eye rolls out and the fish is often surrounded by rocks) and would only be efficient in short-range communication. This would make the signal a poor female attractant, as suggested by our experiments. The eye roll flash may also serve to minimize predator detection while maintaining territorial communication since the signal is short lived, but intense, and can be induced at will. Many fishes have oculomotor movements that expose some part of the eye ball to light (Tamura & Wisby 1963). It is therefore likely that the eye roll behaviour is also used by other species to signal territoriality or for alternative purposes. Similar eye roll flashes have been observed in the turtle, *Trachemys scripta*, where they may play a role in courtship behaviour (Lovich *et al.* 1990).

The high reflectance contrast of the eye signal in the UV (local maximum at 380 nm with respect to the neighbouring head skin; figure 2b) makes it optimal for detection by the UV cones. For a sucker swimming in the water column, the background upwelling light and that reflected from the head skin of a fish settled on the river bottom would preferentially adapt its blue, green and red cone mechanisms and chromatically isolate the action of the

UV cone mechanism (Novales Flamarique & Hawryshyn 1998) as indicated by our quantum catch results. In addition, the sensitivity of the UV cone mechanism in suckers peaks at 380 nm (Novales Flamarique & Hawryshyn 1998) and the UV cones themselves are restricted to the dorsal retina (figure 3), both optimal characteristics to detect the flash signal. The experimental field conditions did not permit us to ascertain the contribution of UV cones to the detection of the flash. This would have required altering the background illumination, which was a physical impossibility. Other cone types, whose combined absorbances (Novales Flamarique & Hárosi 1997) expand the spectral range of the eye roll signal, would also contribute to its detection. The LED emission (figure 2a) did not comprise wavelengths below 400 nm, suggesting that the contribution of the UV cones to the detection of the experimental flash may not have been critical (though UV cone absorbance levels off at approximately 460 nm and stimulation would have occurred throughout the short wavelength region of the peak LED emission). Our calculations suggest that the chromatic adaptation conditions present in the spawning habitat would make the UV cone the primary sensor in detection of the eye roll signal.

It is perhaps no coincidence that the spawning season of the razorback sucker precedes spring runoff, an event that dramatically increases the sediment content and, thus, the turbidity of the water (Mueller 1989; Mueller *et al.* 2000). Ultraviolet wavelengths are highly scattered by dissolved particulates (Novales Flamarique & Hawryshyn 1997) and such an event would diminish signal transmission and the efficiency of the communication system. During the non-spawning season, the razorback sucker is commonly found away from the surface, at depths greater than 20 m (Mueller *et al.* 2000), where UV light does not penetrate in sufficient quantity for visual purposes (Novales Flamarique *et al.* 1992; Novales Flamarique & Hawryshyn 1993). Thus, the preservation of UV cones in this bottom dwelling species appears to fulfil a single ecological role in territorial communication during the spawning season, the only time when suckers are routinely found near the water surface.

The sucker signalling system provides the first ecological evidence for restricted UV cone distributions in the retina of vertebrates. Other fishes, including juvenile salmonids and some cyprinids (e.g. the goldfish), have UV cone distributions that are also restricted to the dorsal retina (Cheng *et al.* 2006). In fishes, this distribution suggests that the primary ecological function of the UV cone is to detect upwelling signals. Such capacity may be primarily related to the detection of aquatic predators, which usually attack from the side or from below, and whose skin (e.g. as per the scales of fishes; Rowe & Denton 1997) would reflect UV wavelengths to produce a high-contrast signal with respect to the longer wavelength background.

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