Video Article

Assays to Detect UV-reflecting Structures and Determine their Importance in Mate Preference using the Sailfin Molly *Poecilia latipinna*

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Abstract

Many organisms use cues and signals beyond human sensitivity during social interactions. It is important to take into account how organisms perceive their worlds when trying to understand their behavior and ecology. Sensitivity to the ultraviolet spectrum (UV; 300 - 400 nm) is found across multiple genera of birds, fish, reptiles, amphibians, and even mammals. This protocol describes a technique for examining organisms for the presence of UV-reflecting structures and a method for testing whether these cues are used as social signals in the context of mate choice. A spectrophotometer is used to detect the presence of UV reflectance and variation in reflective intensity between individuals and sexes. An example of this technique is presented in which a dichotomous mate choice test exposes sexually receptive individuals to opposite sex individuals whose visual appearance can be manipulated by filters that either transmit full spectrum or block UV wavelengths. This system allowed for the determination that female, but not male, sailfin mollies *(Poecilia latipinna)* were using UV markings as part of their mating decisions. These types of studies serve to expand our knowledge of the range of organisms that utilize UV and provide insight into how UV plays a role in their lives.

Video Link

The video component of this article can be found at http://www.jove.com/video/54453/

Introduction

Understanding the cues and signals used in animal social interactions allows us to comprehend the phenotypic variation both within and among species. This variation plays an important role in evolutionary processes such as population divergence, sexual selection, and speciation. Often, however, researchers are limited to exploring the cues most obvious to human sensory systems, especially those within the visual or auditory realms. Use of spectrophotometry, however, allows us to expand our investigations beyond the human visible spectrum and into wavelengths that may be important in social interactions in other species.

In particular, the short-range communication afforded by ultraviolet (UV; 300 - 400 nm) sensitivity has the potential to be highly advantageous during mate choice[1]. Many visually-hunting predators of birds and fishes, for example, are unable to detect UV radiation. In systems where males display elaborately to females, these males would reduce their risk of predation while maintaining their ability to attract mates by exploiting the UV spectrum rather than developing cues detectable in the visible spectrum[2,3].. If one fails to consider the possibility that organisms are communicating with each other using these "private communication channels", significant drivers of behavior and evolution may be missed.

This protocol outlines an investigation into the use of UV cues for mate choice in the sailfin molly, *Poecilia latipinna*, a polygamous fish that has no previously known ability to detect UV or utilize UV markings. This fish species has a close phylogenetic proximity to other UV-sensitive live-bearing fishes[4] and there is microspectroscopic evidence that *P. latipinna*, along with other molly species such as *P. mexicana* and *P. formosa*, possess a class of cones (photoreceptor cells responsible for color vision) that are most sensitive for UV wavelengths[5]. In this sexually dimorphic species, female choice has played a strong role in the evolution of the males' brightly colored and enlarged fins[6-9]. This methodology allows us to explore whether UV is an additional medium by which females assess male quality.

The detection and measurement of UV markings on *P. latipinna* using a spectrophotometer with a fiber optic probe is detailed here. Further, whether receptive female mollies differentially associate with males viewed through an optical filter transmitting full-spectrum light including UV-A ([UV+]; 320 - 700 nm) and males viewed through an UV-blocking filter ([UV-]; 400 - 700 nm) is discussed. This method has broad applications for discovering UV sensitivity and color patterns in fishes and other organisms, allowing research into a variety of questions involving UV and its role in behavior.

Protocol

All experiments were conducted with the approval of Ohio Wesleyan University's Institutional Animal Care and Use Committee.

1. Recording UV Reflectance of Fish using a Spectrophotometer

1. Calibrate a spectrophotometer and light source with a known white standard over the range of wavelengths to be measured according the recommendations of the instrument or software.
2. Anesthetize fish by placing in a 0.5% solution of ethyl 3-aminobenzoate methane sulfonic acid salt (MS-222) buffered with an equal quantity of sodium bicarbonate until fish becomes unresponsive. Once unresponsive, immediately place the fish on a black, non-reflective background. Note: MS-222 is potentially toxic, and gloves should be worn at all times.
3. Take all measurements in a darkened room to minimize excess light beyond that of the spectrometer light source.
4. Measure reflectance using a fiber optic probe held against the body of the organism. Make sure to form a consistent 45° angle between the fiber optics and the body, as is built into some probe designs. Note: For measurement consistency, it may be desirable to average across multiple scans (2 - 5) per body region.
5. Record reflectance data with appropriate reflectance analysis software. Note: The software used here was set to: integration time = 2,000 sec, average scans = 2, and pixel smoothing = 5. Data was copied and pasted to a spreadsheet.
6. Repeat on multiple body areas to determine presence or absence of UV reflectance and range of phenotypic variation across organisms. Note: Preliminary scans may be necessary to determine which, if any, body areas consistently show UV markings.
7. Return the fish to a well-oxygenated holding aquarium until fully recovered, as evidenced by normal operculum beats and swimming behaviors. Note: A commercial protectant to restore the mucous coating to the body may be added to aid in skin recovery.
8. Repeat across multiple individuals, including both sexes if desired.
9. On spreadsheet where all data have been copied, plot data using a scatterplot, with reflectance on the Y-axis and wavelength on the X-axis. Amplitude peaks in the UV range (300 - 400 nm) indicate the presence of UV reflectance on the fish.

2. Dichotomous Mating Trials

NOTE: Perform control observations, in which focal fish, UV filters, and opaque filters are in place. In these trials, when the opaque filters are removed, focal fish will be exposed to parts of the tank displaying the presence and absence of UV light, but lacking potential mating partners. For details on the validity of dichotomous choice tests in this experiment see Palmer and Hankison (2015)[10], although note that these tests also have drawbacks[11].

1. Use sexually receptive individuals as focal organisms. Use female *P. latipinna* < 48 hr postpartum[5] and males isolated for at least 24 hr prior to testing to improve the likelihood of sexual motivation.
2. Place a single focal individual into a neutral area adjacent to two choice compartments. For *P. latipinna,* use a 75.7 L test aquarium (30.5 cm x 30.5 cm x 75 cm; **Figure 1**) divided into three sections by transparent Plexiglas partitions, forming a central area for the focal individual flanked by two equal-sized choice compartments. Ensure that no water or olfactory cues are shared between individual tank sections. Repeat for each individual to be observed.

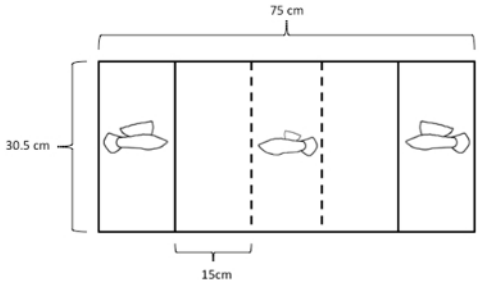

Figure 1. Experimental Aquarium Setup. A rectangular aquarium divided into three sections: a central area that held the focal individual and two end compartments that held object pairs (the figure shows a female in the middle with object males at the ends). Interchangeable filters that either blocked or permitted UV reflectance could be placed over the glass partitions dividing the male and female sections of the aquarium. This figure is modified from Palmer and Hankison (2015) [10]. Please click here to view a larger version of this figure.

3. Under full-spectrum lighting conditions (lighting whose spectrum extends into the UV-A range), isolate one potential mating partner in each end compartment (choice compartment) of the test aquarium. Ensure that potential mating partners resemble one another as closely as possible in size, coloration and display characteristics, so that the main difference between the two isolated mating partners is whether they appear behind a filter that blocks or transmits UV light.
   NOTE: It may be preferable to record reflectance in the visible spectrum to enable the best possible color matching, although here matching was done by eye.
4. Acclimate individuals under visual isolation (opaque filters) for 15 min. Ensure that UV + and - filters are in place at this time. Following acclimation, remove the opaque filters. To ensure that side biases are not present (inherent preference for one side of the tank regardless of object), randomly assign the UV^+ and UV^- sides of the tank for each each trial.

5. Record the amount of time that the focal individual spends within specific preference zones (areas near the choice compartments, in this case approximately two standard body lengths) by using stopwatches. For female fish, this is the amount of time it spends near each of two males, and vise versa for male focal fish.
   NOTE: Event recorder software can also be used for recording. Fifteen minutes trials were used in this experiment and fish that did not visit each preference zone at least once were excluded from the results as being unresponsive.
6. Record and analyze the time that fish spend near empty UV^+ and UV^- compartments to ensure that there is not an inherent bias towards a particular lighting environment that may influence the mating choice of the fish. Use paired t-tests to determine if individuals prefer a particular UV condition.

Representative Results

Figures 1 and **2** show the mating preference aquarium set up and UV measurement sites for our experiments.

Measuring UV reflectance allowed for the determination that *P. latipinna* do possess UV characteristics, especially along the sides of their bodies (**Figure 3**), in addition to individual variation in these traits. Once UV traits were found, testing revealed that female, but not male, *P. latipinna* use these characteristics in their mating decisions (Female: $t_{15} = 4.08$, $p = 0.001$; Male: $t_{14} = 0.67$, $p = 0.517$; **Figure 4**). That both sexes possess UV traits, but that the traits are used only by females as part of mate preference may indicate a role of these traits in other social interactions, such as shoaling or foraging, in *P. latipinna*.

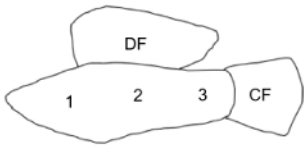

Figure 2. Locations on the *Poecilia latipinna* that were Measured for UV Reflectance. Regions were initially determined from preliminary reflectance trials with reflectance samples taken from across nearly all body regions. 1 - 3, DF (dorsal fin) and CF (caudal fin) refer to body regions shown in **Figure 3**. This figure is modified from Palmer and Hankison (2015)[10]. Please click here to view a larger version of this figure.

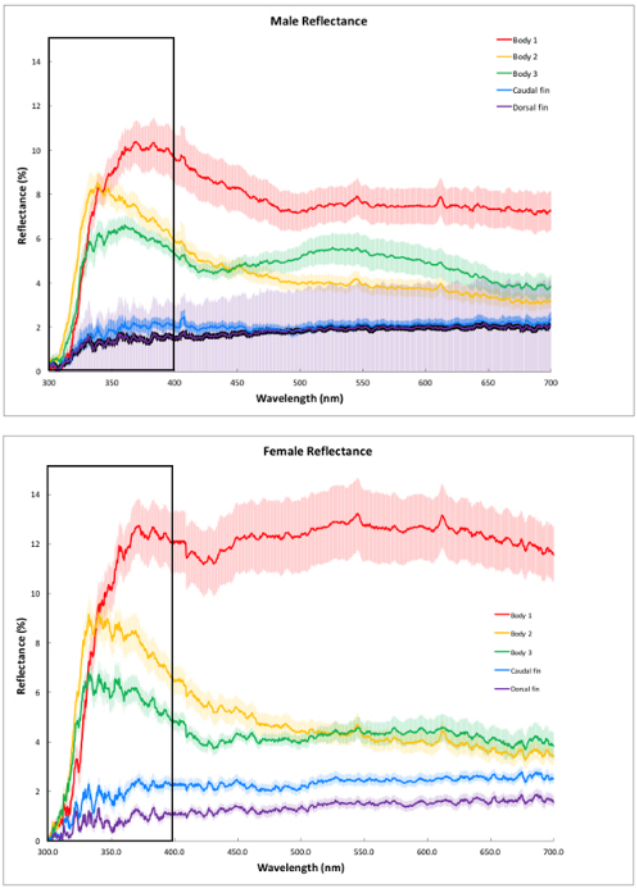

Figure 3. Mean Spectra of Male and Female *Poecilia latipinna*. The graphs show the mean spectral reflectance measured at five locations (side areas, caudal fin, dorsal fin) SE. The areas inside the boxes represent reflectance in the UV spectrum. This figure is modified from Palmer and Hankison (2015)[10]. Please click here to view a larger version of this figure.

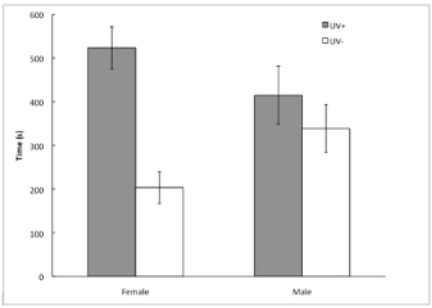

Figure 4. Mean Associate Times of Males and Females. The graphs show the mean time SE that females and males spent near male compartments viewed through UV+ and UV- filters during the control and experimental tests. This figure is modified from Palmer and Hankison (2015)[10]. Please click here to view a larger version of this figure.

Discussion

Spectrophotometry was successful in identifying UV markings on *P. latipinna*. Both sexes of *P. latipinna* possess UV markings along their sides. In addition, some males had UV markings on their dorsal fins, traits previously found to be important in female mating preferences[7].

We recommend using UV spectrophotometry as a mechanism to detect the presence of UV markings. Further testing could determine its role in social interactions, including mating preference (as detailed here). Alternatively, UV characteristics might influence shoaling, individual, sex, or species recognition, or intrasexual interactions[3, 12-15].

Despite the usefulness of spectrophotometry in better understanding the UV characteristics of organisms, especially related to their social behavior, there are some critical steps necessary to fully understand the behaviors, and some limitations to what can be concluded. All experiments should test whether focal individuals have preference for UV^+ or UV^- environments by including controls with no test individuals in the flanking choice tanks, especially as blocking UV may cause changes in the "brightness" or luminance the organisms observe. While

luminance has not been shown to influence female preference in species closely related to *P. latipinna*[16-18] additional data detailing the role of brightness and preference tests was addressed in a previous study[19] and could be an important step in ensuring that preference is not based on brightness changes. Alternatively, differences in the lighting flux (overall brightness per unit of time) can be equalized, allowing for even more control of lighting conditions in systems where this is of concern[2,20].

In addition, for mate choice tests, choice individuals should be matched as closely as possible in size and coloration (aka attractiveness) so that choice more strongly reflects preference for UV^+ or UV^-. Alternative techniques that allow a single individual to be viewed under both UV^+ and UV^- may be useful for some organisms[21]. These studies may allow for control of any differences in individual behavior (as the same individual is viewed under UV^+ and UV^- conditions simultaneously). While we did not see behavioral differences among our fish in our experimental design, this aspect may be important for other organisms or experimental designs. In addition, studies that retest individual females while switching the sides of UV filters[2], or that switch filters mid-way through an individual test would also be useful, both for controlling for individual side bias. Finally, while spectrophotometry and mating preference trials can reveal these aspects of an organism's phenotype, the protocol here does not have the ability to determine fully the meaning of the UV traits and what they may be revealing to focal individuals. Fitness trials to understand how UV traits are impacted by development, inheritance, environmental conditions (such as predictability of food or predators), or other factors would be useful in further understanding the meaning and role of UV characteristics.

Disclosures

The authors have nothing to disclose.

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