

Biofluorescence in Polymorphic Tiger Salamanders (*Ambystoma mavortium nebulosum*)

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Abstract

Animal communication is vital to nearly every species. Serving multiple functions and being conveyed throughout a variety of modes, communication allows animals to make decisions based on information other animals provide. Numerous examples of this can be found across the animal kingdom, with biofluorescence being a newly investigated form of visual communication. Biofluorescence is the absorption and remission of light by a living organism that generates a fluorescent hue of various colors. Heavily studied in marine species, little is known about the mechanism and function of biofluorescence in tetrapods. Recently, biofluorescent sexual dimorphism was discovered in a species of salamander which prompts the question if their fluorescent response could be used as a form of reproductive visual communication. I explored this topic through the study of biofluorescence in *Ambystoma mavortium nebulosum*, a polymorphic salamander subspecies that is capable of successfully interbreeding between morphs. To accomplish this, I photographed both sexes of metamorphic and paedomorphic salamanders under blue-excitation light to visualize fluorescent patterns and compared their torso similarities using pattern recognition software (I3SPattern+). After quantifying pattern similarities, biofluorescent morph differences were found on the dorsal and ventral side of the animal's torso with no differences detected across the sexes. As far as I know, this is the first study investigating biofluorescence in a polymorphic species. While the ecological function of biofluorescence in salamanders remains unknown, understanding the expression of biofluorescence in this subspecies has increased our knowledge of this phenomenon and provided us with more insight into salamander reproductive communication.

Introduction

Animal communication allows individuals to make decisions based on the behavior, physiology, or morphology of others [1]. Amongst a single species, animals can communicate through a variety of modes targeting each other's senses by using olfactory, audible, tactile, and/or visual cues. Biofluorescence has been suggested to aid organisms in communicating with other conspecifics, camouflaging, or perhaps providing no function at all. Often occurring in red, blue, and green hues, the fluorescence's glow is the remission of longer wavelengths of light by a living organism after absorbing light from an abiotic or biotic source [2,3]. Various mechanisms have been proposed to produce biofluorescence involving proteins, pigments, metabolites, and/or mineralization, however, the vast majority of animals demonstrating biofluorescence use unknown mechanisms [4]. Commonly studied in marine animals, this natural phenomenon has begun to be explored in tetrapods, including mammals, reptiles, and birds [2,5]. Most recently, biofluorescence was discovered across numerous families of amphibians including eight families

of salamanders. Living in both freshwater and terrestrial ecosystems and utilizing both chemosensory and visual cues from their environment, salamanders act as a unique study organism to explore the function of biofluorescence [4].

While the Eastern Red-backed Salamander (*Plethodon cinereus*) was the first documented salamander species to demonstrate fluorescence in 2018, the mechanism and function of biofluorescence in any salamander species have yet to be revealed [6]. Upon discovering biofluorescence in twenty-one salamander species (i.e. all of the salamander species included in their study), Lamb and Davis proposed that the fluorescent response is related to pigments in the cutaneous chromatophores or reflective structures containing guanine; however, the authors did not identify a specific mechanism that resulted in the phenomenon. Although Lamb and Davis did not directly address the potential functions of the response, all of the salamander species in the study fluoresced in green wavelengths [4]. Given that salamanders' eyes contain blue-sensitive cones and green rods that were initially thought to assist in color differentiation in low-light environments and maximize their sensitivity to blue light, it is possible that salamanders could also use these photoreceptors to perceive green wavelength fluorescence from other conspecifics [7]. Further, terrestrial salamanders were found to lose blue-sensitive cones and increase their density of green rods after metamorphosis. Given that green rods have a higher light sensitivity than blue cones, this suggests that aquatic and terrestrial salamanders perceive fluorescence wavelengths differently [7]. Thus, biofluorescence could be a potential source of visual communication for salamanders.

One possible driver of biofluorescence is sexual selection. Biofluorescent sexual dimorphism, or rather the fluorescence of sexually dimorphic traits, is an understudied topic with direct implications on reproductive visual communication. Although some studies have shown varying intensities and wavelengths of fluorescence between the sexes, only two cases of distinctly different patterns of fluorescence between the sexes have been documented to my knowledge [2]. The initial discovery was made in a genus of chameleons (*Calluma*) while the most recent documentation of the phenomenon was seen in the Southern Gray-Cheeked Salamander (*Plethodon metcalfi*) with males demonstrating a green speckled pattern down their ventral trunk that the majority of females lacked [2,5]. Taken *P. metcalfi* is a fully terrestrial species and the species' fluorescent pattern was found on its ventral side, the authors of the study do not believe that this pattern is related to visual communication. However, a particular *Plethodon* courtship behavior involving males raising and lowering their limbs could give females the ability to visualize males' fluorescent patterns [2]. Knowing this, further exploration of additional salamander species' potential to display biofluorescent sexual dimorphism is warranted.

Four salamander species within the *Ambystoma* genus have been found to demonstrate biofluorescence with larger intensities than other salamander genera; however, the response has yet to be observed in polymorphic species, such as the Arizona Tiger Salamander (*Ambystoma mavortium nebulosum*) [4]. Uniquely, *A. m. nebulosum* commonly demonstrates the ability to undergo facultative paedomorphosis, a polyphenism in which salamanders can metamorphose

into a terrestrial form (i.e. metamorph) or retain their gills and live out their lives in an aquatic environment (i.e. paedomorph) [8,9]. Further, courtship experiments have found that metamorphs and paedomorphs are capable of successfully interbreeding, suggesting that salamanders may distinguish between the two morphs during courtship [10]. This being said, if the function of biofluorescence in salamanders is ultimately for reproductive visual communication, then this polymorphic species will demonstrate biofluorescent sexual dimorphism. Further, differences in fluorescent patterns between morphs could signify an individual's ability to distinguish between morphs during courtship.

The objective of this project was to explore the possibility of biofluorescence in *Ambystoma mavortium nebulosum*. After observing a fluorescent response, I also investigated two other questions: (1) do *A. m. nebulosum* demonstrate biofluorescent sexual dimorphism; and (2) do *A. m. nebulosum* demonstrate different fluorescent patterns across morphs? To my knowledge, this is the first study investigating biofluorescence in a polymorphic species with the potential to provide evidence for the ecological function of biofluorescence in salamanders.

Methods

Since the 1950s, the Mexican Cut Nature Preserve has consistently used various observational and experimental methods to study *Ambystoma mavortium nebulosum* across more than 25 subalpine ponds [11]. Beginning in 1990, the Mexican Cut has generated a long-term database through a mark and recapture study that has allowed researchers to monitor population dynamics and individual measures of life history such as age, morph, body size, and survival [11]. Knowing this, the Mexican Cut acts as an ideal study site for exploring biofluorescence in this subspecies by being able to apply long-term historical data on individuals to present fluorescent observations. Salamanders were captured from ponds using long-handled nets before being processed in a nearby Weatherport. Processing included checking for a passive integrated transponder (PIT) tag or for a unique toe clip to identify the individual, measuring weight, snout-vent length (SVL), and total length, and assessing body condition, morph, sex, and cloaca condition. If a new individual was captured, a new PIT tag will be inserted and the same measures and assessments were recorded.

Following processing, individuals had their fluorescent response photographed under blue light (Nightsea Xite Flashlight). Although ultraviolet light (360-380 nm) is often used in tetrapod biofluorescence surveys, it reduces rapidly with increases in depth in freshwater and marine environments. Alternatively, blue light (440-460 nm) can penetrate deeper depths making it commonly used in marine animal fluorescence surveys, and has been found to elicit more intense fluorescence in salamanders [4,12]. Thus, each individual was placed in a black-backdropped container within a dark room. An iPhone 13 was used to capture dorsal side pictures under visible light (Energizer - LED Headlamp) before the fluorescent response was photographed through a 500 nm long-pass lens. The individual was then placed in a glass-bottomed tank within the same dark room before the photography process was repeated to capture the ventral side of the animal. Throughout the photography process, the camera and light

sources were held 16 cm away from the individual. After being photographed, the salamander was released back into its pond of origin. Throughout the study, twenty salamanders of each morph and sex were photographed.

Upon completion of photographing all of the individuals in this study, a visual comparison of fluorescent patterns and quantification of fluorescent pattern similarities was conducted. While qualitative in nature, the visual comparison allowed the determination of any obvious fluorescent responses between the sexes and morphs. To quantify similarities between morphs' and sexes' dorsal and ventral torso patterns, Interactive Individual Identification System Pattern Plus (I3SPattern+) was used to create a database of all of the individuals photographed in the study. Traditionally used in mark and recapture studies as a method of photographic identification, I3SPattern+ has the ability to determine key points in an individual's pattern and compare those points to other individuals and generate similarity scores, with lower scores corresponding to a greater amount of similarity between the two photos [15]. For its use in this study, similarity scores were generated between metamorphs, paedomorphs, metamorphic males and females, and paedomorphic males and females while using the left and right base of the head and base of the tail as reference points for the boundaries of the individual's torso. Given the fact that the I3SPattern+ similarity scores were consistently heavily skewed, the median score of each individual's similarity to a particular morph or sex was taken. For statistical analysis, Welch's t-tests were used to evaluate the difference between morphs dorsal and ventral sides while ANOVAs were utilized to determine differences between the sexes across the same morph for each side of the animal.

Results

Morph Differences

Of the 80 adult individuals photographed for biofluorescence (135 total adults and larvae in a companion study), all of the individuals exhibited a biofluorescent response under blue excitation light providing conclusive evidence that the *Ambystoma mavortium nebulosum* subspecies fluoresces. Individual variation in biofluorescent patterns was detected across all of the individuals on both sides of the body. As seen in Figure 1, metamorphs often demonstrated speckling and spotting on the dorsal side while paedomorphs possessed a more marbled pattern.

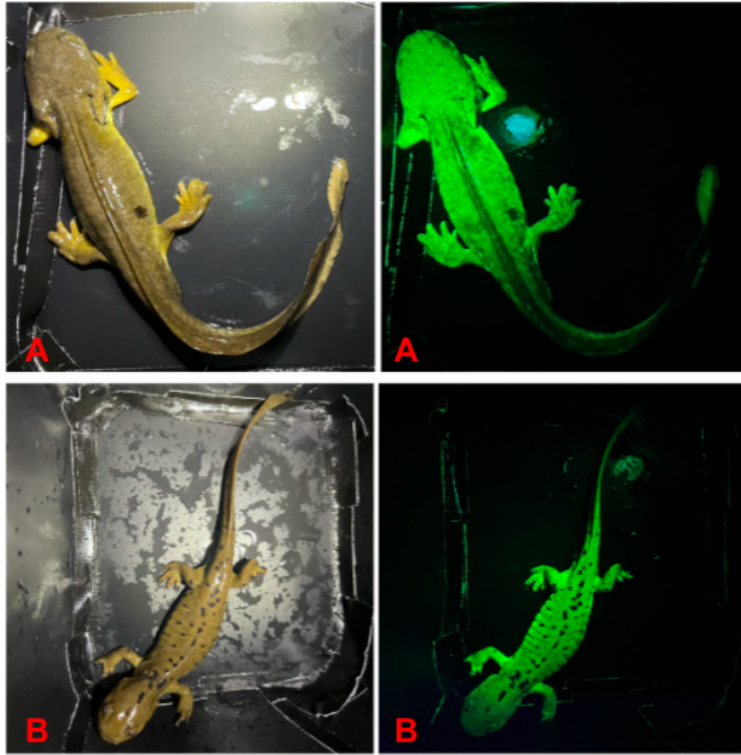


Figure 1. Dorsal View of Paedomorph and Metamorph. (A) Dorsal view of a paedomorph under visible light and blue excitation light through a long pass filter. (B) Dorsal view of a metamorph under visible light and blue excitation through a longpass filter.

On the ventral side (Figure 2), metamorphs would often have a variety of spots and lacked a commonality in pattern while the majority of paedomorphs demonstrated a defined stripe down the center of their torso. Additionally, metamorphs commonly had a fully fluorescing head while a paedomorph's head fluorescence was typically focused more closely around the jaw of the individual.

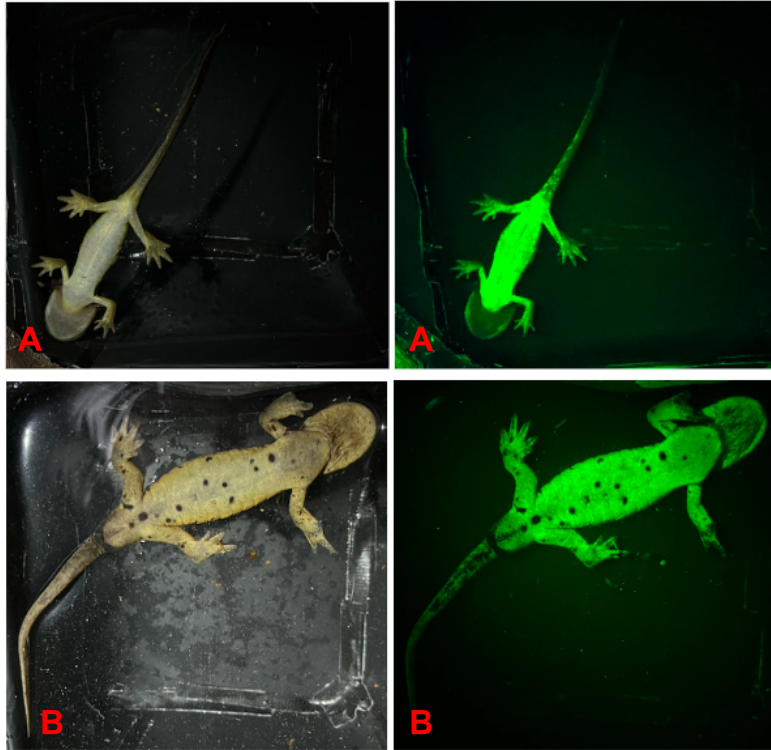


Figure 2. Ventral View of Paedomorph and Metamorph. (A) Ventral view of a paedomorph under visible light and blue excitation light through a long pass filter. (B) Ventral view of a metamorph under visible light and blue excitation through a longpass filter.

Quantification of torso pattern similarity scores supported the notion that biofluorescent differences existed across morphs. As seen in Figure 3, metamorphs were found to have a highly significant difference to paedomorphs on their dorsal side ($p < 0.001$) while no significant difference could be detected between the morphs on their ventral side, meaning that metamorphs displayed more similar torso patterns to each other on their dorsal side with no substantial pattern similarities on individuals' ventral sides.

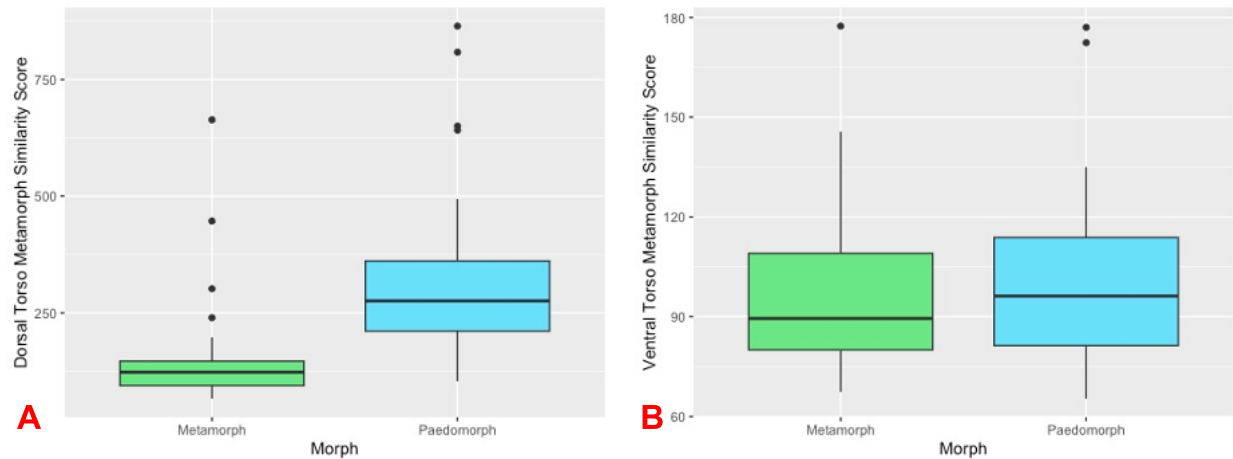


Figure 3. Dorsal and Ventral Metamorphic Similarity Score Comparison. (A) Metamorphs have a highly significant difference ($p < 0.001$) in biofluorescent pattern on their dorsal side in comparison to paedomorphs and have more similar patterns to one another than paedomorphs. (B) No difference can be detected between morphs on an individual's ventral side when comparing all individuals to metamorphs meaning that metamorphs do not possess more similar torso patterns to each other than paedomorphs.

In reference to paedomorphs (Figure 4), a significant difference in torso pattern can be found between morphs on the dorsal and ventral side of the animal. Thus, paedomorphs demonstrated more similar torso patterns to each other on the dorsal and ventral sides than metamorphs.

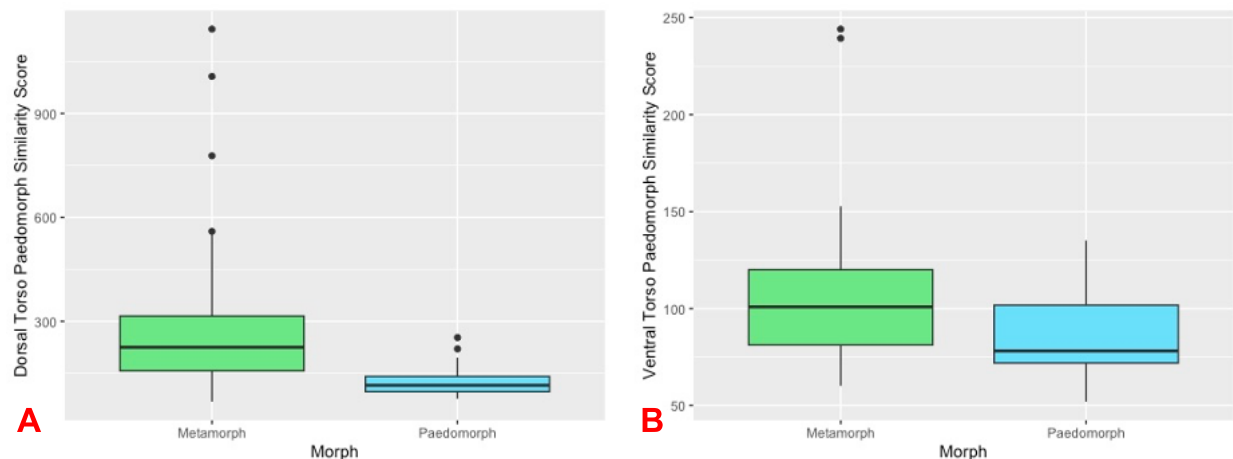


Figure 4. Dorsal and Ventral Paedomorphic Similarity Score Comparison. (A) Paedomorphs have a highly significant difference ($p < 0.001$) in biofluorescent pattern on their dorsal side in comparison to metamorphs and have more similar patterns to one another than metamorphs. (B) Paedomorphs have a significant difference ($p < 0.01$) in biofluorescent pattern on their ventral side in comparison to metamorphs and have more similar patterns to each other than metamorphs.

Sex Differences

Sex differences based on biofluorescence were less evident to the viewer upon visual inspection. While no distinguishable differences between paedomorphic males and females were

observed, metamorphic females often possessed a thin black line that ran through their cloacas and down the base of their tail that metamorphic males lacked (as seen in Figure 5).

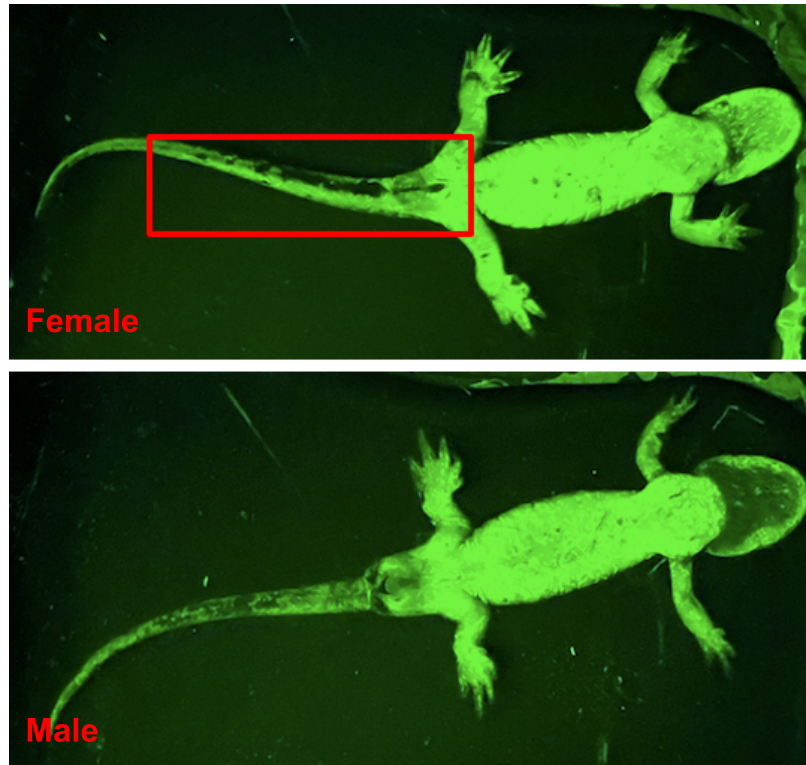


Figure 5. Metamorphic Female and Male Ventral Biofluorescent Comparison. Metamorphic females were commonly found to have black lines through their cloaca and down their tail that metamorphic males lacked.

Looking strictly at the individuals' torsos, no significant differences were found between metamorphic males' and females' dorsal or ventral patterns, meaning that biofluorescent sex differences were not detectable between metamorphs' torso patterns (Figure 6).

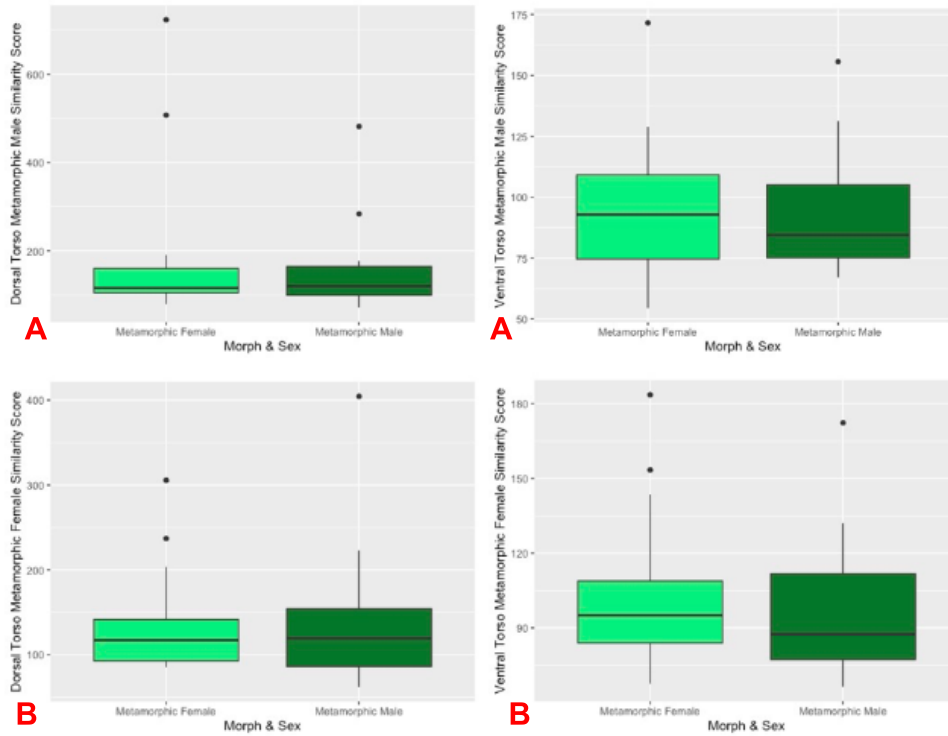


Figure 6. Dorsal and Ventral Metamorphic Sexes Comparison. (A) No significant difference was found between the sexes in reference to metamorphic males on individuals' dorsal and ventral sides. (B) No significant difference was found between the sexes in reference to metamorphic females on individuals' dorsal and ventral sides.

Additionally, no significant differences were found between paedomorphic males' and females' dorsal or ventral patterns (Figure 7). Thus, biofluorescent differences were not detectable between sexes' torso patterns across either morph.

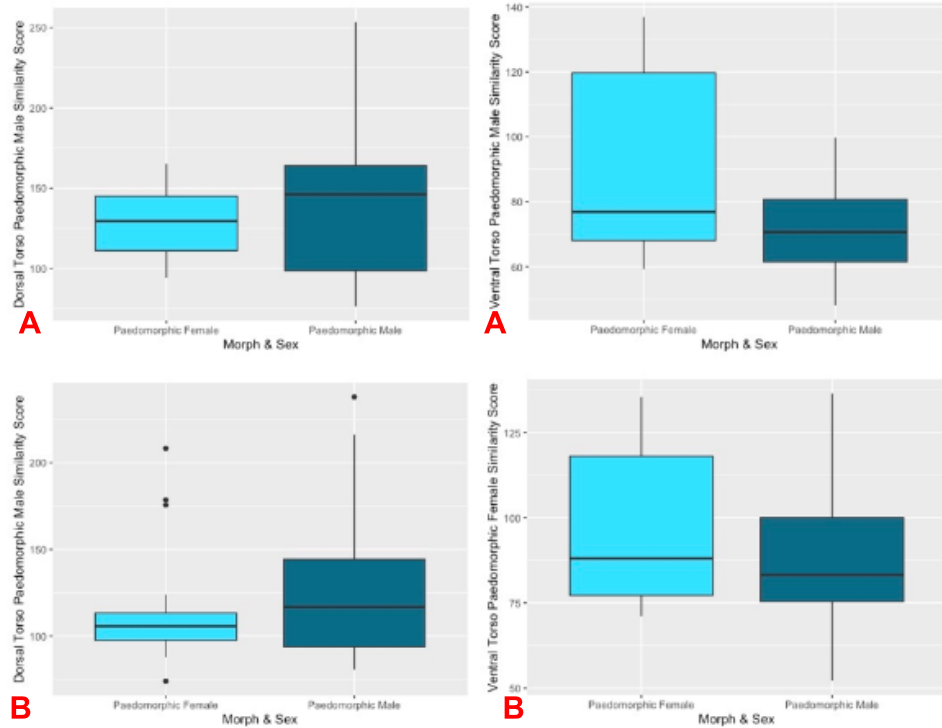


Figure 7. Dorsal and Ventral Paedomorphic Sexes Comparison. (A) No significant difference was found between the sexes in reference to pedomorphic males on individuals' dorsal and ventral sides. (B) No significant difference was found between the sexes in reference to pedomorphic females on individuals' dorsal and ventral sides.

Discussion

The discovery of biofluorescence in *Ambystoma mavortium nebulosum* has continued to expand our knowledge of salamander visual communication. Although no conclusive evidence for the use of biofluorescence in salamanders can be drawn from this study, the previously stated findings do offer support for numerous potential uses. Depending on the individual's life stage, the fluorescent glow could be used for reproductive communication, camouflage, female mimicry, serve a combination of other functions, or potentially be an evolutionary remnant that provides no function to individuals [16]. Given that biofluorescent pattern differences were detected across morphs, it could be suggested that adults have the capacity to differentiate between morphs during sexual selection. Considering that Arizona Tiger Salamanders' (*Ambystoma tigrinum nebulosum*) skin color has been found to correlate to substrate color and water transparency, it could also be suggested that biofluorescence hues in salamanders may also be correlated with these environmental factors [17]. If this is true, biofluorescence could be considered an enhancing pathway that amplifies preexistent visual cues for individuals that live in environments where visual communication strategies are more effective [16].

While biofluorescent torso pattern differences across morphs were able to be quantified on both the dorsal and ventral side of the animal, it should be noted that patterns on other parts of the body (i.e. the head, cloaca, and tail) were unable to be quantified during the time period of this study and could offer more evidence to additional biofluorescent pattern differences. With

this in mind, biofluorescent sex differences were not found when comparing torso patterns but may be detectable in other parts of the body. Moving forward, continued quantification of pattern similarities in other parts of the body would be incredibly useful when determining morph and sex differences across biofluorescent patterns.

Given that salamander fluorescence is a novel phenomenon, further development of lighting and photography methods should be considered when moving forward with this topic. Within the scope of this study, the individuals were photographed to the best of our ability while maintaining as much standardization as possible; however, a variety of improvements could be made to more effectively replicate photographic conditions. For example, the use of a camera model more capable of capturing smaller details would allow for greater amounts of an individual's pattern to be eventually quantified. To make this feasible, a custom 500 nm long-pass lens would be required with the capacity to securely fit around the camera itself rather than being held up to the camera lens. Additionally, the use of tripods or other securing devices would allow for the camera, visible, and blue excitation light to remain in the same position and angle in every photograph to reduce the amount of lighting variation in the photographs.

To my knowledge, this study is the first documentation of biofluorescence in a polymorphic species. Although the function of this phenomenon is still unknown, the findings of this study contribute quantifiable evidence towards its potential uses, with more research on the topic being critical to determining its ecological importance.

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