

Biofluorescence as a Mechanism of Sexual Selection in *Ambystoma mavortium nebulosum*

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ABSTRACT

Biofluorescence, the re-emission of absorbed light at lower energy wavelengths, has been widely documented in marine organisms and recently observed in amphibians. This phenomenon, particularly in amphibians such as frogs, salamanders, and newts, suggests potential roles in ecological interactions, including communication, mate selection, and predator avoidance. Despite advancements, the functional role of biofluorescence, especially regarding sexual selection, remains unclear. This study investigated the role of biofluorescence in sexual selection among Arizona Tiger Salamanders (*Ambystoma tigrinum nebulosum*), focusing on its behavioral functions and implications for conservation at the Mexican Cut in Colorado over the summer of 2024. Using a series of controlled experiments, we examined salamander preferences for different colored light and biofluorescent versus non-biofluorescent potential mates under blue light exposure. Results from glow stick and blue light control experiments indicate no significant sex-based differences in trap choices, but a general attraction to green and blue light was observed. In biofluorescence choice experiments, females showed a significant preference against biofluorescent males, whereas males exhibited no preference for biofluorescent females. These findings suggest that while blue light is a crucial visual cue, biofluorescence may not play a significant role in sexual selection in this species. Understanding the role of biofluorescence in amphibians could enhance non-invasive monitoring techniques and conservation strategies, particularly given the global decline in amphibian populations. This research provides new insights into amphibian behavior and ecology, contributing to improved conservation methods and a deeper understanding of biofluorescence's ecological and evolutionary significance.

INTRODUCTION

Biofluorescence, the re-emission of absorbed light at lower energy wavelengths by organisms, is a phenomenon gaining increasing attention in the study of amphibians. Previously, biofluorescence had been predominantly documented in marine organisms, but recent studies have revealed that this is a more widespread phenomenon than previously known (Taboada et al., 2017, Lamb and Davis, 2020, Witcher et al., 2023). This class, including frogs, salamanders, and newts, exhibit a variety of biofluorescent patterns, which could play significant roles in ecological interactions, including communication, mate selection, and predator avoidance (Lamb and Davis 2020). The discovery of biofluorescence in amphibians highlights its potential importance in their visual perception and behavior. Specifically, studies on biofluorescence in salamanders have revealed that it is widespread and not restricted to any particular family, suggesting a broad ecological and evolutionary significance (Lamb & Davis, 2020).

Despite recent advances in our understanding of amphibian biofluorescence, its functional role remains unknown. It has been hypothesized that biofluorescence may play a role

in communication, predator avoidance, territoriality, and mate selection. For example, a study on the Cope's Gray Treefrog tested the effect of biofluorescence on predation, but found that its role may be limited (Whitcher, et al., 2024). Another study suggested that male fluorescence of the post-cloacal gland may function in the context of both mate choice and territoriality (Anthony, et al., 2023).

Furthermore, there is limited knowledge about the variation in biofluorescence across different life stages and environmental conditions. Most studies focus on adult specimens, leaving a gap in our understanding of how biofluorescence develops and changes from larval stages through to adulthood, and how it might be influenced by ecological factors such as habitat type and light environment. A recent study suggests that adults exhibit more biofluorescence than hatchlings in the Arizona tiger salamander, indicating a potential link with adult reproductive behavior and ecology (Crayton, unpublished data). This underscores the need for further research to elucidate the ecological and evolutionary significance of biofluorescence in amphibians.

Due to amphibians' role as bioindicators, it's important to have a variety of ways to monitor their population health. Understanding biofluorescence in amphibians could provide a novel method for such monitoring, especially given their complex mating behaviors and activities in low-light environments. If biofluorescence is a mechanism of sexual selection in amphibians, variation or amount of biofluorescence could be used as a proxy to measure the reproductive health of the species. Amphibians also serve as key bioindicators due to their permeable skin and sensitivity to environmental changes, making them particularly vulnerable to habitat alterations (Sumanasekara, et al., 2015). Studying biofluorescence could enhance non-invasive monitoring techniques, aiding in the conservation of amphibian populations, which are globally declining due to habitat loss, pollution, climate change, and diseases such as chytridiomycosis (Blaustein and Wake 1990, Beebee and Griffiths 2005). This decline, affecting up to 40% of species, has been documented globally and poses significant threats to biodiversity, ecosystem services, and human health (Stuart, et al., 1783-1786). Amphibians play essential roles in pest control, nutrient cycling, and as prey for other wildlife (Voyles, et al., 2009); their decline could lead to increased insect-borne diseases and disrupt ecosystem stability (West, et al., 2018). By examining the biofluorescent properties of species like the Arizona tiger salamander, research could provide new insights into amphibian behavior and ecology, supporting improved conservation strategies.

My study investigated the role of biofluorescence in sexual selection among paedomorphic Arizona Tiger Salamanders to uncover aspects of their reproductive behavior and ecology. The objective was to determine whether biofluorescence acts as a mechanism of sexual selection, providing insights into its behavioral functions and contributing to amphibian ecology and evolution. Using a three-chamber experiment to investigate mate choice, I aimed to address gaps in previous research by linking biofluorescence to specific behavioral outcomes. Five experiments were conducted: the first established a baseline frequency for salamanders entering traps without stimuli, while the second assessed preferences for traps with different colored glow sticks, hypothesizing a preference for green. The next two experiments investigated if female and male salamanders preferred traps with the opposite sex exhibiting

biofluorescence induced by blue light, compared to non-fluorescent individuals, with hypotheses considering both preferences for and against biofluorescence. The final experiment served as a control to determine general attraction to blue light. These experiments aimed to determine the role of biofluorescence in sexual selection behaviors, enhancing non-invasive monitoring techniques and supporting amphibian conservation.

METHODS

I designed a series of experiments to investigate whether biofluorescence functions as a mechanism of sexual selection in paedomorphic Arizona tiger salamanders. These experiments were conducted at the Mexican Cut, a high-altitude research site known for its pristine conditions and long-term ecological studies. Salamanders were collected using established methods for long-term data collection, ensuring consistency and reliability in our sample (Paton and Crouch 2002).

Salamanders were placed in long, shallow tanks (40 x 20 x 6.12 inches) with traps at either end in a darkened room. A barrier in the middle of the tank prevented light from escaping from one trap to the other. Three colors of glow sticks were used to evaluate salamander attraction. Each experiment included a minimum of 10 trials per treatment combination for robustness and reproducibility. For all experiments, we alternated the side of the glowstick or blue light between each trial.

Data collection involved controlled behavioral experiments using 130 adult chooser salamanders (65 males and 65 females), sampled from specific ponds in the Mexican Cut near Crested Butte, Colorado. Dependent variables included whether salamanders entered a trap, time taken to decide (in seconds) with a maximum of 10 minutes, specific trap selection (green, blue, red, no glow stick, biofluorescent salamander, non-biofluorescent salamander, blue light, no light), colored glowstick or blue light trap positioning (right/left), and salamander sex (male/female).

Experiments were conducted both during and after the breeding season to compare behavioral differences while maintaining consistent environmental conditions within the experimental setting. During the breeding season, the swollen cloacas of salamanders indicated active breeding status, while non-larval salamanders with a mix of swollen and non-swollen cloacas were used in post-breeding season experiments.

For each experiment, we recorded whether a choice was made, the time it took to make the choice, the side of the blue light or certain colored glowstick (switched every trial from right to left and back), which side the salamander chose (e.g., biofluorescent side vs. non-biofluorescent side), the pit tag ID number, which mesocosm was used, the sex of the salamander, the pond collected from for each experiment, and the date of the experiments.

In the control experiment, each trial involved placing a salamander in the center of the tank, with neither side containing any treatment. This setup aimed to determine salamanders'

propensity to enter any trap as well as a possible preference for the right or left trap in each mesocosm. Each salamander had 10 minutes to choose a trap, and the time (to the nearest second) that the salamander's back legs exited the funnel into the trap was recorded. I conducted 10 trials per sex, with different salamanders used for each trial.

In the glow stick choice experiment, each trial involved placing a salamander in the center of the tank. One trap contained a green glow stick, while the other trap contained either a blue, red, or no glow stick. For all experiments, each salamander had 10 minutes to choose a trap, and the time (to the nearest second) that the salamander's back legs exited the funnel into the trap was recorded. 10 trials were conducted for each sex for the green vs. red glow stick and green vs. blue glow stick experiments, while 5 trials were conducted for each sex for the green vs no glowstick experiments, with different salamanders each trial.

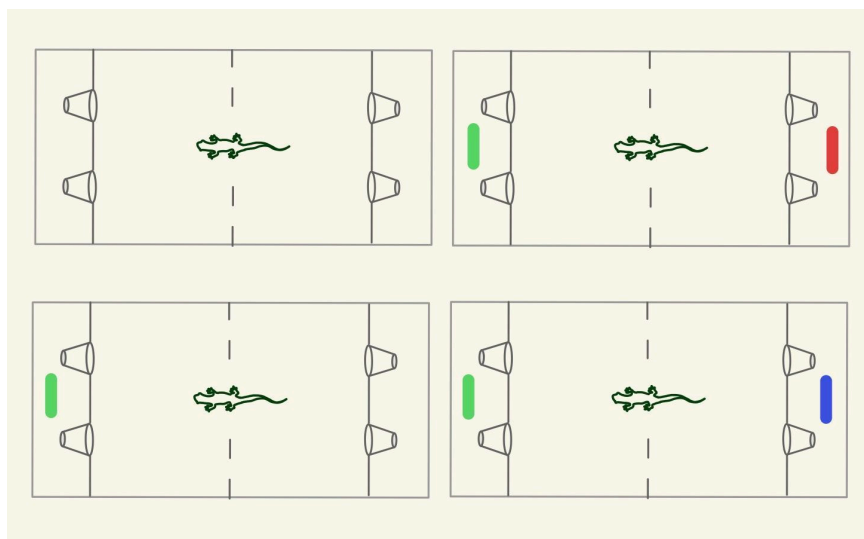


Figure 1. Control (top left) and Glow Stick Choice Experiment Setup: Diagram (top left) illustrating salamanders placed in the center of a long, shallow tank with both traps (represented by the barrier line with conical funnels) remaining empty. The dashed line in the middle of the tank represents the additional light barrier. The three remaining diagrams illustrate the glow stick choice experiments. One trap contains a green glow stick, while the other trap contains either a blue, red, or no glow stick.

In the female biofluorescence choice experiment, each trial involved female salamanders placed in the center of the tank. One trap contained a male not exposed to any light, and the other contained a male exposed to blue light to induce biofluorescence. Blue light flashlights were used to induce biofluorescence. For all experiments, each salamander had 10 minutes to choose a trap, and the time (to the nearest second) that the salamander's back legs exited the funnel into the trap was recorded. Two sets of 10 trials were conducted (20 trials total), using different female chooser salamanders and the same male salamanders for each set of trials for consistency.

In the male biofluorescence choice experiment, each trial involved male salamanders placed in the center of the tank, with one trap containing a female not exposed to any light and the other a female exposed to blue light to induce biofluorescence. For all experiments, each salamander had 10 minutes to choose a trap, and the time (to the nearest second) that the salamander's back legs exited the funnel into the trap was recorded. Similar to the female biofluorescence choice experiment, two sets of 10 trials were conducted (20 trials total), using different male chooser salamanders and the same female salamanders for each set of trials for consistency.

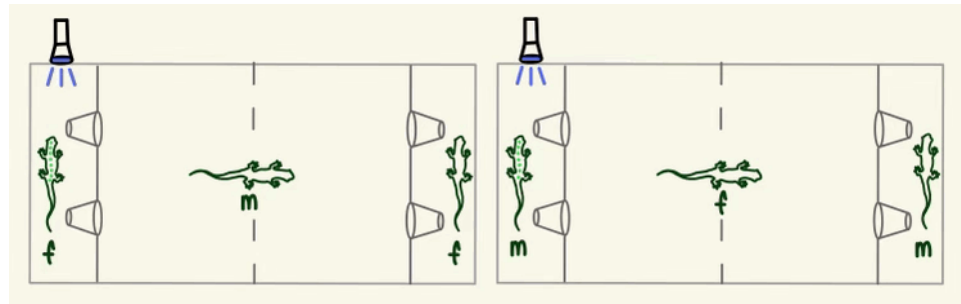


Figure 2. Male (left) and Female (right) Biofluorescence Choice Experiment Setup:

Diagrams illustrating a male or female salamander placed in the center of a long, shallow tank. For the male biofluorescence choice experiment (left), the trap contains a female not exposed to any light, while the other contains a female exposed to blue light to induce biofluorescence. For the female biofluorescence choice experiment (right), the trap contains a male not exposed to any light, while the other contains a male exposed to blue light to induce biofluorescence.

In the blue light control experiment, salamanders of both sexes were placed in the center of the tank. One trap contained blue light, and the other contained no light. For all experiments, each salamander had 10 minutes to choose a trap, and the time (to the nearest second) that the salamander's back legs exited the funnel into the trap was recorded. 10 trials were conducted for each sex, with a different salamander used in each trial.

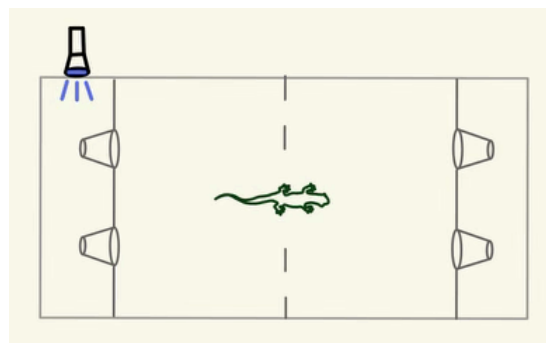


Figure 3. Blue Light Control Experiment Setup: Diagram illustrating salamanders placed in the center of a long, shallow tank. One trap contains blue light, while the other trap contains no light.

Data collection categories for each experiment include variables specific to each setup, such as salamander sex, choice made, time to choose, trap chosen, and trap position. Chi-square tests are used to assess the significance of preference patterns between sexes and for each experimental condition, with significance set at $p < 0.05$ to determine outcomes and implications. The "no choice" option was excluded from the analysis due to inconsistencies in its relevance across different experiments. In some experiments, all salamanders made a choice, rendering the "no choice" category unnecessary. For experiments with only one treatment, salamanders that did not choose were grouped with those that selected the no-treatment side, as both outcomes indicated no preference for the treatment. In experiments comparing two treatments, "no choice" was not a valid option and thus was not analyzed. This approach ensured consistency and clarity in interpreting the results.

RESULTS

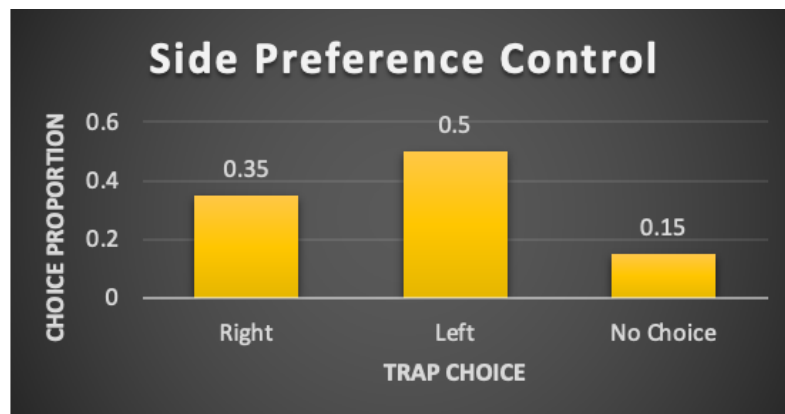


Figure 4. Side Preference Control Experiment Results

There was no significant difference between the sexes in trap choices ($\chi^2 = 0.0843$, $p = 0.771519$). Similarly, when combining data for both sexes, there was no significant difference in right vs. left trap choices ($\chi^2 = 0.529$, $p = 0.4669$), indicating no significant preference in the no light control experiment (Figure 4).

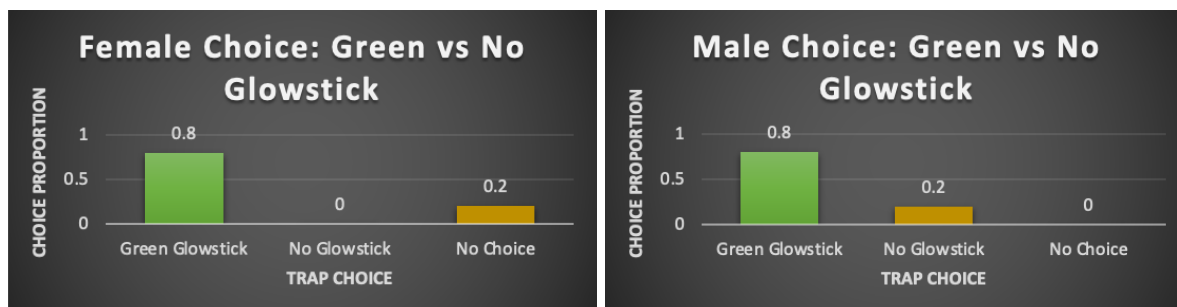


Figure 5. Green vs No Glowstick Experiment Results

The chi-square statistic for the comparison of trap choices between male and female salamanders in the green vs. no glowstick experiment was 0, with a p-value of 1. This indicates no significant difference between the sexes in the trap choices. For the combined sexes, the chi-square statistic for green vs. no glowstick trap choices was 7.200, with a p-value of 0.0073. This result suggests a significant preference for the green glowstick (Figure 5).

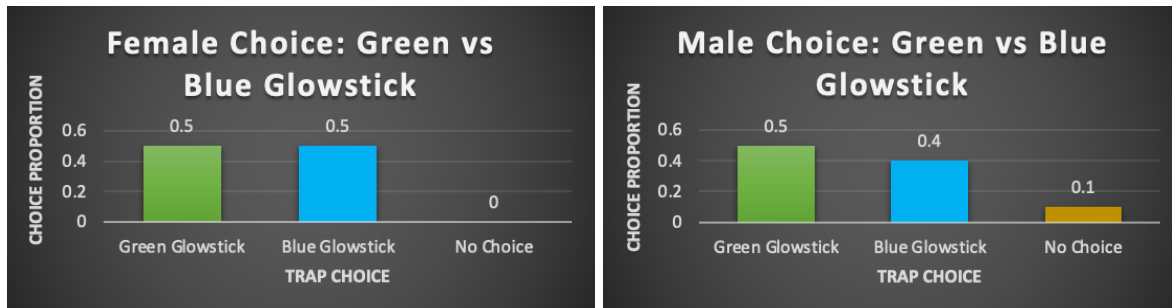


Figure 6: Green vs Blue Glowstick Experiment Results

The chi-square statistic for the comparison of trap choices between male and female salamanders in the green vs. blue glowstick experiment was 0.059 with 1 degree of freedom, and the two-tailed p-value was 0.8087, indicating no significant difference between the sexes in trap choice. For the combined sexes, the chi-square statistic for green vs. blue glowstick trap choices was 0.111 with 1 degree of freedom, yielding a two-tailed p-value of 0.7389, suggesting no significant difference in the trap choices in the green vs. blue glowstick experiment (Figure 6).



Figure 7: Green vs Red Glowstick Experiment Results

The chi-square statistic for the comparison of trap choices between male and female salamanders in the green vs. red glowstick experiment was 0.000 with 1 degree of freedom, and the two-tailed p-value was 1.0000. This indicates no significant difference between the sexes in trap choice. For the combined sexes, the chi-square statistic for green vs. red glowstick trap choices was 3.200 with 1 degree of freedom, and the two-tailed p-value was 0.0736. This result is not significant at an alpha of 0.05 but is significant at an alpha of 0.1, suggesting a preference for the green glowstick (Figure 7).

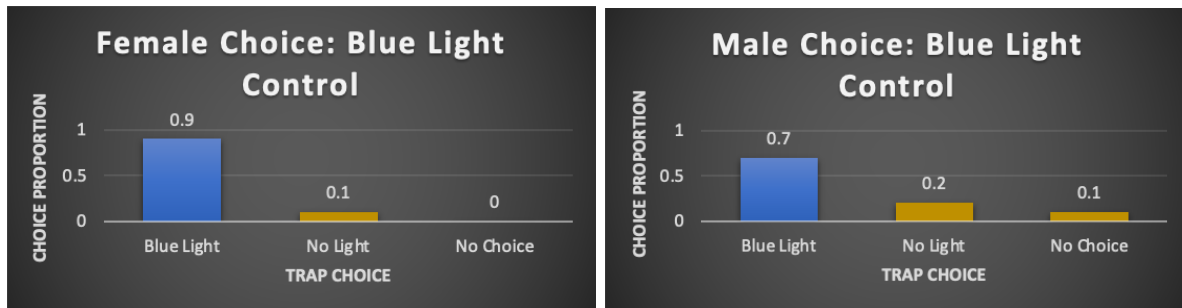


Figure 8: Blue Light Control Experiment Results

The chi-square statistic for comparing trap choices between male and female salamanders in the blue light control experiment was 1.250, with a p-value of 0.2636. This result is not significant at $p < 0.05$, indicating no significant difference between the sexes in the trap choices for the blue light control experiment. When combining data for both sexes, the chi-square statistic for blue vs. no light trap choices was 7.200 with 1 degree of freedom, yielding a two-tailed p-value of 0.0073. This result is significant at $p < 0.05$, indicating a significant preference for the blue light in the blue light control experiment (Figure 8).

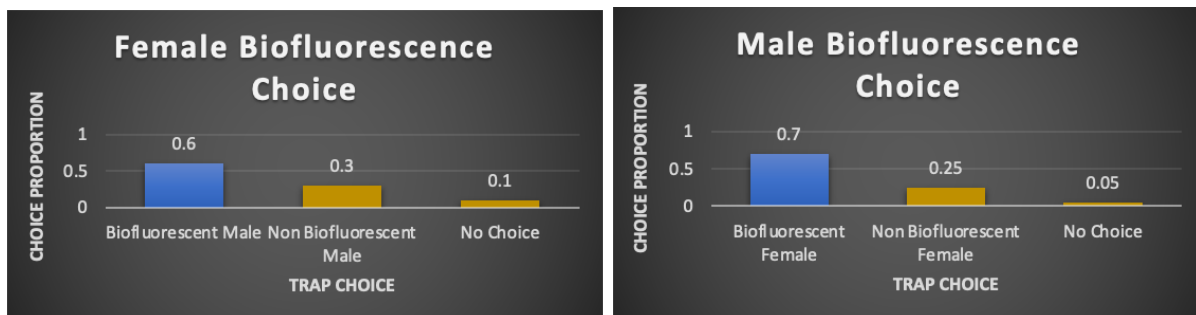


Figure 9: Biofluorescence Choice Experiment Results

The chi-square statistic for comparing trap choices between male and female salamanders in the biofluorescence choice experiments was 0.218 with 1 degree of freedom, and the two-tailed p-value was 0.6407. This result is not significant at $p < 0.05$, indicating no significant difference between the sexes in the trap choices for the biofluorescence choice experiments.

For the female biofluorescence choice experiment, where female salamanders were presented with a choice between a biofluorescing male and a non-biofluorescing male, the chi-square statistic was 10.889 with 1 degree of freedom, yielding a two-tailed p-value of 0.0010 when compared to the male choice blue light control experiment. This result is highly significant at $p < 0.05$, indicating that female attraction to blue light is lower due to male salamander presence (Figure 9, left).

For the male biofluorescence choice experiment, where male salamanders were presented with a choice between a biofluorescing female and a non-biofluorescing female, the

chi-square statistic was 0.123 with 1 degree of freedom, and the two-tailed p-value was 0.7260. This result is not significant at $p < 0.05$, indicating no significant difference between the trap choices for the male biofluorescence choice experiment. The presence of a biofluorescing female did not significantly alter male preference for the trap (Figure 9, right).

DISCUSSION

The results of these experiments provide insights into the potential role of visual cues in the behavior of Arizona Tiger Salamanders. In the no light control experiments, there was no significant difference between sexes or between trap choices (right vs. left), indicating no side preference or sex-based behavior difference without light stimulus. In the green vs. no glowstick experiment, there was no significant sex difference, but a combined significant attraction to green light was observed. The green vs. blue glowstick experiment showed no significant differences between sexes or trap choices, suggesting equal attraction to both colors. The green vs. red glowstick experiment also revealed identical data for both sexes, but a combined preference for green over red.

The glowstick experiments suggest that while there is no significant sex-based difference in trap choice, salamanders showed a possible preference for blue and green glowsticks over red. This is consistent with the known sensitivity of salamanders to blue and green light wavelengths, which are more visually stimulating or attractive to them. In contrast, red light, which they cannot see as effectively, was less preferred. These findings highlight the importance of visual cues in the sensory ecology of these salamanders, indicating that their behavior is influenced by the spectral properties of light. Further research with larger sample sizes and additional colors could provide more definitive conclusions and deepen our understanding of the role of visual stimuli in salamander behavior.

The biofluorescence experiments yielded interesting results regarding the role of visual cues in sexual selection. In the blue light control experiment, there was no significant difference between the sexes in trap choice, indicating that both male and female salamanders did not show a sex-based preference for blue light versus no light. However, when sexes were combined, a moderately significant preference for the blue light trap was observed. This suggests a general attraction to blue light, which may be an important visual cue in the salamander's natural environment, as that is the color of light that causes them to fluoresce.

The biofluorescence choice experiments further explained the role of biofluorescence in mate selection. In the female biofluorescence choice experiment, females showed a significant preference for blue traps without male presence. This finding indicates that females are less likely to choose a trap containing a biofluorescing male. Conversely, in the male biofluorescence choice experiment, there was no significant difference in male trap choice between biofluorescing and non-biofluorescing females. This indicates that the presence of biofluorescence in females does not influence male choice.

These results collectively suggest that while blue light is generally attractive to both male and female salamanders, biofluorescence may not play a significant role in sexual selection in this species. The avoidance of biofluorescing males by females raises intriguing questions about the ecological and evolutionary implications of biofluorescence. For example, why do female salamanders avoid biofluorescing males despite their general attraction to blue and green light? What ecological factors might influence the role of biofluorescence in salamander behavior, and how does biofluorescence affect the survival and reproductive success of salamanders? Additional studies with larger sample sizes and a broader range of conditions could provide insights into these complex interactions.

In addition to repeating these experiments with a larger sample size, replicating all experiments both before and after the breeding season will allow for a comprehensive comparison of behavioral differences across different reproductive states, providing deeper insights into the seasonal influences on mate selection and biofluorescence attraction. Additionally, future studies should consider using UV light instead of blue light in the biofluorescence choice experiments. Since salamanders are unable to sense UV light but still fluoresce under it, using UV light would eliminate the confounding factor of blue light attraction and provide a clearer understanding of the role of biofluorescence itself in mate selection. These changes could reveal whether biofluorescence serves as an important ecological or evolutionary signal, which could influence how we understand the mating behaviors and survival strategies of amphibians.

The use of glow sticks as non-invasive monitoring tools presents a promising method for field studies (Grayson, et al., 2007). Glow sticks can attract salamanders without the need for physical capture, reducing stress on the animals and allowing for more natural observations. If glow sticks prove effective in attracting salamanders consistently, they could become a valuable tool for conservation biologists and environmental managers in monitoring amphibian populations, particularly in challenging or sensitive environments.

This study contributes to our understanding of the potential ecological and evolutionary roles of biofluorescence in amphibians. The findings highlight the importance of visual cues in salamander behavior and suggest that biofluorescence may have a more complex role than previously thought. Repeating and refining this study will not only enhance our understanding of biofluorescence and sexual selection in Arizona Tiger Salamanders but also contribute to the development of innovative and non-invasive conservation strategies.

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