

Color performance on the red eyes tree frog (*Agalychnis callidryas*) tadpoles with two pigment sources

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Abstract. Anuran amphibians display various colorations during different stages of their life, which can be altered by adding pigments to their diet. The study investigated the effects of coloration in *Agalychnis callidryas* hatchlings under laboratory conditions. The study involved 21 experimental units and 210 larvae. The results showed that tadpoles fed with *Arthrospira platensis* had a higher optical density of 161.6 ppp, followed by tadpoles supplemented with a commercial pigment for aquaculture use, with an optical density of 145.2 ppp. Both groups had a 6% pigment inclusion. The percentage of pigmented tadpoles decreased between 27-30 days in treatments with a higher level of pigment inclusion (6%) compared to the control, which took more than 45 days for their skin to become pigmented. These results highlight the importance of using additives such as pigments in diets and nutrition to produce the red-eyed green frog (*Agalychnis callidryas*).

Key Words: amphibian, aquaculture, nutrition, skin.

Introduction. Anuran amphibians, commonly known as toads and frogs, are part of the animal kingdom, they are bred in aquaculture for various purposes, including as a food source, for meat (Assis et al 2009; Mello et al 2006; Gonçalves & Otta 2008; Moreira et al 2013), for biotechnological applications due to compounds found in their skin (Clarke 1997) and for ornamental interest with species from Hylidae, Bufonidae, Ranidae and Dendrobatidae families, known for their vibrant colors (Natusch & Lyons 2012; Licciardi 2014; Pasmans et al 2017; Kaczmarek & Kolenda 2018; Mohanty & Measey 2019; Cavasos et al 2023). One popular species is *Agalychnis callidryas* (Cope, 1862), which is widely distributed throughout tropical climates of America and is valued for its colorful appearance (Savage 2002; Wang et al 2008; Wang et al 2015). Other ornamental species includes *Dendrobates tinctorius* (Rojas & Pašukonis 2019; Jacobson et al 2020; Yaw et al 2020; Ferro et al 2024) and *Ceratophrys cranwelli* (Hou et al 2006; Schalk et al 2014; Serpieri et al 2024), these species are highly sought after for their variety of colorations, achieved through selective breeding (Marchant et al 2015) due to their variety of colorations and tones commonly known as morphos through selective breeding as a tool to obtain certain colorations as well as other aquatic species such as ornamental fish such as the Endler fish (Hernández-López & Luna-Vivaldo 2021), guppies (Hernández-López et al 2024), and even crustaceans such as *Neocaridina davidi* (Luna-Vivaldo et al 2024). The anuran *A. callidryas* has been recently into the pet trade market because of being one of the most beautifully colored amphibians that has a big distribution from Mexico to Panama. Also *A. callidryas* is one of the frog species that has

been recently available in the herpetological pet trade for its beauty and also for its relatively easy captive breeding (Wang et al 2015). This study aims to assess the coloration development of the red-eyed tree frog larvae as they mature into juveniles.

The objective of this study was to measure the optical density of their coloration and how they are pigmented over time using two sources of pigments, a commercial pigment and algae (*Arthrospira platensis*) at different inclusions in the diet (1, 3, and 6 %). These findings are crucial for the commercial production and management of these amphibians, given the increasing popularity of ornamental amphibians in the pet trade (Pineda-Vázquez et al 2022).

Material and Method

Broodstock. The study was conducted at the “Universidad Veracruzana, Facultad de Medicina Veterinaria y Zootecnia” in Veracruz, México, from July to August 2023. Tadpoles were obtained from a controlled sexual reproduction by a rain chamber of 5 males and 10 adult females on *Agalychnis callidryas*. The adult frogs were obtained from a local pet store. Fifteen frogs were used to produce tadpoles, 5 males and 10 females of *A. callidryas*. The frogs underwent a 40-day quarantine period. The adult frogs were kept together, and the breeders fed live crickets (*Acheta domesticus*) and roaches (*Blaptica dubia*). Both live prey species were fed with a diet containing 40% crude protein, which was provided ad libitum to the insects daily before they were offered to the frogs.

The frogs were exposed to artificial rain cycles from 18:00 h to 06:00 h in a rain chamber until the mating pairs laid eggs. The rain chamber was made in a commercial vertical terrarium (Zoo Med®, USA) for reptiles (45 cm x 45 cm x 91 cm) provided with a mechanical filter and a natural plant (*Alocasia odora*). The temperature was maintained at a constant 23°C using 24/7 air-conditioning.

Offspring. The frogs and their habitat were checked daily at 07:00 h for the presence of eggs. When eggs were laid, the adult frogs were immediately separated from the clutch. The eggs were maintained in a high-humidity environment (80–90%) and continuously monitored using a hygrometer until hatching (Figure 1). At hatching, the initial total length of the tadpoles was measured using a digital Vernier caliper to determine baseline size, and body weight was recorded using a laboratory digital balance with a sensitivity of 0.001 g. Three-day-old *A. callidryas* tadpoles were used for the experiments, corresponding to the stage when tadpoles begin exogenous feeding. Ten tadpoles, without sex differentiation, were randomly distributed into 21 containers (40 L) with three replicates per treatment, totaling 210 tadpoles (n = 210). The containers were designed to allow water flow between them, creating a shared water environment for all tadpoles.



Figure 1. Recently laid eggs of *Agalychnis callidryas* (a); 5-day-old embryos inside the eggs gel (b); newly hatched larvae of *Agalychnis callidryas* (c) (original images).

Water parameters. The water parameters were measured weekly using the Nutrafin® (Canada) colorimetric test (Table 1). Water exchanges were conducted twice a week, and evaporated water was replaced to prevent sudden changes in water parameters.

Table 1

Water quality parameters

<i>Parameter</i>	<i>Range value</i>
Oxygen (mg L ⁻¹)	5.5-6
pH	7-7.2
PO4 (mg L ⁻¹)	0
NO3 (mg L ⁻¹)	0
NO2 (mg L ⁻¹)	0
GH (mg L ⁻¹)	110-120
KH (mg L ⁻¹)	90-95
Fe (mg L ⁻¹)	0

Note: GH - general hardness; KH- carbonate hardness.

Feeding. The bioassay involved treatments using freeze-dried microalgae *Arthrospira platensis* (A) analyzed (Figure 2) by spectrophotometry (Arredondo & Voltolina 2007) and a commercial pigment (C) source (*Paracoccus carotinifaciens*, from BulkReefSupply®, USA) with an equivalence of 1.8% of astaxanthin. The experimental treatments consisted of diets containing inclusion levels of 1%, 3%, and 6% of *Arthrospira platensis* (A), and 1%, 3%, and 6% of the commercial pigment source *Paracoccus carotinifaciens* (C). Additionally, a negative control treatment was included, consisting of a diet without pigment supplementation (0% pigment inclusion). Diets were formulated with a mix of ingredients (Table 2) and were provided as a gelled porridge. All treatments were isoproteic (50% PC) and isolipidic (12% EE), with the only variation being the inclusion content of the percentage of pigment for each treatment. The newborn tadpoles were fed until 72 hours post-hatching, and tadpoles were fed four times daily (06:00 h, 11:00 h, 16:00 h, 19:00 h) at 20% of their biomass.

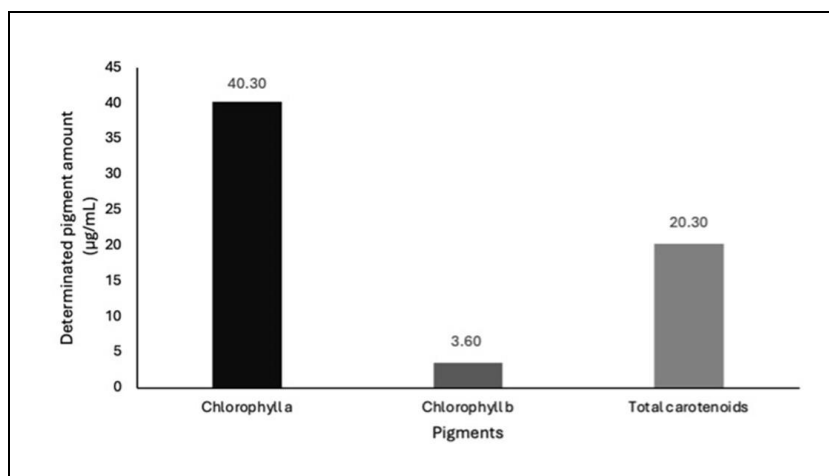


Figure 2. Chlorophyll a, chlorophyll b, and total carotenoid content in the microalgae *Arthrospira platensis* by UV/vis spectrophotometry.

Table 2

Ingredients used to formulate diets during the study

Dietary ingredients ($g\ kg^{-1}$)	Diets (treatments)						
	Ingredients for treatments						
	1% C	3% C	6% C	1% A	3% A	6% A	
<i>Artemia</i> sp. meal ^a	176	176	176	176	176	176	176
Protein mix ^b	352	352	352	352	352	352	352
Soy lecithin	16	16	16	16	16	16	16
Canila oil	45	45	45	45	45	45	45
Energetic mix ^c	206	206	206	206	206	206	206
Oatmeal	103	83	53	103	83	53	113
Mineral premix ^d	20	20	20	20	20	20	20
Vitamin C premix ^e	10	10	10	10	10	10	10
Sodium citrate	2	2	2	2	2	2	2
Vitamin C	10	10	10	10	10	10	10
Gelling agent	50	50	50	50	50	50	50
Pigment	10	30	60	0	0	0	0
Algae	0	0	0	10	30	60	0
Total	1000	1000	1000	1000	1000	1000	1000

Note: ^a *Artemia* sp. flour. (Superior Brine Shrimp Flakes, OCEAN STAR INTERNATIONAL, UTAH, USA). The proximate composition was 50 % crude protein, 15 % ash, 5 % lipids, 2 % fiber, and 9 % moisture.

^b Protein premix contains high-protein ingredients: krill, squid, and fish meal.

^c Energy premix contains high-carbohydrate ingredients: *Taraxacum officinale* flour, corn flour, and *Medicago sativa* flour.

^d Mineral premix contains the following minerals (per kg): 6%, Vitamin D min, 30% calcium, 22,026 IU kg^{-1} .

^e The vitamin premix contains the following vitamins (each per kilogram): Vitamin A 4040,629 IU kg^{-1} , Vitamin D 48,458 IU kg^{-1} , Vitamin E 908 IU kg^{-1} , Vitamin K (Metadione) 66.08 mg kg^{-1} , Vitamin C (L-ascorbyl-2-polyphosphate), 5,506 mg kg^{-1} , Vitamin B1 (Thiamine HCL) 18 mg kg^{-1} , Vitamin B2 (Riboflavin) 31.78 mg kg^{-1} , Vitamin B5 (Pantothenic Acid) 59.02 mg kg^{-1} , Niacin 204.3 mg kg^{-1} , Vitamin B6 (Pyridoxine HCL) 24.97 mg kg^{-1} , Vitamin B12 0.18 mg kg^{-1} , Folic Acid 9.08 mg kg^{-1} , Biotin 0.908 mg kg^{-1} , Choline 2,270 mg kg^{-1} .

Coloration measurement. The images were captured using a stereoscopic microscope (Quasar Qm15) equipped with an 8MP digital USB camera and the analysis system (FutureWinJoe Software) from larva to juvenile stages.

The images of the tadpoles' backs and their coloration were analyzed using the open-access software ImageJ v.1.64. The coloration was measured as optical density (ppp) to obtain skin coloration data for each treatment. The optical density dispersion was considered the dependent variable and the percentage of the pigment source in the diet was the independent variable. For the pigmentation area (ppp), the pictures were analyzed to obtain the amount of pigmented area on the tadpoles' backs from day three when the bioassay starts until they reach the juvenile stage (frogs) after undergoing metamorphosis.

Statistical analysis. The optical density means were analyzed using a completely randomized design and analysis of the variance. The mean optical density was examined using one-way ANOVA, followed by a comparison of a means test to confirm if there was a significant difference between the treatment means at a significance level of $p < 0.05$ for pigment levels, which affect larval coloration and development (SAS®).

Results. The study involved the offspring from two litters obtained from two pairs of frogs, totaling 210 tadpoles. At birth, the tadpoles had an average length of 13.835 ± 0.2 mm. During the larval stage, the tadpoles consumed food with pigments, and a survival rate of $91.5 \% \pm 1$ was registered among all treatments. No significant difference between length and weight was found among the treatments. As the tadpoles transitioned to the juvenile phase, it was observed that 6 % of pigment in both treatments (algae and commercial pigment) resulted in better coloration performance. The *Arthrospira platensis* treatment resulted in coloration performance with an optical density of 161.60 ppp, compared to the commercial treatment of 145.2 ppp. Additionally,

the time taken for 100 % of the organisms to be fed with commercial pigments was 27 days, while the algae treatment took 29-32 days (Figure 3).

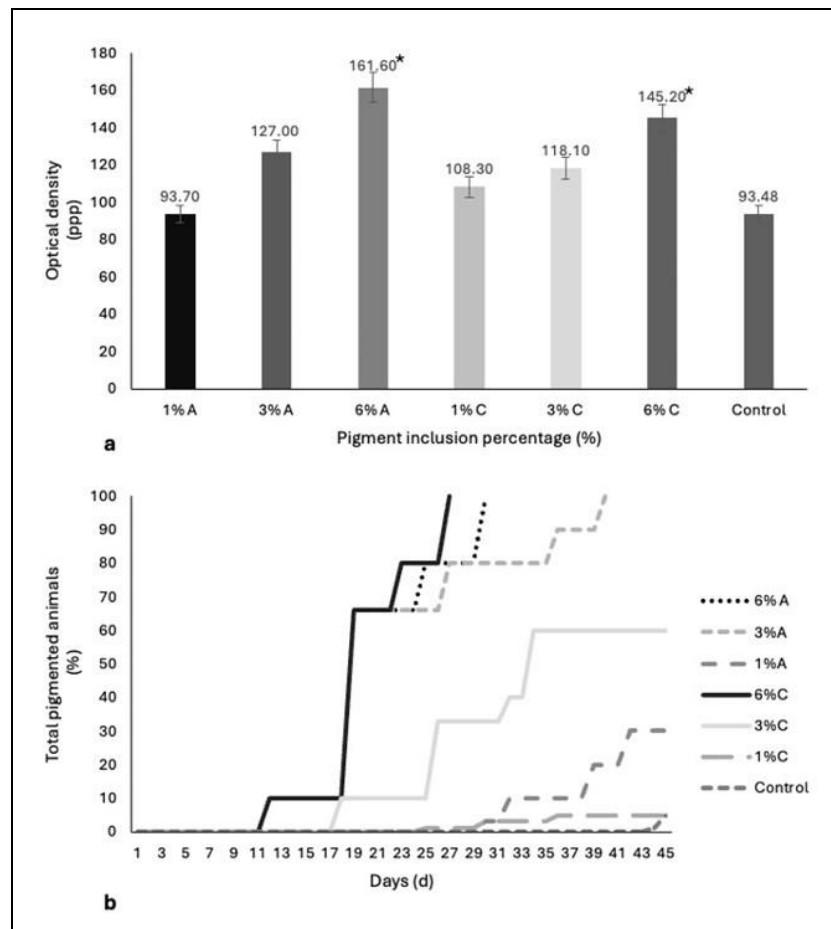


Figure 3. Optical density (a) of *Agalychnis callidryas* fed with different percentages of inclusion of pigment in the diet at 44 days and total pigmented juveniles; n = 210, bars indicate \pm SD.

In comparison to the treatments with a higher pigment content (6%), the other treatments did not achieve a higher optical density, nor did they fully color the organisms after 44 days of the bioassay (Figure 3) for both the commercial pigment and the *A. platensis* as opposed to the control. A significant difference ($p < 0.05$) was observed between the increase in pigment included in the diet with the optical density. The highest value of optical density was recorded when the 6% treatment consumed algae *A. platensis*. The time taken for a percentage of organisms in each treatment to achieve full coloration on their back decreased as the percentage of pigment in the diet increased (Figure 3). Additionally, the colorations observed in the frogs were brown in the control group (without pigment inclusion), green in the treatment with 6% commercial pigment, and bluish-green in the treatment with 6% *A. platensis*. These differences were more evident when the control group was compared with the treatments containing the highest inclusion levels of both pigments (Figure 4).

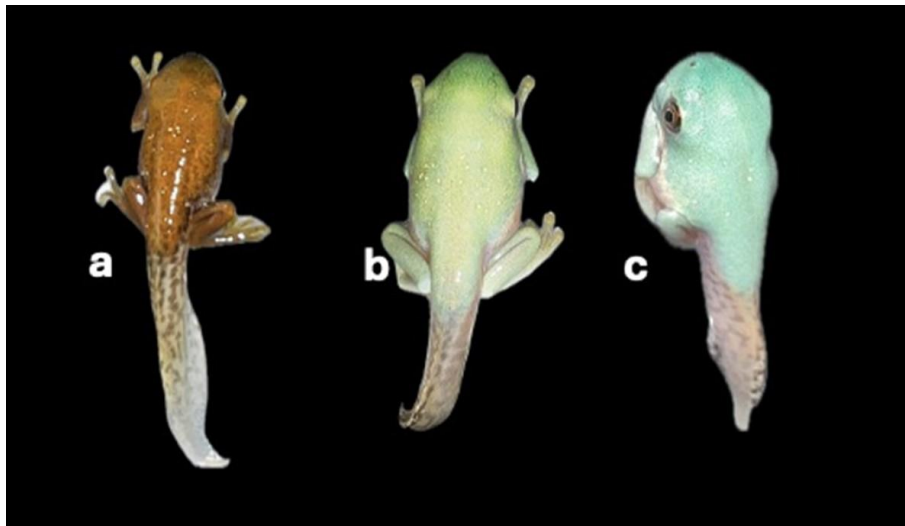


Figure 4. Newly emerged juveniles of *Agalychnis callidryas* from different pigment amounts on diet and source; control (a), commercial pigment (b with 6%), and *Arthrospira platensis* (c with 6%), all at 44 days of life.

The coloration process in *A. callidryas* larvae began on day 13 for the treatments with 6% pigment inclusion (Figure 3), whether using *A. platensis* or a commercial source. There was no coloration in the control treatment with 0% pigments in the larval stage. The pigmentation area of the tadpoles for the highest treatments (6% *A. platensis* and 6% commercial pigment) was completed on the upper back of the tadpoles by day 27. In contrast, the intermediate treatments (3%A and 3%C) took more than 39 days. Tadpoles, with the lowest pigment inclusion percentage (1% A and 1%C) needed more than 45 days to complete the coloring area.

Juveniles fed with pigments from two sources exhibit a noticeable change in color during their larval stage until they reach juvenile status at 50 days old (Figure 5). The level of pigmentation increases as the percentage of pigment inclusion in their diet increases.

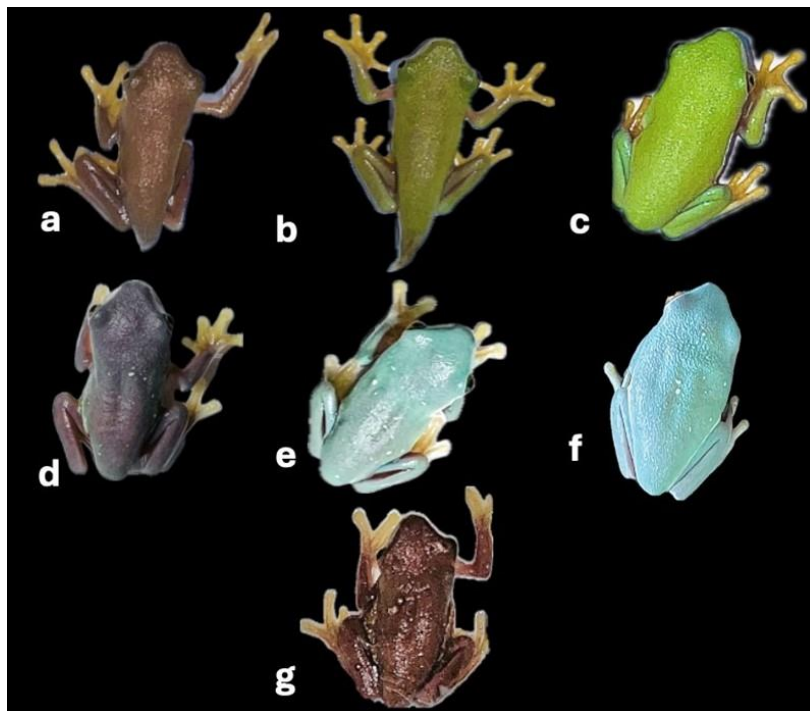


Figure 5. Juveniles of *Agalychnis callidryas* from different pigment percentage amounts in diet and source: commercial pigment with 1, 3, 6% of inclusion (a, b, and c respectively), *Arthrospira platensis* with 1, 3, 6% of inclusion (d, e, and f respectively) and control (g), all at 45-50 life days.

The optical density shows no significant differences between treatments, except for the 6% A and 6% C groups. Additionally, increasing pigment inclusion in the diet reduces the time it takes for frogs to become pigmented after completing the metamorphosis, resulting in earlier frog coloration during the larval stage. The source of the pigment also influences the variation in color tones, ranging from light green (commercial products) to bluish-green colorations (*Arthrospira platensis*).

Discussion. The higher optical density obtained by *Arthrospira platensis* at a concentration of 6% as a pigment source aligns with previous findings indicating that microalgae offer significant nutritional value (Tomas et al 2020). Additionally, these pigments contribute to the coloration of animals, such as the shrimp *Neocaridina davidi*, enhancing their blue-green hues (Luna-Vivaldo et al 2024). This coloration was also evident in the juveniles treated with *A. platensis*, producing light for blue tones that influenced optical density compared to treatment using commercial pigment that presents green tones. Furthermore, it has been demonstrated that feeding microalgae to *N. davidi* affects the intensity of their coloration (Tomas et al 2020), primarily due to their total carotenoid content, which enhances color tones.

It is worth noting that the blueish hues observed in juveniles of *Agalychnis callidryas* are likely attributed to other pigments in *A. platensis*, particularly phycocyanin, which have been shown to influence crustaceans' coloration (Macias-Sancho et al 2014; Kohal et al 2017; Machado et al 2021). This contrasts with findings from Brenes-Soto and Dierenfeld (2014), which suggested a deficiency of carotenoids in the frog *Dyscophus guineti*, in which study of the content of total carotenoids in *A. platensis* supports the observation of coloration in the research.

Our findings align with other research that shows how pigments enhance coloration in various ornamental organisms, such as *Cyprinus carpio* (Sun et al 2012), *Carassius auratus* (Gouveia et al 2003), *Colisa lalia* (Baron et al 2008), *Hyphessobrycon callistus* (Wang et al 2006), and in other amphibians such as *Pseudophryne corroboree* (Silla et al 2016) and *Oophaga pumilio* (Dugas et al 2013). From a commercial perspective, incorporating pigments into the diets of amphibians can enhance their coloration, resulting in more vibrant tones. Effective enhancements on coloration depend on the quality and quantity of the pigments used (Tomas et al 2020). Furthermore, the inclusion of pigments has been explored not only for aesthetic reasons but also for the health benefits of the animals that a pigment-enriched diet can improve fecundity and growth rates (Ogilvy et al 2012).

Conclusions. This study demonstrates that amphibian larvae of the species *Agalychnis callidryas* fed higher concentrations of certain pigments exhibit brighter and more striking colorations than commercial pigments. This feeding regimen modifies the phenotype of the juveniles, resulting in a bluish hue instead of the typical green coloration. It is certain that both sources of pigments promote coloration and reduce the time needed to achieve vibrant colors in a shorter timeframe while diversifying the phenotypes of the same species.

In conclusion, incorporating pigments into the formulated diets of red-eyes tree frogs increase skin color intensity and optical density, improving overall coloration. This enhancement depends on the source of the pigments, which can lead to the development of a bluish phenotype. The research highlights commercial pigments with high carotenoid content, such as astaxanthin derived from *Paracoccus carotinifaciens*, produce a bright emerald-green phenotype, while *Arthrospira platensis* rich in chlorophyll, carotenoids, and phycocyanin produce a blue phenotype on the same species.

Acknowledgements. First author thanks to the scholarship N°848414 granted by CONAHCYT.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 19 June 2025. Accepted: 10 November 2025. Published online: 07 April 2026.

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How to cite this article:

Luna-Vivaldo I., Pinos-Rodríguez J. M., Flores-Primo A., López-Aguirre S., Vicente-Martínez J. G., García-López J. C., Hernández-López M., 2026 Color performance on the red eyes tree frog (*Agalychnis callidryas*) tadpoles with two pigment sources. *AAFL Bioflux* 19(2):597-606.