

The Adaptive Significance of Juvenile Leaf Reddening: A Case Study in *Photinia serratifolia*

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Abstract: Juvenile reddening is a widespread plant physiological phenomenon, which means reddening of young leaves that gradually turn green with maturity as anthocyanins degrade and chlorophyll accumulates. Anthocyanin is a type of common pigment in plants, such as in juvenile leaves, petals, fruits, and stems. Juvenile reddening is a widespread plant physiological phenomenon, and there are two popular hypotheses about the evolutionary significance of juvenile reddening, which is anthocyanin photoprotection hypothesis and aposematic signaling hypothesis. This study aims to verify these two hypotheses on *Photinia serratifolia*. Our results suggest that the red juvenile leaves of *Photinia serratifolia* with 43-fold higher anthocyanins concentration than the green mature leaves showed 9.5-fold smaller rise in MDA concentration after three hours of light-stress treatment, suggesting impressive photoprotection activity and thus supports the photoprotection hypothesis. The larvae of *Actias ningpoana* and *Thosea sinensis* showed no significant difference in preference to green color of in light and dark condition, so the aposematic signaling hypothesis is not supported respect to *P. serratifolia* and *Actias ningpoana* and *Thosea sinensis* larvae.

Keywords: Juvenile Redding; Anthocyanin; Photoprotection; Aposematism; *Photinia Serratifolia*.

1. Introduction

From ancient time to present, plants have evolved various physiological and biochemical characters to survive under abiotic stress conditions. One commonly observed strategy is juvenile reddening [1,2], where young leaves initially appear red and gradually turn green as they mature [3]. This phenomenon is especially common in tropical and subtropical species [1,2,4], including species from the families Moraceae, Anacardiaceae and Rosaceae, and the color transition is typically due to the degradation of anthocyanins and the increased accumulation of chlorophyll during leaf development [3,4]. Anthocyanins are water-soluble flavonoid pigments responsible for the red, purple, and blue coloration in many plant tissues, including leaves, flowers, fruits, and stems [5]. Chemically, anthocyanins are glycosylated forms of anthocyanidins and are synthesized via the flavonoid biosynthetic pathway, which is highly regulated by developmental and environmental factors [5]. These pigments are typically stored in the vacuole and can vary in hue depending on their pH environment, metal ion concentration, and co-pigmentation effects [5]. Beyond their aesthetic and ecological roles in attracting pollinators and seed dispersers [6], anthocyanins are believed to provide a range of physiological functions, particularly under stress conditions [3], such as photoprotection [3,4,7,8,9] and defense against herbivores [1,4,8,10]. Therefore, there are two popular hypotheses about the evolutionary significance of juvenile reddening, specifically, the photoprotection hypothesis and aposematic signaling hypothesis.

The photoprotection hypothesis suggests that anthocyanins function as natural sunscreens, particularly in young or senescing leaves, where chloroplasts are vulnerable [3,4,7,8,9]. According to this view, anthocyanins reduce the amount of light reaching the chloroplasts, thereby protecting the photosynthetic machinery from photoinhibition and photooxidative stress [3,4,7,8,9]. The photooxidative damage can be reflected by the levels of lipid peroxidation products such as malondialdehyde (MDA) [11,12]. MDA is a reactive

aldehyde formed as a byproduct of lipid peroxidation, particularly during cellular aging or when organisms experience oxidative stress under adverse environmental conditions [12]. As a key marker of oxidative damage, MDA levels are closely associated with tissue senescence and stress-induced injury [12]. If the photoprotection hypothesis holds true for *Photinia serratifolia*, a smaller rise in MDA contents is expected in anthocyanin-rich juvenile leaves than anthocyanin-deficient old leaves after light-stress treatment.

The aposematic signaling hypothesis proposes that red or purple pigmentation in leaves serves as a visual deterrent to herbivores. According to this hypothesis, bright coloration may signal toxicity, unpalatability, or low nutritional value, thereby discouraging feeding by insects and other herbivores [3,5,6,7][1,4,8,10]. In some cases, red pigmentation is correlated with elevated levels of defensive compounds such as tannins or alkaloids, further reinforcing its deterrent function [7][1]. If the aposematic signaling hypothesis holds true for *P. serratifolia* and the selected herbivorous larvae (*Actias ningpoana* and *Thosea sinensis*), we would expect the larvae to exhibit a stronger preference for green leaves under light conditions compared to darkness.

To verify these two above-mentioned hypotheses, this study investigated three key aspects: anthocyanin quantification, oxidative stress response, and herbivore preference.

First of all, to confirm the reddening of juvenile leaves is related to higher anthocyanin contents, anthocyanin contents in juvenile and mature leaves were quantified by pH differential method. The pH differential method is a widely used, rapid, and reliable spectrophotometric technique for quantifying total anthocyanin content in plant tissues [13]. This method is based on the unique structural characteristics of anthocyanin pigments, which undergo reversible transformations in response to pH changes. At pH 1.0, anthocyanins exist predominantly in the flavylium cation form, which is intensely colored, whereas at pH 4.5, they are mostly present in the colorless hemiketal form. By measuring absorbance at two wavelengths (typically 520 nm and 700 nm)

under these pH conditions, the difference in optical density can be used to accurately estimate anthocyanin concentration [13].

The photoprotection hypothesis is checked by comparing the rise in MDA concentration in red leaves and green leaves after the same duration of light stress. Under high-temperature and acidic conditions, MDA reacts with thiobarbituric acid (TBA) to form a stable red-colored adduct [14]. This MDA-TBA complex exhibits a characteristic absorbance peak at 532 nm, allowing for spectrophotometric quantification of lipid peroxidation products. To correct for non-specific turbidity and background interference, absorbance at 600 nm is also measured, and the difference between the readings at 532 nm and 600 nm is used to accurately calculate MDA concentration [14].

The aposematic signaling hypothesis is checked by counting the number of larvae feeding on green and red leaves in light and in darkness respectively. A higher percentage of larvae will feed on green leaves in light when they can see the leaf color than in darkness where leaf color is not a factor affecting larvae's preference for leaves.

2. Methods

2.1. Extraction and Quantification of Anthocyanins

Firstly, carefully remove the stems from both old green leaves and new red *P. serratifolia* leaves. Utilize an analytical balance to accurately weigh out 5.0 grams of each type of leaf. Then, cut the leaves into small pieces to facilitate the subsequent grinding process. Transfer the leaf pieces into a clean mortar, add 10 mL of 50% ethanol into it, and grind them thoroughly until a homogeneous slurry is formed. The ethanol serves as an extraction solvent to dissolve anthocyanins from the leaf tissues.

Next, use a disposable plastic dropper to transfer 1.5 mL of the slurry into a 1.5 mL microcentrifuge tube. Centrifuge at 14800 rpm for 10 minutes. After centrifugation, carefully pipette 0.5 mL of the supernatant of each type of leaves into two clean test tubes and label them for standby.

After that, add 10 mL of pH 1.0 KCl-HCl buffer and 10 mL of pH 4.5 sodium acetate-acetic acid buffer to each supernatant sample of old leaves, respectively. Repeat the same operation for the two supernatant samples of new leaves. Finally, analyze these samples in a spectrophotometer at 520 nm and 700 nm after 20-minute equilibration time, with the corresponding buffer solutions as blanks to calibrate the instrument.

2.2. Comparison of MDA Level Change in Red vs. Green Leaves After Light-Stress Treatment

A total of 10 *P. serratifolia* branches were collected from a nearby park in the early morning and separately kept in cups filled with tap water in darkness. On the next day, five of the branches were exposed to strong light from a 100W plant light for five hours, and the rest of branches were kept in darkness. 0.5 grams of the red and green leaves from the two groups were selected, respectively, with the midribs removed to ensure the uniformity of the leaf tissue. The four groups of sample leaves (red leaves with light treatment, red leaves in darkness, green leaves with light treatment, and green leaves in darkness) were respectively ground and homogenized with 5.0 mL of extraction solution from the MDA test kit (Suzhou Grace Biotechnology Co.,Ltd). For each sample, a total of 3.0 mL leaf extract was transferred to two 1.5 mL microcentrifuge tubes with disposable plastic dropper, and then centrifuged at 1200 rpm for 10 minutes. 400 μ L of the supernatant and 600 μ L of operating solution were added to a new 1.5 mL microcentrifuge tube, and incubated in a 90 °C water bath for 30 minutes to allow the reaction to proceed. After cooling, the samples were centrifuged at 1200 rpm for 10 minutes and the absorbance of supernatant at 532nm and 600nm were measured spectrophotometrically.

2.3. Observation of Larvae Feeding Preferences in Relation to Leaf Color

Ten *A. ningpoana* larvae were transferred to transparent plastic box A and B respectively, and ten *T. sinensis* larvae in box C and D respectively. Five fresh juvenile red leaves and five mature green leaves of similar sizes were mixed and placed in each of the four boxes, covering the larvae in the bottom. Box A and C were kept in a closed drawer so that the larvae were in complete darkness, while box B and D were kept on the top of the table with sufficient light. After thirty minutes, count the number of larvae feeding on each type of the leaves. Remove the leaves and leave the larvae in the empty box for thirty minutes. Add the new leaves and repeat the above experiment. A total of three sets of data were collected in around three hours.

3. Results

3.1. Anthocyanin Content

Anthocyanin content in juvenile red and mature green leaves was assessed by measuring absorbance at 500 nm (A_{520}) and 700 nm (A_{700}) under two pH conditions: pH 1.0 and pH 4.5. The difference in absorbance ($A_{520} - A_{700}$) and ΔA (difference between corrected absorbance at pH 1.0 and 4.5) was used to calculate the anthocyanin concentration in the extract and in the leaf tissue.

Leaf Type	pH	A_{520}	A_{700}	$A_{520}-A_{700}$	ΔA	Anthocyanin Conc. in Leaf Extract (μ g/mL)	Anthocyanin Content in Leaves (μ g/g)
Juvenile Red	1.0	0.639	0.050	0.589	0.468	164.1	328.2
	4.5	0.169	0.048	0.121			
Mature Green	1.0	0.470	0.110	0.360	0.002	0.7	1.4
	4.5	0.443	0.085	0.358			

Fig 1. Anthocyanin content was determined by pH differential method, which quantifies total anthocyanins based on the color change of pigments at pH 1.0 and pH 4.5. Absorbance was measured at 520 nm and 700 nm by a colorimeter, and results were expressed as cyanidin-3-glucoside equivalents.

As shown in Fig 1, at pH 1.0, juvenile red leaves had an A_{520} of 0.639 and A_{700} of 0.050, giving a corrected absorbance

of 0.589. At pH 4.5, the corrected absorbance dropped to 0.121. The resulting ΔA was 0.468. For mature green leaves,

the ΔA was only 0.002 (corrected absorbances: 0.360 at pH 1.0 and 0.358 at pH 4.5).

The anthocyanin concentration ($\mu\text{g/mL}$) in the extract was calculated using the following formula [13]:

$$\text{Anthocyanin concentration } (\mu\text{g/mL}) = \Delta A * \text{MW} * \text{DF} * 1000 \backslash (\epsilon * l)$$

Where:

$$\Delta A = (A_{500} - A_{700}) \text{ at pH 1.0} - (A_{500} - A_{700}) \text{ at pH 4.5}$$

MW = molecular weight of cyanidin-3-glucoside = 449.2 g/mol

DF = dilution factor = 21 (0.5 mL extract added to 10 mL buffer)

ϵ = molar absorptivity of cyanidin-3-glucoside = 26,900 $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$

l = path length = 1 cm

Using this formula, juvenile red leaves had an anthocyanin

concentration of 164.1 $\mu\text{g/mL}$, corresponding to 656.5 $\mu\text{g/g}$ fresh weight, and mature green leaves had an anthocyanin concentration of 0.7 $\mu\text{g/mL}$, corresponding to 2.8 $\mu\text{g/g}$ fresh weight. This means the juvenile red leaves contained approximately 234 times more anthocyanin than the mature green leaves ($656.5 \div 2.8 \approx 234.1 \times$), suggesting that the phenomenon of juvenile redding could be caused by anthocyanin accumulation.

3.2. MDA Content

The MDA content, an indicator of lipid peroxidation and oxidative stress, was measured in juvenile red and mature green leaves under two light conditions: darkness and light stress. The absorbance values at 532 nm and 600 nm were used to calculate ΔA ($A_{532} - A_{600}$), and MDA content was expressed in $\mu\text{g/g}$ fresh weight.

Leaf Type	Light Condition	A_{532}	A_{600}	ΔA	MDA Content in Leaves ($\mu\text{g/g}$)
Juvenile Red	Darkness	0.113	0.015	0.098	15.8
	Light Stress	0.130	0.028	0.102	16.5
Mature Green	Darkness	0.112	0.016	0.096	15.5
	Light Stress	0.169	0.027	0.142	22.9

Fig 2. MDA content was determined by pH differential method, which quantifies total MDA based on the difference in absorbance values at 532 nm and 600 nm.

As shown in Fig 2, the MDA content (nmol/g fresh weight) was calculated using the following formula [14]:

$$\text{MDA content (nmol/g fresh weight)} = [\Delta A \div (\epsilon \times d) \times V_2 \times 10^9] \div (W \times V_1 \div V) = 161.2 \times \Delta A$$

Where:

$$\Delta A = A_{532} - A_{600}$$

V = total volume of the extraction solution = 5.0 mL

V_1 = volume of sample extract used in the reaction = 0.4 mL

V_2 = total reaction volume (sample extract + working solution) = 1×10^{-3} L

ϵ = molar extinction coefficient of MDA = 155,000 $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$

d = path length of the cuvette = 1 cm

W = mass of the fresh sample (in grams) = 0.5 g

Using this formula, under dark conditions, juvenile red leaves exhibited a lower baseline MDA content of 15.8 nmol/g , compared to 17.1 nmol/g in mature green leaves. Upon exposure to light stress, MDA levels in juvenile red

leaves increased slightly to 16.5 nmol/g , a 4.4% increase, whereas mature green leaves showed a more substantial rise to 22.9 nmol/g , representing a 33.9% increase. The absolute increase in MDA was 0.7 nmol/g in juvenile red leaves and 5.8 nmol/g in mature green leaves, indicating that the oxidative response in mature green leaves was approximately 8.3 times greater than that of juvenile red leaves. These findings suggest that juvenile red leaves not only have a lower baseline level of oxidative stress but are also significantly less susceptible to light-induced oxidative stress, potentially due to the protective role of anthocyanins.

3.3. Larvae Feeding Preferences

To assess whether the light condition (Darkness vs. Light Stress) influenced the distribution of *A. ningpoana* and *T. sinensis* larvae on green leaves, red leaves, or no leaves, Chi-square tests of independence were performed.

Larvae Species	Light Condition	Box	Count (on green leaves : on red leaves : not on leaves)			Total Count	Overall Ratio
			1	2	3		
<i>A. ningpoana</i>	Darkness	A	6 : 1 : 3	7 : 2 : 1	7 : 3 : 0	20 : 6 : 4	3.3 : 1 : 0.7
	Light Stress	B	8 : 2 : 0	5 : 2 : 3	6 : 1 : 3	19 : 5 : 6	3.8 : 1 : 1.2
<i>T. sinensis</i>	Darkness	C	7 : 3 : 0	8 : 2 : 0	8 : 1 : 1	23 : 6 : 1	3.8 : 1 : 0.2
	Light Stress	D	8 : 0 : 2	5 : 3 : 2	6 : 3 : 1	19 : 6 : 5	3.2 : 1 : 0.8

Fig 3. Each treatment group (species $\times$ light condition) included three replicate boxes, each containing equal amounts of green and red leaves and 10 larvae. After exposure to the designated light condition, the number of larvae observed on green leaves, red leaves, or not on any leaf was recorded.

As shown in Fig 3, under both darkness and light stress conditions, larvae of both species showed a general preference for green leaves over red leaves. A binomial test proved that the preference for green leaves was statistically significant in all four groups. However, Chi-square test of

independence indicated that the difference in feeding preference between light conditions was not statistically significant for either species ($p > 0.05$).

For *A. ningpoana*, under darkness, larvae were distributed as 20 on green leaves, 6 on red leaves, and 4 not on leaves.

Under Light Stress, the distribution was 19 on green leaves, 5 on red leaves, and 6 not on leaves. The Chi-square test of independence revealed no statistically significant difference in leaf preference between light conditions ($\chi^2 = 0.517$, $df = 2$, $p = 0.772 > 0.05$).

For *T. sinensis*, under darkness, larvae were distributed as 23 on green leaves, 6 on red leaves, and 1 not on leaves. Under Light Stress, the distribution was 19 on green leaves, 6 on red leaves, and 5 not on leaves. The Chi-square test indicated no statistically significant difference in leaf preference between light conditions ($\chi^2 = 3.047$, $df = 2$, $p = 0.218 > 0.05$).

4. Discussion

This study investigated the evolutionary significance of juvenile reddening in *P. serratifolia* by testing the photoprotection hypothesis and aposematic signaling hypothesis. Our results demonstrate strong support for the photoprotection role of anthocyanins but no evidence for aposematic signaling in deterring the tested herbivores (*A. ningpoana* and *T. sinensis*).

The significantly higher anthocyanin concentration in juvenile red leaves (234-fold greater than in mature green leaves) correlated with a markedly reduced oxidative stress response under light stress. While green leaves exhibited a 33.9% increase in MDA after light exposure, red leaves showed only a 4.4% rise, suggesting anthocyanins effectively mitigated photooxidative damage. This aligns with prior studies showing anthocyanins act as light attenuators, absorbing excess blue-green light and scavenging reactive oxygen species (ROS). The 8.3-fold smaller MDA increase in red leaves underscores their superior photoprotective capacity, supporting the hypothesis that juvenile reddening in *P. serratifolia* adaptively shields developing chloroplasts from photoinhibition. However, this observational evidence alone cannot confirm a causal relationship—anthocyanins might co-occur with other unmeasured protective mechanisms such as enhanced antioxidant enzymes that independently reduce photooxidation.

Contrary to the aposematic hypothesis, larval preference for green leaves was unaffected by light conditions. Both *A. ningpoana* and *T. sinensis* consistently preferred green leaves regardless of illumination (binomial tests: all $p < 0.05$), with no significant shift in preference between darkness and light (Chi-square tests: $p > 0.05$). This implies that red coloration does not deter herbivores in this system, at least for these larval species, and visual cues may be secondary to other factors such as texture, volatile organic compounds in driving feeding choices. This is probably because the selected larvae species are more sensitive to chemical and sensational signals than visual signals.

Future studies could use anthocyanin-deficient mutants of *P. serratifolia* or apply biosynthetic inhibitors (e.g., phenylalanine ammonia-lyase inhibitors) to test if MDA levels rise when anthocyanins are absent. Additional herbivore species could be tested for more comprehensive study about aposematism. Laboratory conditions may not fully replicate natural herbivore behavior so field experiments could be conducted to validate these findings. To further strengthen the study's conclusions and better understand the ontogenetic trajectory of photoprotection and herbivore deterrence, future experiments should include multiple age groups of leaves (e.g., very young, intermediate, mature, and senescing leaves). This would allow us to track anthocyanin dynamics and photoprotection across development, as well as

test herbivore preferences across leaf ages.

5. Conclusion

Our experiments suggested that juvenile reddening in *P. serratifolia* likely functions as a light stress adaptation rather than an anti-herbivore signal, highlighting the context-dependent roles of plant pigments. Further work should integrate anthocyanin manipulation, developmental gradients and herbivore diversity to refine these evolutionary interpretations.

References

- [1] Kursar, T. A., & Coley, P. D. (1992). Delayed Greening in Tropical Leaves: An Antiherbivore Defense? *Biotropica*, 24(2), 256–262. <https://doi.org/10.2307/2388520>.
- [2] Chapman, C. A., Wrangham, R. W., Chapman, L. J., Kennard, D. K., & Zanne, A. E. (1999). Fruit and flower phenology at two sites in Kibale National Park, Uganda. *Journal of Tropical Ecology*, 15(2), 189–211. <https://doi.org/10.1017/S0266467499000759>.
- [3] Karageorgou, P., & Manetas, Y. (2006). The importance of being red when young: anthocyanins and the protection of young leaves of *Quercus coccifera* from insect herbivory and excess light. *Tree physiology*, 26(5), 613–621. <https://doi.org/10.1093/treephys/26.5.613>.
- [4] Lee, D. W., Brammeier, S., & Smith, A. P. (1987). The Selective Advantages of Anthocyanins in Developing Leaves of Mango and Cacao. *Biotropica*, 19(1), 40–49. <https://doi.org/10.2307/2388458>.
- [5] Cappellini, F., Marinelli, A., Toccaceli, M., Tonelli, C., & Petroni, K. (2021). Anthocyanins: From Mechanisms of Regulation in Plants to Health Benefits in Foods. *Frontiers in plant science*, 12, 748049. <https://doi.org/10.3389/fpls.2021.748049>.
- [6] Goodwin, T. W., T. W. Goodwin editor, 19651607717, English, Book, Chemistry and biochemistry of plant pigments, (xiii + 583 pp.), Academic Press, London & New York, Chemistry and biochemistry of plant pigments., (1965). <https://www.cabdigitallibrary.org/doi/full/10.5555/19651607717>.
- [7] Ranjan, S., Singh, R., Singh, M., Pathre, U. V., & Shirke, P. A. (2014). Characterizing photoinhibition and photosynthesis in juvenile-red versus mature-green leaves of *Jatropha curcas* L. *Plant physiology and biochemistry : PPB*, 79, 48–59. <https://doi.org/10.1016/j.plaphy.2014.03.007>.
- [8] Chapman, C. A., Wrangham, R. W., Chapman, L. J., Kennard, D. K., & Zanne, A. E. (1999). Fruit and flower phenology at two sites in Kibale National Park, Uganda. *Journal of Tropical Ecology*, 15(2), 189–211. <https://doi.org/10.1017/S0266467499000759>.
- [9] Zhang TJ, Chow WS, Liu XT, Zhang P, Liu N, Peng CL. A magic red coat on the surface of young leaves: anthocyanins distributed in trichome layer protect *Castanopsis fissa* leaves from photoinhibition. *Tree Physiol*. 2016 Oct;36(10):1296–1306. doi: 10.1093/treephys/tpw080. Epub 2016 Sep 10. PMID: 27614357. <https://pubmed.ncbi.nlm.nih.gov/27614357/>.
- [10] Gong, W. C., Liu, Y. H., Wang, C. M., Chen, Y. Q., Martin, K., & Meng, L. Z. (2020). Why Are There so Many Plant Species That Transiently Flush Young Leaves Red in the Tropics?. *Frontiers in plant science*, 11, 83. <https://doi.org/10.3389/fpls.2020.00083>.
- [11] Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative medicine and cellular longevity*, 2014, 360438. <https://doi.org/10.1155/2014/360438>.

- [12] Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutrition, metabolism, and cardiovascular diseases: NMCD, 15(4), 316–328. <https://doi.org/10.1016/j.numecd.2005.05.003>.
- [13] Jungmin Lee, Robert W Durst, Ronald E Wrolstad, Collaborators, Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study, Journal of AOAC INTERNATIONAL, Volume 88, Issue 5, 1 September 2005, Pages 1269–1278. <https://doi.org/10.1093/jaoac/88.5.1269>.
- [14] Hodges, D. M., DeLong, J. M., Forney, C. F., & Prange, R. K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta, 207(4), 604–611. <http://www.jstor.org/stable/23385611>.