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Trade-offs Shape Carotenoid-based Color Variation in Redheaded Pine Sawfly (*Neodiprion lecontei*) Larvae

Maranda Gaines

Mentor: Catherine Linnen

Abstract

Carotenoids serve various ecological roles in animals including coloration, immune responses, and vision. Carotenoid-derived coloration is greatly emphasized in the literature, particularly relating to mate choice and aposematic warning. However, the trade-offs between the color and non-color functions of carotenoids are not thoroughly explored. In the redheaded pine sawfly (*Neodiprion lecontei)*, some larval populations have yellow pigmentation, using carotenoids derived from their diets for aposematic warning coloration. Other larval populations are white in color, having genetically lost the ability to produce the yellow pigment. Because carotenoids are essential to life functions in both the yellow and white populations, we aim to explore the selective pressures favoring a loss of yellow coloration in white populations. We hypothesize that trade-offs between color and non-color functions have driven the white populations to stop producing yellow pigment, thereby retaining carotenoids for other life functions. Through analyses of field trip notes, we find that white larvae are more common on pitch pine (*Pinus rigida*) than on other pine hosts. After extracting and quantifying the carotenoids in multiple *N. lecontei* hosts, we find *P. rigida* has lower carotenoid content than other hosts. Our findings support the hypothesis that selection favors white populations who use limited carotenoid sources for non-color functions despite an increased predation risk.

Introduction

Variation in color phenotypes can be seen in nature between individuals of the same species, between populations of a species, and between closely related species. Scientists have attempted to understand the complex interplay between the genetics, ecology, and evolution of color variation over the past century (Cain et al. 1950; Gerould 1921). While there are many types of pigment that produce color in animals, we can group them into two categories: endogenous pigments synthesized by the animals themselves and exogenous pigments acquired from the diet. The exogenous pigments require a precursor from the environment, providing a source of influence on the dynamics of color evolution in these species depending on environmental precursors. Carotenoids are one of these exogenous pigments that, for most animals, must be taken in the diet. Animals cannot synthesize carotenoid molecules themselves but can modify them once obtained through the diet (Feltwell and Rothschild 2009, Ahmad and Pardini 1990). Some of the bright yellows, reds, oranges, and pinks visible in the plumage of birds, the coloration of butterfly wings, and other features of animals is attributed to carotenoids. Carotenoid-derived coloration serves an array of ecological functions. Specifically, carotenoid-derived coloration plays a critical role in mate choice, like in sexually dimorphic butterfly species and many bird species, acting as an indicator of good health and nutrition (Nijhout 2001; Shawkey et al. 2009; Pryke et al. 2002; Hill 1999). Carotenoid-derived coloration can also serve as warning signal, seen in prey species such as the ladybird beetle and her eggs (Blount et al. 2012; Winters et al. 2014). Outside of the function that carotenoids serve in coloration, research has also been devoted to their role in non-color life functions. Carotenoids are shown to play a role in pheromone production and precursors in butterflies and in vision of *Drosophila* and other invertebrate species (Andersson et al. 2007; Stavenga 1979). Scientists have shown that carotenoids are essential to life in all oxygenated environments (Britton 1993). Their role in oxygenated life may be in creating an immune response and reacting with reactive oxygen species (OJala et al. 2007; Babin et al. 2010; Smilanich et al. 2010). However, while there is an understanding of what ecological functions carotenoids are essential to, the trade-offs between these color and non-color functions have not been explored.

One system where carotenoid-derived coloration is observed in a non-mating context is in pine sawflies. *Neodiprion lecontei*, a species of redheaded pine sawfly, utilizes carotenoids in coloration at the larval stage. Present-day populations can be seen displaying a bright, highlighter yellow color, like their ancestral populations (Figure 1A). Because carotenoids cannot be synthesized by insects, they must be taken in through the diet to end up in the tissues of the insect. From ingestion, carotenoids are transported and processed from the gut into the hemolymph and finally deposited into the proper tissues. However, not all present-day populations of *N. lecontei* across the mid-Atlantic region utilize the carotenoids in their diet to achieve the yellow color. In fact, this pine sawfly species displays variation in color between its populations, ranging from bright yellow to soft white (Figure 1). It can be imagined that numerous genes are involved in processing and regulating carotenoids at each step from the diet and into the target tissues. Previous work demonstrates that the color difference between the yellow and white populations is genetically based, stemming from two or more mutations of these processing and regulatory genes in the white morph, resulting in loss of the ability to produce yellow pigment (Lindstedt et al. in prep). Another previous study addresses the function that larval color serves in this species: aposematic warning signaling. Both the yellow and white

populations of *N. lecontei* are shown to be effective warning signals against their avian predators, demonstrating the importance of larval coloration (Lindstedt et al. in prep).

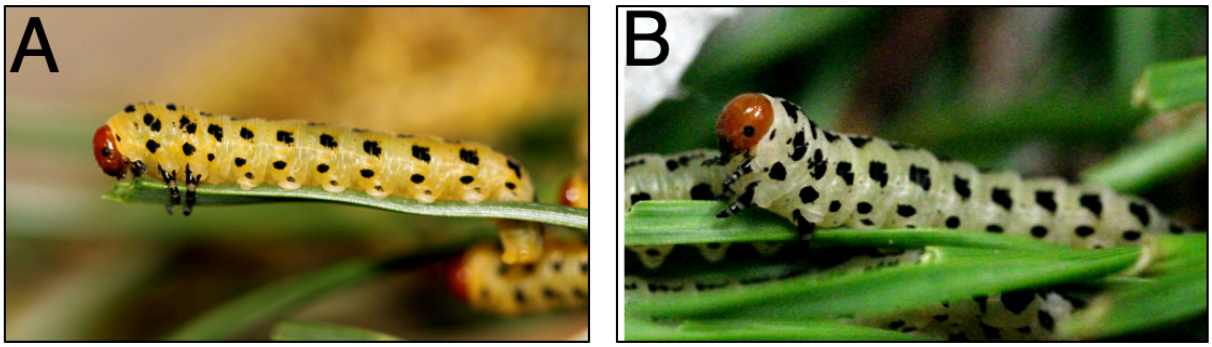

Figure 1. A. Yellow *N. lecontei* larva. **B.** White *N. lecontei* larva.

Despite an understanding of the genetics underlying how color variation exists between populations of *N. lecontei*, the puzzle remains: why did the white allele(s) rise to a high frequency in the mid-Atlantic area of *N. lecontei* amongst yellow populations? In successful aposematic warning signals, natural selection should strongly select against any deviations away from the norm, because aposematic warning signaling relies on strength in numbers (Cott 1940). In other words, selection should have acted against the white mutations when they arose in the yellow populations. What conditions in nature would allow this change in color rather than eliminate the white individuals standing out from the yellow aposematic populations? There are two possible explanations for this seemingly paradoxical existence of the white morph. The first is the principle of drift, which explains the formation of a new phenotypically different population from an older one by chance events of an isolated, small population (Wright 1970). This theory suggests that the white population was created by mutated individuals of the ancestral population surviving a random event to pass on their genes by chance, not because they were advantageous and selected for. All individuals of the new population would be white, simply because of inherited genes. However, the genetic drift explanation was ruled out by poopulation genetic data (Lindstedt et al. in prep). Because the principle of drift is not a possible explanation for the existence of a white morph, we focus on the second possible explanation: the persistence of the new white morph is due to the existence of an additional source of selection, acting in a way that compensates for the potential increase in predation. This suggests that after mutation, the new morph is favored due to a new benefit of losing the ability to produce yellow pigment and outweighs the cost of standing out from the yellow morph. Unlike the principle of drift, this explanation suggests that the white morph is advantageous, offering a benefit to the organism that would not exist as a yellow morph. Our research focuses on potential sources of selection outside of predation that may be responsible for driving the formation of the new white morph.

While the existing literature approaches the underlying causes of these color differences between populations of *N. lecontei* within the lab setting using genetics, we aim to bring the ecological factors of the field into our understanding of the existing variation. Since the white morph results from a mutation for loss of yellow pigment, their carotenoid resources may be devoted to other non-color functions. This brings to light the existing trade-offs between color and non-color functions of carotenoids in this system of pine sawflies. What pressures outside of predation

would shape color evolution of this species, affecting their allocation of carotenoids? We look to differences in the carotenoid sources amongst these populations as potential sources of selective pressures. The host trees where mating takes place and eggs are laid eventually comprise the diets of the larvae. This reasoning directs our research focus to possible differences in hosts between differently colored populations and therefore differences in diets between them. Carotenoid availability in host species across populations of *N. lecontei* may be the driving selective pressure favoring a change in carotenoid use and allowing the existence of a new color morph.

The purpose of our research is to understand why color variation exists between populations of *N. lecontei*. Because drift alone has been ruled out as the cause to the additional white color morph, we focus on the possibility that the more recently diverged white morph provides a fitness advantage that outweighs the cost of standing out amongst the aposematic warning coloration of the yellow morph. We look to the sources of carotenoids in this system, the pine tree hosts, and their role in influencing color evolution. As previously stated, carotenoids are essential to oxygenated life and play a vital role in both color and non-color functions. Warning coloration is costly and if carotenoids are a limited and valuable resource, devoting them to other life functions becomes a more efficient use by the organism rather than in coloration, resulting in a loss of yellow pigment. With this reasoning, we therefore hypothesize that selection favors larvae that avoid using carotenoids for pigmentation on hosts with reduced levels of carotenoids, resulting in the existence and persistence of white-bodied *N. lecontei* populations. To test this hypothesis, we must address two predictions. The first prediction is that the white populations are consistently found on the same host species. We analyze field notes of previous collection trips over the mid-Atlantic region to determine the relative number of yellow and white populations found on each type of host. If the white populations are consistently found on the same host species, we are able to address the second prediction, which states the hosts of the white population have fewer carotenoids than do the other hosts. To test this prediction, we extract and quantify the carotenoid content of all *N. lecontei* host species. The literature suggests that carotenoid content in some conifer species changes across seasons, with maximum carotenoid content levels reached during the winter months (Lewandowska 1977; Gerold 1959; Kaloudin 1967). To ensure that any differences in carotenoid content between species of pine are not due to the season in which the pine are sampled, we sample the same species and individuals in both the summer and fall. We also sample from the current and previous year's foliage of the same individual to ensure differences in carotenoid levels are not due to aging. We conclude with data analyses to determine the distributions of white and yellow larval populations across different hosts, and the relative amounts of carotenoids between these hosts. If the white populations are all found on the same host and this host has fewer carotenoids than do the other hosts in which yellow *N. lecontei* populations can be found, then it is possible that selection has favored these populations to reserve their limited carotenoids for other life functions rather than the yellow warning coloration.

Materials and Methods

Colony Distributions

We examined field notes from collecting trips of 2001-2004 and 2009-2016 to describe the distributions of white and yellow *N. lecontei* larvae populations. Because each colony typically includes offspring of one mated female and has a range in number of individuals, colony rather than individual was used as the unit of replication. Colonies of non-uniform color were noted, so three color categories were assigned to 823 colonies: white-bodied ($N = 26$), mixed-color ($N = 7$), or yellow-bodied ($N = 790$). Significance was determined using Fisher's Exact tests.

Pine Host Carotenoid Quantification

From the University of Kentucky Arboretum of Lexington, Kentucky, we sampled three common *N. lecontei* host species of pine: *Pinus rigida* or pitch, *Pinus echinata* or shortleaf, and *Pinus virginiana* or Virginia (Wilson et al. 1992 and Benjamin 1955). Three individuals from each host species were clipped at 5 random parts of the tree to account for any shading and height effects. This sampling was replicated in both the summer and fall of 2018 with the same individuals. Each clipping contained both old and young foliage, shown by a change in bark color from dark to light, often having lighter colored needles in the most recent year's foliage. Clippings were put in paper bags and then stored in a cooler bag at 4°C until processed.

To prepare for pigment analyses, needles from the oldest and youngest foliage of each clipping were separated from the bark. 2-5mm cuttings of the old and young needles were separately stored in 1.5 mL test tubes at -20°C until pigment extraction. Each host individual gave 5 clippings, with old and young needles on each, making 10 samples per host individual.

In order to quantify the carotenoids in each clipping sample, the carotenoids had to be extracted from the needle samples. We used a protocol adapted from Minocha et al. (2009). We separated 15 mg (±3 mg) of each sample of cuttings into 2.0 mL SealRite Microcentrifuge Tubes, and added 1500 μL of 100% ethanol. Samples were heated for 24 hours at 65°C in complete darkness. After 24 hours, samples were briefly vortexed at maximum speed and centrifuged for 5 minutes at 13500g (rcf). 200 μL of each supernatant was pipetted onto Fisher brand Flat Bottom 96 well clear, PS, Non-sterile plates and then replicated for a total of 3 wells of each supernatant. The plates were read using Gen5 (version 2.00.18) Biotek software at wavelengths of 664, 649, and 470 nm. Using the absorbances at the different wavelengths, chlorophyll A, chlorophyll B, and carotenoid content were calculated through predetermined equations for using 95% ethanol (Table 1).

Table 1. Equations used with each of the four solvents to calculate chlorophyll and total carotenoid concentrations.

Solvent	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	Total carotenoids
95% ethanol (Lichtenthaler 1987)	(13.36 ×A664) – (5.19 × A649)	(27.43 × A649) – (8.12 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A470 – 2.13Ca – 97.64Cb)/209
80% buffered acetone (Lichtenthaler 1987)	(12.25 × A664) – (2.79 × A647)	(21.50 × A647) – (5.10 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A470 – 1.82Ca – 85.02Cb)/198
100% DMSO Wellburn 1994	(12.47 × A665) – (3.62 × A649)	(25.06 × A649) – (6.50 × A665)	Chl *a* + Chl *b*	Cx+c = (1000A480 – 1.29Ca – 53.78Cb)/220
100% DMF (Porra et al. 1989); carotenoids (Wellburn 1994)	(12 × A664) – (3.11 × A647)	(20.78 × A647) – (4.88 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A480 – 1.12Ca – 34.07Cb)/245

Note: Cx+c, concentration of xanthophylls and carotenes; Ca, chlorophyll *a*; Cb, chlorophyll *b*.

Carotenoid Data Analysis

The absorbency readings were exported to Microsoft Excel (2018 version 16.16.7). We averaged the readings for each of the 3 repeats belonging to a single sample, leaving a single average each for young and old foliage for each clipping. These averages were used for significance tests. Using R software (RStudio version 1.1.462), data was tested for normality by a Shapiro-Wilk normality test to conclude that all datasets were normal before significance tests. Tukey's HSD tests were used to determine significance when comparing old and young foliage carotenoid content among hosts in both the summer and fall seasons. Paired t-tests were used to compare old and young foliage in hosts in both the summer and fall, and also to compare changes in hosts between seasons in both the old and young foliage.

Results

Colony Distributions

White larval populations were found on pitch pine more significantly than on shortleaf or Virginia ($p < 0.001$, Figure 2).

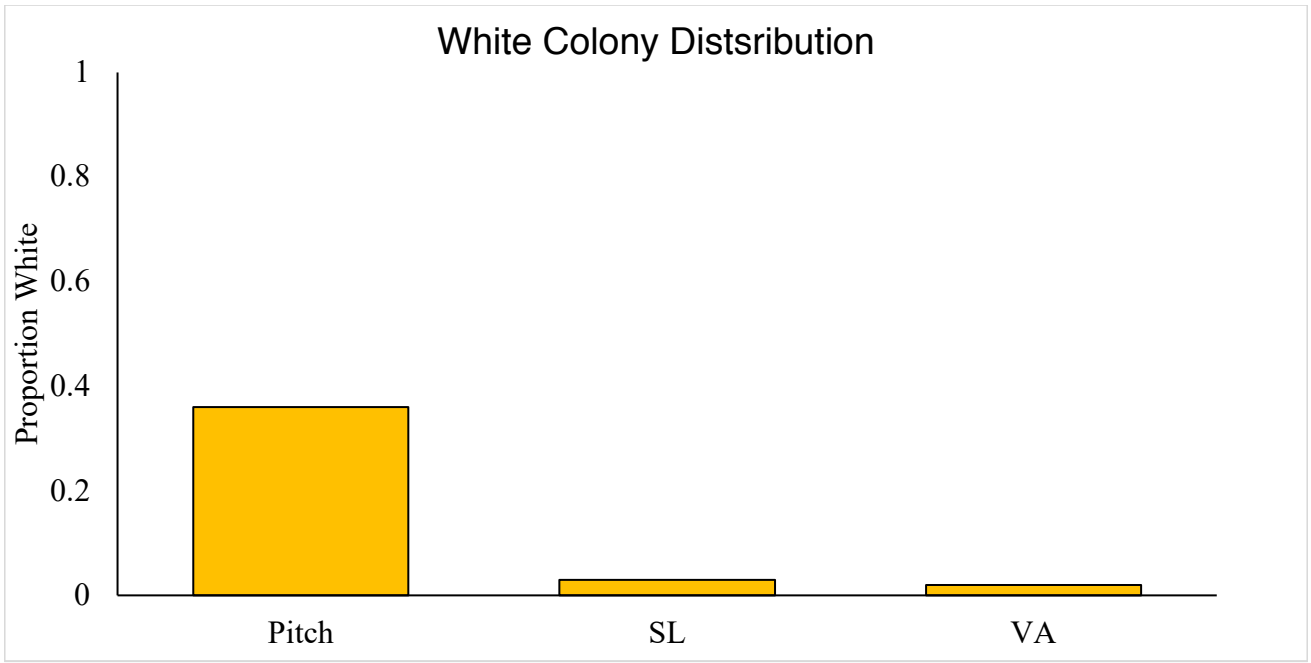

Figure 2. The proportion of white-bodied colonies (n=26) found on N. lecontei hosts *P. rigida* (pitch),*P. echinata*(SL) and *P.virginiana*(VA) across the mid-Atlantic coastal US region. The white larval colonies are found significantly more often on pitch pine than on either shortleaf or Virginia pine ($p < 1 \times 10^{-5}$, Fisher's exact tests).

Pine Host Carotenoid Quantification

In the summer, carotenoid content of young foliage was not significantly different between pitch and shortleaf ($p = 0.482$, Figure 3A) or between pitch and Virginia ($p = 0.599$, Figure 3A). However, the carotenoid content of old foliage showed to be significantly lower in pitch pine when compared to shortleaf and Virginia ($p < 0.001$, Figure 3B). Likewise, in the fall, the carotenoid content in the young foliage was not significantly different between pitch and shortleaf ($p = 0.064$, Figure 4A) or Virginia ($p = 0.132$, Figure 4A) but was significantly less in pitch when comparing to shortleaf and Virginia in the old foliage ($p < 0.001$, Figure 4B).

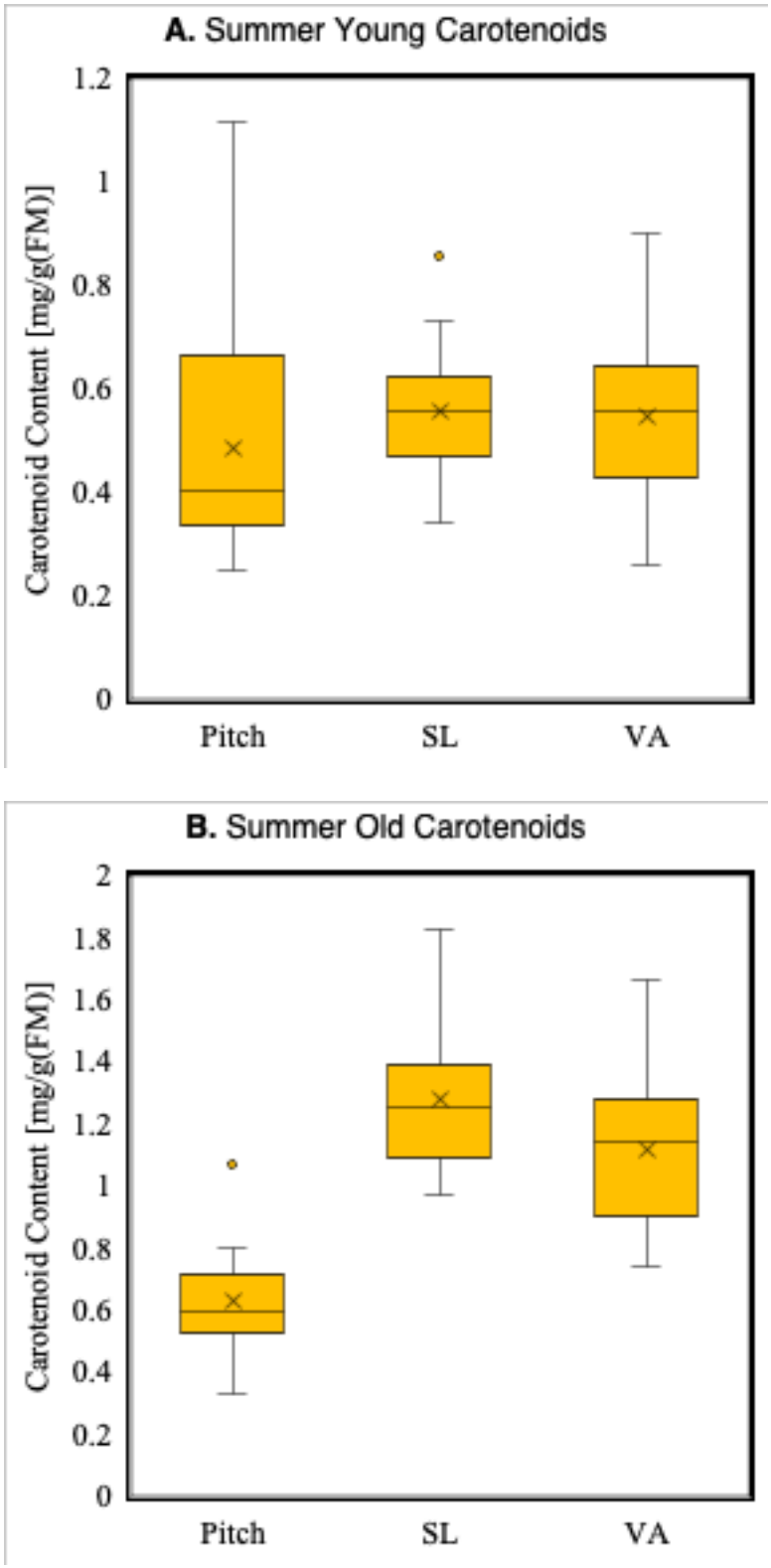

Figure 3. Carotenoid content of *P.rigida* (pitch),*P. echinata*(SL) and *P. virginiana*(VA) in pine needles collected in the summer of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum.

A. The carotenoid content of young foliage did not differ between pitch and shortleaf ($p = 0.482$, Tukey's HSD), pitch and virginia ($p = 0.599$, Tukey's HSD), or between shortleaf and virginia ($p = 0.980$, Tukey's HSD).

B. The carotenoid content of old foliage on pitch pine was significantly less than that of old foliage of both shortleaf and Virginia pines ($p < 0.001$, Tukey's HSD). Old foliage of shortleaf and Virginia pines did not differ significantly ($p = 0.121$, Tukey's HSD).

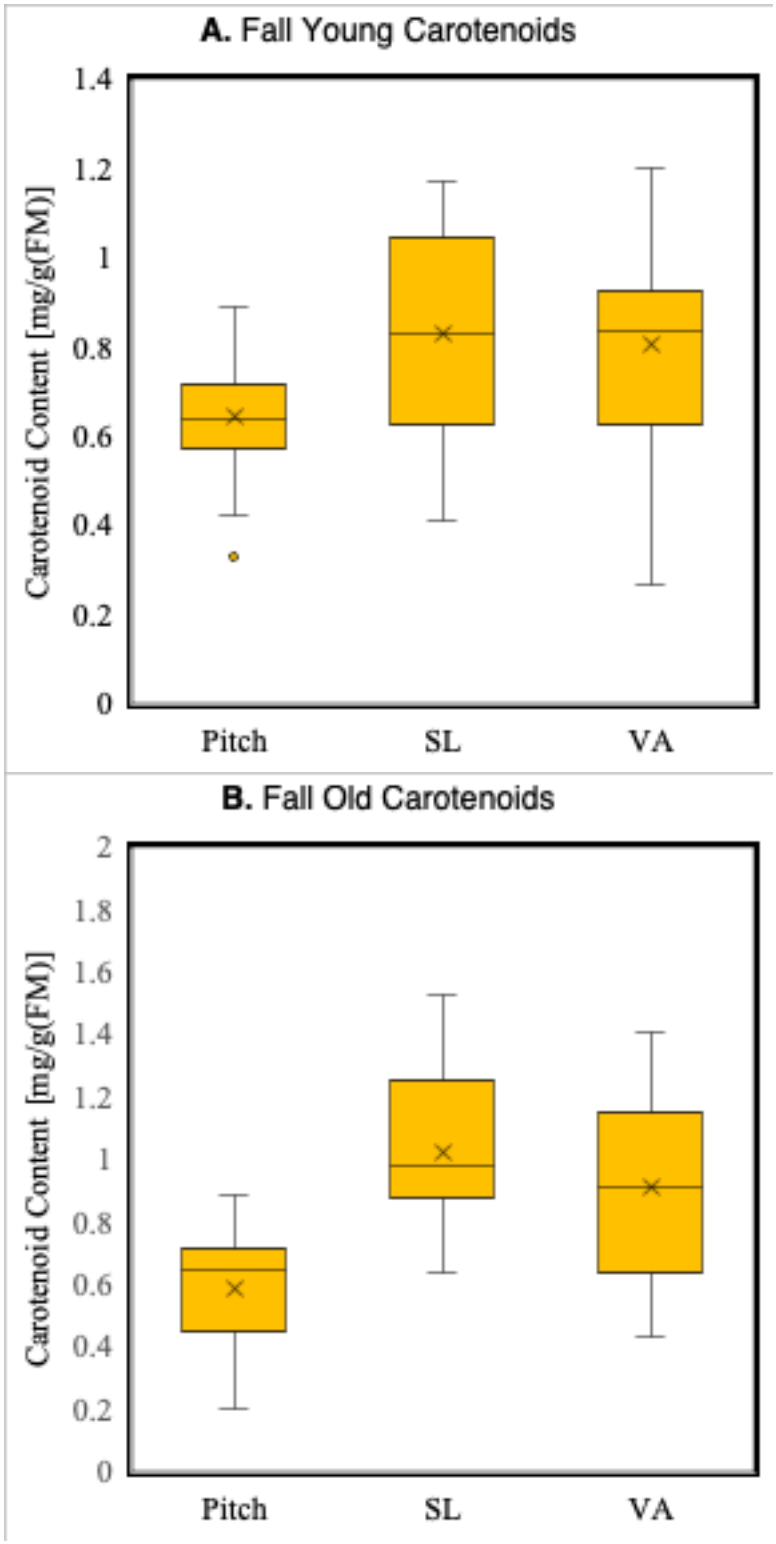

Figure 4. Carotenoid content of *P.rigida* (pitch),*P. echinata* (SL) and *P. virginiana*(VA) in pine needles collected in the fall of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum.

A. The carotenoid content of young foliage did not differ between pitch and shortleaf ($p = 0.064$, Tukey's HSD), pitch and virginia ($p = 0.132$, Tukey's HSD), or between shortleaf and virginia ($p = 0.935$, Tukey's HSD).

B. The carotenoid content of old foliage on pitch pine was significantly less than old foliage of shortleaf pine ($p < 0.001$, Tukey's HSD) and Virginia pine ($p = 0.002$, Tukey's HSD). Old foliage of shortleaf and Virginia pines did not differ significantly ($p = 0.463$, Tukey's HSD). Shortleaf and Virginia pines did not differ significantly ($p = 0.121$, Tukey's HSD).

In the summer season, all host species had significantly greater carotenoid content in the old foliage than in the young (pitch: $p = 0.013$, shortleaf: $p < 0.001$, Virginia: $p < 0.001$, Figure 5). By the fall season, only shortleaf had significantly greater carotenoid content in old foliage than in young foliage ($p = 0.036$, Figure 6) while pitch and Virginia did not have a significant difference between old and young foliage (pitch: $p = 0.424$, Virginia: $p = 0.081$, Figure 6).

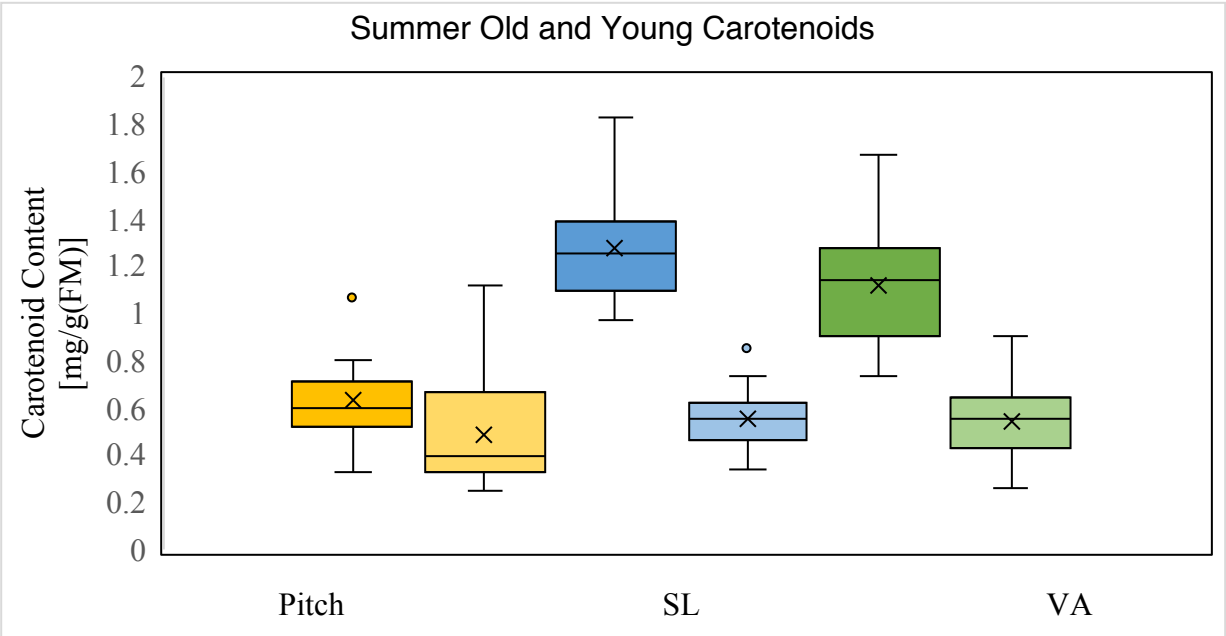

Figure 5. Carotenoid content of old and young foliage from *P.rigida* (pitch),*P. echinata*(SL) and *P. virginiana*(VA) in pine needles collected in the summer of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum. For each host, the left and darker box plot indicates old foliage while the right and lighter box plot indicates young foliage. The carotenoid content in pitch was significantly greater in old foliage than in young foliage ($p = 0.013$, paired t-test). The carotenoid content in shortleaf was significantly greater in old foliage than in young foliage ($p < 0.001$, paired t-test). The carotenoid content in Virginia was significantly greater in old foliage than in young foliage ($p < 0.001$, paired t-test).

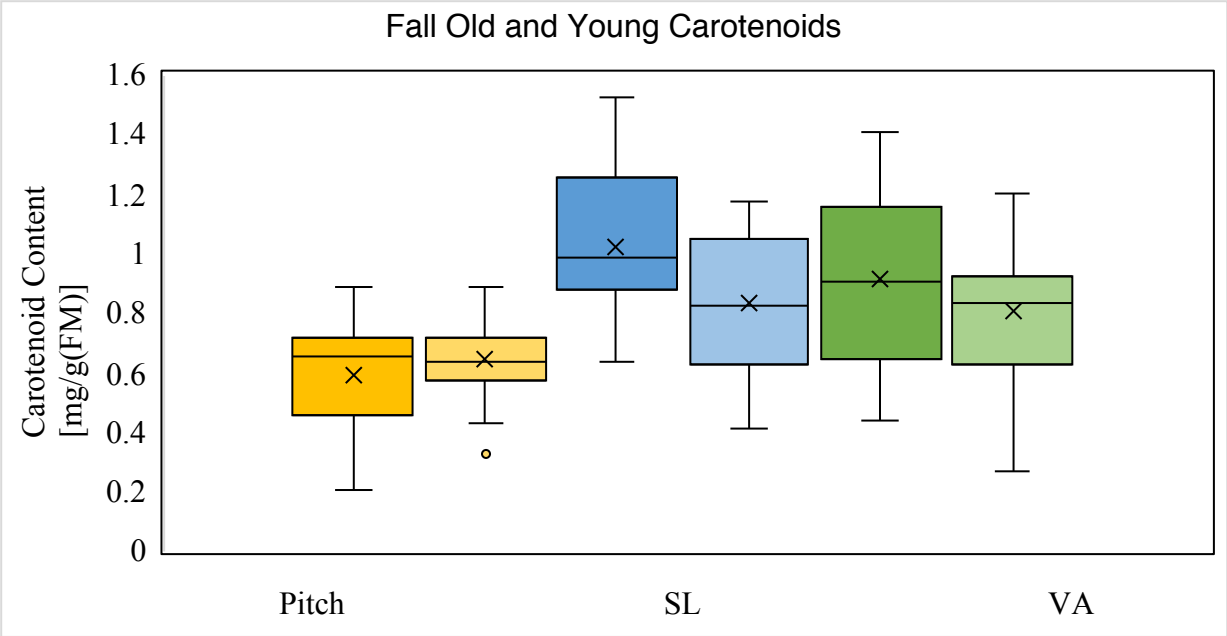

Figure 6. Carotenoid content of old and young foliage from *P.rigida* (pitch),*P. echinata*(SL) and *P. virginiana*(VA) in pine needles collected in the fall of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum. The carotenoid content in the old and young foliage did not differ significantly in pitch ($p = 0.424$, paired t-test). The carotenoid content in shortleaf was significantly greater in old foliage than in young foliage ($p = 0.036$, paired t-test). The carotenoid content in the old and young foliage did not differ significantly in Virginia ($p = 0.081$, paired t-test).

When comparing the host changes in carotenoid content between the summer and fall seasons, pitch pine old foliage did not significantly change between summer and fall ($p = 0.594$, Figure 7). The old foliage for both shortleaf and Virginia was significantly greater in the summer than in the fall ($p = 0.011$, $p = 0.025$, Figure 7). When looking at young foliage, all host species show significantly greater carotenoid content in the fall than in the summer (pitch: $p = 0.046$, shortleaf: $p = 0.002$, Virginia: $p = 0.005$, Figure 8).

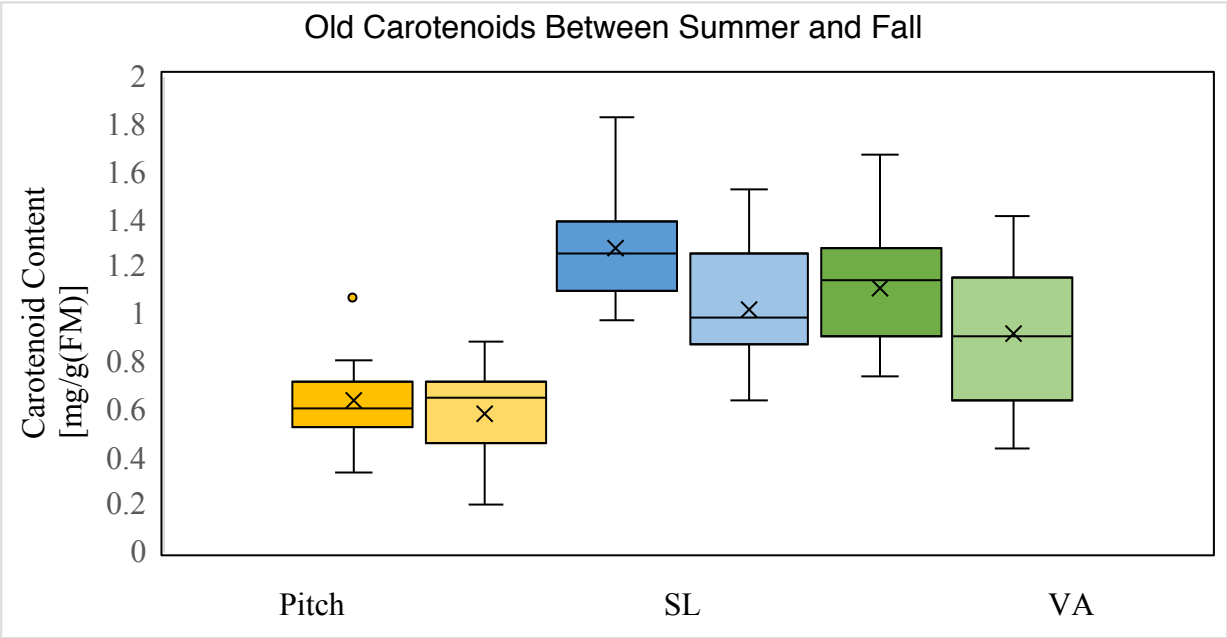

Figure 7. Carotenoid content of old foliage from *P.rigida* (pitch),*P. echinata*(SL) and *P. virginiana*(VA) in pine needles collected in the summer and fall of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum. For each host, the left and darker box plot indicates summer foliage while the right and lighter box plot indicates fall foliage. The carotenoid content between summer and fall did not differ significantly in pitch ($p = 0.594$, paired t-test). The carotenoid content from summer to fall decreases significantly in shortleaf ($p = 0.011$, paired t-test). The carotenoid content from summer to fall decreases significantly in Virginia ($p = 0.025$, paired t-test).

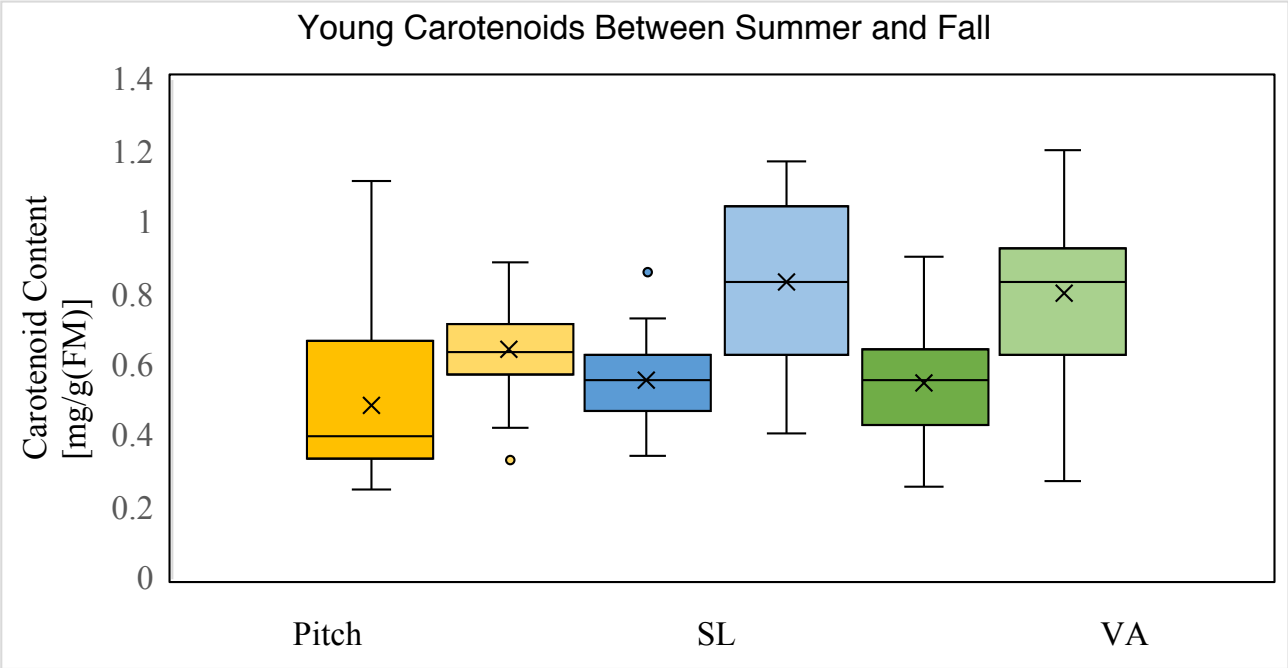

Figure 8. Carotenoid content of young foliage from *P.rigida* (pitch),*P. echinata*(SL) and *P. virginiana*(VA) in pine needles collected in the summer and fall of 2018 (N = 15 clippings each consisting of young and old foliage from 3 individuals of each host). Box plots show maximum, 75thpercentile, median (line), mean (x), 25thpercentile, and minimum. For each host, the left and darker box plot indicates summer foliage while the right and lighter box plot indicates fall foliage The carotenoid content from summer to fall increases significantly in pitch ($p = 0.046$, paired t-test). The carotenoid content from summer to fall increases significantly in shortleaf ($p = 0.002$, paired t-test). The carotenoid content from summer to fall increases significantly in Virginia ($p = 0.005$, paired t-test).

Discussion

Carotenoids play a key role in many color and non-color life functions such as aposematic warning coloration, coloration in mate choice, immune response, and vision. In our *Neodiprion lecontei* redheaded pine sawfly system, carotenoid-based coloration serves as yellow aposematic warning coloration in the larval populations. However, the more recently diverged white morph found in larval colonies across the mid-Atlantic US region have genetically lost the ability to produce this yellow pigment. To answer the question of the seemingly paradoxical existence of the white larval populations diverging from the ancestral yellow and aposematic larval populations, we look to additional selective pressures outside of predation. Specifically looking at host use, an additional selective pressure may favor the loss of the ability to produce carotenoid-derived coloration for a new benefit. Because the pine tree hosts comprise the diets of the pine sawfly, they in turn supply all the carotenoids available to the larvae. When

carotenoids are a limiting resource, there exists trade-offs between color and non-color functions. We hypothesized that trade-offs between color and non-color functions have driven the fixation of mutations that prevent white populations from producing yellow pigment, thereby retaining carotenoids for other life functions. It was predicted that white larval populations would consistently be found on the same host species, and that particular host species would have fewer carotenoid content than the other hosts, limiting the availability of carotenoids in the diets of the white larval populations.

In support of our hypothesis, our results showed that white larval colonies were proportioned more significantly on *P. rigida*, pitch pine (Figure 2). These results support that a shift in host use is a possible selective pressure driving the color evolution in this species. The results also show that carotenoid content of pitch pine was lower in both foliage sample collection seasons (summer and fall) than in shortleaf or Virginia pine when comparing old foliage (Figures 3B and 4B). This supports our hypothesis that selection has favored the mutation for a loss in the ability to produce carotenoid-based coloration because carotenoids are a limiting resource, allowing for the devotion of carotenoids for other non-color life functions. However, there was not a significant difference in any host species when comparing the young, most current year's foliage in neither the summer nor fall seasons (Figures 3A and 4A). Because we hypothesized that a limited carotenoid content in the diets of the larval populations would influence their color evolution, this points to the old, previous year's foliage being the source of carotenoids in the diet rather than the most recent year's foliage since these carotenoid content levels do not differ amongst the host pine species. Consistent with this finding, the literature indicates that *N. lecontei* females tend to lay their eggs in the previous year's foliage where the larva will eventually hatch and begin their feeding (Wilson, et al. 1992). Additionally, it has also been noted that the females will lay eggs in current year's foliage in the most southern regions where the growing season is longer and multiple new foliage blooms during the season (Wilson, et al. 1992).

Not only did our results address the color evolution of the redheaded pine sawfly, but they also provide further insight into animal evolution and the coevolution experienced with closely related species, such as its host. We can further examine our results in two different ways: (1) how the old and young foliage carotenoid content differ within each season separately, and (2) how summer and fall seasons compare in carotenoid content in each type of foliage, old or young, separately. In looking at our results through the first lens, we see that carotenoid content between the three hosts showed the old foliage to be greater than the young foliage in all host species during the summer season, further supporting the *N. lecontei* preferential use of old foliage (Figure 5). However, by the fall season, carotenoid content in old and young foliage tended to level out to be the same, with the exception of shortleaf (Figure 6). This presents a question as to how and why carotenoid levels change in plants, specifically in conifer species. The literature lacks an understanding in the seasonal changes in carotenoid content, although Lewandowska and Jarvis suggest that carotenoids reach a maximum in the winter in conifer species (1977). Despite this contrasting result, the disappearance we see in our results in the difference between young and old foliage as the season progresses could be attributed to the aging of the new foliage to more closely resemble the previous year's, and the trend may continue through the winter. This also warrants further investigation as to pine tree carotenoid functioning, regulation, and overall production relative to the season changes.

We can also attempt to understand our results through the second lens, comparing seasonal changes within foliage age. We find that when looking at the old foliage, the summer carotenoid content is greater than in the fall, with the exception of pitch carotenoid levels remaining the same (Figure 7). In contrast, in the young foliage, the opposite trend is observed as the carotenoid content is greater in the fall than in the summer (Figure 8). However, all values for the carotenoid content in young foliage are relatively lower than in old foliage with respect to season. With the female preference to lay eggs in old foliage in mind, this potentially comments on the idea of coevolution of the pine sawfly life cycle along with the host for the feeding stage to occur when its host contains a maximum level of carotenoids for the diet. Consistently, the pine sawflies are in a prepupa cocoon stage in winter, emerge in the spring, and adults appear several weeks later allowing mating and oviposition to occur (Wilson, et al. 1992). Roughly a month after oviposition, larvae appear and feed for several weeks, placing their most active and feeding stage in mid-summer, with a larger or smaller window depending on how south or north the region is (Wilson, et al. 1992). Our results may provide insight into the coevolution of animals and their hosts, as the life cycle of the species of interest and the seasonal changes of the host complement each other.

Limitations in our study include data collection, specifically in the randomization in the heights for foliage collection. The literature indicates variation in heights of female selection sites for egg laying. For instance, in red pine, eggs laid in shorter trees are usually in the lower half of the available foliage, whereas taller trees have eggs laid in the upper half of the foliage (Wilson, et al. 1992). Randomization of our selection for foliage at various heights and depths to limit any shading or height effects may or may not have been beneficial in collecting foliage that was actually a potential for larval feeding sites. Further studies could investigate variation in carotenoid content levels not only between species and among species, but within the same individual as well to be able to comment on why certain heights are favored over others on certain trees. In addition, our data set is limited by a non-representative sampling location and a small sample size. Our pine host sample collections took place at only one site in Lexington, Kentucky. While the Kentucky region is relatively in the middle of the northern most and southern most mid-Atlantic coastal US region, gathering samples from various locations across the region would expand our data set. A future direction could include sampling along the entire mid-Atlantic coastal US region to assess any differences within the host species.

Whereas the literature traditionally focuses on carotenoid-derived coloration in the context of mate choice and aposematism, our findings touch the surface of additional selective pressures shaping color evolution. Future directions for our study could explore the phenotypic plasticity of the color morphs, determining the extent to which yellow larval populations can brighten or dull their coloration with additional or limited carotenoid supplements in their diets.

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