Spring 5-1-2017

Comparison of Feather Pigments in Red-Winged Blackbirds (Agelaius phoenicus) Between Historical and Modern Samples

Genevieve Nuttall
University of Connecticut - Storrs, genevieve.nuttall@uconn.edu

Comparison of feather pigments in red-winged blackbirds (*Agelaius phoenicus*) between historical and modern samples

Genevieve N.B. Nuttall
Ecology and Evolutionary Biology
Spring 2017

ABSTRACT

Coloration is vital to birds; it is involved in mating, territorial display, communication, camouflage, and predation. Birds rely on their environment for the raw materials necessary to make most colors present in their feathers. As a result, habitat quality can have lifetime fitness consequences through the color pathway. Widescale habitat change has affected the quality of habitats accessible to birds worldwide. Consequently, the availability of pigment-containing resources within many altered habitats has shifted, leading to modification in the coloration of some birds' feathers. I hypothesized that the red pigmentation in the feather shoulders, or epaulets, of red-winged blackbirds (*Agelaius phoenicus*) may have changed in Connecticut's population over time. To test this hypothesis, I compared the carotenoid pigments of epaulet feathers from red-winged blackbird specimens collected around 1970 to those of feathers from birds captured in 2016. Historical feathers were sampled from museum specimens and were evaluated relative to new feathers taken from live birds. Feathers were visually scored for pigmentation intensity, and then carotenoids were extracted and identified using high performance liquid chromatography and spectrophotometry. The pigment profiles of the museum specimens show that the historical birds were able to retrieve and produce the expected pigments in their epaulets whereas the live birds lacked one of the two major carotenoids that constitute epaulet coloration. The habitat of the modern birds may not have had the adequate resources for the individuals to build all of the expected carotenoids in their epaulets. This observation could indicate the effect of habitat quality on carotenoid pigmentation in birds from 1970 to present day. To better understand this effect, analysis of temporal habitat change must be paired with a robust study of pigmentation change over time.

INTRODUCTION

The rainbow of colors that we see in birds' feathers is a complex entity, having origins in both feather structure and deposited pigments. Structural coloration, which often produces blue and green hues in birds, results from interactions between light and nanostructures in the integumentary tissues (McGraw et al. 2004). Pigmentary colors are primarily due to three types of compounds: melanins, porphyrins, and carotenoids (Hill and McGraw 2006). In birds, melanin compounds create brownish hues and are often used to provide structural integrity to flight feathers. Porphyrins are less common pigments that result in fluorescent feathers. Carotenoid compounds produce bright red, yellow, and orange hues that are often associated with male display in territorial ownership and mating for sexually dimorphic species (McGraw et al. 2004, Hill and McGraw 2006). These flashy colors are correlated with success of mating and territorial displays which can impact the reproductive success of birds (Olson et al. 1998, Saks et al. 2003, LaFountain et al. 2015).

Of the different pigment classes, carotenoids are the only class that birds do not produce themselves. Melanins are derived as byproducts of blood or bile, and porphyrins are built from amino acids (Hill and McGraw 2006). Carotenoids, which are produced by many plants and consumed by many organisms, are present in the diet of most passerine bird species. These birds forage on seeds, berries, and insects that contain carotenoids. An individual that uses carotenoid pigments in its feathers must consume the appropriate amount of carotenoid pigments to successfully produce feather coloration (Olson et al. 1998, Saks et al. 2003, Hill and McGraw 2006).

Once consumed by a bird, some carotenoids undergo biochemical transformation into ketocarotenoids before entering feathers (Hill and McGraw 2006). During this process,

carotenoids are first carried by blood to the intestinal mucosa where they are partitioned by micelles. They then move into the lymphatic system where they are partnered with lipoproteins which circulate through the body (Brush 1990, McGraw et al. 2004, Hill and McGraw 2006). Carotenoids bound to lipoproteins can be directed to adipose tissue, ovaries, egg yolk, and to the integument, which includes feathers (Smith 1972, Hill and McGraw 2006). In females of many species, carotenoids are needed to support reproductive physiology, while in males, the carotenoids are deposited in feathers which aid in sexual or territorial signaling (Smith 1972, Olson et al. 1998, Blount et al. 2002). Through this deposition process, the feather color can reflect the availability of carotenoids in the environment (Smith 1972, Saks et al. 2003).

There is evidence that the historical carotenoid sources available to birds in their environment have been altered due to habitat change. Habitat change can have a direct effect on bird pigmentation if it involves a shift in types or abundances of various carotenoid compounds that are used in feather coloration. One such example is habitat change via invasive plants that alter the plant community and offer new dietary sources, such as foreign berries, to birds. These new food sources may either increase the overall supply of carotenoids to animals, or may introduce altogether novel carotenoids to local ecosystems. It has been shown that the carotenoid pigmentation of cedar waxwings (*Bombycilla cedrorum*) has been affected by the invasion of Asian honeysuckle species (genus *Lonicera*) (Hudon and Brush 1989, Mulvihill et al. 1992, Witmer 1996). Cedar waxwings normally have yellow tail feather tips that are colored by canary-xanthopyll, a type of yellow-class carotenoid. In the late 1900s, some cedar waxwings started producing orange tail feathers that contained a different carotenoid called rhodoxanthin. Rhodoxanthin, which produces reddish hues in feathers, was detected in the berries of the invasive honeysuckle species that had become a new food source for cedar waxwings (Mulvihill

et al. 1992, Witmer 1996). This finding exemplifies the powerful link between habitat conditions and carotenoid pigmentation in birds. I aimed to determine if this link was evident in another system involving agricultural expansion and feather pigmentation.

Red-winged blackbirds (*Agelaius phoenicus*) are common birds to Connecticut. They reside in wetlands during the summer and use wetlands as their breeding habitat. Before the breeding season, males develop patches of bright red shoulder feathers, called epaulets. Males dynamically flash their red epaulets to other males in order to signal their territory and to display aggression. The epaulets therefore influence the success of a male in establishing territory and attracting females to mate (Smith 1972, McGraw et al. 2004). Once the breeding season has completed, red-winged blackbirds molt their feathers and migrate to their wintering grounds. Throughout the winter, these birds forage on seeds with carotenoids that contribute to the reconstruction of red epaulets for the next breeding season (Meanley and Bond 1969, Fraga 2017).

The epaulets of red-winged blackbirds are known to contain five carotenoid pigments (McGraw et al. 2004). Astaxanthin and canthaxanthin are the two most prevalent pigments, and they are responsible for the red coloration. These two pigments are derived from basic dietary carotenoids that the birds process in their bodies before depositing into feathers. The other three pigments, lutein, zeaxanthin, and canary-xanthopyll-A, are all associated with yellow coloration. Most lutein and zeaxanthin molecules remain unconverted when deposited into feathers. However, some lutein and zeaxanthin molecules may serve as precursors for the chemical transformation into the red keto-carotenoids, astaxanthin and canthaxanthin, which are then deposited in feathers (McGraw et al. 2004). This unique array of both dietary and transformed carotenoids in the epaulets of male red-winged blackbirds displays the importance of a male's

ability to retrieve multiple types of carotenoids from the environment. If the necessary carotenoid compounds are not sufficiently collected in the diet, a male may not be able to process the compounds in its body to make the bright red epaulets that are so crucial to its fitness.

Since the era of human development in North America, red-winged blackbirds have faced habitat change in both their wintering and breeding habitat. Historically, red-winged blackbirds fed on seeds of plants such as native filarees (genus *Erodium*) and grasses (genus *Echinochloa*) in the winter and various insects and seeds in the summer (Fraga 2017). These historical food sources offer the suite of carotenoids needed to create the expected epaulet pigmentation of males.

The expansion of agriculture has altered much of the natural habitat of red-winged blackbirds. During the winter, red-winged blackbirds now commonly forage in agricultural fields where they feed on seeds from maize (*Zea mays*), domesticated sunflowers (*Helianthus annuus*), invasive foxtail (*Alopecurus pratensis*), and other species associated with agriculture (Linz et al. 1984). These plants, or breeds, are not native, and they represent new food sources to red-winged blackbirds. It is possible that these plant species have different carotenoids in their seeds than the historical food sources of red-winged blackbirds. Since these food sources are consumed during the period in which males develop their epaulets for the upcoming breeding season, they have the potential to impact the coloration of epaulets (Meanley and Bond 1969, Homan et al. 1994, Fraga 2017).

The breeding habitat of red-winged blackbirds has also undergone major changes due to agricultural expansion. Huge proportions of wetlands were converted to farmland during the 1800s and 1900s to compensate for the growing human population. The wetland habitat of red-winged blackbirds was depleted in some areas and fragmented in others, leaving few natural

wetland areas left (United States Geological Survey 1996). In 1985, the United States government enacted the Farm Bill, which provided some protection to natural wetland habitat and promoted mitigation of converted wetlands (Glaser 1986). After this time period, many wetlands were restored by various degrees to a more "natural" state. Despite efforts to reverse wetland damage, the wetlands left in modern times are not fully representative of the environments that occurred before human interference (Tallisa et al. 2015). Today, many restored wetlands suffer invasions of non-native plant species such as purple loosestrife (*Lythrum salicaria*) and common reed (*Phragmites australis*) (Dahl 2000). These invasive plants can alter the ecosystem and disrupt the native plant and insect communities of wetlands (Batzer and Wissinger 1996, Chambers et al. 1999, Whitt et al. 1999, Saltonstall et al. 2005). Both wetland plants and insects are a food source for red-winged blackbirds in the summer, and changes in the plant or insect community could influence carotenoid availability.

The environmental change that has occurred in both the wintering and breeding grounds of red-winged blackbirds has the potential to shift the dietary, and thus carotenoid, sources available to these birds. To investigate this process, I examined whether carotenoids in epaulets of red-winged blackbirds have changed over time. Evidence of change in pigmentation could be indicative of habitat change and dietary alterations. To test this hypothesis, I evaluated how the epaulet color of male red-winged blackbirds has changed over the last fifty years by comparing the pigments in epaulets from preserved museum specimens that were collected around 1970 in Connecticut to living birds existing in similar locations. Different carotenoid pigment profiles suggest a differentiation in ability between past and present red-winged blackbirds to retrieve carotenoid sources from their altered environment.

METHODS

Museum Specimens

I sampled epaulets from three red-winged blackbird specimens held by the UConn Biodiversity Research Collections. I received permission from the curators of the vertebrate collections on February 9, 2016 to remove feathers from epaulets of three specimens. I chose three adult males in full breeding plumage that had been collected from Tolland County, Connecticut, USA between 1962 and 1973. To minimize destructive sampling of specimens, I limited sampling to approximately five individual epaulet feathers per specimen that were tucked beneath the black dorsal coverts of the folded specimen's right wing. This process left the overall epaulet visibly intact on the study skin. The sampled specimens were identified by accession number as UCMB 7116, UCMB 7247, and UCMB 5778. After collection, feathers were stored in a cool, dark environment to preserve the integrity of the light-sensitive carotenoids.

Live Birds

During the summer of 2016, I captured live birds using a standardized mist-net protocol (IACUC A14-009). With the help of technicians of the Saltmarsh Habitat and Avian Research Program, I captured birds in three locations in Connecticut. Our first study site was Barn Island, Stonington in New London County (N 41.329814, W 71.868392). At this site, one juvenile and two adult male red-winged blackbirds were captured. Our second site was Hammonassett State Park in Madison, New Haven County (N 41.256147, W 72.541155). One adult male was captured at this location. Our final site was located on Horsebarn Hill in Storrs, Tolland County (N 41.815211, W 72.251172). Here, I attempted to use a decoy to attract other males to the net. I caught five red-winged blackbirds at this location, but all five were either female or juvenile

birds. I used the three samples from adult male birds captured in Stonington and Madison for the pigment comparison analysis.

Visual Color Scoring

Before extracting carotenoids from the feather samples, I scored the color of the feathers based on a visual scale that I designed (Table 2). These feather colors were scored relative to each other from dullest to brightest, with a description to supplement the scoring. This information was used for qualitative comparison for simple observation on which males had the dullest or brightest feathers and what kind of color variation existed.

Carotenoid Extraction

To extract carotenoids from the feather samples, I followed a standardized protocol for extracting and analyzing carotenoids in museum specimens (LaFountain et al. 2010), which is reliable based on studies that exhibit the stability of carotenoids in well-preserved specimens (Armenta et al. 2008b, Doucet and Hill 2009, LaFountain et al. 2010). This procedure involves collecting approximately 1.5 mg of feather for each sample. The feathers were then covered with 2 ml of acidified pyridine and heated in boiling water for an hour. This step separated out the carotenoids from the epaulet feathers, leaving the feather keratin a brown color from the remaining melanin and the extract a red color from the carotenoids. To ensure that the extract was free of debris and acidified pyridine, I washed the feathers with hexane/acetone and water. Once the extract was cleaned, I transferred the solution to a lightproof vial to avoid pigment degradation by light. The samples were then dried down to a film and stored in a -20º C freezer.

Chromatography

To analyze the pigments in the samples, I used normal-phase high-performance liquid chromatography (HPLC). HPLC separates molecules in a mixture based on their chemical

structure and allows identification of the molecular components of the mixture. HPLC is commonly used to identify pigments, but it also has many other applications in identifying components of various biological samples, drugs, and DNA mixtures (Lindsay 1992). In this study, HPLC was used to separate astaxanthin and canthaxanthin – the two most prevalent carotenoids in red-winged blackbird epaulets – from feather samples. Pigments in the carotenoid class have similar carbon-based backbones with differing branched chains for each specific pigment. These differences in pigment structure result in slight differences in polarity between molecules, which is exploited via HPLC. During HPLC runs, each distinct pigment separates at a different retention time thus dividing a mixture of carotenoids in a sample into peaks of distinct molecular structure. The molecular peaks can then be identified by comparing the retention time and behavior of the HPLC results to a standard mixture containing the target carotenoids, astaxanthin and canthaxanthin.

The samples were diluted using a solvent of 86% hexane and 14% acetone and run through a HPLC machine (Agilent 1100 Series). After separation, UV spectra graphs were created for each peak and for the standards, and comparison to absorbance peaks was used to match molecules to components of the standard mixture. For example, the UV absorbance curve with a peak wavelength of 480 nm matches to canthaxanthin and a sample curve with similar behavior can be identified as canthaxanthin. All significant HPLC peaks that signaled a pigment were matched to spectra from carotenoid standards for identification. For each feather sample, some peaks could not be identified (i.e., no absorbance match). In these cases, the additional peaks can represent either other pigments (e.g., lutein) for which I did not have a standard, or cis-isomers of either canthaxanthin or astaxanthin that are degradation byproducts and were consequently ignored.

Data Processing

The HPLC instrument produced varying degrees of noise in each sample. To compensate for this noise, which inhibits the ability to detect significant peaks on the chromatogram, I created a background for the noisy baselines using a running median filter (Fried et al. 2014). Then, I subtracted the background noise level from the original chromatogram which gives a mean noise level. I calculated the square root of the median of the square to find an approximate standard deviation which was ~1.0 for each sample. I then figured out which peaks were "significantly" larger than the zero mean baseline noise level by taking approximately ten standard deviations. After this statistical transformation, the chromatogram clearly shows the peaks that are confidently large enough to represent a signal from a pigment or cis-isomer, rather than noise (Figure 1).

RESULTS

Of the 11 live birds captured and sampled, only three males exhibited full adult plumage. Of the three adult male samples, only two were successfully analyzed. The third live specimen sample had an unsuccessful extraction and analysis. These feather samples were compared to feather samples of three museum specimens (Table 1).

Visual Color Scoring

The results of the visual color scoring show that the feathers from the museum specimens had a brighter hue than the feathers from the three live samples. The three museum specimens had visual color ranks of 10, 10, and 8, respectively, with 10 being the brightest score (Table 2). All museum specimens were collected in either April or May. Of the live bird samples that were successfully analyzed, labeled L3 and L4, the color ranks were 6 and 7, respectively, while the

male bird that was unsuccessfully analyzed had a color rank score of 9. All live specimens were collected in June (Table 2).

Chromatography

HPLC separated pigments from feather samples based on retention time. For both of the live specimen samples, there was one strong pigment peak between 10 and 12 minutes (Figure 1). No other true carotenoid signals were present in these samples (additional peaks were identified as cis-isomers). The three museum specimens each produced more pigment signals. UCMB 5788 has true signal peaks around 9 and 15 minutes, UCMB 7116 near 6 and 11 minutes, and UCMB 7247 around 5 and 11 minutes (Figure 1). Due to run inconsistency in the HPLC machine, it is difficult to tell which peaks are associated with which pigments based on retention times, requiring identification based on the UV spectra (Figure 2, 3).

Based on UV spectra of samples and standards, the historical specimens contained carotenoids more similar to those expected from previous work on red-winged blackbirds. Canthaxanthin was detected in all three of the museum specimens but none of the live specimens (Figure 4a). Anthaxanthin was found in one of the museum specimens, UCMB 7247, and both of the live specimens (Figure 4b). This was the only carotenoid signal detected in either of the live specimens. In the two museum specimens that did not exhibit astaxanthin (UCMB 5788 and 7116), there was a signal from a different pigment that was neither canthaxanthin or astaxanthin. This unidentified signal was the same for both samples, and may represent a signal from the third most common carotenoid, lutein (Figure 4c). The true identification remains unknown, however, as a standard for lutein was not available for this study.

DISCUSSION

The results of this study show that there is variation in the environmentally-derived pigments deposited into epaulets by red-winged blackbirds. Although the sample size was not large enough to determine widescale trends, it is clear that the 1960s-1970s birds were able to retrieve, process, and deposit the pigments that are expected to be present in the feathers of red-winged blackbirds based on previous studies (McGraw et al. 2004). All three museum specimens had strong signals from either or both astaxanthin and canthaxanthin, which are the two pigments previously shown to be of highest concentration in blackbird epaulet feathers. They also have signals that most likely indicate known additional carotenoids found in epaulets, such as lutein. These patterns tell us that red-winged blackbirds of Tolland County were able to successfully acquire the expected carotenoids in their epaulet feathers around the year 1970. This result may indicate a healthy wetland environment around the area in which these birds were collected since they were able to find enough food sources with carotenoids to contribute to their bright pigmentation. This finding could be supported by proof of wetland habitat in the collection areas around the year 1970, but the available GIS information from the National Wetlands Inventory was incomplete and therefore no further inference could be made (National Wetlands Inventory 2017).

The results from the live specimen samples tell a different story. First, I was able to determine that females and juvenile red-winged blackbirds do not concentrate carotenoids in their epaulets. The feathers removed from female or young birds ($n = 8$) were lacking in coloration and even after melanin was removed from the feathers, the extract was clear, indicating that there were no carotenoids present in the feather samples.

The males from the live specimen samples had red feathers, but all were duller in color than the feathers from ~50-year old museum specimens. This observation suggests that there may be a difference in the ability of historical verses current birds to retrieve carotenoids from their environment.

It is possible that the environment of the red-winged blackbirds caused the differences in pigmentation between past and present individuals. The birds collected between 1962 and 1973 were from Tolland County, Connecticut which consists of inland wetland sites. The birds sampled in 2016 were captured in two coastal sites of Connecticut – one of which is a restored coastal wetland. The two wetland types may offer different dietary sources to red-winged blackbirds. Additionally, effects of the salt water environment in coastal wetlands could have caused feather wear in the epaulets of the live birds used for this study. As such, it is impossible with this small sample to separate the potential effects of time on carotenoid availability with the potential effects of space.

There also may be biological variation causing the feathers of live males collected in this study to be noticeably duller than feathers of male specimens. This difference could be attributed to the time of year of collection. The museum specimens were collected in April or May, when red-winged blackbirds are establishing their breeding territory and preparing to mate. It is possible that males allotting more energy to maintain carotenoid pigments for epaulet coloration during this critical time of year. The brighter a male's epaulet, the higher the chance of its success in establishing and defending a territory and therefore attracting mates (Smith 1972). Once a male has completed its mating season, it may spend less energy maintaining the hue of the epaulet in preparation for the inevitable molting of epaulets that occurs in autumn (Meanley and Bond 1969). Because the live males were caught towards the end of the breeding season in

June, it is possible that the brightness of their epaulets was no longer being maintained. This speculation requires further exploration and may provide insight on feather pigment retrieval patterns in red-winged blackbirds.

To explore this possibility further, the pigments found in the feathers from live samples should be further identified. The presence of the three yellow-producing carotenoids that serve as a precursor to canthaxanthin and astaxanthin in epaulet feathers may provide more information on which dietary carotenoids are available to red-winged blackbirds. Feathers from individual males could also be analyzed throughout the breeding season to determine how pigments change over the season within an individual. Using this information, it could be determined how much variation is caused by biological verses habitat factors.

Limitations

Through this experiment, I determined that the three specimens collected in the 1960s-1970s were able to retrieve and process the expected carotenoids from their environment, and the pigment profile from these samples match well to the known pigment profile stated in the literature. The two successfully analyzed samples from live birds showed that these birds did not have the same pigment profile as the museum specimens. The small sample size tells us some information about the trends we expect with pigment differentiation between historical and modern birds. However, to truly understand this system, we need to expand this study and sample more of both live birds and museum specimens.

To enhance the results from this study, all standards expected from the pigment profile stated in the literature should be run against the samples. In this study, only the two most concentrated expected pigments were used as samples. To determine if the samples have other

pigments, lutein, zeaxanthin, and canary-xanthopyll-A should also be run as standards. This addition would give us a better idea of which birds were able to retrieve which pigments.

Finally, the supplementary information on wetland habitat was not as available as planned when developing this project. There was no adequate GIS layer available for historical wetlands in Connecticut, so a comparison between historical land use and current land use could not be made. Without this information, we had no information about how the habitat of sampled live birds versus museum specimens had changed over time.

Conclusions

There was variation in the pigmentation of red-winged blackbirds that existed in different areas between 1970 and the present-day. This variation may be attributed to the widespread habitat changes that red-winged blackbirds have experienced. Although speculative, this possibility suggests that further exploration of the effect of habitat change on bird pigmentation would be worthwhile. As habitat change continues to threaten our planet, it is important that we understand how species will respond (Sinclair et al. 1995). This study provides a piece of information that will contribute to our understanding of this greater concept.

ACKNOWLEDGEMENTS

I would like to thank the advisors and mentors who made this project possible. First and foremost, I would like to thank Dr. Morgan Tingley who supported my project from start to finish. I also want to acknowledge Amy LaFountain, Randy Hamchand, Dr. Christian Bruckner, and Dr. Harry Frank for teaching and allowing me to use the chemistry equipment required for the project. Additionally, I want to thank Dr. Chris Elphick, Katharine Ruskin, and Samantha Apgar of the Saltmarsh Habitat & Avian Research Program who implemented the field work necessary to capture red-winged blackbird. I would also like to recognize Susan Hochgraf and Eric Schultz, members of the UConn Biodiversity Research Collections, for allowing me to access the red-winged blackbird samples. And, finally, I want to thank my lab mates, Nicholas Russo, Andrew Stillman, Austin Spence, Jacob Socolar, Manette Sandor, and Sarah Rumsey for helping me develop aspects of this project and thesis, and my friends and family for continuous support.

REFERENCES

Armenta, J., P. Dunn, and L. Whittingham. 2008b. Effects of specimen age on plumage color. The Auk 125:803-808.

Batzer, D.P. and S.A.Wissinger. 1996. Ecology of insect communities in nontidal wetlands. Annual Reviews Entomology 41:75-100.

Blount, J.D., P.F. Surai, D.C. Houston, and A.P. Moller. 2002. Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. Functional Ecology 16:445-453.

Brush, A.H. 1990. Metabolism of carotenoid pigments in birds. The FASEB Journal 4:2969-2977.

Chambers, R.M., L.A. Meyerson, and K. Saltonshall. 1999. Expansion of *Phragmites australis* into tidal wetlands of North America. Aquatic Botany 64:261-273.

Dahl, T.E. 2000. Status and trends of wetlands in the conterminous United States 1986 to 1997. U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C.

Doucet, S. M. and G.E. Hill. 2009. Do museum specimens accurately represent wild birds? A case study of carotenoid, melanin, and structural colours in long-tailed manakins *Chiroxiphia linearis*. Journal of Avian Biology 40:146-156.

Fraga, R. 2017. Red-winged Blackbird (*Agelaius phoeniceus*). Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona.

Fried, R. K. Schettlinger, and M. Borowski. 2014. robfilter: Robust Time Series Filters. R package version 4.1. https://CRAN.R-project.org/package=robfilter

Glaser, L.K. 1986. Provisions of the Food Security Act of 1985. United States Department of Agriculture Agriculture Information Bulletin Number 498.

Hill, G.E., and K.J. McGraw (Eds.). 2006. Bird Coloration, vol. 1: Mechanisms and Measurements. Harvard University Press, Cambridge, Massachusetts.

Homan, H.J., G.M. Linz, W.J. Bleier, and R.B. Carlson. 1994. Dietary comparisons of adult male common grackles, red-winged blackbirds, and yellow-headed blackbirds in North Central North Dakota. USDA National Wildlife Research Center - Staff Publications. Paper 840.

Hudon, J. and A. Brush. 1989. Probable dietary basis of a color variant of the cedar waxwing. Journal of Field Ornithology 60:361-368.

LaFountain, A.R., S. Kaligotla, S. Cawley, K.M. Reidl, S.J. Schwartz, H.A. Frank, and R.O. Prum. 2010. Novel methoxy-carotenoids from burgundy-colored plumage of Pompadour Contina *Xipholena punicea*. Archives of Biochemistry and Biophysics 504:142-153.

LaFountain, A., R.O. Prum, and H.A. Frank. 2015. Diversity, physiology, and evolution of avian plumage carotenoids and the role of carotenoid–protein interactions in plumage color appearance. Archives of Biochemistry and Biophysics 572:201-212.

Lindsay, S. 1992. High Performance Liquid Chromatography. Analytical Chemistry by Open Learning. Retrieved from http://books.google.com.

Linz, G.M., D.L. Vakoch, J.F. Cassel, and R.B. Carlson. 1984. Food of red-winged blackbirds, *Agelaius phoenicus*, in sunflower and corn fields. Canadian Field-Naturalist 98:38-44.

McGraw, K.J., K. Wakamatsu, A.B. Clark, and K. Yasukawa. 2004. Red-winged blackbirds *Agelaius phoeniceus* use carotenoid and melanin pigments to color their epaulets. Journal of Avian Biology 35:543-550.

Meanley, B. and G.M. Bond. 1969. Molts and plumages of the red-winged blackbird with particular reference to fall migration. Bird-banding 41:22-27.

Mulvihill, R., K. Parkes, R. Leberman, and D. Wood. 1992. Evidence supporting a dietary basis for orange-tipped rectrices in the cedar waxwing. Journal of Field Ornithology 63:212-216.

National Wetlands Inventory. 2017. Download Seamless Wetlands Data by State. U.S. Fish and Wildlife Service. Web.

Olson, V.A., and I.P.F. Owens. 1998. Costly sexual signals: are carotenoids rare, risky, or required? Trends in Ecology and Evolution 13:510-514.

Saks, L., K. McGraw, and P. Horak. 2003. How feather colour reflects its carotenoid content. Functional Ecology 17:555-561.

Saltonstall, K., D. Burdick, S. Miller, B. and Smith. 2005. Native and non-native *Phragmites*: challenges in identification, research, and management of the common reed. National Estuarine Research Reserve Technical Report Series 2005.

Sinclair, A.R.E., D.S. Hik, O.J. Schmitz, G.G.E. Scudder, D.H. Turpin, and N.C. Larter. 1995. Biodiversity and the need for habitat renewal. Ecological Applications 5:579-587.

Smith, D.G. 1972. The role of the epaulets in the red-winged blackbird (*Agelaius phoeniceus*) social system. Behaviour 41:251-268.

Tallisa, H., C.M. Kennedy, M. Ruckelshausc, J. Goldsteinb, and J.M. Kieseckerb. 2015. Mitigation for one & all: An integrated framework for mitigation of development impacts on biodiversity and ecosystem services. Environmental Impact Assessment Review 55:21–34

United States Geological Survey. 1996. National Water Summary on Wetland Resources. Water Supply Paper 2425.

Whitt, M.B., H.H. Prince, and R.R. Cox. 1999. Avian use of purple loosestrife dominated habitat relative to other vegetation types in a Lake Huron wetland complex. The Wilson Bulletin 1:105-114.

Witmer, M.C. 1996. Consequences of an alien shrub on the plumage coloration and ecology of cedar waxwings. The Auk 113:735-743.

TABLES AND FIGURES

Table 1 A list of live birds and museum specimens incorporated in this study. The age ASY refers to after second year, or adult, bird. HY is a hatch year bird, and SY is a second year bird.

Sample Number	Location	Town	County	State	Sex	Age
L1	Barn Island	Stonington	New London	CT	Male	SY
L2	Barn Island	Stonington	New London	CT	Male	ASY
L3	Barn Island	Stonington	New London	CT	Male	ASY
L4	Hammonassett	Madison	New Haven	CT	Male	ASY
L5	Horsebarn Hill	Mansfield	Tolland	CT	Female?	Juvenile
L6	Horsebarn Hill	Mansfield	Tolland	CT	Female	ASY
L7	Barn Island	Stonington	New London	CT	Unknown	HY
L9	Horsebarn Hill	Mansfield	Tolland	CT	Female	Juvenile
L10	Horsebarn Hill	Mansfield	Tolland	CT	Female	Juvenile
L11	Horsebarn Hill	Mansfield	Tolland	CT	Unknown	Juvenile
UCMB 7116	Unknown	Mansfield	Tolland	CT	Male	ASY
UCMB 7247	Unknown	Willimantic	Tolland	CT	Male	ASY
UCMB 5788	South Eagleville Road	Mansfield	Tolland	CT	Male	ASY

Table 2 Color score ranks of sampled feathers that were successfully analyzed. Extractions from all other samples were unsuccessful, possibly due to a low carotenoid concentration in some cases.

Sample Number	Color Rank	Visual Color Description	Date Collected
L3	6	Light orange	6/27/2016
L4	7	Orange	6/7/2016
UCMB 7116	10	Bright red	4/1/1969
UCMB 7247	10	Bright red	5/18/1973
UCMB 5788	8	Bright orange	4/15/1962

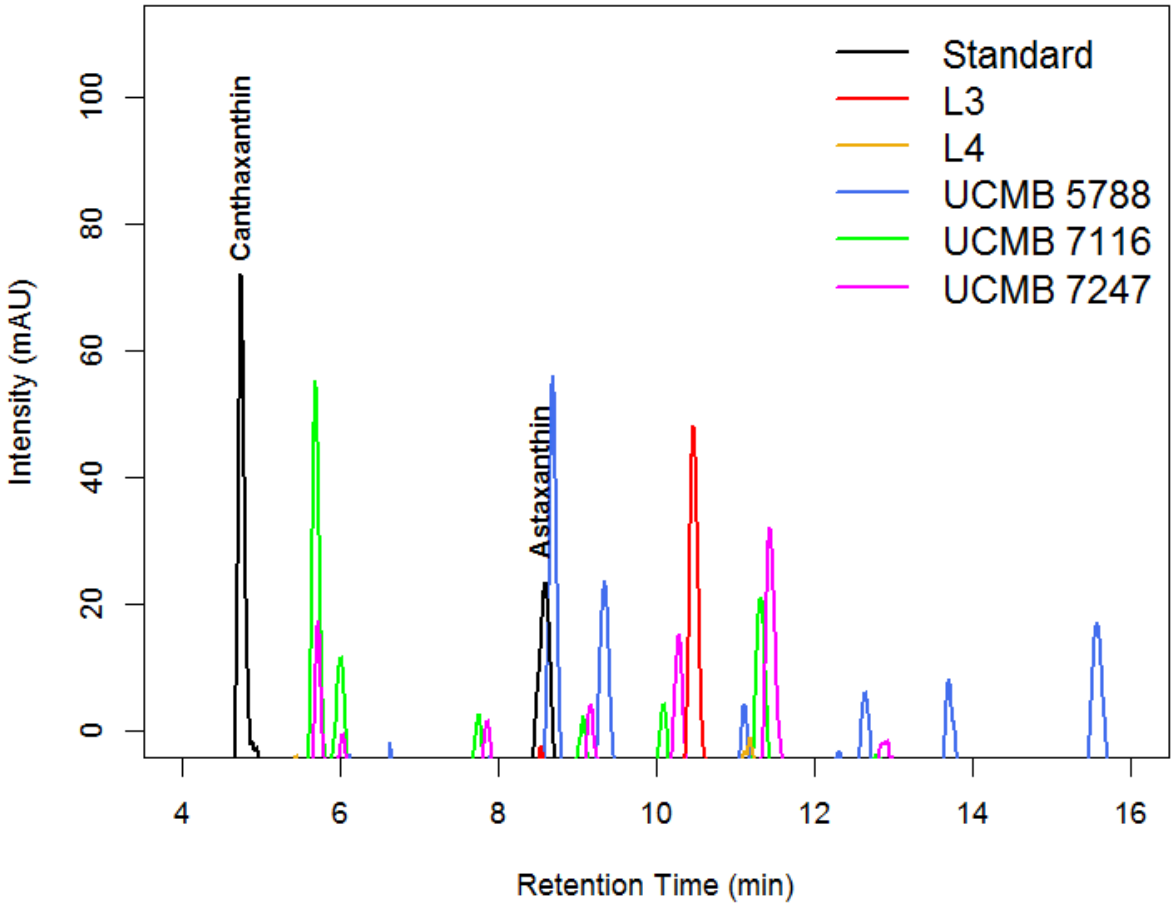

Figure 1 Chromatograms of the pigment separation from samples of red epaulet feathers of red-winged blackbirds in Connecticut. All chromatograms were compared to the standard sample, which consisted of canthaxanthin and astaxanthin. The two samples collected from live birds are labelled L3 and L4. The three samples collected from museum specimens are labelled by their museum accession number, UCMB 5788, UCMB 7116, and UCMB 7247. The chromatographic result for an individual sample is distinguishable by a distinct color. The tallest peaks for each sample represent true carotenoids signals. Smaller peaks are indicative of cis-isomers, which are degraded components of the true carotenoid parent molecule and can be ignored for this study. Pigments can be identified by comparing the retention time to that of one of the peaks in the standard. This procedure is not fully reliable and must be supplemented with additional comparison of UV spectra.

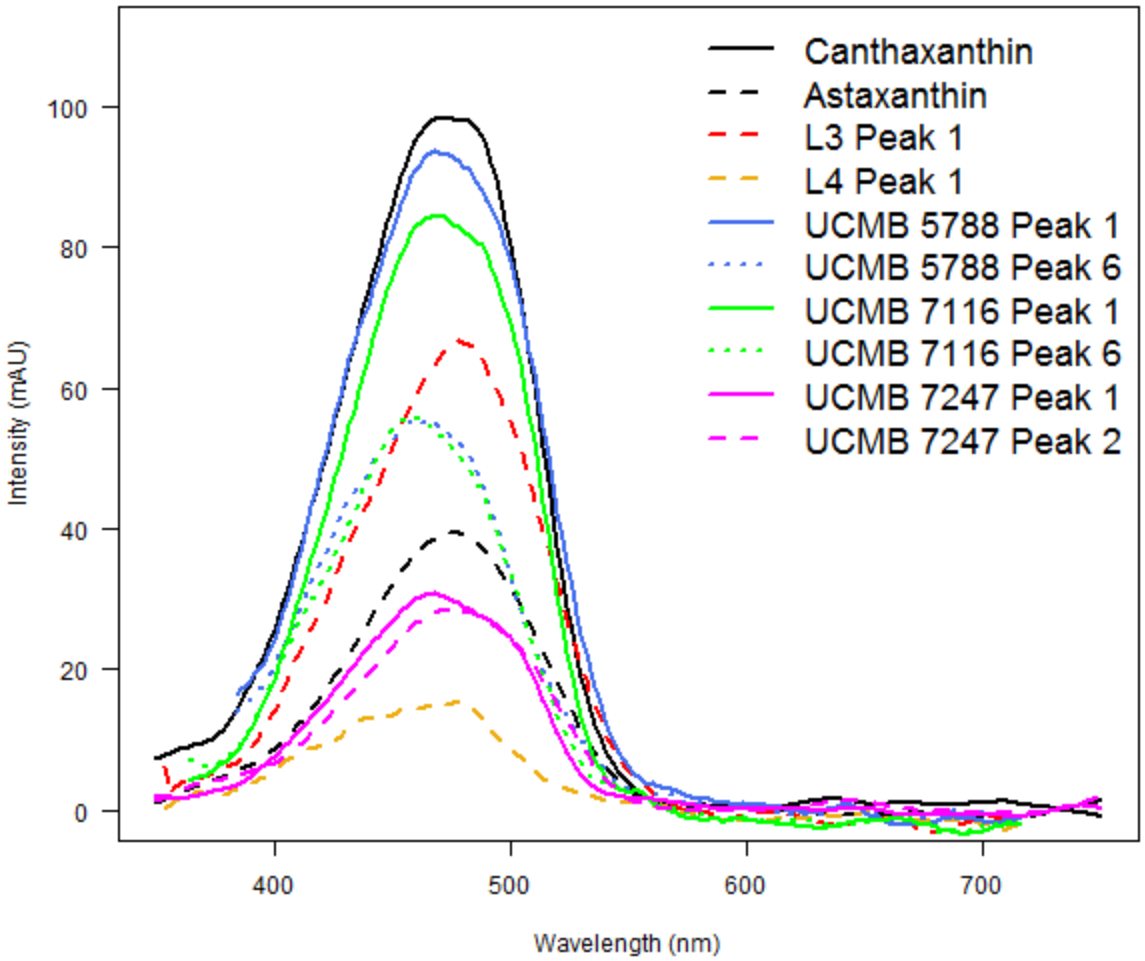

Figure 2 The UV Spectra graphs for each sample of epaulet feathers from red-winged blackbirds. The spectra graphs are combined into one plot and compared to the standards. For each of the tallest peak identified in the chromatogram, the spectra behavior of this peak identifies it as a standard pigment. Any peak that matches in wavelength and behavior to the standard can be identified as that standard pigment. There was one pigment found in each of the two live samples (L3 and L4). There were two pigments found in each of the museum specimens (UCMB 5788, UCMB 7116, and UCMB 7247). Any solid lines within the spectra for a given sample are identified as canthaxanthin. Any dashed lines are identified as astaxanthin. The dotted lines in UCMB 5788 and UCMB 7116 may be signals from lutein, another carotenoid found in the epaulets that was not tested for in this study.

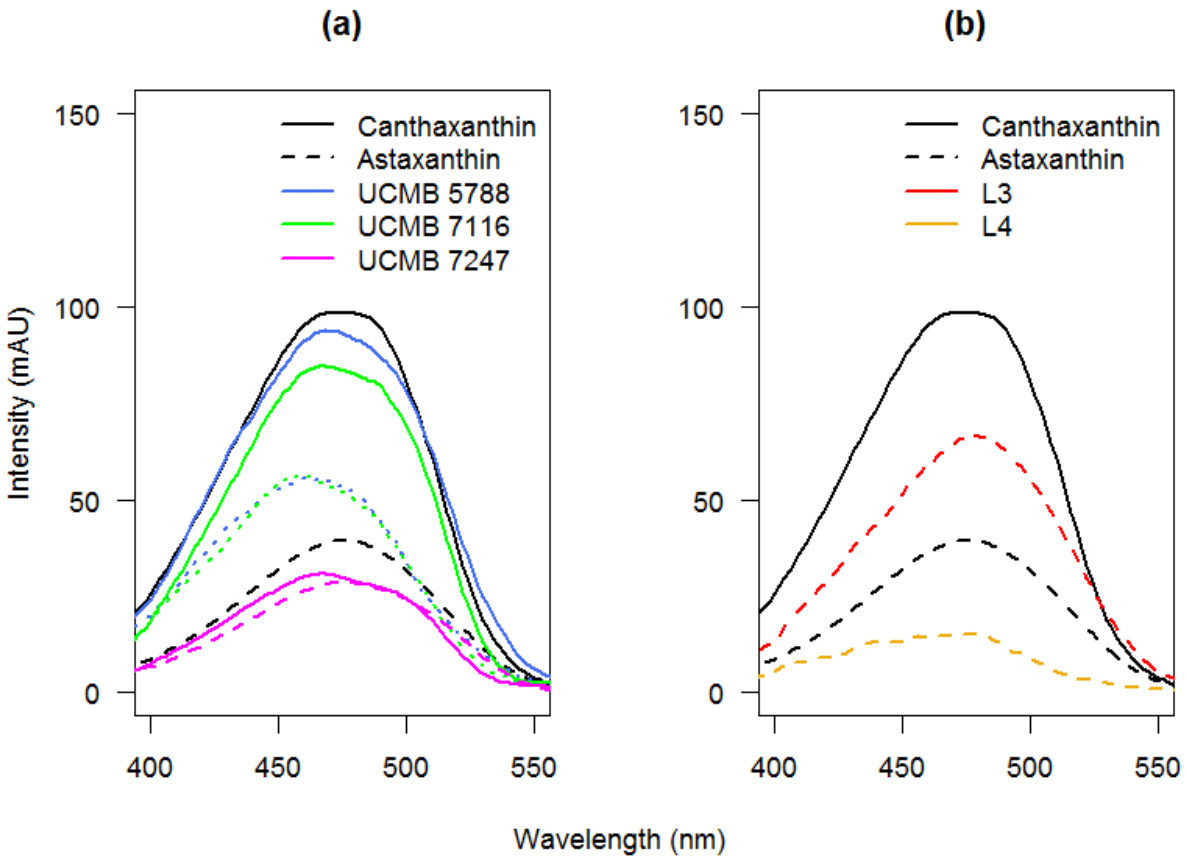

Figure 3 The components of the UV Spectra of the red-winged blackbird epaulet pigments for **(a)** the museum specimens and **(b)** the live specimens. The solid line represents canthaxanthin in a sample, and the dashed line represents astaxanthin. The dotted line represents a pigment that shows up in two of the museum specimens and may represent lutein, or another carotenoid in the epaulet feathers.

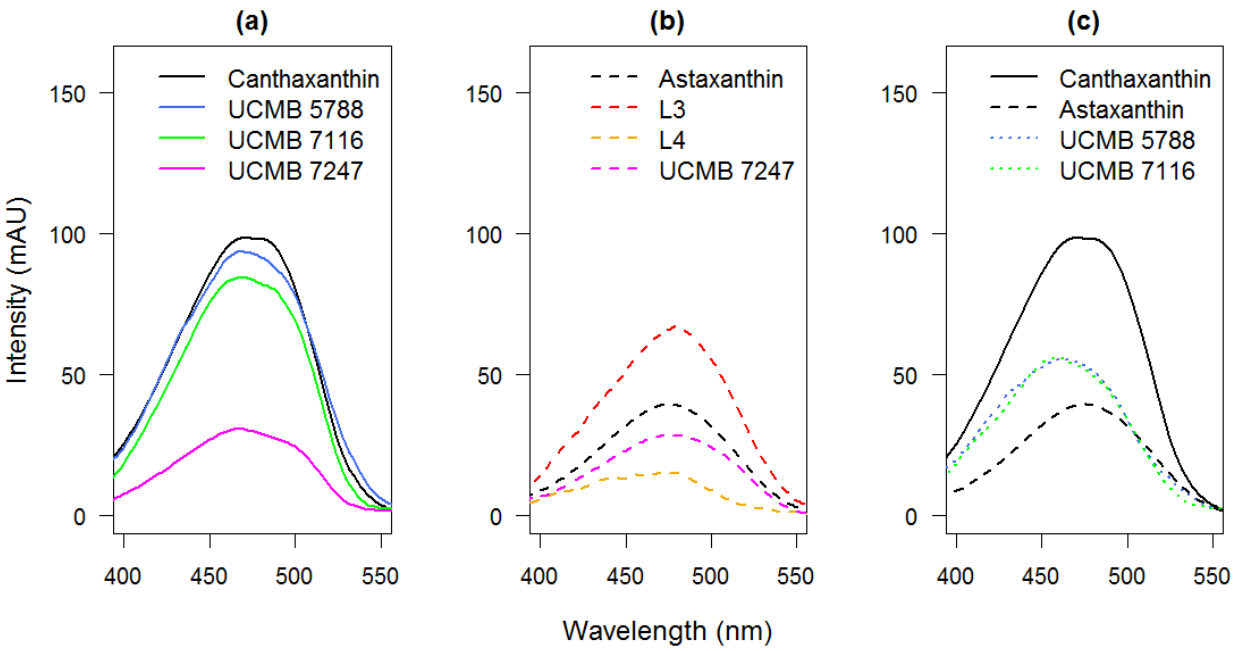

Figure 3 The results by pigment for the samples of red-winged blackbird epaulets. **(a)** Canthaxanthin was detected in all three museum specimens (UCMB 5788, UCMB 7116, and UCMB 7247) and none of the live samples (L3 and L4). **(b)** Astaxanthin was found in both live samples and one museum specimen (UCMB 7247). **(c)** A third pigment was identified in the epaulets of both UCMB 5788 and UCMB 7116. This third pigment is neither canthaxanthin nor astaxanthin and may represent another carotenoid, such as lutein, found in red-winged blackbird epaulets.