COMPARISON OF PLUMAGE INTENSITY IN FLORIDA GRASSHOPPER SPARROWS (*AMMODRAMUS SAVANNARUM FLORIDANUS*) WHEN GIVEN NEKTON-S OR NEKTON-GELB DIETARY SUPPLEMENT AT WHITE OAK CONSERVATION, WITH EVIDENCE OF SEXUAL PLUMAGE VARIATION

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Abstract

The Florida grasshopper sparrow (*Ammodramus savannarum floridanus*) is an endangered subspecies of the grasshopper sparrow complex endemic to the dry prairies of central Florida. Since 2019, White Oak Conservation (hereafter White Oak) has joined the U.S. Fish and Wildlife Service in an *ex situ* captive breeding program to save this grassland subspecies and has released approximately 300 birds into the wild to date. Florida grasshopper sparrows are predominantly brown, buff, and white, although they also bear yellow lores and wrists pigmented by carotenoids. Anecdotal evidence in the last three years suggests that wild Florida grasshopper sparrows show brighter yellow hues and darker overall plumage than their captive-bred counterparts. Here, we use a captive flock of Florida grasshopper sparrows at White Oak to evaluate whether the beta-carotene enriched dietary supplement NEKTON-GELB is more effective in encouraging bright plumage than the NEKTON-S dietary supplement currently used at White Oak. Thirteen hatch-year individuals were supplemented with NEKTON-S while seven hatch-year individuals were supplemented with NEKTON-GELB from August to the following February or March at which time all birds were photographed. Each individual was evaluated in a blind one-to-one comparison to evaluate which individual possessed brighter lores. This evaluation method was repeated for wrist intensity and overall plumage darkness. The respective win-ratios for each of the three characteristics were then reassociated with the individuals and their treatments. We found that NEKTON-S individuals had significantly yellower wrists than Nekton-GELB individuals (t_{17} = 2.11, P = 0.04), though lore intensity and overall darkness did not significantly differ. Having identified variation in lore and wrist intensity anecdotally associated with sex, we produced linear models for each characteristic utilizing sex and supplement as independent variables. Two-way ANOVAs revealed no interaction between sex and treatment nor any impact of treatment on plumage, but sex was found to significantly impact lore and wrist intensity (F_1 = 6.30, P = 0.02; F_1 = 8.26, P = 0.01). We found that males have significantly brighter lores and wrists than females, and that NEKTON-S and NEKTON-GELB do not differ significantly in encouraging intense plumage. Further research should continue the comparative study of NEKTON-S and NEKTON-GELB through the pre-basic molt to fully capture the plumage development cycle of Florida grasshopper sparrows. Additional future research should include validating the plumage differences between male and female Florida grasshopper sparrows and exploring the ecological and evolutionary source behind this variation.

Introduction

The Florida grasshopper sparrow (*Ammodramus savannarum floridanus*) is a subspecies of the greater grasshopper sparrow complex and has been described as the most endangered bird in North America (Jannot, 2017; USFWS, 2019). This sedentary subspecies is dependent on and endemic to Florida's dry prairie habitat and has suffered an estimated 81% loss of prairie habitat from 1900 to 1999, primarily to agricultural and silvicultural land conversion (Pranty & Tucker, 2006; Shriver & Vickery, 1999). Available habitat has continued to decline, and a fragmented distribution of 45,000 ha of appropriate habitat remain for the Florida grasshopper sparrow (Delany *et* al., 2007). The subspecies was classified as endangered by the U.S. Fish and Wildlife Service in 1986 (USFWS, 2019). Despite its listing, the Florida grasshopper sparrow continued to decline through the 1990s and 2000s (Hewett Ragheb *et al.,* 2019). In 2015, state and federal officials launched a captive breeding program, which White Oak Conservation joined in 2016 (USFWS, 2019). Beginning in 2019, captive-raised birds have been released into the wild population each summer, now totaling approximately 300 birds released (A. Schumann, personal communication, May 25, 2021).

As a ground-dwelling species, these birds are primarily feathered in shades of brown, tan, and black with ochraceous lores and wing edges concentrated at the carpal joint (hereafter wrists) (Vickery, 2020). These yellow feathers are pigmented by carotenoids, a compound Florida grasshopper sparrows cannot synthesize on their own but instead must ingest through their arthropod-heavy diet (Hõrak *et al*., 2001; Delany *et al*., 2000). Many species of songbirds are known to utilize melanin- and carotenoid-based sexual signaling to reflect mate-quality in breeding, including Great Tits (*Parus major*), American Goldfinches (*Spinus tristis*), Greenfinches (*Chloris chloris*), Zebra Finches (*Taeniopygia guttata*), and House Finches (*Haemorhous mexicanus*; Griffith *et al*., 2005; Hill, 2002). Carotenoid-based ornamentations are thought to be an honest signal in many bird species as they are part of the metabolic pathways for several immune-related compounds and have been shown to accurately predict parasite loads in birds (Griffith *et al*., 2005; Hill, 2002; Hõrak *et al.*, 2004). Because yellow pigments have been shown to be important indicators of individual quality in many species of birds, it is possible that the ochraceous wrists and lores of Florida grasshopper sparrows serve similar signaling functions. However, anecdotal evidence collected by White Oak has shown that the Florida grasshopper sparrows bred in captivity at White Oak show duller plumage than even the same individuals recaptured after a year in the wild (Figure 1).

Here we utilized a captive flock of Florida grasshopper sparrows at White Oak to determine if offering a diet higher in carotenoids results in producing birds with brighter ochraceous wrists and lores or darker overall body plumage. Thirteen sparrows received NEKTON-S dietary supplement from hatch while seven individuals received NEKTON-GELB dietary supplement, which is advertised to promote yellow feather growth and contain high levels of carotenoids (NEKTON, n.d.). In order to determine if NEKTON-GELB is a more effective supplement for encouraging plumage brightness, photographs of these birds were compared in a series of blind one-to-one comparisons and judged for the yellow hue intensity of the wrists and lores as well as overall body darkness.

Materials and Methods

Diet and Supplementation

All sparrows in this study were offered at least 1 tsp of commercial passerine seed and insectivore grain (Higgins Supreme Finch Seed, Higgins Premium Pet Foods, Miami, FL 33167; Mazuri Insectivore Diet, Mazuri Exotic Animal Nutrition, Richmond, IN 47374). For birds in enclosures with multiple other birds, additional finch seed and insectivore diet was offered such that all birds received at least 1 tsp finch seed and at least 0.5 tsp insectivore diet when diets were divided evenly between birds. Additionally, two types of feeder insects were offered twice daily: crickets (*Acheta domesticus*) and mealworms (larval beetle *Tenebrio molitor*) supplied by Premium Crickets (Winder, GA 30680). All insect diet components were dusted with NEKTON-S (n = 13 individuals) or NEKTON-GELB (n = 7 individuals) beginning August 8, 2020 (Nekton USA, Arcata, CA 95521). Prior to August 8, all individuals were receiving NEKTON-S dusted insects from hatch to the trial start date.

NEKTON-S supplement was given at a rate of 1 g of supplement per 100 g of insect diet. NEKTON-GELB supplement was given at the higher rate of 1.33 g of supplement per 100 g of insect diet to compensate for the decreased concentration of vitamins per g of supplement compared to NEKTON-S (Table 1).

Plumage Photography and Comparison

All individuals were photographed as second-years during their pre-shipment veterinary exams prior to transfer from White Oak in February and March of 2021 while in pre-alternate molt or partial alternate and basic plumage. Photographs were taken with a Canon EOS20D with a Canon 75mm lens of birds held in photographer's grip. All birds were photographed in front of a white tri-fold background in the same room a standardized distance from the camera and background. All birds were photographed to show the anterior, dorsal, and lateral view (Figure 2).

Photographs were edited to obscure the identifying color bands and the hands of the handler, and each individual was assigned a random four-digit identifying number. One-to-one blind comparisons were made determining individually which of the two birds possessed 1) brighter lores, 2) brighter wrists, and 3) darker overall plumage (Figure 2). Comparisons were conducted in two batches aligned with the two sets of veterinary exams. In the first, all seven NEKTON-GELB individuals were compared with five NEKTON-S individuals. In the second set of comparisons, four NEKTON-GELB individuals were reassigned new identification numbers and compared with eight new and two repeated NEKTON-S individuals. Once all individuals were compared to all other individuals, the birds were identified using their identification numbers.

Statistical Analysis

Birds were scored based on the number of comparisons "won" out of total number of comparisons for each of the three considered traits. These win-ratios for lores, wrists, and overall darkness between NEKTON-S and NEKTON-GELB individuals were analyzed using the two-sample t-test assuming unequal variance in R using the package "tidyverse" (R Core Team, 2021; Wickham *et al*., 2019). Secondary analyses were conducted treating sex as an additional variable. Using the package "lmtest," data for each characteristic were evaluated for normality and homogeneity of variance using the Shapiro-Wilk normality test and the Breusch-Pagan Test for Heteroscedasticity (Zeileis & Hothorn, 2002). Then, three two-way ANOVAs were run evaluating how sex,

supplement, and the interaction of sex and supplement impact lore intensity, wrist intensity, and overall darkness.

Results

NEKTON-S individuals had significantly yellower wrists than Nekton Gelb individuals ($t_{17} = 2.25$, $P = 0.04$). NEKTON-GELB and NEKTON-S individuals did not differ significantly in lore intensity or overall darkness ($t_{14} = 0.47$, $P = 0.65$; $t_{13} = 1.38$, $P = 0.19$, respectively; Figure 3).

The win-ratios for lore intensity were found to have both normal distribution and homogeneity of variance ($W = 0.95$, $P = 0.34$; $BP_3 = 1.78$, $P = 0.62$). A two-way ANOVA revealed no significant interactions between sex and treatment ($F_1 = 0.49$, $P = 0.49$), and found sex but not treatment significantly impacted lore intensity ($F_1 = 6.30$, $P = 0.02$; $F_1 = 0.06$, $P = 0.81$, respectively; Figure 4).

The win-ratios for wrist intensity were found to have both normal distribution and homogeneity of variance ($W = 0.94$, $P = 0.28$; $BP_3 = 7.54$, $P = 0.06$). A two-way ANOVA revealed no significant interactions between sex and treatment ($F_1 = 0.20$, $P = 0.66$), and found sex but not treatment significantly impacted wrist intensity ($F_1 = 8.26$, $P = 0.01$; $F_1 = 3.79$, $P = 0.06$, respectively; Figure 4).

The win-ratios for overall darkness were found to have both normal distribution and homogeneity of variance ($W = 0.91$, $P = 0.06$; $BP_3 = 4.18$, $P = 0.24$). A two-way ANOVA revealed no significant interactions between sex and treatment ($F_1 = 0.02$, $P = 0.88$), and found neither sex nor treatment significantly impacted overall darkness ($F_1 = 2.95$, $P = 0.11$; $F_1 = 1.43$, $P = 0.25$, respectively; Figure 4).

Discussion and Conclusions

Preliminary analysis indicated that NEKTON-S individuals developed yellower wrists than NEKTON-GELB individuals. However, considering the minute but noticeable plumage differences observed between males and females, the varying sex ratios in each batch of comparisons and in each treatment group posed a confounding variable that necessitated further analysis. When sex was evaluated as another variable impacting plumage intensity, the only variable found to impact lore and wrist intensity was sex, not treatment. With this relationship identified, the findings of the initial t-test between all NEKTON-S and all NEKTON-GELB individuals become less robust as the sex ratios of the two groups vary, likely belying the perceived significant differences. This study does however present novel evidence that male Florida grasshopper sparrows possess brighter lores and wrists than their monomorphic female counterparts. Finally, no variables were identified that significantly impacted overall plumage darkness.

This study found that in spite of the carotenoid-content of NEKTON-GELB, birds supplemented with NEKTON-GELB did not produce more vibrant plumage than those supplemented with NEKTON-S over the course of their hatch-year fall, second-year winter, and second-year early spring pre-alternate molt. Considering that NEKTON-GELB contains lower proportions of several key vitamins than NEKTON-S, this study supports the use of NEKTON-S during this phase of an individual's life. However, further studies on the impact of NEKTON-GELB on plumage intensity throughout a full molt cycle could provide more robust conclusions, particularly considering that

Florida grasshopper sparrows replace most of their feathers in the pre-basic molt, which this study does not cover. Additionally, though Florida grasshopper sparrows are monomorphic, the variation in plumage intensity between males and females implies a sexually selective pressure that merits further investigation (Hill, 2006).

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Table 1. Nutrient content of NEKTON-GELB and NEKTON-S avian dietary supplements.

Nutrient	**NEKTON-GELB[1]**	**NEKTON-S[1]**
Vitamin A, IU	4,950,000	6,600,000
Vitamin D3, IU	7,500	10,000
Vitamin E (all-rac-alpha-tocopherylacetate), mg	5,000	6,664
Vitamin B1 (thiamine hydrochloride), mg	500	670
Vitamine B2 (riboflavin), mg	1,260	1,680
Calcium-d-pantothenate, mg	2,500	3,300
Niacinamide, mg	7,500	10,000
Vitamine B6 (pyridoxine-hydrochloride), mg	500	670
Folic Acid, mg	168	225
Vitamin B12 (cyanocobalamine), µg	1,500	2,000
Vitamine C (L-ascorbic acid), mg	12,500	16,670
Vitamin K3 (menadione-sodiumbisulfite), mg	1,000	1,330
Biotin, µg	30,000	30,000
Fe (ferrous(II)-sulfate, monohydrate), mg	3,000	3,000
Zn (zinc sulfate, monohydrate), mg	1,220	1,220
Mn (mandanese(II)-oxide), mg	1,250	1,250
Cu (cupric(II)-sulfate, pentahydrate)	250	250
I (calcium iodate, free of water), mg	100	100
Co	Trace	Trace
L-alanine	P[2]	P
L-arginine	P	P
L- aspartic acid	P	P
L-cystine	P	P
L-glutamine	P	P
L-glycine	P	P
L-histidine	P	P
L-isoleucine	P	P
L-leucine	P	P
L-lysine	P	P
DL-methionine	P	P
L-phenylalanine	P	P
L-proline	P	P
L-serine	P	P
L-threonine	P	P
L-tryptophan	P	P
L-tyrosine	P	P
L-valine	P	P
Beta-Apo-8 carotenoic acid ethyl ester	P	A[3]
Technological additive: silicic acid	P	P

[1]Values given per kg of supplement, [2]P: present at unknown values, [3]A: absent

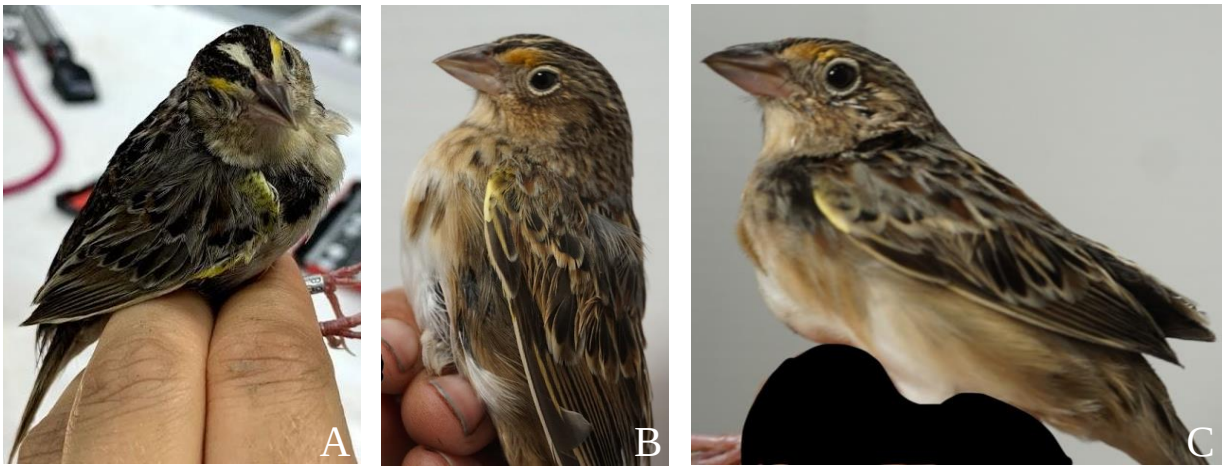

Figure 1. A) After second year female that was released into the wild as a second year and recaptured after 11 months. **B**) Captive second year male and **C**) Captive second year female photographed at White Oak for this study. The recaptured female displays notably yellower lores and wrists, as well as generally darker plumage. This trend of wild birds showing brighter plumage has been anecdotally observed in hatch year and second year males and females throughout the captive-release program.

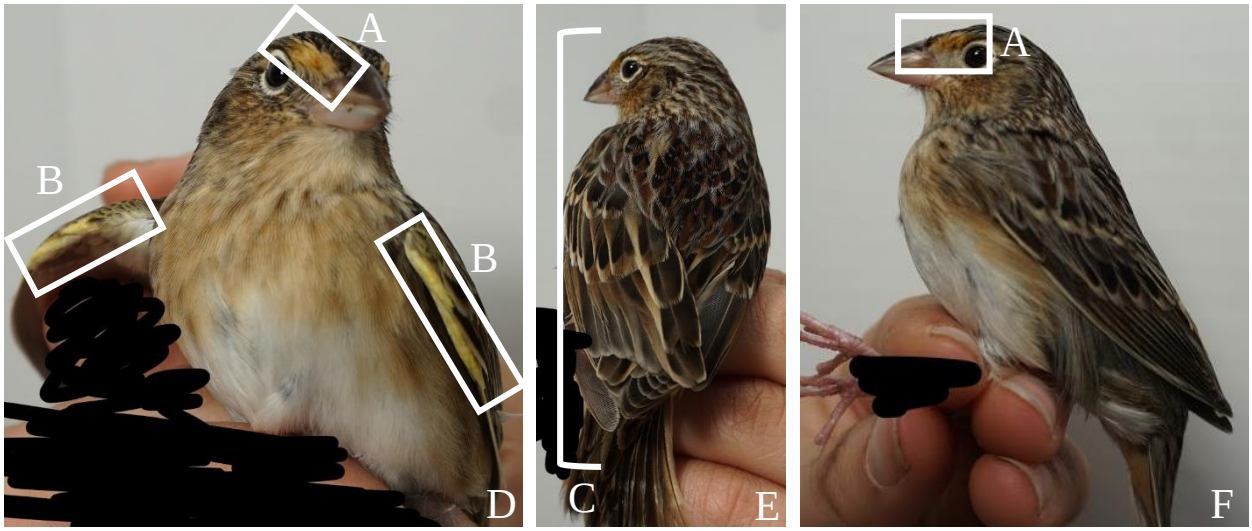

Figure 2. D) anterior, **E**) dorsal, and **F**) lateral views photographed for each bird and utilized in each set of comparisons. The lores (**A**), wrists (**B**), and overall darkness (**C**) are indicated in white. Identifiable features such as the keeper's hands or the birds' color bands were blacked out to maintain blind comparison trials.

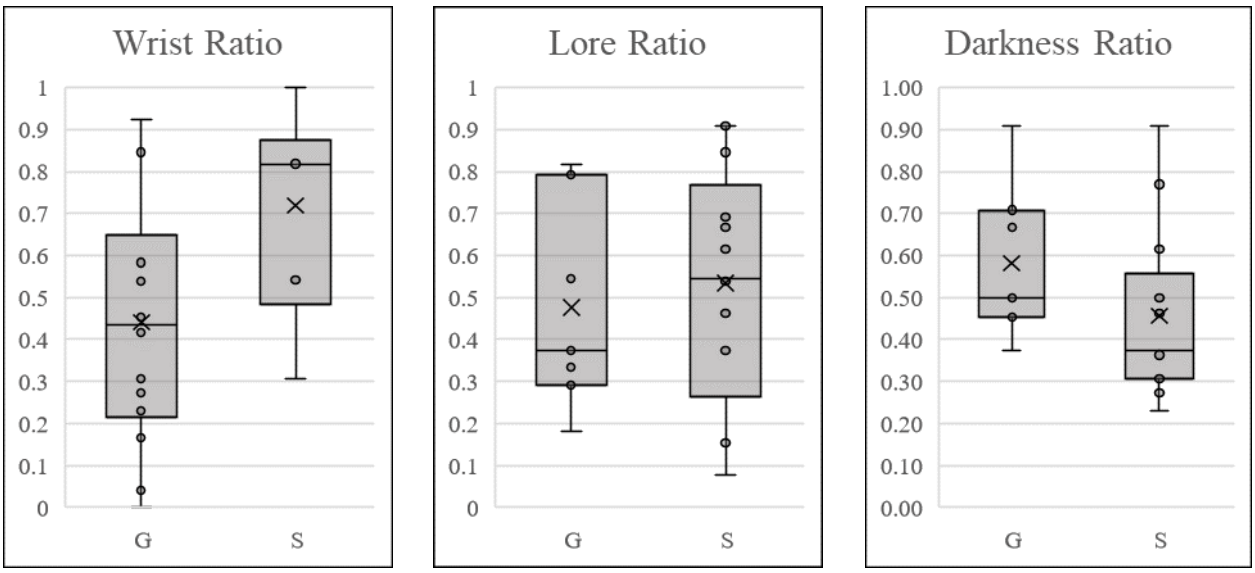

Figure 3. Variation between NEKTON-GELB (G) and NEKTON-S (S) supplemented individuals when analyzed in one-to-one comparisons of lore intensity, wrist intensity, and overall darkness. Wrist intensity varied significantly (P = 0.04), with NEKTON-S individuals judged to have brighter wrists than NEKTON-GELB individuals. Lore intensity and overall darkness did not differ (P = 0.65).

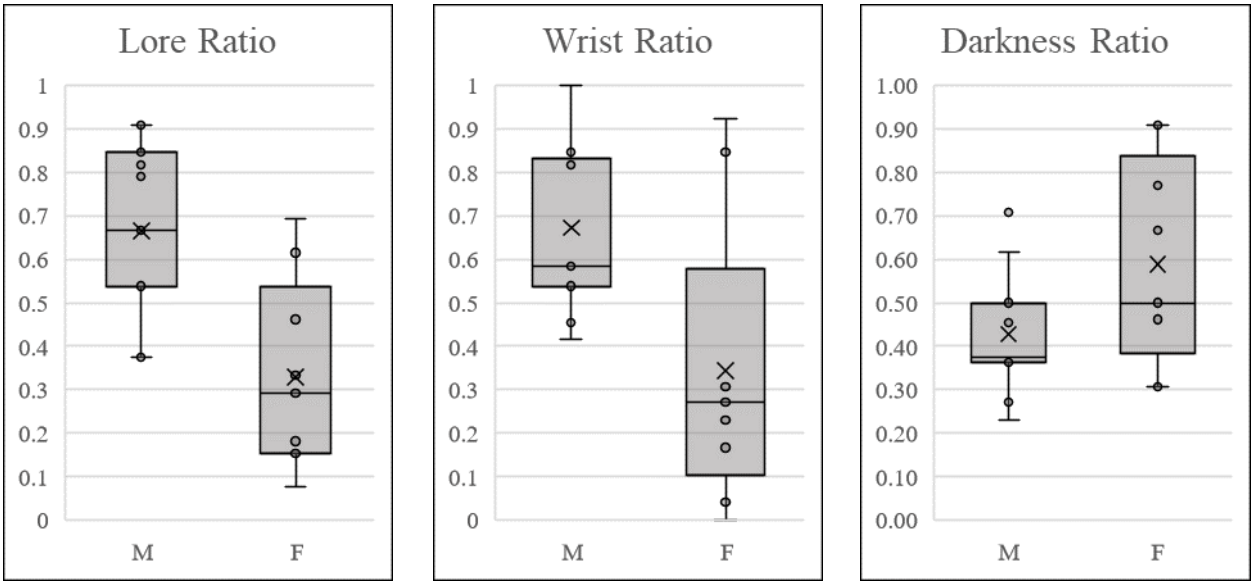

Figure 4. Variation between males and females in one-to-one comparisons of lore intensity, wrist intensity, and overall darkness. Males were found to possess significantly brighter lores (P = 0.02) and wrists (P = 0.01) than females, and no statistical difference (P = 0.11) was observed between sexes in regard to overall plumage darkness.