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ABSTRACT: In late summer, red-winged blackbirds forage heavily on ripening sunflower crops in the Dakotas. Sunflower achenes have a distinct fatty acid profile that should influence the fatty acid composition in tissues of these birds. To determine if fatty acid composition in tissue could be used as a biomarker indicating dietary history, we fed 18 red-winged blackbirds a sunflower diet for 2 weeks and compared fatty acid profiles in their muscle and liver tissues to a control group of red-winged blackbirds ($n = 15$) fed a birdseed mix supplemented with safflower seed. Three subjects from each treatment group were sacrificed at Day 0, 7, 14, and 21, with Day 0 the day the treated group was switched to sunflower. The remaining birds were sacrificed on Day 35. Breast muscle and liver tissue were collected, extracted, and analyzed for levels of linoleic, oleic, palmitic, and stearic acids. Differences existed in levels of all 4 fatty acids between treatment groups pooled across time ($P \leq 0.05$, ANOVA). When comparing fatty acid profiles between treated and controls by day sacrificed, we observed differences in levels of ≥ 1 of the fatty acids at Day 7, 14, and 21 in breast muscle, and Day 7 and 14 in liver tissue ($P \leq 0.05$, t-test). Within-bird comparisons of fatty acid levels in liver and breast indicated temporal lags in metabolism between tissue types ($P \leq 0.05$, paired t-test). Our results demonstrated that fatty acids profiles in body tissues can be used as biomarkers to verify recent foraging in sunflower by red-winged blackbirds.

KEY WORDS: *Agelaius phoeniceus*, biomarkers, blackbirds, breast muscle, diet, fatty acids, *Helianthus anus*, liver tissue, red-winged blackbirds, sunflower

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INTRODUCTION

Sunflower (*Helianthus anus*) fields are a preferred source of high-calorie food for millions of premigratory blackbirds (Icteridae) staging in the Northern Great Plains in late summer. Three species are responsible for the majority of damage to the sunflower crop: red-winged blackbirds (*Agelaius phoeniceus*), common grackles (*Quiscalus quiscula*), and yellow-headed blackbirds (*Xanthocephalus xanthocephalus*). Peer et al. (2003) estimated that blackbird damage in the Northern Great Plains cost sunflower producers $5.4 million for all three species in aggregate, with red-winged blackbirds accounting for 52% of the loss. Blackbird damage represented 1.7% of the dollar value of the 1999 sunflower harvest in the Northern Great Plains. If the damage was evenly distributed, the loss would be inconsequential; however, bird damage is often localized forcing some growers to plant alternative albeit less profitable crops. Management of the damage has proven to be a long-term challenge.

About 15 million red-winged blackbirds establish their breeding territories in the major sunflower growing regions of central North Dakota and north-central South Dakota (Homan et al. 2004). With recruitment of young-of-the-year birds, this population swells to 20 million by August. Because a large portion of sunflower damage occurs early in the damage season (Cummings et al. 1989), it is believed that most of this damage is caused by resident birds (Dolbeer 1978, Linz et al. 1983). One possible management strategy is to reduce the resident population after it has left the sunflower growing area. The reasons for delaying management action until the population has departed are two-fold: first, while sunflower is still available, the foraging behavior of blackbirds precludes use of applicable management tools; and second, nontarget avian risks from management actions are high in sunflower fields during late summer because most species are starting to migrate into the sunflower growing region (Gamble et al. 2003, Linz et al. 2003, Schaaf 2003).

By late fall, large numbers of red-winged blackbirds arrive at feedlots in Nebraska and Kansas; it is conceivable that some of these birds are from the major sunflower growing regions to the north. If we can develop a method for identification of target populations at feedlots in Kansas and Nebraska through dietary history, population management could be effectively focused and efficiently implemented.

Most of the sunflower crop is grown for cooking oil. The oil is composed mainly of unsaturated triacylglycerols (also known as triglycerides), which are molecules containing three fatty acids linked to a glycerol backbone. The dominant fatty acids found in sunflower oil are linoleic acid at 66.2% [9, 12-octadecadienoic acid, 18:2 (n-6)]; oleic acid at 25.1% [9-octadecenoic acid, 18:1 (n-9)]; palmitic acid at 5.6% (hexadecanoic acid, 16:0); and

stearic acid at 2.2% (octadecanoic acid, 18:0) (Weast and Lide 1990). Triacylglycerols are important molecules for metabolic processes, energy storage, and cell building in organisms. The composition of fatty acids in ingested foods often influences the fatty acid profile in body tissues (Blem 1976).

The use of a fatty acid profile in the diet is complicated by the fact that denovo synthesis of fatty acids occurs in tissues, and fatty acids acquired in the diet undergo significant modification (Cook 1985). For example, during denovo synthesis, all organisms will produce palmitic and stearic acid from carbohydrate in the diet. These can then be modified to oleic acid by desaturating the Δ^9 position in the carbon backbone. Thus, three of the four fatty acids in sunflower achenes can be synthesized denovo.

Linoleic acid is an essential fatty acid in the diet as only plants can desaturate a carbon chain beyond the Δ^9 position (Cook 1985). The linoleic acid acquired from the diet is modified by additional desaturation at the Δ^4, Δ^5, and Δ^6 positions in conjunction with chain elongation to form other required metabolites. Linoleic acid is highly conserved following consumption due to its essential nature and is not generally utilized for energy production (Cook 1985). However, the concentration of linoleic acid in a tissue will be governed by other metabolic needs.

We predicted that blackbirds that had recently depredated standing sunflower would have distinctive fatty acid profiles in their body tissues. Blackbirds, depending on their dietary history and metabolic needs, will have produced storage lipid in liposomes that has a distinct fatty acid distribution. Therefore, it may be possible using tissue concentrations of palmitic, stearic, oleic, and linoleic acids (singly or in combination) to keep track of the Northern Great Plains blackbird population – a population unique among others through regional food availabilities and diet.

MATERIALS AND METHODS

To test our hypothesis, we kept a group of red-winged blackbirds on a sunflower diet for two weeks and compared levels of extracted fatty acids against a similar reference group fed a non-sunflower diet (control group). We extracted tissues from the breast and liver and analyzed them for mass percent of palmitic, oleic, stearic, and linoleic acids. The liver is responsible for regulating fatty acid metabolism, while breast muscle is a site of triacylglycerol utilization and storage.

A mixed gender- and age-based sample (n = 33) of red-winged blackbirds was captured in North Dakota and brought to the Animal Research Building (ARB) at NWRC, Fort Collins, CO. The birds were randomly divided into two groups (n = 18, 15) and housed in free-flight aviary rooms in the ARB. A 14-h light/10-h darkness diurnal cycle was maintained for both groups. The rooms were kept at 23°C. For approximately eight weeks, the two groups were offered a daily ration consisting of 255 g birdseed mix (corn:millet:milo 1:2:1), supplemented with 136 g safflower seed, and 50 g Layena (Purina Mills, LLC., St. Louis, MO). Each group was also provided daily access to mealworms. After eight weeks, the treated group (n = 18) was switched to a diet of sunflower (500 g, offered daily), while the control group (n = 15) remained on the initial diet of birdseed. Three birds from each treatment group were randomly selected and euthanized at time = 0, 7, 14, 21 days. At Day 35, we euthanized the remaining birds (treated n = 6, control n = 3). Until analyzed, the carcasses were labeled, frozen, and stored at -70°C.

Breast muscle and liver tissue were removed from each carcass and extracted using a modified Folch procedure (Hamilton et al. 1992). The extracted triacylglycerides were hydrolyzed to release the fatty acids and methyl esterified in 3N HCl in methanol (Anonymous 1997). The fatty acid methyl esters were analyzed on an Agilent 6890 GC/5973 MS (Agilent Technologies, Palo Alto, CA) according to David et al. (2003). We used a Varian VF-23MS GC column (Varian Inc., Lake Forest, CA) to separate methyl esters. An internal standard, tridecanoic acid (13:0) methyl ester, was used to calculate fatty acid methyl ester concentrations in tissue extracts. The concentrations of fatty acid methyl esters in the extracts were converted to mass percents based on total mass of the four fatty acid methyl esters recovered.

Mass percent values of the four fatty acids were averaged by treatment group by sampling interval (i.e., day sacrificed) for breast muscle and liver tissue. A t-test was used to compare treatment means by sampling interval after testing for homogeneity of variance between treatment groups (Steel and Torrie 1960). A paired t-test was used for assessing differences in mean mass percents for each of the four fatty acids in liver and breast tissue within birds within treatment group. An ANOVA was used to determine if mass percents of each fatty acid differed for breast muscle and liver tissue over the five sampling intervals by treatment group. Statistical analyses were performed with Excel software in Office 2000 (Microsoft®, Richmond, WA). Statistical significance was accepted at $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Fatty acid profiles of treated and control groups were generally similar and were as follows: oleic < linoleic < stearic < or = palmitic. By comparison, the fatty acid profile for sunflower oil is stearic < palmitic < oleic < linoleic (Weast and Lide 1990). The profiles between bird tissue and sunflower oil were approximately reversed, with the two highest concentrations in sunflower (linoleic and oleic acid) becoming the two lowest in bird tissue. This difference reflects the underlying anabolic and catabolic processes in organisms.

Liver Extract Analyses

Within a week of switching to the sunflower diet, treatment effects were observed for stearic and palmitic acids (Table 1). Concentrations of stearic acid were greater for birds on the sunflower diet (i.e., treated group), while palmitic acid was greater in the control group. Oleic acid occurred at greater concentrations in the treated group during the second week of treatment (i.e., Day-14 sample). No difference was detected for linoleic acid for either treatment group. None of the fatty acids

Table 1. Average mass percent and standard deviation (SD) of four fatty acids extracted from livers of red-winged blackbirds fed sunflower for two weeks (T) and a similar control group (C) fed birdseed mix (corn:millet:milo 1:2:1) and safflower. Three birds from each treatment group were analyzed for extracted fatty acids at Day = 0, 7, 14, 21. At Day 35, the remaining birds were analyzed (treated, $n = 6$; control, $n = 3$). Day 0 was the day that the treated group was switched to sunflower.

Day	Average mass percent of fatty acids extracted from liver							
	Palmitic		Stearic		Linoleic		Oleic	
	T*	C	T*	C*	T	C	T	C
0	39.7 (4.8)	36.6 (2.9)	33.5 (4.1)	35.4 (2.2)	17.3 (2.5)	21.3 (1.0)	9.6 (3.6)	6.7 (0.7)
7	31.2 (3.1)	43.8 (4.4)+	40.6 (1.9)	31.1 (4.3)+	18.7 (1.5)	16.9 (2.3)	9.5 (0.8)	8.2 (2.6)
14	28.5 (1.5)	38.2 (1.6)+	40.9 (1.1)	36.4 (0.4)+	18.4 (1.0)	17.8 (1.3)	12.1 (1.4)	7.6 (1.8)+
21	49.9 (2.2)	42.8 (6.4)	27.5 (1.6)	30.3 (1.8)	12.9 (2.0)	18.8 (7.7)	9.6 (2.0)	8.1 (3.1)
35	38.1 (2.9)	38.9 (3.5)	26.6 (2.0)	29.0 (3.1)	16.4 (1.7)	17.6 (2.5)	10.5 (1.2)	9.2 (2.1)

* significant difference ($P \leq 0.05$) among mean mass percents over five sampling intervals ($F_{critical\ 4,13} = 3.18$ for treated group; $F_{critical\ 4,10} = 3.48$ for control group; palmitic acid: $F = 23.31$; stearic acid$_T$: $F = 35.73$; stearic acid$_C$: $F = 4.50$).

+ significant difference ($P \leq 0.05$) in mean mass percents between treated and control groups by sampling interval (*t*-test; $n = 6$ for Day 0, 7, 14, 21, and $n = 9$ for Day 35).

Table 2. Average mass percent and standard deviation (SD) of four fatty acids extracted from breast muscle of red-winged blackbirds fed sunflower for two weeks (T) and a similar control group (C) fed birdseed mix (corn:millet:milo 1:2:1) and safflower. Three birds from each treatment group were analyzed for extracted fatty acids at Day = 0, 7, 14, 21. At Day 35, the remaining birds were analyzed (treated, $n = 6$; control, $n = 3$). Day 0 was the day that the treated group was switched to sunflower.

	Average mass percent of fatty acids extracted from breast							
	Palmitic		Stearic		Linoleic		Oleic	
Day	T*	C	T*	C	T*	C	T*	C
0	39.6 (2.4)	38.4 (3.2)	27.3 (0.8)	26.5 (2.6)	26.2 (2.9)	28.1 (1.5)	6.9 (0.4)	6.9 (0.4)
7	32.9 (4.1)	37.7 (3.2)	33.8 (5.7)	32.4 (4.3)	22.2 (3.8)	22.1 (3.4)	11.0 (1.2)	7.8 (1.6)+
14	28.7 (0.8)	39.8 (1.7)+	38.3 (0.4)	30.8 (1.0)+	19.9 (0.6)	22.7 (1.4)+	13.2 (0.1)	6.7 (0.2)+
21	38.3 (2.4)	38.8 (4.4)	30.0 (3.5)	30.0 (1.2)	23.1 (1.6)	24.7 (3.7)	8.4 (0.5)	6.6 (0.5)+
35	26.5 (1.7)	28.9 (3.4)	26.5 (1.7)	28.9 (3.4)	24.8 (4.0)	21.4 (5.8)	10.5 (1.6)	10.8 (3.7)

* significant difference ($P \leq 0.05$) among mean mass percents over five sampling intervals ($F_{critical\ 4,13} = 3.18$ for treated group; $F_{critical\ 4,10} = 3.48$ for control group; palmitic acid$_T$: $F = 8.79$; stearic acid$_T$: $F = 10.00$; linoleic acid$_T$: $F = 3.81$; oleic acid$_T$: $F = 20.81$).

+ significant difference ($P \leq 0.05$) in mean mass percents between treated and control groups by sampling interval (*t*-test; $n = 6$ for Day 0, 7, 14, 21, and $n = 9$ for Day 35).

Table 3. Within-subject differences between breast muscle and liver tissue extractions for the percent mass of the four fatty acids. The values are the difference in average mass percents of fatty acids recovered from breast muscle minus liver tissue. Tissues were extracted from red-winged blackbirds fed sunflower for 2 weeks (T) and a similar control group (C) fed birdseed mix (corn:millet:milo 1:2:1) and safflower. Three birds from each treatment group were analyzed for extracted fatty acids at Day = 0, 7, 14, 21. At Day 35, the remaining birds were analyzed (treated, $n = 6$; control, $n = 3$). The treated group was switched to sunflower on Day 0.

	Breast muscle minus Liver tissue (%)							
	Palmitic		Stearic		Linoleic		Oleic	
Day	T	C	T	C	T	C	T	C
0	-0.1	1.8	-6.2	-8.9*	+8.9*	+6.8*	-2.7	+0.2
7	+1.7	-6.1*	-6.8	+1.3	+3.5	+5.2*	+1.5*	-0.4
14	+0.2	+1.6*	-2.6*	-5.6*	+1.5*	+4.9*	+1.1	-0.9
21	-11.6*	-4.0	+2.5	-0.3	+10.2*	+5.9	-1.2	-1.5
35	-11.6*	-10.0	-0.1	-0.1	+8.9*	+3.8	0.0	+1.6

* significant difference (paired *t*-test, $P \leq 0.05$) in the amount (%) of fatty acid extracted from breast muscle and liver tissue of each red-winged blackbird by treatment group within sampling interval.

was significantly different in liver tissue one week after returning the treated group to the control diet of birdseed and safflower.

Using ANOVAs to compare the mean mass percent distributions for each fatty acid in liver tissue across the five sampling intervals (i.e., Day 0 to Day 35), we found a difference in stearic acid mass in the control group (Table 1). Stearic acid showed a slight downward trend in its mass percents over the study period. There were differences in the mean mass percents of palmitic and stearic acids across time for the treated group. This probably reflects the high calorie diet of sunflower available to the treated group. Levels of stearic acid dropped over 13% in the Day-21 sample, after the treated group had returned to the control diet (Table 1). In contrast, palmitic acid levels in the treated group rose nearly 21% after the sunflower diet was stopped. Palmitic and stearic acids are metabolic precursors to other fatty acids, in addition to being the principle fatty acids in lipids stored as triglycerides for later use in metabolic energy production (Stryer 1981).

Breast Extract Analyses

Excepting oleic acid, treatment effects did not appear until the second week after switching diets (Table 2). Similar to the results from the liver extracts, stearic acid was greater in breast muscle of birds fed sunflower, while palmitic acid was greater for controls. Linoleic concentrations in the control group exceeded the treated group in the Day-14 sampling interval. Oleic acid was greater in treated birds for three weeks following the switch to sunflower. Thus, oleic acid was the only fatty acid to retain a treatment effect beyond the time the treated group was returned back to the control diet on Day 14. No difference was detected in oleic acid concentration between treatments by Day 35.

There were significant differences in mass percents for all four fatty acids across time in breast tissue of the treated group (Table 2). For palmitic acid, it appeared that the sharp decline in levels in the Day-35 sample (which had been preceded by the rise in levels between Day 14 and Day 21) contributed toward detection of a significant difference.

Comparison of Fatty Acids in Breast and Liver

Excepting linoleic acid, within-bird comparisons of fatty acid concentrations in breast muscle and liver tissue showed varying results, both by sampling interval and treatment group (Table 3). Mass percents of linoleic acid were consistently greater in breast muscle than liver across all sampling intervals, with significant differences occurring in the majority of the sampling intervals in treated and control groups (Table 3). For the other three fatty acids, the trend is for the muscle tissue to have lower levels compared to the liver where there is a significant difference between levels. This is consistent with the role of the liver in lipid uptake and distribution in the body.

SUMMARY AND CONCLUSIONS

Based on these results, it appears that the four fatty acids can be used as biomarkers for sunflower seed consumption by red-winged blackbirds. Breast tissue is the preferable matrix for using the fatty acids as biomarkers, as the fatty acids all show mass distributions across time that are significantly different from controls. Moreover, the differences tend to last longer in this tissue compared to liver tissue. The mass percents of all four fatty acids in the treated group rapidly returned to control-group levels and probably reflected the presence of safflower seed in the control diet. That we were able to detect treatment effects, given the overall similarities in mass percents of the four fatty acids between sunflower and safflower (palmitic acid <7%, stearic acid <7%, oleic acid 18.6%, and linoleic acid 70.1%; Weast and Lide 1990), is a demonstration of the potential power of fatty acid analysis to indicate the nature of recent dietary habits.

Our results showed that all four of the fatty acids had distinct profiles in treated birds, and these differences were measurable against a control group fed birdseed and safflower. We hypothesized that the differences might have been significant over a longer period except that the control diet contained safflower, which was nearly identical in fatty acid composition to sunflower. We believe this approach does have potential to identify bird populations that have recently fed on sunflower and should be useful in developing effective management strategies.

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