Dietary fatty acids and flight-training influence the expression of the eicosanoid hormone prostacyclin in songbirds

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A B S T R A C T

Diet shifts can alter tissue fatty acid composition in birds, which is subsequently related to metabolic patterns. Eicosanoids, short-lived fatty acid-derived hormones, have been proposed to mediate these relationships but neither baseline concentrations nor the responses to diet and exercise have been measured in songbirds. We quantified a stable derivative of the vasodilatory eicosanoid prostacyclin in the plasma of male European Starlings (*Sturnus vulgaris*, $N = 25$) fed semisynthetic diets with either high (PUFA) or low (MUFA) amounts of n6 fatty acid precursors to prostacyclin. Plasma samples were taken from each bird before, immediately after, and two days following a 15-day flight-training regimen that a subset of birds ($N = 17$) underwent. We found elevated prostacyclin levels in flight-trained birds fed the PUFA diet compared to those fed the MUFA diet and a positive relationship between prostacyclin and body condition, indexed by fat score. Prostacyclin concentrations also significantly decreased at the final time point. These results are consistent with the proposed influences of precursor availability (i.e., dietary fatty acids) and regulatory feedback associated with exercise (i.e., fuel supply and inflammation), and suggest that prostacyclin may be an important mediator of dietary influence on songbird physiology.

1. Introduction

Micronutrients are dietary components that constitute relatively small proportions of overall diet composition, but have a disproportionately large impact on organism function (Berdanier, 1998). Such influence on function can occur because a micronutrient may itself be bioactive (e.g., the antioxidant properties of α-tocopherol and other vitamins; Beaulieu and Schaefer, 2013; Frankel, 1996), may limit the synthesis and maintenance of functionally important compounds and tissues (e.g., how iron deficiency affects blood hemoglobin levels (Bainton and Finch, 1964)), or may trigger regulatory cascades along various signaling pathways (e.g., polyphenols activation of estrogen receptors; Penttinen et al., 2007). Minerals and vitamins are the most commonly recognized micronutrients, but there is evidence that certain building blocks of macromolecules (e.g., the amino acid leucine; Lim et al., 2005; Wu, 2009), can exert a comparable level of influence on

organismal function. Among macromolecules, recent controlled or focused studies have demonstrated notably large effects of small quantities of essential fatty acids on flight performance (McWilliams et al., 2020; Pierce and McWilliams, 2014; Price, 2010), an energy-intensive activity (Butler, 2016; Tobalske et al., 2003) that for birds relies on fat as fuel (Guglielmo, 2010, 2018). Multiple studies have found that diet composition influences the fatty acid composition of songbird tissues and particularly the composition of essential or conditionally essential polyunsaturated fatty acids (PUFAs, contrast with monounsaturated fatty acids, or MUFAs; McCue et al., 2009; McWilliams et al., 2022; Pierce and McWilliams, 2014; Twining et al., 2016). Furthermore, related work has found that the content of certain n-6 PUFA in fat stores can influence metabolism in songbirds, including effects on peak metabolic rate (Carter et al., 2020; Pierce et al., 2005; Price and Guglielmo, 2009) and sustained energy expenditure (Carter et al., 2020; McWilliams et al., 2020). Together, these results suggest that certain

dietary PUFA act as micronutrients, but evidence for how they affect metabolism remains inconclusive.

Although the biophysical properties of PUFAs could allow molecules destined for catabolism to diffuse more readily through cells (Price et al., 2008; Raclot, 2003), functional effects have been inconsistently correlated to the PUFA content of adipose tissue and intramuscular triacylglycerols (Carter et al., 2020; Pierce et al., 2005). Similarly, evidence for a relationship between lipid membrane composition in flight muscles and energy metabolism has been mixed (Carter et al., 2020; Price and Guglielmo, 2009), despite a proposed link between the properties of PUFA-rich membranes and metabolic enzyme activity (Giroud et al., 2013; Nagahuedi et al., 2009; Price et al., 2018). Somewhat more promising has been the proposed regulation of energy metabolism by PUFAs via the activation of peroxisome proliferator activated receptor (PPAR) signaling pathways (McClelland, 2004; Pierce and McWilliams, 2014; Weber, 2009), which have shown mixed responses to diet in songbirds (DeMoranville et al., 2020; Dick and Guglielmo, 2019a). However, direct stimulation of PPARs by PUFA is not the only mechanism by which fatty acids can regulate physiological function: PUFA also serve as the precursors for eicosanoids, a class of circulating hormones (Berdanier and Berdanier, 2015; Price, 2010; Watkins, 1991), which have almost exclusively been studied in human and model mammal and poultry systems. Thus, at present, a potentially key micronutrient mechanism for dietary PUFA in songbirds remains unexamined.

Eicosanoids encompass several classes of molecules, including prostaglandins, leukotrienes, and thromboxanes, which are synthesized from PUFA located in cell membranes (Berdanier and Berdanier, 2015; Zhou and Nilsson, 2001). These compounds have short half-lives (often less than ten minutes; Zhou and Nilsson, 2001), thus concentrating their effects in localized tissues and are responsible for a wide range of functions, typically related to inflammation (Berdanier and Berdanier, 2015). These functions can oppose one another and eicosanoids derived from n-3 PUFA generally have anti-inflammatory effects while those derived from n-6 PUFA generally have pro-inflammatory effects (Russo, 2009; Schmitz and Ecker, 2008). Prostaglandins, in particular, are associated with the functions of vasodilation and vasoconstriction (Clapp and Gurung, 2015; Craig-Schmidt et al., 1987; Medzhitov, 2008), which might have special relevance for songbirds as energy metabolism during flight may be limited by respiratory gas transport and exchange and fatty acids transport to muscle tissue via the circulatory system (Guglielmo, 2018; Jenni and Jenni-Eiermann, 1998; McClelland, 2004). In addition, prostaglandins can themselves act as ligands for PPARs (Fujimori, 2012; Marion-Letellier et al., 2016) and have been implicated in the regulation of feeding (Madison et al., 2008; Tachibana et al., 2018) and tissue development (Massiera et al., 2003; Watkins, 1991), all of which are highly relevant to physiological performance in songbirds, such as successful preparation for migratory flight.

Despite the potential relevance of eicosanoids and particularly prostaglandins for shaping flight capabilities and the evidence from humans and other taxa that dietary PUFA (Craig-Schmidt et al., 1987; Mourente et al., 2007; Russo, 2009; Stupin et al., 2019), and exercise training (Simopoulos, 2008; Stupin et al., 2019; Zoladz et al., 2010) can influence their circulation no previous studies have documented potential effects of dietary fatty acids and flight exercise on the circulating concentrations of prostaglandins in songbirds. Here we present the first evidence for a relationship between dietary PUFA and prostaglandin concentrations in a songbird, as well as evidence of potential downstream effects on aerobic performance. Specifically, in European Starlings (*Sturnus vulgaris* Linnaeus 1758) we investigated the relationship between levels of dietary linoleic acid (18:2n6; # carbon: # double bonds, position of first double bond) and concentrations of a stable metabolite of prostacyclin (prostaglandin series I2, PGI_2), which is derived from an elongated byproduct of linoleic acid, arachidonic acid (20:4:n6), via the enzyme cyclooxygenase (Berdanier and Berdanier, 2015; Clapp and Gurung, 2015) and noted in mammals to be a potent

vasodilator and potentially involved in appetite and tissue synthesis (Clapp and Gurung, 2015; Madison et al., 2008; Massiera et al., 2003). The function of prostacyclin as a vasodilator makes it particularly likely to influence the circulation of oxygen and fatty acids. We hypothesized that circulating concentrations of prostacyclin metabolite would be positively related to dietary n6 PUFA content and body composition, as birds with greater access to and reservoirs of n6 PUFA could face fewer constraints on prostacyclin signaling.

2. Materials and methods

2.1. Animal care, housing, and diets

This study took place as part of a broader experiment on the exercise physiology of songbirds and additional details on methods as well as results concerning whole-animal energetics and oxidative status can be found in McWilliams et al. (2020). All animal care procedures and experimental techniques were approved by the University of Rhode Island IACUC (Protocol #AN08–02-014) and the government of Upper Bavaria, Germany (AZ 55.2–1-54-2532-216- 2014). Between April – May 2015 we collected 60 male 5–8 day old Starlings from nest boxes in a native colony in Upper Bavaria, Germany (47°55′16.9"N 11°08′32.5"E). Nestlings were brought to the animal care facility of the Max Planck Institute for Ornithology (MPIO) in Seewiesen, Germany and were hand raised with a high-protein diet consisting of crickets, bee and fly larvae, wax worms, and beef heart and supplemented with vitamin mixture (AIN-76) and calcium carbonate powder. This diet has previously been used to successfully hand raise starlings (Engel et al., 2006; Schmidt-Wellenburg et al., 2008). While nestlings, we used blood samples to confirm individual sex based on differences in chromo-helicase DNA binding genes (only CHD-Z for males). At ca. 35 days old, fledgling starlings were moved to four 4 m × 3 m × 2 m (L x W x H) outdoor aviaries with a natural light cycle and were transitioned to a MPIO standard diet of insect powder, live mealworms, lettuce, fresh fruit, and dried fruit pellets. Starlings had ad libitum access to maintenance diets throughout this initial upbringing period (i.e., April–May to early-August).

Between August 8th and August 23rd 2016 (Fig. 1), we transitioned all birds from the maintenance diets to semi-synthetic diets (Table 1), randomly assigned each individual to one of two experimental groups, and collected morphometric data on each individual (McWilliams et al., 2020). Both experimental diets (Table 1) were isocaloric and had the same macronutrient composition (ca. 42% carbohydrates, 23% protein, 20% fat) which was similar to natural high-lipid fruit diets (Johnson et al., 1985; Smith et al., 2007). The experimental diets differed only in the relative amounts of certain mono- and polyunsaturated fats (MUFA and PUFA, respectively): 18:1n9 (oleic acid; MUFA = 71%, PUFA = 49%), 18:2n6 (linoleic acid; MUFA = 9%, PUFA = 28%), and 18:3n3 (linolenic acid; MUFA = 1%, PUFA = 3%; Table 1). These three fatty acids along with 16:0 and 18:0 comprised >95% of the dietary fatty acids (Table 1; all others were < 1%) and these five fatty acids are also the most common in natural fruits eaten by songbirds (Pierce and McWilliams, 2014) as well as in songbirds that eat fruits during migration (McWilliams et al., 2022; Pierce et al., 2005; Pierce and McWilliams, 2014). Starlings had ad libitum access to these experimental diets throughout the experiment except for overnight fasts during flight-training (see below). Concurrent with the start of the diet shift, we resorted the starlings so that all individuals were in single-diet aviaries. Thus, after August 23rd there were two MUFA aviaries (N = 29) and two PUFA aviaries (N = 31). Depending on the timing of subsequent experimental flight training (see below) birds spent between 28 and 110 days on experimental diets. Outdoor aviaries were kept at ambient temperature and on a natural light cycle until September 7th 2016, when the cycle was fixed at 13 h: 11 h (L: D) for the remainder of the experiment, by supplementing artificial light.
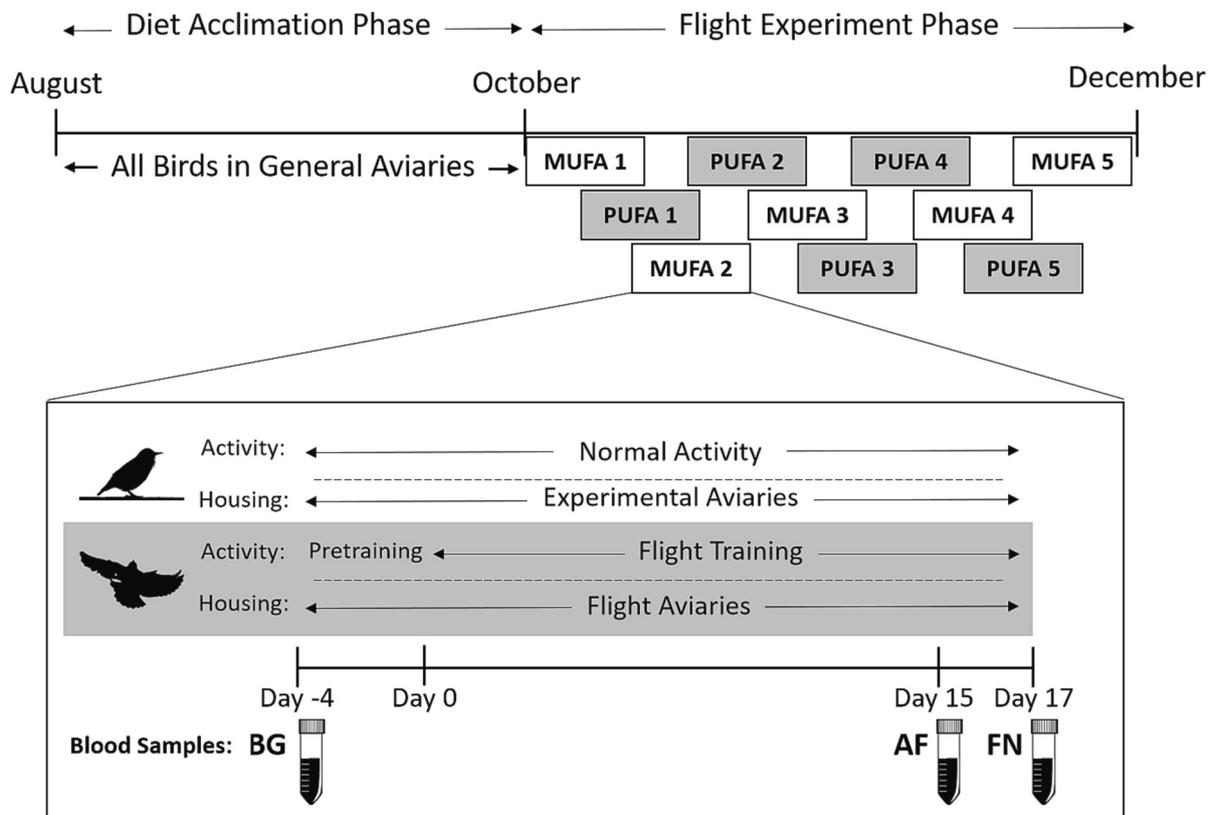
Fig. 1. Timeline of the experimental procedure used in this study. In the diet acclimation phase (August–October), hand raised European Starlings were fed one of two semisynthetic diets differing only in fatty acid composition (high monounsaturated fatty acid [MUFA] or high polyunsaturated fatty acid [PUFA]). In the flight experiment phase, cohorts of starlings underwent a 22-day experimental procedure with trained birds (flying symbol) flying in a wind tunnel and untrained birds (perched symbol) restricted to aviaries. Three blood samples were collected for analysis: a background sample (BG), a post flight sample (AF), and a final/recovery sample (FN).

2.2. Flight-training and blood sampling

Flight-training for a given bird involved five days of acclimation to the recirculating wind-tunnel and then 15 days of daily flying for prescribed amounts of time (see McWilliams et al., 2020 for details). Starlings from a given diet group flew in small groups of 2–3 individuals while 1–2 birds from the same diet group remained in their aviaries and so were untrained, sedentary 'controls'. We refer to these small groups of up to 5 birds as a 'cohort'. Prior to October 20th, 22 starlings in the PUFA group and 20 starlings in the MUFA group were stratified by the extent of Pre-basic I molt (Ginn and Melville, 1983) and then randomly assigned to one of 10 single-diet cohorts of 4–5 individuals as well as flight-training and control groups within cohorts. This stratified random sampling of individuals ensured that all birds had completed flight feather molt prior to flight-training. Starting on October 20th and repeating every 3–6 days thereafter until November 28th we transferred the birds in a given cohort from the outdoor aviaries to 2.5 m × 1.5 m × 2.5 m experimental aviaries in the MPIO wind tunnel facility (Fig. 1). Experimental aviaries were also kept on a 13 h: 11 h (L: D) light cycle, but unlike outdoor aviaries were maintained at a temperature of 18–22 °C. For the first five days of the experimental procedure, each cohort acclimatized to the new environment and on days −3 to 0 was gradually familiarized to the wind tunnel with 30-min test sessions in which they were provided a perch and experienced low wind speeds (5–10 m/s). Starting on day 1 of the experimental procedure, each cohort began a 15-day training regimen that involved flights of increasing duration (30–180 min) in the wind tunnel at 12 m/s and without a perch. This training procedure has been successfully used in prior studies to elicit long duration flights in starlings (Engel et al., 2006; Schmidt-Wellenburg et al., 2008). On the final day of flight-training (day

15 of the experimental procedure) birds flew for as long as they would voluntarily do so, up to six hours in duration. Control birds in each cohort were fasted concomitantly with their flight-trained counterparts. Following the long flight on day 15, each cohort was given two days to recover. Ten individuals in the MUFA group and 13 individuals in the PUFA group failed to complete flight-training and were excluded from subsequent analyses.

We collected blood samples from flight-trained and control birds in each cohort at three sampling points over the course of the above experimental procedure: a background (BG) sample on the morning of day −4, a post-flight (AF) sample collected within 10 min following the long flight on day 15, and a final (FN) sample collected on the morning of day 17 (Fig. 1). All birds were blood sampled at each time point after being fasted for at least 12 h (i.e., after an overnight without food). Post-flight and final samples were taken within 3 min of bird capture, but the BG sample occurred after birds were transferred indoors from the outdoor aviaries, an approximately 15-min process. Blood samples were approximately 400 μL in volume and were taken by puncturing the ulnar vein with a needle, collecting blood in heparinized capillary tubes, and immediately transferring blood to heparinized Eppendorf tubes. Within 10 min of collection, the sample was centrifuged at 10,000g for five minutes to isolate plasma, which was then separated and stored at −80 °C until analysis. Following blood sampling, we evaluated the body condition of each individual by weighing birds (Table 2) and by assessing both fat score (0–8 scale, no furcular fat coverage to complete coverage; Kaiser, 1993) and muscle score (flight muscle depth at a width of 10 mm, measured in mm; Bauchinger et al., 2011). Study animals were subsequently released or euthanized for tissue sample collection as part of the larger experimental protocol.

Table 1

Ingredients and estimated fatty acid composition of experimental diets used in this study, adapted from Tables 1 and 2 in McWilliams et al. (2020). Only fatty acids with concentrations >1% are presented.

	MUFA	PUFA
Ingredients	% dry mass	% dry mass
Glucose[a]	39.35	39.19
Casein[b]	19.20	19.12
Cellulose[c]	4.99	4.97
Salt mixture[d]	4.80	4.78
Olive oil[e]	18.24	9.60
Soybean oil[f]	0.96	9.60
Amino acid mix[g]	2.69	2.68
Vitamin mix[h]	0.38	0.38
Ground meal worms[i]	6.19	6.16
Agar[j]	3.20	3.19
Fatty Acid[k]	%	%
16:0	10.73	11.16
18:0	3.51	3.73
18:1n9	70.70	49.07
18:2n6	9.48	28.42
18:3n3	1.24	3.21

[a] Glucose, VWR International GmbH, Darmstadt, Germany.
[b] Casein, Affymetrix UK Ltd., High Wycombe, UK.
[c] Alphacel, MP Biomedicals, Solon, OH, USA.
[d] Brigg's salt mix, MP Biomedicals, Solon, OH, USA.
[e] Tip Native brand Olive oil (glass bottle, Vandemoortele Deutschland GmbH).
[f] Soya oil, Sojola-brand Soja Oil; Vandemoortele Deutschland GmbH.
[g] Amino Acid Mix, Sigma-Aldrich, St. Louis, MO, USA.
[h] AIN vitamin mix,, MP Biomedicals, Solon, OH, USA.
[i] Freeze dried: Exotic Nutrition, Newport News, VA.
[j] Agar, Ombilab-laborenzentrum GmbH, Bremen, Germany.
[k] Estimated concentration (percent by mass) based on measured composition of oils and mealworms.

Table 2

Mean (± SE) body masses (M_b) and fat scores (FS) of male European Starlings at three points (background, post-flight, final/recovery) during a 22-day experimental flight-training procedure. Means and standard errors are separated for factorial combinations of diet and training treatments.

Diet	Group	Background		Post-Flight		Final/Recovery	
		M_b	FS	M_b	FS	M_b	FS
MUFA	Trained	79.5 ± 1.6	2.6 ± 0.2	70.0 ± 0.7	1.4 ± 0.1	71.3 ± 0.8	1.7 ± 0.2
	Untrained	76.3 ± 2.2	2.3 ± 0.2	70.9 ± 2.9	1.6 ± 0.3	70.3 ± 1.0	2.3 ± 0.3
PUFA	Trained	81.8 ± 2.2	3.2 ± 0.3	71.4 ± 1.2	1.3 ± 0.3	71.9 ± 0.2	2.1 ± 0.2
	Untrained	77.0 ± 2.3	2.3 ± 0.3	68.8 ± 1.9	1.9 ± 0.4	69.5 ± 1.8	1.9 ± 0.2

2.3. Prostacyclin assay

We analyzed the concentration of prostacyclin metabolites in matched BG, AF, and FN plasma samples from a random subset of individuals (MUFA: $N = 13$, PUFA: $N = 12$) using a competitive enzyme-linked immunoabsorbance assay kit (#501100; Cayman Chemical, Ann Arbor, Michigan USA). While this assay may detect prostacyclin itself, it has the greatest affinity for downstream metabolites which are derived from the intermediate 6-keto $PGF_{1\alpha}$ and typically excreted in urine. Briefly, we diluted samples 1:10 with 0.2 M potassium phosphate assay buffer and incubated 100 μL of sample in goat anti-mouse IgG coated 96-well plates with 50 μL of tracer solution (prostacyclin metabolite conjugated with acetylcholinesterase) and 50 μL of monoclonal prostacyclin metabolite antibody for 18 h at 4 °C. Following incubation, we emptied each plate and rinsed it five times with potassium phosphate wash buffer. Finally, we developed plates at room temperature for 90 min with 200 μL of Ellman's reagent in each well and measured the

absorbance of wells at 420 nm with a Biotek Synergy HTX plate reader (Biotek Instruments, Winooski, VT USA). Absorbances were standardized by measurements of maximum tracer binding in wells without any sample, and sample concentrations were calculated by comparison with a standard curve ranging from 39 pg/mL to 5000 pg/mL created by serial dilution. Samples were analyzed in duplicate and the intra-assay coefficient of variation was 13.7%, comparable to manufacturer estimates for the kit.

2.4. Statistical analyses

Prior to analysis, we confirmed that all variables were normally distributed and that the assumption of equal variances across diet, flight-training and sampling groups was met. We used a linear mixed effects model to evaluate the influence of design variables (diet, flight-training, and sampling point) on concentrations of prostacyclin metabolites in plasma samples. While fitting this model we considered and removed non-significant three- and two-way interactions between design variables. We also evaluated the influence of body condition on prostacyclin metabolite concentration by including the fixed effects of fat score and muscle score. The model controlled for variation in body size and the spread of samples over the fall season by including tarsus length and julian date as covariates and included individual ID as a random effect. We evaluated fixed effect significance with type-III Wald Chi-square tests and compared different timepoints via post-hoc Tukey HSD tests with Kenward-Rogers approximations of degrees of freedom. All statistical analyses were conducted in R version 3.6.2 (R Core Team, Vienna Austria) using the packages *lme4* and *lmerTest* and used an α level of 0.05.

3. Results

Concentrations of prostacyclin metabolites in starling plasma were positively related to fat score (Fig. 2, Table 3; $X_1^2 = 6.51$, $P = 0.011$), but unrelated to muscle score (Table 3; $X_1^2 = 1.44$, $P = 0.230$). Circulating concentrations were also influenced by a combination of diet and flight-training (Fig. 3, Table 3). Starlings fed the PUFA diet had higher metabolite concentrations than birds fed the MUFA diet when in the flight-trained group (diet effect; $X_1^2 = 8.60$, $P = 0.003$), but had similar concentrations when in the untrained group (diet X flight-training interaction; $X_1^2 = 6.76$, $P = 0.009$). Regardless of diet and flight-training, prostacyclin metabolite levels were lower in plasma samples collected at the final sampling point (Table 3; $T_{53.1} = 3.14$, $P = 0.008$). Metabolite concentrations were unrelated to tarsus length or Julian date
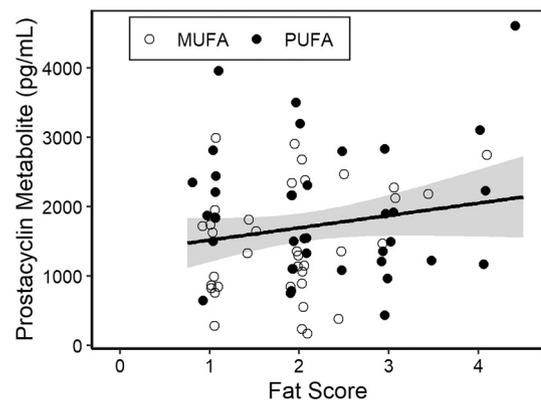

Fig. 2. Positive relationship between circulating prostacyclin metabolites and body condition in male European Starlings. Body condition is represented by fat score, measured on a 0–8 scale (no furcular fat coverage to complete coverage) and was positively related to concentrations of circulating prostacyclin metabolites in plasma ($T_{60.6} = 2.55$, $P = 0.013$). Filled (PUFA; $N = 13$) and unfilled (MUFA; $N = 12$) symbols indicate experimental diets.

Table 3

Type III ANOVA results for fixed effects from a mixed effects model[a] of factors influencing prostacyclin metabolite concentrations in male European Starlings. Estimates represent slopes associated with continuous variables (Julian Date, Muscle Score, Fat score) or differences from a reference group (MUFA for diet, Trained for Training), while statistics are from Wald Chi-square tests. Levels of the timepoint factor (BG = background, AF = post-flight, FN = final/recovery) were compared via pairwise Tukey HSD tests with Kenward-Rogers approximations of degrees of freedom.

Parameter		Estimate	SE	DF	X^2	*P*-value
Intercept		−5558.1	7049.4	1	0.62	0.430
Julian Date		−11.6	9.1	1	1.63	0.202
Tarsus		323.4	196.4	1	2.71	0.100
Muscle Score		219.9	183.1	1	1.44	0.230
Fat Score		294.9	115.6	1	6.51	0.011
Timepoint				2	10.96	0.004
	Contrast				(*T*-ratio)	
	BG–AF	−529.0	321	85.8	−1.65	0.232
	BG–FN	309.0	309	83.1	0.29	0.956
	AF–FN	617.5	197	53.1	3.14	0.008
Diet		819.7	189.2	1	8.60	0.003
Training		412.8	385.4	1	1.15	0.284
Diet X Training		−1289.8	496.1	1	6.76	0.009

[a] The estimated standard deviation of the random effect of individual in this model was 385.4, while the estimated residual standard deviation was 627.2.

(Table 3).

4. Discussion

In this study we demonstrate for the first time that diet quality and flight exercise influenced a representative hormone of the eicosanoid signaling pathway in a migratory songbird. We found that flight-trained birds fed an experimental diet enriched with n6 PUFA had consistently higher circulating levels of the prostaglandin eicosanoid prostacyclin (PGI$_2$) compared to MUFA-fed birds, although the influence of diet was not detectable in untrained birds (Fig. 3). We also found evidence for a consistent decline in PGI$_2$ at the final sampling point (Fig. 3) and a positive effect of body condition on PGI$_2$ regardless of diet or training (Fig. 2). These results support the proposed impact of certain dietary fatty acids on eicosanoid signaling and expand our understanding of the potential impact of dietary fatty acids in songbirds.

The circulating concentrations of bioactive compounds such as prostacyclin may be influenced by several broad factors that are relevant to interpreting our results. For one, concentrations may be constrained by the availability of precursor compounds necessary for the synthesis of the target molecule (Dröge, 2002; Fernstrom, 1977). Importantly, these constraints can be both global, as in the negative effect of tryptophan-poor diets on dopamine concentrations (Fernstrom, 1983), and localized to specific tissues, as with the influence of local ligand availability on thyroid hormone signaling (Mullur et al., 2014). Alternatively, concentrations of bioactive compounds can be adjusted by regulation of the synthesis, interconversion, and degradation of molecules in the relevant metabolic pathway (Braun and Sweazea, 2008; Kim and Lee, 2010; Tretter and Adam-Vizi, 2000). Particularly relevant is the potential for feedback mechanisms that link downstream products with regulation of the focal compound, leading to context-specific physiological phenotypes (Inoue et al., 2000; Naidu et al., 2010). Our results suggest that a combination of these factors likely determined the patterns of prostacyclin expression that we observed. Specifically, dietary fatty acid composition and body condition may constrain the availability of prostacyclin precursors (global constraints), flight-training associated fasting may alter precursor availability in specific tissues, thereby producing acute changes over time in prostacyclin concentrations (a local constraint), and regulatory feedback may lead to associations between body condition or flight-training, and circulating prostacyclin. We discuss each of these cases below.

Polyunsaturated fats include essential or conditionally essential nutrients for vertebrates (Klasing, 1998; Stevens, 2004), and studies have consistently found that diet fatty acid composition strongly influences body PUFA composition in songbirds, (Carter et al., 2020; McCue et al., 2009; McWilliams et al., 2020; Twining et al., 2016), closely matching trends in other taxa (Cherian et al., 2009; Russo, 2009; Toft et al., 2000). These changes in body composition have been linked to corresponding changes in eicosanoid signaling for mammals (Schmitz and Ecker, 2008; Zhou and Nilsson, 2001), as well as poultry (Cherian et al., 2009; Watkins, 1991), but until now those downstream effects have not been documented in non-domesticated birds. Thus, our results provide direct evidence that dietary fatty acid composition exerts a similar influence on eicosanoid signaling in songbirds as mammals and poultry (Cherian et al., 2009; Craig-Schmidt et al., 1987; Russo, 2009; Stupin et al., 2019).
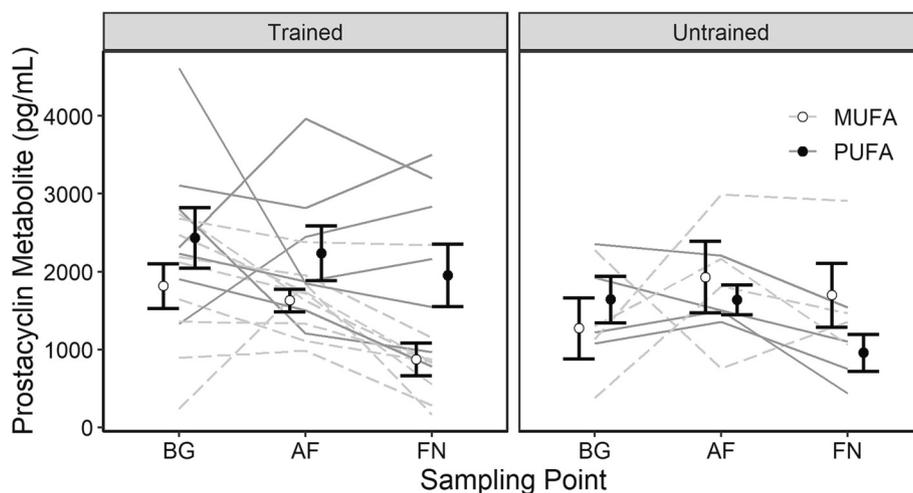

Fig. 3. Changes in circulating prostacyclin metabolites in relation to flight-training and diet. Filled and unfilled symbols and whiskers represent mean ± SE for each of the two diet groups at three different time points; lines represent the responses of individual male starlings. Prostacyclin metabolite concentrations measured in plasma samples collected on days −4 (background, BG), 15 (after flight, AF), or 17 (final/recovery, FN) of an experimental flight-training procedure were significantly lower at the FN sampling point ($T_{47.9} = -3.26$, $P = 0.002$) and were influenced by an interaction between diet and flight-training. Starlings fed a diet high in polyunsaturated fatty acids (PUFA; filled symbols, solid lines; $N = 13$) had higher metabolite concentrations than birds fed a monounsaturated fatty acid diet (MUFA; open symbols, dashed lines; $N = 12$) when flight-trained (diet effect; $T_{26.3} = 2.93$, $P = 0.007$), but similar concentrations when untrained (diet X flight-training interaction; $T_{22.5} = -2.6$, $P = 0.016$).

Although our diet manipulation did involve changes in multiple fatty acids, changes in prostacyclin matched the direction and magnitude of changes in the precursor 18:2n6 and moreover occurred despite any inhibitory effects of 18:3n3, which was also higher in the PUFA group. Among flight-trained birds this effect was relatively substantial, resulting in a 59% increase in plasma prostacyclin metabolite concentration, although this falls short of the >100% increases that have been observed in poultry (Cherian et al., 2009; Watkins, 1991). However, some of the difference between studies in effect size may be the result of differences in focal tissue (whole blood or lung tissue in poultry versus songbird plasma) or developmental stage (early developmental and growth for poultry versus approaching maturity for starlings in our study). Meanwhile, although birds fed the PUFA diet had higher levels of oxidative damage (McWilliams et al., 2020) which could have triggered upregulation of prostacyclin pathways associated with inflammation (Dröge, 2002; Marion-Letellier et al., 2016), this is inconsistent with the lack of effect observed in untrained birds. Ultimately, diet is a generalized interface between the organism and its environment that regularly drives system-wide changes (Cohen et al., 2012; Karasov et al., 2011; Pierce and McWilliams, 2014), and so the restriction of the highest prostacyclin metabolite levels in our study to birds fed a PUFA-rich diet does suggest that at least one species of songbird experiences global constraints on their capacity for eicosanoid signaling. Future studies that relate circulating prostaglandins to diet in wild birds that vary in their intake of precursor fatty acids would be especially informative.

Although the observed diet effect provides evidence for global constraints on eicosanoid signaling, these constraints were not apparent in untrained birds where prostacyclin metabolite concentrations were not significantly different between diet groups. The noticeably higher circulating prostacyclin concentrations in plasma from the flight-trained PUFA-fed birds matches effects of exercise observed in humans and other mammals (Spier et al., 2007; Stupin et al., 2019; Zoladz et al., 2010) and suggests that flight-training prompted the upregulation of the prostacyclin signaling pathway, similar to the influence of exercise on muscle growth (Price et al., 2011), lipid metabolism (DeMoranville et al., 2020; Sasaki et al., 2014), and antioxidant activity (Ji et al., 2006; Nikolaidis et al., 2012). Such upregulation would then expose flight-trained birds to the global constraints on prostacyclin signaling imposed by diet. A probable target for this upregulation would be the enzyme cyclooxygenase, which catalyzes the rate-limiting step in prostacyclin synthesis by converting arachidonic acid to the intermediate prostaglandins PGG_2 and PGH_2 (Berdanier and Berdanier, 2015; Clapp and Gurung, 2015). While variation in cyclooxygenase expression and activity in songbirds should be a target for future research, there are several reasons to suspect that it is relevant here. For one, cyclooxygenase expression can be mediated by a cyclic AMP mechanism (Madsen et al., 2008; Maldve et al., 2000), which is also involved in the regulation of energy metabolism (McClelland, 2004; Weber, 2011), and so cyclooxygenase upregulation could occur based on feedback from the high fuel demands of flight. Meanwhile, cyclooxygenase is also upregulated as part of an inflammation response to tissue damage (Wang et al., 2003), meaning that an increase in cyclooxygenase expression could be part of the process songbirds use to recover from training-induced muscle damage (Dick and Guglielmo, 2019b; Guglielmo et al., 2001). Such inflammation-driven upregulation would most likely be a response to mechanical damage, as trained birds in our study did not have appreciably higher levels of oxidative damage (McWilliams et al., 2020). Thus, there are multiple possible feedback mechanisms by which prostacyclin synthesis could be upregulated in flight-trained compared to untrained birds, with this upregulation most observable in birds fed the PUFA diet.

Whereas the effects of diet were dependent on flight-training, we detected a consistent decrease in prostacyclin metabolite concentrations in both flight-trained and untrained control birds at the final sampling timepoint. Since our experimental design and statistical analysis accounted for potential changes over the fall migration period (i.e., the flight-training procedure was repeated over 10 separate cohorts), explaining this reduction in prostacyclin metabolite concentrations requires a factor that affected both flight-trained and untrained birds within each cohort. Two days before the final sampling timepoint, both untrained and flight-trained birds were fasted longer than any other period during training during the long flights on day 15. This longer fast could have prompted a downregulation of prostacyclin synthesis in a reversal of the energy metabolism feedback mechanism described above, but also may have introduced a localized depletion of prostacyclin precursors. The liver is a major site of lipid metabolism and also exhibits very high turnover rates of membranes (e.g., 2–4 days; Carter et al., 2019; Klasing, 1998), which could contribute to the rapid depletion of prostacyclin precursors (sourced from membrane phospholipids; Berdanier and Berdanier, 2015; Zhou and Nilsson, 2001) over a relatively short amount of time. As with any metabolite, it takes time for prostaglandin products to be excreted (Willis, 1987) and glomerular filtration itself may be reduced during flight (Scanes, 2015, but see Gerson and Guglielmo, 2013), so this depletion would likely not be apparent in samples collected immediately after flight, but could still be detectable two days later at our final sampling point. One other possibility is that concentrations of prostacyclin metabolites may be responsive to stress and handling similar to other plasma metabolites such as corticosterone and triacylglycerols (Guglielmo et al., 2002; Müller et al., 2006; Smith et al., 2015). Although sampling took place in the morning at both the BG and FN time points, there was slightly more handling time prior to blood collection at the BG point (up to an hour), and so it is also possible that low levels of prostacyclin metabolite in FN samples were a product of reduced stress immediately prior to sampling. As before, these explanations are non-exclusive and studies that establish more precise timelines of prostacyclin metabolite levels and impacts of handling will be necessary to parse their contributions.

Finally, we also observed a positive relationship between body condition, in particular body fat rather than muscle development, and prostacyclin metabolite concentration. On the one hand, this finding could provide support for a proposed impact of fat reserves on the availability of precursors to prostacyclin. However, this explanation is potentially complicated by the presence of diet and timing effects on availability that would plausibly overlap with this influence. A non-exclusive but independent explanation is that obesity is regularly associated with chronic inflammation in mammals (Berdanier and Berdanier, 2015; Medzhitov, 2008; Mohite et al., 2011) and prostaglandins are classic mediators of inflammation responses (Guglielmo, 2018; Medzhitov, 2008; Russo, 2009). Accordingly, individuals with high fat scores in our study could be experiencing an ongoing inflammatory response that involves the upregulation of prostacyclin metabolism, likely via oxidative damage-based feedback mechanisms (Craig-Schmidt et al., 1987; Dröge, 2002; Marion-Letellier et al., 2016). Thus, as for the trends described above, there are multiple plausible explanations for this effect of body condition, and, to the extent that migration requires extensive fattening, this suggests that the migratory state in songbirds shares characteristics with more generalized inflammatory states. Taken overall, it appears that a combination of constraints on PUFA precursors and regulatory feedback on eicosanoid metabolism are responsible for the patterns of prostacyclin concentration we observed. In the future, carefully designed mechanistic studies will be necessary to elucidate the specific chains of events that produce these patterns and to provide a full account of their impacts.

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CRediT authorship contribution statement

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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