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Research paper

Lipid signaling and fat storage in the dark-eyed junco

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ABSTRACT

Seasonal hyperphagia and fattening promote survivorship in migratory and wintering birds, but reduced adiposity may be more advantageous during the breeding season. Factors such as photoperiod, temperature, and food predictability are known environmental determinants of fat storage, but the underlying neuroendocrine mechanisms are less clear. Endocannabinoids and other lipid signaling molecules regulate multiple aspects of energy balance including appetite and lipid metabolism. However, these functions have been established primarily in mammals; thus the role of lipid signals in avian fat storage remains largely undefined. Here we examined relationships between endocannabinoid signaling and individual variation in fat storage in captive white-winged juncos (*Junco hyemalis aikeni*) following a transition to long-day photoperiods. We report that levels of the endocannabinoid 2-arachidonoylglycerol (2-AG), but not anandamide (AEA), in furcular and abdominal fat depots correlate negatively with fat mass. Hindbrain mRNA expression of CB_1 endocannabinoid receptors also correlates negatively with levels of fat, demonstrating that fatter animals experience less central and peripheral endocannabinoid signaling when in breeding condition. Concentrations of the anorexigenic lipid, oleoylethanolamide (OEA), also inversely relate to adiposity. These findings demonstrate unique and significant relationships between adiposity and lipid signaling molecules in the brain and periphery, thereby suggesting a potential role for lipid signals in mediating adaptive levels of fat storage.

1. Introduction

Many species accumulate energy stores in anticipation of prolonged periods of increased energy demand or decreased food availability. In passerines, this primarily occurs prior to autumn or vernal migration and during winter (Baldwin and Kendeigh, 1938; Berthold, 1993). Benefits conferred by greater fat storage (e.g., fasting capacity) are tempered by costs that include increased energetic demands of flight (Freed, 1981), loss of agility (Blem, 1975), and greater predation risk with higher foraging rates (Lima, 1986). Thus, individuals must effectively balance this trade-off to maximize survivorship benefits at minimal cost (Rogers, 2015). As a result, fat stores are reduced as birds complete gonadal development and enter the breeding life-history stage (Williams, 2012). The timing and extent of winter fattening are driven by environmental factors such as temperature (Rogers, 1995), food abundance and predictability (Rogers and Smith, 1993; Witter et al., 1995; Biebach, 1996), and climate (Nolan and Ketterson, 1983), whereas migratory fattening follows a circannual rhythm predominantly synchronized by changes in photoperiod (Gwinner, 1986; Berthold, 1996).

The neuroendocrine mechanisms that mediate seasonal changes in fat are less clear. Studies have typically focused on few physiological systems, and results often suggest complex relationships that may vary by species as well as life-history stage. For example, corticosterone has been suggested to drive changes in feeding and fat deposition during winter in the migratory slate-colored race of the dark-eyed junco (*Junco hyemalis hyemalis*), a common North American songbird (Gray et al., 1990; Rogers et al., 1993). In contrast, evidence for relationships between corticosterone and pre-migratory fattening in this subspecies have been mixed, and may further be complicated by differences between migratory and resident subspecies (Ramenofsky et al., 1999; Holberton et al., 2007, 2008; Bauer et al., 2016; Fudickar et al., 2016). In addition to glucocorticoids, hormones such as testosterone (Lofts and Marshall, 1961; Morton and Mewalt, 1962;

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Stetson and Erickson, 1972; Schwabl and Farner, 1989; Deviche, 1995) and prolactin (Meier and Farner, 1964; Buntin and Tesch, 1985; Boswell et al., 1995; Holberton and Dufty, 2005) have been associated with feeding and fat regulation in birds. However, neuroendocrine regulators classically implicated in mammalian energy balance such as neuropeptide Y, insulin, and glucagon have been examined to a lesser extent (Totzke et al., 1997; Holberton and Dufty, 2005; Ramakrishnan et al., 2007).

Similarly, little is known of energy regulatory functions of endocannabinoids in birds, despite well-established roles of these ubiquitous neuromodulators in mammalian food intake (Pagotto et al., 2006), lipogenesis (Osei-Hyiaman et al., 2005; Vettor and Pagano, 2009), and energy expenditure (Cluny et al., 2010; Quarta et al., 2010; Tam et al., 2010). Endocannabinoids are produced locally and on demand in the brain and periphery. They are capable of fine-tuning synaptic transmission by activating CB_1 receptors on presynaptic axons to inhibit transmitter release (Di Marzo et al., 2005). The appetite-stimulating effects of endocannabinoids are in part due to such retrograde inhibition of classical hypothalamic circuits (Jo et al., 2005). Moreover, *in vivo* and *in vitro* models suggest that peripheral endocannabinoid signaling promotes fat deposition by stimulating glucose uptake and lipogenesis via adipocyte CB_1 receptors (Cota et al., 2003; Jbilo et al., 2005; Pagano et al., 2007). Although relatively few studies have examined endocannabinoid signaling in birds, existing reports suggest similar functions to those observed in mammals, including promotion of food intake in chickens (Novoseletsky et al., 2011) and inhibition of hypothalamo-pituitary-adrenal (HPA) activity in European starlings (Dickens et al., 2015). Because hyperphagia is the primary means to increase fat stores in birds (King et al., 1963; King, 1972; Baggott, 1975; Blem, 1980; Piersma and Jukema, 1990), the endocannabinoid system is a prime candidate to maintain plasticity and seasonally appropriate levels of food intake and fat storage.

In the present study, we examined the endocannabinoid system in relation to fat stores in captive dark-eyed juncos acclimating to long days. Specifically, we determined whether individual variation in adiposity of two major fat depots relate to local concentrations of the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA). To determine whether central endocannabinoid signaling contributes to fat levels, we quantified mRNA expression of CB_1 receptors in the hindbrain, a brain region that regulates feeding in birds (Dubbeldam, 1984; Boswell et al., 1995; Moghaddam et al., 2010) and mammals (Berthoud, 2004). Given CB_1 receptors' ability to promote food intake in birds (Novoseletsky et al., 2011), we predicted that central (CB_1) and peripheral (2-AG; AEA) endocannabinoid signaling would relate positively to fat levels in the present study. We also predicted that concentrations of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), two structurally-related lipids with opposing effects on energy balance from those of endocannabinoids (Rodriguez de Fonseca et al., 2001), would correlate negatively with adiposity. However, if the relationship between lipid signaling and adiposity is dependent on current energetic need, then an alternative prediction is that fat levels would negatively correlate with orexigenic lipid signaling in contexts of fat loss, such as during preparation for breeding.

2. Methods

2.1. Animals and housing

Thirty-five male and female dark-eyed juncos (subspecies: white-winged junco, *Junco hyemalis aikeni*) were captured as juveniles in the Black Hills National Forest near Custer, South Dakota (43° 46′ N, 103° 36′ W) at the end of the 2009 breeding season. Juncos were caught using baited mist-nets and potter traps. The birds were banded, measured, bled from the wing vein upon capture, and group-housed in a temporary outdoor aviary in the field. In early August, they were transported to Indiana University (39° 09′ N, 86° 31′ W) where they were group-housed in an indoor aviary held around 16 °C and maintained on a diet of *ad libitum* millet, sunflower seeds, orange slices, mealworms (*Tenebrio molitor*), and a mixture of puppy chow, hardboiled eggs, and carrots. From August through February, the free-flying birds were captured approximately every 2–3 weeks (17 ± 1 days) and subcutaneous fat was visually scored using established criteria (Rogers, 1991) as outlined in Table 1. Photoperiod was adjusted every two weeks to match the naturally changing photoperiod at their capture site. This allowed birds to molt and become photosensitive during spring 2010. In the wild, this subspecies overwinters at lower elevations extending from the breeding range in the Black Hills into the foothills of the nearby Rocky Mountains (Whitney, 1968; eBird, 2016). Thus, the duration of this study tracked a period of time during which birds naturally fatten over winter and lose fat as they enter breeding condition.

During the first week of March 2010, before natural photoperiod reached a stimulatory day length, biweekly photoperiod adjustments were discontinued and photoperiod was increased by 1 h every two days until a 16:8 h light:dark cycle was achieved (March 10). Birds remained on this long-day photoperiod for the duration of this study (through mid-April). During this time, birds were individually housed across four similarly-sized, mixed-sex housing rooms. This rapid increase in photoperiod is sufficient to initiate gonadal growth and loss of winter fat (Dawson et al., 2001; Bergeon Burns et al., 2014; Rosvall et al., 2016), but allows for variation in the rate at which birds adjust fat levels. For this study, we capitalized on this individual variation to examine birds that vary in degree of fat storage and pre-breeding fat loss. Juncos had high survivorship and maintained excellent physical condition in captivity. Thirty-two individuals (19 males, 13 females) were used in this study. All procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University.

2.2. Tissue collection

Prior to tissue collection for the present study, all birds participated in a separate experiment in which they received a series of three hormone challenges (two of chicken gonadotropin-releasing hormone, one of ovine luteinizing hormone) separated by five-day intervals. Small blood samples (100 μl) were collected before and shortly after hormone administration to assess transient hormone responses (Rosvall et al., 2013; Bergeon Burns et al., 2014). All birds were exposed to the same manipulations and these hormone challenges evoke only a short-term hormonal response that returns to basal condition within hours (Jawor et al., 2006; Rosvall et al., 2016). Basal hormone levels do not differ significantly between challenges separated by five-day intervals (Bergeon Burns et al., 2014), suggesting that five days is of

Table 1
Criteria used to assign fat scores.

Fat score*	Criterion
1	Fat present in abdomen or furculum (one place)
2	Fat present in both abdomen and furculum
3	Fat flush with body curvature in abdomen or furculum (one place)
4	Fat bulging in abdomen or furculum (one place)
5	Fat bulging in both abdomen and furculum

* some scores were assigned an additional + or − 0.25 for greater accuracy.

sufficient duration for birds to return to basal physiological condition. Variation in the present measures of interest at the time of sample collection can therefore be reasonably attributed to natural variation among individuals.

Five days after the final hormone challenge (in mid-April; 38 d after reaching 16L:8D), birds were overdosed with isoflurane inhalation and decapitated. Brains and gonads were quickly extracted under RNase-free conditions and immediately frozen on crushed dry ice. Visual analyses of gonads revealed that all birds were in reproductive condition and had testosterone concentrations comparable to free-living birds in the early breeding season (Rosvall et al., 2013; Bergeon Burns et al., 2014). Each bird was assigned a fat score by visual assessment, as before. Fat depots from the furculum and abdomen were then excised, weighed, and frozen on dry ice. Samples were stored at −80 °C until further microdissection (brains) or processing (fat).

2.3. Compound extraction from fat samples

Tissues were processed based on a protocol previously described (Bradshaw et al., 2006). Briefly, fat samples (~0.08 g) were incubated on ice with 50 volumes of 100% HPLC-grade methanol for 1 h. One hundred pmol [$^2H_8$]-anandamide (Cayman Chemical, Ann Arbor, MI) was added to the methanol/tissue sample and used as an internal standard to track recovery of the test compounds. Following incubation, samples were maintained on ice and homogenized (Polytron PT 10–35) for 1–2 min. Samples were centrifuged at 42,858 *g* and 24 °C for 20 min. Supernatants were collected and diluted to a 25% organic solution with HPLC-grade water. Compounds were partially purified from the supernatant using 500 mg C18 solid phase extraction columns (Varian #12113027, Harbor City, CA), conditioned with 5 ml 100% HPLC-grade methanol and 2.5 ml HPLC water prior to addition of the water/supernatant solution. Columns were washed with 2.5 ml of HPLC-grade water and three elutions were collected (1.5 ml each of 65, 80, and 100% HPLC-grade methanol). Elutions were stored at −20 °C until mass spectrometric analysis. Individual furcular and abdominal fat samples were processed separately with both sexes analyzed within each batch.

2.4. HPLC/MS/MS analysis and quantification of lipid signalers

Each elution was warmed to room temperature and vortexed prior to mass spectrometric analysis. Rapid separation was obtained with 10–20 µl injections of analyte (CBM-20A prominence controller and SIL-20AC prominence autosampler; Shimadzu, Columbia, MD; maintained at 24 °C) onto a Zorbax eclipse XDB 2.1 × 50 mm reversed phase column. Gradient elution (methanol and water; 200 µl/min) was formed under pressure on a pair of 10AdVP pumps (Shimazdu). Mass spectrometric analysis was performed using electrospray ionization with a triple quadrupole mass spectrometer (API3000, Applied Biosystems/MDS Sciex, Foster City, CA). Levels of each compound were analyzed by multiple reactions monitoring on the HPLC/MS/MS system. In this mode, detection of each compound is based on fragmentation of a precursor ion $[M+H]^+$ or $[M-H]^+$ to yield a prominent product ion. Mass spectrometric conditions were optimized for each compound using direct flow injection of synthetic standards of each compound (Cayman Chemical). Test compounds of interest were the endocannabinoids 2-AG and AEA as well as non-cannabinoid but structurally-related *N*-acyl ethanolamides, OEA and PEA.

2.5. Hindbrain RNA isolation and cDNA synthesis

Brains were microdissected into functional regions following Soma et al. (1999), and hindbrain was reserved for this study. Hindbrain tissue was homogenized for 30–45 s to isolate total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer instructions. RNA concentration was determined using a spectrophotometer and RNA integrity was verified by gel electrophoresis. All RNA samples were stored at −80 °C until further analysis. Following DNase treatment (Invitrogen), 1 µg RNA was reverse transcribed into cDNA in 20 µl total volume according to manufacturer instructions using oligo dT primers and Superscript III Reverse Transcriptase (Invitrogen), yielding a stock concentration of 50 ng/µl.

2.6. Primer design

To ensure high specificity of primers, a portion of the gene for CB_1, the primary endocannabinoid receptor in the brain, was sequenced for this species. Initial primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) from zebra finch (*Taeniopygia guttata*) CB_1 (GenBank accession no. AF255388.1): forward, 5′-AGTGGGACTTCCTCCGATGCATTT-3′; reverse, 5′-ACGATAGCGATGGTCCACATCACA-3′. A 10 µl PCR reaction was performed using 1X GoTaq Flexi DNA polymerase (Promega, Madison, WI), 2.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1 U taq, distilled water, and cDNA as a template at a concentration of 5 ng/µl. The reaction was carried out at 95 °C for 2 min, then 30 cycles of 95 °C for 30 s/54 °C for 30 s/72 °C for 1.5 min, followed by 72 °C for 5 min. The reaction amplified an 809-bp product which was confirmed on a 1% TBE agarose gel containing ethidium bromide. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer instructions, sequenced at the Indiana Molecular Biology Institute (Bloomington, IN), and deposited in GenBank (accession no. KX574508). Product sequences were confirmed to be highly homologous to the CB_1 sequences for zebra finch (97%) and the closely-related white-throated sparrow (*Zonotrichia albicollis*; 99%) using NCBI BLAST. Junco-specific qPCR primers for CB_1 were then designed based on this sequence using PrimerQuest: forward (300 nM), 5′-TTACGTGGGCTCCAACGATATCCA-3′; reverse (300 nM), 5′-TGATGTTGACCTGCTCTGAGGGAA-3′ (amplifying a 179-bp product). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a reference gene and its primers were based from zebra finch sequence (GenBank accession no. AF255390): forward (300 nM), 5′-TGACCTGCCGTCTGGAAAA-3′; reverse (300 nM), 5′-CCATCAGCAGCAGCCTTCA-3′ (amplifying a 70-bp product). Standard curves with serial dilutions of cDNA resulted in >0.99 correlation coefficients and amplification efficiency at 100.1% and 109.3% for CB_1 and GAPDH, respectively.

2.7. Quantitative PCR

Quantitative PCR was performed on a Stratagene MX3000P qPCR machine by using PerfeCta SYBR Green SuperMix with Low ROX (Quanta BioSciences, Inc., Gaithersburg, MD). Twenty-five µl reactions were carried out containing half the reaction volume (12.5 µl) of SYBR, 12.5 ng cDNA, and forward and reverse primers at a concentration of 300 nM. Samples were run in duplicate at 95 °C for 5 min, 40 cycles of 95 °C for 30 s/60 °C for 1 min/70 °C for 30 s, followed by dissociation curves of PCR products to confirm primer specificity.

Samples were run across two plates and a common calibrator run on each confirmed low inter-plate variability (% CV = 8.25). No template controls were also run in duplicate on each plate and demonstrated no amplification. To quantify CB_1 mRNA expression, mean C_T values were determined between reaction duplicates for each sample. Mean CB_1 C_T values were then normalized to

those of an internal reference gene (GAPDH) using the $2^{-\Delta\Delta CT}$-method (Schmittgen and Livak, 2008).

2.8. Statistical analyses

Statistical analyses were conducted using SPSS 17.0 (Chicago, IL) and JMP 12.0 (Cary, NC). Pearson correlation coefficients were calculated for all correlational data except for the correlation between fat score and combined fat mass, for which Spearman's rho was calculated instead due to the ordinal nature of fat score. Outliers were determined by Grubbs' test (GraphPad Software, La Jolla, CA) and excluded from analyses. Data from one individual for which lipid concentrations were not detectable in furcular fat were also removed from analyses, resulting in final sample sizes of 30–32. Changes in adiposity over time were quantified using a repeated-measures MANOVA. A post-hoc Welch's ANOVA was used to contrast fat scores in the last score before light advancement (Feb 24) and the final score at the end of the study (April 17). Because males and females did not differ in any outcome measure after light advancement, data were collapsed across sex for all end-point analyses. Findings were considered significant when $p < 0.05$, and we report means ± SEM unless otherwise noted.

3. Results

3.1. Adiposity

There was a strong positive correlation between abdominal and furcular fat mass (Fig. 1A; $r = 0.936$, $p < 0.001$) and between combined fat mass (i.e., abdominal + furcular fat mass) and visual fat score (Fig. 1B; $r = 0.912$, $p < 0.001$) thereby validating the use of this measure in estimating fat levels. White-winged juncos responded to light advancement with significant reductions in adiposity (Welch's ANOVA: $F = 6.24$, $p = 0.01$; Fig. 2). Just prior to light advancement, mean fat score was 3.72 ± 0.30 and differed significantly between males and females (Repeated measures MANOVA, Date: $F = 33.05$, $p < 0.001$; Sex: $F = 7.38$, $p = 0.012$; Sex * Date: $F = 2.9256$, $p = 0.008$). After 38 d in a long-day photoperiod, mean fat score was 2.76 ± 0.16 and did not differ between sexes ($t = 1.12$, $p = 0.27$). Based on the relationship between fat score and mass (Fig. 1B), this decline in fat score amounts a loss of almost one gram of fat (average: 0.87 g), with some birds gaining up to 0.35 g and others losing as much as 1.54 g.

3.2. Lipid signals in adipose tissue

In general, concentrations of lipid signals corresponded significantly with adiposity levels but did not differ between sexes (all $p > 0.1$). Individuals with lower amounts of fat tended to have higher levels of 2-AG and OEA. Specifically, furcular 2-AG levels correlated negatively with all three measures of adiposity (i.e., furcular, abdominal, and combined fat mass) ($r = -0.442$, $p = 0.014$ for combined fat mass; Fig. 3A), and similar correlations were observed with OEA ($r = -0.462$, $p = 0.010$ for combined fat mass; Fig. 3C). In contrast, furcular PEA trended towards a positive correlation with combined fat mass (Fig. 3D; $r = 0.322$, $p = 0.082$), whereas no associations were observed between furculum-derived AEA and any adiposity measure (Fig. 3B; $p > 0.05$). Concentrations of lipid signals in abdominal fat demonstrated somewhat similar patterns as in the furculum: abdominal 2-AG and OEA correlated negatively with all adiposity measures ($r = -0.557$, $p = 0.001$; 2-AG and combined fat mass; Fig. 4A), ($r = -0.535$, $p = 0.002$; OEA and combined fat mass; Fig. 4C) whereas abdominal AEA and PEA showed little relation to adiposity (Fig. 4B and D; $p > 0.05$). In both fat depots, 2-AG and OEA were the only compounds that correlated significantly with each other (furculum: $r = 0.371$, $p = 0.048$; abdominal fat: $r = 0.509$, $p = 0.004$; all others: $p > 0.09$, $r < |0.326|$).

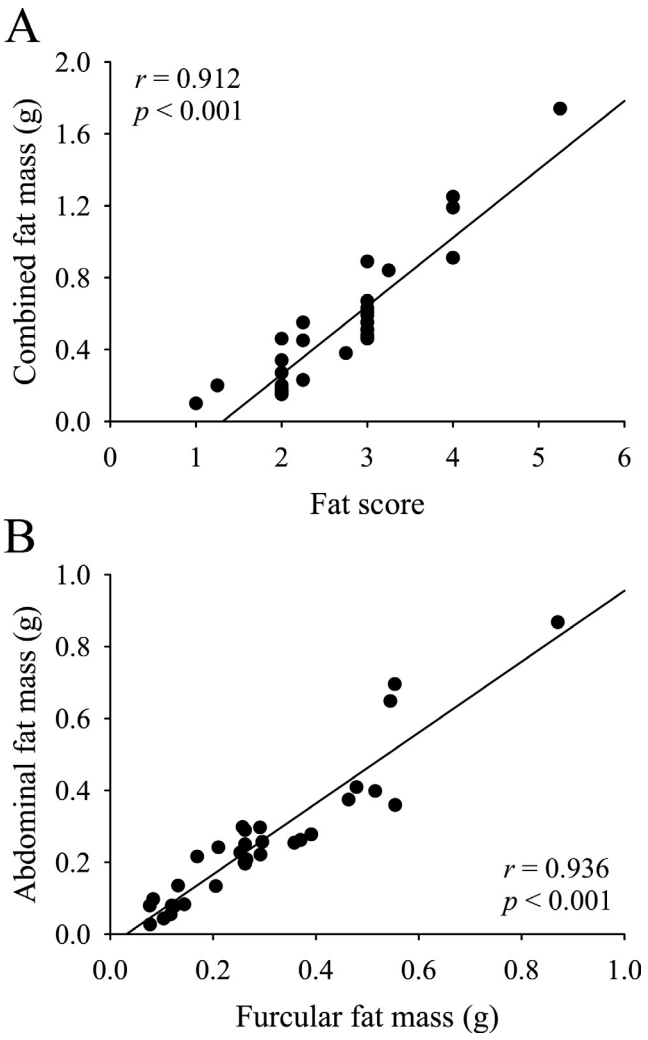

Fig. 1. Relationships between visual fat score and combined mass of abdominal and furcular fat depots (A) and between abdominal and furcular fat mass (B). $p < 0.05$, Pearson's correlation coefficient and Spearman's rho.

3.3. CB_1 expression in hindbrain

Hindbrain CB_1 mRNA expression did not differ between sexes ($p > 0.05$) and correlated significantly and negatively with abdominal fat mass ($r = -0.364$, $p = 0.044$). A similar pattern was observed between CB_1 mRNA and furcular fat levels ($r = -0.338$, $p = 0.063$), resulting in a significant negative correlation between hindbrain CB_1 mRNA expression and overall adiposity (Fig. 5; $r = -0.357$, $p = 0.048$).

4. Discussion

Seasonally appropriate fat storage has important consequences for survival and reproductive success. Given its role in mammals to promote food intake and lipogenesis (Osei-Hyiaman et al., 2005; Pagotto et al., 2006; Vettor and Pagano, 2009), endocannabinoid signaling may be one mechanism by which rapid modulation of feeding and fattening is achieved in seasonally-breeding birds. In the present study, we used photostimulation of captive birds to

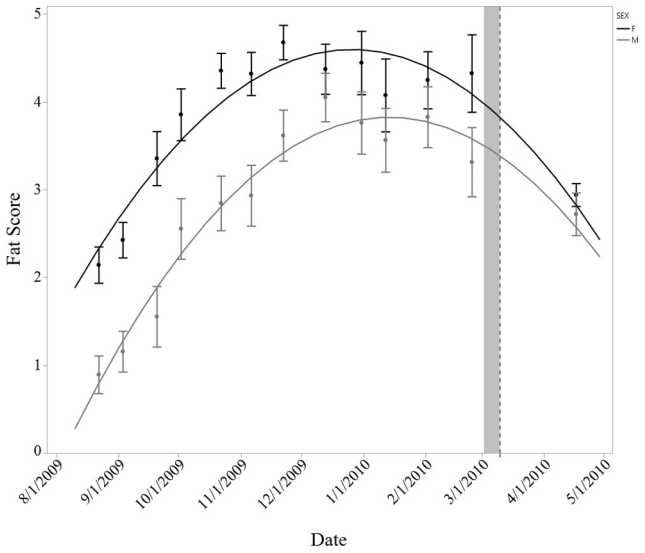

Fig. 2. Relationship between adiposity and date, as birds gain fat over-winter and lose fat as they enter breeding condition. Error bars represent one standard error from the mean. The shaded bar highlights the period of light advancement, and the dashed line indicates when birds reached a long-day photoperiod (16L:8D). Although the sexes differed in overwinter fat levels, males (gray) and females (black), they did not differ after light advancement. Repeated measures MANOVA, Date: $F = 33.05$, $p < 0.001$; Sex: $F = 7.38$, $p = 0.012$; Sex * Date: $F = 2.926$, $p = 0.008$. Post-hoc t-test compared sexes in April: $t = 1.12$, $p = 0.27$.

induce physiological readiness to reproduce and concurrent fat reduction. We report negative correlations between adiposity and local 2-AG and OEA concentrations, as well as with hindbrain mRNA expression of CB_1 receptors. Our results suggest that different lipid signals serve different functions that may depend on the context under which fat storage occurs.

The birds in this study exhibited highly variable and in some cases extremely pronounced fat while under winter-like photoperiods with *ad libitum* access to food (Fig. 2). The experimental increase in photoperiod was sufficient to trigger an overall reduction in adiposity, as well as gonadal growth and marked steroidogenic capabilities demonstrating that birds were in reproductive condition (Rosvall et al., 2013, 2016; Bergeon Burns et al., 2014). Mean fat score of birds at study end remained elevated relative to initial measures in August when birds were on natural long-day photoperiods, suggesting that birds may still have been losing fat at the time of terminal measures. At the time of collection, there remained high variation in adiposity among individuals (CV = 31.9%).

Such variation in fatness revealed negative relationships between 2-AG concentrations and furcular and abdominal fat mass, indicating that fatter birds have less 2-AG content in fat depots. These findings are consistent with a previous study of seasonal adiposity in which lower 2-AG was observed in subcutaneous white adipose tissue of fat, summer-acclimated Siberian hamsters compared with lean, winter-acclimated counterparts (Ho et al., 2012). However, these findings sit in contrast to our predictions and reports of elevated 2-AG concentrations in adipose tissue of obese humans and diet-induced obese mice (Matias et al., 2006). One possible explanation for the discrepancy between studies may be the context in which adiposity occurs. In the context of seasonal adaptation, the negative relationship between endocannabinoid signaling and adiposity may reflect a drive towards fat loss or maintenance once sufficient stores are achieved. Although food intake was not measured in the present study, low 2-AG levels may reflect an appropriate response for individuals with sufficient fat stores by inhibiting further food intake and lipogenesis, and perhaps even promoting continued fat loss in preparation for breeding. In contrast, high 2-AG levels in clinical obesity may result from dysregulation of regulatory systems that include endocannabinoid signaling. Because our findings provide only a snapshot of dynamic changes in lipid signaling, studies that compare signaling across life-history stages (such as pre- and post-migratory fattening, winter fattening, and/or breeding) are needed

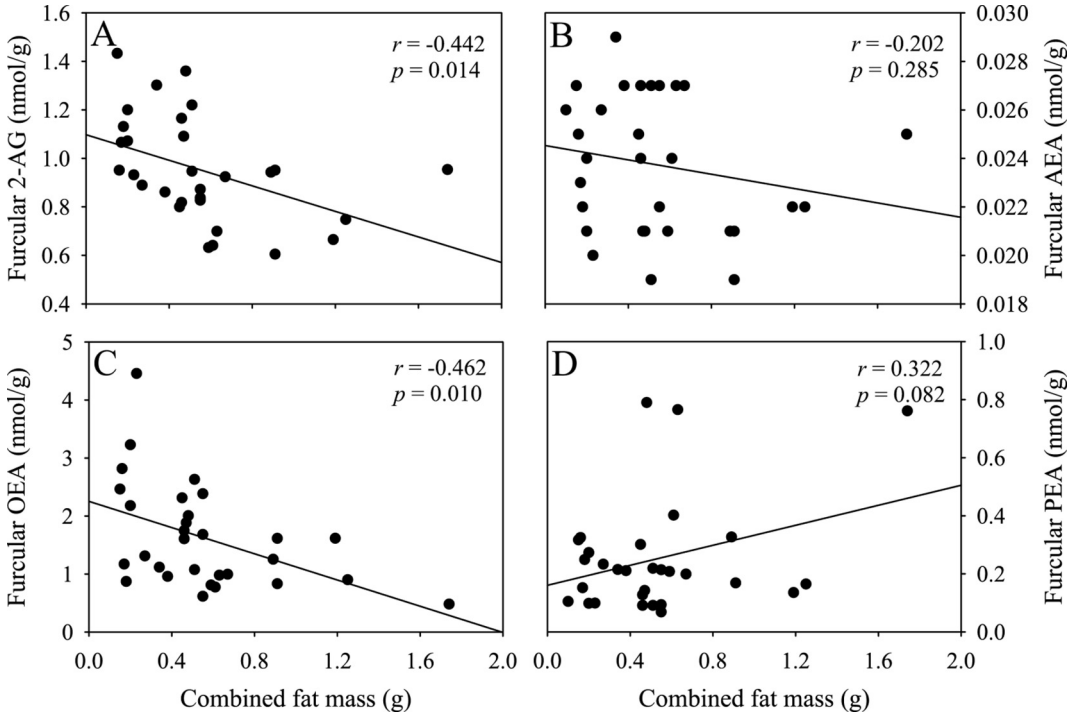

Fig. 3. Relationships between combined abdominal and furcular fat mass and furcular levels of 2-arachidonoylglycerol (2-AG) (A), anandamide (AEA) (B), oleoylethanolamide (OEA) (C), and palmitoylethanolamide (PEA) (D). Pearson's correlation coefficient.

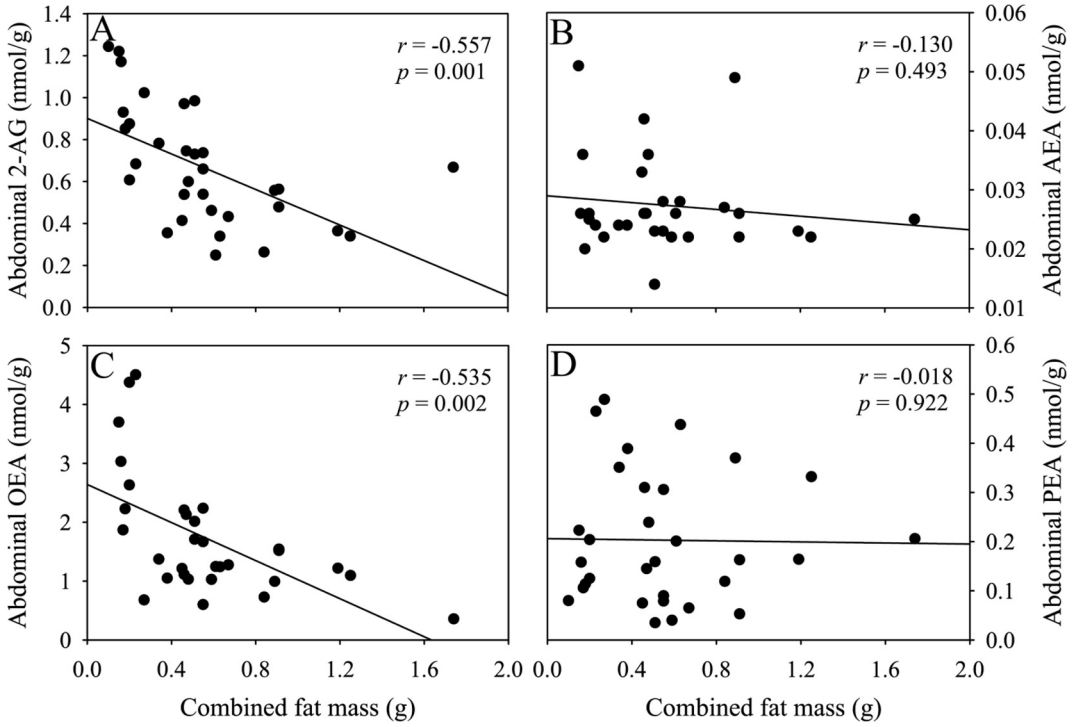

Fig. 4. Relationships between combined abdominal and furcular fat mass and abdominal levels of 2-arachidonoylglycerol (2-AG) (A), anandamide (AEA) (B), oleoylethanolamide (OEA) (C), and palmitoylethanolamide (PEA) (D). Pearson's correlation coefficient.

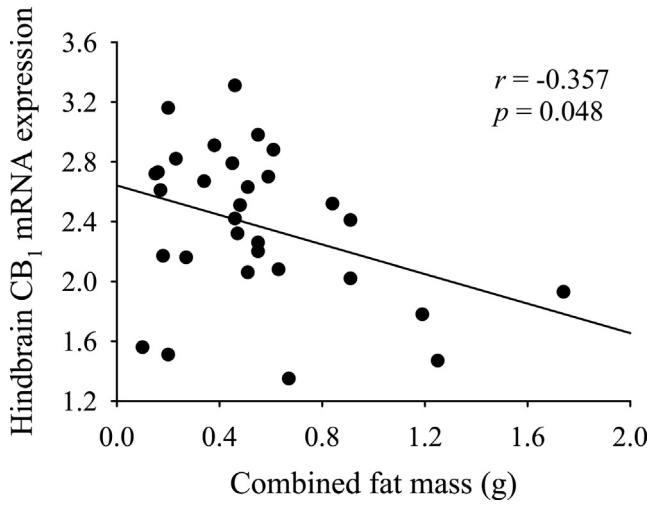

Fig. 5. Relationship between combined abdominal and furcular fat mass and hindbrain CB_1 mRNA expression levels. Expression levels were calculated relative to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized to calibrator values. $p < 0.05$, Pearson's correlation coefficient.

to determine how 2-AG concentrations relate to adiposity in natural conditions.

No correlations were observed between the other major endocannabinoid ligand, AEA, and any measure of adiposity, indicating a functional separation between endocannabinoid signals. This finding is not surprising, as dissociations between 2-AG and AEA have been reported across a variety of physiological contexts (Di Marzo and Maccarrone, 2008) and concentrations of 2-AG correlated significantly with OEA, but not AEA, in this study. Indeed, concentrations of 2-AG, but not AEA, are significantly elevated in epididymal fat of diet-induced obese mice and in visceral fat of obese humans in comparison with their respective lean controls (Matias et al., 2006). Moreover, each ligand uses a different set of enzymatic machinery (Dinh et al., 2002; Sugiura, 2008), and AEA also interacts with non-cannabinoid receptors (Zygmunt et al., 1999). Our findings suggest that as with mammals, avian 2-AG and AEA are independent from one another in form and function.

The patterns we observed in the brain mimic those seen for 2-AG in the periphery: individual juncos demonstrating greater adiposity had less hindbrain CB_1 mRNA expression. In mammals, this brain region includes the dorsal vagal complex, which sits at the interface of the gut-brain axis and integrates meal-related satiety signals (Berthoud, 2004). To the extent that mRNA abundance is predictive of receptor expression, less hindbrain CB_1 mRNA in fatter birds is suggestive of less endocannabinoid suppression of satiety signals that emerge from this brain region, thereby promoting satiety. These results may once again reflect a drive to maintain or reduce energy intake in fat birds that have already achieved sufficient energy stores. These results stand in contrast to previous studies in Siberian hamsters that demonstrate endocannabinoid signaling fluctuates seasonally in the hindbrain, such that high summertime adiposity is associated with higher protein levels of hindbrain CB_1 and 2-AG relative to lean, winter-like states (Ho, 2011; Ho et al., 2012). Again, further studies that address how hindbrain CB_1 signaling relates to appetite and adiposity with greater temporal resolution will help clarify its role in regulating adiposity.

Because endocannabinoids are part of a family of biologically active lipids, we examined two additional lipids found in the fat depots that are structurally related to AEA and relevant to energy regulation, although with very different functions than endocannabinoids. OEA and PEA do not act at cannabinoid receptors, but instead activate peroxisome proliferator-activated receptors (PPARs) that regulate lipid metabolism. OEA attenuates body mass gains and stimulates breakdown of fat through PPAR-α (Fu et al., 2003), but its peripherally-based anorectic effects (Rodriguez de Fonseca et al., 2001) may be in part due to its ability to activate GPR119 and elicit glucagon-like peptide-1 (GLP-1) secretion (Lauffer et al., 2009; Moss et al., 2016), which itself has anorectic

effects in the hindbrain (Hayes et al., 2008). Thus we predicted that OEA would function in a direction opposite to that of endocannabinoids with respect to fat regulation; instead, we found that OEA concentrations correlated negatively with adiposity, much like our 2-AG findings. Interestingly, OEA concentrations were notably higher than any other lipid examined. While it is not yet known how changes in OEA signaling alter adiposity in birds, these patterns suggest that this compound may be important and should be studied further.

PEA also activates PPAR-α (Fu et al., 2003; LoVerme et al., 2006) and inhibits food intake in rodents, albeit to a lesser extent than OEA (Rodriguez de Fonseca et al., 2001). In our study, furcular PEA correlated somewhat positively with combined fat mass, but no relationship was observed between abdominal PEA and fat mass. It is presently unclear why patterns of PEA expression contrast with those of OEA given their similar effects on appetite, but these patterns may relate to PEA's involvement in other functions such as inflammation (Solorzano et al., 2009) and analgesia (Calignano et al., 1998). Moreover, relationships between adiposity and the other lipids examined in this study were generally consistent between furcular and abdominal depots, thus the functional significance of these particular findings are unclear.

5. Conclusions

Considered together, our findings reveal several significant relationships between lipid signaling and adiposity that collectively suggest a role for peripheral and central endocannabinoid signaling in the regulation of fat storage in birds. The patterns we have identified lay the groundwork for future studies that are essential to understanding relationships between fat storage and lipid signaling, including those that ultimately determine how lipid signaling relates to temporal changes in adiposity. Further studies that experimentally manipulate lipid signaling, examine food intake and energy expenditure, and define temporal changes across periods of fat gain and loss will help determine the functional effects of these lipid signals and whether they causally relate to seasonal changes in adiposity. Our study is the first to report OEA and PEA concentrations in birds and demonstrates significant associations between lipid signaling and adiposity. As a consequence, these results add to the wealth of knowledge on the adaptive function of fat regulation in birds by shedding new light on the lesser known, proximate mechanisms involved in this process. The present study takes an important step towards establishing a potential role for lipid signaling in regulating adiposity in birds.

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