Received: 10 October 2017 | Revised: 22 August 2018 | Accepted: 27 August 2018

DOI: 10.1002/cne.24535

WILEY JCN RESEARCH IN SYSTEMS NEUROSCIENCE THE JOURNAL OF COMPARATIVE NEUROLOGY

RESEARCH ARTICLE

Neuropeptide Y and orexin immunoreactivity in the sparrow brain coincide with seasonal changes in energy balance and steroids

H. Bobby Fokidis[1] | Chunqi Ma[2] | Benjamin Radin[1] | Nora H. Prior[2,3] | Hans H. Adomat[4] | Emma S. Guns[4,5] | Kiran K. Soma[2,6,7]

[1]Department of Biology, Rollins College, Winter Park, Florida

[2]Department of Psychology, University of British Columbia, Vancouver, British Columbia, Canada

[3]Program in Neuroscience and Cognitive Neuroscience, University of Maryland, College Park, Maryland

[4]The Prostate Centre, Vancouver General Hospital, Vancouver, British Columbia, Canada

[5]Department of Urological Sciences, University of British Columbia, Vancouver, British Columbia, Canada

[6]Graduate Program in Neuroscience, University of British Columbia, Vancouver, British Columbia, Canada

[7]Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver, British Columbia, Canada

Correspondence
H. Bobby Fokidis, Department of Biology, Rollins College, 1000 Holt Avenue, Winter Park, FL 32789-4499.
Email: hfokidis@rollins.edu

Funding information
Canadian Institutes of Health Research, Grant/Award Number: 133606; Centre of Excellence in Commercialization of Research; Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: postdoctoral fellowship

The transition between the breeding and nonbreeding states is often marked by a shift in energy balance. Despite this well-known shift in energy balance, little work has explored seasonal differences in the orexigenic neuropeptides that regulate food intake in wild animals. Here we tested the hypothesis that free-living male song sparrows (*Melospiza melodia*) show seasonal changes in energetic state, circulating steroids, and both neuropeptide Y (NPY) and orexin (OX) immunoreactivity. Nonbreeding song sparrows had more fat and muscle, as well as a ketone and triglyceride profile suggesting a greater reliance on lipid reserves. Breeding birds had higher plasma androgens; however, nonbreeding birds did maintain androgen precursors in circulation. Nonbreeding birds had more NPY immunoreactivity, specifically in three brain regions: lateral septum, bed nucleus of the stria terminalis, and ventral tegmental area. Furthermore, nonbreeding birds had more OX immunoreactivity in multiple brain regions. Taken together, the data indicate that a natural shift in energy balance is associated with changes in NPY and OX in a region-specific manner.

KEYWORDS

β-hydroxybutyrate, cholesterol, hypocretin, mass spectrometry, RRID: AB_518504, RRID: AB_653610, songbird, steroid profiling

1 | INTRODUCTION

Energy balance is a primary driver of animal life-histories, as metabolic demands differ across the course of a year (Guglielmo & Williams, 2003; Malik, Singh, Rani, & Kumar, 2014) and this imposes varying metabolic challenges on breeding and nonbreeding individuals (Broggi et al., 2007; Swanson, Zhang, Liu, Merkord, & King, 2014). In seasonal breeders, the potential benefits of increased food availability may be offset by greater energy expenditure through reproductive activities (Hinsch, Pen, & Komdeur, 2013). In contrast, nonbreeding animals face other metabolic challenges including: low temperatures and inclement weather (De Bruijn & Romero, 2013; Metcalfe, Schmidt, Bezner Kerr, Guglielmo, & MacDougall-Shackleton, 2013; Romero, Reed, & Wingfield, 2000); reduced food availability and less foraging time with short photoperiods (Cahill, Tuplin, & Holahan, 2013; Watts & Hahn, 2012); and longer overnight fasts (Smith, Reitsma, & Marra, 2011). Seasonal differences in energetic balance are well-documented (Fokidis, Orchinik, & Deviche, 2009; Wu et al., 2014).

The central mechanisms coordinating energy balance are located within the hypothalamus, where visceral and hormonal inputs regulate neuropeptides, such as neuropeptide Y (NPY) and orexin (OX; Ebling & Barrett, 2008; Langhans, 2002). In the arcuate nucleus, NPY acts to increase food intake and fat storage (Joly-Amado et al., 2014). OX, present as both A and B isoforms, promotes food intake and wakefulness (De la Herrán-Arita, Equihua-Benítez, & Drucker-Colín, 2015; Ohno & Sakurai, 2008; Teske, Billington, & Kotz, 2010; Thompson & Borgland, 2011) as well as thermogenesis (Morrison, Madden, & Tupone, 2014; Tupone, Madden, Cano, & Morrison, 2011).

The orexigenic effect of NPY is mediated in part through synaptic interactions with OX cells in the lateral hypothalamus (Gorissen, Flik, & Huising, 2006; Li, Xu, Rowland, & Kalra, 1994; Martins, Marques, Tufik, & D'Almeida, 2010; Mercer, Chee, & Colmers, 2011). Furthermore, both OX and NPY respond to lipid infusion and higher serum triglycerides with higher gene expression and c-Fos immunoreactivity in relevant brain regions (Chang, Karatayev, Davydova, & Leibowitz, 2004), and a higher access to dietary saturated fatty acids decreases NPY expression in the arcuate nucleus (Barson, Karatayev, Gaysinskaya, Chang, & Leibowitz, 2012). Previous studies have demonstrated seasonal shifts in diet alter NPY and OX (Archer et al., 2002; Clarke, Scott, Rao, Pompolo, & Barker-Gibb, 2000; Kirsz et al., 2012; Kirsz, Szczesna, Dudek, Bartlewski, & Zieba, 2014; Kirsz, Szczesna, Molik, Misztal, & Zieba, 2017; Reddy, Cronin, Ford, & Ebling, 1999; Striberny, Ravuri, Jobling, & Jorgensen, 2015), suggesting the environment can drive patterns in orexigenic neuropeptide abundance and distribution. However, despite experimental studies proposing strong links between energy balance and orexigenic neuropeptides, the effects of natural fluctuations remain essentially unstudied.

Natural shifts in energy balance are associated with reproduction. Orexigenic neuropeptides generally suppress the reproductive axis by lowering gonadotropin-releasing hormone (GnRH) neuronal activity (Gaskins & Moenter, 2012); decreasing luteinizing hormone release (Furuta, Mitsushima, Shinohara, Kimura, & Funabashi, 2010); and reducing vascularization of the gonads (Allen et al., 2011). There are also potential effects of gonadal NPY in the regulation of steroidogenesis (Priyadarshini & Lal, 2018a, 2018b). Collectively, these observations suggest that orexigenic neuropeptides may vary between the breeding and nonbreeding states.

Songbirds are excellent models for understanding the links between steroids and orexigenic neuropeptides, because of well-documented seasonal changes in steroid-sensitive behaviors, such as territoriality and mating, and in relevant brain regions (Saldanha, Remage-Healey, & Schlinger, 2011). The songbird brain regions associated with social behaviors and sex steroid actions are well-described (Goodson, Wilson, & Schrock, 2012), as are the distributions of both OX and NPY (Godden, Landry, Slepneva, Migues, & Pompeiano, 2014; Miranda et al., 2013; Singletary, Deviche, Strand, & Delville, 2006; Singletary, Hayworth, & Delville, 2010).

Here, we tested the hypothesis that free-living male song sparrows (*Melospiza melodia*) in a temperate environment face increased metabolic challenges during the nonbreeding season compared to the breeding season, and this alters circulating steroid levels and brain NPY and OX immunoreactivity. First, we assessed metabolic state, by measuring body morphology, fat and muscle stores, and circulating concentrations of glycerol, triglycerides, and ketone bodies. Second, we used liquid chromatography tandem mass spectrometry (LC–MS/MS) to profile circulating steroids, especially androgens and androgen precursors, as well as cholesterol and associated esters. Third, we examined NPY and OX immunoreactivity in multiple brain regions associated with social behavior or metabolism. This is the most comprehensive study of seasonal differences in NPY and OX in the avian brain and the first to examine both steroid and metabolic profiles.

2 | METHODS

2.1 | Animals

Adult male song sparrows were captured within the Pacific Spirit Regional Park and the surrounding University of British Columbia Endowment Lands Ecological Reserve (49.253°N, 123.216°W) in the Point Grey area of Vancouver, BC, Canada, using mist nets and conspecific song playback during the nonbreeding season (N = 10; January 30–February 9, 2012) and breeding season (N = 8; April 18–24, 2012). All subjects were captured within 3 hr of sunrise (mean ± *SEM*: 1.09 ± 0.23 hr), following <9 min of playback (3.27 ± 1.26 min), and all subjects responded in a manner consistent with territorial defense, including rigorous singing and calling and approaches to the speaker, although the possibility of vagrant birds cannot be entirely excluded. Within 4 min of capture (2.16 ± 0.24 min), a blood sample was collected from the right jugular vein using a heparinized 3 cc insulin syringe and stored on ice, until centrifugation in the laboratory to remove plasma, and aliquots were then stored at −80 °C. Subjects were weighed (±0.5 g), scored for furcular fat and pectoralis muscle (both on a 0–5 scale, with 0 indicating no fat or muscle regressed to completely expose the keeled sternum), and the length of their tarsus was measured (±0.1 mm). To assess breeding condition, the cloacal protuberance and both the right and left testes were measured (±0.1 mm), after birds were sacrificed (see below).

2.2 | Tissue processing

Subjects were then immediately anesthetized with isoflurane and transcardially perfused with 300 ml of 0.75% avian saline, followed by 150 ml of 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Brains were removed within 16 min of capture (12.11 ± 0.51 min), post-fixed overnight at 4 °C in 4% paraformaldehyde, rinsed 3 times with PBS, and cryoprotected in 10% and then 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 24 hr each. Brains were then frozen at −80 °C, until coronally sectioned on a cryostat at 40 μm. Separate alternating series for NPY and OX staining were collected and stored in cryoprotectant solution at −20 °C until immunohistochemistry was performed.

2.3 | Glycerol, triglycerides, and β-hydroxybutyrate analyses

Plasma free glycerol and total triglycerides were measured using a sequential color endpoint assay (reagents F6428 and T2449, Sigma–Aldrich, St Louis, MO; for details, see Butler, Lutz, Fokidis, & Stahlschmidt, 2016; Fokidis et al., 2012; Fokidis, Hurley, Rogowski, Sweazea, &

Deviche, 2011; Guglielmo, O'Hara, & Williams, 2002). Plasma free triglycerides were estimated as the difference between the total triglycerides and free glycerol concentrations. Free triglycerides were used in subsequent analyses. Plasma concentrations of the ketone body β-hydroxybutyrate were determined using a commercial colorimetric assay (#700190, Cayman Chemical Co., Ann Arbor, MI). All concentrations are expressed in mM and assay sensitivities were 1.41 mM (glycerol and free triglycerides) and 0.025 mM (β-hydroxybutyrate). The mean intra- and interassay coefficients of variation are 2.3 and 9.3% (glycerol); 4.1 and 8.0% (free triglycerides); and 3.3 and 12.6% (β-hydroxybutyrate). Validations include tests for parallelism between a standard curve and a serial dilution of a plasma pool (all $p \leq .016$) and analyte recovery from spiked plasma samples (91%, 82%, and 76% for glycerol, free triglycerides and β-hydroxybutyrate, respectively).

2.4 | Cholesterol analysis

Plasma cholesterol was analyzed using LC–MS/MS according to (Liebisch et al., 2006). Briefly, cholesterols in 5 μl of plasma were extracted using 1 ml of 1:4 (v/v) methanol:methyl *tert*-butyl ether (MeOH:MTBE). As an internal standard, 0.2 μg of deuterated cholesterol (d6-CHOL, C/D/N isotopes) was added to all samples, and then 500 μl of water was added to provide a clear phase separation. Plasma was further extracted with 500 μl of MTBE and then dried down in a vacuum concentrator (Centrivap, LabConco Inc., Kansas City, MO). Resulting residues were derivatized with 200 μl of 1:5 (v/v) acetyl chloride:chloroform ($CHCl_3$) to improve ionization capability and dried down. Samples were then resuspended in 60 μl of MeOH:$CHCl_3$ (70:30, v/v), further diluted with 140 μl MeOH, and transferred to a 300 μl LC insert.

Analysis of cholesterol and cholesteryl esters was conducted on a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer (Waters Corp., Milford, MD) equipped with a 2.1 mm×50 mm, BEH 1.7 μM C18 column. The mobile phases for separation of cholesteryl esters consisted of acetonitrile:0.1 M ammonium acetate (9:1, v/v; mobile Phase A) and isopropanol (mobile Phase B; gradient: 0.2 min, 25% B; 5–8 min, 70% B, 8.1 min, 25% B; 10 min total run length). Data were collected in electrospray (ES+) mode by multiple reaction monitoring (MRM) for cholesterol, as the acetyl derivative (acetyl-cholesterol), or by precursor scanning (PC) for cholesteryl esters. Instrument parameters were optimized for the *m/z* ratios and corresponding fragments of the acetyl-cholesterol monitored for each MRM with PC parameters, similar to the MRM for cholesterol. Data were analyzed with Quanlynx (Waters Corp.) and normalized to sample volume. Putative cholesteryl esters were identified based on precursor mass with calibration against cholesterol oleate for all esters. The calibration range for cholesterols was 0.2–10 μg/ml (all R^2 values >0.98). Recoveries and conversions to derivatized species were greater than 90% for cholesterol and greater than 75% for cholesterol oleate.

2.5 | Steroid analysis

Steroid analysis has been previously described (Fokidis, Adomat, et al., 2015; Fokidis, Yieng Chin, et al., 2015; Locke et al., 2009, 2010; Lubik et al., 2011; Prior et al., 2016). In brief, steroids were extracted from 50 μl of plasma using 20 μl of 1 M NaOH with 2,000 μl of 60:40 (v/v) hexane:ethyl acetate (extracted 3 times). Deuterated testosterone, dihydrotestosterone (DHT) and androstanediol (ADIOL; d3-T, d3-DHT, d3-ADIOL, and C/D/N isotopes) were included as internal standards. The three extracts were pooled together, and then split into two samples prior to dry down in a vacuum concentrator (Centrivap). Each of the resulting residues (two per sample) was derivatized using either hydroxylamine (HA) or 2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate (FMP), which enhance sensitivity for ketone and hydroxyl steroids, respectively (Locke et al., 2010). For HA, samples were derivatized using 50 μl of 50 mM HA in 50% MeOH, centrifuged at 20,000 *g* for 2 min, transferred to an LC insert, heated to 65 °C for 30 min, and then loaded onto the LC–MS/MS. For the other half of the sample, FMP (Sigma, Oakville, Ontario, Canada) was dissolved in $CHCl_2$ to yield a 20 mM solution, and then 4 μl/ml of triethylamine was added. This solution was prepared immediately prior to use. Samples were then derivatized using 400 μl of the FMP solution, left to incubate at room temperature for 1 hr, and then dried down. The dried extracts were then reconstituted in 50 μl of 50% MeOH and transferred to an LC insert for loading onto the LC–MS/MS.

Analysis was carried out with a Waters Acquity UPLC Separations Module coupled to a Waters Quattro Premier XE Tandem Mass Spectrometer. 2.1 mm × 100 mm BEH 1.7 μM C18 columns were used for the derivatized steroid samples. Steroid mobile phases consisted of water (mobile Phase A) and 0.1% formic acid in acetonitrile (mobile Phase B), with the following gradient used: 0 min, 10% B; 0.5 min, 10% B; 1 min, 20% B; 7 min, 30% B; and 13 min, 35% B. This was followed by a column flush of 95% acetonitrile and re-equilibration, for a total run length of 18 min. Column temperature was 35 °C and injection volumes were 15 μl. The MS was set at unit resolution, capillary was 1.5 kV, source and desolvation temperatures were 120 °C and 300 °C, respectively, desolvation and cone gas flows were 1,000 L/hr and 50 L/hr and the collision cell pressure was held at 4.6×10^{-3} mbar. All data were collected in ES+ by MRM for steroids. The calibration range for steroids was 0.01–10 ng/ml (all R^2 values >0.95). Instrument parameters were optimized for the *m/z* ratios and corresponding fragments of the oxime-steroids monitored for each MRM. Data processing was done with Quanlynx (Waters Corp.) and normalized to sample volume. Recoveries and conversions to derivatized steroid species were greater than 80% for each steroid.

2.6 | Immunohistochemistry

Brain sections from each series were rinsed in 0.1 M Trizma-buffered saline (TBS: 3 × 10 min). Next, sections were incubated in 0.5% hydrogen peroxide in TBS for 30 min and then rinsed in TBS (5 × 5 min). Sections were then blocked by incubating for 2 hr in either 5% normal goat serum (NGS: for NPY) or 5% normal donkey serum (NDS: for OX staining) in TBS with 0.2% TritonX-100 (TBS-T). After blocking, sections were incubated either overnight (for NPY) or for two nights (for OX) in primary antibody in either 2% NGS (for NPY staining) or 2% NDS (for OX staining) in TBS-T.

Incubation in primary antibody (see below) was followed by 5 × 10 min rinses in TBS; incubation in biotinylated secondary antibody (NPY in a 1:2,000 goat antirabbit IgG for overnight at 4 °C and for OX in a 1:500 donkey antigoat IgG for 2 hr at room temperature; both from Vector Laboratories, Burlingame, CA); followed by 5 × 10 min rinses in TBS; and then 1 hr in Vectastain ABC solution (Vector Laboratories). After 5 × 5 min rinses in TBS, immunolabeling was visualized using a DAB peroxidase substrate kit (Vector Laboratories) for ~10 min, and then sections were washed twice in TBS. Immunolabeled sections were mounted onto gelatin-coated microscope slides and allowed to dry for 24 hr at room temperature, then dehydrated with ethanol, cleared in xylene, and coverslipped using Permount mounting medium (Fisher Scientific Co., Pittsburgh, PA).

2.7 | Antibody characterization

Details on the primary antibodies are in Table 1. The anti-NPY antibody (RRID# AB_518504) was generated against rat NPY, and previous validations report specificity for human, rat, and porcine NPY, but no cross-reactivity with peptide YY, vasoactive intestinal peptide or somatostatin (Belenky, Yarom, & Pickard, 2008). Here, preadsorption with 5 μg/ml of NPY peptide (Bachem Inc. H-6375, Torrance, CA) eliminated immunostaining.

The anti-OX A antibody (RRID# AB_653610) was raised in goat against an epitope within the C-terminus of human prepro-OX, which is identical to that of the mouse. This anti-OX antibody has been previously validated for specificity in songbirds (Singletary et al., 2006). Preadsoprtion with 1 mg/ml of OX A or OX B peptide (Santa Cruz Biotechnologies sc-8070P and sc-8071P, respectively) eliminated immunostaining.

Additional negative controls included the omission of both the primary and the secondary antibodies, which each resulted in a complete loss of immunoreactivity (*ir*) for all peptides, within the brain regions where abundant immunostaining was expected.

2.8 | Image analysis

Images of brain sections were digitized using a camera (Leica DFC 420C, Leica Microsystems GmbH, Wetzlar, Germany) attached to a light microscope (Leitz Laborlux S, Leica Camera AG, Wetzlar, Germany) at 40× magnification and using fixed microscope, camera and software settings (i.e., equivalent contrast, hue, and brightness).

We investigated NPY and OX across brain regions associated with: (a) metabolism: lateral hypothalamus (LHy), locus coeruleus (LoC), and nucleus of the solitary tract (NTS), (b) social behaviors: paraventricular nucleus of the hypothalamus (PVN) infundibulum (IN); substantia nigra pars compacta; medial hippocampus (HP), the medial bed nucleus of the stria terminalis (BST) and nucleus taeniae of the amygdala; lateral septum (LS); medial preoptic area; anterior hypothalamus; ventromedial hypothalamus (VMH); periaqueductal gray (PAG); and ventral tegmental area (VTA); and (c) song learning and production: HVC (abbreviation used as a proper name); robust nucleus of the arcopallium (RA); Area X (X); lateral magnocellular nucleus of the anterior neostriatum. We also included the median eminence, as an indicator of neuropeptide release from the hypothalamus (Panzica, Plumari, Garcia-Ojeda, & Deviche, 1999).

Images were analyzed using ImageJ v. 4.0 (National Institutes of Health, MD). Briefly, all images were first converted to black-and-white (grayscale 16 function with black = 0 and white = 255) and flattened (filter enhancement function). The background was determined from an adjacent hypothalamic area free of immunopositive cells and fibers that was imaged out of focus, to smooth variation in gray across the image (Fokidis & Deviche, 2012; Kabelik, Weiss, & Moore, 2008; Sewall, Dankoski, & Sockman, 2010). This was done for each subject, and measures of this background staining, based on the average optical density (OD), did not vary more than 4.6% among subjects. This was used to obtain a threshold value, and only pixels with an OD exceeding the threshold being used as indicators of immunoreactivity (background correction function) and this was kept constant across subjects and brain regions sampled. ImageJ software conditions of image brightness, hue and saturation were kept constant throughout the study. Following background correction, images were then quantified for immunoreactivity, with each brain region being analyzed together for all subjects at a time.

First, we determined the integral optical density (IOD) of the immunolabeled brain regions. This IOD is defined as the integral sum of the pixel area within an area of interest (AOI) drawn around the respective brain region in both hemispheres (Fokidis & Deviche, 2012; Kabelik et al., 2008; Sewall et al., 2010) based the major neuroanatomical landmarks defined in (Stokes, Leonard, & Nottebohm, 1974) and (Balthazart et al., 1996). The location of the BST was further defined based on (Aste et al., 1998). The nomenclature used subsequently is based on current revisions in birds (Reiner et al., 2004a, 2004b; Reiner, Perkel, Mello, & Jarvis, 2004). The IOD value represents a sum across multiple sections (separated by 120 μm) and for both hemispheres within the respective brain area, and incorporates both the intensity of the DAB staining (both cells and fibers) exceeding background, and the total immunolabelled area within the AOI (Fokidis & Deviche, 2012; Kabelik et al., 2008; Sewall et al., 2010).

TABLE 1 Characteristics of primary antibodies

Antigen	Description of immunogen	Host	Clonality	Source and catalog #	RRID #	Dilution
NPY	Amino acids 30–65 of rat NPY; antigen sequence: H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2	Rabbit	Polyclonal	Bachem Inc., T-4070	AB_518504	20,000
OX-A (C-19)	Amino acids 48–66 of the human OX precursor; antigen sequence: Leu-Tyr-Glu-Leu-Leu-His-Gly-Ala-Gly-Asn-His-Ala-Ala-Gly-Ile-Leu-Thr-Leu	Goat	Polyclonal	Santa Cruz Biotechnologies, sc-8070	AB_653610	15,000

Second, the number of immunoreactive perikarya (hereafter cell number) was counted using an automatic cell count function optimized to detect and quantify cells within the same AOI used in the IOD analysis above, based on cell OD and shape. This was done using the same threshold and illumination parameters as above. Immunoreactive cells from each section for each brain region were summed to provide a total cell number for each bird based on the same number of sections per individual (Fokidis & Deviche, 2012; Kabelik et al., 2008). When necessary, cells missed by the automatic count function were counted manually. As consecutive sections used for analyses were 120 μm apart, no cell was counted twice. Cell number was corrected for the total number of brain sections examined and included both hemispheres, thus providing a mean cell count per subject.

2.9 | Statistical analysis

For both IOD and cell number, mean measurements for each individual were used in statistical analyses that compared breeding and nonbreeding birds. All data were tested for normality using Kolmogorov–Smirnov tests and for equal variance (i.e., homoscedasticity) using Levene tests. When necessary, data were log-transformed to satisfy the above assumptions. Morphometric data were compared using two-sample *t* tests. Energy substrates, cholesterol and steroid concentrations, and neuropeptide immunoreactivity data were compared using multivariate analysis of variance (MANOVA) with reproductive season (breeding vs. nonbreeding) as the between-subjects (main) factor. The statistically significant α-level of .05 was used for these comparisons. Pearson correlation analyses between NPY and OX measures and concentrations of steroids, cholesterols and metabolites that differed by season were also conducted to elucidate specific relationships. All data are presented as means ± standard error of the mean, and all analyses were conducted using SPSS for Windows: v. 22 (IBM Corp. Armonk, NY).

3 | RESULTS

3.1 | Morphology

The time of day (i.e., time since sunrise); the time taken to capture; and times taken to collect blood and brain did not differ between the breeding and nonbreeding seasons (all $p \geq .081$). As expected, testis widths and cloacal protuberance lengths were much larger in the breeding season (Table 2); all breeding subjects were in full reproductive condition. There were no seasonal differences in body mass and tarsus length, but nonbreeding subjects had higher scores for furcular fat and pectoralis muscle (Table 2).

3.2 | Energy substrates

No seasonal difference in plasma glycerol concentration was observed (MANOVA $F = 0.94$, $p = .741$; Figure 1). Breeding subjects had higher circulating concentrations of free triglycerides compared to nonbreeding subjects (MANOVA $F = 6.38$, $p = .017$; Figure 1). Nonbreeding subjects had higher circulating levels of the ketone β-hydroxybutyrate than breeding subjects (MANOVA $F = 3.59$, $p = .046$; Figure 1).

3.3 | Cholesterols

In all plasma samples, cholesterol and cholesteryl esters were above the limit of quantification (LOQ: 10× above background). Total and free cholesterol concentrations in plasma did not differ between seasons (MANOVA $F = 1.06$, $p = .533$), but nonbreeding subjects had higher absolute concentrations of specific cholesteryl esters: those containing linoleic acid, stearic acid, and eicosenoic acid (Figure 2).

3.4 | Steroids

After derivatization using either FMP or HA, all steroids examined were above the limit of detection (3× above background). For nearly every steroid examined, the majority of samples (>70% of total samples) were above the LOQ (10× above background), with the exception of pregnan-3,20-dione ($N = 6$ samples below LOQ) and 4-pregnan-3,17-diol-20-one ($N = 5$ samples below LOQ). Values below the LOQ were set to 0 for further statistical analysis.

Overall, plasma steroid concentrations were significantly elevated in breeding subjects compared to nonbreeding subjects (MANOVA $F = 4.04$, $p = .032$). The steroids showing the greatest difference between breeding and nonbreeding subjects were the androgens associated with the Δ^4 pathway and the "backdoor" pathway to DHT (Figure 3a). As expected, plasma testosterone (T) was significantly higher in breeding subjects than nonbreeding subjects ($p \leq .001$; Figure 3b). Furthermore, plasma concentrations of androstenedione, 5α-DHT, androsterone, and ADIOL were also higher in breeding subjects than nonbreeding subjects (all $p < .006$; Figure 3b). In contrast, steroids earlier in the androgen synthesis pathways (e.g., pregnenolone [PREG], progesterone) were similar between seasons ($p > .14$

TABLE 2 Morphological characteristics of wild male song sparrows captured during the breeding and nonbreeding seasons

	Breeding $N = 8$	Nonbreeding $N = 10$	t	p
Body mass (g)	23.69 ± 0.49	24.95 ± 0.58	1.551	.141
Tarsus length (mm)	22.88 ± 0.40	22.90 ± 0.32	0.047	.963
Fat score (0–5)	0.75 ± 0.28	3.56 ± 0.17	8.047	**.0001**
Muscle score (0–5)	3.50 ± 0.17	4.44 ± 0.17	3.663	**.002**
Cloacal protuberance (mm)	9.84 ± 0.33	3.47 ± 0.11	11.745	**.0001**
Right testes length (mm)	10.50 ± 0.24	0.73 ± 0.14	34.445	**.0001**
Left testes length (mm)	11.13 ± 0.36	0.71 ± 0.17	25.985	**.0001**

Note. Data are presented as means ± standard errors. Bold indicates statistical significance at $p \leq .05$.

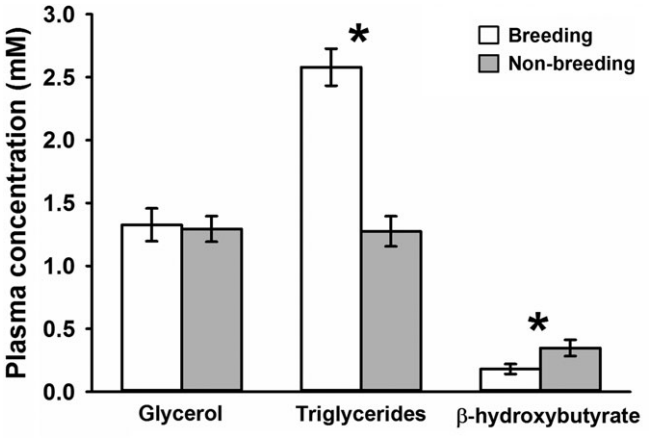

FIGURE 1 Plasma concentrations of glycerol, free triglycerides and the ketone β-hydroxybutyrate in breeding (N = 8) and nonbreeding (N = 10) male song sparrows. Data are presented as means ± standard error. *$p \leq .05$

Figure 3b). Levels of dehydroepiandrosterone (DHEA), which is a steroid in the Δ^5 pathway, were not significantly different between the seasons (p = .14), and plasma DHEA levels were higher than plasma T levels during the nonbreeding season (Figure 3b).

3.5 | NPY immunoreactivity

There were only four discrete populations of NPY-*ir* perikarya (Table 3, Figure 4): (a) large NPY+ cells in the HP, hyperpallium apicale (HA) and parahippocampal area; (b) a dense population of NPY+ cells in the VMH and IN (Figure 4a); (c) NPY+ cells along the ventromedial border of the nucleus rotundus (Rt), consistent with the tractus tectothalamicus; and (d) a sparse population of small NPY+ cells in the VTA (Figure 4b). In contrast, NPY-*ir* fibers were widespread throughout the song sparrow brain including the PAG (Figure 4c,d; Table 3).

Nonbreeding subjects had a higher IOD in three brain regions: LS (F = 4.63, p = .036; Figure 5a); BST (F = 3.99, p = .041; Figure 5a); and VTA (F = 4.11, p = .028; Figure 5b). In addition, nonbreeding subjects had significantly more NPY-*ir* cells in the HP than breeding subjects (F = 5.39, $p \leq .001$). A significant effect of season on overall NPY-*ir* was observed, with nonbreeding subjects having a higher IOD than breeding subjects (MANOVA, F = 3.17, p = .042; Figure 5c).

3.6 | OX immunoreactivity

OX-*ir* perikarya were observed in the PVN and VMH (Figure 6a,b). By contrast, OX-*ir* fibers were extensive throughout the song sparrow brain, including the PAG and VTA (Figure 6c,d; Table 3).

Nonbreeding subjects had significantly higher overall OX-*ir* IOD than breeding subjects (MANOVA, F = 8.04, $p \leq .0001$; Figure 7a); and, specifically, seven regions showed a significant seasonal difference (all $p \leq .014$; Figure 7a). The largest seasonal difference was observed in the PAG (Figure 7b). Nonbreeding subjects had more OX-*ir* cells in the PVN than breeding subjects (F = 5.09, p = .004; Figure 7c,d).

4 | DISCUSSION

The present results suggest that nonbreeding song sparrows have a higher metabolic demand, compared to breeding subjects. Evidence for this includes higher circulating β-hydroxybutyrate ketone levels, indicative of fat store oxidation (McGuire, Fenton, Faure, & Guglielmo, 2009; Newman & Verdin, 2017) and lower triglyceride concentrations, suggestive of low fat deposition (Bairlein, Zajac, Cerasale, & Guglielmo, 2002; Cerasale & Guglielmo, 2006). Coinciding with these different energetic states is a shift in circulating steroids, with breeding birds having higher androgens associated with the Δ^4 and "backdoor" pathways. Furthermore, in the brain, nonbreeding birds showed increased NPY-*ir* within three regions and increased OX-*ir* within seven regions. Taken together, these data from free-living animals support the hypothesis that natural seasonal changes in metabolic state are associated with changes in NPY and OX in the brain.

4.1 | Differences in energetic state between the seasons

Song sparrows in our study area do not migrate and instead defend territories year-round, including the winter. In captivity, song sparrows

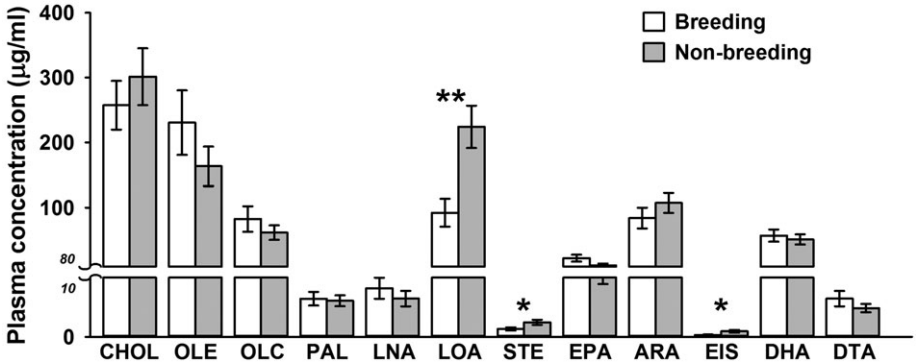

FIGURE 2 Plasma concentrations of free cholesterol and esterified cholesterols (i.e., cholesterols bound to fatty acids) in breeding (N = 8) and nonbreeding (N = 10) male song sparrows. Abbreviations (esterified cholesterols are identified by the conjugated fatty acid): CHOL = free cholesterol; OLE = oleaic; OLC = oleic; PAL = palmitic; LNA = linolenic; LOA = linoleic; STE = stearic; EPA = eicosapentanoic; ARA = arachidonic; EIS = eicosenoic; DHA = docosahexanoic; DTA = docosatetraenoic. Data are presented as means ± standard error. *$p \leq .05$, **$p \leq .01$

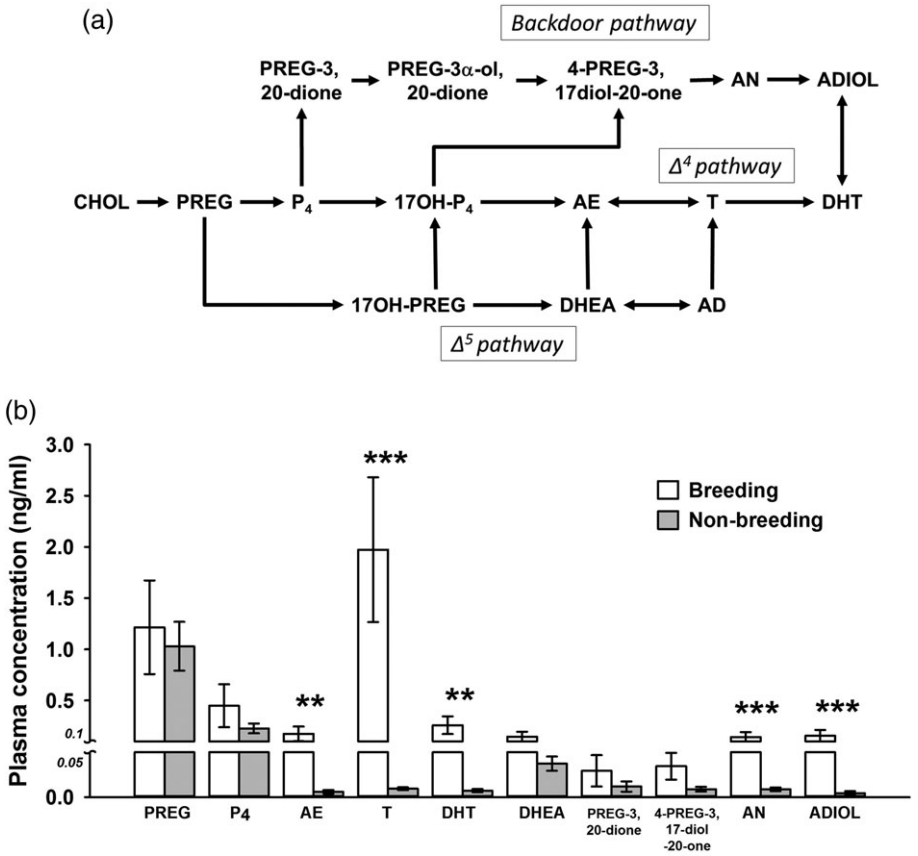

FIGURE 3 (a) The three pathways for androgen synthesis: Δ^4 pathway, Δ^5 pathway and "backdoor" pathway and (b) plasma concentrations of steroids in breeding (N = 8) and nonbreeding (N = 10) male song sparrows. Abbreviations: PREG = pregnenolone; P4 = progesterone; AE = androstenedione; T = testosterone; DHT = dihydrotestosterone; DHEA = dehydroepiandrosterone; AN = androsterone; ADIOL = androstanediol. Data are presented as means ± standard error. $^{**}p \leq .01$, $^{***}p \leq .001$

show reduced body mass and fat scores in response to cold overnight temperatures (Heimovics, Fokidis, & Soma, 2013), illustrating the major energetic demands exerted by cold ambient temperatures on these small songbirds with a body temperature of 41 °C. Furthermore, food availability and foraging opportunities are greatly limited in winter because of reductions in invertebrates and fresh vegetation, shorter daylight hours for foraging, increased inclement weather and potentially increased predation pressure (Brown & Sherry, 2008).

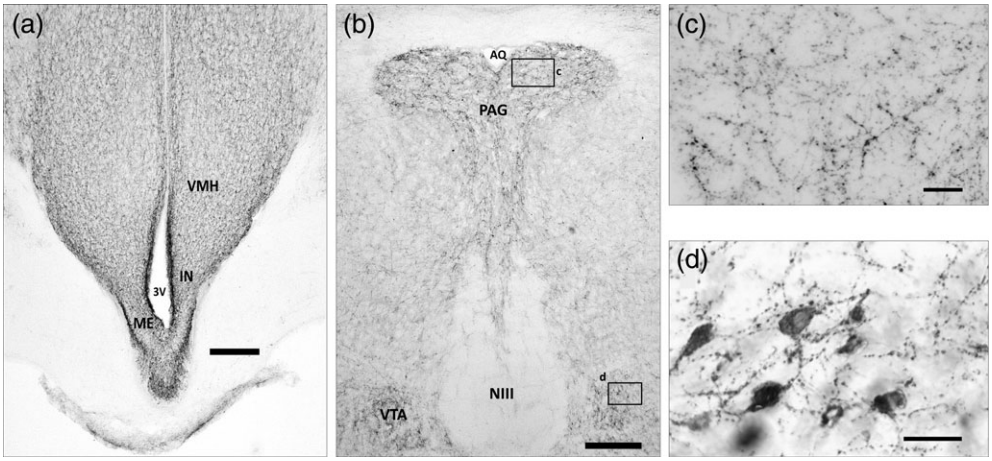

FIGURE 4 (a) Low-power (40×) photomicrograph of neuropeptide Y (NPY) immunoreactive (*ir*) cells and fibers in the ventromedial hypothalamus (VMH), median eminence (ME) and infundibulum (IN) along the third ventricle (3V); (b) low-power (40×) photomicrograph of NPY-*ir* cells and fibers in the ventral tegmental area (VTA) and the periaqueductal gray (PAG) adjacent to the cerebral aqueduct (AQ) and the oculomotor nerve (NIII). Inset photomicrographs at high-power (200×) depicting NPY-*ir*; (c) fibers within the PAG; and (d) both cells and fibers in the VTA. Scale bars indicate 500, 500, 20, and 20 μm, respectively. Overall brightness and contrast of the whole images were adjusted (brightness = 20 and contrast = 10) using adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA)

TABLE 3 Presence (+) or absence (–) of immunoreactive (*ir*) cell bodies or fibers for neuropeptide Y (NPY) and orexin (OX) in brain regions of the song sparrow. Data are presented as: (–) no labeled cell bodies or fibers; (+) labeled cell bodies or fibers sparsely distributed; (++) labeled cell bodies or fibers densely distributed; or (+++) labeled cell bodies or fibers very densely distributed

	NPY-*ir*		OX-*ir*	
	Cells	Fibers	Cells	Fibers
Telencephalon				
MSt	–	+++	–	+
X	–	++	–	–
LMAN	–	+	–	–
LFB	–	+	–	+
HA	+	++	–	+
Hp	++	++	–	+
APH	+	+++	–	–
HVC	–	+	–	–
TnA	–	++	–	–
RA	–	+	–	+
NCM	–	+	–	+
LSt	–	++	–	–
POA	–	+++	–	+++
Diencephalon				
AH	–	++	–	–
VMH	+++	+++	–	+++
PVN	–	+++	+++	++
LHy	–	+++	++	+
IN	–	+++	–	–
ME	–	+++	–	++
Rt	–	+	–	–
TT	++	+	–	–
BST	–	++	–	+++
LS	–	++	–	+++
Midbrain and brainstem				
OT	–	+	–	+
PAG	–	++	–	+++
VTA	+	+++	–	+++
SNc	–	++	–	++
LoC	–	++	–	+++
NTS	–	+	–	–
CB	–	++	–	+

Abbreviations: MSt = medial striatum; X = area X; LMAN = lateral magnocellular nucleus of the anterior nidopallium; LFB = lateral forebrain bundle; HA = hyperstriatum accessorium; Hp = medial hippocampus; APH = area parahippocampalis; HVC (abbreviation is official name); TnA = nucleus taeniae of the amygdala; RA = robust nucleus of the arcopallium; NCM = caudal medial nidopallium; LSt = lateral striatum; POA = preoptic area; AH = anterior hypothalamus; VMH = ventromedial hypothalamus; PVN = paraventricular nucleus; LHy = lateral hypothalamus; IN = infundibulum; ME = median eminence; Rt = nucleus rotundus; TT = tractus tectothalamicus; BST = bed nucleus of the stria terminalis; LS = lateral septum; OT = optic tectum; PAG = periaqueductal gray; VTA = ventral tegmental area; SNc = substantia nigra pars compacta; LoC = locus coeruleus; NTS = nucleus of the solitary tract; CB = cerebellum.

Nonbreeding subjects had greater fat and muscle reserves than breeding subjects, but body mass did not differ, which is consistent with other avian species (Bairlein et al., 2002; Rogers, 2015; Rogers & Rogers, 1990). Increased energy reserves promote survival during winter, yet the high metabolic costs during winter require that small birds forage nearly constantly during daylight hours (Bairlein et al., 2002; Bednekoff & Krebs, 1995). Compared to breeding subjects, nonbreeding subjects had higher circulating levels of β-hydroxybutyrate, a fuel that is an oxidized ketone derivative of fatty acids, and lower circulating levels of triglycerides, suggesting higher fat oxidation and lower fat deposition. No difference in plasma glycerol levels was detected, suggesting fat reserves are not directly used for gluconeogenesis (Cerasale & Guglielmo, 2006; Guglielmo et al., 2002; Guglielmo, Cerasale, & Eldermire, 2005). However, higher β-hydroxybutyrate levels in nonbreeding subjects may indicate use of fat reserves for ketone production. These wild subjects were not fasted, which limits our ability to interpret these data (Zajac, Cerasale, & Guglielmo, 2006). Glucose was not measured here because circulating glucose levels rarely fluctuate unless subjects are fasted (Fokidis et al., 2011; Fokidis et al., 2012). Subjects were captured soon after sunrise (2.49 ± 0.16 hr) in both seasons; however, the duration of the overnight fast was longer during the nonbreeding season, which may impact circulating metabolite levels. Taken together, these data suggest nonbreeding song sparrows allocate more energy to fat stores, which is compatible with the life histories of seasonally breeding species.

4.2 | Seasonality alters circulating steroid profiles

Song sparrows are affected by steroids in both the breeding and nonbreeding seasons (Heimovics et al., 2013; Heimovics, Ferris, & Soma, 2015; Soma, Tramontin, & Wingfield, 2000). The songbird brain expresses the requisite enzymes for steroid synthesis (Balthazart, 2010; Pradhan et al., 2010; Schlinger & Remage-Healey, 2012). Neurosteroids can be derived de novo from cholesterol but also produced from circulating prohormones (Fokidis, Adomat, et al., 2015; Fokidis, Yieng Chin, et al., 2015). In the case of androgens, synthesis can occur through three pathways: the Δ^4, Δ^5, and backdoor pathways (Auchus, 2004; Mostaghel & Nelson, 2008). Using LC–MS/MS, we quantified multiple circulating steroids in these pathways. Circulating androgens were generally higher in breeding subjects, as expected; however, the seasonal differences were primarily for steroids in the Δ^4 and backdoor pathways. Circulating steroids in the Δ^5 pathway, such as DHEA, did not change seasonally and were higher than circulating testosterone during the nonbreeding season. This is consistent with studies showing DHEA promotes nonbreeding aggression in some avian and mammalian species (Soma, Rendon, Boonstra, Albers, & Demas, 2015). Although environmental factors influencing DHEA concentrations are not well known, in songbirds, an acute 6-hr fast elevates plasma DHEA (Fokidis, Prior, & Soma, 2013) and DHEA concentrations are impacted by both acute stress and season (Fokidis, 2016; Wright & Fokidis, 2016).

De novo sex steroid synthesis is possible in the avian brain (Tsutsui, Matsunaga, Miyabara, & Ukena, 2006) and cholesterol conversion to PREG might be the rate-limiting step (Goncharov & Katsya, 2013; Hu, Zhang, Shen, & Azhar, 2010; Miller & Bose, 2011). In this study, no seasonal differences in total or unconjugated cholesterol levels were observed and cholesterol concentrations were far higher than steroid concentrations. This suggests access to cholesterol

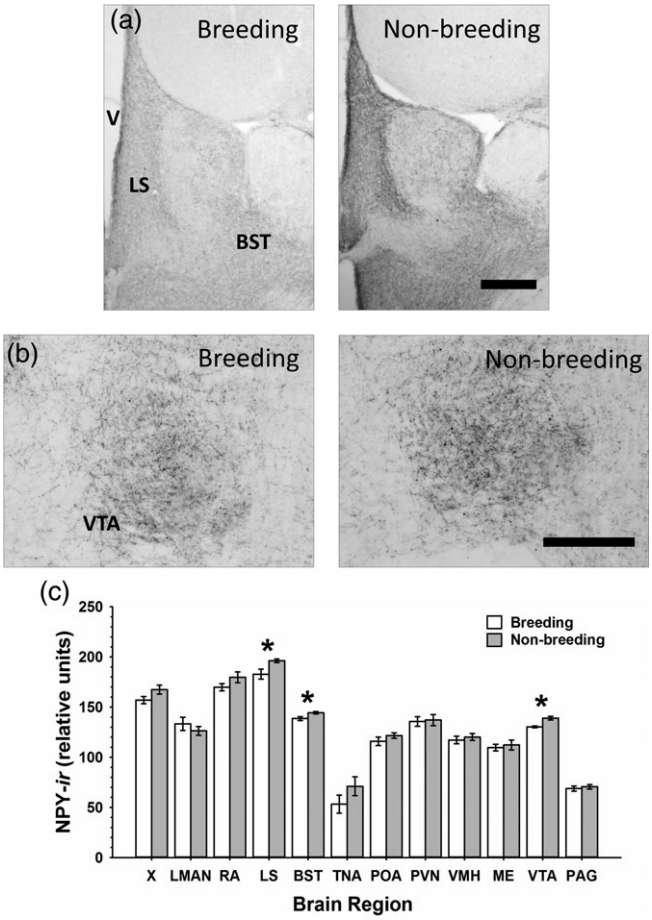

FIGURE 5 Medium-power (100×) photomicrographs depicting differences in neuropeptide Y (NPY) immunoreactivity (*ir*) between breeding and nonbreeding song sparrows within the (a) lateral septum (LS) and medial bed nucleus of the stria terminalis (BST) along the ventricle (V); (b) ventral tegmental area (VTA). Scale bars indicate 200 μm; and (c) NPY-*ir* in various brain regions in breeding and nonbreeding song sparrows. Abbreviations: X = Area X; LMAN = lateral magnocellular nucleus of the anterior nidopallium; RA = robust nucleus of the arcopallium; LS = lateral septum; BST = medial bed nucleus of the stria terminalis; TnA = nucleus taeniae of the amygdala; POA = preoptic area; PVN = paraventricular nucleus; VMH = ventromedial hypothalamus; ME = median eminence; VTA = ventral tegmental area; PAG = periaqueductal gray. *$p \leq .05$. Overall brightness and contrast of the whole images were adjusted (brightness = 20 and contrast = 10) using Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA)

is likely not a limiting factor for steroid production. Nonbreeding subjects had small increases in three cholesteryl esters, and this likely reflects seasonal changes in diet (Caron-Jobin et al., 2012; Hu et al., 2010).

4.3 | Region-specific differences in brain NPY and OX between the seasons

The distributions of NPY and OX in song sparrows are consistent with those of other songbirds (Aste et al., 1991; Boswell, Li, & Takeuchi, 2002; Boswell, Millam, Li, & Dunn, 1998; Den Boer-Visser & Dubbeldam, 2002; Esposito, Pelagalli, De Girolamo, & Gargiulo, 2001; Fiore et al., 1999; Godden et al., 2014; Gould, Newman, Tricomi, & DeVoogd, 2001; Miranda et al., 2013; Phillips-Singh et al., 2003; Singh et al., 2013; Singh et al., 2016; Singletary et al., 2006, 2010; Zhou, Murakami, Hasegawa, Yoshizawa, & Sugahara, 2005). Nonbreeding subjects had higher NPY-*ir* based on IOD measurements than breeding subjects, specifically in the LS, BST, and VTA. These differences are likely related to increased fiber staining, since NPY-*ir* cells were absent from the LS and BST, with only a few in the VTA, suggesting increased connectivity in nonbreeding birds within these regions. These brain regions regulate emotional responses, particularly in the context of fear, stress, and sociality, and regulate cognitive processes involving goal-directed behaviors and conditioned learning in vertebrates (Aston-Jones et al., 2010; Briand, Vassoler, Pierce, Valentino, & Blendy, 2010; Lungwitz et al., 2012; Mahler & Aston-Jones, 2012). Thus seasonal differences in NPY (Ando et al., 2001; Lecklin et al., 2002) in these brain regions suggest that seasonal shifts in energy balance can affect a variety of behaviors.

NPY levels in the PVN, a central regulator of glucocorticoid secretion, tracks fasting-refeeding regimes in rodents (Bertile, Oudart, Criscuolo, Maho, & Raclot, 2003; Zhou et al., 2005). However, previous research on this species has suggested that corticosterone concentrations are higher during the breeding season, presumably to mobilize energy for reproductive efforts (Newman, Pradhan, & Soma, 2008; Newman & Soma, 2009). Nonetheless, despite some research implicating a direct link between NPY and adrenal glucocorticoid release (Krysiak, Obuchowicz, & Herman, 1999) and here a higher amount of DHEA (an adrenal androgen) in nonbreeding subjects, the absence differences in NPY-*ir* within the PVN limits our ability to infer a role of glucocorticoids in regulating seasonal shifts in metabolism.

Regardless of energetic state, NPY increases the motivation to feed (Brown, Fletcher, & Coscina, 1998; Jewett, Cleary, Levine, Schaal, & Thompson, 1992). The VTA, a brain region critical for motivated behaviors, including feeding, expresses several NPY receptors in rodents (Korotkova, Brown, Sergeeva, Ponomarenko, & Haas, 2006; Liu & Borgland, 2015; Pandit, La Fleur, & Adan, 2013) and NPY directly injected into the VTA increases feeding rates (Pandit, Luijendijk, Vanderschuren, la Fleur, & Adan, 2014). In rodents, NPY inhibits dopaminergic neuron firing in the VTA through the NPY-Y_1 receptor (West & Roseberry, 2017); however, these effects depend on the distinct neural subpopulation sampled, and the net effect of NPY on food motivation is not clear (West & Roseberry, 2017). Injections of NPY-receptor agonists into the LS reduce anxiety-related behaviors in rats (Lach & de Lima, 2013; Trent & Menard, 2011) and comparative studies of voles reveal interspecific differences in NPY-*ir* consistent with variation in social behaviors (Hostetler, Hitchcock, Anacker, Young, & Ryabinin, 2013). A lack of such studies in birds limits our abilities to assign direct functional roles of NPY across a seasonal context.

Nonbreeding subjects had more OX-*ir* across multiple brain regions, driven primarily, as in NPY, by differences in fiber staining, except for the PVN. The hypothalamic production of OX is largely confined to the PVN and LHy, where it regulates feeding, reward seeking, wakefulness, and fat deposition (Gao & Horvath, 2014). The higher OX-*ir* in nonbreeding subjects is consistent with an increased demand to forage during the winter. Alternatively, higher OX-*ir* in

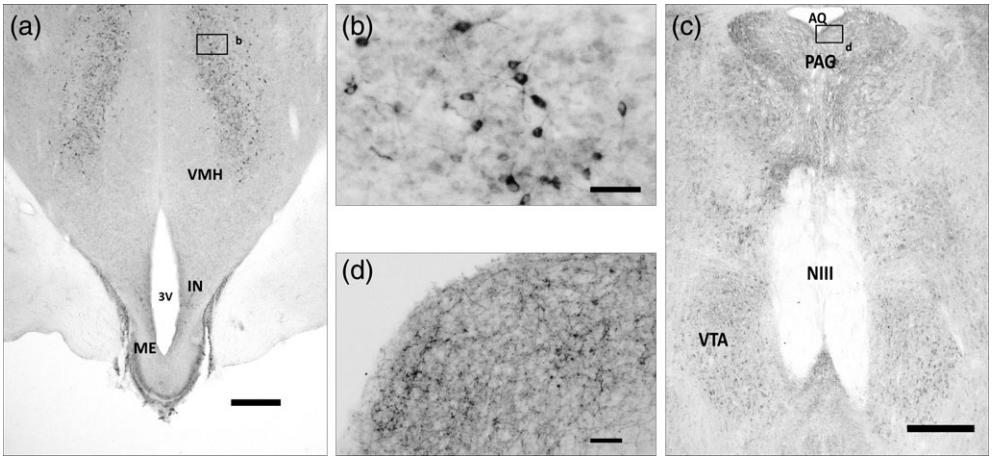

FIGURE 6 (a) Low-power (40×) photomicrograph of OX immunoreactive (*ir*) cells in the ventromedial hypothalamus (VMH) and limited OX-*ir* fiber staining in the median eminence (ME). Also depicted is the infundibulum (IN) and third ventricle (3V); (b) high-power (200×) inset photomicrograph of OX-*ir* cells and fibers within the VMH; (c) low-power (40×) photomicrograph of OX-*ir* in the periaqueductal gray (PAG) and ventral tegmental area (VTA) along with the cerebral aqueduct (AQ) and oculomotor nerve (NIII); and (d) high-power (200×) inset photomicrograph of OX-*ir* fibers along the border of the PAG and AQ. Scale bars indicate 20, 250, and 500 μm, respectively. Overall brightness and contrast of the whole images were adjusted (brightness = 20 and contrast = 10) using Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA)

nonbreeding subjects may be the result of higher concentrations necessary to maintain arousal and wakefulness during shorter photoperiods, since previous research has shown intracerebroventricular injection of OX induces arousal in neonatal chickens (Katayama, Hamasu, Shigemi, Cline, & Furuse, 2010) and reduces bouts of sleep in pigeons (da Silva et al., 2008). However, OX mRNA levels did not differ in broiler chickens collected during the day or night (Miranda et al., 2013), but those sampling times only differed by 2–4 hr and not across seasons. Another explanation for the seasonal difference in OX-*ir* may be the role of OX in reproduction. However, previous studies have reported that OX upregulates GnRH and gonadotropin release in mammals (Cataldi, Lux Lantos, & Libertun, 2014; Martynska et al., 2014), and may facilitate the expression of sexual behaviors (Di Sebastiano & Coolen, 2012; Di Sebastiano, Yong-Yow, Wagner, Lehman, & Coolen, 2010). The opposite effect observed here, of higher levels during nonbreeding, contradicts a role for OX in regulating reproduction, since this species breeds initiates breeding in spring (Soma et al., 2000). However, previous research has shown a negative

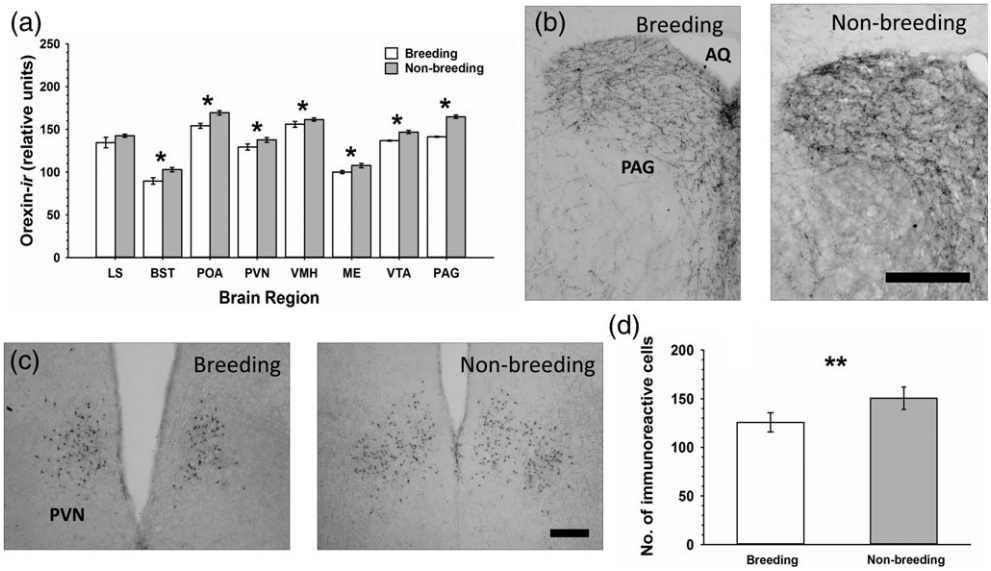

FIGURE 7 (a) Orexin (OX) immunoreactivity (*ir*) in various brain regions in breeding and nonbreeding song sparrows. Medium-power (100×) photomicrographs depicting differences in OX-*ir* between breeding and nonbreeding song sparrows within the (b) periaqueductal gray (PAG) adjacent to the cerebral aqueduct (AQ); (c) paraventricular nucleus (PVN) with scale bars indicating 100 μm; and (d) number of OX-*ir* cells in the PVN of breeding and nonbreeding song sparrows. Abbreviations: LS = lateral septum; BST = medial bed nucleus of the stria terminalis; POA = preoptic area; VMH = ventromedial hypothalamus; ME = median eminence; VTA = ventral tegmental area. *$p \leq .05$. **$p \leq .01$. Overall brightness and contrast of the whole images were adjusted (brightness = 20 and contrast = 10) using Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA)

effect of OX on reproduction in fish (Hoskins, Xu, & Volkoff, 2008; Zhao, Singh, Prober, & Wayne, 2016) and GnRH secretion in mice (Gaskins & Moenter, 2012). Thus, further research on the roles of OX in reproduction in birds is necessary and clearly warranted.

4.4 | Neuropeptides as potential regulators of steroids in the brain

The transition from nonreproductive to reproductive state in vertebrates involves altered steroid metabolism, and it is tempting to relate neuropeptide changes with direct actions of steroids within the brain. Both NPY and OX could alter neural steroid metabolism and as the entire suite of steroidogenic enzymes are present in the brain, this is a potential fruitful area of study. NPY regulates the sulfation of neurosteroids in amphibians, which limits their bioavailability (Beaujean et al., 2002). Furthermore, most of the brain regions showing seasonal differences in NPY and OX are known to express aromatase, the enzyme that converts androgens to estrogens (Heimovics et al., 2013; Saldanha & Schlinger, 2008), and aromatase regulates nonbreeding aggression in the song sparrow (Heimovics et al., 2013). Nonetheless, whether NPY or OX regulate neural steroid metabolism remains unclear. This study highlights the need to examine energetic influences on neuropeptides in a manner that emphasizes the ecological context.

ACKNOWLEDGMENTS

We thank Dr. Matthew Taves for laboratory and field assistance. This work was supported by a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada (NSERC) to HBF, funds from the Office of the Dean of Arts and Sciences at Rollins College to HBF, funds from the Centre of Excellence in Commercialization of Research (CECR) to ESG, and Operating Grant #133606 from the Canadian Institutes of Health Research (CIHR) to KKS.

ORCID

H. Bobby Fokidis http://orcid.org/0000-0003-2386-4066

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Fokidis HB, Ma C, Radin B, et al. Neuropeptide Y and orexin immunoreactivity in the sparrow brain coincide with seasonal changes in energy balance and steroids. *J Comp Neurol*. 2018;1–15. https://doi.org/10.1002/cne.24535