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Animal behaviour

Differential gene expression in seasonal sympatry: mechanisms involved in diverging life histories

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In an era of climate change, understanding the genetic and physiological mechanisms underlying flexibility in phenology and life history has gained greater importance. These mechanisms can be elucidated by comparing closely related populations that differ in key behavioural and physiological traits such as migration and timing of reproduction. We compared gene expression in two recently diverged dark-eyed Junco (*Junco hyemalis*) subspecies that live in seasonal sympatry during winter and early spring, but that differ in behaviour and physiology, despite exposure to identical environmental cues. We identified 547 genes differentially expressed in blood and pectoral muscle. Genes involved in lipid transport and metabolism were highly expressed in migrant juncos, while genes involved in reproductive processes were highly expressed in resident breeders. Seasonal differences in gene expression in closely related populations residing in the same environment provide significant insights into mechanisms underlying variation in phenology and life history, and have potential implications for the role of seasonal timing differences in gene flow and reproductive isolation.

1. Introduction

In preparation for migration and reproduction, birds undergo changes in muscle, brain and digestive structures among other things [1,2]. Comparisons of closely related populations of 'seasonally sympatric' migrants and residents can provide a powerful approach to understanding the genetic and physiological bases of traits associated with these stages [3]. Comparative studies have examined expression of candidate genes [4–10]. However, for complex traits, transcriptomic studies (RNA-seq) have the advantage of capturing a large number of functionally relevant genes that might be missed with a candidate gene approach. Comparing gene expression in closely related migrants and residents experiencing the same environment provides an opportunity to identify a large number of genes involved in life-history transitions.

Here we examined the pectoral muscle and blood transcriptomic profiles of two seasonally sympatric, closely related subspecies of dark-eyed juncos (*Junco hyemalis*), one of which (*J.h. hyemalis*) is migratory and the other (*J.h. carolinensis*) sedentary, in order to identify differential expression of genes associated with variation in the seasonal timing and performance of migratory and reproductive behaviour and physiology (table 1). In early spring each year, as the sedentary population prepares to reproduce, the migrants delay reproduction and undergo behavioural and physiological changes that support migration [3,11,12]. Because migratory juncos transition into the migratory stage of the

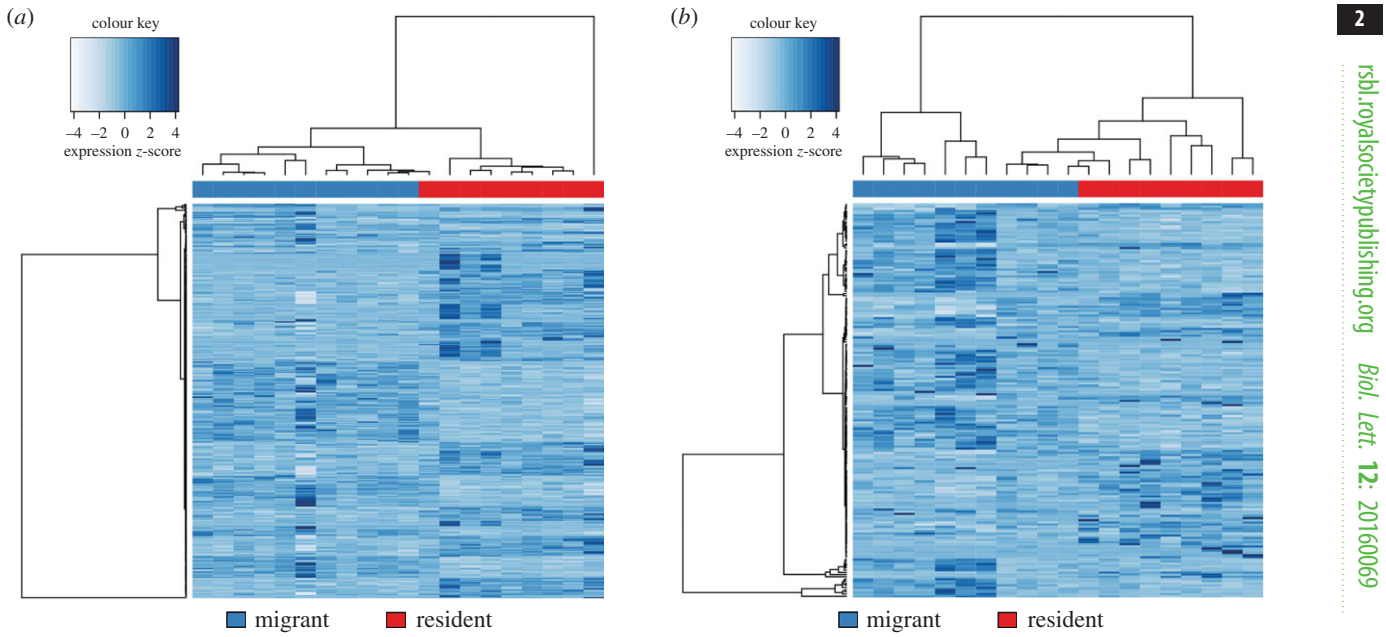

Figure 1. Blood and muscle heat maps. Heatmaps showing significantly DE genes in muscle (*a*; 366 genes) and blood (*b*; 181 genes). Each column represents an individual (blue, migrants; red, residents) and each row represents a DE gene. Expression scores for each gene are z-score normalized and darker colours represent higher expression of that gene in that individual. Dendrograms represent hierarchical clustering for visualization.

Table 1. Predicted changes in muscle and blood during preparations for migration.

trait	predicted change	tissue
prolonged flight	muscle hypertrophy	muscle
increased fuelling	lipid oxidation	muscle, blood
demands	lipid transport	muscle, blood
increased aerobic	erythropoiesis	blood
capacity	elevated haemoglobin	blood

annual cycle during our sampling period, we predicted that genes associated with greater muscle growth, lipid transport and metabolism, and aerobic capacity would be more highly expressed in migrants than in residents, whereas for residents we predicted increased expression of genes involved in reproductive processes (table 1).

2. Material and methods

From 4 to 12 December 2013, we captured 11 migratory and 9 sedentary male dark-eyed juncos (*Junco hyemalis*) at the University of Virginia's Mountain Lake Biological Station (MLBS) in Giles County, VA (37.37° N, 80.52° W) using baited mist nets. We determined migrant (*J.h. hyemalis*) versus resident (*J.h. carolinensis*) subspecies status using bill coloration, plumage and wing chord differences [11]. On 14 December, birds were transported to Indiana University and housed in mixed flocks in two climate-controlled indoor aviaries (6.4 × 3.2 × 2.4 m).

On 27 February, we individually housed birds in 61 × 46 × 46 cm cages. We adjusted lights every 3 ± 1 days to simulate natural seasonal changes in day length at MLBS (9.6 h of light on 14 December to 12.6 h of light on 1 April). The temperature in the aviary was maintained at 16 ± 2°C. From 4 to 26 March, we took weekly measurements of subcutaneous fat and cloacal protuberances (CPs). Each week we also measured circulating testosterone, followed by an injection with a standardized dose of gonadotropin releasing hormone (GnRH), which is an established method for assessing a male's ability to produce a short-term increase in testosterone [13]. Extensive prior work using 'GnRH-challenges' to study reproductive ecology and behaviour in juncos found no evidence for lingering physiological or behavioural effect beyond the initial and transient (approx. 30 min.) stimulation [13]. On 31 March and 1 April, we euthanized birds using a lethal dose of isoflorane. Tissues were extracted, flash frozen and stored at −80°C. Subsequent generation of RNA libraries was carried out at the Center for Genomics and Bioinformatics at Indiana University following established protocols [14]. For additional information on RNA extraction and RNA libraries, see the electronic supplementary material.

We identified differentially expressed (DE) genes using rnaseqWrapper [15] and DESeq [16] packages for R [17] with a false discovery rate threshold of 0.05. We determined which gene ontology (GO) terms were over-represented among DE genes using the 'weight' algorithm from topGO [18] R package with a significance threshold of 0.05. We required at least three significant genes within a GO term to be reported to avoid biases caused by GO terms with small numbers of genes.

3. Results

In total, 547 genes were DE between migrants and residents. Of 8822 genes that were expressed in the pectoral muscle, 366 genes were significantly DE between migrants ($n = 11$) and residents ($n = 9$) (figure 1*a* (heat map); see the electronic supplementary material for genes). Twenty-seven GO categories were significantly over-represented among the DE genes (table 2). Out of 7211 genes expressed in blood, 181 were DE among migrants and residents (figure 1*b*; see the electronic supplementary material for genes). Seven GO categories were significantly over-represented among DE genes.

A prior study reported that residents had larger CPs and higher baseline and GnRH-induced plasma testosterone, while migrants had more subcutaneous fat than residents, confirming that residents transitioned into the reproductive

Table 2. GO processes with a significant number of genes DE in pectoral muscle (PM) and blood in early spring in migratory and sedentary dark-eyed juncos held in a common garden. Expression was measured at 8822 genes in PM and 7211 genes in blood in 11 migrants and 9 residents. The category (migration (M), residency (R)) with more genes upregulated is listed in the group bias column. A full list of genes and processes can be found in the electronic supplementary material. ER, endoplasmic reticulum.

gene ontology description	GO #	tissue	functional hypothesis	annotated	DE	*p*-value	group bias
Wnt signalling pathway	GO:0016055	blood		28	3	0.040	M
structural molecule activity	GO:0005198	blood	erythropoiesis	147	8	0.045	M
ribosome	GO:0005840	blood	erythropoiesis	75	8	0.013	M
large ribosomal subunit	GO:0015934	blood	erythropoiesis	27	3	0.038	M
peptidase inhibitor activity	GO:0030414	blood	inhibit protein catabolism	30	3	0.047	M
Golgi-associated vesicle	GO:0005798	blood	lipid synthesis	17	3	0.011	M
appendage development	GO:0048736	blood	reproduction	28	3	0.029	R
mitochondrial part	GO:0044429	PM		166	15	0.004	M
cell-substrate adherens junction	GO:0005924	PM		45	6	0.010	M
phosphotransferase activity, for other substituted phosphate groups	GO:0016780	PM		14	3	0.018	M
protein targeting	GO:0006605	PM		118	11	0.019	M
ER-nucleus signalling pathway	GO:0006984	PM		20	3	0.045	M
fatty acid catabolic process	GO:0009062	PM	migratory fuel	34	8	$<$0.001	M
C-acyltransferase activity	GO:0016408	PM	migratory fuel	12	3	0.011	M
sterol metabolic process	GO:0016125	PM	migratory fuel	31	4	0.035	M
lipid transporter activity	GO:0005319	PM	migratory fuel	18	3	0.036	M
lipid transport	GO:0006869	PM	migratory fuel	40	5	0.042	M
peroxisomal part	GO:0044439	PM	migratory fuel	32	4	0.043	M
organelle envelope	GO:0031967	PM	muscle growth	343	26	0.003	M
regulation of cellular ketone metabolic process	GO:0010565	PM	sugar metabolism	14	3	0.017	M
ion binding	GO:0043167	PM		1263	71	0.004	R
microtubule cytoskeleton organization	GO:0000226	PM		66	8	0.011	R
response to abiotic stimulus	GO:0009628	PM		112	10	0.015	R
negative regulation of apoptotic process	GO:0043066	PM		96	9	0.015	R
cell death	GO:0008219	PM		394	28	0.016	R
centrosome	GO:0005813	PM		27	4	0.025	R
mRNA binding	GO:0003729	PM		16	3	0.026	R
single-organism cellular localization	GO:1902580	PM		28	4	0.029	R
response to peptide hormone	GO:0043434	PM		59	6	0.031	R
sensory perception of light stimulus	GO:0050953	PM		44	5	0.031	R
spindle	GO:0005819	PM		48	5	0.049	R
multicellular organismal reproductive process	GO:0048609	PM	reproduction	111	14	$<$0.001	R
developmental process involved in reproduction	GO:0003006	PM	reproduction	93	11	0.001	R
hexose metabolic process	GO:0019318	PM	sugar metabolism	91	12	0.001	R

state, while migrants delayed reproduction and prepared for migration during the course of our experiment [12].

4. Discussion

Analysis of differential gene expression can elucidate organismal and evolutionary mechanisms by which phenotypes, individuals and populations diverge [19,20]. This study demonstrates that closely related seasonally sympatric migratory and sedentary dark-eyed junco populations (*J.h. hyemalis and J.h. carolinensis* subspecies) held in a common garden in early spring differentially express genes in pectoral muscle and blood that are associated with metabolic, aerobic and reproductive processes.

Genes involved in lipid transport and fatty acid catabolic processes were expressed more in pectoral muscles of migrants than residents (tables 1 and 2). As the primary fuel for most migratory songbirds, lipids provide more energy per wet mass than other sources of energy [21]. Lipids are stored in muscle and adipose tissue in preparation for migration and are mobilized in the form of fatty acids to fuel migratory flight [21–23] and transported in circulation [24,25]. L-3-hydroxyacyl-Coenzyme A dehydrogenase and long-chain-acyl-CoA dehydrogenase, which are involved in fatty acid oxidation, were expressed more in migrants. Fatty acid binding protein, which facilitates fat transport in migratory birds [22], Acyl-CoA synthetase long-chain family member 1, which is involved in lipid biosynthesis, and carnitine O-palmitoyltransferase, which is involved in the metabolism of fatty acids, were all expressed more in migratory juncos. Small muscle protein, which is associated with muscle growth, was highly expressed in the pectoral muscles of migratory juncos, suggesting it could aid muscle growth for migration.

Eleven genes associated with ribosomal structures were highly expressed in migrant blood, suggesting that the machinery for protein synthesis in migratory blood elevates in preparation for migration. Repeated sampling of migrant blood prior to migration could reveal whether blood ribosomal genes could be used as biomarkers to assess migratory state in the wild, which could be extremely useful in studies on free-living migrants.

In resident juncos, the highly expressed genes often were associated with multicellular organismal reproductive and developmental processes (table 2). The testes of residents in the current study were more developed than testes of migrants when blood and muscle tissues were collected, confirming phenological differences in timing of reproduction [12]. Furthermore, CPs, the primary site of sperm storage in birds, were at a more advanced stage in resident juncos when tissues were collected [12]. Genes associated with appendage development were expressed more in resident than migrant blood, which may relate to seasonal CP and gonad growth. Further study is needed to confirm this. Comparison of differential gene expression in the gonads of the birds in the current study will be important for identification of more genes associated with reproduction.

Although the proximate mechanisms that resulted in the differential expression we observed are unknown, our work has implicated several genes that may underlie the alternative phenology of these two subspecies. Future studies examining the role of development and/or differences in transcription factors (e.g. activated sex steroid receptors) will lead to a greater understanding of potential ontogenetic and proximate regulation of these varying phenotypes. Also needed is further investigation of seasonal shifts in these, and other, genes in both subspecies. However, the findings here provide an important insight into the role these genes may play.

Ethics. All procedures were approved by the Indiana University IACUC (protocol 12–050) and conducted under scientific permits issued by the Virginia Department of Game and Inland Fisheries (permit no. 47553) and the US Fish and Wildlife Service (permit no. MB093279).

Data accessibility. All data are available in the electronic supplementary material.

Authors' contributions. A.M.F., E.D.K., T.J.G., J.W.A., M.P.P. and E.S.B. helped design the study. A.M.F., J.W.A. and E.D.K. performed the experiment. M.P.P. analysed the data. A.M.F. drafted the manuscript. All authors commented on the manuscript and gave approval for/are accountable for the final version of the manuscript.

Competing interests. We have no competing interests.

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