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Seasonal gene expression in a migratory songbird

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Abstract

The annual migration of a bird can involve thousands of kilometres of nonstop flight, requiring accurately timed seasonal changes in physiology and behaviour. Understanding the molecular mechanisms controlling this endogenous programme can provide functional and evolutionary insights into the circannual biological clock and the potential of migratory species to adapt to changing environments. Under naturally timed photoperiod conditions, we maintained captive Swainson's thrushes (*Catharus ustulatus*) and performed RNA sequencing (RNA-Seq) of the ventral hypothalamus and optic chiasma to evaluate transcriptome-wide gene expression changes of individuals in migratory condition. We found that 188 genes were differentially expressed in relation to migratory state, 86% of which have not been previously linked to avian migration. Focal hub genes were identified that are candidate variables responsible for the occurrence of migration (e.g. *CRABP1*). Numerous genes involved in cell adhesion, proliferation and motility were differentially expressed (including *RHOJ*, *PAK1* and *TLN1*), suggesting that migration-related changes are regulated by seasonal neural plasticity.

Keywords: *Catharus ustulatus*, circadian rhythm, circannual rhythm, migratory bird, photoperiod, Swainson's thrush

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Introduction

Circannual rhythms are ubiquitous and include seasonal fluctuations in diverse biological processes such as reproductive status (Nakane & Yoshimura 2014), metabolism (Bairlein 2003; Ebling 2014) and the immune system (Dopico *et al.* 2015). One of the most remarkable examples of circannual rhythms is avian annual migration, the seasonal movement between breeding and nonbreeding areas in response to seasonal change (Dingle & Drake 2007). Migration involves striking seasonal alterations in physiological, morphological and behavioural characteristics (Berthold *et al.* 2003; Dingle 2006), such as feeding behaviour and metabolism to fuel flight muscles (Bairlein 2003), reduction and rebuilding of organs and muscle (McWilliams & Karasov 2005) and traits involved in sleep deprivation in nocturnal migrants (Rattenborg *et al.* 2004; Piersma *et al.* 2005; Fuchs *et al.* 2006). Molecular studies of migration can provide insight into the mechanisms by which such circannual rhythms are coordinated (Gwinner 1996; Dawson *et al.* 2001) and the adaptive potential of migratory species to changing environments. Currently, the molecular underpinnings of migration are poorly understood, and their degree of similarity across avian taxa has not been evaluated (Dingle 2006; van Noordwijk *et al.* 2006; Dingle & Drake 2007; Jones *et al.* 2008; Liedvogel *et al.* 2011; Mueller *et al.* 2011; Lundberg *et al.* 2013; Boss *et al.* 2016; Fudickar *et al.* 2016).

Regulation of seasonal migration potentially involves a variety of dynamic processes in the brain. Many of these processes likely occur in the hypothalamus, which integrates signals of circadian and circannual time (Majumdar *et al.* 2015) and regulates hormone release, energy balance, feeding and reproductive status (Yasuo

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et al. 2003; Ebling & Barrett 2008; Barrett & Bolborea 2012; Dardente *et al.* 2014; Trivedi *et al.* 2014). An endogenous circannual clock, hypothesized to be linked to two or more of the master circadian pacemakers in the suprachiasmatic nuclei (SCN), pineal gland and retina (Kumar *et al.* 2004; Bartell & Gwinner 2005; Trivedi *et al.* 2016), is thought to ensure that seasonal changes occur at appropriate times of the year (Yasuo *et al.* 2003; Kumar *et al.* 2004; Bartell & Gwinner 2005; Rani *et al.* 2006; Dardente *et al.* 2014; Wood *et al.* 2015). Seasonal cellular remodelling in the hypothalamus, regulated by photoperiod, has been observed in birds (discussed in Yoshimura 2013; Migaud *et al.* 2015) and suggested to play roles in controlling hormone release (Yamamura *et al.* 2004, 2006). Changes in hormone signalling may contribute to alterations in multiple traits, such as impacts of hypothalamic thyroid hormone signalling on seasonal weight gain and increased energy consumption (Woods *et al.* 1998; Ebling & Barrett 2008; Ross *et al.* 2009; Barrett & Bolborea 2012), and, in spring migrants, effects of thyroid hormone signalling and gonadotropin releasing hormone on reproductive status (Dawson *et al.* 2001; Schwartz & Andrews 2013; Yoshimura 2013; Dardente *et al.* 2014; Tavolaro *et al.* 2015). In general, many cellular and physiological signals result in specific alterations of gene expression (Cheung & Kraus 2010). Therefore, assessment of transcriptome-wide gene expression changes in the brain provides the opportunity to evaluate migration-related regulatory processes at molecular, developmental and physiological levels.

Here, we evaluate gene expression changes associated with migration in the Swainson's thrush (*Catharus ustulatus*), a Neotropical, long-distance migratory songbird. We assessed expression of two geographically and genetically distinct subspecies (*C. u. swainsoni* and *C. u. ustulatus*, referred to here as 'inland' and 'coastal', respectively), which diverged approximately 10 000 years ago and exhibit dramatically different migratory pathways separated by a distinct migratory divide (Ruegg & Smith 2002; Ruegg *et al.* 2006a,b, 2014; Ruegg 2008; Delmore *et al.* 2012; Delmore & Irwin 2014). Migratory behaviour of the two subspecies is suggested to be under divergent selection (Ruegg *et al.* 2014; Delmore *et al.* 2015), but knowledge of which genes are involved in migration in this species is unknown and critical for evaluating this inference.

To examine transcriptome-wide expression of genes associated with migratory status, we performed RNA-Seq of Swainson's thrushes during nonmigratory and migratory states in controlled, captive conditions. First, we assessed migration-related gene expression and used network analyses to identify central processes and genes associated with migration, controlling for subspecies and time of day. Second, we tested for migratory expression in pathways hypothesized to be important for migration, including hormone signalling pathways and the circadian system. Finally, we compared migration-related gene expression between subspecies and evaluated overlap of migration-related genes in Swainson's thrushes with genes associated with migration in other bird species. This study provides functional insight into annual migration and reveals novel evidence of seasonal neural plasticity as a mechanism for regulating migration-related changes in physiology and behaviour.

Materials and methods

Bird capture and housing

The capture and handling of Swainson's thrushes was performed under federal Migratory Bird permit MB758364 and state permits SC-11246 (California) and 10–132 (Alaska) and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern Mississippi (USM; protocol 09012601). Hatch-year birds were captured in Humboldt County, California (coastal birds), in June–July 2010 and near Fairbanks, Alaska (inland birds), in July 2010 with mist nets using both playbacks and passive netting. Individuals were housed at the USM animal facilities. Birds were caged individually and fed 70–75 g daily a semisynthetic diet consisting of hard-boiled eggs (25%), insects (freeze-dried crickets and shrimp, waxworms; 20.8%), cottage cheese (17.5%), blueberries (16.7%), crackers (12.5%), egg shells (5%), red meat (2.5%) and a vitamin supplement. Throughout the entire period of animal captivity, photoperiod was adjusted weekly to mimic each population's natural environment (Fig. S1, Supporting information). Using data collected from past research (Mack & Yong 2000), we estimated the timing of departure for fall migration from coastal (August 1) and inland breeding areas (August 15). To mimic the photoperiod that birds would typically experience during migration, changes in photoperiod during autumn migration were estimated using data collected from wild thrushes during migration, based on the estimated weekly location of each population (range: 800–1250 km/week, using an average speed of migration of 265 [±192] km/night; Cochran & Wikelski 2005]. In addition, we supplemented this information with migration passage dates from the literature (Mack & Yong 2000) and from long-term migration-banding data collected along the Gulf of Mexico (F. R. Moore, unpublished). During the year prior to this study, individual body weight, fat level, plasma metabolites levels, plasma hormone levels and caloric content of faeces

were measured weekly for a separate study (K. L. Paxton & F. R. Moore, unpublished).

Migratory activity and sampling time

To classify birds as nonmigratory or migratory, we quantified individual proportion nightly activity for the 2 weeks prior to sample collection using infrared motion detectors which record activity via data loggers (JoAC Electronik, Lund, Sweden) and activity analysis software (NI LabVIEW National Instruments, Austin, TX, USA). Proportion nightly activity was quantified as the proportion of 10-min intervals, between 30 min after lights were turned off and 30 min before lights were turned on, with >20 movements. Birds were considered nonmigratory if they never exhibited more than 0.05 proportion nightly activity during each night of the 2 weeks prior to animal sacrifice and tissue collection. Five birds from each subspecies were sacrificed and tissues collected within 2 days during summer (August 7–8, 2011; $N = 10$; Table S1, Supporting information). Six coastal and five inland birds were sacrificed and tissues collected within 2 days during autumn (October 3–4, 2011; $N = 11$; Table S1, Supporting information). Completion or moderate presence of post-breeding moult by August 1, as well as regressed testes in males, indicated that birds were post-reproductive photorefractory at the time of sampling except one coastal bird sacrificed on October 3 (individual Y56), which had enlarged testes and was fully excluded from the study. Of the remaining birds, birds sacrificed during summer were classified as nonmigratory except for individual R182, which exhibited migratory restlessness during the 2 weeks prior to tissue collection and therefore classified as migratory (Table S1, Supporting information). Birds sacrificed during the autumn were classified as migratory except individual Y57, which showed minimal nightly activity during the 2 weeks prior to tissue collection and therefore classified as nonmigratory (Table S1, Supporting information). The sample sizes of the study treatments were therefore: nonmigratory inland ($N = 4$), nonmigratory coastal ($N = 6$), migratory inland ($N = 6$) and migratory coastal ($N = 4$). All animals were sacrificed during daylight hours, ranging from 2.5 to 8.57 h after lights on (Table S1, Supporting information). As samples were not collected through the full 24-h cycle, we use 'circadian' gene expression in the adjective sense (relating to a 24-h day).

Tissue collection

Birds were deeply anesthetized with isoflurane and decapitated following USGS National Wildlife Health Center guidelines and with approval from the IACUC of USM. The brain was dissected following guidelines described for the zebra finch (Comito *et al.* 2016). Briefly, each brain was removed from the skull and placed ventral side up on a chilled petri dish. The hindbrain was removed, and two parasagittal cuts were made just medial to each optic tectum to remove the optic tecti. To ensure collection of the SCN of the hypothalamus, which is just dorsal to the optic chiasma, we collected the total tissue located medial to the two parasagittal cuts to a depth of approximately 2 mm. Thus, the tissue collected included the ventral hypothalamus and optic chiasma. The tissue was immediately flash frozen on dry ice, with no more than 10 min passing from the time each animal was sacrificed to the time the tissue was frozen on dry ice. Samples were shipped to UCLA in a liquid nitrogen tank and stored at −80 °C until RNA extraction.

RNA extraction and sequencing

Total RNA was extracted from tissues using the Trizol Plus RNA Purification Kit and included DNase treatment with the Ambion RNase-Free DNase Set and column cleanup with the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Complimentary DNA (cDNA) libraries were generated with the TruSeq RNA Sample Preparation Kit v2 which includes poly-A selection (Illumina, San Diego, CA, USA) and sequenced as 100-base pair paired-end reads on the Illumina HiSeq 2000 at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley. Five barcoded samples were pooled per sequencing lane.

Quality filtering and mapping

A range of 31.2–48.8 million 100-base pair read pairs were generated for each Swainson's thrush sample (Table S1, Supporting information). Reads were trimmed with Trim Galore (available at www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove adaptors (read ends with three or more base pairs matching adapter sequence) and base pairs with Phred score <20 at the ends of reads. Read pairs with either read shorter than 25 base pairs were removed, resulting in 29.8–46 million high-quality read pairs per sample. We used Tophat2 (Kim *et al.* 2013) to map reads to the collared flycatcher genome (*Ficedula albicollis*, version 1.4.74) (Ellegren *et al.* 2012). We used the collared flycatcher genome as a reference because it contains fewer gaps and is better annotated than the draft assembly of the Swainson's thrush genome (Delmore *et al.* 2015). Moreover, because of its availability in Ensembl, the flycatcher genome is accessible for analytical tools that utilize the Ensembl genome database [e.g. gene ontology (GO) analysis with g:profiler]. In order to

allow for base pair differences due to cross-species divergence while maximizing the number of uniquely mapped reads, we used the following parameters for read mapping: read-mismatches 16, read-gap-length 10, read-edit-dist 28, splice-mismatches 1, max-insertion-length 12, max-deletion-length 12, max-multihits 5, b2-D 15, b2-N 1, b2-L 20, b2-i S,1,0.50. After aligning reads, we filtered for only uniquely mapped reads for further analyses. Overall, an average of 58.5% of the total sequenced reads per sample passed all filters and were used for gene expression quantification, ranging from 15 to 28.5 million read fragments per sample.

Gene expression quantification

Gene expression was quantified using HT-Seq (Anders *et al.* 2014) with the 'union' mode. If only one mate of a read pair mapped uniquely within the transcriptome, that read was kept and was counted equally as a read pair for performing gene expression counts, as both a single-end read and a paired-end read represent one RNA molecule. Expression values were normalized using the trimmed mean of *M*-values method in the DESEQ package (Anders & Huber 2010) in R (R Core Team 2013) and adjusted for gene length and GC content using the R package Conditional quantile normalization (CQN; Hansen *et al.* 2012). Genes were included in the analyses if they were annotated as protein coding and if their CQN-normalized and transformed expression values were ≥25 in at least 14 individuals. To identify sample outliers, we used the $Z.k_u$ score in the R package WGCNA (Langfelder & Horvath 2008), which specifies the number of standard deviations a sample's gene expression network connectivity is below the mean of all samples (Horvath 2011). Individual t22 was identified as an outlier and removed from further analyses because its $Z.k_u$ score was greater than the cut-off of 3.

Modelling

We first used principal components analysis of the normalized, log2-transformed expression data to identify variables potentially structuring overall expression data. Variables that significantly correlated with overall normalized expression data included subspecies [correlated with PC1 ($r = 0.7$, $P < 0.001$) and PC2 ($r = 0.596$, $P = 0.007$) of the normalized expression data], time of day [correlated with PC9 ($r = -0.756$, $P < 0.001$)], sex [correlated with PC3 ($r = 0.627$, $P = 0.004$)] and sequencing lanes [correlated with PC2 ($r = -0.6$, $P = 0.007$) and PC11 ($r = 0.602$, $P = 0.006$)]. Sequencing lanes and sex were regressed from the gene expression data (i.e. from the response variables) prior to linear modelling. Migration status, time of day of tissue collection and subspecies were included as covariates in the linear model. Specifically, we performed gene by gene multiple linear regression, with the explanatory variables defined as migration status, time of day, and subspecies, and the response variable defined as expression level of each of the 11 727 genes that passed the sequence coverage criteria. As individuals R182 and Y57 did not show typically timed migratory behaviour, the consistency of results in relation to the migratory classification of individuals R182 and Y57 was evaluated by performing the linear modelling excluding R182 and Y57. *P*-values were corrected for multiple testing using the *q*-value method in R (Storey & Tibshirani 2003). Given the limited sample size, we used a false discovery rate (FDR) threshold of 0.2 after performing 100 random permutations of each explanatory variable of interest (i.e. migration status, time of day and subspecies) to ensure that the empirical null distributions of *P*-values were uniform (Fig. S4, Supporting information). Gene annotations and orthologs for comparison of migration-related genes across species were acquired from Ensembl release 81.

Network analysis

Coexpression of genes significantly associated with migration or time of day (*Q*-value < 0.2) was analysed using the WGCNA package in R. Gene clusters were visualized using the plotNetworkHeatmap function with a network of signed correlations and the topological overlap matrix, which is a measure of gene network interconnectedness based on the number of neighbouring genes shared between two genes (Yip & Horvath 2007; Horvath 2011). Gene connectivity was measured by the K-within metric produced by the intramodularConnectivity function in WGCNA.

Gene ontology analysis

To identify molecular functions and biological processes represented in the gene expression data, we performed GO analyses using g:profiler version r1488_e83_eg30 (Reimand *et al.* 2007). Ensembl identification numbers of genes associated with migration, time of day or subspecies (*Q*-value < 0.2) were input as query lists, and all genes analysed ($N = 11\,727$; see criteria above) were used as the background gene list. Query lists were divided into significantly upregulated and downregulated genes. The minimum allowed overlap between query genes and genes belonging to a GO term was set to 2. *P*-values were corrected using the Benjamini–Hochberg FDR method (Benjamini & Hochberg 1995).

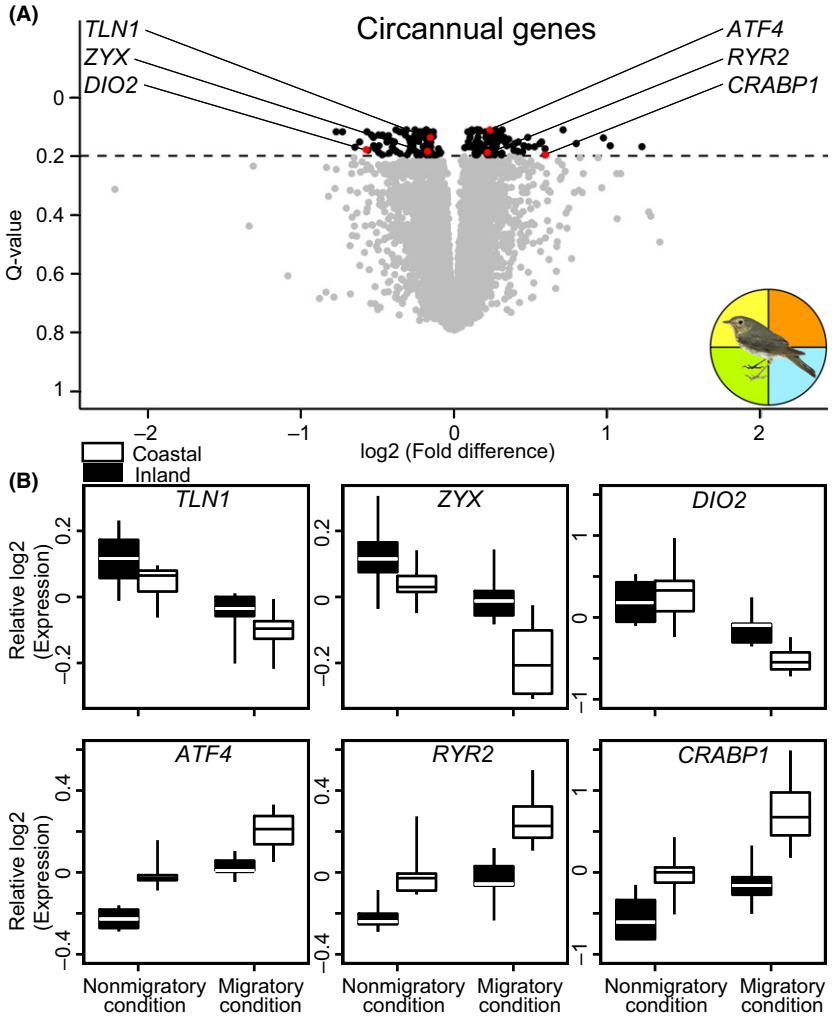

Fig. 1 Gene expression level differences with migratory status. (A) Volcano plot depicting differential gene expression of 11 727 protein-coding genes in Swainson's thrushes in the migratory state relative to the nonmigratory state. Significant genes (Q-value <0.2) are represented by black and red dots. Genes highlighted in red are depicted in (B). See Table S1 (Supporting information) for sample information and Table S2 (Supporting information) for comprehensive gene results. (B) Examples of genes exhibiting expression levels significantly associated with migratory status. Boxes represent the first through third quartiles of gene expression across birds. Horizontal lines represent the mean across birds, and vertical lines extend to the minimum and maximum expression values across birds.

Results

Migratory activity

To assess migratory status of Swainson's thrushes in captivity, we measured individual migratory restlessness, or *zugunruhe*, nightly during summer and fall prior to tissue sampling (Fig. S1, Supporting information). Individuals classified as migratory exhibited significantly more average migratory restlessness activity (mean of migratory group = 0.251, mean of nonmigratory group = 0.003) and greater body weight (mean of migratory group = 45.21 g, mean of nonmigratory group = 34.88 g; Welch's t-test; $N = 20$; d.f. = 17.376, $P = 0.0052$) than nonmigratory birds (Table S1, Supporting information). Within birds in the migratory condition, inland Swainson's thrushes exhibited significantly higher average nightly migratory restlessness activity than coastal Swainson's thrushes (mean of inland = 0.34, mean of coastal = 0.11; Welch's t-test; $N = 10$; d.f. = 7.387, $P = 0.0448$), concordant with the greater natural migration distance of inland Swainson's thrushes (Delmore *et al.* 2012).

Signature of cellular plasticity with migration

To evaluate transcriptome-wide gene expression changes associated with migration in the SCN, we performed RNA-Seq on the ventral hypothalamus and optic chiasma, located just ventral to the hypothalamus (see Materials and methods). We assessed expression differences with migratory state, controlling for time of day and subspecies, using a Q-value threshold of 0.2 (Storey & Tibshirani 2003). We detected 188 genes differentially expressed between Swainson's thrushes in nonmigratory and migratory states (Fig. 1; Table S2, Supporting information). These results were robust to the exclusion of two individuals that did not exhibit typically timed migratory behaviour (see Materials and methods; 91.5% genes still significant; Table S3, Supporting information). Genes upregulated in birds exhibiting migratory activity were

Table 1 Examples of enriched gene ontology (GO) terms and genes differentially expressed with autumn avian migration

Enriched GO term	GO corrected *P*-value	Gene symbol	Ensembl gene ID	Fold difference	*Q*-value
Cell migration	9.51E-3	*EMX2*	ENSFALG00000002201	1.507	0.176
		GLI3	ENSFALG00000005113	1.220	0.118
		EPHB1	ENSFALG00000013390	1.253	0.137
		FGF13	ENSFALG00000003780	1.228	0.118
Establishment of localization	6.77E-3	*CRABP1*	ENSFALG00000010635	1.511	0.196
		RYR2	ENSFALG00000013270	1.162	0.188
		RASL10B	ENSFALG00000009711	1.240	0.190
		IWS1	ENSFALG00000013574	1.066	0.153
Actin filament-based process	3.27E-3	*TLN1*	ENSFALG00000002197	0.897	0.137
		ZYX	ENSFALG00000004531	0.886	0.185
		RHOJ	ENSFALG00000003356	0.743	0.131
		PAK1	ENSFALG00000007597	0.815	0.141
		MYO1F	ENSFALG00000013814	0.784	0.118
		MYO1C	ENSFALG00000006676	0.837	0.129
		MSN	ENSFALG00000004282	0.830	0.168
		ARPC1B	ENSFALG00000013811	0.736	0.137
		TRIOBP	ENSFALG00000012797	0.826	0.165
Regulation of cell motility	4.31E-3	*PDGFA*	ENSFALG00000013959	0.882	0.137
		LAMA4	ENSFALG00000010965	0.756	0.150
		ADAM10	ENSFALG00000012254	0.866	0.189

Multiple linear regression was used to identify genes associated with avian migration controlling for subspecies and time of day. Genes were annotated based on Ensembl 81 of the *Ficedula albicollis* genome. See Table S4 (Supporting information) for a comprehensive list of GO terms. FD, fold difference.

significantly enriched with genes related to cell migration and cell development and include *EMX2*, *GLI3*, *FGF13* and *EPHB1* (*Q*-values <0.2; Tables 1, S2 and S4, Supporting information). Similarly, genes downregulated during migration were significantly enriched with genes related to cell adhesion and motility of cells, including *RHOJ* and its effector *PAK1*, *myosin IC* (*MYO1C*) *and myosin IF* (*MYO1F*), and adhesion complex molecules *talin-1* (*TLN1*), *zyxin* (*ZYX*) and *moesin* (*MSN*) (*Q*-values <0.2; Tables 1, S2 and S4, Supporting information).

Weighted gene coexpression network analysis (WGCNA) results indicate that within the 188 migration-related genes is a core cluster of 34 highly co-expressed genes (Fig. S2, Supporting information). Gene ontology analysis reveals that these genes are enriched for three groups of GO terms (Table S4, Supporting information), relating to 'anatomical structure morphogenesis' (*P* = 2.23E-2), 'neural precursor cell proliferation', (*P* = 3.13E-3) and 'protein localization to organelle' (*P* = 3.77E-2). This cluster is also enriched for genes involved in action potential and includes potassium channel gene *KCNB1*, potassium channel modulator *DPP6* and the calcium channel component *RYR2*.

Endocrine signalling associated with migration

We examined genes found to be migration-related (*N* = 188) for components of endocrine signalling pathways. *DIO2* was significantly downregulated with migration (*Q*-value = 0.179; fold difference (FD) = 0.671). This gene is known to be photo-responsive and regulates thyroid hormone by converting the prohormone T_4 to the active form of thyroid hormone, T_3 (Kohrle 1999), supporting a potential role for thyroid hormone in regulating seasonal changes in migratory birds. *CRABP1*, which is regulated by T_3 and regulates retinoic acid signalling, was significantly upregulated in migratory birds (*Q*-value = 0.196; FD = 1.511). Further, *CRABP1* had the third highest connectivity of all 188 migration-related genes after *SBK1* and *MTUS2* and was found to be a central hub gene within the core cluster of migration-related genes (Table S2, Supporting information).

Circadian system expression associated with migration

Given the potential role of the circadian system in regulating circannual rhythms (Kumar *et al*. 2004; Bartell & Gwinner 2005; Rani *et al*. 2006; Yoshimura 2013; Dardente *et al*. 2014; Singh *et al*. 2015), we tested whether expression of known avian circadian clock genes were altered in birds in migratory condition. We did not detect migration-related expression differences in the seven genes previously identified in the avian circadian clock [*PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *ARNTL* (or *BMAL1*) and *ARNTL2* (or *BMAL2*; Kumar *et al*. 2010]. However, additional genes are

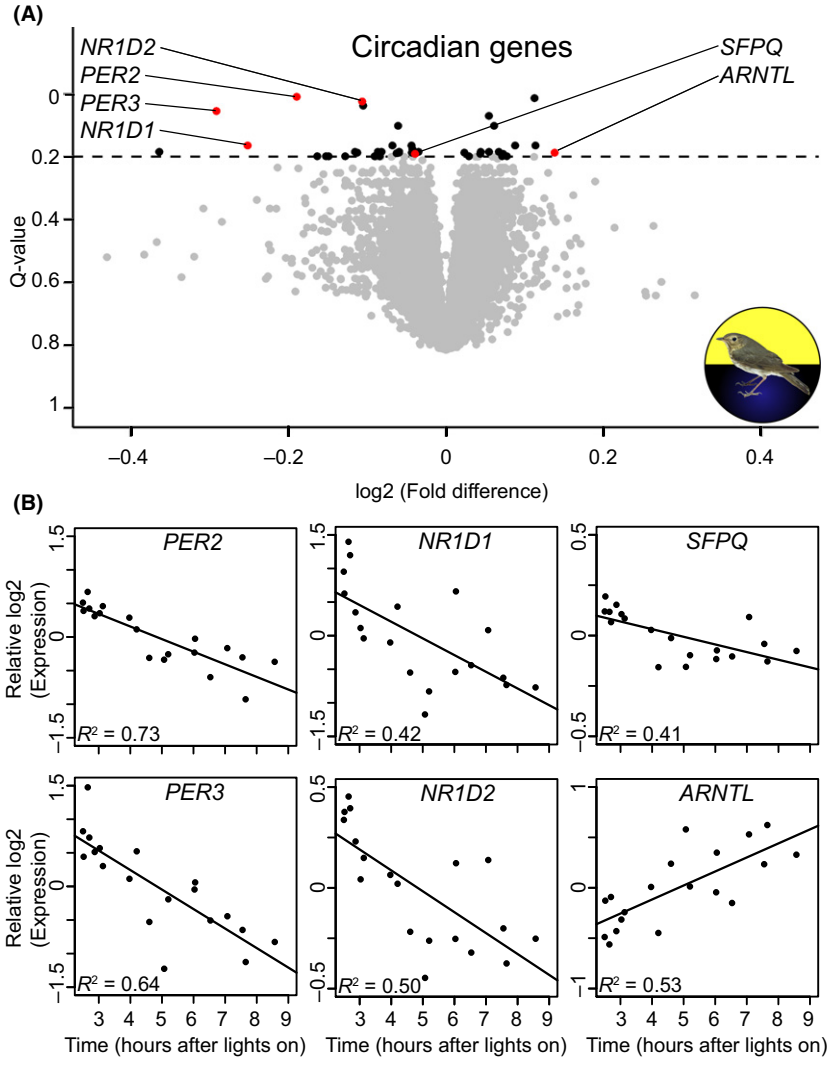

Fig. 2 Gene expression level differences with time of day. (A) Volcano plot depicting changes in gene expression per hour of day (measured only during lights on) in 11 727 protein-coding genes in Swainson's thrushes. Significant genes (*Q*-value <0.2) are represented by black and red dots. Genes highlighted in red are depicted in (B). See Table S2 (Supporting information) for a comprehensive gene list. (B) Examples of genes exhibiting expression levels significantly associated with time of day. Each dot represents the normalized, log2-transformed expression level of an individual Swainson's thrush.

likely involved in the avian clock, as several avian clock components are proposed based on knowledge of the mammalian clock, but have not been empirically studied (reviewed in Kumar *et al.* 2010). Therefore, we evaluated genes for expression level differences with time of day to identify novel components of the avian circadian clock and its target genes. We observed circadian-related expression in 43 genes, encompassing *PER2*, *PER3*, *ARNTL*, and 40 genes that have not been previously established as components or targets of the avian circadian clock (*Q*-values <0.2; Fig. 2; Table S2, Supporting information). Novel genes exhibiting circadian-related expression included *SFPQ* (also called *PSF*), which encodes a component of the PER complex in the negative loop of the mammalian circadian clock (Duong *et al.* 2011), and *NR1D1* (also called *Rev-erbα*) and *NR1D2* (also called *Rev-erbβ*), which are components of an accessory circadian feedback loop linking the negative and positive limbs of the mammalian circadian clock (Fig. 2) (Preitner *et al.* 2002; Cho *et al.* 2012). We then used WGCNA to identify genes that may be central drivers of the avian circadian clock. *PER2*, *NR1D2* and *PER3* had the highest intramodular connectivity of genes exhibiting circadian expression and were at the centre of a core cluster of coregulated genes also including *NR1D1* and *SFPQ* (Fig. S3, Supporting information). This is the first evidence that *SFPQ*, *NR1D1* and *NR1D2* are involved in the avian circadian clock. Outside this module, we also detected circadian expression of seven genes encoding heat shock and heat shock-related proteins: *HSF2*, *HSPA5*, *HSPH1*, *DNAJA1*, *DNAJA4*, *AHSA2* and *ENSFALG00000015235* (which belongs to the *HSP20* family) (*Q*-values <0.2; Table S2, Supporting information). We did not detect migration-related expression differences

in any of the 43 genes exhibiting circadian expression in Swainson's thrushes.

Expression differences between subspecies

Controlling for migration status and time of day of sample collection, 610 genes were differentially expressed between the inland and coastal subspecies (Q-value <0.2; Table S2, Supporting information). Genes differentially expressed between subspecies were highly enriched for migration-related genes, with 47 genes differentially expressed with migration and between subspecies (hypergeometric test; $N = 47$; $P = 4.7E\text{-}20$; Table S2, Supporting information). These 47 genes were over-represented with GO terms relating to regulation of gene expression ($P = 3.51E\text{-}0.5$), cell motility ($P = 7.85E\text{-}3$) and central nervous system neuron axonogenesis ($P = 6.84E\text{-}5$; Table S4, Supporting information). The enrichment of subspecies differences for cellular processes is specific to genes associated with migration, as overall subspecies differences are instead enriched for the opioid receptor signalling pathway ($P = 0.0015$; Table S4, Supporting information). Specifically, the inland subspecies exhibited upregulation of all four known opioid receptors (*OPRD1*, *OPRK1*, *OPRM21* and *OPRL1*), which have central roles in sensory perception (e.g. Mueller *et al.* 2010) and have been implicated in avian behaviours such as birdsong (Kelm *et al.* 2011).

Migration-related genes across species

We compared the 188 migration-related genes to genes with expression associated with migration in other bird species for which homologous annotations are available (Table S2, Supporting information), including willow warblers (*Phylloscopus trochilus*; $N = 1079$ genes) (Lundberg *et al.* 2013; Boss *et al.* 2016), dark-eyed juncos (*Junco hyamalis*; $N = 141$ genes) (Fudickar *et al.* 2016) and white-crowned sparrows (*Zonotrichia leucophrys*; $N = 11$ genes) (Jones *et al.* 2008). Genes differentially expressed in Swainson's thrushes during migration were significantly enriched for candidate migration genes identified in willow warblers (hypergeometric test; $N = 25$ genes; $P = 0.039$) and include the calcium channel component *RYR2* and the transcription factor *ATF4*, which acts as the culmination of multiple pathways to integrate stress signals to impact energy metabolism, amino acid transport, oxidative stress resistance and skeletal myofibre atrophy in mammals (Harding *et al.* 2003; Rutkowski & Kaufman 2003; Yoshizawa *et al.* 2009). One gene, *TMEM132B*, was associated with migration in dark-eyed juncos (Fudickar *et al.* 2016). No migration-related genes were shared with those identified in white-crowned sparrows (Jones *et al.* 2008). We did not detect migration-related expression of *ADCYAP1* or *CLOCK*, whose allele lengths have previously been associated with measures of migratory distance and phenology in blackcaps (*Sylvia atricapilla*) (Mueller *et al.* 2011; Mettler *et al.* 2015), dark-eyed juncos (Peterson *et al.* 2013), Wilson's warblers (*Cardellina pusilla*) (Bazzi *et al.* 2016), nightingales (*Luscinia megarhynchos*) and tree pipits (*Anthus trivialis*) (Saino *et al.* 2015).

Discussion

We identified 188 genes associated with fall migratory restlessness activity. The highly significant enrichment of migration-related genes for cell motility, cell adhesion and cell development provides evidence that migration-related circannual changes involve seasonal neural plasticity. Determining which molecular and cellular processes are necessary for and unique to migration will require functional comparative analyses across migratory and nonmigratory species.

The theoretical 'threshold model of migration' proposes that the tendency to migrate develops when a currently unknown continuous variable (e.g. the concentration of a protein or hormone), termed a 'liability' variable, reaches a certain threshold (Pulido *et al.* 1996; Pulido 2011). Given our focus on the hypothalamus and optic chiasma, where signals of photoperiod are thought to be integrated to elicit downstream responses, the genes we present here are candidate 'liability' variables involved in triggering the occurrence of migratory behaviour. Our utilization of WGCNA, combined with available information on specific functions of genes, highlights particularly strong candidate 'liability' genes. *DIO2*, via T_3 production, may be a driver of neural plasticity, as has been suggested in the nonmigratory Japanese quail (*Coturnix japonica*) for regulating gonadal growth and regression (Yamamura *et al.* 2004, 2006). *CRABP1* may also play a role in regulating the suite of expression changes associated with cellular plasticity, given that: (i) *CRABP1* was a hub gene with high connectivity to other migration-related genes; (ii) *CRABP1* is known to be regulated by T_3, the product of DIO2 activity (reviewed in Balmer & Blomhoff 2002; Mey & McCaffery 2004; Park *et al.* 2005; Maden 2007; Shearer *et al.* 2012); and (iii) thyroid hormone and retinoic acid signalling have been found to drive seasonal neural plasticity in other species (reviewed in Balmer & Blomhoff 2002; Park *et al.* 2005; Yamamura *et al.* 2006; Maden 2007; Horn & Heuer 2010; Shearer *et al.* 2010, 2012).

Elucidating the cellular changes that specifically occur between nonmigratory and migratory states will require additional evidence, such as ultrastructure imaging of

the hypothalamus. The observed differential expression of genes involved in cell motility, adhesion and development may reflect cellular morphological changes (discussed in Migaud *et al.* 2015; Wood *et al.* 2015). One possibility is that glial cells surrounding nerve terminals undergo morphological changes, which has been suggested to regulate hormone secretion in Japanese quail (Yamamura *et al.* 2004, 2006). Another possibility, which could be evaluated with immunohistochemistry, is neurogenesis. Neurogenesis has been reported in the adult hypothalamus of diverse species including zebrafish, sheep and hamsters (reviewed in Sousa-Ferreira *et al.* 2014). Supporting this potential process, we detected expression of markers for stem cells and cell proliferation known to be expressed in the neurogenic niche in the mammalian hypothalamus (Migaud *et al.* 2010, 2015) including *SOX2*, *NES*, *DCX* and *PCNA* (results not shown). Adult hypothalamic neurogenesis is thought to be important in regulating food intake in response to environmental and physiological signals (reviewed in Sousa-Ferreira *et al.* 2014) and could potentially play a role in regulating the striking hyperphagia of birds during migratory fattening.

Among the 188 genes differentially expressed with migration, a significant proportion of genes exhibited expression differences between the two subspecies (N = 47 genes; 25% of migration-related genes). Genetic divergence likely contributes to the observed expression differences between subspecies, given that the subspecies also exhibited expression differences in 558 genes regardless of migration state or time of day (i.e. independent of temporal measures). This possibility supports previous research suggesting divergent selection on migration in the Swainson's thrush (Ruegg *et al.* 2014; Delmore *et al.* 2015). However, the subspecies expression differences could also partly be due to gene expression responsiveness to the difference in photoperiod that each subspecies experienced (see Materials and methods). Research on the molecular evolution of these genes across natural populations, combined with further work on birds in controlled conditions, will be essential in understanding the roles of adaptive and acclimatory responses in generating interindividual variation in migration-related phenotypes.

Expression differences of 188 genes between birds in migratory and nonmigratory states, combined with enrichment of genes associated with cellular plasticity, demonstrate the extensive changes that may seasonally occur in the brain for this remarkable behavioural phenomenon. We suggest the possibility that seasonal neural plasticity has a greater role in this physiological and behavioural transition than previously thought. Network analyses reveal hub genes, potential 'liability' variables, whose expression may be pivotal in regulating the onset of migratory behaviour. Understanding the expression thresholds of such variables for actuating migration, and their sensitivity to environmental conditions, will be critical for predicting the response of migratory animals to future climate and environmental changes.

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Data accessibility

The sequence data are available in the NCBI Gene Expression Omnibus repository (Edgar *et al.* 2002) and are accessible through GEO: GSE87549.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Photoperiodic conditions and migratory restlessness activity of Swainson's thrushes during the project.

Fig. S2 Covariation heatmap of gene expression variation across Swainson's thrushes of genes associated with migration.

Fig. S3 Covariation heatmap of gene expression variation across Swainson's thrushes of genes associated with circadian time of day.

Fig. S4 Distribution of *P*-values from the linear analysis vs. the empirically derived null for each variable of interest: (A) migration status, (B) time of day, and (C) subspecies.

Table S1 Biological and technical information of all Swainson's thrushes used in the RNA-Seq analyses.

Table S2 Gene-by-gene results of all 11 727 genes analyzed in the study.

Table S3 Gene-by-gene results excluding samples r182 and y57.

Table S4 Results from all GO analyses performed in g:profiler.