Wintering molecular changes in the brain of Calidris pusilla at lower latitude

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Abstract

Background:

Migrant birds prepare differently to fly north for breeding in spring and for the flight to lower latitudes during autumn avoiding the cold and food shortages of the north hemisphere's harsh winter. The molecular events associated with these fundamental stages in the life history of migrants include the differential gene expression in different tissues. Semipalmated sandpipers (*Calidris pusilla*) are Artic breeding shorebirds that migrate to the coast of South America during non-breeding season. Thus, the adaptive molecular changes in the brain of these birds at lower latitudes have not yet been investigated in detail.

Results:

Here, we searched for differential gene expression in the brain of semipalmated sandpiper, of recent arrived birds (RA) from autumnal migration and that of individuals in the premigratory period (PM) in the spring. All individuals were collected in the tropical coastal of northern Brazil. We generated a *De novo* neurotranscriptome for *C. pusilla* individuals and compared gene expression across libraries for neurotranscriptome. To that end we mapped RNA-seq reads to the *C. pusilla* neurotranscriptome in a total of 4 brain samples each group. A total of 266,414 transcripts were reconstructed that yielded 615 differentially expressed genes in the brain of both groups.

Conclusions:

The present report revealed a remarkable differential gene expression in the brain of recently arrived and premigratory individuals. It also revealed molecular brain changes associated with the recovering of the 4 to 5 days long-distance uninterrupted flight across Atlantic Ocean and preparation for the long-distance multiple stopover spring migration.

1 Background

Each year, migratory birds undergo behavioral changes associated with reproduction (Costa et al., 2021; Kimmitt, 2020; Lifjeld et al., 2022; Sharma et al., 2020; Yoshimura, 2013), and wintering, pre-migration and migration (Sharma & Kumar, 2019; Sharma, Singh, Malik, et al., 2018; Trivedi, Kumar, Rani, & Kumar, 2014), which are accompanied by molecular (Frias-Soler, Kelsey, Villarín Pildaín, Wink, & Bairlein, 2022; Frias-Soler, Pildaín, Pârâu, Wink, & Bairlein, 2020), cellular (Carvalho-Paulo et al., 2017; de Almeida Miranda et al., 2021; DeMoranville et al., 2019; Henrique et al., 2020), and systemic changes (Catry, Granadeiro, Gutiérrez, & Correia, 2022; Elowe & Gerson, 2022; Guglielmo, 2018; Schmaljohann, Eikenaar, & Sapir, 2022). Indeed, to promote adaptive responses for spring and autumnal migrations, migrating birds change between two life story states (LHS) (Malik, Singh, Rani, & Kumar, 2014) with contrasting seasonal
phenotypic profiles emerging before and after breeding, respectively (Trivedi et al., 2014). These states are connected to significant brain molecular changes including differential hypothalamic gene expressions leading to distinct regulatory strategies at the transcriptional level in the autumn and in the spring (Sharma et al., 2020; Sharma, Singh, Das, & Kumar, 2018). For example in the Black-headed Bunting songbird, (Emberiza melanocephala) after photoperiodical induced seasonal LHS, it has been described contrasting differences in the activity-rest pattern, body fattening and weight gain, testis size, heart and intestine weights, blood glucose and triglyceride levels between spring premigratory and post-migratory phenotypes (Trivedi et al., 2014).

Spring migration requires preparation for prolonged migratory flights by fuelling at wintering sites, when fat accumulation, metabolic enzymatic changes, and lipogenesis in the liver with subsequent transport to skeletal muscle indicate readiness for departure (Sharma et al., 2021).

Low latitude climate zones around the equator are chosen for wintering by many species of shorebirds to escape harsh northern winter weather and lack of food. This tropical region remains stable between years, is a rich food resource area, and show warmer temperatures with little variation in the annual range, enabling migrant birds to prepare for (in spring) and recover from (in fall) long migratory flights. (Albert et al., 2020; Gratto-Trevor et al., 2012; PW Hicklin, 1987; Peter Hicklin & Gratto-Trevor, 2010). Because the environment on winter grounds is annually stable, endogenous rhythms imposed by internal clocks in association with weather at the wintering and stopover grounds, temperature rise and more favorable wind conditions, may determine the individual’s timing of vernal migration (Gwinner, 1996; Haest, Hüppop, & Bairlein, 2018, 2020; Horton et al., 2019; Hüppop & Hüppop, 2003; Marra, Francis, Mulvihill, & Moore, 2005).

The semipalmated sandpiper C. pusilla, performs a remarkable five to six days nonstop flight across the Atlantic Ocean from James Bay (Ontario, Canada) or from Bay of Fundy (between New Brunswick and Nova Scotia, Canada) to coastal South America, Caribbean and Central America, before moving on to his wintering area in Brazil (Brown, 2014; PW Hicklin, 1987). This species arrives on the coast of Venezuela (Mata, Marin, Rodriguez, Yurai Guerrero, & Cardillo, 2009) and in the Brazilian Coast (Larrazábal, AZEVEDO-JÚNIOR, & Pena, 2002) between the middle of August and early September and stay at resources-rich locations until April/May when birds start vernal migration (Mata et al., 2009). In these areas, these migratory shorebirds spend a large portion of the non-breeding season (Linhart et al., 2022), where they exchange feathers, increase body mass (Fedrizzi, Azevedo Júnior, & Larrazábal, 2004) and decreased corticosterone levels (Mata et al., 2009) maximizing fitness in preparation for the next long flight of spring migration.

There is not a single report related to brain molecular changes associated with the wintering period following the long uninterrupted migratory flight across the Atlantic Ocean (in fall) or preparation for the multiple stopover migratory flight for breeding (in spring) in this species. Similarly, brain transcriptome before and after reproduction in this species remain to be investigated. Because the ribonucleic acids represent the genomic expression, linking the genotype to the phenotype (Buccitelli & Selbach, 2020), we
compared two snapshots of transcripts in the brain of recently arrived and pre-migratory semipalmated sandpiper (*Calidris pusilla*) captured respectively at two-time windows of wintering period: August/September (fall) and April/May (spring). In the absence of a sequenced genome to guide the reconstruction process, the transcriptome was assembled *De novo* based on RNA-sequencing reads (RNA-Seq) and annotation (Raghavan, Kraft, Mesny, & Rigerte, 2022). Using RNA-Seq we searched for differential gene expression in the brain of this latitudinal migrant species, and the results were used to interpret the functional implications of the genomic expression (Wang, Gerstein, & Snyder, 2009).

### 2 Results

#### 2.1 Sequencing assembly of semipalmated sandpiper transcriptome

The RNA telencephalon tissues of *C. pusilla* were sequenced in Ion Proton Sequencer. Eight samples generated a total of 130,275,514 of single end raw reads, from these 66.1% survived the trimming/short reads removal phase and were used for transcriptome assembly (Fig. 1), producing a total of 266,414 transcripts.

#### 2.2 Gene expression between experimental groups

The transcript expression data were obtained mapping back the reads to the assembled transcripts. The volcano plot in Fig. 2 exhibits the statistical significance of the difference relative to the amplitude of difference for every single gene in the comparison between recently arrived and premigratory birds' brain, through the negative base-10 log and base-2 log fold-change, respectively. Since the P-values have a negative transformation, the higher along the y-axis a data point is, the smaller the P-value. The volcano plot indicates which genes are considered statistically differentially expressed based on the adjusted P-value of their difference. The log fold-change along the x-axis displays more considerable differences in the extreme values, with data points closer to 0 representing genes that have similar or identical mean expression levels. The wider dispersion indicates RA and PM genes that have a higher level of difference regarding its gene expression; for recent review of interpretation of expression level results and pairwise magnitude of difference for each gene using RNA-seq data, see (McDermaid, Monier, Zhao, Liu, & Ma, 2019). The present report revealed 615 differentially expressed genes (DEGs) in the brain of recently arrived and premigratory groups: 356 upregulated and 259 down-regulated genes (Fig. 2 and supplementary File S1).

Figure 3 is a large-scale snapshot of genomic differential expression in the brain of RA and PM *C. pusilla* data sets. The heat map of expression shows two clusters of genes with opposed expression patterns, showing a unique profile for each cluster among experimental groups (Figure 3, Figure S1 shows an expanded version of heatmap presented in Figure 3 for a more detailed genes annotation identifications).

#### 2.3 Gene ontology and functional analysis
Gene ontology (GO) annotation analysis was performed for brain transcriptome of RA and PM *C. pusilla* (Full annotation report are in supplementary File S1). We found a total of 4656 enriched terms for recently arrived birds and 1859 terms for pre-migratory birds. From these numbers, 923 belongs to Cellular Component (CC), 1730 to Molecular Function (MF) and 3862 to Biological Process (BP). The terms annotated in the gene ontology (GO) for the transcriptome showed different functional roles that may reflect pre- and post-breeding fundamental stages in the life history of this long-distance migrant specie.

Table 1 list the top 20 enriched GO terms related to biological process exhibiting significant differential expression.
**Table 1**

*Calidris pusilla* top 20 enriched Biological Process GO terms showing significant differential expression in the brain of autumn recently arrived and spring premigratory semipalmated sandpipers.

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Gene Symbol</th>
<th>GO IDs</th>
<th>GO Names</th>
</tr>
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<tr>
<td>TRINITY_DN40544_c0_g1</td>
<td>GABBR2</td>
<td>GO:0007186; GO:0007214;</td>
<td>G protein-coupled receptor signaling pathway; Gamma-aminobutyric acid signaling pathway;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRINITY_DN3467_c1_g1</td>
<td>MAN1A2</td>
<td>GO:0005975; GO:0006491;</td>
<td>Carbohydrate metabolic process; N-glycan processing;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRINITY_DN41739_c0_g1</td>
<td>BCAT1</td>
<td>GO:0009082; GO:0009098; GO:0009099;</td>
<td>Branched-chain amino acid biosynthetic process; Leucine biosynthetic process; Valine biosynthetic process;</td>
</tr>
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</tr>
<tr>
<td>TRINITY_DN56325_c0_g1</td>
<td>NXPE3</td>
<td>GO:0008150;</td>
<td>encode a member of neurexophilin family of neuropeptide-like glycoproteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRINITY_DN62709_c0_g1</td>
<td>FGF9</td>
<td>GO:0000122; GO:0001525; GO:0001649; GO:0001654; GO:0001934; GO:0002053; GO:0002062; GO:0006606; GO:0008543; GO:0008584; GO:0010628; GO:0030178; GO:0030238; GO:0030326; GO:0030334; GO:0030949; GO:0032927; GO:0042472; GO:0045880; GO:0048505; GO:0048566; GO:0048706; GO:0050679; GO:0051781; GO:0060045; GO:0060484; GO:0090263; GO:1904707;</td>
<td>Negative regulation of transcription by RNA polymerase II; Angiogenesis; Osteoblast differentiation; Eye development; Positive regulation of protein phosphorylation; Positive regulation of mesenchymal cell proliferation; Chondrocyte differentiation; Protein import into nucleus; Fibroblast growth factor receptor signaling pathway; Male gonad development; Positive regulation of gene expression; Negative regulation of Wnt signaling pathway; Male sex determination; Embryonic limb morphogenesis; Regulation of cell migration; Positive regulation of vascular endothelial growth factor receptor signaling pathway; Positive regulation of activin receptor signaling pathway; Inner ear morphogenesis; positive regulation of smoothened signaling pathway; Regulation of timing of cell differentiation; Embryonic digestive tract development; Embryonic skeletal system development; Positive regulation of epithelial cell proliferation; Positive regulation of cell division; Positive regulation of cardiac muscle cell proliferation; Lung-associated mesenchyme development; Positive regulation of canonical Wnt signaling pathway; Positive regulation of vascular associated smooth muscle cell proliferation;</td>
</tr>
</tbody>
</table>
Previous analysis of the genes illustrated in Table 1 in other species revealed their involvement in a variety of biological processes that, when examined from the perspective of differential gene expression in the brain of *C. pusilla*, raise relevant questions about the contribution of the wintering period for spring migration. Indeed, the top 6 upregulated (GABBR2, MAN1A2, BCAT1, NXPE3, FGF9 and TRDMT1) and the top 4 downregulated (PLXNA1, BICRAL, ARHGEF9 and MVB12B) differentially expressed genes in the brain of RA and PM individuals, are involved in various biological processes, e.g. signaling pathways, carbohydrate metabolism, N-glycan processing including protein folding, trafficking, and signal...
transduction (Hirata & Kizuka, 2021), remodeling of cytoskeleton and cell shape, regulation of GTPase activity, vesicular trafficking process, etc.

3 Discussion

Differential upregulation of transcript expression may be an appropriate starting point in the search for potential physiological changes associated with genetic or environmental changes (Pereira et al., 2020). In this study, we performed De novo assembly of RNA extracted from brain of C. pusilla collected before and after breeding, seven months apart, during the wintering period, and compared transcriptomic changes. Samples were collected in September/October, when birds have just completed their autumunal migration, and April/May, when birds become ready for spring migration. We found 259 up and 357 down regulated genes, differentially expressed in the brains of recently arrived and pre-migratory birds.

In the present work we used information from genomic studies of other species to speculate about the functional significance of genes of C. pusilla that are being differentially expressed in wintering recently arrived (in the fall) and premigratory (spring) individuals. The underlying assumption of comparative genomics is that functional regions are conserved through evolution (Guigo & de Hoon, 2018) and because of that speculative views about functional genomic is reasonable as starting point.

3.1 GABBR2 and ARHGEF9 differential expressed genes in wintering C. pusilla

In the volcano plot of Fig. 2 we highlighted significant differences between gene expressions in the brains of recently arrived and pre-migratory groups of C. pusilla. Here we discuss GABBR2 and ARHGEF9 gene expressions as an example of two contrasting differential gene expressions related to inhibitory activity in the brain of C. pusilla. The upregulation of the synthesis of Subunit 2 of the Gamma-Aminobutyric Acid Receptor Type B may provide an increase in GABAergic transmission in the brain as the spring migration approaches. Important to highlight that hippocampal circuits involved in the learning and memory (Barkan, Yom-Tov, & Barnea, 2017) and social interaction (Rytova et al., 2019) seem to be important for the long flights of migrations. Social interaction between birds of the same group may facilitate collective behavior to form flocks and organize flights during migration for energy savings (Ling et al., 2019). Of note, in a previous study we found in a shorebird of the same family (Scolopacidae), a significant increase in parvalbuminergic neurons in the hippocampal formation of this species, in the premigratory groups captured in the wintering period, at the same time point and place (Guerreiro et al., 2022). If this observation about the PV neurons of the hippocampal formation extends to C. pusilla, it is reasonable to raise the hypothesis that GABAergic adaptive changes may be required for the spring migratory flight. In contrast, because autumn migration has been left behind, and long-distance flights will not be required during the wintering period, GABAB expression may not be required to the same extent. In addition, parvalbumin-expressing basket-cell network plasticity induced by experience regulates adult learning (Donato, Rompiani, & Caroni, 2013), and hippocampal early-born and late-born PV neurons are recruited in rule consolidation and new information acquisition through excitation and inhibition respectively (Caroni,
2015a, 2015b). Learning and memory is modulated by hippocampal GABAergic activity through GABAB receptor and metabotropic glutamate receptor-dependent cooperative long-term potentiation, suggesting that GABAergic synapses may contribute to functional synaptic plasticity in adult hippocampus (Patenaude, Chapman, Bertrand, Congar, & Lacaille, 2003; Patenaude, Massicotte, & Lacaille, 2005). Indeed, the synaptic plasticity of inhibitory neurons provides long-lasting changes in the hippocampal network, and this is a key component of memory formation (Honoré, Khlaifia, Bosson, & Lacaille, 2021; Topolnik & Tamboli, 2022). Distinct interneuron types contribute to the temporal binding of hippocampal ensembles, synaptic plasticity, and the acquisition of spatial and contextual information (Chamberland & Topolnik, 2012; Topolnik & Tamboli, 2022). This hippocampal activity is part of the neuronal network used to integrate the multisensory navigational information (magnetic field, celestial cues, and geographical cues) in the hippocampal formation and this is important to define flight direction and stopovers recognition during the spring long-distance migration, back to the breeding niches (Grönroos, Muheim, & Akesson, 2010; Mouritsen, Heyers, & Güntürkün, 2016; Tyagi & Bhardwaj, 2021).

In line with these findings is the differential downregulation of Guanine Nucleotide Exchange Factor ARHGEF9 that is essential for the synaptic localization and maintenance of GABAA receptors in the postsynaptic neuronal membrane in the hippocampus (Chiou et al., 2011; Ibaraki et al., 2018; Papadopoulos et al., 2008). This gene synthesizes collybistin, a guanine nucleotide exchange factor that seems essential to materialize this operation (Papadopoulos et al., 2008). Important to remember that GABA type A receptor is a ionotropic ligand-gated chloride channel which mediates fast inhibitory signals through rapid postsynaptic hyperpolarization, whereas GABA type B is a metabotropic receptor producing slow and prolonged inhibitory signals via G proteins and second messengers involved in pre- and postsynaptic inhibition, regulation of Ca\(^{2+}\) and K channels (Smart & Stephenson, 2019).

Thus, related to brain inhibition in this species, many questions emerge to be explored in future studies. For example: what is the functional role of such contrasting differential gene expressions for GABAA and GABAB receptors found in the brains of newly arrived and pre-migratory individuals? It is important to highlight that although our samples were separated by 7 to 8 months (wintering period), the time windows studied were close to the reproductive period. In fact, autumn migration takes place two months after breeding (August and September), and spring migration back to the breeding site takes place two months before breeding (April and May). Because of this proximity to the reproduction, we can ask whether the physiological changes induced before and after reproduction differentially modulate these receptors in the brain? As the expression of GABA\(_A\) receptor subunit transcriptional regulation is affected by sexual hormones (Agrawal & Dwivedi, 2020), and hormone levels may be not the same before and after reproduction (Blank et al., 2020), the differential expression of GABAA receptors may change. It remains however to be investigated in detail, the physiological implications of these differential gene expression regulating GABAergic receptors in the wintering C. pusilla at lower latitude.

Finally, because our sample did not distinguish the different areas of the nervous system, nor did it examine the expression in different neuronal types, it is worth asking whether these receptors are differentially expressed in different brain areas and in different neuronal types.
4 Conclusion

In the present work we compared brain transcriptomes of *C. pusilla* at two time points of the winter period: after breeding, and a long-distance uninterrupted autumnal flight across the Atlantic Ocean, and before breeding, at the end of winter period, before the spring migratory flight back to the reproductive site (Frias-Soler et al., 2020; Frias-Soler et al., 2018). We quantified differentially expressed genes of recently arrived and premigratory individuals and used information from genomic studies of other species to speculate about the functional significance of this differential expression. The underlying assumption was that genomic functional regions are conserved in birds and mammals through evolution (Colquitt, 2022; Guigo & de Hoon, 2018). Although this is a limited way to interpret the results (Colquitt, 2022; Prather, Okanoya, & Bolhuis, 2017), it is reasonable to use this approach as a first step towards a detailed molecular-based guide for formulating and testing new hypotheses. Because functional genomic analysis referred in the present report is based on different species and the whole genome of *C. pusilla* is not available, a deeper understanding of the biology of its transcriptome and functional implications are necessary.

5 Methods

5.1 Bird sampling and ethics recommendations for the use of animals in research

We used a total of eight *C. pusilla* individuals all collected in Otelina Island (0°45′42.57″S and 46°55′51.86″W), four of which collected during the period between September and October (Recently arrived birds) and other four between April and May (Premigratory birds). Birds were captured under license Nº 44551-2 from the Chico Mendes Institute for the conservation of Biodiversity (ICMBio). All procedures were carried out in accordance with the Association for the Study of Animal Behavior/Animal Behavior Society Guidelines for the Use of Animals in Research. All efforts were made to minimize the number of animals used, stress, and discomfort.

5.2 Transcardiac perfusion with RNA Later and RNA-Seq Sequencing

The birds were transcardially perfused with saline solution followed by RNA Later® Solution, the brains were removed from the skull and stored at -20°C prior to sequencing. Telencephalic tissues were homogenized for extraction and sequencing. The total RNA was extracted according to the manufacturer’s suggested protocol for isolating RNA from tissues and the mRNA was isolated and purified using the Dynabeads™ mRNA DIRECT™ Micro Purification kit (Thermo Fisher Scientific) for the conversion of RNA into cDNA we used the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific). The template preparation was performed into Ion One Touch 2 Instrument with the OT2 200 kit (Life Technologies). The fragments sequencing was performed into Ion PI chip v2 through Ion Proton System.
Instrument. Eight single ends read FASTQ files were generated that reference the eight biological replicates (four individuals from the RA group and four from the PM group).

5.2.1 Filtering, Trimming and Transcriptome Assembly

To verify the quality of the sequenced transcriptome we used the software FastQC 0.18 (FastQC, 2016) and for clean up the low quality reads we used the Trimmomatic 0.36 (Bolger, Lohse, & Usadel, 2014).

5.2.2 De novo assembly, differential expression discovery and functional annotation

To perform the De novo assembly we used the Trinity 2.11 (Grabherr et al., 2011) according to the default parameters and the Salmon v1.0 software to quantify the transcript expressions (Patro, Duggal, Love, Irizarry, & Kingsford, 2017). We used the EdgeR v3.38.4 package to run the differential expression analysis (Robinson, McCarthy, & Smyth, 2010) and Blast2GO (Conesa et al., 2005) to identify differentially expressed transcripts through several Blast+ (Altschul, Gish, Miller, Myers, & Lipman, 1990) searches. In attempt to identify the maximum number of valid hits we blasted our differentially expressed transcripts to Uniprot SwissProt and TrEMBL (UniProt Consortium, 2018), NCBI non redundant proteins and nucleotides (nr and nt) (Pruitt, Tatusova, & Maglott, 2007), RefSeq Protein and RNA (Pruitt et al., 2007), Uniref (Suzek, Huang, McGarvey, Mazumder, & Wu, 2007) and GenPept (Sayers et al., 2019). After Blast+ phase we ran the Blast2GO functional annotation protocol according to user manual and default parameters (Conesa et al., 2005; Götz et al., 2008). We normalized data for total read count and gene length expressed as transcript per million (TPM) and for cross samples normalization we used Trimmed Mean of M values (TMM).

Abbreviations

RA = Recent Arrived Birds
PM = Premigratory
DEGs = Differentially Expressed Genes
FDR = False Discovery Rate
Log2FC = Log2 Fold Change
GO = Gene Ontology
CC = Cellular Component
MF = Molecular Function
BP = Biological Process
IDs = Identifications

TPM = Transcript Per Million

TMM = Trimmed Mean of M values

Declarations

Ethics approval and consent to participate

Birds were captured under license Nº 44551-2 from the Chico Mendes Institute for the conservation of Biodiversity (ICMBio). All procedures were carried out in accordance with the Association for the Study of Animal Behavior/Animal Behavior Society Guidelines for the Use of Animals in Research. All efforts were made to minimize the number of animals used, stress, and discomfort. The use of animals in this work was approved by Prof. Barbarella de Matos Macchi Coordinator of the Ethics Committee on the Use of Animals at the Federal University of Pará, Brazil.

Consent for publication

Not applicable

Availability of data and materials

Authors declare that under request, all qualitative and quantitative data will be shared and genetic information such as raw RNASeq data will be deposited in the NCBI SRA after publication.

Below the reviewer link, notice that the public release of the data will be done after publication


Competing interests

Not applicable

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Authors' contributions

All listed authors contributed substantially to the conception or design of the work; the acquisition, analysis, or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; and/or final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Authors' information (optional)

Not applicable

References


Figures

Read length distribution

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<tr>
<td>Total reads (raw data)</td>
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<td>Recently arrived</td>
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<td>Pre-migratory</td>
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Figure 1
Descriptive data of *C. pusilla* brain transcriptome of recently arrived and pre-migratory wintering birds. Frequency distribution of high-quality reads is expressed as base pairs length (BP).

**Recently arrived vs Premigratory**
Differential gene expression
Down-regulated = 259
Up-regulated = 357

![Volcano plot of differentially expressed genes between the pairwise comparison of premigratory and recently arrived groups.](image)

**Figure 2**
Volcano plot of differentially expressed genes between the pairwise comparison of premigratory and recently arrived groups. The vertical axis shows -log10 adjusted p value (FDR logarithmized significant p values) and the horizontal axis the Log2Fold Change (change in the gene expression level). Since the FDR values were transformed to their -log10, the higher the position of a point, the more significant its value is (y axis). Points with positive fold change values (to the right) are Up-regulated and points with negative fold change values (to the left) are down-regulated (x axis) genes. The vertical line at log 2 ratio = 0 indicates fold change = 1. Thresholds were based on cutoffs as adjusted P-values below 0.01 and log fold-changes above 1. In this plot each dot (regardless of color) represents a gene, and the color of each dot defines its significance in relation to the levels set in the graph. Gray dots represent genes that did not show differential expression (not significant), and blue dots are significant only for Log2FC >1, whereas red and green dots above the horizontal dotted line are significant for both Log2FC and FDR and highlight...
genes with FDR <0.01 and fold changes above 4. Eleven of the greater top 20 differentially expressed genes are identified indicating between brackets, normalized correspondent values between samples comparisons for recently arrived (RA) and premigratory (PM) groups. They are expressed as TMM (Trimmed Mean of M-values; M=log₂RA/PM). GABBR2 = Gamma-Aminobutyric Acid Type B Receptor Subunit 2; NXPE3 = Neuroexophilin and PC-Esterase domain family member 3; FGF9 = Fibroblast Growth Factor 9; TRDMT1 = TRNA Aspartic Acid Methyltransferase 1; BCAT1 = Branched Chain Amino Acid Transaminase 1; ARHGEF9 = Cdc42 Guanine Nucleotide Exchange Factor 9; MVB12B = Multivesicular Body Subunit 12B; BICRAL = BICRA like Chromatin Remodeling Complex Associated Protein; PLXNA1 = Plexin A1.
Hierarchical cluster analysis of the differential gene expressions (DEGs) and transcriptome RNA sequencing heat maps of these genes in the brains of recently arrived (RC) and pre-migratory (PM) *Calidris pusilla* (adjusted P-cutoff of 0.01 for classification as differentially expressed). Top: Dendrogram of the distribution of individuals according to differentially expressed genes in the brain of RC (red bar) and PM (blue bar) (same data set in figure 2). The detailed dendrogram on the left identifies Up-regulated
(green) and down-regulated (red) genes in each biological replicate of distinct RC and PM individuals. The brighter the color the more differentially expressed a gene is. Colored bars under the left dendrogram (light green and blue) indicate the two groups of genes with contrasting expression patterns (see Figure 4). Bottom: individual identifications on subclusters 1 and 2. GABBR2 = Gamma-Aminobutyric Acid Type B Receptor Subunit 2; NXPE3 = Neuroexophilin and PC-Esterase domain family member 3; FGF9 = Fibroblast Growth Factor 9; TRDMT1 = TRNA Aspartic Acid Methyltransferase 1; BCAT1 = Branched Chain Amino Acid Transaminase 1; ARHGEF9 = Cdc42 Guanine Nucleotide Exchange Factor 9; MVB12B = Multivesicular Body Subunit 12B; BICRAL = BICRA like Chromatin Remodeling Complex Associated Protein; PLXNA1 = Plexin A1.

**Figure 4**

Differential gene expression on sub-clusters 1 and 2 indicated in Figure 3. The expression in recently arrived (red) and pre-migratory (blue) individuals is expressed in Y axis as TMM (Trimmed Mean of M-values; M=log2RA/PM); for detailed explanation see (Zhao et al., 2021).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1d1.pdf
- FileS1.xlsx