

RESEARCH ARTICLE

Seasonal changes in the song control nuclei of the Rufous-bellied Thrush, *Turdus rufiventris* (Oscine, Passeriformes, and Turdidae)

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Abstract

In vocal learning birds, memorization and song production rely on a set of telencephalic nuclei referred to as the song control system. Seasonal changes in song production are correlated with changes in the volume of the song control nuclei and are influenced by photoperiodic conditions and hormonal cues. The seasonal volume changes in the avian brain that controls singing are thought to involve regulation of neuronal replacement, which is a striking example of neuronal plasticity. The Rufous-bellied Thrush (*Turdus rufiventris*) is a seasonally breeding bird that actively sings during the spring and summer (breeding season) and is relatively silent in the fall, yet possible mechanisms behind the periodic changes in song production remain unknown. Here, we have examined two song control nuclei: High vocal center (HVC) and robust nucleus of arcopallium (RA) in fall males, spring males, and fall females of Rufous-bellied Thrush. The cytoarchitectonic organization was analyzed and quantified from Nissl-stained sections, and gene expression of song nuclei markers was examined by in situ hybridization during breeding and nonbreeding seasons. We observed a reduction in HVC volume and reductions in parvalbumin, and RGS4 expression in HVC and RA in males during the nonbreeding season. These findings provide evidence of seasonal changes in the song system of a representative tropical-breeding Turdidae species that does not maintain territories or mate bonding, setting the histological and molecular groundwork for future studies aimed at better understanding of song nuclei changes in seasonally breeding songbirds.

KEYWORDS

bird, brain, in situ hybridization, PVALB, thrush, vocal learning

1 | INTRODUCTION

Vocal learning is a trait present in three groups of birds: Hummingbirds, parrots, and songbirds. Vocal learning and song production rely on a set of specialized brain nuclei known as the song

control system, which shares important similarities with vocal control systems in humans. Two main pathways connect the song nuclei and control vocal learning and production in vocal learning birds: The posterior and anterior pathways. The posterior vocal motor pathway is involved in song production and includes two song nuclei: HVC and

robust nucleus of arcopallium (RA; Figure 1). The anterior pathway, implicated in song learning and plasticity, starts with a projection from HVC to striatal area X and also includes the lateral part of the magnocellular nucleus of the nidopallium (Figure 1; Bolhuis, Okanoya, & Scharff, 2010; Bottjer, Brady, & Walsh, 1998; Doupe, 1993; Nottebohm, Stokes, & Leonard, 1976).

Seasonal reproductive behaviors are associated with photoperiodic changes and are triggered by increases in circulating sex steroids such as testosterone (Brenowitz, Nalls, Wingfield, & Kroodsma, 1991; Buttemer, Astheimer, & Wingfield, 1991; Tsutsui, Wingfield, & Bottjer, 1991). Day length is the primary cue for the onset of the breeding season, and its influence on testosterone levels correlates with increases in song nuclei volume and singing behavior (Bernard, Wilson & Ball, 1997; Boseret, Carere, Ball, & Balthazart, 2006; Riters, Eens, Pinxten, & Ball, 2002; Sartor, Balthazart, & Ball, 2005; Small, Brenowitz, Wojtenek, & Moore, 2015; Smith, Brenowitz, Beecher, & Wingfield, 1997; Wennstrom, Reeves, & Brenowitz, 2001). Increases in the size of HVC have been observed during the breeding season in several songbird species but the mechanisms underlying this change are unclear. Although increased plasma levels of testosterone are tightly associated with increased HVC volume (Nottebohm, 1981), this correlation has not always been observed. For example, castrated males of *Spizella arborea* show increased HVC and RA volumes in a conditioned long-day environment, despite the lack of high serum levels of testosterone (Bernard & Ball, 1997). Further studies of seasonal changes of vocal nuclei in seasonal breeders where the photoperiod is less dramatic would be helpful to further clarify these relationships.

The Rufous-bellied Thrush is a tropical thrush and a seasonal singing songbird found in South America. In Brazil, its breeding is generally from August to January (spring and summer). During the breeding season, males defend territories and sing loud, continuous, and varied songs. These songs are substitute by few descending notes used in nonbreeding territorial defense (Sick, Haffer, Alvaranga, Pacheco, & Barruel, 1997). Its melodious and complex songs are characteristic of the spring season and accompany changes in reproductive behavior. The seasonal control of singing in this species has not yet been studied, thus the mechanisms that govern its singing behavior are largely unknown. Most of the endocrine studies on the relationship between tropical species reveal that they have relatively low levels of circulating steroid hormones, yet are able to show seasonality and will increase aggression if confronted by a conspecific male (Hau, Wikelski, Soma, & Wingfield, 2000). Although we still do not know the hormone profiles of Rufous-bellied Thrush, it represents a possible comparison where there are a number of other closely related species that occur in both the tropics and temperate latitudes. Tropical species, including the species of the present study exhibit similar patterns of seasonality and aggressive behaviors, thus Rufous-bellied Thrush is a very suitable model to understand seasonality in the hormonal status, singing behavior, and neurobiology of a tropical bird.

Here, we used histology and in situ hybridization to examine the song nuclei of wild-captured Rufous-bellied Thrushes during

the breeding and nonbreeding seasons. Our main focus was the HVC, because this nucleus connects the anterior and posterior pathways and is known to undergo major seasonal morphological changes, based on the studies in other seasonal breeders (Brenowitz & Larson, 2015; Brenowitz et al., 1991; Nottebohm et al., 1976; Wingfield, Hegner, & Lewis, 1992). We found that HVC volume changes substantially between the breeding (spring and summer) and nonbreeding seasons (fall and winter), and is accompanied by changes in the expression of the markers parvalbumin (PVALB) and the regulator of G-protein signaling 4 (RGS4).

2 | MATERIALS AND METHODS

2.1 | Animals and tissue collection

A total of 10 specimens of *Turdus rufiventris* were captured at the Instituto de Pesquisas Veterinárias Desidério Finamor (30°03' S and 51°18' W), in the Rio Grande do Sul-Brazil. Males and females were collected in two seasons: Fall (June and July, nonbreeding season) and spring (October, breeding season). Birds were killed by decapitation and the brains were immediately dissected out and flash frozen in Tissue-Tek OCT compound (Sakura, Alphen aan den Rijn, Netherlands) on dry ice and 100% isopropanol. The brains were stored at -80°C and later transferred to the Universidade Federal do Pará (UFPA) on dry ice. This study was approved by the Ethics Committee at the Instituto de Ciências Biológicas-UFPA and performed in accordance with the special license 20902-1 issued to MPDS by IBAMA/SISBIO.

2.2 | Riboprobe synthesis

The complementary DNA (cDNA) clones for PVALB and for the RGS4 were from the zebra finch brain cDNA ESTIMA collection, cloned in psBluescript II KS plasmids. Polymerase chain reaction (PCR) amplification was performed using the forward and reverse primers with a T3 overhang (reverse: 5'-GTA AAA CGA CGG CCA GTG AG-3' and forward: 5'-ATG ACC ATG ATT ACG CCA AG-3') to amplify a fragment containing the cDNA insert and T3 polymerase promoter using the vector as the template. PCR was performed in 50 µl reaction volumes containing 39.2 µl of RNase-free water, 1.5 µl of 10 mM MgCl₂, 5 µl of 10X buffer, 1 µl of each primer (0.5 µM), 1 µl of dNTP mix (10 mM), 0.3 µl of Taq DNA polymerase, and 1 µl of DNA template. The temperature profile consisted of preheating at 94°C for 3 min, 32 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, followed by a final extension step at 72°C for 10 min. Then, antisense riboprobes were synthesized using T3 RNA polymerase (Lide Technologies, Carlsbad, CA) and DIG-labeling mix (Roche Applied Science, Mannheim, Germany). The riboprobe reaction (Life Technologies) was performed in 20 µl reaction volumes containing 0.5 µl of RNase inhibitor, 2 µl of dithiothreitol, 2 µl of digoxigenin (DIG), 2 µl of 10X reaction buffer, 5 µl of the template, 2 µl of T3 enzyme mix, and 6.5 µl of nuclease-free water.

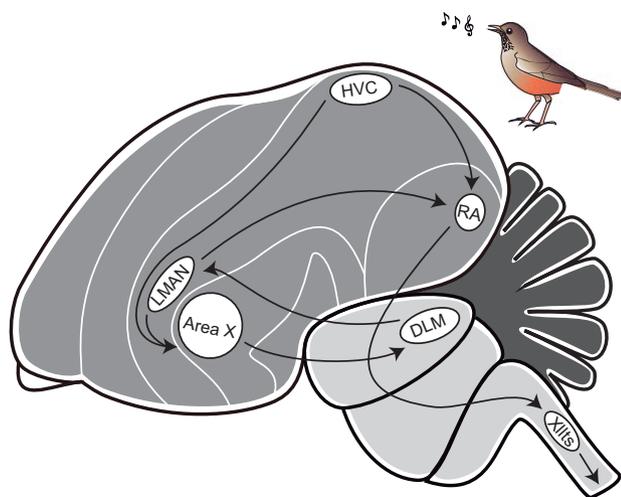


FIGURE 1 The song control system of the Rufous-bellied Thrush, *Turdus rufiventris*. Schematic representation of the song control pathways. DLM: Dorsolateral Medial Nucleus; HVC: high vocal center; LMAN: lateral magnocellular nucleus of the nidopallium; RA: robust nucleus of arcopallium [Color figure can be viewed at wileyonlinelibrary.com]

2.3 | Brain sectioning and Nissl staining

The brains were cut (20 μ m) on the parasagittal plane on a Leica CM1850 UV cryostat (Nussloch, Germany). Sections were mounted onto color frost plus microscope slides (Fisher Scientific, Waltham, MA), fixed in 3% paraformaldehyde and stored at -80°C until further use. Selected serial sections were stained in an aqueous solution of 0.1% cresyl violet. After staining, the slides were dehydrated in absolute ethanol for 2 min and cleared with two washes in xylene for 2 min each. The sections were then cover-slipped using Cytoseal XYL (Richard-Allan Scientific, San Diego, CA) mounting medium.

2.4 | In situ hybridization

In situ hybridization was performed according to a previously established protocol, with modifications (Carleton et al., 2014; Lovell, Carleton, & Mello, 2013). Serial brain sections from three fall males, four spring males, and three fall females were acetylated for 10 min in a solution of 0.33% acetic anhydride in water, rinsed twice with 2X Sodium Chloride-Sodium Phosphate-EDTA (SSPE) buffer (0.02 M ethylenediaminetetraacetic acid and 2.98 M NaCl in 0.2 M phosphate buffer) and dehydrated in 70, 95, and 100% RNase-free ethanol. Each section was then hybridized with a solution (16 μ l) containing 50% formamide, 2X SSPE (Sodium phosphate, NaCl and EDTA) 1 μ g/ μ l bovine serum albumin (BSA), 1 μ g/ μ l poly-A (SIGMA, Worli, Mumbai) in diethylpyrocarbonate-treated water, and 1 μ l of DIG-labeled riboprobe. Slides were cover-slipped, sealed by immersion in mineral oil, and incubated overnight at 62°C . On the following day oil was removed with chloroform and slides were decoverslipped in 2X SSPE, followed by incubation for 1 hr and 10 min at 62°C in 2X SSPE containing 50% formamide, and then twice in 0.1X SSPE for 30 min at 62°C . Sections were then rinsed briefly in Tris, NaCl + Triton-X 100 (TNT)

(TN: 100 mM Tris, pH 7.5; 150 mM NaCl/TNT: TN + 0.3% Triton-X 100), demarcated using a pap pen (Life Technologies) and blocked for 30 min at room temperature in 200 μ l of Tris-NaCl-blocking buffer (TNB; 0.1 M Tris, 150 mM NaCl, and BSA), blocking buffer (200 μ l TNB/slide, 1% skim milk), and incubated overnight in TNB with an alkaline phosphatase conjugated anti-DIG antibody (TNB + 0.3% skim milk+anti-DIG-AP; 1:600 dil.; Roche). Then, the slides were washed twice for 15 min in Tris-MgCl₂-NaCl buffer (TMN; 100 mM Tris, pH 9.5; 150 mM NaCl, 0.05 M MgCl₂), and incubated for 1–3 days in a detection solution containing the alkaline phosphatase substrates nitro blue tetrazolium chloride (Roche) and 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt (Roche) in a TMN solution with 1:1 chromogenic proportion. Slides were washed overnight in distilled water to remove salts, fixed in 4% paraformaldehyde, washed twice in distilled water, and cover-slipped with Cytoseal (Thermo Fisher Scientific). Slides were imaged using a Nikon SMZ1500 microscope and processed on NIS-elements D program (v. 4.10.01; Nikon). For qualitative assessment of messenger RNA (mRNA) expression in brain tissue, we compared the neuroanatomical expression of these two selected genes with data retrieved from the ZEBRA database (www.zebrafinchatlas.org).

2.5 | Statistical analysis and quantification of HVC

The area of HVC from three nonbreeding and three breeding males was estimated using ImageJ. The border of HVC was outlined in each Nissl-stained section containing HVC (viewed at $\times 40$ magnification). The total volume of HVC was estimated by multiplying the outlined HVC area in one brain section by the interval between measured sections (400 μ m), and then adding all partial volumes, as in Olson, Rodrigues, Jeong, Prael, and Mello (2011). Student's *t* test (unpaired, parametric, and two-tailed test) was performed using the GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla, CA, www.graphpad.com. Statistical significance was set at an α of 0.05 (Tables S1 and S2).

2.6 | HVC reconstruction

Images were captured with NIKON-DS Ri1 and NIS-elements program, and ImageJ (NIH image program) was used to analyze the images. Image files in tag image file format were opened in the stack mode and were scaled based on the scale bar in each image (210 px = 0.25 mm). HVC was outlined in each section and the area was measured. HVC volume was estimated by calculating the area of each section and multiplying by the distance to the next section. Adobe Illustrator was used to generate serial reconstructions of HVC. Scale bars represent 0.25 mm.

3 | RESULTS

3.1 | HVC reconstruction reveals seasonal changes in the volume

The nuclei comprising the song control system of the Rufous-bellied Thrush show conserved position and organization relative to that

seen in canary and zebra finch atlases. To examine seasonal changes in HVC volume, we performed Nissl staining on brain sections of females and males during nonbreeding and breeding seasons. Females showed a discrete HVC, consisting of a small nucleus at the expected position in the dorsal-caudal nidopallium, displaying the large cells characteristic of neurons in this nucleus. The HVC was significantly larger in males during the breeding season compared with the nonbreeding season (Figure 2). Whole-HVC volumetric reconstructions corroborate this trend with a p value of 0.0125 (Tables S1 and S2). In summary, the Rufous-bellied Thrush shows strong seasonal plasticity of HVC, a forebrain structure associated with seasonal singing behaviors in temperate species.

3.2 | PVALB and RGS4 expression in the HVC and RA

The reduced volume of HVC in males during the nonbreeding season that was observed by Nissl staining led us to investigate the expression pattern of PVALB and RGS4, two genes that are robust markers of several song nuclei in other songbird species, particularly of HVC in zebra finches (Lovell, Clayton, Replogle, & Mello, 2008; Mello, 2004; Mello, Nottebohm, & Clayton, 1995). These confirmed that the molecular markers are allowed for a detailed examination of this nucleus in serial sections, thus providing a strong evidence to identify these brains structures in this thrush. In females, the expression for both markers was very low or undetected (Figure 3a,d). In males during the breeding season, the increase in HVC volume is accompanied by an increased expression of these two markers within the HVC area (Figure 3b,c,e, and f).

We also examined RGS4 and PVALB expression in RA nucleus, where we observed less cells expressing these markers from the nonbreeding season (Figure 4b,e), and more cells are expressing RGS4 and PVALB in males during breeding season (Figure 4c,f). Cells expressing these genes were not observed in females (Figure 4a,d). Despite the apparent seasonal difference in gene expression in males, the cross-sectional area of the RA was very similar between males collected during the breeding and nonbreeding seasons.

4 | DISCUSSION

In this study, we report the cytoarchitectonic organization of the song control system of a tropical new-world *Turdus* thrush. Whereas this species is representative of low-latitude neotropical forest ecosystems (Sick et al., 1997), it is also a member of a large genus with a cosmopolitan distribution, including thrush species that breed in arctic ecosystems as well as those that are island endemics (Gill & Donsker, 2018). The reproductive biology of this species, and perhaps the vocal neuroanatomy, is potentially influenced by selective forces characteristic of tropical ecosystems, yet it is a migratory thrush within the tropics in a genus with global representation. In addition, to represent the first brain examination of this exceptional singer, it adds to the few studies that examine the song system or brain of a member of the Turdidae family (de Lima et al., 2015; Iwaniuk et al., 2006). Our Nissl-stained sections demonstrated seasonal plasticity of song nucleus HVC, namely a significant decline in volume in males from the breeding to the nonbreeding season. HVC nucleus reconstruction analysis provided further confirmation of difference in volume between males in both the seasons. The seasonal and sex differences in song circuit neuroanatomy are consentient with patterns of seasonal and sex-specific singing behavior.

Previous studies have linked increased HVC volume during the breeding season to high levels of testosterone in the blood (Nottebohm, 1981), and indeed many populations of songbirds have been shown to have vocal nuclei associated with seasonal fluctuations in steroid levels. However tropical songbirds generally lack the surges in circulating testosterone that is associated with the reproductive behaviors such as territory maintenance (Hau et al., 2000; Hau, Stoddard, & Soma, 2004), or the surges are disassociated from the onset of the song. One putative mechanism to stimulate increases in vocal nuclei of equatorial species is to increase the androgen sensitivity of cells within the vocal nuclei by the upregulation of androgen receptors (Quispe et al., 2016), thus allowing for the development and maintenance of song year-round, the dissociation of singing behavior from territoriality, and possibly even between sexes, allowing both males and females to sing. Furthermore, the relative contributions of hormonal signal versus tissue-level sensitivity may vary greatly among species, and

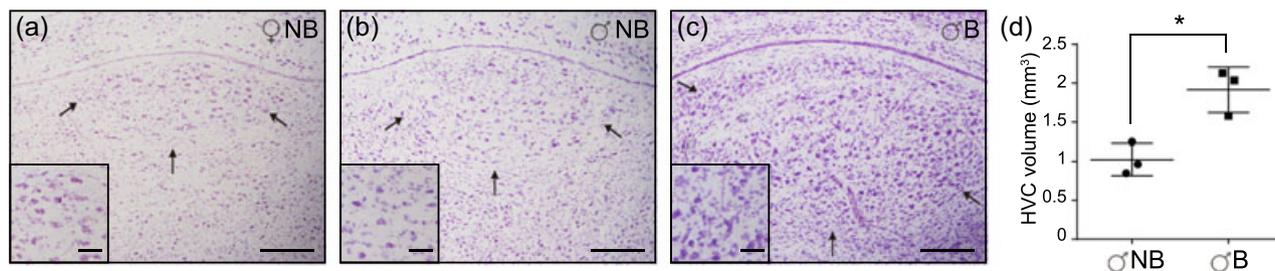


FIGURE 2 The song control nucleus HVC of *Turdus rufiventris*. Nissl-stained brain sections of *T. rufiventris* showing the HVC nuclei of female (a), and male during nonbreeding (b) and breeding (c) seasons. The average volumes of the HVC were compared between nonbreeding and breeding males. A single asterisk denotes a significant difference (p value of 0.0125) between designated groups. Error bars represent the standard error of the mean (d). Arrows denote the HVC nuclei. Scale bars are 0.2 mm (a–c) and 0.05 mm on close-up squares at the bottom left of each image. HVC: high vocal center; NB: nonbreeding [Color figure can be viewed at wileyonlinelibrary.com]

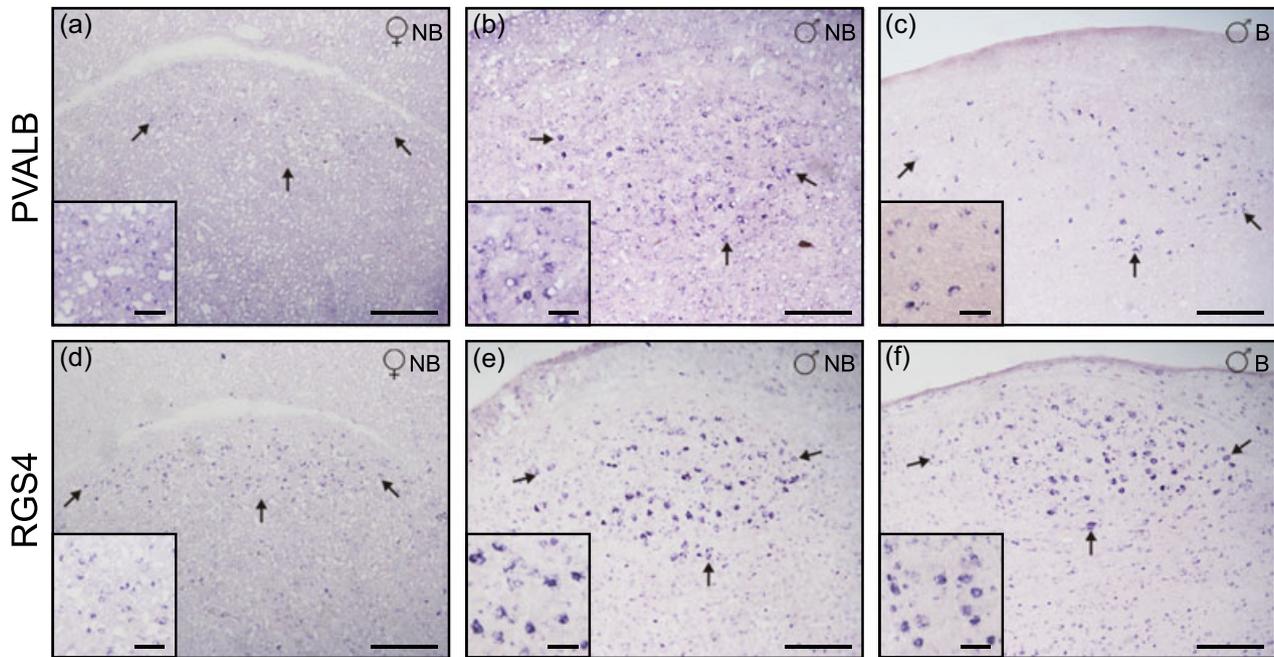


FIGURE 3 Expression pattern of the HVC song nuclei markers PVALB and RGS4. HVC of female (a,d), and males in nonbreeding (b,e) and breeding (c,f) seasons. Arrows highlight the HVC nuclei. Scale bars are 0.25 mm (a–f) and 0.05 mm on close-up squares at the bottom left of each image. HVC: high vocal center; NB: nonbreeding; PVALB: parvalbumin; RGS4: regulator of G-protein signaling 4 [Color figure can be viewed at wileyonlinelibrary.com]

depend in part on other hormone-dependent behaviors that make up the species life-history strategy. Although we cannot directly infer the physiological mechanism underlying the difference in HVC volume we observed in the Rufous-bellied Thrush, its lack of territorial behavior in the nonbreeding season, and male-only singing behavior suggest that these observed changes in HVC

volume may be due to more increases in hormone signal rather than sensitivity, similar to what we know occurs in high-latitude songbirds. Furthermore, the small HVC volumes found in females, which do not sing, suggests that male sex hormones (*ergo* the signal) are directly responsible for the increased HVC volume in this species.

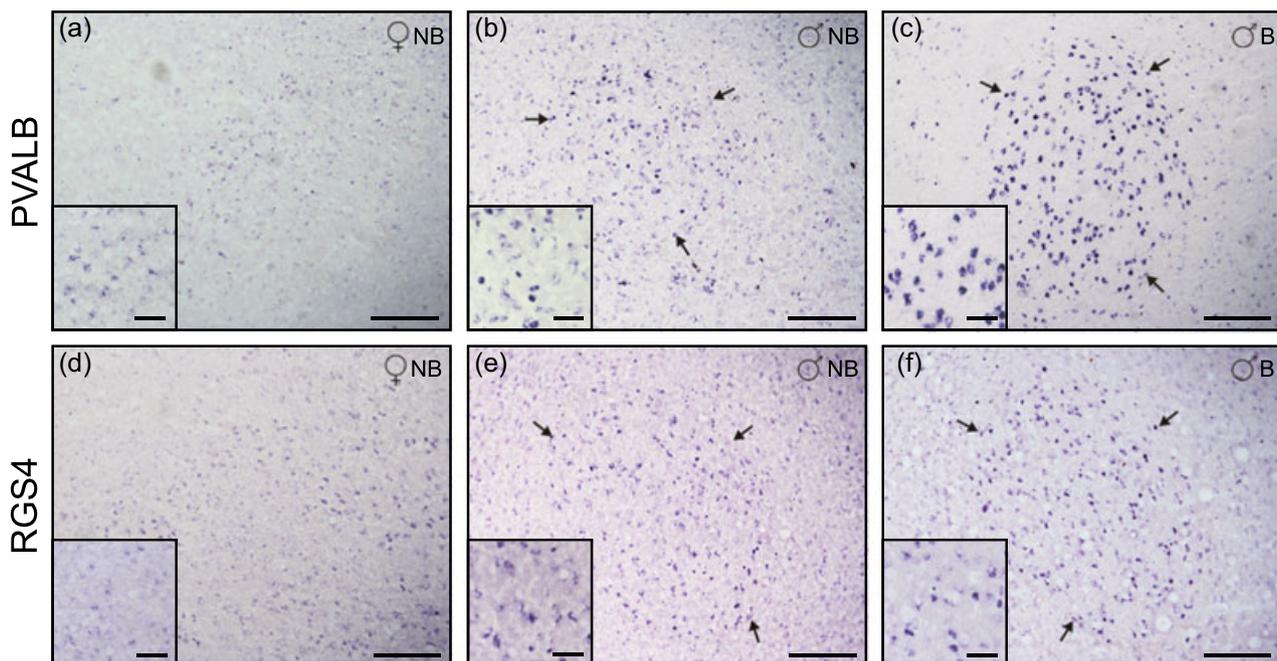


FIGURE 4 Expression pattern of the RA song nuclei markers PVALB and RGS4. RA nuclei within the arcopallium of female (a,d), and males in nonbreeding (b,e) and breeding (a,f) seasons. Arrows indicate the RA nuclei. Scale bars are 0.2 mm (a–c) and 0.05 mm on close-up squares at the bottom left of each image. NB: nonbreeding; PVALB: parvalbumin; RA: robust nucleus of arcopallium; RGS4: regulator of G-protein signaling 4 [Color figure can be viewed at wileyonlinelibrary.com]

Using *in situ* hybridization, we analyzed the expression of two song nuclei markers: RGS4 and PVALB. RGS4 is an intracellular regulator of G-protein signaling known to control the expression of NMDA-receptors in response to elevated glutamate signaling (Saugstad, Marino, Folk, Hepler, & Conn, 1998) and previously found to be expressed in the forebrain of songbirds with high enrichment in HVC and RA (Lovell et al., 2008). PVALB is also a marker of these song system nuclei (Braun, Scheich, Heizmann, & Hunziker, 1991) and plays a critical role in neuroprotection. PVALB is a marker of vocal circuitry in male but not in female zebra finches, which undergoes developmental increases during song learning (Olson et al., 2011) and its expression is positively related to the amount of singing that occur (Zengin-Toktas & Wooley, 2017). We found enriched mRNA expression of RGS4 and PVALB within the HVC and RA in breeding males compared with nonbreeding males, showing that the seasonal effects on the vocal circuit differ in a molecular-functional basis as well as in volume. Although we do not have singing data for the thrushes in this study, the reduction of PVALB during the nonbreeding season suggests a reduced neuroprotective capacity in the vocal circuitry, and reduced RGS4 suggests a reduced degree of glutamate signaling in the vocal circuit during the nonbreeding season associated with the less singing behavior. Similarly, females had low expression of these markers within HVC and RA.

5 | CONCLUSIONS

This study provides a seasonal study of the structural and molecular changes in song control nuclei in *T. rufiventris*, demonstrating significant plasticity of the brain nuclei in males between the breeding and nonbreeding seasons. More specifically, we have shown that gene expression in song nuclei changes between these periods, and two song nuclei, HVC and RA are significantly reduced during the nonbreeding season of this tropical species. We suggest that this reduction in vocal brain structures is related to the lack of territorial behavior and the increase in migratory or seminomadic behaviors during the nonbreeding season during which song is not exhibited.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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

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