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FEMALES INFLUENCE BRAIN EVOLUTION:
DANCE COMPLEXITY RELATED TO VOLUME OF A SENSORIMOTOR REGION IN MANAKINS

By
Wilson Helmhout

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
2016

Approved by

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ABSTRACT

WILSON HELMHOUT: Dance Complexity Related to Volume of a Sensorimotor Region in Manakins
(Under the Direction of Lainy Day)

Manakins are a family of birds the males of which use acrobatic, non-vocal display behaviors to attract females to mate. Across the manakin family (Pipridae), species perform displays of varying complexities with variation in the number and type of display sites, acrobatics, and number of mechanical sounds. Females of at least one species, select males on the basis of 10s of ms differences in performance of certain male display elements suggesting strong sexually selection. Additionally, recent studies show a positive relationship between display complexity and relative brain weight in manakins. This overall association of complexity with brain weight could be due to growth of many regions of the brain, concerted evolution, or individual brain regions may be driving this correlation, mosaic evolution. The arcopallium (AP), is a region likely to be specialized for display complexity. AP has both motor and limbic functions, and in oscines (songbirds), a specialized portion of the AP, the robust nucleus of the arcopallium (RA), is known to function in song production of vocal courtship displays. Manakins are suboscines that do not appear to have vocal learning or an RA. However, the AP has been shown to be larger in male golden-collared manakins which perform displays than in females that do not display. In addition, the AP in golden-collared manakins contains numerous androgen receptors similar to those found in the RA of songbirds, a trait not seen in other suboscines that do not have complex display. Thus, the AP in manakins is capable of responding to testosterone (T), and because display in manakins in known to be activated by T, the AP could play a role in the courtship behaviors of manakins. Another area, the Nucleus Taeniae (Tn) of the AP, could also be implicated in display complexity. Tn has been shown to have high concentrations of androgen receptors, and though considered part of AP, it is exclusively limbic and may have distinct function from AP. The volume of the nucleus rotundus (Rt), a visual thalamic nucleus, was used as the control since it was unlikely to be related to display complexity. We compared AP, Tn, and Rt volume of 12 different manakin species and the closely related ochre-bellied flycatcher; species were chosen for their varying display complexities. We found a significant positive relationship between AP volume and display complexity of the manakins' non-vocal courtship behaviors, but no relationship between Tn or Rt with display complexity.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

AR	Androgen Receptor
AP	Arcopallium
CE	Coefficient of Error
GLM	General Linear Model
Tn	Nucleus Taeniae
PGLS	Phylogenetic Generalized Least Squares
RA	Robust Nucleus of Arcopallium
Rt	Nucleus Rotundus

Manakins are a group of neotropical suboscine birds (family *Pipridae)*, non-songbird passerines. Lekking male manakin species are known to perform acrobatic, physical courtship displays used to attract females to mate. Females of several manakin species are known to select mates based on the qualities of particular display elements (Barske et al., 2011, Duval, 2007, Uy and Endler, 2004). Courtship display repertoires vary widely across the family pipridae and include many different non-vocal elements such as flips and jumps, mechanical sounds made with the wings and tail feathers, cooperative display between males, and specialized display arenas (Prum 1990, 1994, 1998, Lindsay et al., 2015). By comparing sexes within species or comparing a few behaviorally distinct manakin species, it has been possible to identify a number of morphological and physiological specializations for display characteristics including hypertrophied muscles (Schultz et al., 2001), bone (Fusani et al., 2014) and feather modifications (Fusani et al., 2014), specific patterns of steroid hormone regulation (Schlinger et al., 2013), specialized steroid receptor distribution (Schlinger et al., 2013), and sex-specific neural phenotypes (Day et al., 2011). Examination of a broad range of manakin species that vary greatly in display complexity is needed in order to determine if trait specializations studied in a few species show correlated evolution with increasing display complexity and the particular traits known to be highly developed among a few species.

Since each species has a different courtship display, to capture species variation in display complexity objectively we previously developed a manakin display complexity scoring system (Lindsay et al., 2015). These complexity scores are based on identifying the presence or absence of 40 distinct display elements, numbers of mechanical sounds produced, presence and level of cooperation among males, and intensity of arena gardening. For twelve species of manakins and a closely related flycatcher, we identified traits from previously published display descriptions (Bostwick and Prum, 2003; Castro-Astor et al., 2007; Chapman, 1935; Day et al., 2006; Duraes, 2009; Duval, 2007; Fusani et al., 2007a; Prum 1990, 1994; Rosselli et al., 2002; Skutch, 1949; Tello, 2001; Théry, 1990; Westcott and Smith, 1994) and gathered high-speed and high definition video to quantify display complexity further (Lindsay et al., 2015).

Our lab has found that display complexity among the thirteen species studied is positively associated with brain weight and brain volume; either absolute brain size or brain size relative to body weight depending on the methods used (Day & Lindsay, 2016, Gutierrez et al., 2016, Lindsay et al., 2015). The displays of manakins are physically complex sexual displays (Lindsay et al., 2015) that likely require specializations of several brain regions involved in motoric, endocrine, motivational, and cognitive aspects of the display. Previous research suggests enlargement of the hippocampus, in male compared to female *M. vitellinus* likely related to the need of males' to recall spatial relationships for their court perches (Cocoon et al., 2012, Day et al., 2011). Similar sexual dimorphism has occurred for the cerebellum, which governs motor planning and sequencing, and for the arcopallium, a sensorimotor and limbic region (Day et al., 2011). In addition, our lab has demonstrated that the sexual dimorphism seen in *M. vitellinus* is a generalized phenomenon in that we find correlated evolution of increased cerebellar volume with courtship display complexity in 12 species of manakin and a closely related flycatcher (Pano, 2015). Finding sexual dimorphic brain regions suggest these brain areas could be specialized for courtship display, and the recent finding of a positive association between cerebellar volume and display complexity demonstrates that at least one brain region related to motor planning and sequencing abilities has coevolved with complexity.

In the current study, we examine the relationship between arcopallium (AP) volume and display complexity in manakins. In addition to AP volume, we also study the relationship between nucleus taeniae (Tn), a sub nucleus of the AP, and display complexity. The arcopallium is involved in behaviors that may be relevant to courtship display, such as limbic type functions like emotion, memory (Reiner et al., 2004; Saint-Dizier et al., 2009; Yamamoto et al., 2005) and motivation (Charlier et al., 2005; Silva et al., 2009) as well as control of relevant motor behaviors such as song production and calling (Jarvis et al., 2006, Manley, Popper, & Fay, 2004). Tn is a portion of the arcopallial complex that is strictly limbic rather than motoric in nature (Reiner et al., 2004)

The AP is considered to be homologous to the mammalian amygdala due to its limbic functions, neural connectivity (Reiner et al., 2004; Yamamoto et al., 2005), and neurochemistry (Yamaoto et al., 2005). Like the mammalian amygdala, AP likely plays a role in sexual and social interactions (Charlier et al., 2005). AP functions in both nonsexual (Campanella et al., 2009; Silva et al., 2009) and sexual (Charlier et al., 2005) motivational behaviors. Lesions of AP alter ingestive behaviors of pigeons (Campanella et al., 2009; Silva et al., 2009). High expression of the immediate early genes, c-fos and ZENK, in AP of Japanese quail during copulation, shows that AP is activated during sexual behavior (Charlier et al., 2005). In oscines, there is a motor pathway descending from AP in which the intercollicular nucleus, receiving signal from AP, serves as an intermediary between auditory and motor pathways to aid in call production (Manley, Popper, and Fay, 2004). In owls, AP has been show to serve a motor function, helping to control their gaze fields (Reches and Gutfreund, 2008).

In oscine songbirds, one region of the AP, the robust nucleus of the arcopallium (RA), is particularly well known for its role in motor production of song within the song circuit. RA is crucial in the song pathway of oscine songbirds. RA innervates the vocal muscle, the syrinx via the X cranial nerve (Jarvis et al., 2006; Suthers et al., 1999). Many regions of the song-control pathway, unlike surrounding tissues, have high densities of steroid receptors, ostensibly due to the sexual nature of the courtship display (Brenowitz, 2013). In particular, RA is known to have a high density of androgen receptors (AR) (Jarvis et al., 2006). Because song regions, such as RA, are surrounded by areas activated by motor behavior, it has been proposed that the song system was co-opted from surrounding motor control regions (Jarvis et al., 2006). In addition, the volume of RA, is known to be larger in male songbirds with more complex vocal courtship displays (Zeng et al., 2007). The relationship of an arcopallial nucleus with vocal courtship display complexity in oscines is further reason to hypothesize that AP or a sub-nucleus of AP like Tn could be related to non-vocal courtship display complexity in sub-oscines like manakins.

Unlike RA in songbirds, no nucleus in manakin AP is an obviously distinct nucleus based on gross morphological features in nissl stained tissue (Day et al., 2011). In addition, no particular nucleus in AP has greater cell density or cell size in males than in females (Day et al., 2011). However, patterns of cell size and density in the subnuclei of AP and in Tn did vary between males and females suggesting some sexual dimorphic patterns of nuclei in AP could be related to male manakin display (Day et al., 2011). Like RA in oscines, the AP and Tn of *Manacus vittelinus* have high concentrations of androgen receptors compared to other sub-oscines (Fusani et al., 2014). In particular, Tn has a high density of androgen receptors when compared with the rest of AP. The display of manakins is known to be activated by androgens (Schlinger et al., 2013), and therefore, androgen receptors in the AP and Tn suggests that these regions, like RA in oscines, could be a target of androgen activation of display.

In addition to heightened expression of androgen receptors (Fusani et. al, 2014), Tn could be implicated in display complexity due to its limbic nature. Tn is comparable chemically and topologically to the mammalian amygdala (Yamamoto et. al, 2005) and it plays roles in nonsexual motivation (Campanella et. al, 2009; da Silva et. al, 2009). Furthermore, Tn is activated during pair bonding of zebra finches, demonstrating the potential for Tn in sexual and social behaviors (Svec et. al, 2009).

Thus, the AP and Tn appear to regulate behaviors associated with limbic and motor aspects of courtship in both oscines and non-oscines, to play a specialized hormone dependent role in manakin display, and appear to be a likely region for evolution to act upon to create a specialized display control region. Therefore, demonstrating that there is relationship between the volumes of AP or one specific nucleus in AP, Tn, of manakins and the complexity of display would support the idea that AP neuromorphology is specialize for control of acrobatic display in manakins. We tested this idea by examining the volume of the arcopallium, nucleus taeniae, and a control brain region in 12 species of manakin that vary in the complexity of non-vocal display and in the closely related ochre-bellied fly-catcher.

Methods

Collection of Manakins

We collected 37 birds of 12 species (2-3 individuals of each species) of manakins (*Pipridae)* and a closely related ochre-bellied flycatcher (*Mionectes oleagineus*) in Panama and Guyana using mist nets from June to August 2012 and January to June 2013. Sex and breeding status were confirmed before sacrifice in phenotypically monomorphic birds by collecting semen via cloacal massage. Birds were weighed to the nearest 0.1g using a Pesola spring balance.

Determining Display Complexity

The manakins have species-specific displays of varying complexity (Figure 1), and so it was necessary to quantify their display elements for comparison. The closely related ochre-bellied flycatcher was chosen as an out-group because it is a member of the family Tyrannidae, which is closely related to manakins and is a sub-oscine that performs a simple courtship display. Both the manakins and the ochre-bellied flycatcher employ a lek breeding strategy and both are frugivorous. Details of capture sites are given in Lindsay et al. 2015. Figure 1 shows a phylogeny of 12 manakin species and closely related ochre-bellied flycatcher with illustrations of display for each genus.

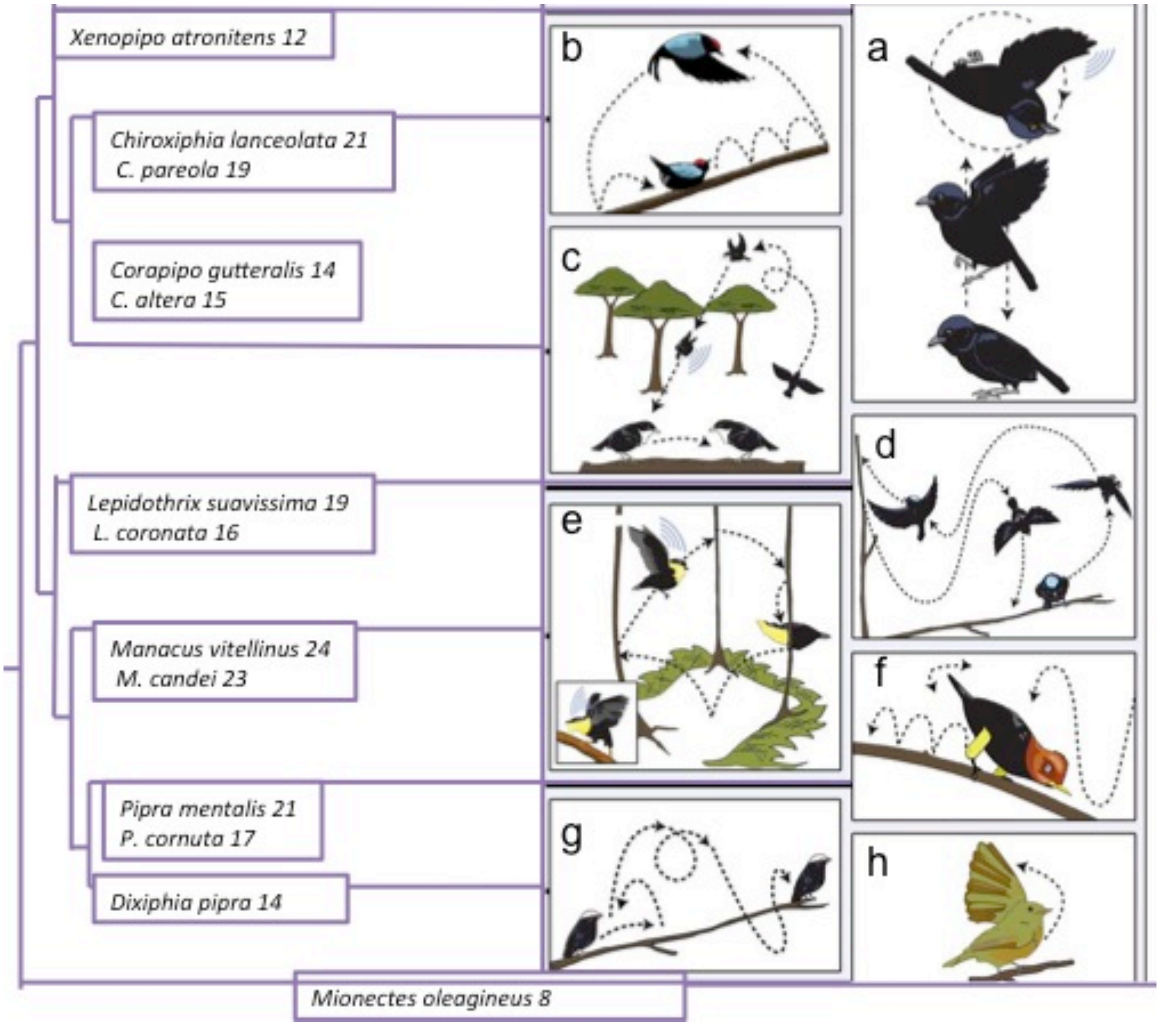


Figure 1: Only one illustration per genus was included because within each genus displays vary only slightly. Figure and figure legend are taken from Lindsay et al., 2015 with minor alterations. a. *X. atronitens* display includes a wingsnap and a backflip (Lindsay et al., 2015). b. *C. lanceolota* display includes two males cooperating in which one will flutter backwards over the other as he hops forward towards the female in a "cartwheel" motion taking turns in each role though only one male will mate with the on looking female (Duval et al., 2007, Prum, 1990). *C. Pareola* performs a very similar display (Prum, 1990). c. *C. altera* pictured demonstrate the male's flight above the canopy and subsequent plummeting down to his prepared display sight and doing a swift

"about face" (Prum, 1990, Roselli et al., 2002). *C. gutturalis* display, though similar, also includes exposure of white throat ruff and wing patches (Prum, 1990, Théry, 1990). d. *L. coronata* display includes "frenzied flights" and "butterfly flights" between perches and an aerial turn to face the opposite way or sometimes "bowing" or pivoting "about face" (Duraes, 2009, Lindsay et al., 2015). *L. suavissima* display, though similar, also includes a "slide down" on a vertical perch and wing sonations (Théry, 1990). e. *M. vitellinus* males flip or hop between perches and the ground of a prepared arena and also perform display elements such as wing snaps, "beard out" landings, slides, "roll snaps" and "grunts" with his wings (Chapman, 1935, Day et al., 2006, Fusani et al., 2007, Lindsay et al., 2015). *M. candei* performs a similar display. f. *P. mentalis* descends onto his perch in an "S" shape flight pattern, wiggles his tail, and takes tiny backwards jumps giving the illusion of a "moonwalk". Often he will rapidly turn around and moonwalk the other way (Prum, 1990, Skutch, 1949, Tello, 2001). *P. cornuta* performs a similar display except he uses small steps to "moonwalk" rather than hops (Lindsay et al., 2015, Tello, 2001). g. *D. pipra* display includes rapid jumps forward and backward on a perch and short between perch flights (Castro-Astor et al., 2007, Lindsay et al., 2015, Prum, 1990). h. *M. oleagineus* display is simply wing lifts of a single side plus "hops" or "flicks" between perches and occasional undulating flights like the "butterfly flights" of manakins (Westcott and Smith, 1994).

To score the discrete elements of the display complexities, we referenced previous documentation of manakin displays (Bostwick and Prum, 2003; Prum 1986, 1990, 1994, 1998) and used high-definition, high-speed cameras to record courtship behaviors outside of the standard movements associated with perching and flight (Lindsay et al., 2015). Our complexity score provides an objective measure of the physical complexity of manakin courtship display. In the 13 species we studied, 40 discrete display elements were identified. Each species was given one point given for each of these distinct elements such as butterfly flights, cartwheels, and hops (Prum, 1990). Birds with a greater variety of movements have higher complexity scores. Points were given for cooperation between males in the display; 1 point when displays are simultaneous but not synchronized, and 2 points for species where males coordinate their displays. The use of lek breeding strategy garnered 1 point. All of our species are lek breeders, but adding this into the system allows for comparison with non-lekking species in other research (Fuxjager, 2015). The courtship display site was scored for spatial complexity; 1 point for using one or more horizontal perches or a fallen log, 2 points for a loosely organized court with multiple horizontal perches without a cleared ground arena, and 3 points for a true court with a cleared ground arena. Finally, points were added for the number of distinct mechanical sounds produced (0-5 points) with an added point to the total score when the sound production required multiple pulses and for complexity of posture when the sound is produced; 1 point for perched sonations, 2 in-flight sonations, and 3 if the species used both production postures. Two individual researchers calculated display complexities separately, and inter-rater agreement was high ($r=0.899$; $p= <0.001$).

Table 1: Scoring of nonvocal courtship display in manakins

	n	Elements	Lekking	Mechanical	Cooperation	Arena	Complexity
Manacus vitellinus	3	10	1	9	1	3	24
Manacus candei	3	9	1	9	1	3	23
Pipra mentalis	6	8	1	10	1	1	21
Pipra cornuta	5	8	1	6	1	1	17
Chiroxiphia lanceolota	5	12	1	5	2	1	21
Chiroxiphia. pareola	5	9	1	6	2	1	19
Lepidothrix suavissima	3	9	1	5	2	2	19
Lepidothrix coronata	3	11	1	0	2	2	16
Corapipo altera	4	9	1	4	0	1	15
Corapipo gutturalis	3	8	1	4	0	1	14
Dixiphia pipra	5	12	1	0	0	1	14
Xenopipo atronitens	4	3	1	7	0	1	12
Mionectes oleagineus	4	7	1	0	0	0	8

Ethics Statement

All of the birds were collected in Guyana with approval from the Guyana Environmental Protection Agency. The birds were collected in Panama with approval of Smithsonian Tropical Research Institute IACUC and by the Autoridad Nacional del Ambiente and the Autoridad del Canal de Panamá. In Amerindian tribal lands, the collections were approved by the Guyana Ministry of Amerindian Affairs. The University of Mississippi IACUC approved all procedures. All species are common in the area in which they were collected and none are listed as endangered or threatened.

Tissue Preparation

Birds were overdosed with isoflurane and transcardially perfused with 30mls of 0.1M phosphate-buffered saline (PBS) followed by 40 mls of 10% neutral-buffered formalin (NBF) at 3mL/min. Perfused brains were weighed to the nearest 0.001g. A gas powered portable cooler was used to keep tissue and solutions cool in the field. Brains were placed in NBF for 24 hours to complete fixation of the tissue and then transferred to cold 20% (w/v) sucrose in phosphate-buffered saline (PBS) for cryoprotection until they sank. Brains were then transferred to PBS and kept cool from 1-4 days depending on timing of access to dry ice or -80 refrigeration. Brains were placed in a gel block (8% w/v gelatin and 16% w/v sucrose in RO‑H20, and the gel block was placed in NBF for 24 hours to harden in 30% w/v sucrose until it sank, and brains were finally frozen on dry ice and stored in a -80 freezer in the country of origin until transferred to a -80 °C freezer at the University of Mississippi.

Slide Preparation

Brains were cut sagittally in a cryostat at 30 µm, and every third slice was placed on slides for volumetric stereology. Tissues on slides were stained with cresyl violet, which stains nissl bodies allowing for nuclear and cytoplasmic visualization.

Measurements of Brain Region Volumes

To estimate the volume of selected brain regions using stereology, the area of the region on every third section on the slides (every ninth section, 270 μm apart) was measured using Axiovision (University of Mississippi) software (Carl Zeiss, Inc., Thornwood, N.Y., USA) via light microscopes (Zeiss) equipped with AxioCam digital cameras (Zeiss) and using Mouton stereology software to measure surface area with the Cavalieri point counting system with a grid of 1.00 in AP and 0.25 in Tn and Rt (Gundersen, 1999). The effectiveness of the sampling strategy was analyzed in a pilot study used to determine the amount of sampling required to obtain an acceptable coefficient of error. The coefficient of error (CE) is a prediction of stereological accuracy that accounts for the shape of the region of interest and the number of points measured within the region. For accurate stereological measurement, the stereology program takes into account the size of the region and the thickness of the tissue. The objective for determining boundaries was 1.25x, and the objective for determining depth through the z-axis was 100x while the phototube to the camera magnifies objectives by 10x resulting in a final magnification of 10.25 for areas and 1000x for depth of sections. Three regions were measured: the arcopallium, nucleus taeniae, and nucleus rotundus (Rt). It should be noted that the measurement for AP also includes Tn since it is a sub-nucleus of the AP, thus statistical analysis of AP volume must remove Tn volume to create independence of these variables. Rt is a visual thalamic nucleus that served as the control region as it is not expected to differ in size among manakins. Figure 2 shows AP boundaries used for stereology at three different points from when the region is first visible laterally until the region ends more medially. Figures 3 and 4 show the boundaries for TnA and Rt respectively, each at 3 different points from more lateral where they begin to more medial where they end. Acceptable coefficients of error in stereology are lower than 5-10%

(Marcos et al., 2012), and in this study, the coefficients of error for AP, TnA, and Rt respectively were 0.04, 0.06, 0.07.

Figure 2: Arcopallium Boundaries

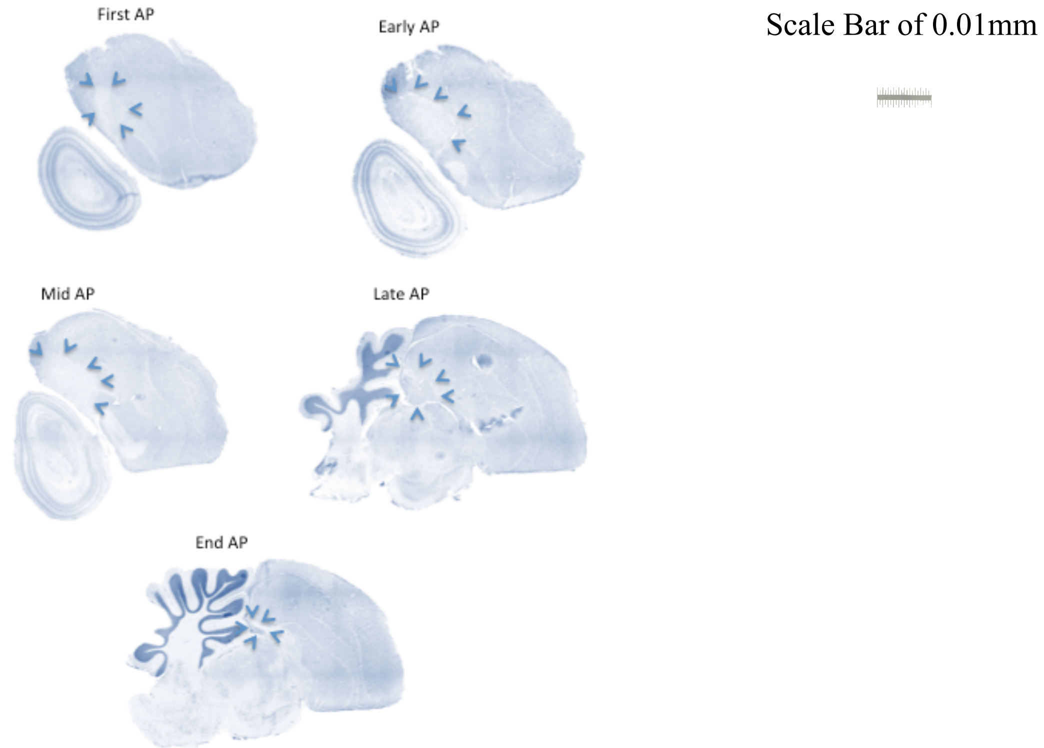


Figure 3: Nucleus Taeniae Boundaries

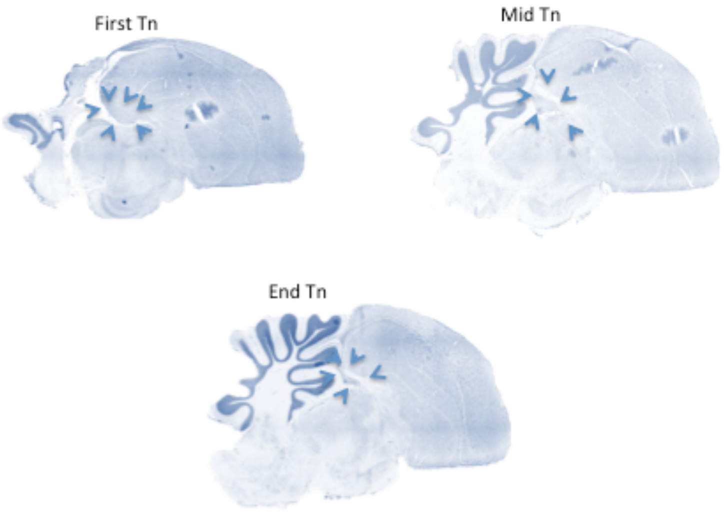
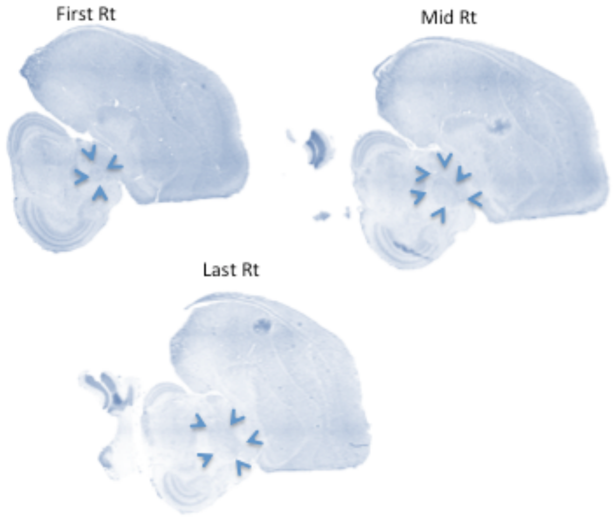


Figure 4: Nucleus Rotundus Boundaries



Statistical Analysis

Because birds vary in overall size, brain region volumes must be corrected for this allometric scaling of brain and body size of the bird. To determine what morphological measurements varied allometrically with each brain region we examined correlations between brain region volume and body size or brain size variables. In addition to considering which scaling variable is correlated with each brain region, we must also make sure that the independent variable (species) does not have a significant interaction with the chosen covariate. If there is an interaction, it implies some difficulty in untangling whether values obtained from a size correction process represent the region of interest or the covariate. We examined simple correlations of brain regions with areas that subsumed the area of interest or used a measure of body size. For example, prospective covariates for acropallium would include brain volume minus arcopallium volume (br.vol min AP,), tarsus (cm^3), and body weight (bod.wt) (grams). AP region was subtracted out from Br vol to create independence of variables. We used stepwise linear regressions to examine which variables accounted for the most variation in the brain region volume. We then ran a general linear model (GLM) with species as a fixed factor, brain region of interest as the dependent variable, and the area that accounted for the most variation in the stepwise linear regression as the covariate. We first ran the GLM with an interaction term between species and the covariate, if the interaction was not significant, we proceeded with the GLM without the interaction. If the interaction was significant, we choose another correlated morphological variable and tested interactions again until we found a covariate that was correlated with the brain region volume, was a logical correction factor for allometry, and did not have an interaction with the independent variable. The GLM adjust the region of interest for the covariate and calculates estimated marginal means that adjust for the allometric associations. The adjusted means from the GLM are then regressed on display complexity to determine if there is an association between the adjusted brain region volume and display complexity (see Table 2 for a summary of covariates used). AP was adjusted for brain volume minus AP and Tn to obtain results for just AP volume independent of Tn contributions to AP volume. Tn was measured relative to brain volume minus Tn volume as using AP minus Tn volume produced interactions of this covariate with species. By subtracting Tn from brain volume but not subtracting out the remainder of AP, we are examining Tn volume relative to all remaining brain structures. For Rt, we tried several covariates that all produce interactions with species. Since we know that brain volume and cerebellar volume differ between species (Lindsay et al., 2015, Pano, 2015) and because our results suggested AP volume also differed substantially between species, Rt was measured relative to brain volume minus AP, cerebellum, and Rt volumes (see Table 2).

For Ap, Tn and Rt, in addition to calculating marginal means from a general linear model (adjusted volumes), we also obtained mean residuals from a least squares regression analysis (region of interest regressed on covariate) to obtain residual region volumes. This type of "residuals analysis" is another method besides GLM that is commonly used to adjust for allometry. We performed both analyses (see Table 2) to ensure our results were robust to statistical analysis. However, residual analyses are known to be overly conservative (Darlington & Smulders, 2001), thus; we interpret our results in terms of the significance of the tests performed with marginal means. We include the residual results to show that similar values are obtained with both methods.

Since all species measured are closely related, it is necessary to correct for phylogenetic relatedness. To correct for a lack of phylogenetic independence, adjusted and residual volumes were regressed on complexity scores using phylogenetic generalized least squares (PGLS) for AP and Tn. PGLS allows for the maximal likelihood estimation of λ, the phylogenetic scaling parameter: a measure of the phylogenetic dependence of trait covariance. A λ of 1 indicates strong phylogenetic signal between and a λ of 0 indicates phylogenetic independence of trait (specifically evolution by Brownian motion or random walk). Note that a PGLS analysis has not yet been performed for Rt and the values given are for a simple linear regression for allometrically corrected Rt and complexity. See Table 2 in Results for statistical values.

Results

PGLS analysis showed that adjusted AP volume was positively associated with display complexity (see Table 2 for statistical tests). Neither adjusted Tn nor Rt volumes were related to display complexity (Table 2). We present results of analyses using both marginal means analyses and residuals analyses in Table 1 and Figures 5-8.

Table 2: Statistical values for allometric and phylogenetic correction

		Allometric correction GLM species effects			**Phylogenetic Generalized Least Squares species x complexity**			
Independent Variable (Ln)	**Allometric Factor (Ln)**	**R2**	**F(df)**	**p**	**R^2**	**t (df)**	**p**	**ML λ**
Arcopallium Marginal Means	Brain – AP -Tn	0.69	1.83 (12,24)	0.10	0.25	2.24 (12,24)	0.05	0
Arcopallium Residuals	Brain – AP -Tn	0.41	25.3 (1,36)	<0.001	0.18	1.91 (1,36)	0.08	0
Nucleus Taenia Marginal Means	Brain – Tn Vol	0.77	4.28 (12,24)	0.001	-0.02	0.89 (12,24)	0.39	0*
Nucleus Teania Residuals	Brain – Tn Vol	0.28	13.73 (1,36)	0.001	0.09	-0.06 (1,36)	0.96	0
Nucleus Rotundus Marginal Means	Brain–Cb-Ap-NR	0.50	0.85 (12,24)	0.60	0.01	0.38 (12,24)	0.73	NA
Nucleus Rotunds Residuals	Brain–Cb-Ap-NR	0.29	14.92 (1,36)	<0.001	-0.08	0.35 (1,26)	0.71	NA

*Maximum likelihood lambda did not differ significantly from 1 indicating phylogenetic independence of trait. For each brain region measured the brain region used to correct for allometry (allometric factor), the statistical values for the GLM, and the values for the PGLS are given. The "-" in the table signifies that the volumes of these regions were subtracted from the whole brain volume.

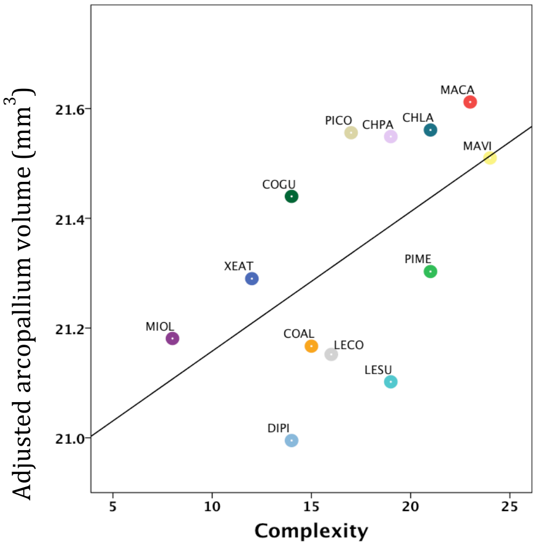


Figure 5: Marginal means analysis of adjusted arcopallium volume vs. display complexity

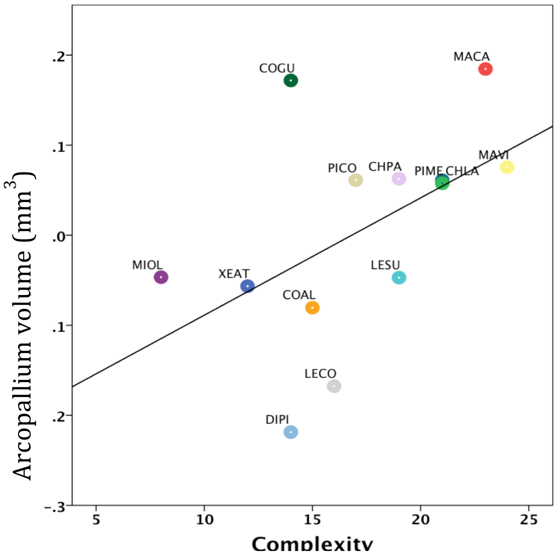


Figure 6: Residuals analysis of arcopallium volume vs. display complexity

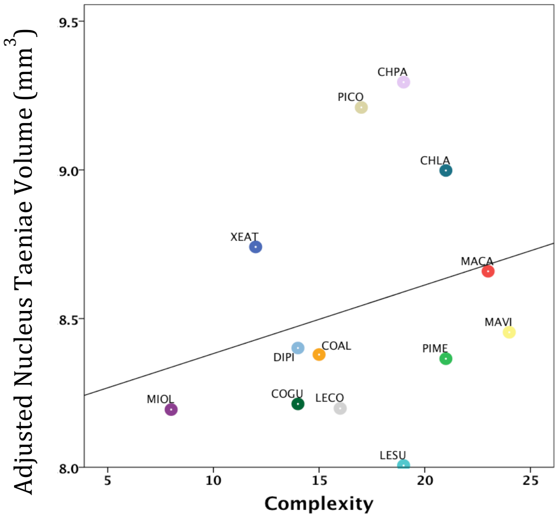


Figure 7: Marginal means adjusted nucleus taeniae vs. display complexity

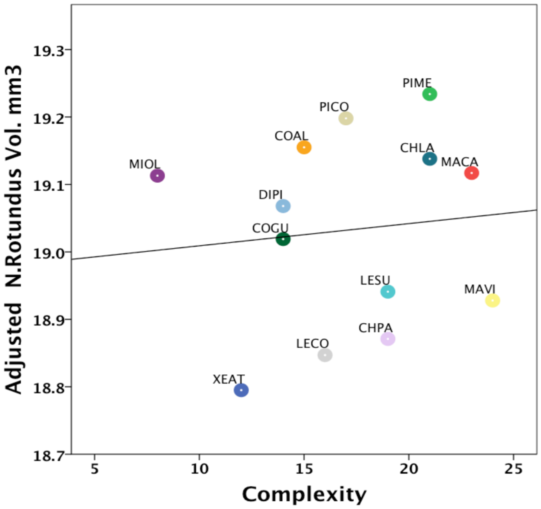


Figure 8: Marginal means adjusted nucleus rotundus volume vs. display complexity

Figures 5-8. Analysis of adjusted AP, Tn, and Rt volumes vs. display complexity. Species data points are labeled with 4-letter abbreviations (MAVI = *M. vitellinus;* MACA = *M. candei;* CHLA = *C. lanceolata;* CHPA = *C. pareola;* PICO = *P. cornuta;* PIME = *P. mentalis;* LESU = *L. suavissima;* LECO = *L. coronata;* COGU = *C. gutturalis;* COAL = *C. altera;* DIPI = *D. pipra;* XEAT = *X. atroni- tens;* MIOL = *M. oleagineus).* Results show a significant relationship between adjusted AP and display complexity but no relationship between Tn and display complexity or Rt and display complexity. Results for AP and Tn but not Rt have been corrected for phylogenetic relatedness.

Discussion

We found a positive relationship between adjusted AP, but not Tn or Rt volume and courtship display complexity. Our data suggest that AP volume coevolved with display complexity and suggest AP volume was sexually selected for non-vocal courtship in manakins.

The function of the RA of the AP in the vocal courtship displays of oscines has long been known (Jarvis et al., 2006), but only recently have studies suggested AP has a role in non-vocal courtship displays (Day et al., 2011). The parallels between AP, but not Tn, specialization in manakins and RA specialization for song suggest conserved motoric functions of the AP in passerines but that RA and innervation of the song production pathway are derived characteristics in oscines and AP function in non-vocal courtship is a derived characteristic in suboscines. Our current study is significant since it is the first to implicate a role for AP in non-vocal courtship display of suboscines.

As previously stated, whole brain size predicts display complexity in 12 species of manakins and a close relative (Lindsay et al., 2015). Based on this study, manakin display complexity could have coevolved with whole brain size by concerted evolution of many brain regions increasing in volume in tandem or a few particular regions that perform discrete functions important to different aspects of the display could underlie the whole brain/complexity relationship, mosaic evolution.

The results of our study indicating that AP but not Tn or Rt coevolved with display complexity in manakins suggest that mosaic evolution is more likely than concerted evolution to explain the positive relationship between brain size and complexity described by Lindsay.

In manakins, regions specifically involved in motor planning are likely candidates for regulating aspects of the courtship display. A previous study has shown that cerebellar morphology predicted display complexity in 12 species of manakin and an ochre-bellied flycatcher (Pano, 2015). The cerebellum of manakins having a positive relationship with display complexity suggests the idea of mosaic evolution, and the results of our study further confirm this idea. The results of our study indicating that AP but not Tn or Rt coevolved with display complexity in manakins suggest that mosaic evolution is more likely than concerted evolution to explain the positive relationship between brain size and complexity described by Lindsay.

Tn, though considered a nucleus of the AP, does not have a relationship to display complexity. Tn is a limbic region mostly involved in motivational behaviors, and has been shown to be activated during sexual behaviors (Svec et al., 2009, Yamamoto et al., 2005). Tn is also very dense in androgen receptors in manakins (Fusani et al., 2014). The lack of relationship between Tn and display complexity in this study could be due to the fact that Tn is involved specifically in sexual motivation: all 13 species of sub-oscines studied here were motivated to perform a display, yet the difference in display occurs not in motivation to perform, but in the complexity of the display. In other words, Tn more likely regulates motivation to display than the ability to perform the display, which would be more likely regulated by a sensorimotor region.

AP volume, on the other hand, did vary with display complexity. This relationship is likely due to the motoric nature of AP. Many studies demonstrate AP innervates muscles involved in performing courtship display (Jarvis et al., 2006, Manley, Popper, and Fay, 2004, Reches and Gutfreund, 2008, Suthers et al., 1999) and even having a positive relationship with the complexity of vocal courtship display in oscines (Zeng et al., 2007). The ability of AP, but not Tn, to innervate muscles likely drives the positive relationship between AP and display complexity. In such acrobatic, athletic displays, great demand is placed on motor systems, and in manakins, muscles involved in the displays are hypertrophied and are dense in androgen receptors (Schlinger et al, 2013). Thus, a brain region suited for innervating muscles involved in display, such as AP, would likely be larger in species with more complex display.

In the future, we will measure cell size and gather cell counts of the AP of manakins. The AP can be divided into distinct regions, such as the dorsal, ventral, lateral, and medial AP, that are known to possess specific functions (Reiner et al., 2004). However, the boundaries of these individual regions are extremely difficult to discern in nissl stained sections. We thus cannot accurately measure the volume of these nuclei. We can however gather cell size and cell number measures from the central areas of these nuclei. Measurements of cell size and cell number in the AP and in the sub-nuclei will help us gain a better understanding of the mechanism by which volume increases in AP in manakins with more complex displays and will potentially allow us to isolate the specific functions that are likely specialized in the acrobatic species should sub-nuclei have regionally specific morphometry.

Though lacking cell count and size measurements, this study is novel not only because it is one of the first demonstrating coevolution of a motor region in nonvocal courtship display but also because it furthers understanding of how brain and motor systems evolve in concert in vertebrate species.

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