

Lateralization of the song nucleus in the zebra finch *Taeniopygia guttata*

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Abstract

The vocal control system of songbirds has a significant laterality in song output control, and the adult zebra finch *Taeniopygia guttata* has a significant advantage in the right high vocal center (HVC) in song frequency domain and intensity control. We investigated the allometric relationship between song control nucleus and brain scale in zebra finches, investigated whether HVC and the robust nucleus of the archistriatum (RA) had similar differences in volume, cell density and cell number, and whether Testosterone (T) played a role in these differences. The results of Nissl staining and Immunohistochemistry show that although there is no significant difference between left and right HVC in nucleus volume, cell density and cell number ($P > 0.05$), the correlation parameters on the right side are always higher than those on the left side. On nucleus volume and cell number, both sides of the RA have no significant difference ($P > 0.05$), but on the cell density has a significant ($P < 0.05$). Testosterone receptors (TR), on both sides the HVC and RA, have no significant difference ($P > 0.05$). The growth rate of left and right HVC and RA is consistent with that of brain ($K > 1$). HVC and RA are well fitted to brain length, width and height, and the intercept is also very close. What's more, the song nucleus always show negative growth relationship with the three-dimensional parameters of the brain. Therefore, while the living environment remains relatively constant, songbirds will continue to strictly follow the existing allometric growth pattern, and any individuals that deviate seriously from this development pattern will show more inadaptability.

Introduction

Allometric growth is the nonlinear growth between the size of an organism and its body structure (Mosimann 1970). Organisms in the process of development continuously control the variation of body structure so as to achieve individual coordination, the integration process does not change with the change of natural environment (Gould 1966), but its integration under strict control, character changes to body size always follows the certain proportion (Mirth and Shingleton 2012). With the development of different individuals of the same species, a large number of variations deviating from their predetermined procedures are often produced, such variations are considered to be the raw materials for genetic evolution and natural selection, and organisms achieve diversification by controlling such effects (Stern and Emlen 1999). We have been studying allometric growth for more than a century, but it is still unclear how developmental restriction and natural selection play a role in allometric evolution (Peters and Wassenberg 1983; Niklas 1994). By observing different levels of allometry, we can divide allometry into three types (Cock 1966): Ontogenetic allometries refer to the relative size of two traits during the growth of an individual; The growth trajectories described by ontogenetic allometries result in a static allometry, which is the scaling relationship among individuals within species, populations, sex, or other groups; Changes in ontogenetic allometries can affect the slope or intercept of static allometries to yield derived scaling relationships, called evolutionary allometries (Klingenberg and Zimmermann 1992; Shingleton et al. 2007).

Human language is composed of a certain number of characters or words arranged according to certain grammatical rules. When hearing incorrectly arranged statements, certain parts of the brain are activated to identify the wrong grammatical rules (Abe and Watanabe 2011). This kind of grammar that integrates language into sentences and arranges sentences into paragraphs with certain hierarchical relationships is called recursive grammar. Most people believe that this language pattern is unique to humans (Hauser et al. 2002), but in some aspects, the communication between humans and animals have certain similarities (Eliot et al. 2010). For example, songbirds in birds have surprising similarities in their song learning with human language learning (Doupe and Kuhl 1999), and both need to go through stages of language perception and speech imitation (Doupe et al. 1997). Songbirds perceived, learned, and produced birdsong through special telencephalon, basal ganglia and thalamus circuits (Brenowitz and Beecher 2005), which also provided the ordering ability need to produce and perceive other songs. At the same time, anatomical studies have proved that songbirds and mammals have significant homology in this circuit (Doupe. 2005). Songbirds that have a great similarity in function and structure with humans are often used as one of the model animals for studying the occurrence and evolution of language (Rocha et al. 2019).

Songbirds have a significant lateral advantage in song output, and the forms of output are various among diverse songbirds. The lateral advantage of song nucleus control on song was first found in Common Chaffinch (*Fringilla coelebs*) (Nottebohm 1970; Nottebohm 1972a), and was also observed in white-crowned sparrows (*Zonotrichia leucophrys*) and White-throated Sparrow (*Zonotrichia albicollis*) (Lemon 1973; Nottebohm and Nottebohm 1976). Nottebohm et al. (1976) described the left sublingual dominance of Island Canary (*Serinus canaria*) control sound through unilateral injection experiments. In 1997, Wild et al. (1997) concluded that the left sirinx subnucleus (nXII TS) was 6.4% larger in males and 11.4% larger in females. This suggests that the generation of side dominance is related to the size of the nucleus, and the larger nucleus can integrate more information. Zhang et al.(2014) obtained that the HVC of zebra finches had a right-sided advantage in controlling the characteristics of sound intensity and frequency domain through the electrical damage experiment. Testosterone (T) could promote various types of neuroplasticity in this system (DeVoogd and Nottebohm 1981) but whether testosterone affects lateral advantage of the warbler nucleus has not been reported. The laterality of the song system was closely related to long-term genetic control and sexual selection, but whether macro evolution caould be explained by micro-structure remains a major question in developmental evolution (Eldredge et al. 2005; Futuyma 2010; Varotsos et al. 2011). Developmental limitations were generally defined as resistance factors that prevent or slow the organism's structure from reaching optimal parameters (Pélabon et al. 2014). Allometric relationships were defined as one of the manifestations of developmental restriction in which phenotypic evolution was often inhibited (Smith et al. 1985), but some studies have suggested that developmental restriction itself is also the result of natural selection (Wagner et al. 2000; Pélabon et al. 2014). In this study, we measure the static allometric relationship between the parameters of the right and left sound control nuclei and the brain scale of songbirds to explore the mechanism influencing the evolution of bilateral sound control systems.

Material And Method

Experimental material

This experiment selected 20 adult zebra finch (120 d-150d), of which 13 birds were used for Nissl staining to count the community volume of the song nucleus, 5 birds were used for immunohistochemistry of the testosterone receptor (TR), and 2 birds died during feeding. The average weight and body length of zebra finches are $11.74 \pm 0.83\text{g}$ and $10.44 \pm 0.38\text{cm}$, respectively.

Experimental method

Zebra finches were anesthetized by intraperitoneal injection of 25% ethyl carbamate at 0.4 ml/kg. Morphological parameters of zebra finches were measured with vernier caliper. The anesthetized zebra finches were instilled with 0.75% normal saline, followed by 10% formaldehyde phosphoric acid buffer solution. After the instillation, the birds' brains were decapitated and transplanted into the original fixation solution for 2 days. After fixation, they were placed in 15% and 30% sucrose phosphate solution for gradient dehydration. After dehydration, they were placed in frozen section embedding box, and OCT embedding glue was dropped for embedding. Then they were placed in -80 refrigerator. When used, the slides were removed and placed in a frozen slicer for rewarming for 30min, and then continuous sections were made with a thickness of $30\mu\text{m}$ ($20\mu\text{m}$ immunohistochemistry). Slices were taken at intervals, and the slides after the placement were dried overnight or stored in frozen slice protection solution. Nissl staining was performed with 0.1% tar purple.

The sections in immunohistochemistry were washed 2min×2 times with PBS, immersed in fresh 3% H_2O_2 solution, treated at room temperature for 30min to inactivate endogenous enzymes, washed with distilled water for 2min×3 times. Immersed in 1%Triton-100 for 25min to increase cell membrane permeability, and washed with distilled water for 2 min×3 times. Compound digestive solution was dropped and washed with PBS for 2 min ×3 times at room temperature for 10 min. Normal goat serum was sealed at room temperature for 60 min and then dumped. Primary antibody was dropped and incubated at 4°C for 18–24 h or reacted at 37°C for 4h, then washed with 0.1mol/L PBS for 2 min×3 times. The secondary antibody was ready-to-use sheep anti-rabbit IgG, incubated at room temperature for 45min, and washed with 0.1 mol /L PBS for 2 min×3 times. SABC complex was added by dropping, and incubated at room temperature at 37°C for 20 min. Then, 0.1mol /L PBS was washed for 5min×4 times. Finally, DAB was used for 8min and PBS was fully rinsed to stop the reaction. After the reaction, gradient dehydration was carried out in 100%, 95%, 90%, 70% and 50% ethanol solution for 2min each time, xylene ethanol mixture for 3min, xylene pure solution for 3min, and neutral adhesive was sealed. Due to quantitative detection of optical density involved in the statistical process, hematoxylin was not used to re-stain the nucleus to reduce errors.

Selection of the song nucleus

The song of songbirds is a learned behavior, which is continuously strengthened through auditory input-learning-memory-vocalization-feedback correction process. The vocal control system consists of a series

of discrete brain regions with complex nerve fiber connections. Anatomical studies have revealed the composition of the nuclei in this system and the relationships between the nuclei. The sound control system of songbirds mainly consists of two neural pathways, the descending motor pathway controlling the syrinx and the forebrain circuit controlling the singing learning. Descending motor pathways is composed of the high vocal center HVC, the ancient striatum crude nuclear RA and hypoglossal nerve tracheal syrinx subnuclear (nXIIIts). It was found that HVC and RA neurons had synchronicity in their excitations and warbling, any inhibition of neurons in either of them would affect the warbling system, and after damaging the HVC, songbirds trill recognition ability would be greatly reduced. There was also a complex synaptic connection between HVC and RA. A separate partition of HVC projected to the X area of the basal ganglia. Then reached the lateral LMAN of the new striatum giant cell nucleus via dorsolateral nucleus of the lateral anterior thalamus (DLM), and LMAN emitted fibers projecting to RA. The HVC→X region→DLM →LMAN→RA formed the song learning circuit, and damaged to any of the nucleus in the circuit would hinder normal song learning of young birds and the perception of song of adults. Both the vocal control pathway and the learning pathway are involved in the higher vocal center (HVC) and the paleo striatum nucleus (RA), so it is of great significance to discuss the allogenic growth of these two neural nuclei.

Data statistics

The sealed slices were taken under a photographic microscope. We need to find the target nucleus HVC and RA at the expected location, make a mark, and input the mark photo into Image J to obtain the target nucleus area. The stereological formula is as follows:

$$V = \sum_{i=1}^{N=13} (S_i \times D) \quad 1$$

S_i is the sectional area of the target nucleus, and D is the section thickness.

The cell density of HVC and RA was statistically analyzed. Since the calculated cell size was relatively small compared with section thickness, Therefore, the results obtained by direct counting method are not significantly different from those obtained by stereoscopic techniques such as optical analyzer (Tramontin et al. 1998). During the sampling process, random sampling was carried out in the ventral, middle and caudal nuclei, with a total of 6 sampling frames, each containing 300–400 brain cells, as shown in Fig. 1. The counting software used Image J. According to the Principle of Nissl staining, the resulting figure included deeply stained nucleoli and cytoplasm with uniform particle distribution. Threshold was adjusted through Threshold window. Because some sections had a certain thickness, overlapping cells would appear, so the watershed method was used to separate overlapping cells and operate (add or delete) each point to reduce the counting error. Ensure that each cell was counted and derive the cell count after completion of the count. To deal with immunohistochemical chips after taking pictures, using the Average Optical Density (AOD) to quantitatively measure the immunohistochemical staining intensity, this value could reflect the target substance concentration per unit area, the stereological formula is as follows:

$$AOD = IOD / Area \quad 2$$

IOD is the total optical density of the target structure, and Area is the area of the measurement area. This formula is mainly for a specific section. When comparing the average optical density of different sections, the influence of errors caused by some non-human factors on the determination of results should be considered. Therefore, the existence of these errors should be eliminated or reduced. The following is the optical density calculation formula:

$$AOD_{Calculate} = AOD_{Truevalue} + AOD_{Background} + AOD_{Refraction} \quad 3$$

$AOD_{Background}$ can be estimated by negative control of the same batch of sections, while $AOD_{Refraction}$ needs to take images of the same section under the same conditions without cells for calculation.

ALLOMETRIC GROWTH MODEL

The relationship between two representative parts x and y of a growing individual, thus forming the relative growth formula:

$$y = bx^a \quad 4$$

In order to facilitate the calculation and comparison of allometric growth index, the linear equation was obtained by converting Formula 4 to 1og:

$$\log y = \log b + a \log x \quad 5$$

A and b are constants. A is the ratio of the growth rates of the two parts, and is the relative growth coefficient. B is the y value when x part is set to 1, which is called initial growth index. Extrapolate the logarithmic equation as if it were a close unary equation ($y = kx + b$). Slope K, intercept A. When $k = 1$, it is called isometric growth, that is, the change between relative shapes is constant speed; when $K > 1$, it is called positive allometric growth, that is, the change rate of Y is faster and faster than that of X; otherwise, it is called negative allometric growth. It is important to note the above derivation characteristics of Y and X is the premise of measurement scale is in the same dimensions. For example, the allometric relationship between brain weight and body weight needs to be transformed when the two discussed trait parameters are not in the same dimension. Such as, when fitting the relationship between insect chest length and body weight, chest circumference needs to be calculated to the third power to achieve the same dimension with body weight (Shingleton et al. 2008). In order to more accurately measure the influence of multiple factors on traits and eliminate the influence of different orders of magnitude of sample data on the determination of results, correlation coefficient was used to measure.

$$\rho_{XY} = \frac{\text{cov}(X, Y)}{\sqrt{\sigma(X)\sigma(Y)}}$$

The ρ_{XY} represents the correlation coefficient between shapes X and Y, $\sigma(X)$ and $\sigma(Y)$ are standard errors.

Results

Analysis of community volume and cell density of the song nuclei

The localization of the target nucleus has been revealed by a large number of studies (Nottebohm 1992; Alvarez-Buylla et al. 1997), and the results of section observations showed that the song nucleus had clear boundaries in the brain of songbirds, which were favorable for the calculation of the target nucleus (Fig. 1). The estimated brain HVC and RA volumes were calculated by stereological formula. T-test was used to analyze whether left and right HVC and RA volumes have significant difference. The results showed that there were no significant differences in both sides HVC and RA volumes ($P_{HVC1}=0.284$, $P_{RA1}=0.425$). The ipsilateral singing nuclei groups have significant differences ($P_{HVC2}=0.0046 < 0.05$, $P_{RA2}=0.0057 < 0.05$). Although there was no significant difference between the both sides of the volume, it was found that the volume of right HVC was $5.755 \pm 1.251\%$ larger than that of the left side, and the volume of right RA was $3.962 \pm 1.020\%$ larger than that of the left side, which supported the conclusions of DeVoogd and Nottebohm that there was no significant morphological difference in the distribution of the song system on both sides of the brain (Nottebohm 1981; Devoogd et al. 1993). In the same way, on both sides of the cell density of the nuclei were compared, the results for about there was no significant difference between the HVC cell density ($P_{HVC3}=0.311$), RA cell density has significant difference ($P_{RA3}=0.041 < 0.05$). The cell number of HVC and RA was estimated by nucleus volume and cell density, and there was no significant difference in the cell number of HVC and RA ($P_{HVC4}=0.139$, $P_{RA4}=0.092$). For cell density calculation was based on the assumption that the distribution of nerve cells in the HVC and RA was uniform, Brenowitz et al.(2010) later proved to be correct. Here, it was found that RA has a significant difference in cell density between the two sides. More neurons per unit volume meant that RA would have more advantages in the process of information processing, and meanwhile, the utilization rate of individual space of the brain was improved in the process of brain refinement (Devoogd and Nottebohm 1981). Of course, it may also be caused by too small sample size and sampling error. But it's clear that RA on the right side of the brain would perform more integrative functions than RA on the left.

Allometric growth analysis of song nuclei

In this study, we first measured the static allometric relationship between the song nucleus mass and brain weight in male zebra finches at the same developmental stage. The fitting degree of left HVC and right HVC to brain weight was close. Within the error range, it could be considered that the changes between brain weight and both sides HVC are in equal proportion ($K_{right}=1.240 \pm 0.1234$, $K_{left}=1.255 \pm$

0.148). $K > 1$ indicated that the song nucleus increased with the increase of brain mass, and its growth rate would gradually accelerate before reaching the threshold or selecting the critical value. This result may be caused by a special sample group (two-thirds of adult male songbirds). Because for male songbirds, the complexity of singing was often positively correlated with the HVC, that is, having a large volume of HVC was beneficial for the editing of complex songs (Airey et al. 2000). The more complex and high frequency song output may affect female zebra finches' mate selection and lead to genetic preference for traits with larger song centers, which may be one of the factors that natural selection influences the allometry of songbirds. In RA, there were some differences between both sides RA and static allometry of the brain ($K_{\text{right}} = 1.300 \pm 0.381$, $K_{\text{left}} = 1.080 \pm 0.448$) (Fig. 2). The growth slope of right RA was faster than that left side. However, static allometric growth evaluation not only depended on the growth relationship of relative traits, but also considered the growth accumulation amount of related traits (Gould 1966). And, an experiment on selecting the forewing and hindwing of butterflies showed that the growth accumulation could affect the static allometric growth process by changing the intercept (Frankino et al. 2007). For RA, the growth accumulation could be regarded as the initial volume of left and right RA nuclei, and the results of significance analysis showed that there was no significant difference between the two initial volumes, so that the main factors influencing the slope may be other neural structures for the left side of the inhibition of RA. The RA as control one of the important nuclear group of muscle activity, its relative to the growth of the brain in different speed, showed that the development potential of left RA was lower than that of right RA, which was consistent with the right-sided dominance of zebra finches in song (Williams et al. 1992). From the perspective of the static allometric growth to prove it, and whether the HVC or RA, its relative to the growth of brain always have $k > 1$, indicating that the song control system has abundant variation among individuals, which is often beneficial to the evolution within the population, because the static allometric relationship could be regarded as the response mode of individuals to environmental changes (Franklin 2010).

In addition, the static allometric relationship between brain three-dimensional parameters and vocal nucleus community volume was explored. For left and right HVC, the calculated results were similar to those of brain weight, On both sides HVC with length, width and height of the brain all had good fitting and the intercept were very close. The fitting relationship of left and right RA to brain three-dimensional parameter was the same as the allometric relationship of brain weight (Fig. 3). By comparing the allometric relationship between the mean value of HVC, RA and three-dimensional brain parameters, the result of brain length with the HVC and RA were the lowest among brain three-dimensional parameters, that is, the changes of the song nuclei were the slowest to be affected by changes in brain length. And brain length was often closely related to the degree of evolution of species, so it was not difficult to make such a conclusion: the song nucleus had a certain lag in the evolution of ontogeny, that is, it was relatively stable in the evolution process and was not changed by the direct influence of natural selection. Meanwhile, a study have shown that natural selection only plays a role in the overall body size, and the change of traits was passive selection for the change of individual scale (Van Buskirk et al. 1997).

Finally, we calculated whether there was a certain co-mutation mode between HVC and RA (Fig. 4), and the fitting results showed that $K_{\text{Right}} > 1 > K_{\text{Left}}$, that is, for zebra finches, right song control pathway between RA and HVC presented positive allometric growth relationship while left song control pathway showed a negative allometric relationship. The results showed that allometric growth between left and right HVC was synergistic. When HVC volume increases, right RA has a faster response than that on the left side, and the change amount has a greater trend than that on the left side.

Immunohistochemical results

There are two types of neurons in HVC, one is the neuron that projects to Are X and the other is the neuron that projects to RA. Both types of neurons contain testosterone cells, and testosterone receptors in HVC increased rapidly during song learning (Bottjer et al. 1997). The effects of testosterone on songbirds' seasonal plasticity and sexual dimorphism have long been proved (Smith et al. 1997), which mainly promoted the survival and migration of newborn neurons in songbirds, increases the cell size of vocation-related nucleus neurons, and the growth of dendrites and synaptic generation (DeVoogd and Nottebohm F 1981). Immunohistochemical staining marked the distribution of TR in bilateral HVC and RA (Fig. 5) after excluding background staining (non-specific staining) and bright interference, the average optical density (AOD) of right HVC was $0.300 \pm 0.044 \text{ IOD}/\mu\text{m}^2$, and RA was $0.253 \pm 0.028 \text{ IOD}/\mu\text{m}^2$. Left HVC was $0.297 \pm 0.042 \text{ IOD}/\mu\text{m}^2$, RA was $0.253 \pm 0.25 \text{ IOD}/\mu\text{m}^2$. There was no significant difference in the distribution of TR in both sides HVC and RA ($P_{\text{HVC}}=0.905$, $P_{\text{RA}}=0.992$), which was consistent with the comparison of the left and right nucleus volume, that is to say, songbirds did not showed the dominance of right side of song control at the morphological level. In terms of individual zebra finch, controlling the size of song nucleus was't directly related to the complexity of song nucleus, because male zebra finches that encode more song nucleus had no significant changes in the size of song nucleus and the number of neurons, besides the development process of song nucleus on time scale was't affected (Burek et al. 1991). The same results have been found in male marsh wrens (*Cistothorus meridae*) (Beecher and Brenowitz 2005).

Discussion

Studies have found that the complexity of songbirds' song was positively correlated with song nucleus, neuron density and neuron number (DeVoogd et al. 1993; Garamszegi and Eens 2004). The experimental resulted showed that there was no significant difference in the nuclear population volume and cell number of left and right HVC, It was proved that the endocrine system of songbirds played an equal and stable role in male birds with stable neurodevelopment in adulthood. Immunohistochemical results showed that the significant changes in songbirds' response to hormones may exist in the early stage of songbirds' song learning and development. Zhang et al. (2014) described that the right HVC had a significant advantage in the control of the frequency domain and intensity of singing through the click-damage experiment, but some studies have shown that the control of singing melody required the joint action of both HVC. It was not difficult to see that the right side of the song system was not an absolute control advantage over the song, but showed advantages in some signal output, that is to say, the output

of song depended on the synergistic effect between the two sides of the song nuclei. Sound production depended on the auditory feedback, HVC was not only an important structure for song generation and learning, which was located at the highest level of singing motor pathway, but also a gated nucleus that received auditory information input (Nottebohm 1971), and integrate the new code was passed to give in to the front of the brain Are X or downstream muscle to produce the chirping of information feedback. The control level of HVC in the song system was higher than that of RA, but the nerve impulses generated by HVC could not directly control the activity of the song muscle. RA, as the efferent nucleus of HVC, was also the premotor nucleus that directly controlled the activity of nXII TS. RA could process neural signals from HVC, generated neural codes that could coordinate the interaction of the vocalis muscle and respiratory muscle, outputed to nXII TS and other related vocal motor nuclei to control singing behavior (Fee et al. 2004; Wang et al. 2018). There was no significant difference in nuclear population volume and cell number between left and right RA, but there was a difference in cell density. The cell density of right RA was significantly higher than that of left RA. If this result was caused by sample size and sampling error, the error would be further enlarged when the number of RA neurons was estimated, resulting in a significant difference in the number of neurons in left and right RA. However, the calculation results showed that there was no statistically significant difference in the number of RA cells (Fig. 6). So, another possible explanation of this phenomenon without considering the error was that the auditory processing and error feedback mechanism was biased to the right (Toyomura et al. 2007; Tourville et al. 2008), which led to the right HVC bearing more output of neural coding in the process of singing. Therefore, the right RA needs to integrate and output more regulatory information to regulate the movement of the vocalis muscle. On the other hand, In RA on the right, the number of nerve cells per unit volume increases in order to complete more complex information processing than that on the left, and more neurons and synaptic connections per unit volume can control the encoding of some special syllables in the process of singing. Right RA can accept more from the ipsilateral HVC and the lateral part of the striatum giant cell nucleus (LMAN), resulting in stronger regulation of the descending nucleus, which was consistent with the partial lateralization of its function. Whether right HVC has a stronger projection to ipsilateral RA has not been proved due to limited samples.

The production of complex songs were closely related to sexual selection, and males with more song editing ability were more likely to be approached by female songbirds (Spencer et al. 2005). This trait could be preserved and evolved under the restriction of genetic selection. Studies have shown that allometric relationships between ontogeny, static growth and developmental evolution were all influenced by a common factor, namely the growth process (Warton et al. 2006). Therefore, the degree of correlation between traits could be tested by estimating the covariance between relative traits. However, as the order of magnitude between traits may be different, phenotypic correlation coefficient was used in this experiment to compare multiple pairs of traits so that all parameters were compared at the same level. The growth rates of both sides HVC and RA were consistent with that of the brain ($K > 1$), indicating that in the upstream nucleus of the song learning and control system, the song control system had abundant variation among individuals, which was often beneficial to the evolution of the population, because the static growth relationship could often reflect the adaptation mode of individuals to environmental

changes. Through experimental studies, Bondar et al. found that positive allometric growth seems to have a stronger inhibition on shape development in individuals (Meter et al. 2020). After comparing the correlation between HVC and RA, it was found that there was a higher correlation between right RA and HVC. What's more, There was a positive allometric relationship between RA and HVC, It showed that the right RA in the process of development was the growth inhibition of mainly comes from the HVC's three-dimensional shape, so as to brain size, but the ultimate factor in maintaining this relationship was natural selection. However, left RA showed negative allometric growth compared with HVC. It was generally believed that directional selection plays a role through the negative allometric growth between allometric growth. HVC and RA had a good fitness values and intercept was close with brain length, brain width and brain height. The singing nucleus always showed a negative growth relationship with three-dimensional parameters of the brain, which maybe because the brain always acts as a restriction to the neural nuclei occupying a certain spatial dimension, so as to improve the adaptability of individuals to environmental changes. Therefore, it was not difficult to find that the growth of the left and right nuclei was restricted by a variety of factors through static allometric growth of the singing nuclei. Among them, HVC was mainly influenced by natural selection, and there was mutual inhibition between HVC and ipsilateral RA, which was higher on the left than the right. The development process of RA had a certain delay on the individual level, so only the change of genetic covariance caused by natural selection or sexual preference for a long time can change its nuclear cluster development pattern. Therefore, when the living environment remained relatively constant, songbirds would continue to strictly follow the existing allometry growth pattern, and any individuals who seriously deviated from this development pattern would show more inadaptability. It was not hard to understand because, on an evolutionary scale, brain size's use of internal space required a measure of resource input and individual contribution, so the brain had always acted as a constraint on the number of neurons occupying a certain spatial dimension (Székely et al. 1996).

Conclusions

Our data suggest that there were significant differences between the ipsilateral singing nuclei groups. Although there was no significant difference in volume between the both sides of the singing nuclei, it was found that the volume of HVC on the right side was $5.755 \pm 1.251\%$ larger on average than that on the left side. Right RA was $3.962 \pm 1.020\%$ larger than left RA on average, and the cell density of right RA was significantly higher than that of left RA. In addition, through the static allometric relationship between left and right RA and brain, it was found that the developmental potential of left RA was lower than that of right RA, which was consistent with the partial lateralization of function. There was no significant difference in TR receptor distribution between HVC and RA. The growth rates of left and right HVC and RA were consistent with that of the brain ($K > 1$), indicating that in the upstream nucleus of the song learning and control system, the song control system had abundant variation among individuals, which was often beneficial to the evolution of the population. Comparing the relationship between HVC and RA, it was found that the correlation between right RA and HVC was higher, and there was a positive allometric relationship. Those results indicated that the growth inhibition of right RA in the development

process mainly came from the three-dimensional morphology of HVC, as well as the brain volume. However, RA on the left showed negative allometric growth compared with HVC. It was generally believed that directional selection plays a role through the negative allometric growth between allometric growth. HVC and RA had a good fitness results and intercept were close with brain length, width and height. The singing nucleus always showed a negative growth relationship with three-dimensional parameters of the brain, which may be because the brain always acted as a restriction to the neural nucleus which occupies a certain spatial dimension, so as to improve the adaptability of individuals to natural selection.

Declarations

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Data availability Data can be made available upon request to the authors.

Author contributions Statement Xiaoxing Yang and Cheng Zhang wrote the main manuscript text and Zhaohui Xie, Shaobin Li prepared figures 1-3. All authors reviewed the manuscript.

Conflict of interest The authors have no relevant financial or non-financial interest to disclose.

Ethics approval All experimental protocols and handling, use, and care of laboratory animals were conducted in compliance with the current normative standards of Henan University of Urban Construction.

Consent for publication All authors consent.

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Figures

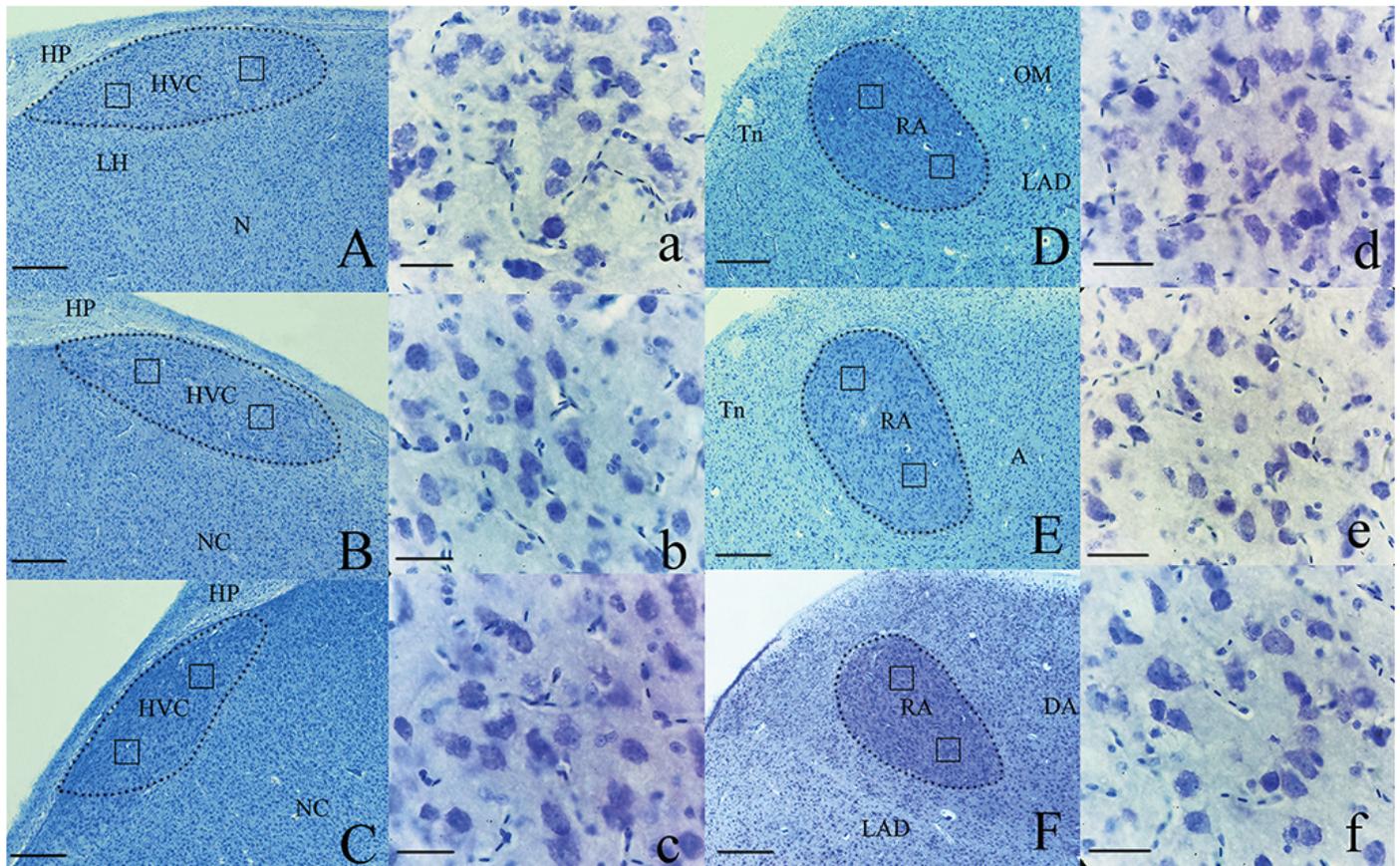


Figure 1

Diagram of Nissl staining of Higher vocalization center (HVC) and Robust nucleus of the archistriatum (RA) in zebra finches. Each sample site contains 300-400 cells. A, B, C, D, E and F, scale: 200µm. a, b, c, d, e, and f, scale :5µm. HVC, High vocal center; RA, Robust nucleus of the archistriatum; HP, Hippocampus; N, Neostriatum; NC, Neostriatum caudale; LH, Lamina hyperstriatica; A, Archistriatum; Tn, Nucleus taeniae; DA, Tractus archistriatalis dorsalis; LAD, Lamina archistriatalis dorsalis. Scale:

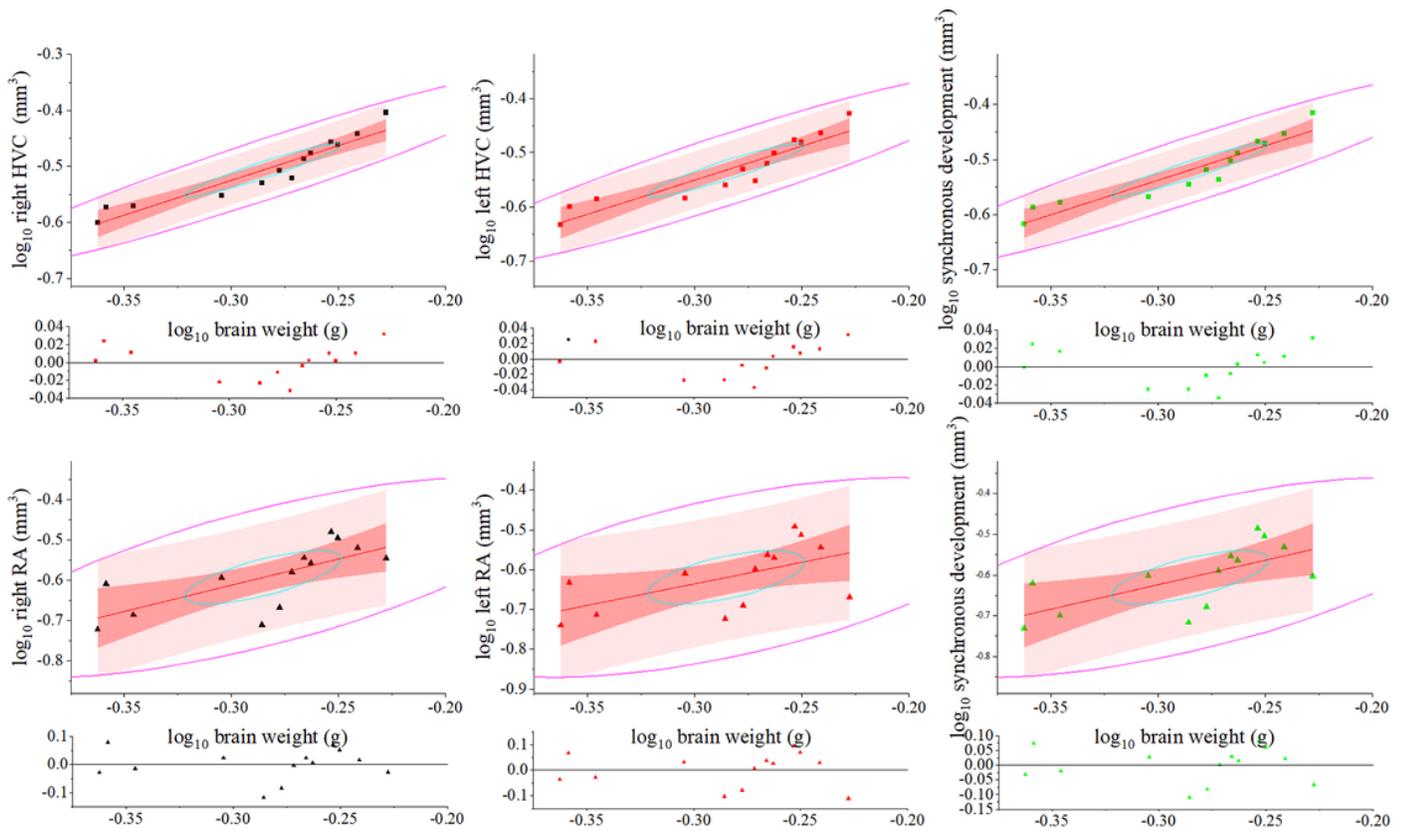


Figure 2

Static allometric relationship between song nucleus mass and brain weight in zebra finches. The figure above shows the relationship between brain weight and bilateral HVC, and the figure below shows the relationship between brain weight and bilateral RA

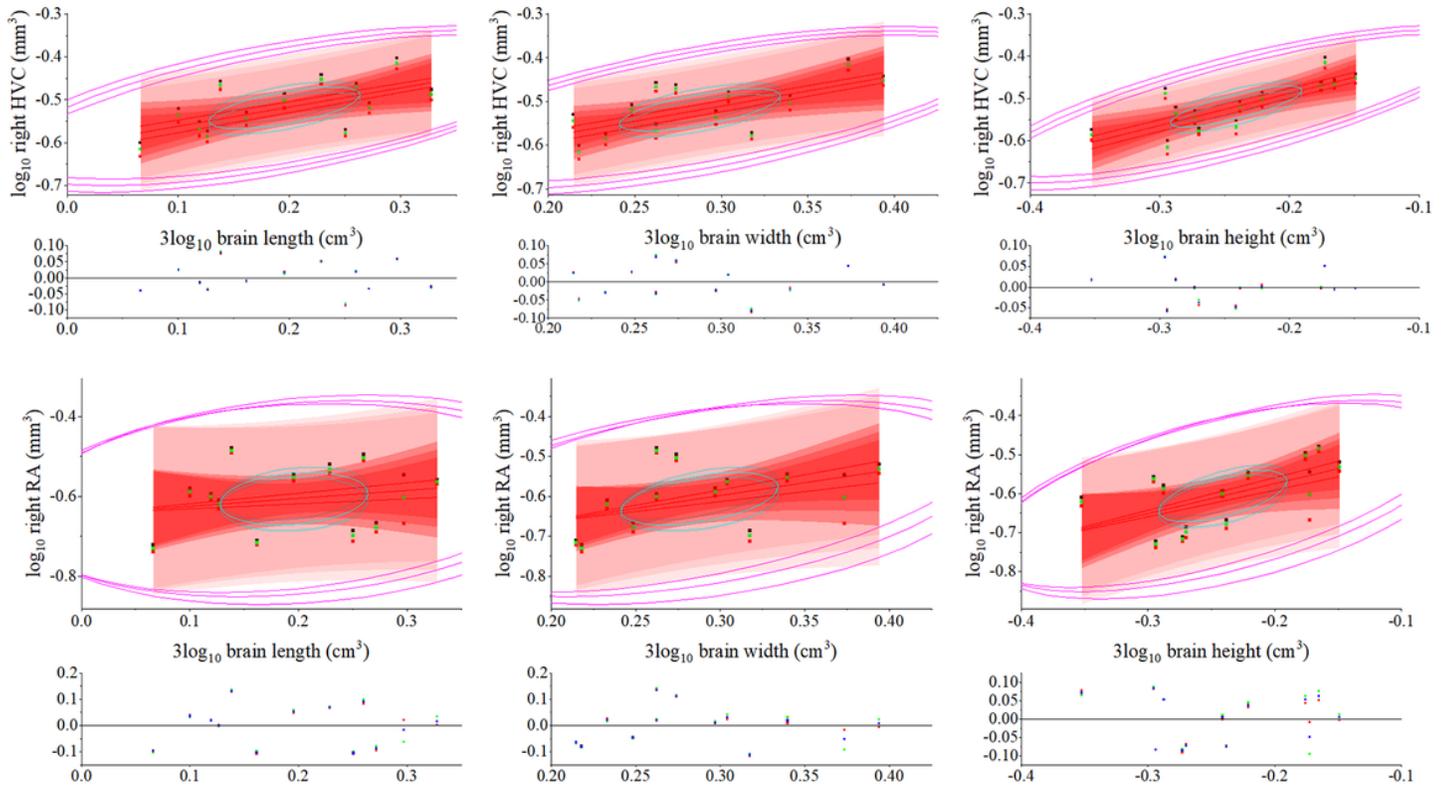


Figure 3

Static allometric relationship between three-dimensional brain parameters and song nucleus mass in zebra finches. Brain parameters are brain length, brain width and brain height

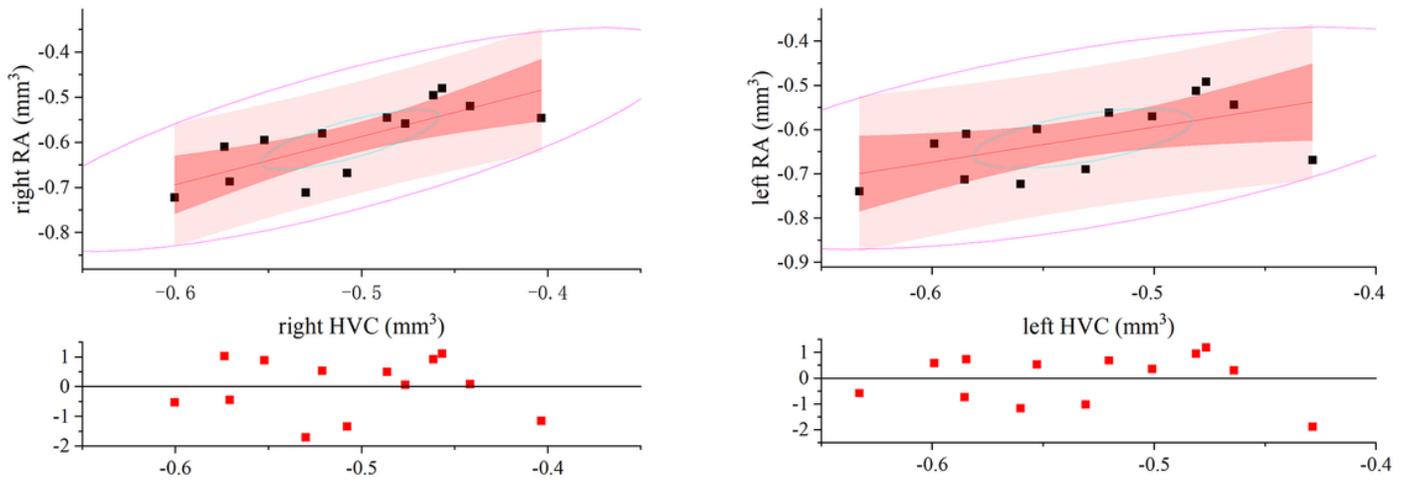


Figure 4

Static allometric relationship between HVC and RA in zebra finches. The left figure shows the linear fitting between HVC and RA on the right, and the right figure shows the linear fitting between HVC and RA on the left.

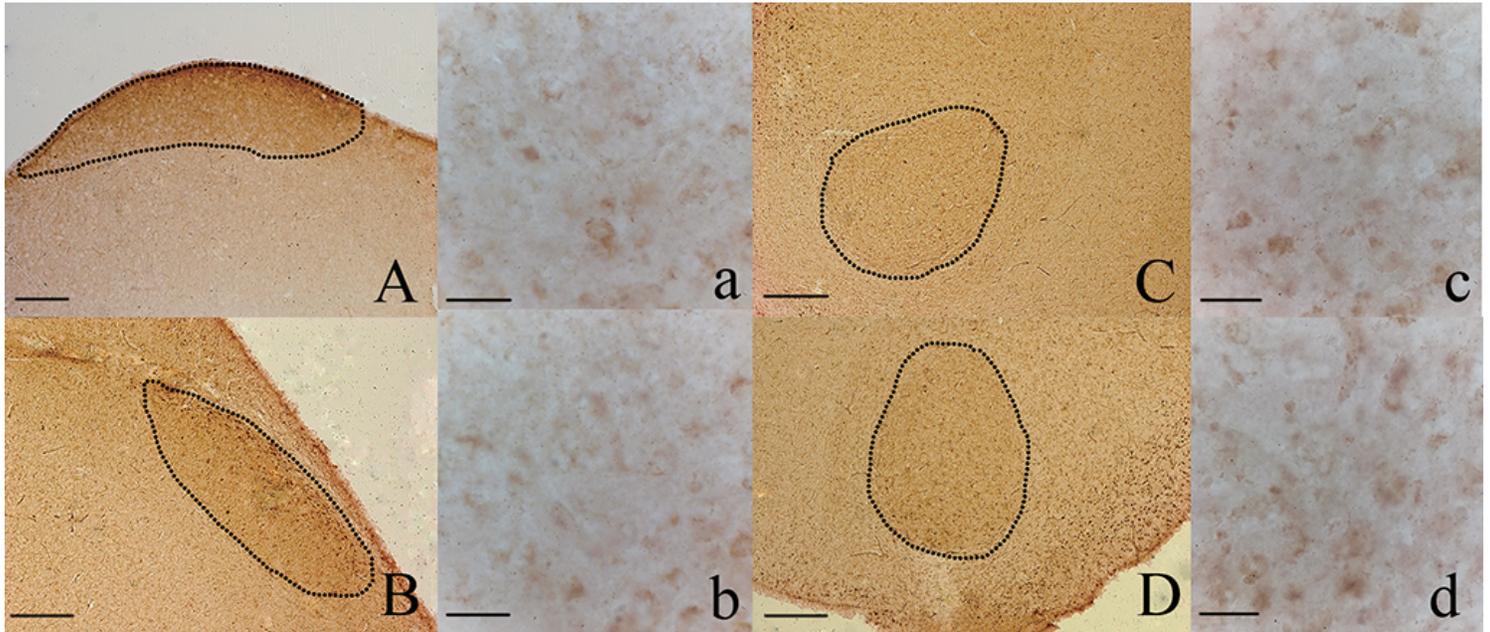


Figure 5

Immunohistochemical schematic diagram of testosterone receptor (TR) in higher vocalization center (HVC) and robust nucleus of the archistriatum (RA) of zebra finch brain. In figure A and B, dotted lines are the immunohistochemical diagrams of HVC on the right and left sides of the brain respectively. Figure C and D are the immunohistochemical diagrams of RA on the right and left sides of the brain respectively. A, B, C and D, scale: 200 μ m. a, b, c and d, scale: 5 μ m.

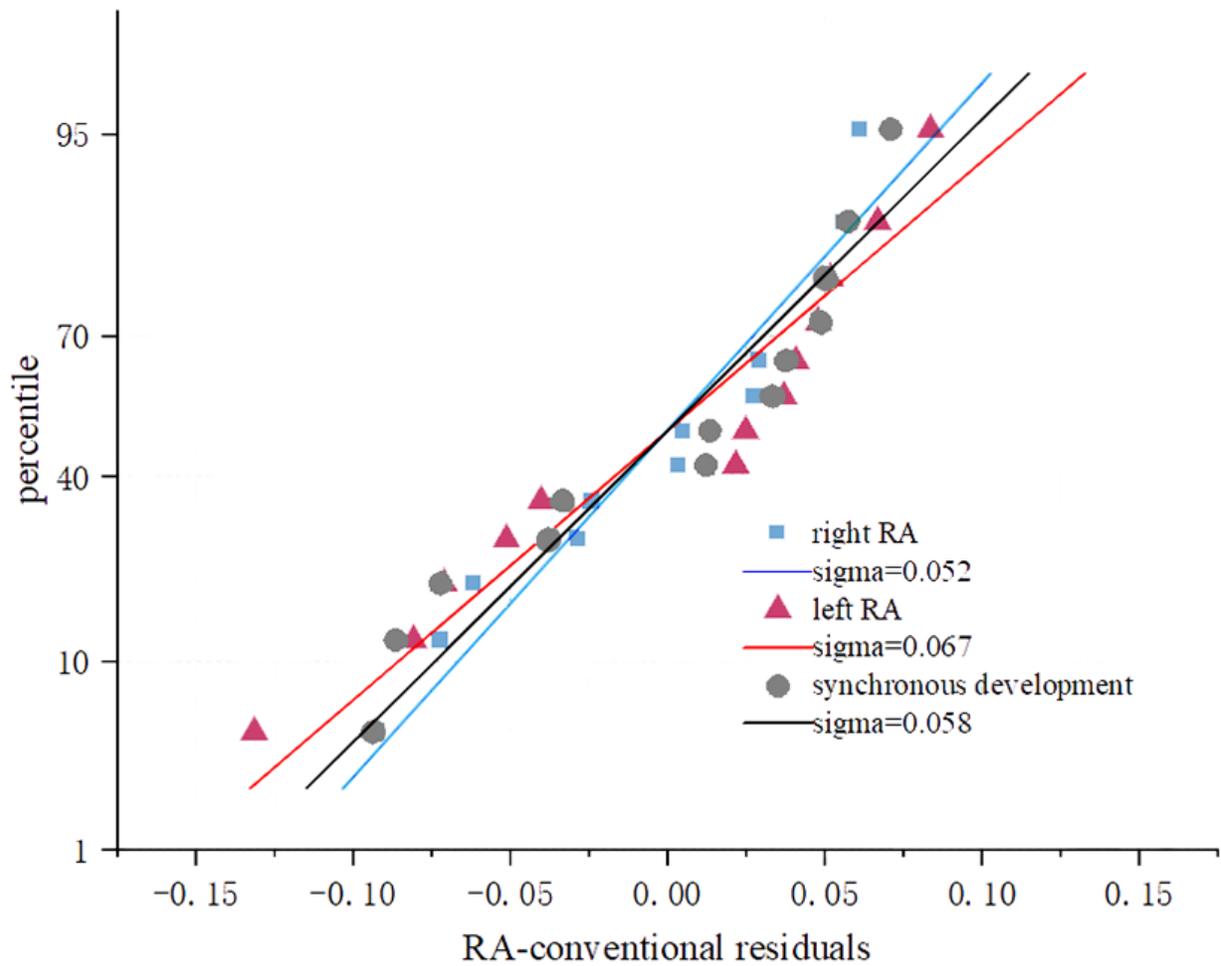


Figure 6

The conventional residual diagram of RA, the blue square is the RA percentile on the right, the red triangle is the RA percentile on the left.

Supplementary Files

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