



Short communication

Variation in auditory neural activation in response to strain-specific songs in wild and domesticated female Bengalese finches

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ABSTRACT

Female songbirds identify and prefer conspecific male songs. Songs are an important cue for species discrimination. Bengalese finches are domesticated species and their male songs seem to have evolved as they comprise more complex sequences and tonal sounds than the songs of their wild ancestors, white-rumped munias. Previous research suggested that the degeneration of song functionality for species identification may have been one of the factors that promoted the evolution of song complexity in domestic strains. We hypothesized that female responses to conspecific songs have changed between the two strains: white-rumped munias could distinguish songs of their own species more readily than Bengalese finches. Because the song discrimination is affected by developmental experiences, we used adult female Bengalese finches and white-rumped munias reared with or without exposure to songs of their own strains (*i.e.*, socially-reared or untutored). To evaluate their song discrimination, we quantified *zenk*-labeled cells in the auditory areas after exposure to song stimuli, either with songs of own strains or those of other strains. Socially-reared white-rumped munias exposed to songs of Bengalese finches showed lesser *zenk* expression than munias exposed to songs of their own strain. However, there were no significant differences among the groups in Bengalese finches. The result suggests that white-rumped munias could distinguish songs of their own species more strictly than Bengalese finches.

Animal domestication is an important factor that leads to phenotypic changes including behavior and neural mechanism between wild and domesticated species as the selection pressure in the wild differs from that in captive environments [1,2]. Domestic Bengalese finches, *Lonchura striata domestica*, are well suited for exploring the relationship between song phenotype and neural changes associated with domestication. Bird songs, which are mating signals necessary for species recognition, are well-known traits that evolve by natural and sexual selection [3]. Life history or ecological variables also influence the evolution of song features; birds in urban environments have evolved to have a higher pitch and louder vocalizations due to anthropogenic noise [4]. The mating songs in male Bengalese finches evolved as they have more complex sequences and tonal sounds than the songs of the wild ancestors, white-rumped munias, *Lonchura striata* [5,6]. In addition, song learning strategies are different between these two strains [7]. Passerine birds learn songs through their auditory experiences based on

innate constraint in early life. White-rumped munias show a strong bias for learning accurately from songs within their strains. In contrast, Bengalese finches do not show a tight constraint for learning conspecific songs. There are no records of artificial selection for more complex songs during the Bengalese finch domestication. Instead, it has been recorded that birds were selected by other traits, including parenting abilities and feather colors [5]. Therefore, artificial selection could not directly affect song evolution from white-rumped munias to Bengalese finches. Previous ecological studies have suggested that the evolutionary background, which induces differences in songs between the strains, could have led to the degeneration of the song functionality under a domestic environment [8]. The munias might need to differentiate between their own and related species to prevent crossbreeding in the wild environment. In contrast, domestic Bengalese finches might be relaxed from such constraints leading to the hypothesis that song variability might have evolved during domestication. Their song

Abbreviations: CMM, caudomedial mesopallium; NCM, caudomedial nidopallium

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change could be due to the change in their environmental conditions.

Further, it is possible that song discrimination of females changed during the domestication of white-rumped munias into Bengalese finches. In most passerine birds, only males sing songs to advertise their territories and attract a conspecific mate. Sexual selection is also an important factor for song evolution, since females identify and prefer conspecific songs that contribute to their adaptive mating behavior. Therefore, songs are evolved by natural and sexual selection because females chose mates depending on the songs. In domestic environments, the functionality of songs for species identification is not strongly restricted to song features. Owing to increased male song variability under domestication, the female discrimination to conspecific songs may have decreased in Bengalese finches compared with that in white-rumped munias.

Neural activation of the secondary auditory regions indicates song discrimination in female birds. The second auditory regions, including the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM), play important roles in the processing and perceiving of conspecific vocalizations [9,10]. Females with lesions to the CMM lose the discrimination to conspecific songs [11]. The expression of the immediate early gene *zenk* in the CMM and NCM increased in response to conspecific songs compared with that in response to heterospecific songs [12]. The difference in responses were correlated with the results of electrophysiological and functional magnetic resonance imaging (fMRI) studies, which indicated neural activation increased in response to conspecific songs compared with that in response to heterospecific songs [13,14].

Here we compared female responses to conspecific songs between Bengalese finches and white-rumped munias using *zenk* expression in the forebrain auditory regions. Because female song discrimination is affected by experiences in early development and based on innate constraints [15], we used adult females reared with or without exposure to songs of their strains to examine early experience-dependent and -independent song discrimination. We compared the number of *zenk*-labeled cells in the auditory areas after birds were exposed to song stimuli of self-strains or those of other strains.

We used a total of 36 adult female birds aged > 100 days post-hatching. Socially-reared female Bengalese finches (n = 12) were purchased from local pet breeders or bred in our laboratory. Socially-reared female white-rumped munias (n = 12) were maintained for > 1 year after being captured from the wild in Taiwan, or were bred in our laboratory from the captured birds. They were maintained in large metal cages with either related or unrelated birds. Untutored subjects (6 birds in each strain) were bred in our laboratory and raised only by their mothers from approximately day 7 post-hatch in a soundproof chamber to isolate them from conspecific songs. From day 35 post-hatch, they were kept with only female siblings in a metal cage placed in a soundproof chamber. Genetic relatedness was different among socially reared birds. Some of the untutored birds shared born from the same parents (Table S1). All birds were fed three types of millet, calcium supplements, green leaves, and freely available vitamin-enhanced water. The environment was maintained at a controlled temperature of < 25 °C, humidity of < 60 %, and 13 h of daylight exposure. Experiments complied with the Animal Care and Use Committee of RIKEN (#H20-2-231 and #H22-2-217) and National Institutes of Health Guidelines.

We recorded undirected songs of adult Bengalese finches (n = 12) and white-rumped munias (n = 12) in a soundproof chamber to prepare the song stimuli as per the method described previously [16].

Birds were exposed to the unfamiliar song stimuli. The song stimuli were different among subjects in the same groups (Table S1). We used undirected songs because the differences between undirected and directed songs were expected to be slight, since that in other finch species, such as the Zebra finch, tempo and frequency consistency were reported to change little when songs were directed to females [17]. In addition, female Bengalese finches discriminate stimuli between familiar and

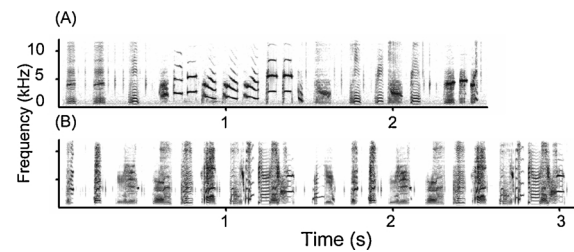


Fig. 1. Examples of parts of song stimuli of Bengalese finches (A) and white-rumped munias (B). Typical parts of the songs were used as stimuli.

unfamiliar undirected songs [16,18]. Songs were stored as wav files; the typical sequences were analyzed by categorizing song elements. Typical parts within the songs were used as stimuli (Fig. 1). The mean stimulus duration was 6.59 s (0.42 SD) in Bengalese finches and 6.13 s (1.50 SD) in white-rumped munias. Song stimuli were different between the two strains in relation to complexity of song sequences measured using linearity index, sound tonality measured by entropy of sound spectrums and variation of song note duration (see the details in Table S2, Table S3, Figure S1). Three repetitions in song phrases were edited and the phrases were separated by silent intervals. The duration of the song stimuli was approximately 20 s. We presented the song stimulus once a minute, a total of thirty repetitions for 30 min.

We exposed birds to playback song stimuli to assess the differences in neural activation in response to strain-specific or other-strain songs in females. Birds in each strain were divided into three groups based on types of song stimuli and their rearing condition. First group consisted of socially reared birds that were exposed to song stimuli by own strain songs. Second group consisted of socially reared birds that were exposed to song stimuli by the other strain songs, e.g., Bengalese finches were presented to white-rumped munias' songs. Third group consisted of untutored birds that were exposed to song stimuli composed of own strain songs. Females were transferred to an individual sound proof chamber one day before playback was started (9:00–11:00 a.m.). All birds were exposed to unfamiliar song stimuli, which were presented using Sanwa (MM SPL2) speakers in the experimental cage and at an average of 70 (range 60–80) dB. Songs were presented 2 h after the lights were turned on in the experimental chamber. Birds were decapitated after 30 min of song presentation. Brains were extracted immediately after the song presentation and embedded into an optimal cutting temperature (Sakura Fine Technical, Tokyo, Japan) medium and frozen at -80°C . Brain tissues were sectioned into 20- μm parasagittal slices and mounted on a slide glass for *in situ* hybridization.

Digoxigenin-labeled probes were generated by PCR amplification of *zenk* cDNA cloned into the pDNR-LIB vector (accession No. DC289531) using pDNR-LIB universal primers. The T7 promoter sequence was added to the antisense primer to synthesize antisense probes (sense primer: 5'-AGTCGACGGTACCGGACATA-3'; antisense primer: 5'-TAA TACGACTCACTATAGGGGC CAAACGAATGGTCTAGAAAG-3'), and the SP6 promoter sequence was added to the sense primer to synthesize sense probes for the negative control (sense primer: 5'-ATTAGGTGACACTATAGAAAGTTCGACGGTACCGGACATA-3'; antisense primer: 5'-GCCAAACGAATGGTCTAGAAAG-3'). PCR products were purified using Wizard SV Gel and a PCR Clean-up System (Promega, Madison, Wisconsin, USA). The probes were synthesized using T7 or SP6 RNA polymerase (Roche Diagnostics, Rotkreuz, Switzerland) with digoxigenin RNA labeling mix (Roche Diagnostics). Sections from three experimental groups within one batch were treated to avoid variability in staining. The samples of the same batches were processed at the same time. The total of incubation duration was 7 h for all batches. The procedures of *in situ* hybridization were performed following methods in the previous study that suggested the differential activation to song stimuli between familiar and unfamiliar songs in female Bengalese finches [16,19].

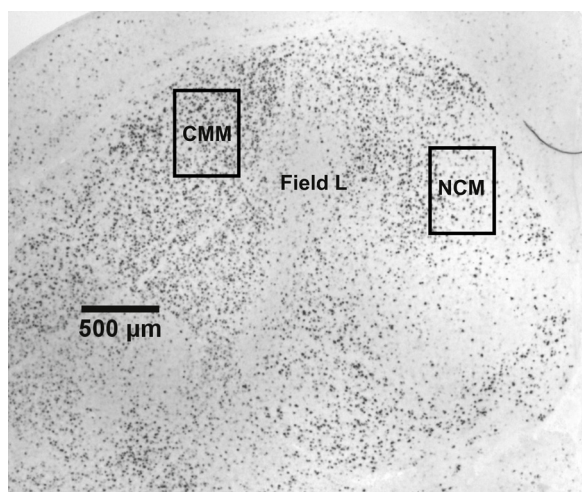


Fig. 2. Placement of the counting frames to quantify *zenk*-labeled cells within the CMM and NCM.

zenk-labeled cells were quantified by analyzing microscopic images of each selected area (Fig. 2, microscope: Eclipse E600, Nikon, Tokyo, Japan, software: NeuroLucida, MicroBrightfield, Williston, Vermont, USA). The actual image size was $420 \times 560 \mu\text{m}$ (magnifications: $\times 20$ objective and $\times 10$ eyepieces). We defined the image area using anatomical landmarks as described previously [16]. For the CMM, we recorded images in the most dorsal and caudal area between Field L and the lamina mesopallialis. For the NCM, we placed the image corner on the most caudal and dorsal edge (Fig. 2). *zenk*-labeled cells were automatically counted in the images using Image J software (version 1.38). The counting threshold was defined as the stained cells were detected. The threshold was fixed in all sections of the tissue. To determine the optimal counting threshold of the automatic function, we randomly selected 8 sections in each strain per staining batch in a total of 32 sections. The number of *zenk*-labeled cells was visually counted by an observer blind to all experimental conditions and sectional areas. We calculated the correlation between the number of cells in all selected section between the manual and the automatic counting. The threshold of the automatic function with the highest correlation value (> 0.9) to manual counting was selected to count the number of the stained cells in all sections using Image J. The average number of *zenk*-labeled cells was obtained by counting the number of consecutive sections in the CMM and NCM in each female. The mean number of counted slices per bird was 11.88 ± 2.08 in the CMM and 11.75 ± 2.08 in the NCM. The sampling region was approximately $400\text{--}900 \mu\text{m}$ from the midline.

We performed generalized linear mixed model analysis to compare *zenk*-labeled cells among the three groups including 6 birds in each strain based on rearing conditions and song stimuli; socially-reared birds exposed to songs of their own strains, socially-reared birds exposed to songs of the other strains, and untutored birds exposed to songs of their own strains. The body sizes were different between Bengalese finches and white-rumped munias. Therefore, we did not directly compare the groups across the two strains. We performed all analyses using the R 3.4.2 software system. We constructed a model with Poisson distribution and logit-link function, and with individual as a random factor using package lme4. We compared models using chi-square statistics. We tested the significance of group contrasts using the multcomp package as post-hoc tests.

Based on our analyses, we observed that the number of *zenk*-labeled cells in response to song stimuli in the CMM was not significantly different among the three different groups in Bengalese finches; however, significant differences were observed in the number of *zenk*-labeled cells in the CMM of white-rumped munias (glmm, $\chi^2 = 6.56$; $p < 0.05$, Fig.3 (A), (B)). Post-hoc tests indicated that socially-reared birds

exposed to songs of their own strains had significantly higher *zenk*-labeled cells than socially-reared birds exposed to stimuli of other strains (Tukey's test, $p < 0.01$).

Furthermore, no significant differences were observed in response to song stimuli in the NCM among the three groups of Bengalese finches. However, consistent with the observations in the CMM, the number of *zenk*-labeled cells in the NCM was significantly different among the groups of the white-rumped munias (glmm, $\chi^2 = 10.21$; $p < 0.01$, Fig.3 (C), (D)). Post-hoc tests indicated that socially-reared and untutored bird groups exposed to song stimuli of their own strains had significantly greater *zenk*-labeled cells than socially-reared groups exposed to the song stimuli of other strains (Tukey's test, socially-reared; $p < 0.05$, untutored; $p < 0.001$).

Bengalese finch's songs evolved to comprise more complex and tonal features throughout domestication than those of their wild strains, white-rumped munias. It is possible that female song discrimination have also changed between the two strains. We conclude that female white-rumped munias were more responsive to song stimuli of their own compared with those of other strains. However, the female Bengalese finches did not show a neural activity bias for songs of their own strains. This suggests that white-rumped munias possess higher sensitivity for identification of songs of own strains than the Bengalese finches.

Relaxed or weakened selection pressures in environment are considerable factors to understand the evolutionary background of phenotypic differences [2,20]. Song discrimination in Bengalese finches may change throughout domestication, which is critical for accurate species identification for appropriate breeding in wild settings. Conversely, domestic birds could readily find their mate without accurate species identification for breeding success. Relaxed environmental pressures during domestication may have weakened the functionality of songs as species recognition in Bengalese finches. Detailed understanding of the neural mechanisms underlying the differences in song discrimination remains to be determined. It is possible that neural mechanisms, such as sensory gating for processing of songs of own species, may also change throughout domestication.

There are other possibilities to explain why Bengalese female finches did not discriminate songs of their own strains from those of white-rumped munias. Possibly, Bengalese female finches may have preserved song discrimination for their wild ancestors as the short domestication period might not have allowed the diversification of the song recognition system. We need to conduct studies using females birds of both strains exposed to heterospecific songs and compare their responses. In addition, it should be considered the possibility that the song divergence between the two strains has been encouraged not only by female song responses but also by male song production abilities. We could not determine which factor is primary to promote the song evolution between the two strains.

Previous studies have suggested that the amount of *zenk*-labeled cells decreased when birds were reared without hearing conspecific songs [21]. Our result did not indicate the differences in the amount of *zenk* expression in response to conspecific songs with respect to rearing condition between socially-reared birds and untutored birds in the two strains. Previous studies have reported that untutored birds did not have song discrimination to conspecific songs [22]. Our results suggest that untutored white-rumped munias exposed to their own songs have greater *zenk*-labeled cells in the NCM than socially-reared groups exposed to the other strains' song. We could not statistically compare the responses to the song stimuli of other strain of untutored birds owing to the limited number of subjects. Unfortunately, it was difficult to breed wild birds to get enough subjects. Therefore, we could not determine whether the song strongly responses to the own strains songs in white-rumped munias were acquired or innate. Although the data was lacked, our result indicated important suggestions that listening to conspecific songs during juvenile could not affect to suppress *zenk* expression to the other strain's songs in Bengalese finches.

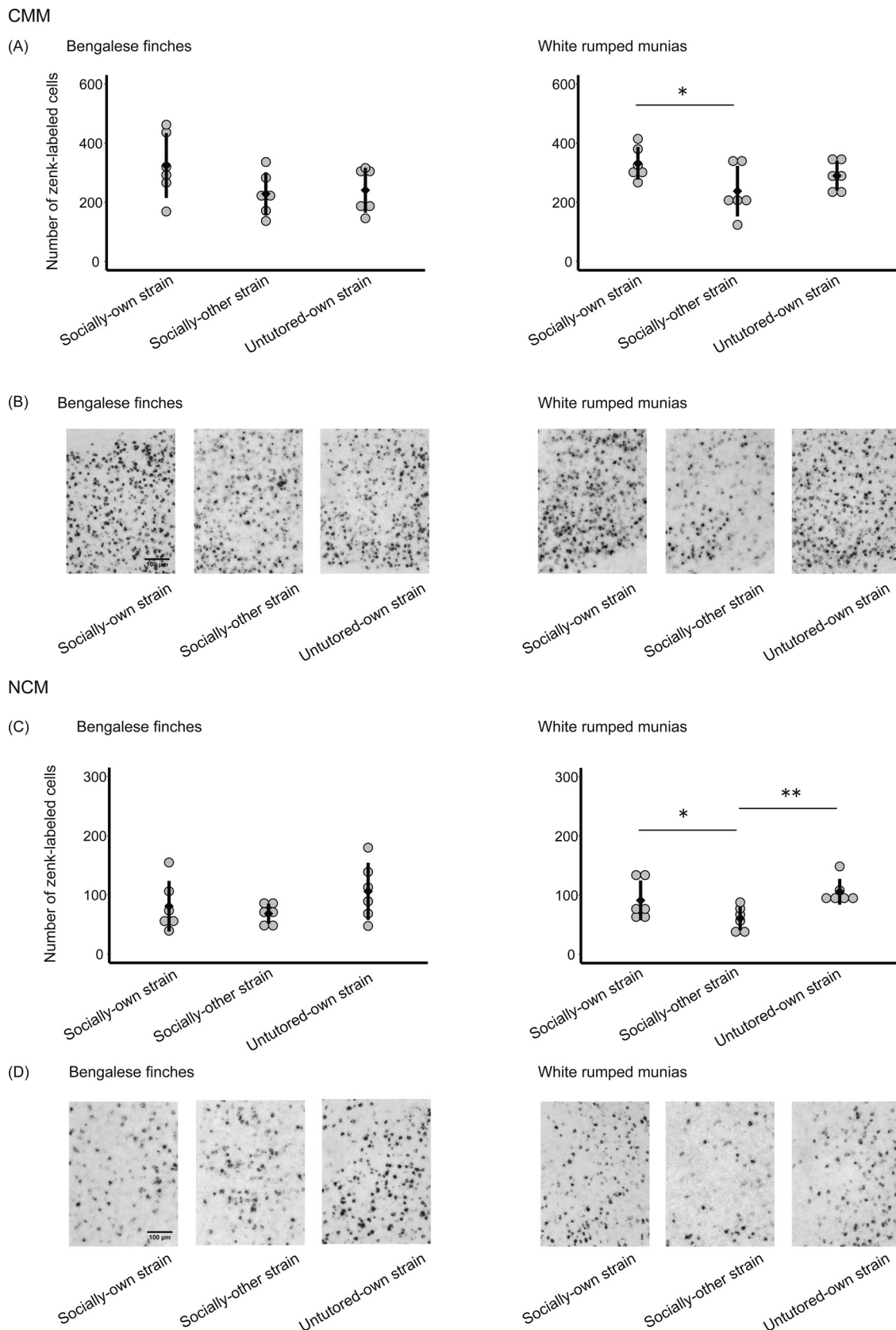


Fig. 3. Neuronal activation in CMM and NCM after exposure to each song stimuli in each group of Bengalese finches and white-rumped munias. Black circles and error bars indicate mean values and standard deviations of the group (* $p < 0.05$, ** $p < 0.01$, posthoc-test).

(A) Mean number of *zenk*-labeled cells in the CMM. Socially-reared white-rumped munias exposed to own songs have significantly greater *zenk*-labeled cells than socially-reared munias exposed to other strain's songs ($\chi^2 = 6.56$; $p < 0.05$). There are no significant differences in Bengalese finches. (B) Examples of *zenk* expression in the CMM for each stimulus in the two strains. (C) Mean number of *zenk*-labeled cells in the NCM. Socially- and untutored-reared white-rumped munias exposed to songs of own strains have significantly greater *zenk*-labeled cells than socially-reared munias exposed to songs of other strain ($\chi^2 = 10.21$; $p < 0.01$). There were no significant differences in Bengalese finches. (D) Examples of *zenk* expression in the NCM for each stimulus in the two strains.

We observed that white-rumped munias responded more to songs of own strains compared with those of other strains. Our study agreed with previous findings in the cross-fostering study, which indicated differences in song learning strategy in males between the two strains [7]. White-rumped munias learn their songs more accurately than the songs of other strains. Conversely, Bengalese finches don't have strong learning bias for songs of their own strains. Therefore, males and females could have common neural system for song memory. Future studies should focus on female song discrimination in cross-fostered birds.

CRedit authorship contribution statement

Hiroko Kagawa: Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Yoko Kato:** Investigation, Conceptualization, Methodology, Writing - review & editing. **Kenta Suzuki:** Resources, Investigation. **Masaki Kato:** Resources, Methodology, Writing - review & editing. **Kazuo Okanoya:** Conceptualization, Supervision, Funding acquisition.

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