Expanded song learning capacity in interspecies hybrid songbirds

（異種間雑種歌鳥の歌学習能力拡張についての研究）

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Contents

General introduction

Species-diversity in vocal learning capacity in songbirds

Learning is defined as sustained behavioral changes based on experiences (Manning & Dawkins, 2012; B. R. Moore, 2004; Pearce, 2013; Thorpe, 1956; Warren, 1973). While modulation or acquisition of behaviors through learning helps animals adapt to the environment coping with unpredictability in their habitat such as predator species or foraging sites (Brown, 2003; Ferrari et al., 2007, 2008; Fischer, 2020; Herzog & Hopf, 1984; Hollén & Manser, 2006; Holzhaider et al., 2010; Manassa & McCormick, 2012; P. R. Marler, 1982; Mateo, 1996; McQuoid & Galef, 1992; Mineka & Cook, 1988; Seyfarth et al., 1980), learning incorrectly or more than necessary is rather a waste of energy and results in a decrease in fitness (Laland & Williams, 1998). Thus, the learning abilities of each species have evolved under genetic constraints in several ways (Manning & Dawkins, 2012; B. R. Moore, 2004; Pearce, 2013): (1) the types of behaviors that can be acquired through learning, such as foraging site selection or predator avoidance; (2) the sensory stimuli relevant to learning, such as vision, audition, or olfaction; (3) the capacity for learning variation, including how many different stimuli can be remembered or how many behavioral repertoires can be acquired; (4) the speed of learning; (5) memory retention, and (6) the time window (e.g., critical period) during which effective learning can occur.

Vocal communication in animals either relies on innate or learned vocalization patterns. Vocal learning is limited in several mammalian species and almost half of bird species (Catchpole & Slater, 2003; Kroodsma & Miller, 1982; P. Marler & Peters, 1977; P. Marler & Tamura, 1964; Tyack, 2020). Oscine songbirds are one of the representative groups of vocal learners, which includes more than 5,000 species (Goller & Shizuka, 2018; Xu et al., 2019) with prominent diversity in songs (Catchpole & Slater, 2003; Kroodsma & Miller, 1982; Okanoya, 2004; Poulsen, 1959). Except for some dueting species in which females also learn songs (Catchpole & Slater, 2003; Garamszegi et al., 2007), usually males learn songs. Functionally, songs are a part of courtship behavior and serve as informative signals for conspecific recognition (Becker, 1982; Bremond et al., 1968; P. Marler, 1958) and for the evaluation of the fitness of males by females (Thorpe, 1958; Vallet et al., 1998; Vallet & Kreutzer, 1995). Besides, songs contain other information such as individual identity (Lind et al., 1996; D. B. Miller, 1979) which is used for territorial defense (McDonald, 1989; Peek, 1972), or local dialects of song reflect information about their habitat (Bjerke and Bjerke, 1981; Baptista 1975). While conspecific recognition based on courtship sound does not requires learning in other animals (in

crickets *Teleogryllus*: Hoy and Paul, 1973; Walker, 1957; in *Drosophila*: Wheeler *et al.*, 1991; Kyriacou & Hall, 1982; in *Xenopus*: Yamaguchi and Peltier, 2023; in gibbons *Hylobates*: Brockelman & Schilling, 1984), learning aspects of bird songs are thought to enrich the information that can be acquired from a song by reflecting potential fitness or habitat of each male as mentioned above.

Song learning vary among species regarding rigidity of species specificity, fidelity of copying tutor songs, song/syllable repertoire size (number of different song phrases or syllables which one bird can sing), learning speed, and timing of the critical period for song learning (Eales, 1985; Eens et al., 1992; Hultsch & Kopp, 1989; Hultsch & Todt, 1989; D. E. Kroodsma, n.d.; D. E. Kroodsma & Pickert, 1980; Kroodsma & Miller, 1982; P. Marler, 1970a, 1984; P. Marler & Peters, 1982a, 1982b, 1988; Mountjoy & Lemon, 1995; Nordby et al., 2001; Nottebohm et al., 1986; Nottebohm & Nottebohm, 1978; Payne, 1981, 1985; Peters et al., 1992; Petrinovich, 1985; Poulsen, 1959; Slater et al., 1988; Todt & Geberzahn, 2003; West & King, 1988). Generally, species with small song repertoires tend to have limited sensitive period for song learning (known as "closed-ended learners") and strong predisposition to learn conspecific songs, while species with large song repertoires retain plasticity to learn new songs as adults and have generalized copying fidelity in learning various sounds including songs of other species, suggesting that the evolution of these multiple aspects of vocal learning abilities are linked to some extent (Catchpole & Slater, 2003; Howard, 1974; Kroodsma, 2012; D. E. Kroodsma & Pickert, 1984; Kroodsma & Miller, 1982; Lovette & Fitzpatrick, 2016; C. M. Robinson et al., 2019; R. A. Zann, 1996). For instance, one of the most well-studied species, the zebra finch (*Taeniopygia guttata*, ZF), has a limited critical learning period as a juvenile, during which each bird learns a single song characterized by species-specific features, and the song remains unchanged throughout its life (Catchpole & Slater, 2003; R. A. Zann, 1996). On the other hand, lyrebirds, one of the most extreme learners, retain the ability to learn new songs as adults and can acquire countless different sounds throughout their lives (around 1000 song repertoire) including heterospecific songs, vocalization of other animal species, and even mechanical noise such as the sound of a chainsaw (Dalziell et al., 2021; Kelley & Healy, 2011; F. N. Robinson & Curtis, 1996; R. Zann & Dunstan, 2008). In most species with a limited size of song repertoire, learning heterospecific songs is not adaptive, as males that learn "wrong" song are less likely to be recognized as attractive by conspecific females and may not be successful in reproduction. Contrary to this, in species with extremely large song repertoires where males commonly mimic various sounds, heterospecific mimicry of other bird species' songs or the vocalizations of predator animals is thought to be useful in some way for repelling rival birds from their territory, and the abundance of the song repertoire itself attracts females (Brenowitz, 1982;

Catchpole & Slater, 2003; Hindmarsh, 1984; Lovette & Fitzpatrick, 2016). Despite the contrasting characteristics, vocal mimicry, an extremely flexible vocal learning, is suggested to have evolved from strict conspecific song learning (Dalziell et al., 2015a; Goller & Shizuka, 2018). Songbirds are an important example of an animal lineage with remarkable species diversity in vocal learning. As one approach to understanding the diversification of learning capacities, studying the neuro-molecular mechanisms underlying species differences in song learning will help clarify how genetic components shape qualitative and quantitative differences in vocal learning capacities within a taxon that shares similar brain structures. Additionally, it will provide insights in uncovering how vocal learning abilities evolved in a limited number of species.

Preserved neural circuit and species genetic constraints in song learning

Despite the existence of species diversity in song-learning, the neural circuits responsible for song learning and production, called the song system, are highly conserved in songbirds (**General introduction Fig. 1**). The song system consists of two major pathways: the vocal motor pathway (VMP), which is necessary for the acquisition and production of syllable acoustics or sequence patterns (Fee et al., 2004; Nottebohm et al., 1976; Yu & Margoliash, 1996) and the anterior forebrain pathway (AFP), which is needed for vocal learning in juveniles and the modulation of vocal variability (Andalman & Fee, 2009; Aronov et al., 2008; S. W. Bottjer et al., 1984; Brainard & Doupe, 2000; Goldberg & Fee, 2011; Kao et al., 2005; Ölveczky et al., 2005; Scharff & Nottebohm, 1991). Each pathway comprises multiple song nuclei, which can be histologically identified and are relatively well studied in terms of function. The VMP consists of the song nuclei HVC and RA, and the AFP consists of LMAN, Area X and DLM, all of these nuclei are common in songbirds (Zeigler & Marler, 2004). Some studies have suggested that species differences and intra-species individual variation in the volume and the number of constitutive neurons in song nuclei, particularly HVC, correspond to song complexity such as song/syllable repertoire size (Canady et al., 1984; Devoogd et al., 1997; J. M. Moore et al., 2011; Nottebohm et al., 1981; Pfaff et al., 2007; Ward et al., 1998). However, this rule cannot be applied to all songbird species (Baker et al., 1984; Brenowitz et al., 1995; Gahr et al., 1998a), indicating that the volume of the song nuclei (or the constitutive neuron number) is not the sole factor determining song complexity or learning capacity.

Learning bias towards conspecific song is guided genetically, which is supported by studies that examined song development of birds reared without auditory experience of tutor songs through social isolation or permanent auditory deprivation (Güttinger, 1981; Konishi, 1964; P. Marler & Peters,

1988; P. Marler & Sherman, 1983a, 1985; Mori et al., 2018; Mori & Wada, 2015). Although songs of those isolated or auditorily deprived birds generally were deficient in acoustic stability and clarity in their song syllables, they still retained species-specific song characteristics to some extent, such as duration of syllables/songs, the inter-syllable interval (ISI), or the syllable/song repertoire size. These studies suggest that each species innately has a motor bias known as "(preactive) song template" (Konishi, 1964, 1965; P. Marler, 1984; Thorpe, 1958) to produce songs with species-specific characteristics, although the auditory experience of tutor songs is still necessary to brush up song quality, such as detailed acoustic structures of each syllable or complex syllable sequence patterns. In a recent study, inter-individual transcriptome variation in a specific neuron type in the song nucleus RA is suggested to underlie the innate vocal motor bias that affects tutor song selection or the successfulness of learning given tutor songs (Toji et al., 2024), which can also be applied to inter-species differences to explain how innate motor bias supports to achieving good imitation of conspecific tutor songs. In addition to the innate motor bias in song production, young males also possess an auditory recognition bias towards conspecific songs, which is known as an "auditory template" (P. Marler, 1970b; J. Soha, 2017). The caudomedial nidopallium (NCM) and several other auditory areas show stronger responses in neural activity to species-specific acoustic sounds (Mello et al., 1992; Terleph et al., 2007; Theunissen & Shaevitz, 2006), which is suggested to facilitate conspecific-selective song learning in juveniles (P. Marler & Peters, 1977; Mundinger & Lahti, 2014; Thorpe, 1958). Auditory predisposition in recognizing species-specific sounds was suggested to more or less rely on genetic background (Hauber et al., 2001; P. Marler & Peters, 1977; Takahasi & Okanoya, 2010), while some other studies suggested that early auditory experience crucially affects establishing that (Jarvis, 2004; Jin & Clayton, 1997). Indeed, juveniles exceptionally learn heterospecific songs in some conditions, even in species that normally show selective learning towards conspecific songs (Baptista et al., 1981; Baptista & Morton, 1988; J. A. Soha & Marler, 2000), demonstrating that auditory bias for conspecific songs does not totally prohibit learning other species songs/syllables, rather it prioritizes learning conspecific songs in response to hearing them. In species with large song or syllable repertoire, both motor and auditory constraints are thought to be "loosened" to become more permmisive towards imitation of various sounds (Dalziell et al., 2015b; Goller & Shizuka, 2018), although the evolutionary process or genetic mechanism underlying the emergence of such loosened constraints remains unknown. Currently, no study has yet revealed the detailed neuro-molecular mechanisms sufficiently to explain how species differences in song learning capacities are regulated.

Because of the consistency of neural circuit anatomy, the histological clarity of the song

system, and the species differences in their songs, songbirds are a good model to study the neurogenetic mechanisms underlying the diversification of vocal learning among species. Previous studies revealed species differences in song nuclei-specific gene expression patterns (Asogwa et al., 2018; Hayase et al., 2021; Ko et al., 2021; Leung et al., 2011; Wada et al., 2013; H. Wang et al., 2019). As species' genetic constraints are suggested to exist in both auditory and motor learning processes in song learning, genetic signatures to explain the constraints should exist in the song system or brain regions related to auditory song perception. However, a comprehensive understanding of species differences in gene expression patterns in the song system and auditory areas is poorly explained, due to the missing links between previous studies focusing on different species, different brain areas and using different experimental strategies. To approach that, comparative studies examining transcriptome patterns among species and among brain areas including the VMP/AFP in the song system and auditory areas are needed to consider whether genetic constraints equally or differently affect all processes in song learning (hearing/memorization of the tutor song, reinforcement learning of the tutor song, or motor adjustment to produce songs similar to the tutor songs). Besides, examinations of the link between such gene expression differences and learning capacity based on a unified behavioral test are important as well.

Inter-species hybrid animals as a source of phenotypic diversity

To elucidate the neuro-molecular basis of species differences in behavior, comparative studies of behaviors and gene expression patterns among species with distinct behavioral patterns are necessary (Katz & Harris-Warrick, 1999). In addition, examining behaviors and gene expression in inter-species hybrids that possess two different species' genome information will be another strong approach (Bentley & Hoy, 1972; Mousseau & Howard, 1998; H. Wang et al., 2019). As hybrids inherit characteristics of each parental species in their behaviors, analysis of their behavioral patterns and changes in gene expression patterns compared to their parental species will provide clues to link phenotype and gene expression, leading to understanding how changes in gene expression patterns caused by crossbreeding affect their behaviors.

Generally, inter-species F_1 hybrid animals tend to show increased among-individual variation in their behavioral phenotypes with varied levels of reflection of parental phenotypes, indicating transcriptomic regulation changes based on interactions between different species' alleles. (Brockelman & Schilling, 1984; Derégnaucourt, 2010). In songbirds, several studies have investigated the song learning of F_1 hybrids between relatively closely related species (Takahasi et al., 2006; Toji

et al., 2024; Vokurková et al., 2013; H. Wang et al., 2019). Wang *et al.* (2019) and Toji *et al.* (2024) found that F_1 hybrids between ZF and owl finch (*Taeniopygia bichenovii*, OF) tend to learn songs with a bias towards one of the parent species or learn songs with intermediate characteristics. Vokurkova *et al.* (2013) reported that F_1 hybrids between the thrush nightingale (*Luscinia luscinia*) and the common nightingale (*L. megarhynchos*) in the wild populations, both species with large song repertoires and can mimic songs of the other species, incorporate songs from both parent species. As such, hybrid songbirds are a good source for studying the gradient diversity of song learning capacities. Investigating how phenotypes and gene expression in the F_1 hybrids change in comparison to the parental species helps to elucidate the neuro-molecular basis that governs the diversity of song learning phenotypes. Furthermore, phenotypic variation and novelty in F_1 hybrid animals tend to increase when crossing relatively distantly related but still breedable species (Atsumi et al., 2021; R. B. Stelkens et al., 2009). Based on that, I expected that a trial crossing more distant species than in previous studies result in the emergence of novel phenotypes that are not observed in either parental species, gaining insights into the genetic mechanism underlying the diversification of song learning.

The aim of this study

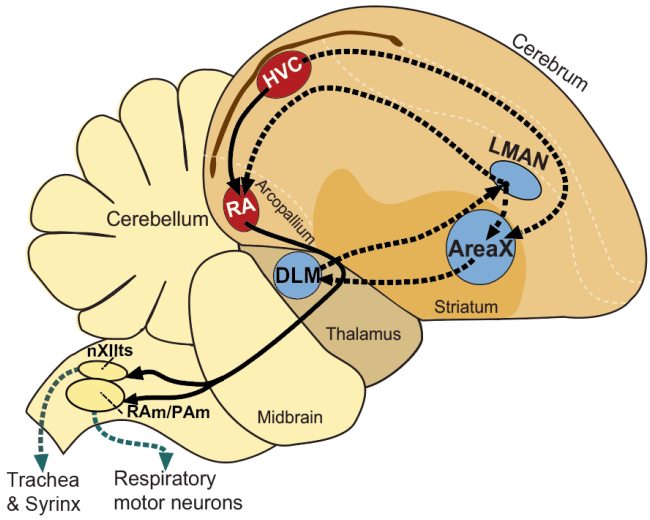
This study aims to elucidate the genetic basis of species-specific learning capacities in songbirds, which exhibit remarkable interspecies diversity. Through the trials of breeding various combinations of inter-species hybrid in Wada laboratory at Hokkaido University, we found that ZF and cherry finch (*Aidemosyne modesta*; CF) can be bred relatively stably. I utilized F_1 hybrid songbirds between these two species, expecting greater phenotypic changes or variability in their song learning capacities. The two species are phylogenetically more distant compared to the inter-species crossbreeding combination in the previous studies (Olsson & Alström, 2020; Payne, 1980; Toji et al., 2024; Vokurková et al., 2013; Wang et al., 2019), except for one study on F_1 hybrids between female ZF and male Bengalese finch (Takahasi et al., 2006), which produced only one hybrid individual. Here, by comparing the results of this study with the results of those previous studies, I also aimed to examine how genetic heterogeneity between the parents affects the song learning capacities of offspring.

In **Chapter I**, to examine how genetic backgrounds from different species interact in the regulation of song learning capacities, I tested song learning capacities in ZFs, CFs, and their F_1 hybrids by tutoring them with the same set of tutor songs under a controlled rearing environment. I found that song learning capacities in the F_1 hybrids expanded compared to either parental species.

In **Chapter II**, to investigate mesoscopic anatomical changes related to changes in song learning capacity in the F_1 hybrid individuals, I examined whether the expanded song learning capacities of the F_1 hybrids are accompanied by anatomical changes such as an increase or decrease in the volume of song nuclei or the number of constitutive neurons in the song system. Although previous studies reported the significance of the correlation between song nuclei size and/or neuron numbers and song complexity (Canady et al., 1984; Devoogd et al., 1993; J. M. Moore et al., 2011; Nottebohm et al., 1981; Pfaff et al., 2007; Ward et al., 1998), no studie has examined such brain anatomy in F_1 hybrid birds. Therefore, I included five major song nuclei from both the VMP and AFP in the comparison of song nuclei size and constitutive neuron numbers among parental species and the F_1 hybrids. These mesoscopic brain anatomical features in the F_1 hybrids were at intermediate levels between the two parental species and were unlikely to be the major factor underlying their expansion of song learning capacity.

In **Chapter III**, to identify the song nuclei and cell types associated with differences in song learning capacity between the parental species and the F_1 hybrids, I examined transcriptome differences in the song nuclei and auditory learning-related areas between the three groups at each cell-type level. Although previous studies found a significant correlation between specific gene expression differences in song nuclei and song acoustic features (Toji et al., 2024; Wada et al., 2013; H. Wang et al., 2019), many other genes remain unexplored. Furthermore, by comparing the transcriptomes of different cell types within the song nuclei, it is possible to investigate which cell types are likely to be involved in functional differentiation. This approach will allow for a more accurate estimation of the neural mechanisms underlying differences in song learning capacities. I performed single-nucleus RNA-seq (snRNA-seq) using brain tissue from the VMP (HVC, RA), AFP (Area X), and auditory area (NCM). I found prominent transcriptome differences among the two parental species and the F_1 hybrids specific to glutamatergic excitatory projection neurons in the VMP.

Finally, based on the overall results, I will discuss the potential mechanisms underlying the regulation of learning capacities in the F_1 hybrids and the possible scenario of the evolution of song learning diversity.



General introduction Fig. 1.

The song system

Black solid arrows and dashed arrows each indicate the VMP and AFP projection.

Red ovals: song nuclei composing the VMP, blue ovals: song nuclei composing the AFP.

Chapter I

Expanded song learning capacity in F_1 hybrids between the zebra finch and cherry finch

1.1 Introduction

Despite sharing a conserved neural circuit, the song system, which is specialized for song learning and production (S. W. Bottjer et al., 1989; Foster et al., 1997; Johnson et al., 1995; Nottebohm et al., 1976, 1982; Vates et al., 1997), songbirds show species diversity in their songs and song learning capacities. Bird songs are characterized by species-specific syllable acoustic features, syllable sequence patterns, and song tempo (Catchpole & Slater, 2003), each of which is suggested to develop through learning guided by genetic background (Güttinger, 1981; Konishi, 1964; P. Marler & Peters, 1988; P. Marler & Sherman, 1983b, 1985; Mets & Brainard, 2018; Mori et al., 2018; Mori & Wada, 2015). In addition, song/syllable repertoire size also characterizes each species, with genetic constraint also involves in determination of it (D. E. Kroodsma & Canady, 1985; P. Marler & Peters, 1982a, 1988).

Species with single song repertoire usually have a stronger bias towards songs that can be learned. If they are forced to learn songs of different species, they don't accurately mimic the heterospecific tutor songs (Clayton, 1989; Takahasi et al., 2006; Takahasi & Okanoya, 2010) or they alter learned heterospecific song patterns into their own species' song pattern afterward (Gardner et al., 2005). Besides, they don't acquire more than two different songs even if they hear songs of multiple conspecific adults, instead, they select one tutor or combine partial copies from multiple tutors into one song (Clayton, 1987; Mann & Slater, 1995; Takahasi et al., 2010). Therefore, in species with a single song repertoire, song features can basically be regarded as a reflection of the learning capacities of the species, although tolerance for heterospecific tutors varies to some extent. In the case of species with large learning capacity, the variation in song features and richness of the repertoire can be more affected by the quality of hearing experience; thus, we cannot precisely estimate their learning capacities in terms of learnable sound or repertoire size based solely on song recordings of wild individuals. What genetic mechanism regulates such species differences in song learning, both qualitatively (in syllable acoustics, sequence or tempo), and quantitatively (in repertoire size)?

Cross-breeding experiments have long been used as a research method to explore the relationship between genetic factors and phenotypes, and they remain a highly effective approach even today. In songbirds, many genes have been identified that exhibit species-specific differences in expression levels within song nuclei (Asogwa et al., 2018; Hayase et al., 2021; Ko et al., 2021; Leung et al., 2011; Wada et al., 2013; H. Wang et al., 2019), suggesting that species-specific song or song learning are regulated by polygenic mechanisms. Therefore, single-gene manipulation experiments do

not necessarily produce detectable changes in song learning or cause some abnormalities that are difficult to interpret in terms of their effect on the regulation of species-specific traits (Asogwa et al., 2018; H. Wang et al., 2019). Here, a close investigation of phenotypes and the transcriptome in interspecies hybrids will help to understand the molecular basis underlying natural phenotypic variation.

Previous studies using captive F_1 hybrids between ZF and the owl finch (OF) found that learned song phenotypes in the F_1 hybrids varied within the range of song features in the two parental species (Toji et al., 2024; H. Wang et al., 2019). A case in which F_1 hybrids show intermediate phenotypes with broader among-individual variation is common in both wild and captive animals (Dowling & DeMarais, 1993; Mallet, 1986; Nolte & Sheets, 2005). In addition, some interspecies crosses lead the hybrids to acquire a novel phenotype that falls outside the range of phenotypic variability of their parental species (Gilbert, 2019; Kagawa & Takimoto, 2018; Parsons et al., 2011; Richey, 1942; Rieseberg et al., 1999; R. B. Stelkens et al., 2009). Such phenotypic novelty tends to increase with the genetic distance between two parental species (Atsumi et al., 2021; R. B. Stelkens et al., 2009). Therefore, the consequences of song learning in F_1 hybrids may also change with the genetic distance between the two parental species.

Expecting a broader variation in song learning capacities, I focused on interspecies F_1 hybrids between ZF and CF (**Chapter I, Fig. 1-A),** toward understanding the genetic mechanisms that generate diversity in song learning. Both species are Australian finches whose wild populations' habitats overlap in the eastern part of Australia. Songs of the two species are acoustically distinct (**Chapter I, Fig. 1-B**): ZF songs are composed of around 3–5 different syllable types aligned in a fixed order (motif) with relatively short inter-syllable gaps (around 50 msec). CF songs (captive-bred individuals) are composed of 4–9 different syllable types, and the sequence includes both chunks with a fixed order of a couple of syllables and repetitions sung relatively more flexibly than in ZF, and the ISI is significantly longer than in ZFs. Quantitatively, fifteen syllable acoustic parameters (see methods) were compared, which contribute to the overall differentiation of syllable acoustics between ZF and CF (**Chapter I, Fig. 1-C).** Four acoustic parameters among the fifteen significantly differed between the two species: mean pitch, mean pitch goodness, variance in pitch goodness, and syllable gap duration (**Chapter I, Fig. 1-D**). The song learning capacity of CF has not been well characterized yet. However, based on reports about their song features (Immelman, 1982) and the song analysis I performed (**Chapter I, Fig. 1-B)**, at least this species has a single song repertoire and shares similar song features regardless of its source (several different local breeders).

In this chapter, I examined the song learning capacities of the two parental species and the F_1 hybrids by tutoring them with typical ZF and CF songs (referred to as "double-species song tutoring"). With partial reference to the previous studies on hybrid phenotypes (Toji et al., 2024; H. Wang et al., 2019), I examined the song learning capacities of the F_1 hybrids to determine whether the hybrid pupils exhibit one of the following patterns: "Dominance," where the pupils learn only ZF or CF song; "Intermediate," where the pupils combine syllables from both species' songs or produce acoustically intermediate syllables that do not resemble the tutor syllables; "Multiple," where the pupils accurately learn both ZF and CF songs; or "Improvisation," where the pupils develop novel songs that are distinct from those of either parental species (**Chapter I, Fig. 2-B**). Furthermore, to distinguish between innate predisposition and learning effects on syllable repertoire size in the F_1 hybrids, I compared the syllable repertoire size of hybrids tutored with double-species songs to those reared under a "non-song tutoring" condition, where they were not exposed to any tutor songs during the song learning phase. Additionally, I conducted two further experiments to investigate song learning capacities in the F_1 hybrids: the "four-song tutoring" experiment, designed to assess the maximum number of different syllable types the F_1 hybrids can learn, and the "heterospecific song tutoring" experiment, aimed at determining whether the F_1 hybrids can learn songs from other species with characteristics distinct from those of either parental species.

1.2 Materials and methods

Animals

ZFs and CFs were bred in our breeding colony established at Hokkaido University to obtain conspecific and F_1 hybrid offspring. Although I tried to obtain reciprocal hybrids, I could not get enough number of F_1 hybrids derived from CF♀ and ZF♂ to perform all the experiments in this thesis. Therefore, in this thesis, I compared the learning capacities of those reciprocal hybrids based on the limited number of tutoring experiments (see results section in this chapter) and for statistical tests in tutoring experiments or further experiments, only F_1 hybrids frim ZF♀ crossed with CF♂ were used. The birds were kept in an indoor aviary under a controlled photoperiod (13h light/11h dark), temperature (23-27℃), with free access to food and water. The sex of juvenile birds was determined by PCR (Soderstrom et al., 2007). All experimental procedures were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University. These guidelines adhere to the national regulations for animal welfare in Japan.

Preparation for song tutoring and recording

Juvenile song tutoring was conducted according to previously described methods (Hayase et al., 2018; Imai et al., 2016; H. Wang et al., 2019). A passive song playback system was implemented within sound attenuation boxes using recorded songs. To record songs, a microphone (XM8500-ULTRAVOICE; Behringer) was suspended near a perch from the top of the cage and connected to a sound amplifier (US-16-08; TASCAM). Songs were recorded at a sampling rate of 44 kHz and a 16-bit amplitude resolution using Sound Analysis Pro (http://soundanalysispro.com/). By 15 post-hatching days (phd), when hatchlings remained in the nest, they were separated from their male parents to prevent exposure to paternal songs. The critical period for song memorization in zebra finches typically begins at approximately 20–30 phd after fledging (Tchernichovski et al., 2001; R. A. Zann, 1996). Therefore, this timeline of father removal prevented juveniles from memorizing their biological father's songs. Note, however, that detailed growth curve in song learning of CF is not have been characterized yet. After removing the male parents, the juveniles were raised solely by their mothers. When the juveniles fledged, their mothers and other siblings were removed, and male juveniles were individually isolated in the sound attenuation box for song tutoring and recording (**Chepter I, Fig. 2-A**).

Double-species song tutoring

42 birds (ZF =12, CF = 9, and F_1 =21 birds) were tutored with the playback of recorded songs of the two parental species (*i.e.*, both ZF and CF songs) (**Chapter I, Supplementary Table. 1**). Song playback started at 33 ± 6 phd (mean ± SD) and continued until adulthood (>147 phd). Fourteen song playback sessions were scheduled daily, with seven sessions occurring each in the morning (8 AM–12 PM) and seven in the afternoon (1 PM–6 PM). In each session, one song file was randomly chosen from a collection of four stocked files with a total duration of up to 15 seconds (**Chapter I, Supplementary Fig. 2**), and was played from a speaker (MM-SPL11UBK; SANWA) at a volume of 55–75 decibels. The timing of song playback was controlled using Sound Analysis Pro, with a playback rate of 0.0025/s and intervals exceeding 20 seconds. During playback of tutor songs, an interval of silence ranging from 2.3 to 4.2 seconds was always interjected between renditions of ZF and CF songs, while keeping the same number of playbacks broadcast each day. The ZF and CF song models used for playback were originally recorded from a single ZF and CF male within our breeding colony, representing typical ZF and CF song vocalizations.

Non-song tutoring

To examine the effect of the auditory experiences of song models on syllable repertoire size, male F_1 juveniles (n = 6), different from those used in the double-species tutoring experiment, were individually housed without exposure to any tutor songs within a sound attenuation box from fledging until adulthood (>150 phd). The syllable repertoire size in adult songs of these F_1 hybrids was compared with that of the pupils in the double-species tutoring group.

Analysis of syllable acoustics

For each bird, an individual song syllable was defined as a vocal element within a song, punctuated from each other by a silent interval exceeding the segmentation threshold (set as 1/3 of the median value of 500 silent gaps in song bouts of the bird). Repetitive introductory notes were excluded from the analysis. High- and low-frequency background noise (below 0.50 kHz and above 18.0 kHz) was removed using the SASlab “Frequency Domain Transformation” function. The Audacity “Noise Reduction” tool was utilized for noise reduction in the middle-frequency range. After noise reduction treatment, the song files were segmented into individual syllable files using the SASlab batch processing function. To analyze species differences in syllable acoustics between ZFs and CFs, adult songs from 10 birds of each species were used. These ZFs were bred in our lab by normal ZF pairs,

and the CFs were bought as adults from breeders in Japan. Fifteen acoustic parameters were analyzed, including the following: unique syllable number (syllable repertoire size), syllable duration, inter-syllable gap duration, the ratio of syllable duration to inter-syllable gap duration, mean pitch, mean frequency modulation (mean FM), the square of mean amplitude modulation (mean AM^2), mean entropy, mean pitch goodness, mean frequency, variance in frequency modulation, variance entropy, variance pitch goodness, variance mean frequency, variance amplitude modulation (variance AM). Syllable identification was performed on randomly selected song recordings from each bird using Avisoft SAS Lab Pro or Song browser. The identified syllables were saved as separate WAV files, and 500 syllables per individual were used for the analysis. Sound Analysis Pro was used for calculating acoustic parameters, except for inter-syllable gap duration and the ratio of syllable duration to inter-syllable gap duration, which were manually calculated. The median value of 500 syllables was used as a representative value for each bird across the acoustic parameters.

Visualization of song sequential property

The syllable similarity matrix (SSM) method was utilized (Imai et al., 2016). Briefly, spectrograms of two different songs of a bird were aligned along the columns and rows of a matrix. For all column versus row syllable combinations, the similarity of the syllable spectrogram was calculated as the peak correlation coefficient through the round-robin comparison using the Avisoft CORRELATOR application. The similarity score (Φ_XY) between syllables a and b was calculated according to the following formula:

$$\Phi_{XY} = \frac{\sum_X \sum_Y ((a_{xy} - m_a) \times (b_{xy} - m_b))}{\sqrt{\sum_X \sum_Y (a_{xy} - m_a)^2 \times \sum_X \sum_Y (b_{xy} - m_b)^2}}$$

where m_a and m_b are the mean values of the spectrograms *a* and *b*, respectively. a_{xy} and b_{xy} denote the intensities of the spectrogram points at the locations x and y, respectively. $\Phi\_XY = 1$ indicates that syllables *a* and *b* are identical, whereas $\Phi\_XY = 0$ means that the two syllables are not similar at all. After generating the SSM on a spreadsheet, the matrix was converted into a binarized pattern consisting of black and white cells. The similarity score threshold of 0.6 was applied, wherein cells with a high similarity score (>= 0.6) were colored black, while those with a low similarity score (< 0.6) were colored white. The prevalence of characteristic patterns within the binarized "2 rows × 2 columns" cells in the SSMs was quantitatively analyzed to examine syllable temporal structures. The R software package was used to identify the most similar binarized pattern for each 2 × 2 cell in the SSM from a total of 12 possible patterns. Subsequently, the percentage of the following three transition types was calculated. Syllable transition type I, referred to as a "paired-syllables transition," indicates

the presence of two consecutive dissimilar syllables with identical sequential order in a pair of songs (e.g., "song bout 1 [··A B·····] vs. song bout 2 [····A B····]"). Syllable transition type II encompassed the "repetitive-syllables transition," occurring when two successive identical or highly similar syllables appeared in two songs (e.g., "song bout 1 [·····A A··] vs. song bout 2 [···A A·····]"). Lastly, syllable transition type III, termed a "nonmatching syllables transition," occurs when two consecutive syllables are different within a song and across two songs (e.g., "song bout 1 [····A B····] vs. song bout 2 [···C D·····]"). A, B, C, and D represent distinct syllables in the given examples.

Unsupervised identification and labeling of unique syllables

The unsupervised clustering method of T-distributed Stochastic Neighbor Embedding (t-SNE) (Maaten & Hinton, 2008) was used to identify unique syllable types in each bird. A total of 500–1,384 syllables per bird were analyzed to determine the syllable repertoire size. The syllables were sampled from multiple song bouts randomly selected from a day during the adult stage (145–184 phd for ZFs, 150–215 phd for CFs, and 130–181 phd for F_1 hybrids). For each syllable, 12 syllable acoustic parameters were measured, including syllable duration, mean pitch, mean frequency modulation, mean amplitude modulation, mean entropy, mean pitch goodness, mean frequency, variance frequency, variance entropy, variance pitch goodness, variance mean frequency, and variance amplitude modulation. Sound Analysis Pro was used to obtain these measurements. The resulting data files containing the values of 12 acoustic parameters were used to generate t-SNE plots. The cluster boundary was determined based on density-based spatial clustering of application with noise (DBSCAN). Syllables that could not be assigned to any cluster or belonged to small clusters comprising fewer than 30 syllables were excluded from further evaluation. The remaining clusters in the t-SNE plots were identified as unique syllable types.

Evaluation of syllable learning achievement

Unique syllables from both tutored model songs (using all syllables) and songs produced by pupil birds (10 syllables per unique syllable type) were employed. In the model songs of the parental species, the ZF and CF songs, there were six and four distinct syllable types, respectively. The similarity score of each tutor-pupil syllable pairing was calculated using Avisoft CORRELATOR. The 75th percentile values of the similarity scores for each syllable type pairing, comparing 10 spectrograms of an identical syllable type in a pupil song to all spectrograms of a specific tutor syllable type, were utilized as the representative similarity score. If the maximum value among the

representative similarity scores exceeded 0.6, the syllable type was classified as a "learned syllable." Conversely, if the maximum value was below 0.6, the pupil syllable was designated as a "novel (not learned)" syllable. The proficiency of syllable learning for each bird was determined using the following formula:

$$Syllable\ learning\ (\%) = \frac{(ZF(n)/ZF(N)) + (CF(n)/CF(N))}{2} \times 100$$

where ZF(n) and CF(n) represent the numbers of learned syllables from the tutored songs of ZFs or CFs, respectively. ZF(N) and CF(N) denote the total number of unique syllable types contained in tutor songs of ZFs and CFs (in the case of tutoring from both parental species' songs, ZF(N) was set at 6 and CF(N) was set at 4). When a pupil learns all syllables from only one of the two parental species but fails to learn from the other species' songs, the syllable learning score is 50%.

Evaluation of the upper limit of syllable learning capacity in F_1 hybrids (four-song tutoring)

Four F_1 hybrid males were tutored using songs from four tutor birds representing the parental species (two ZFs and two CFs) (**Chapter I, Fig. 5-A top**). The songs of the tutor birds were combined into a single playback. Each playback session included all four tutor songs, with 4–7 seconds of silent gaps between them, and the order of songs was randomized. As in the double-species tutoring, fourteen playback sessions were conducted daily, with seven sessions in the morning (8 AM–12 PM) and seven in the afternoon (1 PM–6 PM).

Evaluation of the quality of heterospecific song mimicry (heterospecific song tutoring)

Eight F_1 hybrid males were tutored using songs of genetically unrelated species (4 by OF songs, 2 by BF songs, and 2 by CN songs). For juvenile song tutoring using songs of genetically unrelated species (OF, BF, and CN), each recorded song file was randomly broadcast from a collection of 4–5 prepared song files (**Chapter I, Supplementary Fig. 7**). To evaluate the comprehensive quality of heterospecific song mimicry, syllable acoustics, syllable sequence patterns, and song-bout duration were compared between playback tutor songs and pupil songs. Syllable acoustic similarity between tutor songs/parental species songs and F_1 pupil songs was visually checked using a dimensionality reduction method Uniform Manifold Approximation and Projection (UMAP). Further, the Euclidean distance of syllable acoustics between pupils' songs and their corresponding tutor songs (Δsyllable acoustics) was used for quantitative assessment of syllable similarities, selecting an equal number of syllables from each pupil and the corresponding tutor songs (110–250 syllables, depending on the abundance of playback tutor song syllables). This Euclidean distance, calculated based on 12 acoustic

parameters also used for t-SNE plots, indicates higher acoustic similarity with smaller values. Syllable sequence pattern similarity was assessed by comparing the proportion of syllable transition types I and II (indicating motif-like and repetitive transitions, respectively) within 50 x 50 syllable SSMs of pupil and tutor songs. The differences in syllable sequence patterns were quantified as "Δ syllable sequence" using the formula: Δ Syllable sequence = (|tutor P1 – pupil P1| + |tutor P2 – pupil P2|) / 2, where "tutor P1" and "pupil P1" represent the proportion of syllable transition pattern type I in the SSM of tutor and pupil songs, respectively. Song-bout duration differences (Δsong bout duration) were assessed by calculating the average duration from 10 randomly selected song bouts for each pupil and using all available playback tutor song files for comparison. Differences in song-bout duration were calculated using the formula: Δsong bout duration = |(average tutor bout duration) - (average pupil bout duration)| / (average tutor bout duration). The quality of heterospecific song mimicry among ZF, CF, and F_1 pupils was visualized on a 3D plot of these three measurements. In this plot, a smaller distance from the origin (0, 0, 0) indicates a higher comprehensive similarity between tutor and pupil songs.

1.3 Results

Comparison of song learning capacities among the parental species and the F_1 hybrids

To examine how the F_1 hybrids learn songs when they are exposed to both parental species' songs and to compare song learning capacities between the hybrids and the parental species, double-species tutoring was conducted on juveniles of ZF, CF, and the F_1 hybrids. ZF and CF pupils learned their conspecific song parts at most but failed to mimic the heterospecific parts in the tutor song sets (**Chapter I, Fig. 3-A; Chapter I, Supplementary Fig. 3, 4**). To confirm whether they are unable to learn the heterospecific songs or simply prefer conspecific songs despite having the capacity to learn heterospecific songs, I additionally performed cross-species tutoring on the parental species, in which each pupil was exposed only to the other heterospecific part of the double-species tutor songs. When ZF and CF pupils heard only heterospecific songs, neither of them learned the heterospecific song models; instead, they retained certain features of their own species in their songs, such as ISI (**Chapter I, Fig. 1-E**). On the other hand, some F_1 hybrids exposed to double-species tutor songs learned both parental species' songs and maintained them even at the adult stage, despite the tutor song phrases of ZF and CF being distinct in terms of syllable acoustics, syllable sequence, and rhythm, which is mainly defined by ISI (**Chapter I, Fig. 3-A**). Among F_1 hybrids that learned both species' songs, variability in the rigidity of singing patterns among individuals was observed. For example, some individuals sang the two species' song phrases either in combination, independently, or in a flexible order, whereas others tended to sing the two phrases consistently in a fixed order (**Chapter I, Supplementary Fig. 5**). A similar case was also confirmed in reciprocal hybrids derived from crossing CF females and ZF males (n = 2; **Chapter I, Supplementary Fig. 1**). However, due to the insufficient sample size, those hybrids were excluded from statistical tests or other downstream experiments. Overall, F_1 hybrids (derived from crossing a ZF female and a CF male) showed increased variation in syllable learning achievement among individuals compared to either group of parental species: more than 50% of F_1 hybrids learned syllables from both ZF and CF tutor songs, some of them predominantly learned either the ZF or CF song, and others did not learn either tutor song well (**Chapter I, Fig. 3-B**). This variation in syllable learning achievement among individuals was not due to differences in the song tutoring start time (the range from 20 phd to 43 phd) or variation in the timing of vocal practice initiation (**Chapter I, Supplementary Fig. 6**). The group difference in syllable learning achievement in the double-species tutoring was significant, with the F_1 hybrids exceeding both parental species (Steel-Dwass test, F_1-ZF: $p = 0.017$; F_1-CF: $p = 0.0006$; ZF-CF: $p = 0.004$) (**Chapter I, Fig 3-B**). Note,

however, that the data raised concerns about selecting the best-fitted statistical test, as both normality and homoscedasticity could not be assumed. Therefore, I also examined the significance of group differences using the Kruskal-Wallis test (H = 22.12, p = 1.57e-05), and applied pairwise permutation test with Bonferroni correction as post-hoc test, as a method that can be used for nonparametric and non-homoscedastic data. The number of permutations was set to 10000. Statistical significance also remained between the F_1 and ZF (p = 0.0081), the F_1 and CF (p = 0.0003), and ZF and CF (p = 0.0030).

Examination of innate or learning effects in increased unique syllable numbers in F_1 hybrids

In addition to the number of learned syllable types, similar increases in both average and among-individual variation were found in the syllable repertoire size of F_1 hybrids compared to the parental species. (**Chapter I, Fig 4-A**, left three groups). Here, syllable repertoire size contains not only learned syllables but also improvised syllables that do not resemble any tutor syllables. Moreover, the number of syllable types learned from the tutor songs and the syllable repertoire size of each pupil were positively correlated (Spearman's rank correlation, $p < 0.001$ $r = 0.75$) (**Chapter I, Fig 4-B**).

Based on this result, I considered two possibilities to explain the increased syllable repertoire size in some F_1 hybrids: (i) the F_1 hybrids are innately predisposed to produce more syllables than the parental species regardless of auditory experience, or (ii) the increased syllable repertoire in some F_1 hybrids reflects increased syllable learning capacities rather than innate predisposition. To examine these possibilities, I compared syllable repertoire size between the double-species tutored group and the other group reared without hearing any tutor songs. If syllable repertoire size is innately predetermined, the hybrids should possess similar syllable repertoire sizes regardless of their auditory-rearing environment. As a result, F_1 hybrids without song tutoring developed relatively simple songs (**Chapter I Fig 4-C**). Their syllable repertoire size was similar to the parental species groups and significantly smaller than that of double-species tutored F_1 hybrids (Wilcoxon rank sum test, $*p = 0.012$) (**Chapter I Fig 4-A**). These results support the second possibility that syllable repertoire size in the F_1 hybrids is not genetically predetermined before learning. Instead, some of the F_1 hybrids possess an expanded capacity for syllable learning, which becomes apparent when exposed to tutor songs with a highly abundant syllable set.

The upper limit of unique syllable number learned by F_1 hybrids

A further question about the expansion of song learning capacity in the F_1 hybrids is its limitation: how many different syllables or songs can they learn at most? To examine this, I tutored

four F_1 hybrid males with a new tutor song set, which contained four different songs (two ZF and two CF songs), named "four-tutor songs" (**Chapter I, Fig 5-A, top**). As a result, no pupils copied all the 18 syllables from the tutor song set. However, among the four F_1 hybrid pupils tutored with four-tutor songs, two birds learned syllables from more than three different songs. Those two pupils copied not entire songs but only some parts from each tutor song and combined those "clipped-out" short song phrases into a long bout (**Chapter I, Fig 5-B**). In their songs, the structure of each clipped phrase was highly stable, with syllable order and inter-syllable intervals retained as in the original tutor songs. The transition patterns of those clipped-out phrases also seemed consistent within each pupil, although they sometimes changed the ending point of each bout. For example, sometimes an F_1 hybrid pupil sang the clipped-out phrases [A'-B'-C'-end] in one bout (here A' means clipped song phrase from song A), but at another time the same pupil also sang [A'-B'-end]. The number of repetitions of each clipped-out phrase was relatively flexible. They sometimes sang the same single phrase repeatedly, and the maximum syllable repertoire size of the four pupils was 13 (**Chapter I Fig 5-B**), which was not significantly increased compared to that of F_1 hybrids tutored with double-species songs (**Chapter I Fig 5-C**).

Heterospecific song learning in the F_1 hybrids

Based on the expanded learning capacities of the F_1 hybrids for various syllable acoustics and sequence patterns, finally, I wondered if the F_1 hybrids could even learn unrelated species' songs with different syllable acoustics and sequences compared to the parental species. To examine this, I tutored the F_1 hybrids using the songs of genetically unrelated species ranging from closely related to distantly related species: the OF, BF, and CN (**Chapter I, Fig 6-A**; **Chapter I, Supplementary Fig 7**). In all song tutoring conditions, some F_1 hybrids mimicked these heterospecific tutor songs (**Chapter I, Fig 6-B; Chapter I, Supplementary Fig. 8-A, B, C**): F_1 hybrids tutored with OF songs perfectly mimicked OF songs. The F_1 hybrids tutored with BF songs learned syllable acoustics and sequence patterns from the model songs, although their song sequence was less flexible than real BF songs. The F_1 hybrids tutored with CN songs also mimicked some syllables and repetitive phrase structures from the tutor songs; however, they did not copy high-frequency syllables, and both repetition length and song-bout length were shorter than those of real CN songs. Finally, song similarity among the heterospecific tutor songs, the F_1 pupils, and the songs of the parental species was evaluated based on three features that characterize species-specific songs: syllable acoustics, sequence patterns, and song-bout duration. Syllable acoustic similarities were quantitatively assessed

based on the Euclidean distance of syllable acoustics using 12 acoustic parameters and visually represented by the UMAP, indicating that the syllable acoustics of F_1 hybrids tutored with OF, BF, or CN songs were similar to their own tutor species rather than other tutor species or their parental species (**Chapter I, Fig 6-C**). SSM patterns of F_1 pupils were distinct among pupils that heard different heterospecific songs, and each of them showed SSM patterns similar to those of their tutor songs (**Chapter I, Fig 6-D; Chapter I, Supplementary Fig. 8-D**). Regarding song-bout duration, F_1 pupils tutored with long BF or CN songs tended to sing relatively long bouts, whereas F_1 pupils tutored with short OF songs did not show such elongation in their song bouts. Although two ZF pupils learned syllable acoustics and some repetition of syllables from BF songs, the duration of song bouts of those ZF pupils was much shorter than the original tutor songs and was instead similar to the normal ZF motif. In summary, although the sample size is not large enough to confirm statistical significance, in all three unrelated species' song tutoring conditions, multiple F_1 hybrid pupils learned syllable acoustics, syllable sequence patterns and song bout duration of the heterospecific songs more accurately than their parental species counterparts did (**Chapter I, Fig 6-E**).

Summary of song learning capacities in the F_1 hybrids (ZF female x CF male)

1. The F_1 hybrids had increased song learning capacity in terms of song (phrase) repertoire, with some birds acquiring two distinct song phrases (double-species tutoring, **Chapter I, Fig. 3 and 5)**
2. The F_1 hybrids are suggested to have increased learning capacity in terms of syllable repertoire size as well, which is apparent only when exposed to complex tutor song (double-species tutoring, four-song tutoring, and non-song tutoring, **Chapter I, Fig. 3, 4, and 5)**
3. The F_1 hybrids are capable of learning heterospecific songs that are dissimilar to the songs of either parental species (heterospecific song tutoring, **Chapter I, Fig. 6; Chapter I, Supplementary Fig. 8**) better than either parental species.
4. Points 1 and 2 co-occur, however, further examinations are needed to determine whether the hybrids are capable of learning multiple heterospecific songs (1 and 2 and 3).
5. In all cases, F_1 hybrids showed among-individual variability in the learning capacities mentioned above.

1.4 Discussion

Based on the results of double-species song tutoring, I found that F_1 hybrids between ZF and CF showed expanded song-learning capacities compared to the two parental species. While ZF and CF are single-song singers biased toward learning conspecific songs, their F_1 hybrids learned and produced both parental species' songs composed of different syllable acoustics and sequence patterns. The two learned species' songs were maintained in one-year-old adults (**Chapter I, Supplementary Fig. 9**), suggesting that the increase in learned syllable types differs from "overproduction" in juveniles followed by attrition of syllable repertoire afterward, which was reported in some species such as swamp sparrow (P. Marler & Peters, 1982a). Although I observed a wide range of individual differences in their syllable learning achievements in the double-species song tutoring, with more than 50 % of F_1 hybrid pupils exceeded either parental species in terms of learned syllable numbers.

From a critical point of view, a detailed characterization of CF song learning including development is desirable to support the expansion of song learning capacities in the F_1 hybrids. Currently, the details of the sensory learning period and sensory-motor learning period in CF remain unclear. Based on my observation that CFs did not alter their song once it had crystallized at around 180 phd, they are surely closed-ended learners and should have a critical period during the juvenile stage, similar to ZF. The end of the critical period seemed earliest in ZF, intermediate in the F_1 hybrids, and latest in CF, although I have not analyzed this in detail. In this study, learning achievement in CFs was worse than that of F_1 hybrids or ZFs in all tutoring experiments. If we assume that CF has a similar timeline for the critical period as ZF, one possibility for the low rate of successful learning in CF may be the delay in tutoring (**Chapter I, Supplementary Table. 1**). The reason for the delay was that we had to obtain some CF juveniles from local breeders to compensate for the low success rate in breeding CFs in the lab due to poor parental care of chicks. However, the average age at the start of tutoring was not significantly different from that of the F_1 group (34.2 phd in F_1 and 36 phd in CF), and some hybrid individuals still learned both parental species' songs well in double-species tutoring experiments (e.g. three birds with learning achievements greater than 79% started receiving tutor song playback at 35-41 phd). In addition, CF actually may have had low fidelity in learning songs, based on the following observations: (i) even one CF in double-species tutoring who was born in our lab and started tutoring earlier (23 phd) did not produce an accurate copy of the CF tutor song, and (ii) another CF reared by a foster pair (a ZF female and a CF male) was kept with his foster CF father from before hatching and learned his song from the foster CF but still incompletely, even though learning from a

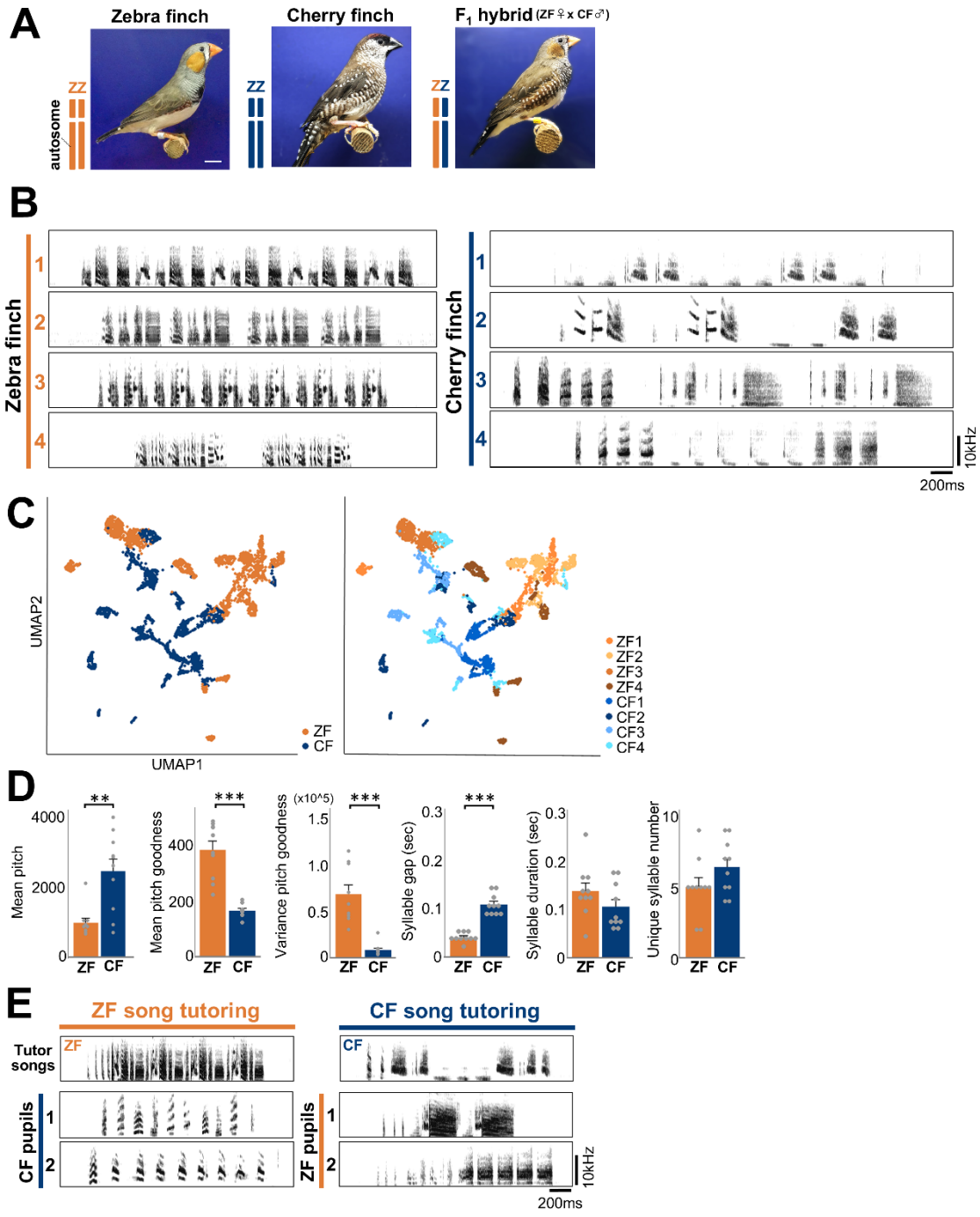
real tutor has a strong effect on better learning compared to passive playback tutoring (Chen et al., 2016; Eales, 1989). In conclusion, even though I cannot deny the possibility that the CF song learning capacity in terms of syllable repertoire might have been underestimated in this study due to some technical limitations, at least the expansion of learning capacitiy in maintaining two distinct song phrases is clearly novel to the F_1 hybrids. A further study comparing the critical period among those two parental species and the F_1 hybrids would provide more insight into how genetics affects the regulation of species-difference in learning time windows.

The phenomenon in which hybrids exceed either parental species' phenotypes is called heterosis (East, 1940; Shull, 1908). In addition to the double-species song tutoring, non-song tutoring and four-song tutoring using songs from two ZFs and two CFs demonstrated that the F_1 hybrids had increased syllable learning capacity. Moreover, the F_1 hybrids even learned genetically unrelated heterospecific songs other than their parental species, which were not seen in their parental species. In animals, heterosis is reported in phenotypes such as body size, stress tolerance, disease resistance, or milk production (e.g., the liger derived from crossing a female tiger with a male lion, the mule derived from crossing a female horse with a male donkey, or hybrid cows produced by crossing different lines) (Bryant et al., 2007; Livesay, 1930; WARREN, 1927). However, only a few studies have examined heterosis in cognitive or learning capacities under well-controlled environments. In several studies focusing on species other than songbirds, three studies reported positive heterosis, in visual discrimination learning in mules (female *Equus caballus* x male *Equus asinus*) (Proops et al., 2009), better spatial memory in F_1 mice between different strains (Lassalle et al., 1991), and a higher success rate in guide dog training for mixed-breed dogs (Ennik et al., 2006), although the last two studies focused on strain-hybrids, not inter-species hybrids. Another study reported that F_1 and F_2 hybrid fish of the family *Poeciliidae* showed an intermediate level between the parental species in associative and reversal learning using visual cues (Vila-Pouca et al., 2022), and another study reported negative heterosis in wild chickadees (*Poecile atricapillus* x *Poecile carolinensis*), where the performance on spatial learning tasks and problem-solving tasks was worse in hybrids than in the parental species (McQuillan et al., 2018; Rice & McQuillan, 2018).

Regarding song learning, this is the first report of heterosis among several studies that have examined song learning in hybrid songbirds (Payne, 1980; Toji et al., 2024; Vokurková et al., 2013; H. Wang et al., 2019). In one of these studies, wild hybrid songbirds between two phylogenetically closely related parasitic finches, dusky indigobird (*Vidua purpurascens*) and paradise whydah (*V. paradisaea*) acquired songs with characteristics of their foster parental species, inheriting the ability

of vocal mimicry from paradise whydah (Payne, 1973). Another study examined song learning capacities in F_1 hybrids between two closely related species, ZF and OF, under controlled experimental conditions in a laboratory (Toji et al., 2024; H. Wang et al., 2019). The two parental species, ZF and OF, have acoustically distinct songs and species-specific song learning capacities, and their F_1 hybrids showed increased individual differences in their learned song patterns despite hearing the same set of tutor songs. Some F_1 hybrids developed songs that highly resembled either parental species' song, while others developed "intermediate" songs, which were not a copy of tutor songs but combined syllables with acoustic characteristics of ZF and OF. Wang *et al.* (2019) found that species-specific differences in the expression of brain-derived neurotrophic factor (BDNF) in HVC and RA between ZFs and OFs, and especially in HVC of the F_1 hybrids, BDNF expression levels varied among individuals and showed a significant correlation with syllable acoustic bias or syllable sequential bias toward either parental species. However, the cell type which shows differential expression of BDNF in HVC was unknown in the study. Toji, Sawai, Wang *et al.* (2024) suggested that glutamatergic projection neurons in RA are the main source of individual transcriptomic variation related to innate individual differences in song patterns among the F_1 hybirds, although the results of song learning in those F_1 hybrid pupils were unknown. Thus, each of the studies found suggestive clues to understanding the genetic basis of species/individual differences in song learning. However, certain points still need further exploration to fully understand species differences in song learning capacities. Specifically, it remains to be clarified which song nuclei or cell types show differential gene expression between species or individuals with varying learning capacities, as well as the identification of such genes. In the following chapters, I examined the mesoscopic anatomy of the song system among ZF, CF, and the F_1 hybrids, and cell-type-specific transcriptome signature.

1.5 Figures



Chapter I, Fig. 1.

Species-specific song acoustics and learning constraints of zebra finch and cherry finch

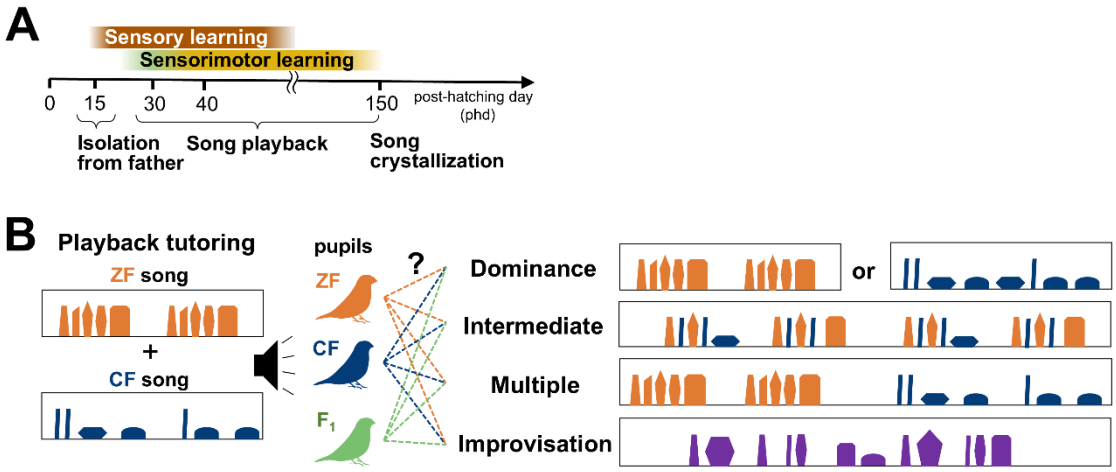
(A) Zebra finch (ZF), cherry finch (CF), and the F_1 hybrids (ZF ♀ x CF ♂)

(B) Species differences in the songs of ZF and CF (n = 4 each).

(C) Visualization of the relative relationship between syllables from 8 birds shown in panel A (500 syllables per bird) by UMAP. The dot colors represent species (left panel) or individuals (right panel).

(D) Four significantly different acoustic parameters and comparison of syllable duration and syllable repertoire size between ZF and CF (500 syllables per bird, 1 dot = the median of 500 syllables per bird, n = 10 birds each, exact Wilcoxon rank sum test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

(E) Sonograms of ZF pupils and CF pupils after cross-species tutoring (n = 2 each).

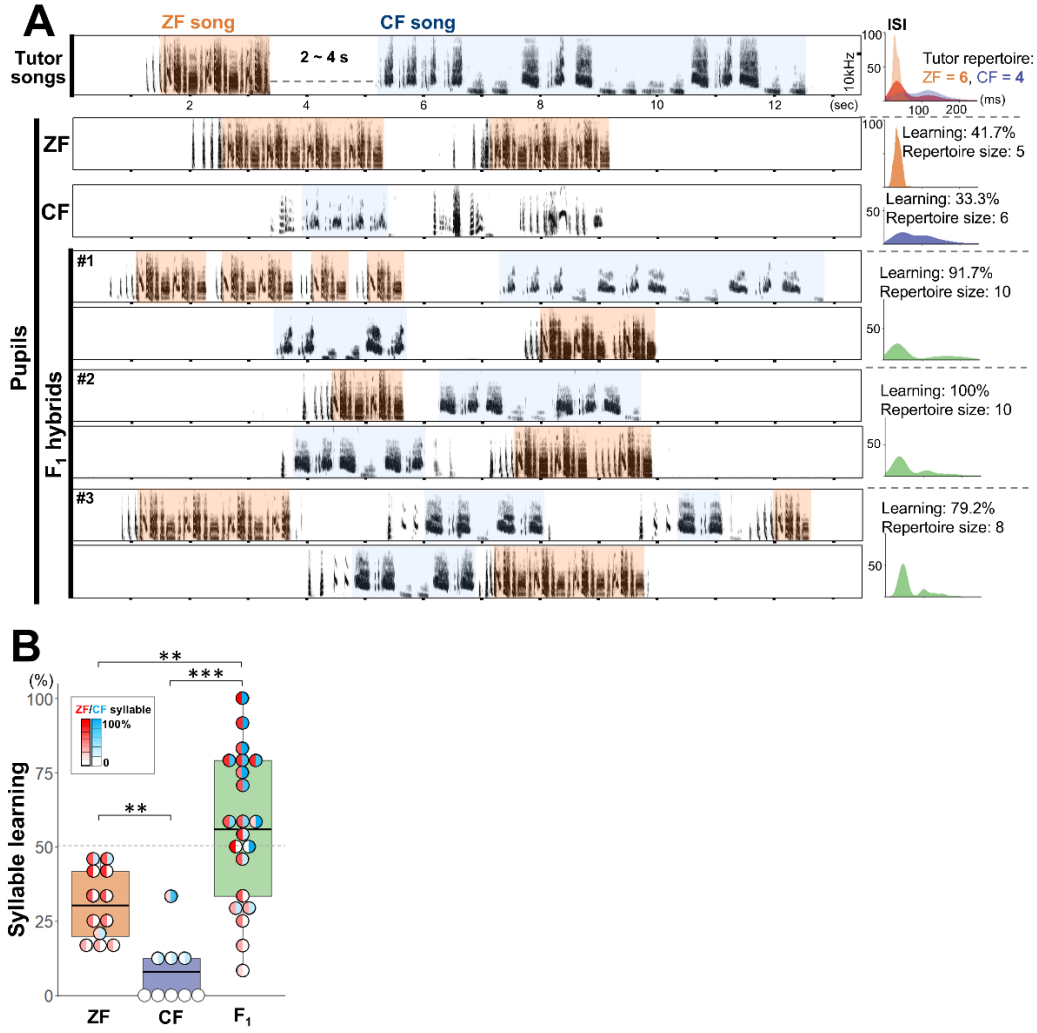


Chapter I, Fig. 2.

Possible results of double-species song tutoring

(A) Experimental timeline of playback-based song tutoring.

(B) Possible results categorized into four groups: "Dominance" (learning only ZF song or CF song), "Intermediate" (mixing syllables from two-species songs or generating syllables that are acoustically intermediate between syllables of ZF and CF), "Multiple" (learning both ZF and CF songs), and "Improvisation," where the pupils develop novel songs that are distinct from those of either parental species.

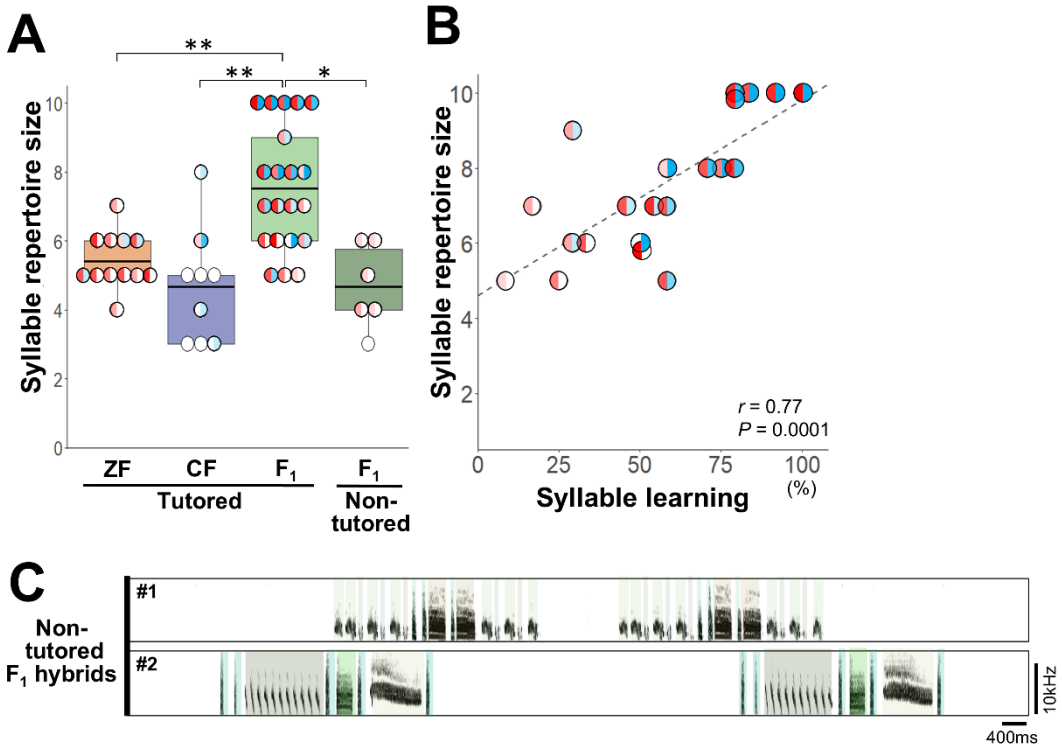


Chapter I, Fig. 3.

Expanded syllable learning capacities and individual differences in song learning in F_1 hybrids

(A) Left panel: Learned songs of ZF, CF, and F_1 pupils in double-species tutoring (ZF: n = 1, CF: n = 1, F_1: n = 3). Orange/blue highlights in the sonograms indicate the ZF/CF tutor song and the corresponding parts in the pupils' songs. Right panel: Distribution of inter-syllable interval (ISI) in tutor and pupil songs. The learning achievement of syllable repertoire (%) and total repertoire size of each pupil are indicated above each ISI distribution curve.

(B) Syllable learning achievement in double-species song tutoring among ZF, CF, and F_1 hybrid pupils (ZF: n = 12, CF: n = 9, F_1: n = 21, $^{**}p < 0.01$, $^{***}p < 0.001$, Steel-Dwass test). The color intensity of semicircles at each point represents the learning percentage of syllable repertoire included in ZF song (red, left half) or CF song (blue, right half).



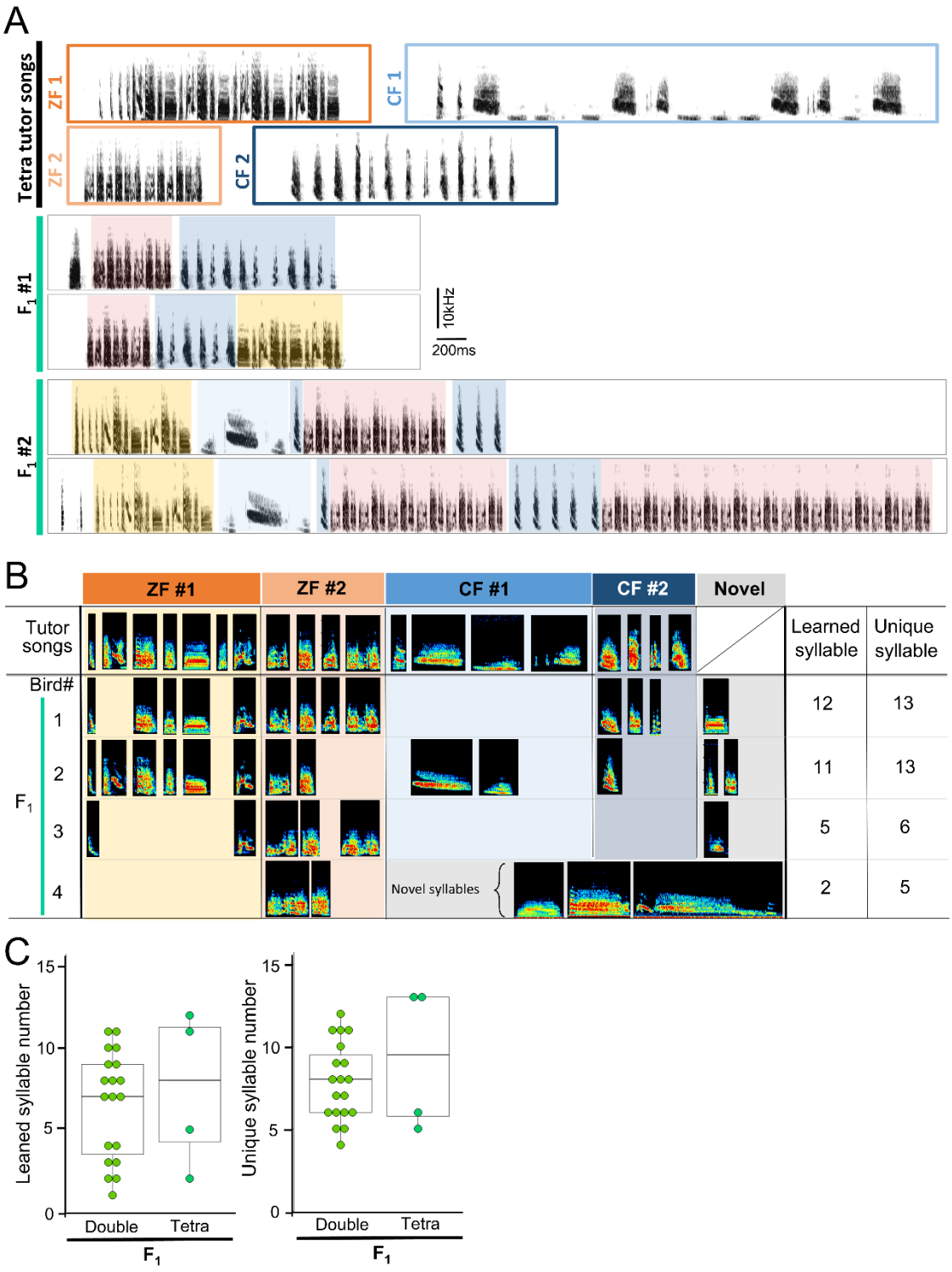
Chapter I, Fig. 4.

Examination of innate and learning effects in increased unique syllable numbers in F_1 hybrids

(A) Number of unique syllables in ZF, CF, F_1 hybrids (double-song tutored), and F_1 hybrids (non-tutored) (ZF: n = 12, CF: n = 9, F_1 double-song tutored: n = 21, F_1 non-tutored: n = 6, $^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Steel-Dwass test). The color intensity of the semicircles at each point represents the learning percentage of the syllable repertoire included in the ZF song (red, left half) or CF song (blue, right half).

(B) Correlation between the number of unique syllables and syllable learning achievement (%) in double-species tutored F_1 hybrids in panel A ($r_s = 0.77$, $p = 0.0001$, Spearman's rank correlation).

(C) Songs of F_1 hybrids reared without hearing any tutor song (n = 2). Highlight colors on the spectrograms indicate identical syllable repertoires.



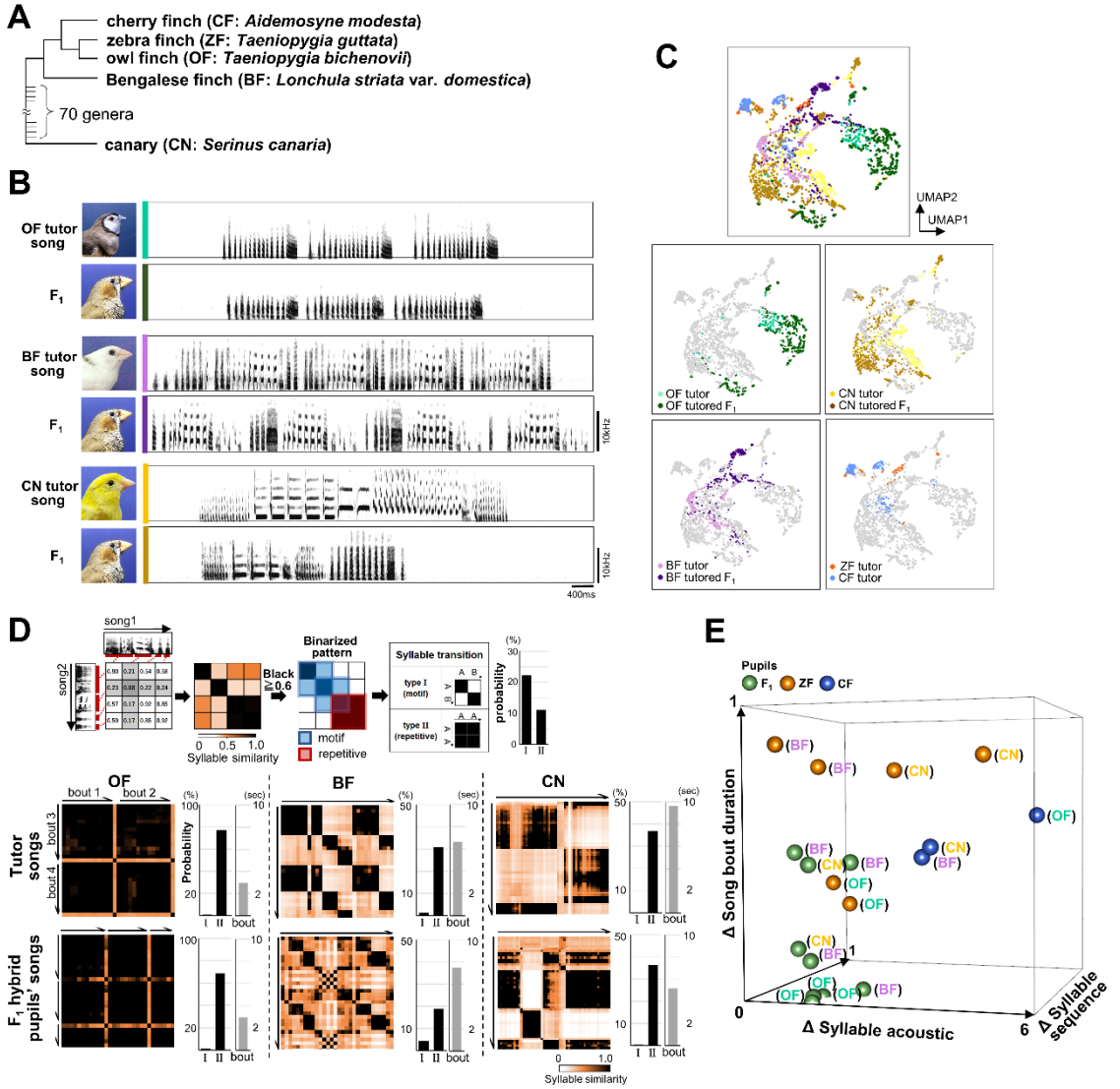
Chapter I, Fig. 5.

Examination of the Upper limit of unique syllable numbers learned by the F_1 hybrids

(A) (Top) Tutor songs used in four-song tutoring (combining two ZF and two CF songs). (Bottom) F_1 hybrid pupil songs tutored with four-songs (n = 2).

(B) Learned syllables by F_1 hybrid pupils after four-song tutoring (n = 4). “Novel” refers to unique syllable types in F_1 pupils that are dissimilar to tutor syllables.

(C) Comparison of the total number of unique syllables in F_1 hybrids tutored with double-species tutor songs and F_1 hybrids tutored with four-tutor songs.



Chapter I, Fig. 6.

Heterospecific song learning in the F_1 hybrids

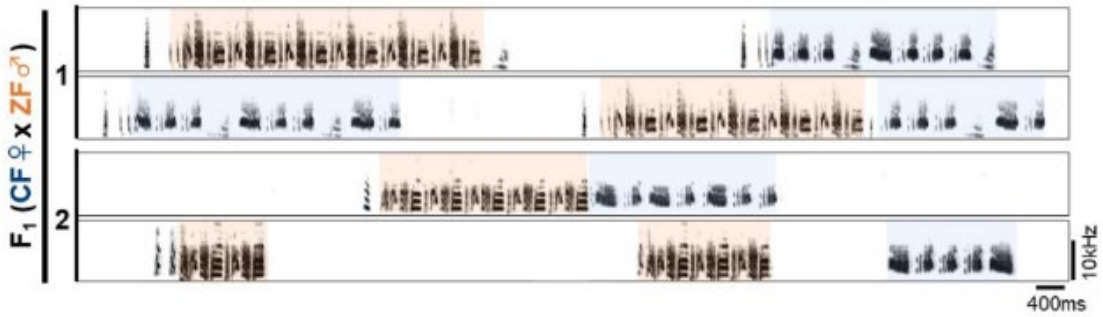
(A) Phylogenetic relationships among ZF, CF, and the species used as heterospecific tutors for F_1 hybrids.

(B) Examples of heterospecific tutor songs (OF, BF, and CN) and learned songs of F_1 hybrid pupils.

(C) UMAP plots of song syllables from F_1 pupils and playback tutor songs (289–300 syllables from F_1 pupils' songs; 66–244 syllables from playback tutor songs). The plot colors correspond to tutor songs and pupil songs as indicated in panel B. Note that plots of F_1 pupils contain multiple individuals, indicated in the same color (OF tutored F_1: n = 3 in dark green; BF tutored F_1: n = 4 in dark purple; CN tutored F_1: n = 2 in khaki)

(D) (Upper panels) Syllable similarity matrix (SSM) analysis for identifying syllable transition types: types I and II as motif- and repetition-syllable transitions, respectively. (Lower left panels) Representative SSMs of the playback tutor songs and songs acquired by F_1 pupils. (Lower right panels) Occurrence rates of syllable transition types I (motif) and II (repetition) in playback tutor and pupil songs.

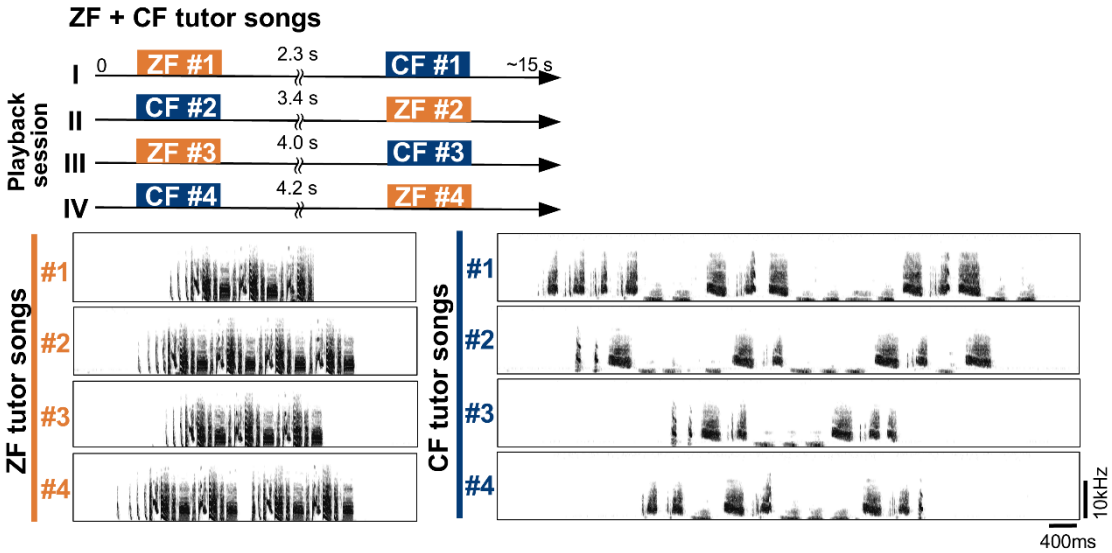
(E) 3D plot showing differences in syllable acoustics, syllable sequence, and song-bout duration between tutor and pupil songs. The species of pupils and tutored songs are represented by the dot colors and the characters in parentheses, respectively. A smaller distance from the coordinate origin indicates a higher similarity in the mimicry of the tutor songs by pupils.



Chapter I, Supplementary Fig. 1.

Songs acquired by F_1 hybrids (CF♀ x ZF♂) tutored with double-species tutor songs

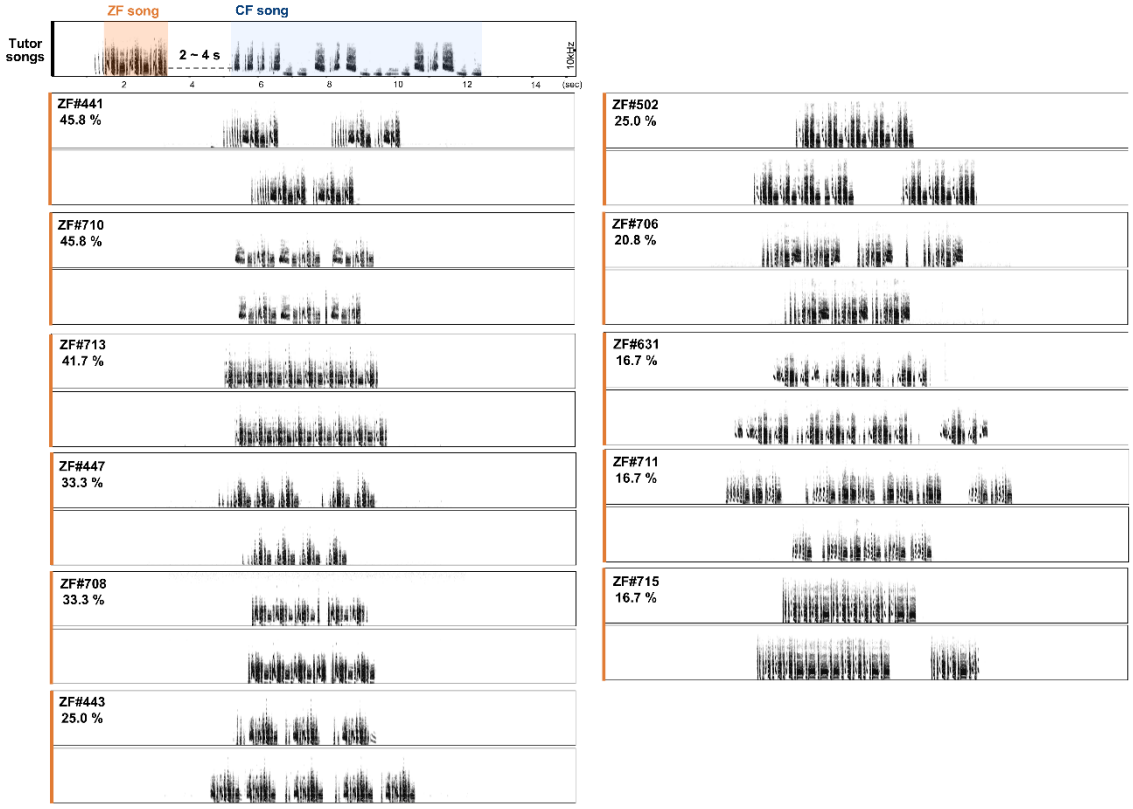
Examples of two hybrid birds (two sonograms per individual). Orange/blue highlights indicate the learned parts from ZF/CF tutor songs.



Chapter I, Supplementary Fig. 2.

Songs used for playback tutoring of two parental species.

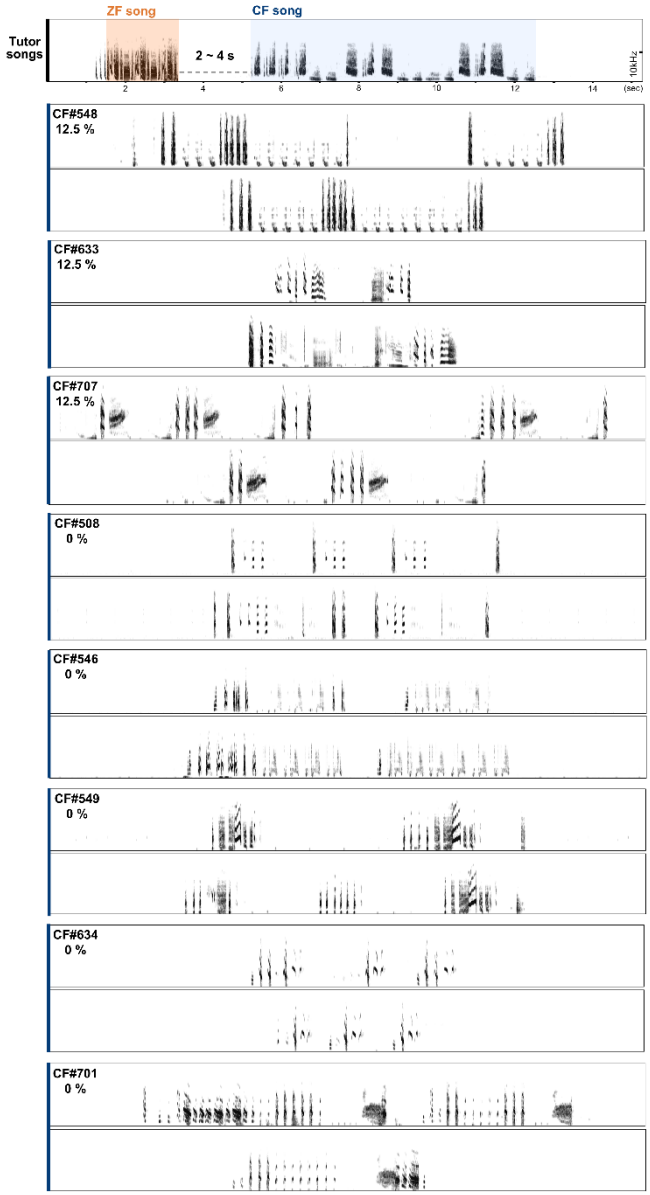
(Top) Song playback paradigm of the two parental species' songs. (Bottom) Spectrograms for playback tutoring of both ZF and CF songs.



Chapter I, Supplementary Fig. 3.

Double-species tutoring results in ZF pupils (n = 11).

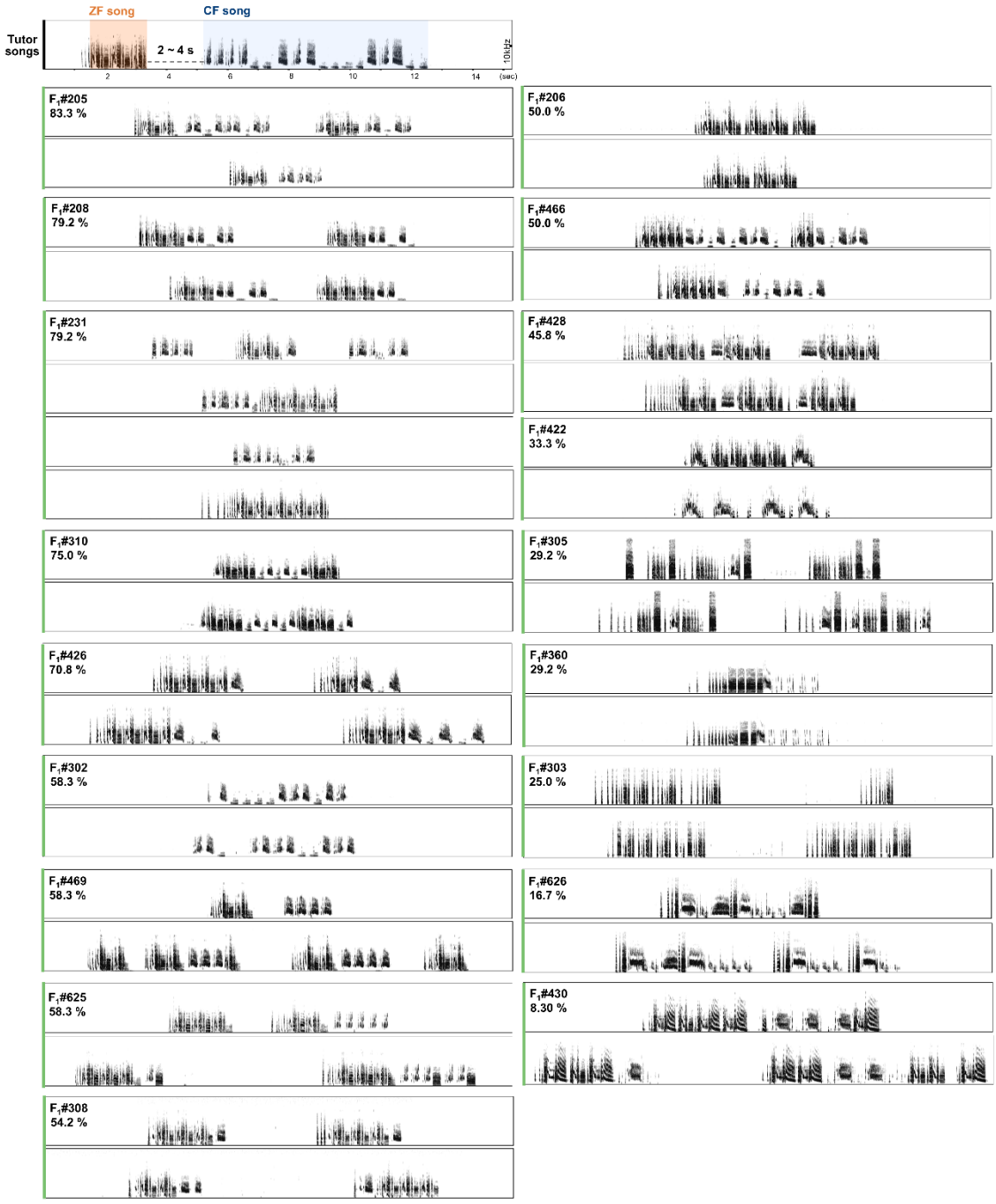
Spectrograms of double-species tutor songs (Top) and adult songs of ZF pupils that are not shown in Chapter I, Fig. 3 (two spectrograms per individual).



Chapter I, Supplementary Fig. 4.

Double-species tutoring results in CF pupils (n = 8).

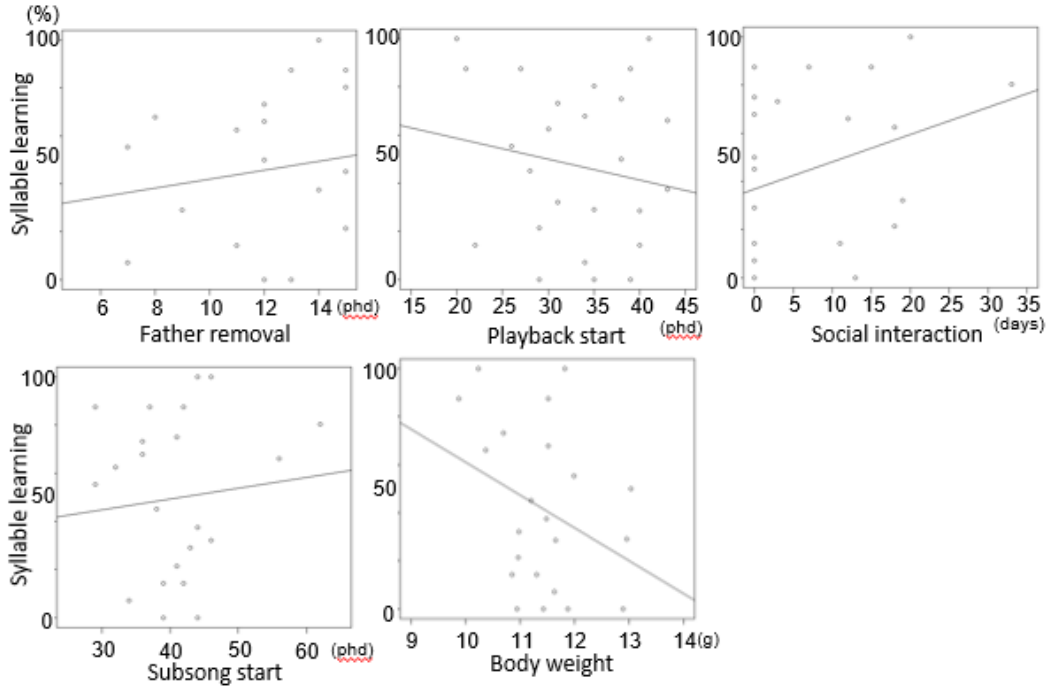
Spectrograms of double-species tutor songs (Top) and adult songs of CF pupils that are not shown in Chapter I, Fig. 3 (two spectrograms per individual).



Chapter I, Supplementary Fig. 5.

Double-species tutoring results in F_1 hybrid pupils (n = 18).

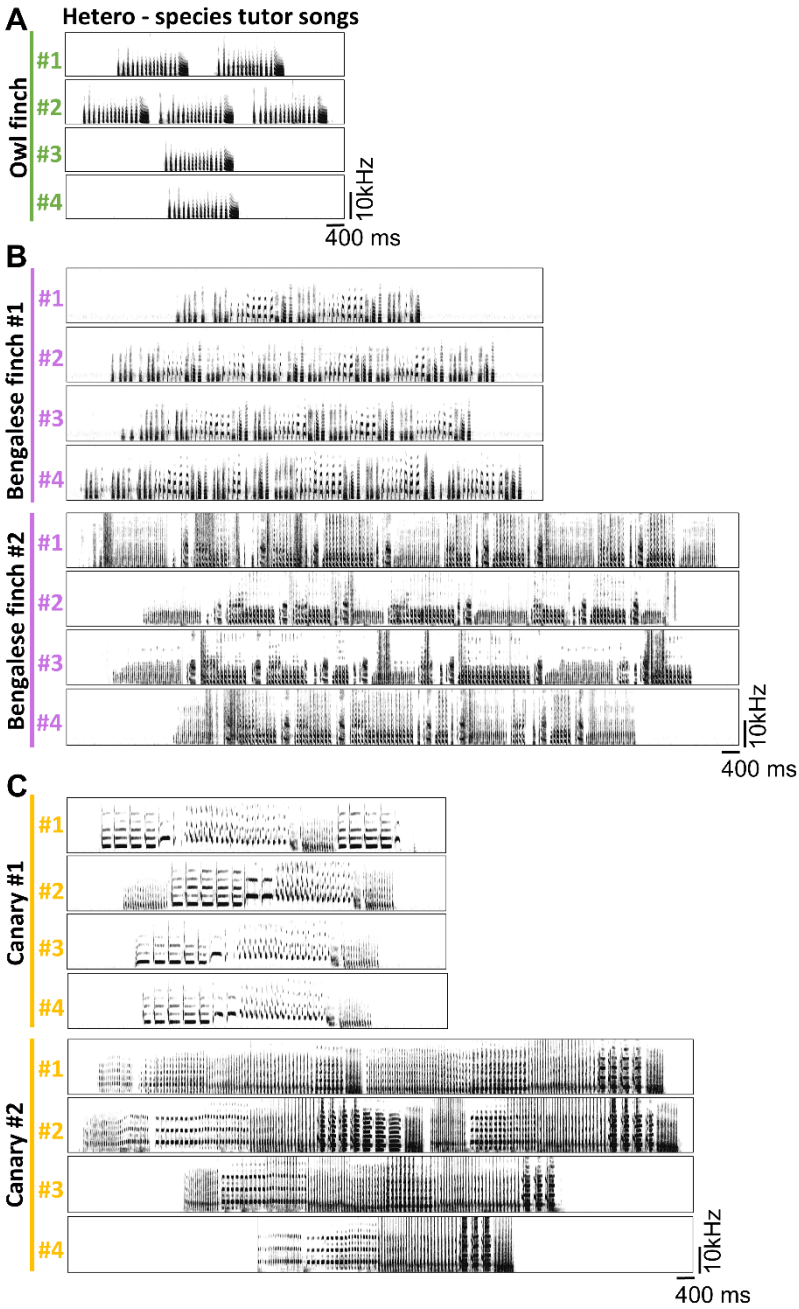
Spectrograms of double-species tutor songs (Top) and adult songs of F_1 hybrid pupils that are not shown in Chapter I, Fig. 3. Spectrograms connected with a green line are derived from one individual.



Chapter I, Supplementary Fig. 6.

Correlation analyses between syllable learning achievement and individual variations in the tutoring environment

No significant correlation was found between father removal (phd), playback start phd, social interaction length with mother or siblings in the same cage (days), subsong start (phd), and body weight (Pearson's product-moment correlation).



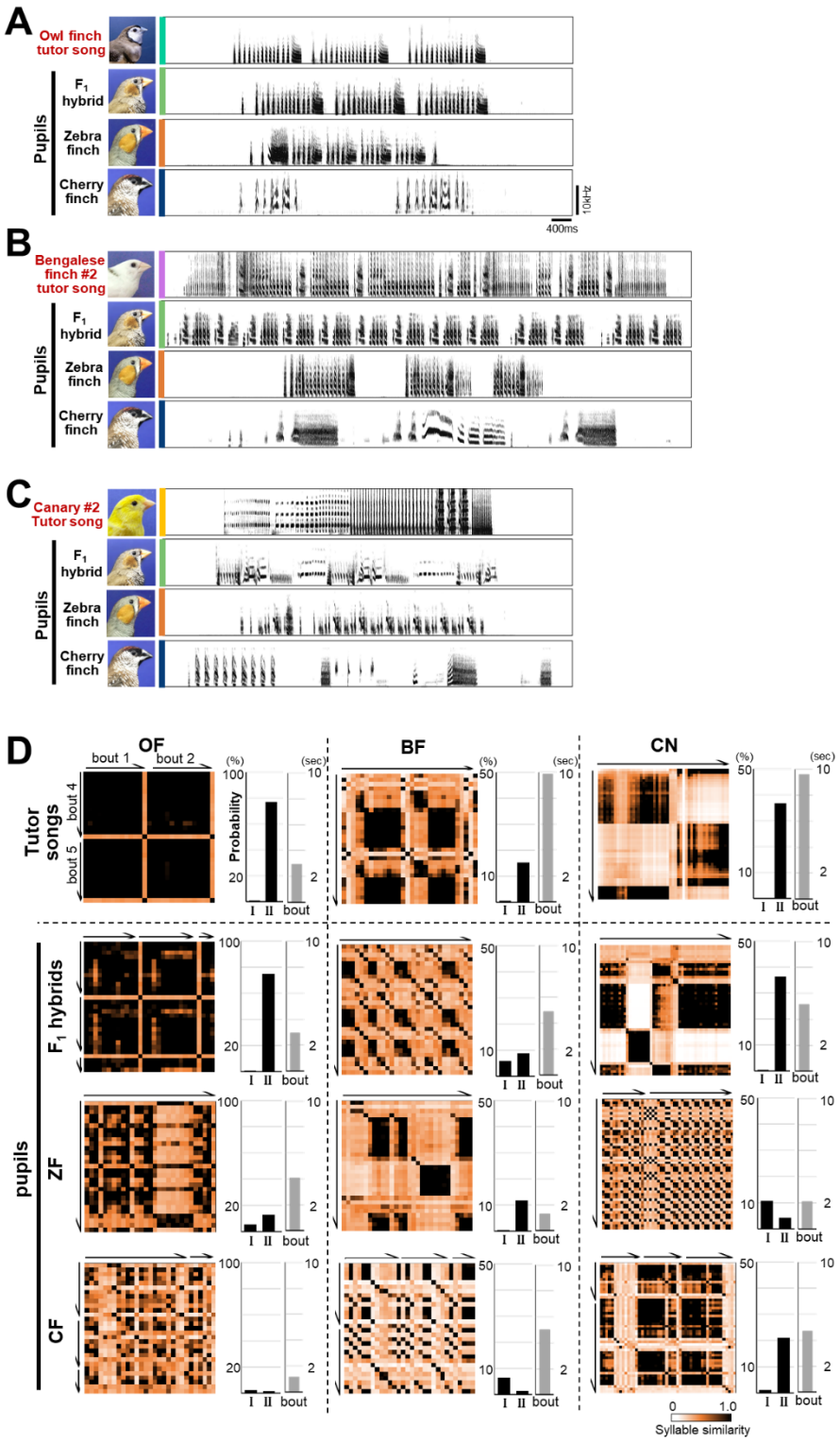
Chapter I, Supplementary Fig. 7.

Tutor songs used for hetero-species song tutoring

(A) OF tutor song set.

(B) BF tutor song sets. Each pupil bird heard the playback of either one set of songs (BF#1 or BF#2, four songs each).

(C) CN tutor song sets. Each pupil bird heard the playback of either one set of songs (CN#1 or CN#2, four songs each).



Chapter I, Supplementary Fig. 8.

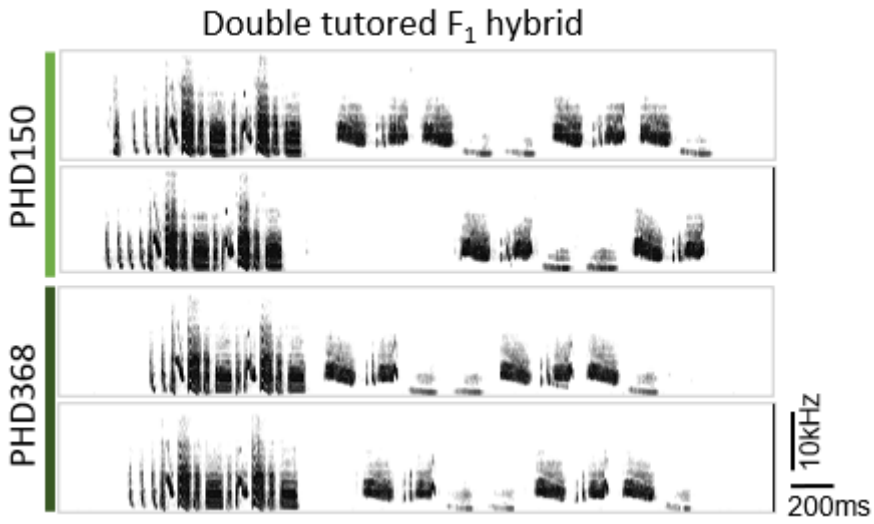
Results of heterospecific song tutoring

(A) Spectrograms of OF tutor songs and pupils (F_1 hybrid, ZF, CF)

(B) Spectrograms of BF tutor songs and pupils (F_1 hybrid, ZF, CF)

(C) Spectrograms of CN tutor songs and pupils (F_1 hybrid, ZF, CF)

(D) Syllable similarity matrix (SSM) and occurrence rates of syllable transition types I (motif) and II (repetition), or song bout duration of each tutor or pupil song. Each tutor song and corresponding pupil songs are aligned vertically (from top to bottom: tutor song, F_1 hybrid, ZF, CF).



Chapter I, Supplementary Fig. 9.

Long retention of two distinct songs in an adult F_1 hybrid after double-species tutoring

An example of double-species songs learned by an F_1 hybrid at the young adult stage (150 phd, shortly after crystallization, top two sonograms) and the mature adult stage (368 phd, bottom two sonograms).

species	ID	father	mother	clutch	foster father	foster mother	Hatched date	playback start PHD	learning achievement(%)
F1	orange209A	CFblack066A	ZForange194A	1	-	-	2017.8.31	35	91.7
F1	orange231A	CForange147A-white99	ZForange183A	2	-	-	2017.10.28	20	79.2
F1	orange302A	CFblack050A	Zfgray	1	-	-	2018.2.26	43	58.3
F1	orange360A	CFblack066A	ZFblue169A	1	-	-	2018.4.21	40	29.2
F1	yellow407A	CForange142A-red98	ZFgray038A	1	-	-	2018.5.21	39	100
F1	orange202A	CFblack050A	ZFred040A	1	-	-	2017.6.5	41	79.2
F1	orange206A	CFblack050A	ZFred040A	2	-	-	2017.8.10	38	50
F1	orange208A	CForange147A-white99	ZForange183A	1	-	-	2017.8.23	27	79.2
F1	orange205A	CForange143A-blue046A	ZForange027A	1	-	-	2017.8.19	31	83.3
F1	orange430A	CForange143A-blue046	ZFgray026A	2	-	-	2019.3.30	34	8.3
F1	orange426A	CFblack050A	ZForange14-26	1	-	-	2019.2.14	34	70.8
F1	orange303A	CForange259A	ZFblue169A	1	ZFblue017A	CZblack252A	2018.7.20	29	25
F1	orange428A	CForange147A-white99	ZForange113A	1	-	-	2019.2.15	26	45.8
F1	orange305A	CForange259A	ZFblue169A	2	-	-	2018.8.26 (putative)	22	29.2
F1	orange422A	CForange143A-blue046	ZFgray026A	1	-	-	2019.1.28	35	33.3
F1	orange466A	CForange142-red98	ZFred398A	1	-	-	2019.4.26 (putative)	28	50
F1	orange310A	CForange258A	ZFblack145A	1	-	-	2018.12.4 (putative)	43	75
F1	orange308A	CFblack050A	ZForange14-26	1	-	-	2018.10.23 (putative)	38	54.2
F1	orange469A	CFblack050A	ZForange14-26	2	-	-	2019.2.14	30	58.3
F1	orange625A	CFred541red035	ZFyellow312A	1	-	-	2020.5.15	31	58.3
F1	orange626A	CFred541red035	ZFyellow312A	1	-	-	2020.5.15	31	16.7
ZF	red441A	ZFblack377A	ZFyellow268A	1	-	-	2018.11.25	23	45.8
ZF	red444A	ZFltb255A	ZFblack328A	1	-	-	2018.12.15	22	41.7
ZF	red443A	ZFltb255A	ZFblack328A	1	-	-	2018.12.12	25	25
ZF	red447A	ZFblack160	ZFyellow392	1	-	-	2018.12.27	32	33.3
ZF	ltb502A	ZFred272A	ZFred11-95	1	-	-	2018.11.16	24	25
ZF	red631	ZForange368A	ZFyellow596	1	-	-	2020.9.14	34	16.7
ZF	red706	ZFltb540A	ZFyellow420A	1	-	-	2021.7.18	32	20.8
ZF	red708	ZFred213A	ZFblue169A	1	-	-	2021.7.21	30	33.3
ZF	red710	ZFred485A	ZForange557A	1	-	-	2021.7.21	30	45.8
ZF	red711	ZFred485A	ZForange557A	1	-	-	2021.7.22	29	16.7
ZF	red713	ZForange115A	ZForange540A	1	-	-	2021.7.23	44	41.7
ZF	red715	ZForange115A	ZForange540A	1	-	-	2021.7.25	28	16.7
CF	red411	CFred244green	CFblack341Ablue150	1	ZCorange204A	ZFred378A	2018.5.18	23	33.3
CF	ltb508	unknown	unknown	-	BF	BF	2019.5.27	52	0
CF	ltb546	unknown	unknown	-	BF black423	BF (died in the next day of arrival)	2019.6.5(putative)	43	0
CF	ltb548	unknown	unknown	-	BF 1238NCC16	BFblack426	2019.6.5 (putative)	43	12.5
CF	ltb549	unknown	unknown	-	BF 1238NCC16	BFblack426	2019.6.5 (putative)	43	0
CF	ltb633	CForange147-white99	CFltb509A	1	BF x SpF blue130A	BF green53	2020.4.16	31	12.5
CF	ltb634	CFred353silver17-19	CFltb509A	1	-	BFblack215A, BFblack1338A	2020.5.3	30	0
CF	ltb701	CFred353A-silver17-19	CFltb509A	2	-	BFblack462A,red556A,black493A	2020.5.12	30	0
CF	ltb707	unknown	unknown	-	ZF x Afsil red522A	ltb600A	2020.6.16	33	12.5

Chapter I, Supplementary Table. 1.

Bird information used for double-species tutoring

The same color indicates siblings. The numbers in the cluch column indicate the order of the cluch in which the individual was born. Individuals in black text indicates that these birds didn't have any siblings within the group of pupils used for this tutoring experiments.

Chapter II

Comparison of mesoscopic anatomy of the song system among the parental species and the F_1 hybrids

2.1 Introduction

Through the experiments in **Chapter I**, I found that inter-species F_1 hybrids between zebra finches (ZF) and cherry finches (CF) showed expanded song learning capacity compared to the parental species in terms of mimicking ability for multiple species' songs, maintenance of two different songs, and the size of their syllable repertoire. Considering the results from non-song tutoring and four-song tutoring experiments, the hybrids had a large syllable repertoire when tutored by songs with a rich syllable repertoire, indicating that the increase in syllable repertoire reflects the expansion of their syllable learning capacity. In **Chapter II**, I aimed to reveal which brain areas are associated with expanding song learning capacities in the F_1 hybrids and whether they involve any changes in brain anatomy.

Higher skills are sometimes reflected in larger volume of specific brain areas involved in the execution of those abilities/skills (Gaser & Schlaug, 2003; Lefebvre et al., 1997; Lewis et al., 2011; Maguire et al., 2000; Schlaffke et al., 2014; Yuan & Raz, 2014). In humans, London taxi drivers, who have passed rigorous exams for a license that requires extensive training to navigate anywhere in the city, have larger hippocampi compared to control groups, and hippocampal volume was positively correlated with the amount of time spent working as taxi drivers (Maguire et al., 2000). All these studies compared individuals within a species or between relatively closely related species sharing similar brain anatomical structures. In birds, a positive correlation between relative brain volume, problem-solving ability, and vocal learning complexity across 23 songbird species was reported (Audet et al., 2023). Some other studies reported a positive correlation between the relative size of the hippocampal complex and spatial memory among food-storing passerine species (Hampton et al., 1995; Krebs et al., 1989).

Because of the histological clarity of the song system, the correlation between the volume of the responsible brain areas and song learning capacity is well studied in songbirds (Bernard et al., 1996; Canady et al., 1984; DeVoogd, 2004; J. M. Moore et al., 2011; Nottebohm et al., 1981; Pfaff et al., 2007; Szekely et al., 1996; Zeng et al., 2007). Neurons with the same function for song learning and production are clustered in each song nucleus, with histologically obvious boundaries. In the VMP, the song nucleus HVC (used as a proper name) is responsible for the regulation of syllable sequence patterns and projects to the robust nucleus of the arcopallium (RA), which regulates syllable acoustics (Fee et al., 2004; Okubo et al., 2015; Sánchez-Valpuesta et al., 2019; Yu & Margoliash, 1996), and also projects to Area X, which plays a role in proper song learning in juveniles (Sánchez-Valpuesta et al., 2019). In the AFP, three song nuclei, the lateral magnocellular nucleus of the anterior nidopallium

(LMAN), Area X, and the dorsal lateral nucleus of the medial thalamus (DLM) form the basal-ganglia thalamocortical loop. LMAN generates vocal variability, contributing to the promotion of vocal exploration in juveniles (S. W. Bottjer et al., 1984; Ölveczky et al., 2005; Scharff & Nottebohm, 1991). Area X is involved in the evaluation of the acoustic quality of a bird's own song by receiving dopamine inputs from the ventral tegmental area (VTA) (Fee & Goldberg, 2011; Kao et al., 2005; Nicholson et al., 2018; Prather et al., 2008). DLM is also required to generate vocal variability (Fee & Goldberg, 2011; Person & Perkel, 2007). Multiple studies have reported species differences in song nuclei size, with the majority of them finding an association between the volume of HVC and song complexity, such as song or syllable repertoire size. This correlation is supported by many studies at both inter-species levels (DeVoogd, 2004; J. M. Moore et al., 2011; Szekely et al., 1996; Zeng et al., 2007) and intra-species levels (Bernard et al., 1996; Canady et al., 1984; Nottebohm et al., 1981; Pfaff et al., 2007; Ward et al., 1998). Moore *et al.* (2011) also estimated the neuron number in several song nuclei and confirmed that the volume of HVC in the VMP is strongly related to the constitutive neuron number, suggesting that more neurons strengthen the computational power in HVC to regulate the production of complex songs. RA, another song nucleus that is part of the VMP, also showed a similar association in some cases (Canady et al., 1984; Nottebohm et al., 1981), while such a tendency was not clear in song nuclei that are part of the AFP.

In **Chapter II**, inspired by the concept of heterosis and previous studies that have reported an association between song nuclei volume and song complexity, such as syllable repertoire size, I formulated a working hypothesis that a heterotic increase in song nuclei volume in the F_1 hybrids underlies the expansion of song learning capacity. To examine if the parental species and the F_1 hybrids with different syllable learning capacities have corresponding anatomical differences in their song system, I compared the volume and constitutive neuron numbers of the five major song nuclei in the song system. The volume of each nucleus was estimated by summing the areas of serial cross-sections, while the neuron numbers were calculated based on the nucleus volume and neuron density. The analyzed nuclei included HVC and RA from the VMP, as well as LMAN, Area X, and DLM from the AFP. I additionally compared the proportional composition of excitatory and inhibitory neurons in these song nuclei, as the coordination of these neuronal populations is generally important for normal brain development and function (Alreja et al., 2022; Sahara et al., 2012). A lack of such coordination can cause disorders in information processing, including learning (Kirischuk, 2022), and has also been suggested to be related to the regulation of species-specific song development (Gogola et al., 2019).

2.2 Materials and methods

Brain sampling

For brain sampling, the bird was placed in a sound-attenuating box overnight under silent and dark conditions. Before light onset the next morning, brains were sampled under non-singing conditions by decapitation and immediately frozen in dry ice after being embedded in OCT Compound (Sakura Finetek Japan).

Evaluation of song nuclei size, neural cell density, and constituent neuron number

To evaluate song nucleus size, *in situ* hybridization was performed using marker genes exhibiting different expression patterns within and outside the song nuclei (HVC, RA, LMAN, Area X, and DLM). Twenty-six adult male birds were utilized, comprising 7 ZFs, 8 CFs, and 11 F_1 hybrids. Four genes were used as *in situ* hybridization probes: *AR* for labeling HVC and LMAN (Wada *et al.*, 2013), glutamate receptor ionotropic AMPA subunit 1 (*GRIA1*) for labeling Area X, glutamate receptor metabotropic type 2 (*GRM2*) for labeling DLM, and glutamate receptor ionotropic kainate type subunit 1 (*GRIK1*) for labeling RA (Wada *et al.*, 2004). The cDNA fragments of *AR* (1,032 bp), *GRIA1* (1,340 bp), *GRIK1* (500 bp), and *GRM2* (1,782 bp) were PCR-amplified using oligo-primers from pGEM-T easy cloning vectors. The amplified cDNA fragments were purified and used as templates for synthesizing antisense ^{35}S-labeled riboprobes through *in vitro* transcription using SP6 or T7 RNA polymerase. During the pre-hybridization step, frozen brain sections were fixed in 4% paraformaldehyde (PFA)/1× PBS, washed in 1× PBS, acetylated, dehydrated in an ascending ethanol series, and air-dried. A total of $1x10^6$ cpm of the ^{35}S-labeled riboprobe was added to 140 µL of hybridization solution (50% formamide, 10% dextran, 1× Denhardt's solution, 12 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.5 mg/mL yeast tRNA, 10 mM dithiothreitol). Hybridization was carried out at 65 ℃ for 14 hours in an oil bath. Subsequently, the slides were washed in chloroform, 2× SSPE, 50% formamide in 2× SSPE, 0.1% SSPE, and ascending ethanol series. Following drying, the slides were exposed to X-ray film (Biomax MR, Kodak) for the appropriate periods and developed.

To count the number of neural cells within the song nuclei, immunohistochemistry using anti-NeuN antibody was performed on the same set of 26 birds used for in situ hybridization. Frozen sections mounted on slide glasses were fixed in 4% PFA/1× PBS for 10 min and washed in 1×PBS. Slide glasses were incubated with anti-NeuN antibody (dilution 1: 800, Millipore, #MAB377) in

blocking solution (0.3% Triton X-100/4% normal goat serum/1% BSA/1× PBS) at 4 C° for 16 hours. The primary antibodies were detected using anti-mouse AlexaFluor 555 conjugated antibody (Thermo Fisher, #A-21422). Finally, the slides were mounted with Prolong Gold with DAPI (Invitrogen).

To estimate the volume of song nuclei and constituent neuron numbers, each sample from the 26 birds used for *in situ* hybridization and immunohistochemistry was randomly labeled with letters A to Z, without any species or bird identification information, to ensure blinding. The size of each song nucleus was quantified by measuring the summed area (mm^2) in eight brain sections collected at 400 μm intervals, spanning from the lateral to medial direction. Brain images exposed on X-ray films from situ hybridization were captured using a stereo microscope with a CCD camera (Leica Z16 AP0 and Leica DFC490). The boundaries of song nuclei were traced in the digital images, and the sizes of those areas were measured using Adobe Photoshop software. The approximate volume of each song nucleus was calculated by summing the products of the area size of each section and interval thickness between every two sections. For neural cell counting, fluorescent signals from the anti-NeuN antibody were acquired using a fluorescence microscope (Keyence, BZ-X810). The NeuN signals, along with cellular nuclei stained with DAPI, were counted within sampling windows measuring 150 × 150 μm^2 for larger song nuclei (HVC, RA, and Area X) or 100 × 100 μm^2 for smaller song nuclei (LMAN and DLM). These counts were used to assess neural cell density (/mm^2). Finally, the constituent neuron number in each song nucleus was estimated as the product of the approximate volume and neuron density.

Signal Amplification by Exchange Reaction-Fluorescent *in situ* Hybridization (SABER-FISH)

To assess the ratio of excitatory glutamatergic neurons and inhibitory GABAergic neurons in song nuclei, SABER-FISH was conducted following the protocol provided at (http://saber.fish/). Six adult birds each from ZFs, CFs, and F_1 hybrids that were used to compare song nucleus size and neuron density were utilized to calculate the ratio between excitatory and inhibitory neurons. The labeling of glutamatergic and GABAergic neurons was achieved using the SABER amplified FISH method. mRNA sequences for solute carrier family 17 member 6 (*SLC17A6*) (glutamatergic neuron marker) and glutamate decarboxylase 1 (*GAD1*) (GABAergic neuron marker) were obtained from the NCBI database, and probes were designed using the OligoMiner pipeline. Fresh frozen brain sections on slide glasses were fixed in cold 4% PFA/1x PBS. The slides were washed in 1x PBS three times at room temperature, followed by washing in buffer-1 (40% formamide/1% tween 20/2x SSC) at 45C°. The first probe set of *SLC17A6* and *GAD1* was hybridized in hybridization solution-1 (40%

formamide/10% Dextran sulfate/0.1% Tween-20/2x SSC) (1 mg/gene/slide) overnight at 42C°. After the first hybridization, the slides were washed in wash buffer-1 at 42C°, 2x SSCT, and wash buffer-2 (30% Formamide/1% tween 20/2x SSC). The second branching probe was hybridized in the hybridization solution-2 (30% Formamide/10% Dextran sulfate/0.1% Tween-20/2×SSC) (1 mg /glass) for 6 hours to overnight at 37C°. After the second hybridization, the slides were washed in wash buffer-2, 2× SSCT, and 1×PBST at 37C°. The fluorescent primer was then hybridized in a fluorescence solution containing Hoechst to stain cell nuclei for 1 hour to overnight at 37C°. Subsequently, the slides were washed in 1×PBST and 1×PBS, and then treated with an autofluorescence quenching solution (Vector Trueview). Finally, the slides were washed in 1×PBS and mounted.

Cell counting was performed using a fluorescence microscope (Keyence, BZ-X810) at a magnification of x40. At this magnification, Area X and RA were larger than the microscopic field; thus, all the signals of *SLC17A6* or *GAD1* merged with 4′,6-diamidino-2-phenylindole (DAPI) were counted as excitatory or inhibitory neurons for each image. For smaller areas such as HVC, LMAN, and DLM, signal-positive neurons were counted within a sampling window (150 × 150 μm^2) to ensure the exclusion of neurons from the surrounding region.

2.3 Results

The size and neuron density in song nuclei of F_1 hybrids compared with parental species

To examine the working hypothesis that heterosis in size and/or constitutive neuron number in the song nuclei of the F_1 hybrids led them to acquire expanded song learning capacities (**Chapter II, Fig. 1-A**), I measured song nucleus size and neuron density in HVC, RA, LMAN, Area X, and DLM in the two parental species and the F_1 hybrids (**Chapter II, Fig. 1-B**), and estimated constitutive neuron number based on the data. In all the examined song nuclei, no heterotic change in size or constitutive neuron number was found in the F_1 hybrids compared to the parental species ($p > 0.05$, Steel-Dwass test; **Chapter II, Fig. 1-C, D**).

The ratio between excitatory and inhibitory neurons in song nuclei of F_1 hybrids compared with parental species

I further examined whether the ratio of glutamatergic excitatory neurons and GABAergic inhibitory neurons differed among the F_1 hybrids and the parental species (**Chapter II Fig 2-A, B)**. In HVC, RA, LMAN, and Area X, there was no significant difference in the ratios between the excitatory and inhibitory neurons among F_1 hybrids and the parental species ($p > 0.05$, Steel-Dwass test; **Chapter II, Fig. 2-C)**. In DLM, only glutamatergic excitatory neurons were detected, and the density of those neurons was similar among the F_1 hybrids and the parental species groups (**Chapter II, Fig. 2-D)**.

Overall, no significant heterotic change was found in the F_1 hybrids regarding song nucleus size, constitutive neuron number, and the ratio of excitatory and inhibitory neurons, which did not support the working hypothesis that the heterotic enhancement of syllable learning capacity in the F_1 hybrids relies on the heterotic change in the anatomy of the song system, at least at the mesoscopic scale examined in this study.

Correlation between constitutive neuron number and syllable learning achievement in the F_1 hybrids

Finally, I examined whether individual variation in song nucleus size among the F_1 hybrids correlates with syllable learning achievement. In all five song nuclei examined, no significant correlation was found between constitutive neuron number and syllable learning achievement in F_1 hybrids ($p > 0.05$ after Bonferroni correction, Spearman's rank correlation coefficient). Only Area X showed negative correlation tendency close to significance (**Chapter II, Fig. 3**; n = 11 F_1 hybrids, r_S = -0.73, p = 0.057, Spearman's rank correlation coefficient). Although song nucleus size and

constitutive neuron number in the F_1 hybrids were at an intermediate level between the two parental species, these results allow for the possibility that among-individual variation in neuron number in Area X, which plays an important roles in song learning during the juvenile stage and song maintenance in adults, may reflect syllable learning capacity in the F_1 hybrids.

2.4 Discussion

Given the previous insights into interspecific variations in mesoscopic brain anatomy of the song system responsible for song complexity such as syllable or song repertoire size (DeVoogd, 2004; J. M. Moore et al., 2011; Szekely et al., 1996; Zeng et al., 2007), I expected heterotic changes in the volumes of song nuclei in the F_1 hybrids. However, the F_1 hybrids did not have significant heterotic changes in the size or estimated total number of constitutive neurons in any of the song nuclei examined in this study. Similarly, no significant difference was found in the ratio of excitatory to inhibitory neurons among the three groups.

Even among previous studies, conclusions about whether song nucleus size and/or constitutive neuron number reflect song complexity or song learning capacity remain controversial. Among the song nuclei, variation in HVC volume or neuron number has been found to positively correlate with species or individual variation in song complexity, such as repertoire size, across multiple studies and species (Canady et al., 1984; Devoogd et al., 1997; J. M. Moore et al., 2011; Nottebohm et al., 1981). However, this correlation is not supported by some other species (Bernard et al., 1996; Brenowitz et al., 1991; Gahr et al., 1998b; Kirn et al., 1989). For instance, in the dueting species African dueting bush shrike (*Laniarius funebris*), both sexes exhibit similar song learning capacities; nevertheless, there is clear sex dimorphism in HVC and RA, with males having twice as many neurons as females (Gahr et al., 1998a). Taken together, the volume or constitutive neuron number in song nuclei is not the sole factor determining song learning capacities.

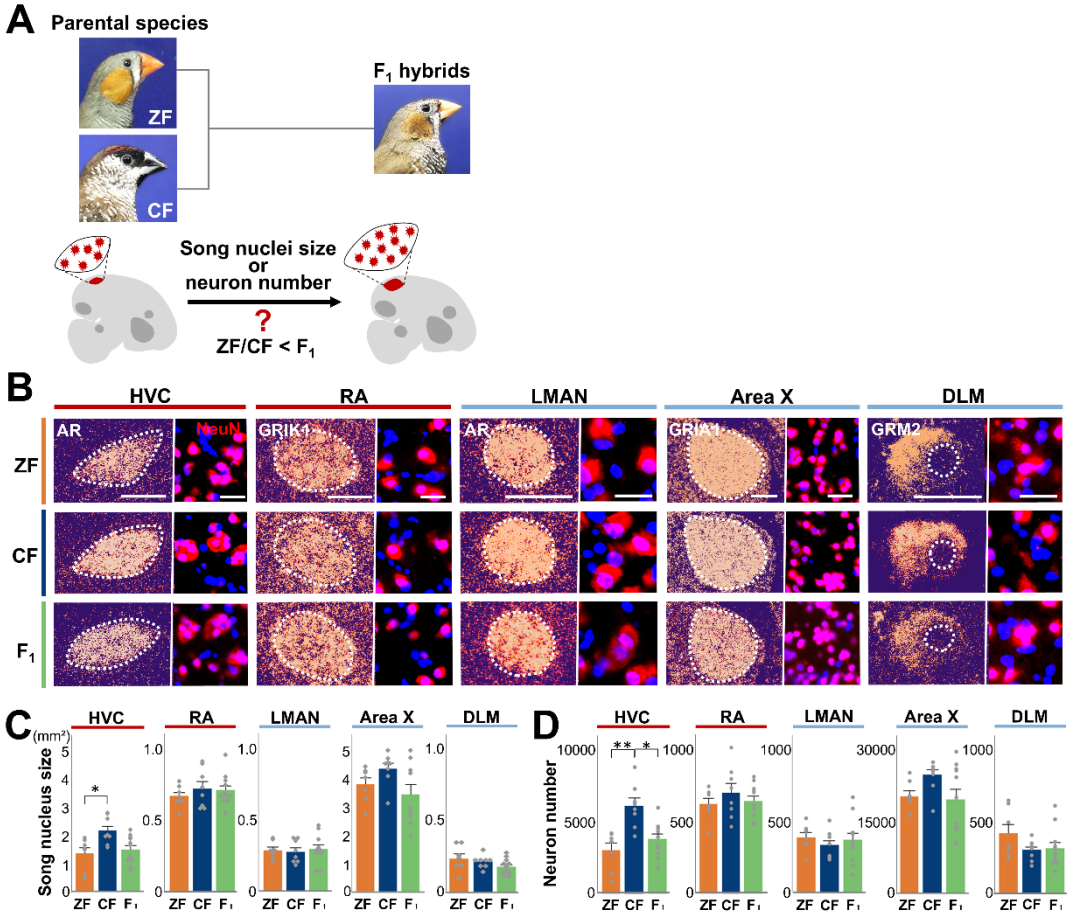
In addition, due to the technical limitations, both previous studies and my experiments did not measure the entire volume or neuron number in song nuclei. Instead, total mass was estimated by measuring the size or neuron number on brain sections. Thus, such technical differences or inter-observer variability in the evaluation of song nuclei volume, neuron number (and song characteristics) should also be considered as potential factors causing inconsistencies in the conclusions of each study.

As a future direction, examining developmental changes in song nucleus size and neuron number or density will be informative for considering how much developmental difference in brain anatomy is likely to affect song learning differences between the parental species and the F_1 hybrids. Species differences in the development of song system anatomy remains largely unexplored. In ZF, each song nucleus exhibits unique developmental patterns in constituent neuron numbers, the growth of individual neurons, and the distribution of neurons (S. Bottjer et al., 1985; S. W. Bottjer et al., 1986). For instance, in HVC, neurons increase drastically, and neurogenesis is active. In RA, neuron density

decreases while soma size increases. In LMAN, the number of neurons decreases. The developmental timing of neural projection also differs among song nuclei: in ZF, projections from LMAN are already innervated in RA by 15 days after hatching, which is the timing when sensory learning starts. Projections from HVC to Area X are formed by 20 dph, and the projection from HVC to RA develops last, being formed around one month after hatching (Mooney & Rao, 1994). Therefore, comparing developmental changes in the mesoscopic brain anatomy of the song system among the parental species and the F_1 hybrids, while considering their song development processes, would help clarify the functional significance of mesoscopic neuroanatomical regulation specific to each song nucleus in species-specific song learning or the expansion of song learning in the F_1 hybrids.

In addition to estimating volume and neuron numbers, an examination of neuroanatomy at a finer scale, which may have been overlooked in this study, will be necessary. At the neural population level, the ratio of excitatory to inhibitory neurons in the VMP and AFP did not show significant differences among ZFs, CFs, and the F_1 hybrids. However, the synaptic excitatory/inhibitory ratio or its dynamics is still worth examining. Synapses are highly plastic, and the formation or removal of synapses occurs more frequently than the replacement of neurons. The synaptic excitatory and inhibitory balance is important for precise higher cortical functions, including learning and memory formation (Barron et al., 2017). Similarly, the precise regulation of spine density is suggested to play an important role in the progression of song learning (Airey et al., 2000; Hayase et al., 2018). In ZF, the pruning of synapses is suggested to have an important role in the stabilization of learned songs (Hayase et al., 2018). In one of the multiple song singers, marsh wren (*Cistothorus palustris*), spine density was higher in HVC neurons in the group tutored with many songs (45 song types) than in the other group tutored with fewer songs (5 types), despite having a similar HVC size. Although these traits are influenced by auditory experience or singing practice, the range of variation may differ among species. In this study, the crystallization of songs seemed to have taken longer in CF, which leads me to predict that spine density in HVC or RA, for instance, remains higher for a longer time in CF than in ZF. Besides, considering the individual variation among F_1 hybrids in syllable learning achievement, variation in spine density or the timing of pruning may have varied among individuals and been influenced innately by variations in interactions between parental alleles, leading them to show varied syllable learning capacities or flexibilities in singing patterns across two song phrases. Finally, in addition to the different types of neurons, the population ratio of non-neuronal cells, such as glial cells, also potentially affects the efficiency of song learning, considering that glial cells support learning and modulate neural activities (Fields et al., 2014; Han et al., 2013).

2.5 Figures



Chapter II, Fig. 1.

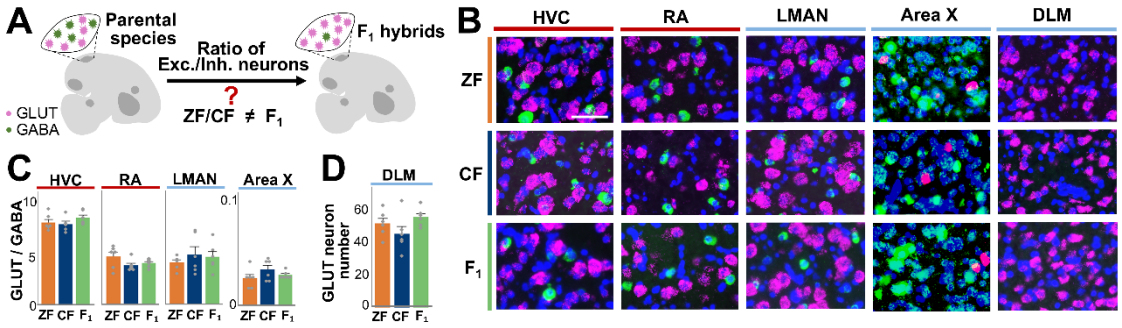
No heterotic changes in song nuclei size/constitutive neuron number in the F_1 hybrids

(A) A possible mesoscopic difference in song nuclei size and/or constitutive neuron number among the parental species and F_1 hybrids.

(B) (Left panels) mRNA *in situ* hybridization image showing the boundary of the song nuclei (white dotted line, marker genes for each song nucleus are indicated in the top panel) in five song nuclei among ZF, CF, and F_1 hybrids (n = 1 each). Scale bar = 0.5 mm. (Right panel) immunohistochemistry image (anti-NeuN for neuronal labeling: red, and DAPI for cell nuclear labeling: blue), scale bar = 25 μm.

(C) Summary data of the size of five song nuclei (mm²) among the three groups (ZF = 7, CF = 8, and F_1 = 11 birds; *$p < 0.05$, Steel-Dwass test).

(D) Summary data of constitutive neuron numbers (song nucleus size × neuron density) in five song nuclei among the three groups (ZF = 7, CF = 8, and F_1 = 11 birds; *$p < 0.05$, Steel-Dwass test).



Chapter II, Fig. 2.

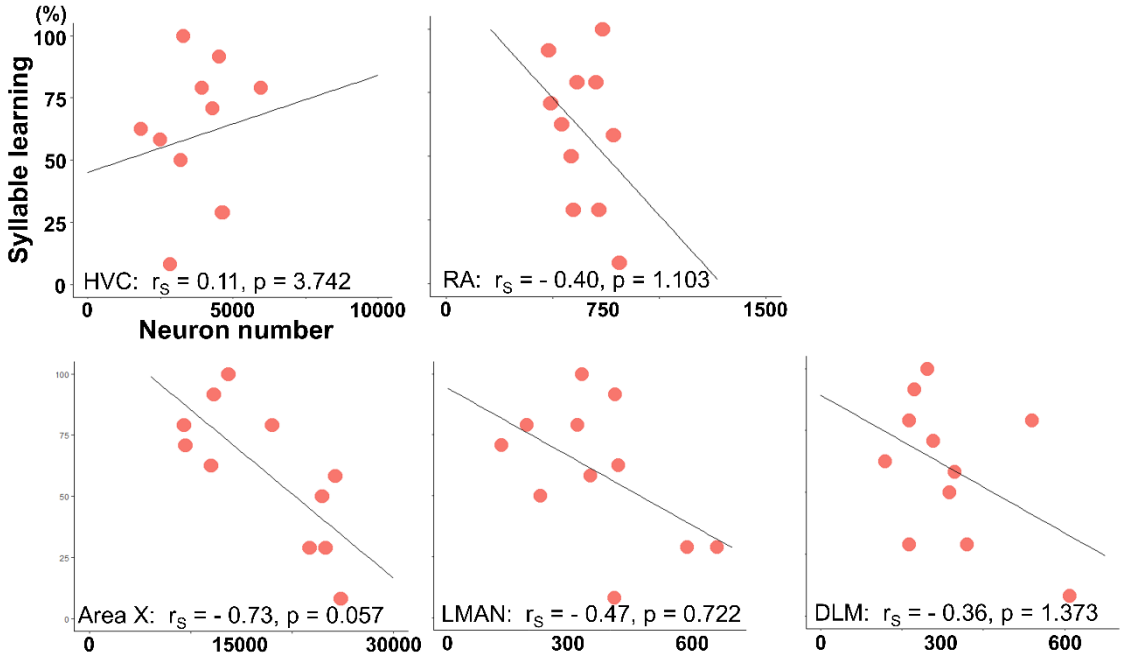
Similarity in the ratio of E/I neurons in song nuclei among ZF, CF, and F_1 hybrids

(A) A possible mesoscopic difference in the ratio of E/I neurons among the parental species and F_1 hybrids.

(B) mRNA *in situ* hybridization images showing the distribution of glutamatergic excitatory neurons (SLC17A6-positive, magenta) and GABAergic inhibitory neurons (GAD1-positive, green) in five song nuclei among ZF, CF, and F_1 hybrids (n = 1 each). Scale bar = 50 μm.

(C) Quantification of the ratio of excitatory and inhibitory neurons in song nuclei (HVC, RA, LMAN, and Area X) of ZFs, CFs, and F_1 hybrids (n = 6 birds/group; $^{NS}p > 0.05$, Steel–Dwass test). The data for Area X are shown on a different scale for the y-axis due to its very small values.

(D) The number of excitatory neurons in DLM of ZFs, CFs, and F_1 hybrids (/mm²) (n = 6 birds/group; $^{NS}p > 0.05$, Steel–Dwass test). In DLM, only excitatory neurons were observed, as GABAergic neurons were absent.



Chapter II, Fig. 3.

Correlation between estimated constitutive neuron number in song nuclei (HVC, RA, Area X, LMAN, and DLM) and syllable learning achievement in the F_1 hybrids

n = 11 F_1 hybrids, r_S: Spearman's rank correlation coefficient with Bonferroni correction applied to p-values

Chapter III

Cell type-specific transcriptome divergence in vocal motor pathway among the parental species and the F_1 hybrids

3.1 Introduction

In **Chapter II,** I compared the brain anatomy of zebra finch (ZF), cherry finch (CF), and the F_1 hybrids to examine if the F_1 hybrids possessed larger song nuclei with increased neurons, which could be associated with the expanding of their syllable learning capacity. However, the F_1 hybrids did not exceed both parental species either in volume, neuron density, or neuron number in the major song nuclei in the song system (HVC, RA, Area X, LMAN, and DLM), suggesting that the expansion of song learning capacities observed in the F_1 hybrids between ZFs and CFs are not likely to be associated with heterotic traits in their mesoscopic brain anatomy. The song system of each species differs not only in song nucleus size but also in transcriptomic pattern. Previous studies suggest that species differences in gene expression in the song system underlie the regulation of species-specific songs (Asogwa et al., 2018; Hayase et al., 2021; Ko et al., 2021; Leung et al., 2011; Wada et al., 2013). Therefore, it is important in this study to also compare the gene expression related to the functional differences among neurons constituting the song nuclei in the parental species and the hybrids.

Heterosis, also known as hybrid vigor, has been a mystery of heredity for a long time. It is a phenomenon in which F_1 hybrids between different lines or species acquire quantitatively and/or qualitatively superior phenotypes compared to either parental species (Shull, 1908, 1948). To date, all of the studies that reported heterosis in the learning capacities of hybrid animals have conducted only behavioral experiments (Ennik et al., 2006; Lassalle et al., 1991; Proops et al., 2009) and have not investigated the underlying neuro-molecular mechanisms. Meanwhile, research on heterosis other than learning suggests that the expression of non-additive genes plays a key role in the emergence of heterotic phenotypes in plants and animals (Fujimoto et al., 2018; Hedgecock et al., 2007; D. Li et al., 2016; Mai et al., 2021; Shiba, 2015; Swanson-Wagner et al., 2006; L. Wang et al., 2015). In general, when comparing gene expression levels in F_1 hybrids (F_1) with those of the two parental species (P1 and P2), most genes in F_1 hybrids are expressed at an average levels between the two parental species. This average, known as the midparent value, is calculated as MPV=(P1+P2)/2, and this pattern of gene expression is referred to as additive gene expression (F_1 = MPV). On the other hand, the expression levels of a small subset of genes significantly deviate from MPV, a phenomenon defined as non-additive gene expression (F_1 > MPV or F_1 < MPV). In F_1 hybrid rice with yield heterosis, non-additively expressed genes were found in multiple quantitative trait loci (QTLs), which drive yield heterosis (D. Li et al., 2016). Similarly, in hybrid *Arabidopsis*, genes in metabolic pathways related to biomass heterosis were non-additively expressed in the F_1 hybrids with larger biomass than either parental line (L. Wang et al., 2015). Note, however, that there is still no consensus on whether the

number of non-additive genes reflects the extence of heterosis (supportive conclusion: Carlson et al., 2022; X. Li et al., 2009; Wu et al., 2016; negative conclusion: Fujimoto et al., 2018). Mechanisms suggested to underlie non-additive gene expression include *cis*-/*trans*- regulatory divergence caused by heterozygous loci, allele-specific gene expression, or changes in epigenetic regulation (Botet & Keurentjes, 2020; Fujimoto et al., 2018; Liu et al., 2022; Shiba, 2015; Stupar et al., 2008; L. Wang et al., 2015). Although no study has investigated the genetic mechanisms of learning heterosis, non-additive gene expression may have occurred in the song system of the F_1 hybrids in this study as well.

Some genes such as cAMP response element binding protein (*CREB*), N-methyl-d-aspartate (NMDA)-type glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, calcium/calmodulin-dependent protein kinase II, histone acetylase /deacetylase, and DNA methyltransferases are well known for their importance in learning across many species (Bailey et al., 1992; Dash et al., 1990; Glanzman, 2010; Halder et al., 2016; Keiser et al., 2024; Levenson & Sweatt, 2006; Lisman et al., 2002; C. A. Miller & Sweatt, 2007; Mitchnick et al., 2015; Morgado-Bernal, 2011; Morris et al., 2014; Tang et al., 1999; Tsien et al., 1996). In regards to songbirds, several other genes in addition to the genes mentioned above, such as androgen receptor (*AR*) (Wada et al., 2013) and cholinergic receptor muscarinic 2 (*CHRM2*) (Asogwa et al., 2018) are also suggested to play some role in species differences in song or song learning, as these genes are differentially expressed across species in specific song nuclei (*AR*: Area X; *CHRM2*: HVC). Wada et al., 2013 showed a correlation between expression levels and species differences in the mean coefficient of variation of the syllable gaps between two wild and domesticated songbird strains. Are these genes also related to the generation of species differences in learning, or are there any other genes involved?

Species-specific behavior is regulated by specific types of neurons (Alder & Rose, 1998; Edwards et al., 2002; Gupta et al., 2021). Differentiation in neural properties of homologous neurons among different species is accompanied by transcriptome differences and changes in neurotransmitters or receptors, or the distribution of such receptors (Katz & Harris-Warrick, 1999; Wright et al., 1996; Young et al., 1998). In songbirds, several studies have performed single-nuclei RNA sequencing (Colquitt et al., 2021, 2023; Toji et al., 2024; Xiao et al., 2021). In a study focusing on F_1 hybrids between ZF and owl finch (OF), Toji, Sawai, Wang et al. (2024) revealed that individual transcriptomic differences are specific to excitatory projection neurons in the RA of the VMP even before song learning, suggesting that these differences may have influenced a predisposed vocal motor bias in certain acoustic properties of songs, such as syllable duration, mean amplitude, mean pitch goodness,

and entropy variance. Although heterotic learning capacity was not observed in these F_1 hybrids, this study highlighted the importance of examining transcriptome differences not at the entire song nucleus level but at the level of each cell type, to gain better insights into the link between differential gene expression and the functional properties of specific cell types.

Therefore, in **Chapter III**, I conducted single nuclei RNA-seq in HVC, RA, Area X, and the caudomedial nidopallium (NCM), an auditory area related to the recognition and memorization of conspecific vocalizations (Mello et al., 1992; Terleph et al., 2007) (**Chapter III, Supplementary Fig. 1**). The upregulation of immediate early gene (IEG) expression, such as Egr1 (a.k.a. ZENK) in response to hearing conspecific songs, is well known in NCM. However, whether NCM also exhibits inherent species differences in gene expression when birds are neither singing or hearing songs has rarely been examined (Louder et al., 2018; Tremere et al., 2009). I examined transcriptome differences among ZF, CF, and the F_1 hybrids in song nuclei and an auditory area at each a cell-type resolution to identify which song nucleus, which cell types, and what kind of genes affect song learning differences among these three groups.

3.2 Materials and methods

Single-nucleus RNA sequencing (snRNA-seq)

For snRNA-seq of HVC and RA, adult males of F_1 hybrids from ZF female crossed with CF male (n = 7, 162–369 phd), ZFs (n = 3, 320–814 phd), and CFs (n = 4, 202–267 phd) were used in total. For Area X snRNA-seq, we used two birds each for F_1 hybrids, ZFs, and CFs, which were the same individuals used for HVC and RA snRNA-seq except for one ZF (phd145). For NCM snRNA-seq, two F_1 hybrids, one ZF, and two CFs were used. All the sampled F_1 hybrids and CFs were tutored with the playback of recorded songs of the two parental species (i.e., both ZF and CF songs), while ZFs were tutored with playback of conspecific ZF songs. Brain sampling was performed as described in chapter II. Frozen brain sections were cut at a thickness of 300 μm in the sagittal plane using a cryostat microtome (Leica Biosystems). HVC, RA, and Area X tissues were punched out with a Rapid core sampling tool (0.5–1.2 mm diameter; EMS) and stored at –80 ℃ until nuclei isolation. For NCM tissue sampling, a brain section with a thickness of 300 μm was collected from 200-500 μm lateral from the midline. A punch biopsy with a diameter of 1.2 mm was performed at the center of the caudal part of the nidopallium, covering approximately 40–60% of the NCM region within the brain section.

The punched tissue samples were separated into seven tubes to generate 12 snRNA-seq libraries [Library #1: F_1-HVC I (n = 4); #2: F_1-RA I (n = 4); #3: CF-HVC I (n = 3); #4 CF-RA I (n = 2); #5 CF-RA II (n = 1); #6: ZF-HVC (n = 3); #7: ZF-RA (n = 3); #8: F_1-HVC II (n = 3); #9 F_1-RA II (n = 3); #10: Area X (n = 6); #11: F_1-NCM (n = 2); and #12: ZF/CF-NCM (ZF = 1 and CF = 2)] based on brain region (HVC, RA, Area X, ad NCM) and sampling timings. Punched tissues were homogenized in 750 μL of ice-cold Nuclei PURE Lysis Buffer by 40–60 strokes of a glass Dounce tissue grinder (Wheaton), incubated for 5 minutes on ice, and transferred into a 2 mL centrifuge tube. The dounce tissue grinder was washed three times with 300 uL of lysis buffer to remove the remaining cell nuclei, and the wash solution was added to the homogenate in the 2 mL tube. The homogenate sample was pipetted 10 times with a 1mL micropipette for further separation of cell nuclei and centrifuged at 400 x g for 10 minutes at 4 ℃. After removing the supernatant, the pellet was resuspended in 1 mL of Nuclei Wash Buffer by pipetting five times with wide-bore 1mL tip and then 15 times with a normal 1mL tip. The suspension was centrifuged at 400 × g for 10 minutes at 4 ℃ to pellet cell nuclei again and remove the wash buffer in the supernatant. Cell nuclei pellets were then suspended in 100 μL of Nuclei Wash and Resuspension Buffers containing DAPI, and then filtered using a 40 μm cell strainers.

Isolated cell nuclei were sorted based on DAPI fluorescence using a cell sorter (SH800, Sony). Following the manufacturer's protocol, 10× Chromium libraries were prepared using Chromium NEXT GEM Single Cell Library Kit v3.1 (PN-1000269, 10x Genomics). The cDNA with cell barcode identifiers was PCR-amplified, and sequencing libraries were prepared. The constructed library was sequenced on either MGI DNBSEQ-G400 (150 bp Paired-end) or the Illumina NovaSeq6000 (28/91PE) platform. The Cell Ranger Software Suite (v6.0.0) was used for sample de-multiplexing, barcode processing, single-cell 3' unique molecular identifier (UMI) counting, and mapping onto the reference genome. The ZF genome (bTaeGut1.4.pri, GCF_003957565.2) was used as a mapping reference genome. For identifying transcripts from each bird, the Souporcell singularity container was used for individual cell de-multiplexing. Cell indexes were clustered based on SNP information to match the number of individuals mixed in the snRNA-seq libraries using "souporcell_pipeline.py." Individual-specific SNPs were identified using the vcf file information from the Souporcell output and a custom Perl script. For library #12(ZF/CF-NCM), species-specific SNPs were used to identify the genotypes of transcripts. To determine which cell index cluster belonged to which individual, the genomic DNA of the sampled birds was sequenced with Sanger sequencing to verify individual-specific SNPs.

Cell cluster Analysis

The R package Seurat 4.2.0 was used for data filtering and analyses. The mapped data was filtered using the "CreateSeuratObject" function with min.cells = 3 and min.features = 200. Cells containing multiple nuclei in one droplet were removed based on the souporcell output. The filtered gene-barcode matrix was then normalized using the "LogNormalize" method with default parameters, and the top 2,000 variable genes were identified using the "vst" method in the Seurat FindVariableFeatures function. Subsequently, the data were scaled for each gene, shifting the mean expression across cells to 0 and adjusting the expression variance among cells to 1. Principal component analysis (PCA) was performed using the top 2,000 variable genes. To visualize the data distribution, UMAP was performed on the top 15–50 principal components to display the cells. Graph-based clustering was performed on the PCA-reduced data using the "FindNeighbors" function (with top 50 principal components) and "FindClusters" function (with a resolution of 0.5) to differentiate between different cell types.

To identify the cell type of each cell cluster, the expression of established marker genes was used (**Chapter III, Supplementary Fig. 2.**)(Asogwa et al., 2022; Colquitt et al., 2021; Toji et al.,

2024). The marker genes used were as follows: Glial cell line-derived neurotrophic factor Family Receptor Alpha 1 (*GFRA1*) for HVC glutamatergic neurons projecting to RA ($HVC_{(RA)}$-PN); Steroid 5 Alpha-Reductase 2 (*SRD5A2*) for HVC glutamatergic neurons projecting to Area X ($HVC_{(X)}$-PN); Adenylate Cyclase Activating Polypeptide 1 (*ADCYAP1*) for glutamatergic neurons in arcopallium surrounding RA; *GAD1* and *GAD2* for GABAergic neurons; solute carrier family 1 member 2 (*SLC1A2*) and Aspartoacylase (*ASPA*) for astrocytes; Platelet Derived Growth Factor Receptor Alpha (*PDGFRA*) for oligodendrocyte precursor cells (OPCs); ST18 C2H2C-type zinc finger transcription factor (*ST18*) for oligodendrocytes; Colony Stimulating Factor 1 Receptor (*CSF1R*) for microglia; Steroid 5 Alpha-Reductase 2 (*SRD5A2*) and signal peptide, CUB domain and EGF like domain containing 1 (*SCUBE1*) for the glutamatergic neurons in RA projecting to the nucleus of cranial nerve XII (RA-PN). Medium spiny neurons (MSN) in Area X were identified based on another previous report (Toji et al., 2024), Forkhead Box P2 (*FOXP2*), Tachykinin Precursor 1 (*TAC1*), and Neuropeptide Y (*NPY*) to identify and classify three clusters of MSNs. Clusters without specific marker genes were filtered out as unknown clusters. Subsequently, to integrate data from multiple libraries and account for differences in cell number among bird groups, Seurat objects from multiple libraries were combined using "IntegrateData" (anchor weighting dimension: 50) with 5,000 anchor genes identified by "FindIntegrationAnchors" (CCA dimension to specify the neighbor search space: top 50). After data integration, 15,958 cells in HVC, 20,129 cells in RA, 5,509 cells in Area X, and 6,943 cells in NCM were obtained for further analysis. To ensure equal representation among species groups and avoid the influence of cell number differences, we randomly selected 1,200 cells per species per song nuclei for generating UMAPs. For categorizing cell cluster distributions in UMAPs (as 'similar,' 'different,' or 'intermediate' between the ZFs, CFs, and F_1 hybrids), the genotypes of 10 neighboring cells for each cell were first examined, considering the UMAP 1 and 2 axes (**Chapter III, Fig. 1. A**). The percentage of cells that share the same genotype with all 10 neighboring cells was then calculated for each cell type. We then classified the cell types as having 'different' distributions when more than 85% of the cells were located with all 10 neighbors of the same genotype. Conversely, if less than 50% of the cells shared the same genotype with their 10 neighbors, such cell types were classified as 'similar' distributions. An 'intermediate' distribution was defined as any categorization not meeting the above criteria.

Differentially expressed genes between two groups were identified using "Findmarkers." According to the population size, an equal number of cells (40-150 cells/group) were randomly

selected from each species group (ZF, CF, and F_1) for each cell type. Cell types with fewer than 40 cells per group due to a shortage of successfully sequenced cells were excluded from this analysis, as they had insufficient cell numbers in each group. Significance was determined using the Wilcoxon rank-sum test (default setting) in the "Findmarkers" function, and *p*-values were adjusted by Bonferroni correction using all genes in the dataset.

Non-additively expressed genes were defined as genes whose expression level significantly differed from the mean expression level of the two parental species groups. In this study, we used "FindMarkers" function in Seurat (with a minimum fold-change (FC) difference setting of 0 and a minimum fraction of cells detected in either population of 0) to identify genes whose expression level significantly differed from at least one of the parental species as non-additively expressed genes. The analysis of non-additively expressed genes was performed for each cell type independently. Furthermore, non-additively expressed genes were classified into four types based on the relationship in gene expression levels among the F_1 hybrid and the two parental species: Type I represented over-dominance (F_1 > ZF & CF), Type II represented under-dominance (F_1 < ZF & CF), Type III represented ZF bias (significant difference in gene expression level between F_1 and CF but not F_1 and ZF), and Type IV represented CF bias (the opposite case of type III).

Gene ontology (GO) analysis was performed to examine gene enrichment of non-additively expressed genes in each cell type using Metascape v3.5.20230501 (https://metascape.org/gp/index.html#/main/step1). All the input Entrez gene IDs were converted to human Entrez gene IDs to perform enrichment analysis. Significance of the enrichment term was calculated after clustering similar GO terms to reduce the redundancy in the interpretation of the result (Zhou et al., 2019).

For the correlation analysis between gene expression levels and both syllable learning and syllable repertoire size, seven F_1 hybrids with varied syllable learning rates and repertoire sizes were examined. Gene expression levels in each individual were represented as average values among 95 cells/bird of $HVC_{(RA)}$-PNs or 71 cells/bird of RA-PNs, calculated using the "AverageExpression" function. The correlation between the average expression level of non-additive genes and both syllable learning rate or syllable repertoire size was examined using Pearson's product-moment correlation in R.

Statistical Analysis

snRNA-seq data were analyzed using Seurat 4.2.0, including statistical tests. Specifically, to

examine significant differences in gene expression levels between two groups, the Wilcoxon rank-sum test and Bonferroni correction were applied. The correlation between gene expression levels and either syllable learning achievement or syllable repertoire size was analyzed using Pearson's product-moment correlation coefficient. GO enrichment analysis for non-additively expressed genes was performed with Metascape v3.5. Briefly, in Metascape, gene enrichment was calculated based on a comparison between the ratio of genes belonging to a given pathway "A" (i.e. gene group based on functional property) contained in gene lists provided by the user. If the ratio was larger than the expected by chance, which was calculated as the ratio of total genes contained in pathway "A" to the background gene list, the enrichment score increased. In addition, based on this concept, the p-value for the significance of enrichment was calculated using a hypergeometric distribution. Although the p value is not adjusted, $\log_{10}p < -6$ would be statistically significant even after Bonferroni correction or BH correction (Based on explanation on the Metascape website, https://metascape.org/blog/?p=122).

3.3 Results

Cell type- and song nuclei-specific transcriptome divergence among the parental species and the F_1 hybrids

After sequencing the snRNA-seq libraries, I defined cell types in each target area using the distribution patterns of cell clusters and marker gene expression patterns on UMAP plots (**Chapter III, Fig. 1-A)**. The UMAP plot visualizes transcriptome similarity among cells based on the top 2,000 highly variable genes. The distance of dots represents the relative transcriptome differences between cells, where identical cell types cluster together. The UMAP plot revealed cell type-specific transcriptome divergence among the ZF, CF, and F_1 hybrids: the clearest separation was observed in glutamatergic projection neurons ($HVC_{(RA)}$-PNs, RA-PNs) and MSNs in Area X, moderate separation in glial cells (astrocytes, oligodendrocytes in RA), and overlapping distributions in microglia, oligodendrocytes in HVC, Area X and NCM, and oligodendrocyte precursor cell (OPC), as well as GABAergic neurons in all four target areas (**Chapter III, Fig.1-A)**. Contrary to the glutamatergic projection neurons in the VMP, glutamatergic neurons in NCM did not show separation among the two parental species and F_1 hybrids. Instead, the three groups were adjacent within a single cluster on UMAP. However, as UMAP plots visualize relative similarities within the same UMAP space, cell distribution patterns in UMAPs are based on relative similarity among the cells in the same UMAP space; thus, comparisons across different UMAP plots are not valid.

To confirm whether the transcriptome divergence levels in a specific cell type differ among the four target areas, I next integrated all the data from those four target areas into one UMAP plot (**Chapter III, Fig.1-B**). The integrated UMAP clearly indicated that transcriptome patterns in glial cells (astrocytes, microglia, oligodendrocytes, and OPC) and GABAergic neurons (except MSNs in Area X) were similar among the four target areas, as each of those cell types clustered together regardless of the origin of the four target areas (**Chapter III, Fig 1-B** left and middle). In contrast, glutamatergic neurons from three different areas—$HVC_{(RA)}$-PN, RA-PN, and NCM glutamatergic neurons—were distributed separately (**Chapter III, Fig 1-B** left and middle). Among each type of glutamatergic neurons, the three genotypes were also separately distributed in $HVC_{(RA)}$-PNs and RA-PNs (**Chapter III, Fig 1-B** right). Similarly, MSNs in Area X were also distributed separately from other GABAergic neurons (**Chapter III, Fig 1-B** left and middle), and cells from the three genotypes were separated but positioned closer together compared to the UMAP in **Fig. 1-A,** which contained only Area X data (**Chapter III, Fig 1-B** right). Among $HVC_{(RA)}$-PNs, RA-PNs, and MSNs, RA-PN

showed the clearest separation by genotype group, followed by $HVC_{(RA)}$-PN, while MSNs showed a relatively adjacent distribution (**Chapter III, Fig 1-B** right). These results further supported the notion that transcriptome patterns were distinct among the two parental species and the F_1 hybrids, specifically in glutamatergic neurons in the VMP and to a moderate extent in MSNs in Area X.

Non-additively expressed genes in glutamatergic projection neurons in HVC and RA

While more than 97% of expressed genes in all cell types of F_1 hybrids were additively expressed, I found cell type-specific expression of non-additive genes. The number and combination of non-additively expressed genes differed in each cell type, with numbers of non-additive genes being prominently larger in glutamatergic projection neurons in the VMP ($HVC_{(RA)}$-PN and RA-PN). I aggregated the number of non-additive genes classified into any of the following four types (F_1, ZF, and CF indicate gene expression levels in each F_1, ZF or CF): (1) 'over-dominance'; F_1 > ZF & CF, (2) 'under-dominance'; F_1 < ZF & CF, (3) 'ZF bias'; $F_1 \fallingdotseq$ ZF $\neq$ CF, and (4) 'CF bias'; $F_1 \fallingdotseq$ CF $\neq$ ZF (**Chapter III, Fig. 2A, B)**. Although the proportion of non-additively expressed genes relative to all expressed genes was small (~2.2%), the ratio of non-additive genes to total expressed genes within each cell type was varied: Non-additive genes were most accumulated in glutamatergic projection neurons of $HVC_{(RA)}$-PN (189 genes, 1.00%) and RA-PN (406 genes, 2.2%). On the other hand, in MSNs, non-additively expressed genes were much fewer (64 genes, 0.4% in MSN1 and 61 genes, 0.4% in MSN2), even though the UMAP cell cluster showed a separated distribution among the parental species and the F_1 hybrids. Glial cells also contained some non-additively expressed genes (maximum ratio: astrocyte in RA, 106 genes, 0.6%), and GABAergic neurons had the least enrichment of non-additively expressed genes (maximum in RA, 40 genes, 0.2%). The detected number of non-additive genes in each cell type largely corresponded to the divergence levels observed in UMAP genotype cluster distribution. To examine whether those non-additive genes contributed to the cell type/song nuclei-specific transcriptome divergence, I removed the gene expression data for non-additively expressed genes from the integrated UMAP plot. As a result, when non-additively expressed genes were removed, glutamatergic projection neurons in the VMP and MSN in Area X from the three group—ZF, CF, and F_1—gathered closely within each cell type cluster (**Chapter III, Fig. 2-C**).

Functional property of non-additive genes in glutamatergic projection neurons in the VMP

Next, to search for the potential functional properties of the non-additively expressed genes in each cell type, I performed GO analysis (**Chapter III, Fig 3-A**). In $HVC_{(RA)}$-PNs, non-additively

expressed genes were significantly enriched with GO terms such as alternative mRNA splicing via spliceosome, potassium ion transmembrane transporter activity, gated channel activity, second-messenger mediated signaling, and calcium ion binding. In RA-PNs, many non-additive genes were related to cell-cell adhesion, and some other significant GO terms were shared with $HVC_{(RA)}$-PNs regarding channel activities. Contrary to the projection neurons in the VMP, in NCM glutamatergic neurons, and MSNs, only a few GO terms were significant, and they were shared with $HVC_{(RA)}$- and RA-PNs. Among other cell types, oligodendrocytes contained some significant GO terms related to neural activity, and the remaining cell types showed hardly any enrichment in GO terms. Finally, for a comprehensive understanding of enriched genes in $HVC_{(RA)}$-PNs and RA-PNs, where many significant GO terms were listed, I visualized the relationship between the top GO terms (Metascape $p < 0.01$) in each of these two cell types as GO networks (**Chapter III, Fig 3-B**).

Potential links between expression levels of non-additive genes and syllable learning outcomes among the F_1 hybrids

To further explore which non-additive genes are likely to be associated with the learned phenotypes, I analyzed the correlation between the expression levels of the non-additive genes in PNs of VMP and syllable learning achievements or acquired syllable repertoire size among seven F_1 hybrid birds tutored by double-species tutor songs. Some non-additive genes such as Potassium Voltage-Gated Channel Subfamily C Member 2 (*KCNC2*), solute carrier family 25 member 13 (*SLC25A13*), and MTSS I-BAR Domain Containing 1 (*MTSS1*) showed a positive correlation tendency with syllable learning achievement, syllable repertoire size, or both (all $p < 0.05$, Pearson's product-moment correlation) (**Chapter III, Fig. 4-A**). Some genes, such as Wingless-Type MMTV Integration Site Family Member 4 (*Wnt4*), showed a negative correlation tendency with syllable learning achievement. In RA-PN, genes such as potassium channel Potassium Voltage-Gated Channel Subfamily H Member 1 (*KCNH1*), Bone Morphogenetic Protein Receptor type 1B (*BMPR1B*), and NIMA Related Kinase 3 (*NEK3*), which are related to cell morphogenesis, showed a positive correlation tendency with both syllable learning achievement and repertoire size. In contrast, glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B*) or SLIT and NTRK-like family member 1 (*SLITRK1*) showed a negative correlation tendency with syllable learning achievement. Finally, for these "correlation genes," I compared the ratio of such genes included in non-additive genes composing enriched GO terms versus additive genes in the same cell type. In both $HVC_{(RA)}$-PNs and RA-PNs, genes correlated with either song learning achievement or syllable repertoire were more abundant in non-additive genes composing

significant GO terms (**Chapter III, Fig. 4-B**).

3.4 Discussion

Since no differences were observed in the mesoscopic anatomy of the song nuclei, next I focused on differences in transcriptome levels in the song system among the F_1 hybrids and the parental species. Some studies have reported species differences in expression levels of some genes, which are either constitutively expressed or induced by singing in song nuclei (Asogwa et al., 2018; Hayase et al., 2021; Ko et al., 2021; Leung et al., 2011; Wada et al., 2013; H. Wang et al., 2019). Including those studies and other studies that have focused on interspecies F_1 hybrids, a significant correlation has been found between expression levels of such genes and specific song acoustic features such as variability of ISI, which is related to species/individual differences in songs (Toji et al., 2024; Wada et al., 2013; H. Wang et al., 2019). However, for most genes with species-specific expression levels, the relationship between expression levels and song or song learning capacity remains unexplored. Additionally, in the studies mentioned above, transcriptome comparisons of the entire song nuclei were conducted using methods such as *in situ* hybridization or bulk RNA-seq, making it difficult to detect significant differences in lowly expressed genes or to determine in which cell type such gene expression differences occurred. Recent studies using snRNA-seq have revealed that transcriptome patterns differ among cell types within a song nucleus, with difference in numbers and composition of those differentially expressed genes (Asogwa et al., 2022; Colquitt et al., 2021; Nevue et al., 2023; Toji et al., 2024; Xiao et al., 2021). In addition to the song nuclei, I also included the auditory area NCM. Based on the fact that *Egr1*, one of the IEGs, is upregulated in NCM in response to auditory stimuli from conspecific songs, this region is thought to be involved in the recognition of species-specific acoustic features and the memorization of tutor songs (Mello et al., 1992; Terleph et al., 2007). The boundary of NCM cannot be traced histologically, and it is unclear if the size of NCM and song learning capacity are related. Besides, little is known about which genes underlie innate sensitivity to conspecific songs in NCM. Therefore, this study also examined the existence of a genetic basis for species differences in auditory learning by comparing transcriptomes in NCM among the ZF, CF, and F_1 hybrids.

Among the four target areas examined in this study (HVC, RA, Area X, and NCM), significant transcriptome differences among ZFs, CFs, and the F_1 hybrids were observed, particularly in excitatory projection neurons ($HVC_{(RA)}$-PNs, RA-PNs) of the VMP, each of which is crucial for the regulation of syllable sequence and the acoustics of each syllable. Non-additive genes (approximately 200–400 genes) contributed to these differences in F_1 hybrids. Additionally, the non-additive genes included genes related to ion channels and signal transduction, which are likely to affect the

physiological activity properties of neurons. Genes related to alternative mRNA splicing in $HVC_{(RA)}$-PNs, and genes related to cell adhesion in RA-PN were the most significantly enriched. Among the F_1 hybrid individuals, the proportion of genes correlated with syllable learning capacity or repertoire size was higher in non-additive genes enriched in significant GO terms compared to additive genes in the same cell type.

There is no consensus on the possible relationship between heterosis and the number of non-additive genes. Some studies have reported that more non-additive genes were expressed in the F_1 hybrids exhibiting heterosis than in those that did not (Lu et al., 2023; Xiao et al., 2021), while other studies have suggested that additive genes also can contribute to heterosis (Fujimoto et al., 2018; Qiao et al., 2024). Thus, I cannot deny the possibility that the cell types with fewer non-additive genes are also related to heterosis. For instance, GABAergic neurons showed the lowest level of transcriptome differentiation between the parental species and the F_1 hybrids among cell types identified in the VMP, while multiple studies support the importance of these neurons in song learning, indicating that proper regulation of inhibitory input to $HVC_{(RA)}$-PNs and RA-PNs is crucial towards the end of the critical period, ensuring precise singing of the learned song (M. N. Miller et al., 2017; Vallentin et al., 2016). In both cases of HVC and RA, the inhibitory inputs come from GABAergic interneurons in the same nuclei, and inhibitory inputs become more specialized as song learning proceeds, suppressing the response of excitatory neurons to hearing songs other than tutor songs, which is thought to "protect" activity patterns that code successfully learned songs. In adult ZF, decreasing the effect of inhibitory input within HVC by pharmacological or optogenetic methods allowed slight song modification with additional call/introduction-like notes at the end of the song bout, but did not disrupt the major structure of the original song (Heim et al., 2024). Based on that, establishment of song structure may be aided but not entirely dependent on the activity of GABAergic neurons. Rather, other factors, such as the formation of connections between $HVC_{(RA)}$-PNs and RA-PNs, also may contribute to the primary establishment of the learned song pattern. Even if the number of non-additive genes does not necessarily reflect the degree of heterosis, the accumulation of non-additive genes with deviation in expression levels from the parental species' average may lead to phenotypic novelty or diversity in F_1 hybrids. In the F_1 population of this study, while more than half of the individuals who received double-species tutoring learned syllables from the song tutors of both parental species, there was large variation among individuals in learning achievement across the population. Moreover, in the GO analysis, a higher proportion of non-additive genes in significant GO terms for both $HVC_{(RA)}$-PNs and RA-PNs showed a correlation between expression levels and syllable learning rates compared to

additive genes. Considering these results, the identified non-additive genes in this study exhibited greater variability in expression levels among F_1 hybrids compared to additive genes. While these genes may not have necessarily caused heterosis in all the F_1 hybrids, they may be related to individual differences in song learning capacity. Further detailed analysis of individual genes will be needed to determine the specific expression levels of genes involved in the expansion of learning capacity in the F_1 hybrids. At least, the heterosis in song learning capacity observed in this study may have resulted from interactions among multiple genes, including both additive and non-additive genes. Therefore, it is possible that expanded song learning capacity arose not from a single gene but from changes in the balance of gene expression.

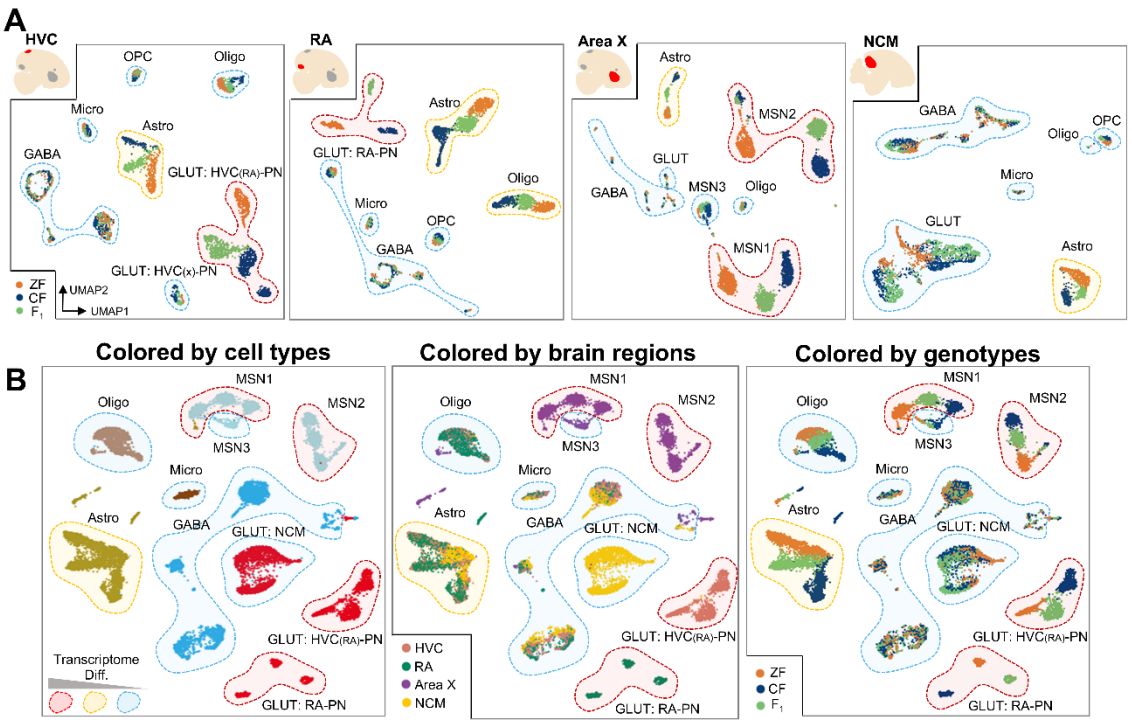
Furthermore, non-additive genes concentrated in $HVC_{(RA)}$-PNs and RA-PNs were both enriched for ion channel subunits and receptors involved in signaling pathways. Interestingly, the most significantly enriched GO terms differed between the two cell types, with “alternative splicing” being the most enriched GO term in $HVC_{(RA)}$-PN and “cell adhesion molecules” being the top enriched term in RA-PN. Alternative splicing is suggested to have an important role in stress response. In the brain, the regulation of alternative splicing is essential for normal neural function, and in the mouse hippocampus, stress-induced alternative splicing of the acetylcholinesterase gene is known to be involved in the enhancement of fear memory (Nijholt et al., 2004). In humans, abnormal alternative splicing is also known to cause various neurological diseases, including those related to memory impairment. Additionally, some studies have reported non-additive expression of genes related to alternative splicing in the heterosis of heat tolerance in abalone (*Haliotis*) and tilapia (*Oreochromi*) (Lu et al., 2023; Xiao et al., 2021). Additionally, cell adhesion molecules are thought to play a role in the formation and modulation of neural circuits related to learning capacities. For instance, N-cadherin attenuates the strength of trans-synaptic adhesion in response to neural activities, and *in vivo* disruption of N-cadherin cleavage leads to enhanced spatial learning in mice (Asada-Utsugi et al., 2021). In songbird brains, the cadherin family shows specific expression patterns in song nuclei, and its expression levels change depending on the stage of song development and auditory stimulation, suggesting its involvement in the formation, maintenance, or modulation of synapses during song learning and development (Matsunaga & Okanoya, 2008; J. M. Moore et al., 2011).

In conclusion, while further investigations are needed to reveal the detailed molecular mechanisms of heterosis in song learning observed in this study, in the VMP of F_1 hybrids, it is suggested that non-additive gene expression related to alternative splicing in $HVC_{(RA)}$-PNs may have been induced in response to the auditory experience of complex song tutors with a rich syllable

repertoire. Besides, non-additive gene expression related to ion channels and receptors, as well as those involved in signaling pathways that affect the functional physiological properties of neurons, may have contributed to the production of a broader range of acoustic characteristics than those of the parental species during song development. In regulating synaptic formation among these projection neurons, the non-additive expression of cell (synaptic) adhesion molecules (SAMs) may have contributed to the capacity of some individuals to maintain and more freely control two distinct songs. There are many families of SAMs, and each SAM protein has specificity in affinity for other adhesion molecules, which is important for the formation of synapses between specific cell types (Rudenko, 2017). The molecular behavior of some SAMs in binding or releasing is also regulated in response to neural activation (Asada-Utsugi et al., 2021). Considering these molecular properties, it is possible that alternations in expression levels or the composition of SAMs in a neuron due to non-additive effects may alter the fate of neurons in determining which neurons form synaptic connections, thus resulting in novel connectome patterns in the VMP of F_1 hybrids that differ from either parental species. It is still unexplored whether the non-additive expression of SAMs at the synapse is rather uniform within each individual or varies from cell to cell in an individual.

Indeed, among the F_1 hybrids tutored with double-species songs, some individuals exhibited greater flexibility in switching ZF and CF song parts during singing. In contrast, others showed more stereotyped singing patterns, such as always singing the ZF song part first and following with the CF song part. Such individual differences might be influenced by the variabilities in affinity among PNs which are caused by the non-additive expression of SAMs. Moreover, since adult birds were used in this study to examine the achievements of song learning, it remains unclear whether a similar tendency of non-additive gene expression would be observed in the transcriptomes of juvenile F_1 hybrids before song learning. If the composition of non-additive genes changes depending on the tutoring conditions, there may be fewer individual variabilities in non-additive gene composition in juveniles before song learning. If innate individual differences in the expression patterns of non-additive genes exist, it would be difficult to compare transcriptomes of song nuclei before and after song learning in the same individuals due to the invasive nature of such experiments.

3.5 Figures

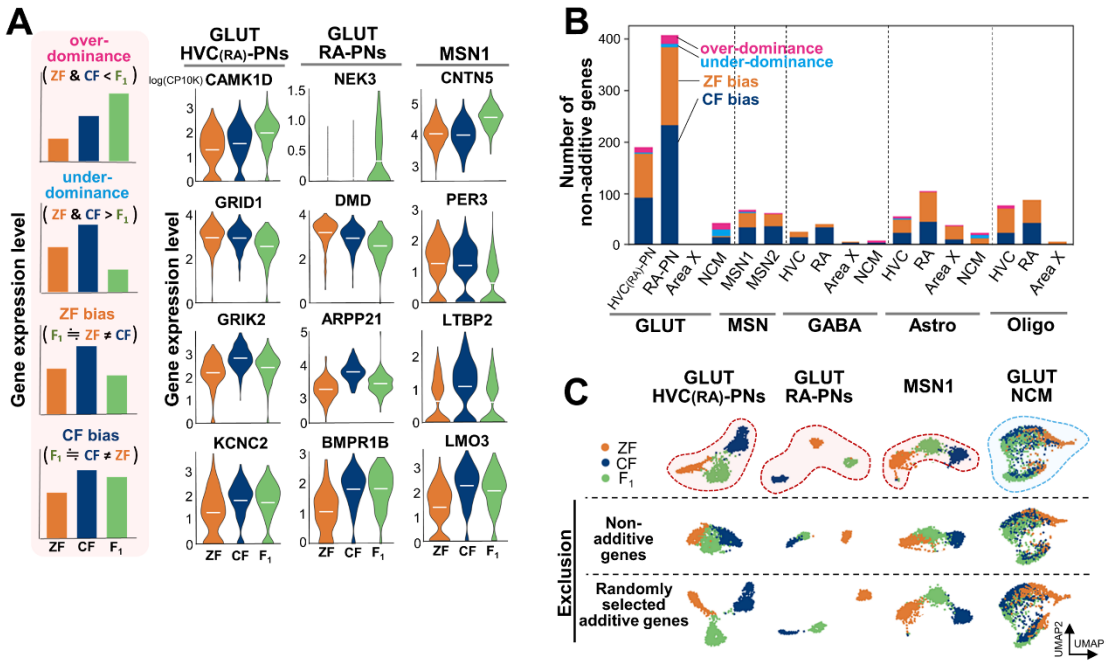


Chapter III, Fig. 1.

Cell type and song nuclei-specific transcriptome divergence among the parental species and the F_1 hybrids

(A) Single-nuclei mRNA transcriptome UMAP plots in HVC, RA, Area X, and NCM. Dot colors: orange (ZF), navy (CF), green (F_1). Shading colors: highly divergent (red), moderately divergent (yellow), merged (blue).

(B) Integrated UMAP, which combines data from four UMAPs in panel A. Dot color indicates different cell types (left panel), song nuclei (middle panel), and genotypes (right panel).



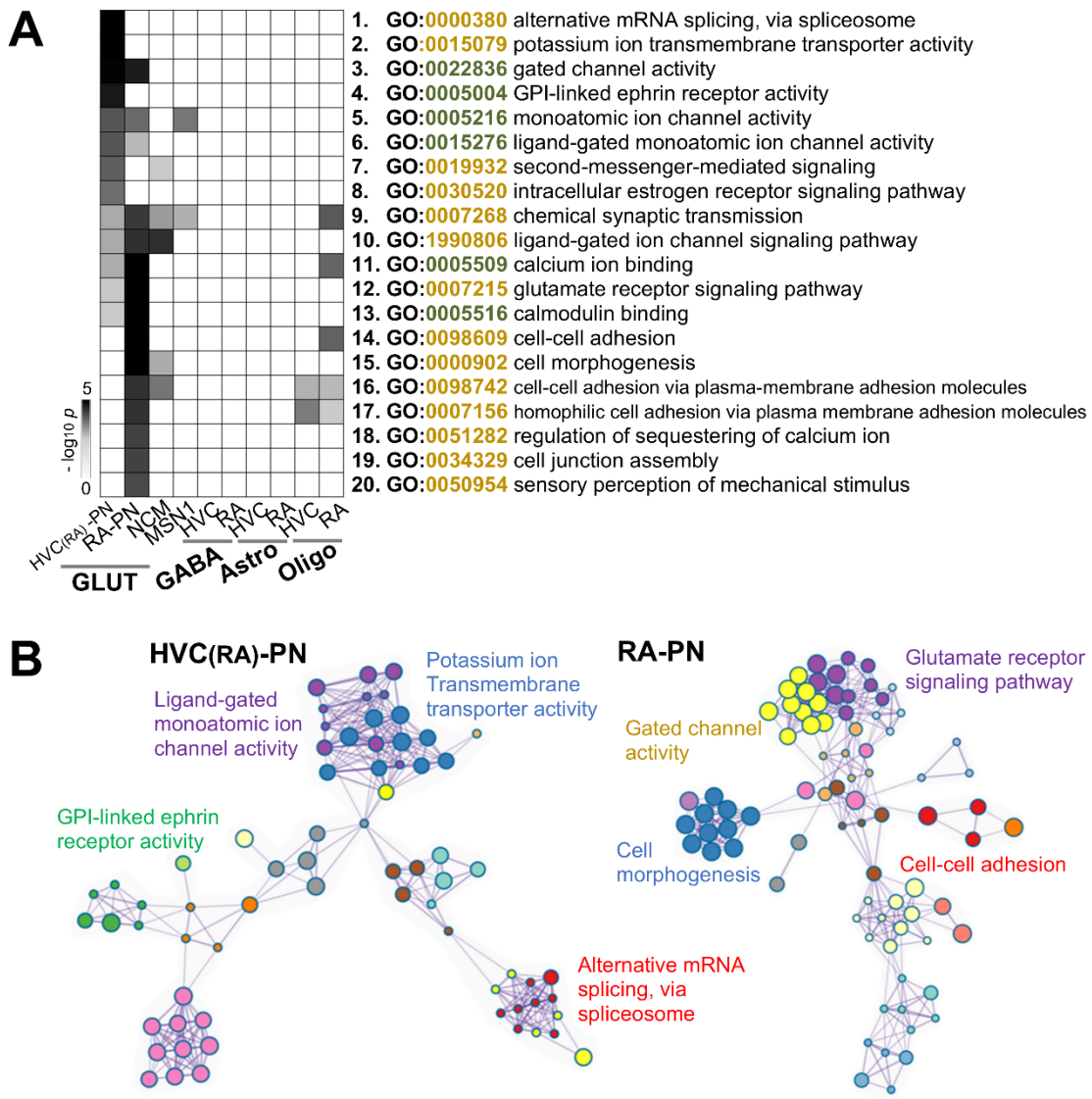
Chapter III, Fig. 2.

Non-additively expressed genes accumulated in Glutamatergic projection neurons in HVC and RA

(A) (Left, pink highlighted) Four types of non-additively gene expression patterns. (Right) Examples of non-additively expressed genes detected in $HVC_{(RA)}$-PN, RA-PN, and MSN.

(B) Number of detected non-additively expressed genes in each cell type within each song nucleus. Colors correspond to the four types of non-additively expressed genes in panel A (left, pink highlighted).

(C) Zoom-in image of cell clusters of GLUT neurons in HVC, RA, NCM, and MSN in Area X. Neurons in song nuclei showed a distribution shift among the three genotypes, while NCM glutamatergic neurons showed no distribution change.

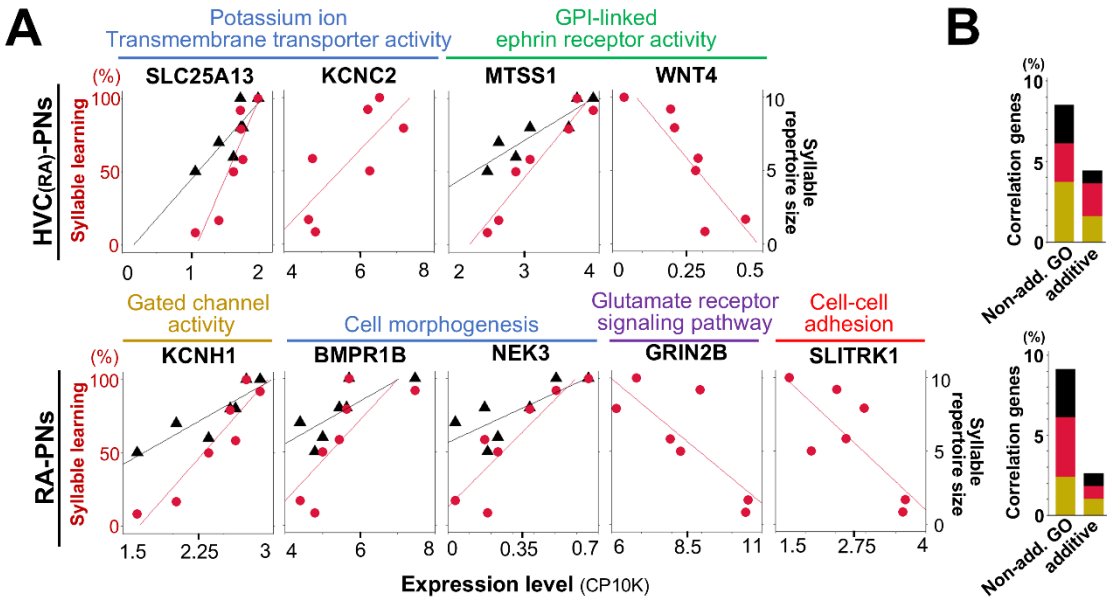


Chapter III, Fig. 3.

Functional property of non-additive genes in glutamatergic projection neurons in the VMP

(A) Enriched GO terms in glutamatergic projection neurons in each cell type with more than 20 non-additive genes. The colors of GO IDs indicate molecular function (green) and biological process (khaki).

(B) GO networks of non-additive genes in $HVC_{(RA)}$-PN and RA-PN. The same color within each network indicates similar GO terms and the distance between clusters indicates the similarity of those clusters.

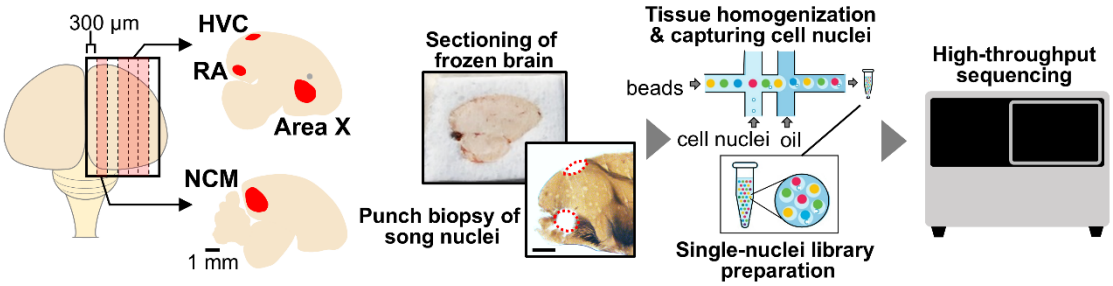


Chapter III, Fig. 4.

Correlation between expression levels of non-additive genes in glutamatergic projection neurons in the VMP and syllable learning achievement or repertoire size

(A) Non-additive genes in $HVC_{(RA)}$-PNs and RA-PNs with significant correlation to syllable learning achievement or syllable repertoire size among F_1 hybrids (Pearson's product moment correlation, $p < 0.05$ for all genes shown). In each graph, the red circle indicates syllable learning achievements (y axis) and gene expression levels (x axis) of each F_1 hybrid pupil, and the black triangle indicates syllable repertoire size (y axis) and gene expression levels (x axis) of the same pupil.

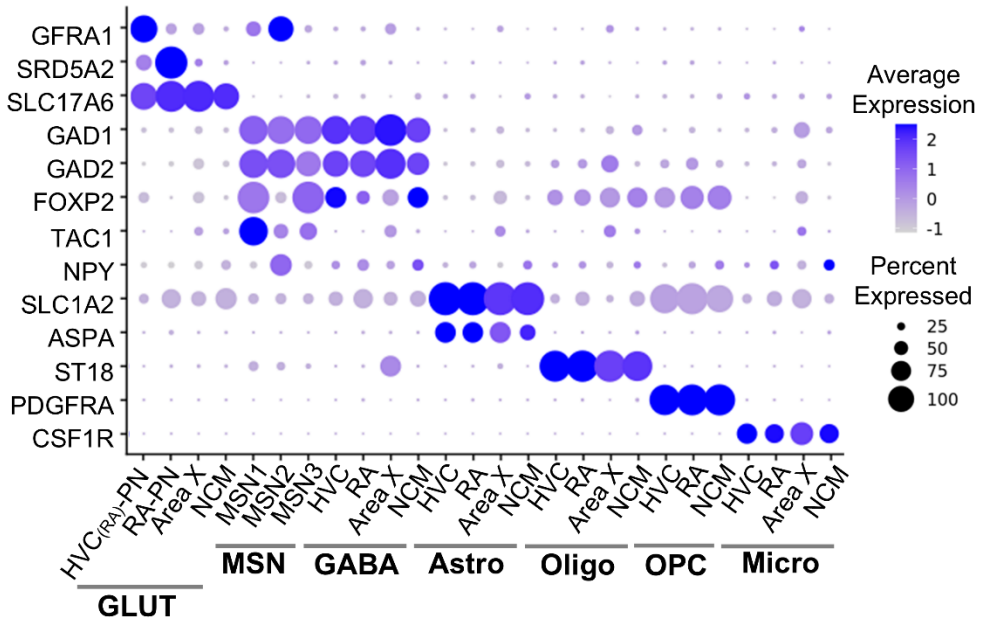
(B) Non-additive genes in $HVC_{(RA)}$-PNs or RA-PNs included in significant GO term ($p < 0.01$), which correlates with syllable learning achievement or syllable repertoire size.



Chapter III, Supplementary Fig. 1.

Workflow of single-nuclei RNA-seq

Frozen tissue of HVC, RA, Area X, and NCM were sampled by punching out fresh frozen brain slices (300 µm thickness). The sample tissues were processed to construct a single-nucleus library and sequenced by the Illumina NovaSeq6000 or DNBSEQ-G400.



Chapter III, Supplementary Fig. 2.

Marker genes used for cell type identification

General Discussion

All animal species possess species-specific learning capacities. This learning ranges from relatively simple conditioning, which elicits stereotypical responses such as innate defensive reactions (Botton-Amiot et al., 2023; Brembs, 2003; Burghardt et al., 1973; Cott, 1936; Giurfa & Sandoz, 2012; Greenlees et al., 2010), to more complex forms, such as human language acquisition, where learned content is used to construct novel, meaningful signals for communication. Although learning does not always guarantee adaptive behaviors (Laland & Williams, 1998), species-specific learning is generally genetically guided. It enables the animals in coping with crucial challenges such as environmental changes, predation avoidance, foraging, and reproduction (Arnold, 1978; Catchpole & Slater, 2003; Dugatkin & Alfieri, 1991; Dukas & Real, 1991; Griffiths, 2003; Kis et al., 2015; Kroodsma & Miller, 1982; Laland & Williams, 1997; Milinski et al., 1990; Rapaport & Brown, 2008; Schnell & Clayton, 2019; Seyfarth et al., 1980; Sheehan & Tibbetts, 2011; Slagsvold & Wiebe, 2011; Smid et al., 2007; Suboski, 1992; R. A. Zann, 1996). Despite numerous studies indicating the existence of genetic constraints on species-specific learning capacities, the genetic basis of species-specific learned behaviors remains incompletely understood.

Towards elucidating the neural and molecular bases that determine species-specific learning, I focused on vocal learning in songbirds, known for remarkable interspecies diversity in learning capacities. Expecting greater phenotypic changes or variability in their song learning capacities, I utilized inter-species F_1 hybrids between the zebra finch and the cherry finch, that are phylogenetically more distant compared to the inter-species crossbreeding combination in the previous studies (Olsson & Alström, 2020; Payne, 1980; Toji et al., 2024; Vokurková et al., 2013; Wang et al., 2019). Through the series of song playback tutoring experiments, I newly discovered that the inter-species F_1 hybrids, had ability to learn two distinct song phrases, more syllable repertoires, and heterospecific songs. These learning results were not observed either parental species, which are both single-repertoire singer with rigid constraints toward learning conspecific songs. Although the F_1 hybrids exhibited large variation in learning success among individuals, this expantion of the song learning capacity in the F_1 hybrids can be regarded as heterosis in vocal learning capacity.

Most studies on heterosis have focused on species domesticated for agriculture, dairy farming, or aquaculture. The genetic mechanisms of heterosis in learning are completely unknown, possibly because the expansion of learning capacities is not visually apparent without a behavioral examination and is less likely to be paid attention to compared to phenotypes with industrial demand

like body size or increased reproductive capacity. Even the few existing studies regarding heterosis in learning are all reports based solely on behavioral observations of a limited number of individuals (Ennik et al., 2006; Lassalle et al., 1991; Proops et al., 2009). This study is the first to examine the behavioral differences between the F_1 hybrids and the parental species using a sufficient number of individuals, as well as the first study to investigate differences in the brain anatomy or transcriptomes of the song nuclei.

Mesoscopic brain anatomical features contribute little to heterosis in song learning capacities

Inspired by the concept of heterosis and previous insights into interspecific variations in mesoscopic brain anatomy of the song system responsible for song complexity such as syllable or song repertoire size (DeVoogd, 2004; J. M. Moore et al., 2011; Szekely et al., 1996; Zeng et al., 2007), I expected heterotic changes in the volumes of song nuclei in the F_1 hybrids. However, the F_1 hybrids did not have significant heterotic changes in the size or estimated total number of constitutive neurons in any of the song nuclei examined in this study. Similarly, no significant difference was found in the ratio of excitatory to inhibitory neurons among the three groups. Considering that some other studies neither support such correlation between song nucleus volume and song complexity (Bernard et al., 1996; Brenowitz et al., 1991; Gahr et al., 1998b; Kirn et al., 1989), the volume or constitutive neuron number in song nuclei is not the sole factor determining song learning capacities. Instead, as a future direction, an examination of neuroanatomy at a finer scale, such as synaptic excitatory/inhibitory ratio or its dynamics, and spine density, will be informative to clarify whether the F_1 hybrids had anatomical change in the song system. Besides, as these traits are dynamically changes through song development, examining developmental changes in synapse composition or spine density, as well as mesoscopic anatomical traits (song nucleus size and neuron number or density) will be important work to explore the link between species-specific song development and anatomical changes in the song system. Finally, examination of the population ratio of non-neuronal cells, such as glial cells, is also worth examining, as grail cells are suggested to affects learning by supporting modulation of neural activities (Fields et al., 2014; Han et al., 2013).

Differentiation of neural properties in excitatory projection neurons in the VMP underlying expanded learning capacities in the F_1 hybrids

SnRNA-seq revealed that the transcriptome pattern is particularly distinct among ZF, CF, and F_1 hybrids in glutamatergic PNs in HVC and RA, as well as in MSNs in Area X. Astrocytes in all

four target areas (HVC, RA, Area X, and NCM) showed moderate divergence. However, other glial cells, glutamatergic neurons in NCM and GABAergic neurons showed small or almost no differences in transcriptome patterns among the three genotype groups. I also confirmed that the accumulation of differentially expressed genes (DEG) between the parental species and non-additively expressed genes in the F_1 hybrids contributed to the prominent transcriptome divergence in some cell types mentioned above.

Based on GO enrichment analysis, non-additively expressed genes in $HVC_{(RA)}$-PNs and RA-PNs were both significantly enriched with genes potentially important for the regulation of learning capacities or the generation of various vocal output patterns. Specifically, genes related to ion transport, channel activity, axon guidance, and synapse formation/plasticity were enriched in both types of neurons. Interestingly, the most significantly enriched gene groups differed between the two types of neurons. In $HVC_{(RA)}$-PNs, mRNA splicing-related genes were the most significantly enriched, whereas in RA-PNs, cell-cell adhesion-related genes were the most enriched. Functionally, $HVC_{(RA)}$-PNs play a crucial role in the regulation of syllable sequences and the rhythm of the entire song. In ZF, each of these neurons fires at a specific time point in a song (Fee et al., 2004; Yu & Margoliash, 1996), and the ablation of a population of $HVC_{(RA)}$-PNs causes transient song degradation, which is recovered through the recruitment of new $HVC_{(RA)}$-PNs via the birds' regenerative ability (Scharff et al., 2000). During normal development, the replacement or net addition of new $HVC_{(RA)}$-PNs occurs at the highest rate during the critical periods in juvenile ZF (a closed-ended learner) or every fall in CN (an open-ended learner), coinciding with the timing when both species acquire new syllable repertoires (Kirn et al., 1989; Nordeen & Nordeen, 1988; Ward et al., 1998; Wilbrecht et al., 2002). RA-PNs are responsible for the precise acoustic regulation of shorter time windows, such as the detailed acoustic structure of each syllable (Fee et al., 2004; Yu & Margoliash, 1996). What do the enriched GO terms mean in terms of their functional importance for these neurons?

Generally, the activity properties of a neural network are determined by the intrinsic firing properties of neurons and synaptic plasticity. Although synaptic modification has long been thought to be crucial for plasticity in neural network activity (Singer, 1995), the modification of intrinsic properties of neurons is suggested to be another important mechanism that underlies song learning and maintenance (Daou & Margoliash, 2021), as it can be altered much more quickly with shorter and fewer stimuli, compared to synaptic changes (Belmeguenai et al., 2010; McKay et al., 2009; Ohtsuki et al., 2012). In addition to this, excitatory projection neurons in HVC projecting to Area X (HVC(x) neurons) are suggested to reflect learning effects rather than innate genetics in their intrinsic firing

properties in adults (Daou & Margoliash, 2020). A separate study found that the intrinsic firing properties in HVC(x) neurons in an adult ZF can be modulated within one day after receiving manipulated playbacks of their own songs (Fukushima & Margoliash, 2015). Thus, the regulation of intrinsic firing properties is suggested to be an important mechanism for the quick adjustment of neural network activities during song learning in juveniles and maintenance of adult songs (Daou & Margoliash, 2021). Once intrinsic firing properties have been quickly modulated in response to auditory stimuli, synaptic plasticity may contribute to stabilizing these changes in the network activity. The intrinsic firing properties of neurons are determined by a combination of multiple types of ion currents. These properties, in turn, are influenced by the distribution patterns of various ion channels or transporters, including molecules found in the enriched GO terms in this study. It should be noted that the activity properties of a neural network cannot be predicted solely based on transcriptome patterns: there are multiple solutions to achieve similar activity patterns through different intrinsic firing properties and different synaptic connections. However, the prominently deviated transcriptome patterns suggest that non-additive gene expression in the F_1 hybrids increased the variation of possible intrinsic neural properties and synaptic connections. This, in turn, may have enabled the hybrids to produce a greater variety of sounds to mimic different songs. In addition, alternative mRNA splicing can enhance diversity in molecular function by transcribing different variants from the same coding region. This process can lead to significant changes in neural circuit performance, resulting in drastic differences in behavior or learning, as demonstrated in some studies (Demir & Dickson, 2005; Franz et al., 2023). As HVC is crucial for coding individual syllables, their order, and their rhythm, molecular diversity caused by alternative mRNA splicing may facilitate the coding of complex songs. However, the method used in this study cannot identify splicing variants; thus, further transcriptome analysis is required to confirm whether non-additive gene expression in the hybrids actually contributes to this expansion of capacity in producing various sounds. In RA-PNs, where projections from HVC form synaptic connections with RA neurons, cell-cell adhesion molecules, which include many genes related to axon guidance and synapse plasticity, were the most enriched gene group. This may have contributed to the increased variation in connection patterns between $HVC_{(RA)}$-PNs and RA-PNs, or connections between neurons within RA, which may also contribute to the flexible regulation of vocalization patterns.

Although the number of non-additive genes may not necessarily be associated with stronger heterosis, it may still contribute to shifting the hybrid transcriptome toward more divergent patterns from the parental species, potentially leading to novel phenotypic traits related to neural activity

patterns in the F_1 hybrids. Since the F_1 hybrids in this study showed prominent individual differences in syllable learning achievements, learning variability may also be linked to variations in comporition of non-additive gene expression among individuals within the hybrid population. Indeed, I found either a positive or negative correlation tendency, with a correlation coefficient greater than 0.75, between the expression levels of some of the non-additive genes and syllable learning or repertoire size in F_1 hybrids. Although the statistical significance of these correlations was not supported after the adjustment of p-values based on the Benjamini and Hochberg method (1995), this may have been affected by the small number of sample individuals.

To further understand the link between transcriptome patterns and song learning tendencies—specifically, which types of songs and syllables each F_1 hybrid can learn—I need more snRNA-seq data from the song system of F_1 hybrids tutored with different types of tutor songs, including those with four tutors or heterospecific tutors.

Impact of interspecies hybridization on song learning diversity in nature

Could such interspecies hybridization, which induces changes in song learning capacity, occur in wild populations? Moreover, if such hybridization were to occur, what kind of impact might it have on the species diversity of song learning capacities? It is generally said that heterosis tends to occur between species that are genetically more distant but still breedable (Atsumi et al., 2021; R. B. Stelkens et al., 2009; R. Stelkens & Seehausen, 2009). In the wild, sympatric songbird species form hybrid zones, where cross-breeding between two species occurs frequently worldwide, especially when the sympatric species are closely related within the same genus with similar appearances. However, in the case of hybridization between species that are visually and vocally very different, even when sympatric, it may be less likely to occur as long as there are many individuals of the same species and sex around. The wild populations of ZF and CF both live in Australia, with their habitat partially overlapping. As far as I searched, no reports in the literature were found stating that the two species naturally hybridize in the sympatric area. As they are clearly different in both appearances and songs, it is unlikely that they hybridize so frequently that the hybrids can be easily observed. However, interspecies hybridization between different genera happens frequently in some other songbirds. In the most extreme case, surprisingly, hybridization between species with completely different appearances and behaviors has been frequently repeated among birds-of-paradise (*Paradisaeidae*) (Blom et al., 2024). Furthermore, several other cases of wild hybrids between species with clearly different appearances have been reported (Toews et al., 2018, 2022). As such, interspecies-hybridization can

occur between relatively distant species in the wild, although female preference might be an important factor in determining the occurrence frequency of such events. Similar to the preference of females for conspecific males with a large song repertoire including heterospecific vocal mimicry in some multiple-song singers (Catchpole & Slater, 2003), richness of vocalization repertoire or noticeable visual ornaments rather than species-specific visual and auditory cues may be more attractive to females in some species, as the abundance of those appealing signals represents the quality of the male (Catchpole & Slater, 2003; Lovette & Fitzpatrick, 2016).

If two sympatric species breed and the F_1 hybrids are fertile, the hybrid male is most likely to breed with one of the parental species (backcross) or pair with a hybrid female. The former case would affect the song learning traits of both parental species slowly over time. In the latter case, if repeated, F_1 hybrids may form a new species with novel song features, to put it extremely simply. It is unknown what kind of songs hybrid females prefer, and for hybrid males, acquiring heterotic song learning capacity would not necessarily be beneficial for their survival if such "abnormal" songs containing acoustics of foreign species were avoided by females of the parental species. However, although this scenario may be an extreme case, if the hybrids acquire more complex songs and hybrid females also prefer such complex songs, it may be possible that expanded song learning capacity and more complex songs than those of their ancestral species could evolve.

Songbirds, which account for about half of all avian species, exhibit remarkable diversity in their song learning traits. However, how this diversity evolved remains not fully understood. This study demonstrated that interspecies hybridization can enable the F_1 hybrid songbirds to acquire expanded song learning capacities beyond the range observed in their parental species. I suggest that interspecies hybridization may have contributed to the diversification of song learning capacities among songbirds. However, many other factors need to be taken into account when assessing the significance of gene introgression caused by hybridization or backcrossing in natural populations, where various reproductive barriers work to maintain species boundaries. Further comparative studies examining species differences in both the genome and transcriptome of the song system would help to examine this hypothesis. Overall, this study provides a perspective for approaching the evolutionary scenario of species diversity in song learning capacity while also offering insights into the underlying neuro-molecular mechanisms.

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Main publication

1. **Shibata, Y.**, Toji, N., Wang, H., Go, Y., Wada, K., (2024). Expansion of learning capacity elicited by interspecific hybridization. *Science Advances*.10, eadn 3409 (2024). DOI:10.1126/sciadv.adn3409

Other publications

1. Asogwa, N. C., Toji, N., He, Z., Shao, C., **Shibata, Y.**, Tatsumoto, S., Ishikawa, H., Go, Y., & Wada, K. (2022). Nicotinic acetylcholine receptors in a songbird brain. *Journal of Comparative Neurology*, 530(11), 1966-1991.

2. Iwamoto, M., **Shibata, Y.**, Kawasaki, J., Kojima, S., Li, Y. T., Iwami, S., Muramatsu, M., Wu, H. L., Wada, K., Tomonaga, K., Watashi, K., & Horie, M. (2021). Identification of novel avian and mammalian deltaviruses provides new insights into deltavirus evolution. *Virus Evolution*, 7(1), veab003.

3. Sanchez-Valpuesta, M., Suzuki, Y., **Shibata, Y.**, Toji, N., Ji, Y., Afrin, N., Asogwa, C. N., Kojima, I., Mizuguchi, D., Kojima, S., Okanoya, K., Okado, H., Kobayashi, K., & Wada, K. (2019). Corticobasal ganglia projecting neurons are required for juvenile vocal learning but not for adult vocal plasticity in songbirds. *Proceedings of the National Academy of Sciences of the United States of America*, 116(45), 22833-22843.

4. Wang, H., Sawai, A., Toji, N., Sugioka, R., **Shibata, Y.**, Suzuki, Y., Ji, Y., Hayase, S., Akama, S., Sese, J., & Wada, K. (2019). Transcriptional regulatory divergence underpinning species-specific learned vocalization in songbirds. *PLoS Biology*, 17(11), 1-27.

Oral presentations

1. Virtual songbird satellite meeting (online), 2023 年 4 月 1 日, 『Unlocking the genetic constraints of vocal learning in interspecies hybrid songbirds』（招待講演）

2. Society for Neuroscience meeting (San Diego), 2022 年 11 月 16 日,『Novel enhanced learning ability elicited by interspecific hybridization of songbirds』Y. Shibata, N. Toji, S. Tatsumoto, H. Ishikawa, Y. Go, K. Wada (ポスター発表）

3. 日本動物学会北海道支部第 67 回大会（旭川）, 2023 年 3 月 18 日,『異種間雑種ソングバードにおける歌学習能の向上』, 柴田ゆき野、田路矩之、辰本将司、郷康広、和多和宏

4. 日本神経科学大会第 45 回大会（沖縄）, 2022 年 7 月 1 日,『異種間ハイブリッドソングバードを用いた発声学習個体差の神経分子基盤』柴田ゆき野、田路矩之、辰本将司、石川裕恵、郷康広、和多和宏（口頭発表）

5. 2021 年比較生理生化学学会大会（オンライン）, 2021 年 12 月 4 日,『Heterosis and individual difference in syllable learning abilities in F1 hybrid songbirds』,Y. Shibata, N. Toji, Y. Go, S.

Tatsumoto, H. Ishikawa, K. Wada（ポスター発表）

6. 第 44 回日本神経科学大会（オンライン）,2021 年 7 月 31 日,『Heterosis in vocal learning traits in F1 hybrid songbirds』,Y. Shibata, K. Wada（ポスター発表）

7. 日本動物学会第 90 回大会（大阪）, 2019 年 9 月,『異種間ハイブリッドソングバードにおける歌学習能力』, 柴田ゆき野, 和多和宏（口頭発表）

8. 日本動物学会北海道支部第 63 回大会（札幌）, 2019 年 3 月 23 日,『ハイブリッドソングバードの歌学習能力に対する両親の遺伝情報の影響についての研究』 柴田ゆき野、和多和宏（口頭発表）

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