NEURAL CONTROL OF VOCAL BEHAVIOR IN SONGBIRDS IN DIFFERENT SOCIAL CONTEXTS

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General introduction

Vocal communication

Vocal communication is a widespread behavior among animal taxa. In this form of communication, the sender can rapidly convey the information to the receiver through acoustic signals over short and long distances (Figure 1A) [1]. The features and function of vocalizations vary significantly between different species, ranging from swift contact calls in frogs [2] to complex courtship displays in birds [3]. Via the individual features of vocal signals, the sender can transmit various information [1, 4, 5], such as hierarchical status, age or identity and emotional state [6]. Moreover, many species can modify their vocal output to provide information to conspecifics about behavioral context [1, 7]. The receiver's presence [8, 9] and identity [10] can also dramatically change the structure of the sender's vocal signals.

Songbirds are highly vocal animals that produce a diverse repertoire of social vocalizations [11]. The vocalization repertoire of songbirds is commonly divided into songs and calls [11, 12]. In this thesis, I will concentrate on singing behavior, because comparable to human speech, song is a learned behavior. Songs are complex vocal displays, which primarily function in territory defense and mate attraction, although both function and song structure vary from species to species. Songs consist of ordered strings of vocal elements. Songbirds exhibit a diverse array of vocal structures in their songs, however, for the majority of birds, the structure typically consists of discrete vocal elements referred to as syllables, which are often organized into cohesive units known as motifs (Figure 1A).

Similar to language acquisition in humans, songbirds learn their songs during development by imitating adult conspecifics [13-16]. Although learned from a tutor, songs in many species are to some degree guided by innate templates, and variation in song composition occurs naturally [17]. During the song learning process, birds can vary the spectral features, the timing, the order and the arrangement of vocal elements in the song [18], which leads to high interindividual variation of internal song organization. Songbirds can modify their vocalizations depending on the social context [19, 20]. The courtship song of the male zebra finch (*Taeniopygia guttata*) is the most studied example of the influence of social context on birdsong [21-24]. When male zebra finches direct their song to a female, they perform a highly stereotyped version of their song, but a more variable song when singing alone [Figure 1A, 8]. Moreover, vocalization can be affected by the "Audience effect", which implies the change of vocalization behavior based on the presence of listeners additional to the intended receiver [25, 26]. Additional listeners (eavesdropper) often extract information by assessing song quality of the overheard individual and shape their behavior accordingly [27]. The final goals of eavesdropping are various, for example: evaluating the fitness of a potential breeding partner [28] or the threat level of a competitor [29].

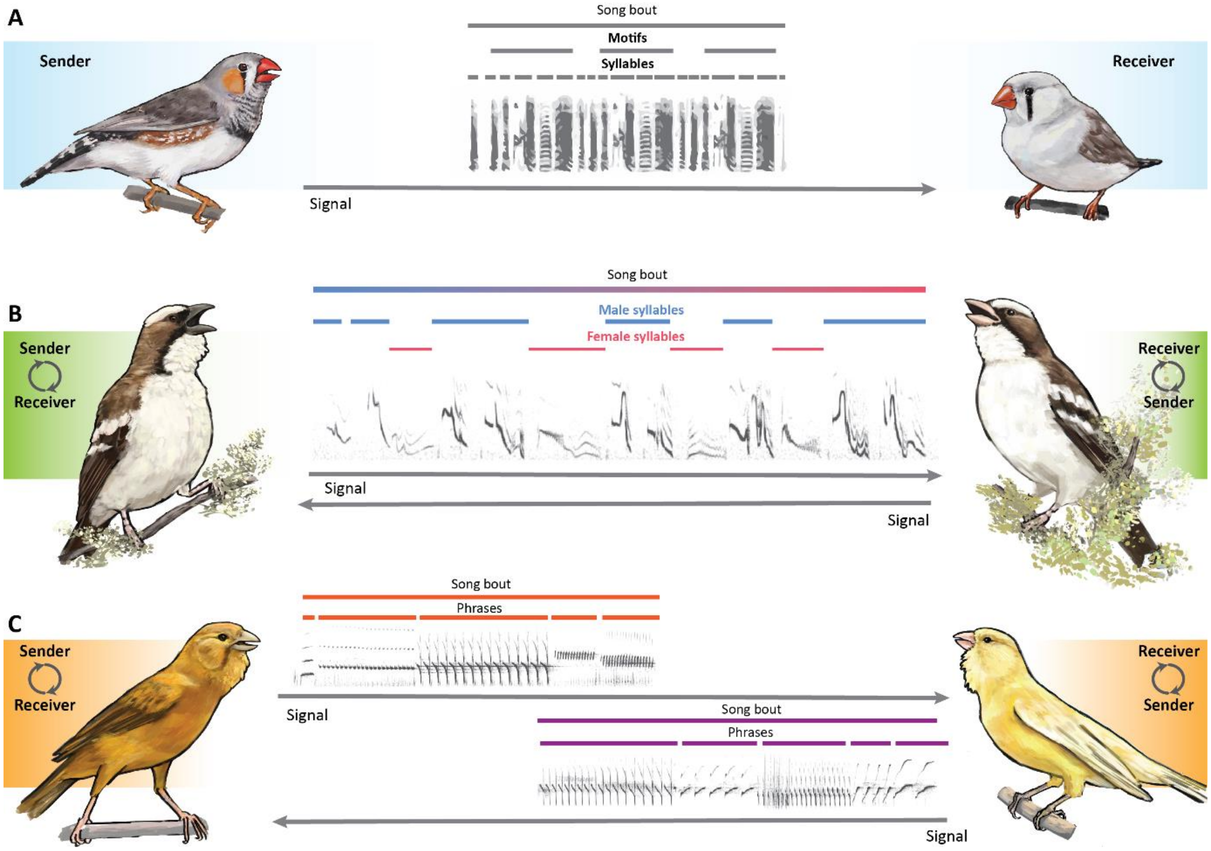

Figure 1 Vocal communication in songbirds. A. Unidirectional vocal communication. Spectrogram modified after Sakata and Birdsong [30]. B. Cooperative dynamic vocal interaction. C. Competitive dynamic vocal interaction.

Dynamic vocal interactions

Dynamic vocal interaction is a type of vocal communication in which sender and receiver actively alternate their roles and mutually adjust their vocal output to the sensory feedback resulting from the receiver's response [31, 32]. Animals heavily rely on auditory feedback not only during learning but also to maintain their vocal output [33-35], and to modify it during dynamic vocal interactions with conspecifics [36]. Auditory feedback from the interaction partner can induce temporal and pattern-specific changes to the focal individual [36].

Dynamic vocal interactions have been studied in many different animal taxa, such as amphibia [37, 38], different groups of mammals [39] including rodents [40, 41], bats [42], whales [43], primates [44] and birds [36]. However, the plethora of terms (turn-taking, duetting, antiphonal singing, counter-calling and countersinging and chorusing) used to describe such interactions, often leads to confusion when comparing interactive vocalizations across different taxa. For instance, competitive vocal interactions between male in anuran Legler's stream frog (*Ptychohyla legl*eri) are termed "duetting" in certain literature [45]. This term is akin to what might be referred to as "counter calling" in songbirds, as within avian literature, duetting is frequently described as coordinated vocalizations displayed by mated pairs [36, 46]. To address this discrepancy, Vanderhoff and Bernal Hoverud [39] proposed the term "temporally coordinated interactive vocalizations" (TCIVs) while studying mammalian, non-primate interactive vocalizations. This term aims to encompass both the dynamics between signaling individuals and the rhythmic and temporal aspects of the vocalizations themselves. The authors delineated dynamic vocal interactions into three primary categories: Antiphonal vocalizations, Duetting, and Counter-Vocalizations. Furthermore, the authors specify that this interaction may entail turn-taking, wherein individuals adapt their vocalizations according to the behavior of the other participant, including the avoidance of overlap. Antiphonal vocalization, as the most extensive category, encompasses a call-and-response pattern occurring at regular intervals among two or more individuals, but does not include precise time coordination of participating individuals. Duetting characterizes coordinated, predictable, repetitive, and stereotypical vocalizations between two individuals, which are often bonded pairs. Counter-vocalizations in the form of counter-calling or counter-singing occurs between individuals, typically involved in territorial dispute. However, the authors distinctly state that during countersinging, participants emit their vocalizations in a non-overlapping manner, which differs from the definition of countersinging in avian literature, where overlapping vocal interactions are encompassed and will be addressed later in this chapter.

Thus far, songbird has been regarded as the best model concerning the function, causation, and ontogeny of dynamic vocal interactions. Generally, in songbirds, dynamic vocal interactions between two individuals that involve singing, fall into two major categories: duetting and countersinging. In songbirds, duetting refers to a behavior where a pair of birds, typically a male and female, sing together in a coordinated manner [46]. This can involve both birds singing simultaneously or taking turns in a synchronized pattern. Countersinging is a behavior where one bird sings in response to the song of another bird either by singing a similar or different song to respond to or challenge an opponent [47].

Countersinging is often observed in territorial species where males use their songs to defend their territory and attract mates. In both types of vocal interactions, timing and vocal element sequence can be influenced by the other participating bird's song [36]. Timing plays a major role in the studies of countersinging behavior. While engaging in countersinging behavior, many species adjust the timing of their vocalizations to avoid overlaps or to actively overlap the sounds produced by the opponent [47, 48]. In case of duetting, partners also demonstrate exceptional control of timing during vocal coordination. Much like in countersinging behavior, some duetting songbird species intentionally overlap their partner, although the reason for this is not to interrupt the partner but to increase the effectivity of signaling [46]. Meanwhile in other duetting species, partners leave sufficient time between their own vocal elements for the other bird's contribution to the cooperative song.

Birds participating in either countersinging or duet singing interactions are able to adjust the ordering of vocal elements or use different types of vocal elements in their vocal response. Countersinging may elicit vocal matching, where birds use similar or identical vocal elements to interact with an opponent [47], while duetting birds use certain vocal element types that can be predicted by the duetting partner [46]. For example, duetting canebrake wrens (*Cantorchilus zeledoni*) learn to adjust their song syntax to the vocalization of a new partner [49] and male Washington song sparrows (*Melospiza melodia*) choose a certain song type that matches the playback when responding in simulated countersinging interactions [50, 51].

Songbirds can participate in dynamic vocal interactions in a reciprocal, competitive, or cooperative manner [36]. This thesis focuses on the investigation of vocal interactions between two individuals, within cooperative and competitive social contexts. Cooperative vocal behavior is defined as the vocal interaction between two or more individuals with a mutually beneficial common goal (Figure 1B) [52-55]. In songbirds, this can include both calls [56] and songs [54] that convey information about the location of food sources [57], about potential predators [58], or about parental care [52]. Cooperative vocal behaviour is commonly observed in species that live in social groups or engage in other cooperative activities, such as hunting or rearing offspring together. Typically, vocal communication complexity increases with the complexity of social structure within species [59]. Cooperative breeding in birds is strongly correlated with the size of their vocal repertoire, particularly in the proportion of contact calls and alarm calls [60]. Social complexity could also shape the structure of vocal sequences: in several songbird species, group size is positively correlated with the number of call/note combinations within songs [61, 62]. It allows individuals to navigate a higher number of social situations and to interact more effectively [59]. Competitive vocal behavior is defined as the vocal interaction between two or more conspecifics, where each animals possesses individual goal (Figure 1C), for instance, to assert dominance [63-65] or establish territorial claims [66-68], or acquire and defending potential mates [69]. The specific mechanisms employed by animals to communicate dominance can vary greatly between species, and are influenced by factors such as social structure and environmental conditions [47].

Sequential organization of birdsong

The rules that govern the arrangement of discrete vocal elements into vocal sequences [18, 70] have been studied across different animal taxa: in amphibia [71], in parrots [72, 73] and songbirds [70, 74] and in many mammals such as hyraxes [75, 76], rodents [77], mongoose [78, 79], primates [80-83], bats [84-87] and cetaceans [88]. Animals implement many different strategies to alter the structure of vocal sequences: they can change the number of vocalizations produced over a certain time period, use different vocal elements or alter the order of vocal elements within the vocal sequence [18, 47, 89, 90]. Non-human animals exhibit the capacity to encode and extract information from vocal sequences, however the sequential arrangement of vocal elements doesn’t always convey information. For instance, fruit bats, while arranging syllables into long sequences to enhance contextual information, do not seem to transmit any relevant information through syllable order [84]. At the same time, titi monkeys (*Callicebus nigrifrons*) are able to convey information about predator location and type with sequences that utilize just two call types [83].

Sequence structure can convey various levels of individual information within one species: For example, in male rock hyraxes (*Procavia capensis*), syntactic structure reflects geographical dialect [75] as well as the individual fitness level [76, 91]. Moreover, animals alter the structure of vocal sequences depending on social context since sequences directed towards different receivers are associated with specific behavioral functions. The Brazilian free-tailed bat (*Tadarida brasiliensis*), dynamically adjusts song sequence in the presence of conspecifics [86]. The structure of vocal sequences plays also a role in competitive vocal interactions in multiple animal species, for example alteration of vocal sequences can be observed in territorial disputes in several frog species [71].

In human language, the organization of vocal elements within sequences is described through syntax, i.e. the rules for arranging items (sounds, words, word parts or phrases) into their possible permissible combinations within a language [70]. The observation of linguistic rules governing statistical patterns in human language is mirrored by similar findings in vocal sequences of non-human animals, revealing the shared emergence of linguistic principles across various vocal communication systems. For example, in several primate species, such as geladas (*Theropithecus gelada*) [92] or gibbon's (cao vit gibbon (*Nomascus nasutus*) and western black-crested gibbon (*Nomascus concolor*) [93], vocal sequences follow Menzerath's law, which states that the size of a sequence's components decreases, as the number of components constructing the sequence increases.

The structural organization of birdsong is considered an analog to syntax in human speech, possessing so-called "phonological syntax" [94]. The difference lies in the semantics – which is the meaning that is assigned to a discrete vocal element. While human language can be described through phonology (meaningless sounds become meaningful sound strings) and syntax (the arrangement of meaningful sound strings into longer sound sequences), birdsong is described only through phonology. Berwick, Okanoya [70] suggest that birdsong appears to be a "graded or holistic system of communication" rather than a compositional system like language, in which different combinations of vocal elements (words) convey different meanings [70, 95]. Although it is impossible to directly compare syntax in birdsong with human speech, the combinatorial system in the structure of birdsong is a sufficient model to study the mechanism of human language syntax [70]. Comparative research demonstrated that both birdsong and human speech possess the hierarchical organization of vocal sequences [96]. This organization is characterized by a nested or multi-level structure of sounds, where sounds at one hierarchical level are influenced by those at a higher level. The hierarchical organization allows the grouping of sounds into smaller units, such as syllables in birdsong or words in human speech, and subsequently, into larger units, such as motifs or sentences. Short-range dependencies between vocal elements are governed mostly by Markovian processes, where one vocal element is mostly determined by the previous one [96]. A power law pattern is observed in the long-range dependencies, characterized by a decrease in the strength of connections between vocal elements as the distance between them increases. Furthermore, the study of bird vocal sequences has become a highly regarded area of quantitative linguistic research, aimed at uncovering universal statistical patterns that govern these sequences [97, 98]. For example, studies of songs from various species have shown that the structure of the songs follows linguistic laws, such as Menzerath's law, however, it may not be indicative of communicative efficiency but rather a consequence of vocal production constraints [98].

Overall, songbird species have multiple strategies for organizing their songs: the arrangement of vocal elements can differ in diversity, repetition, and combination (Figure 1) [18]. The rules of ordering vocal elements into sequences also vary drastically among species, from delivering syllables in a stereotyped and linear order to adhering to complex and variable sequencing rules. Bengalese finches (*Lonchura striata* var. *domestica*), for example, exercise control over the transition between syllables within "branch points" where several alternative syllables might follow a specific syllable [99, 100]. In this case, one or more preceding syllables could predict the selection of the following syllable in the song sequence. The sequential organization of vocal elements shapes the species-specific syntax, which in some species remains fixed upon the initial learning during a sensitive period [70, 101, 102]. In some species, syntax plays a role in learning by "chunks" - groups of specifically ordered syllables [103]. Chunks can organize songs hierarchically and control song interruption [99, 104, 105]. One typical example of chunks is found in Bengalese finches' songs [99]. At the same time, in other species, the syntax does not seem to play an important role. In zebra finches, altering the syllable order did not prevent recognition of the father's song by the offspring [14]. Likewise, adult zebra finches did not pay as much attention to syntax as to the acoustic structure of individual syllables [106].

In species susceptible to the song's sequential features, the individual can convey information about its identity, age, or geographical origin by ordering vocal elements [18]. For example, common nightingales (*Luscinia megarhynchos*) can recognize conspecifics by their song syntax, which can also be altered with age [107, 108], while chickadees (*Poecile carolinensis*) demonstrate distinct dialects in vocal sequences among populations [59]. Additionally, the complexity of sequences in certain species, such as marsh warblers (*Acrocephalus palustris*) [109] or song sparrows (*Melospiza melodia*) [110] can signal male quality. The influence of social context on syntax variation is especially apparent in female-directed courtship songs and male-male countersinging. In the presence of a female, males utilize not only a different selection of syllables but often modulate syllable order. Both zebra finches and Bengalese finches demonstrate less variable syntax in female-directed songs than in undirected songs, preferring to perform the optimal variant [8, 111]. Studies on male countersinging interaction demonstrated that European blackbirds (*Turdus merula*) alter the ordering of song elements depending on the number of countersinging rivals. As the level of aggressiveness in a bird increases, the variability in the order of the song syllables also tends to increase [112].

Duetting

Duetting behavior is characterized by a coordinated exchange of vocal outputs [36, 39, 46, 113]. Research on duetting has encompassed a wide range of taxa, including insects [114], amphibians [115], mammals [39, 43, 44, 116-121], and birds [36, 46, 113, 122], with the latter group being studied most extensively. Amphibian duets have received comparatively less attention, yet initial investigations suggest that male-female pairs engage in duets to facilitate copulation, enabling partners to locate receptive mates through continuous vocal exchanges. For instance, female American clawed frogs (*Xenopus laevis*) emit a 'rapping' vocalization in response to male calls, simulating the male's approach towards the female [115]. In non-primate mammals, duetting is considered a rare phenomenon, with limited studies available [39]. For example, sperm whales engage in duet-like sequences of vocal exchanges to maintain social bonds [43]. Primates represent a significant proportion of studied mammalian duetting species [39, 44], despite duetting primates comprising only slightly over 10% of primate diversity. Duetting is primarily observed in socially monogamous and territorial primates, functioning as a signal for mate guarding or joint territory defense [39, 44, 116, 121, 123].

In songbirds, duet song is a prime example of cooperative behavior, requiring two individuals to coordinate vocalization in a predictable manner. Duet songs can be observed in hundreds of bird species [46, 122]. Most duetting species are non-migratory and can be found in the tropical and subtropical regions [122]. Several behavioral traits are commonly found in duetting birds, such as prolonged pair bonds, year-round territoriality, and cooperative breeding. Depending on the species, duet songs can serve different functions, ranging from pair bond maintenance to territorial defense [46]. Generally, duet songs are defined as a pair-level singing performance in which both partners sing alternating or overlapping song elements [46, 113]. Reaction time in duetting birds is swift, demonstrating how alert they are to when and what their partner is singing [46, 122]. The duets' temporal characteristics vary, with some species displaying antiphonal, non-overlapping duets, while others exhibit simultaneous, overlapping duets (Figure 2) [46, 113].

In my thesis, I will concentrate on antiphonal duets, where the duet partners precisely alternate their vocal outputs to avoid overlap of song elements. Antiphonal singing is performed to avoid acoustic interference and consequently increases transmission quality of the vocal signal, so that both the sender and the receiver perceive the vocal signal more effectively [48]. In addition to temporal complexity, several duetting species use a specific set of interaction rules, i.e. the “duet code,” to construct their duet songs [46, 113]. Duet codes consist of a set of answering rules, and each answering rule represents the link between a certain type of vocalization produced by one bird and a certain type of vocalization produced by the partner as an answer to the first bird's vocalization [54]. For example, if the male syllable M is always followed by the female syllable F, the link between M and F is called an answering rule. Duet codes and answering rules can vary in their complexity, depending on the species [46]. Moreover, pair-specific duet codes are common, and after initial juvenile learning can be learned again as a result of re-mating [49].

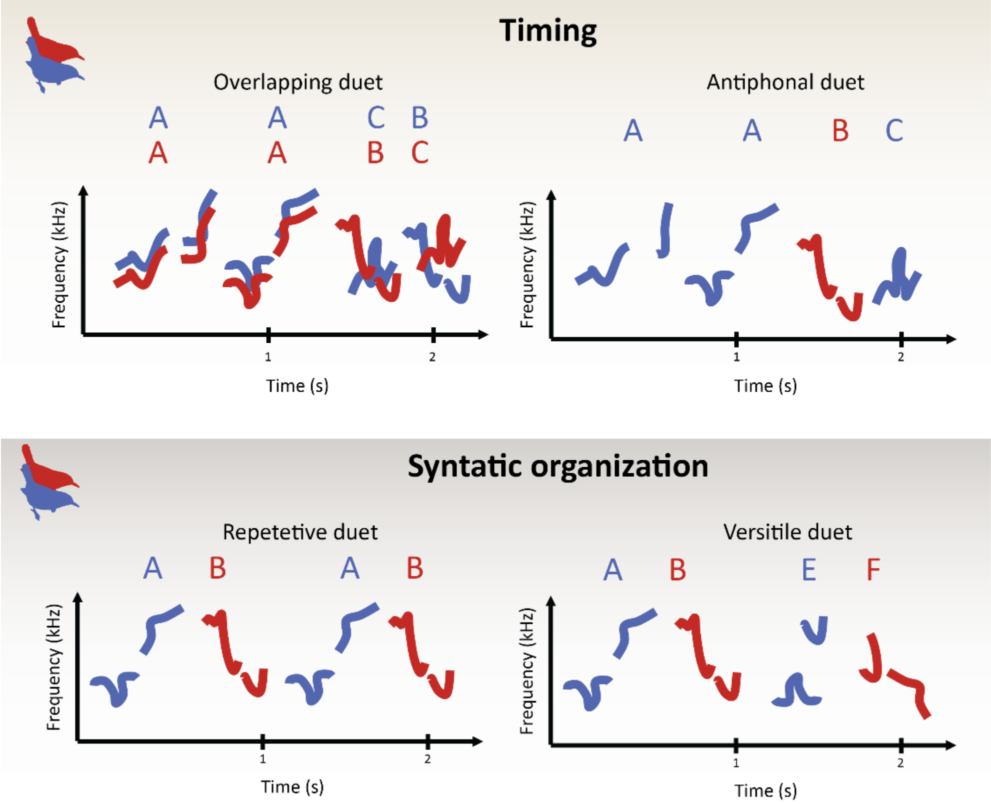

Figure 2 Different organizational structures of duet song.

Countersinging

In contrast to cooperative duetting behavior, countersinging is often assumed to be a competitive behavior [36, 47]. Countersinging is defined as dyadic male-male vocal interactions manifesting through songs, typically aimed to mediate territorial conflicts with rival males or attract females [36, 39, 47]. Territorial songbirds engage in countersinging with one or more conspecifics to advertise their presence [36, 39, 47]. Although both sexes theoretically can participate in countersinging, it is mostly studied in males [47]. The research on countersinging predominantly centers on songbirds [39, 47]. This emphasis arises from the fact that, while many vertebrate species depend on vocal signaling for territorial disputes and sexual advertisement [39], only a limited number of species participate in countersinging via vocalizations structured similarly to songs. In non-human mammals, males engaged in countersinging by producing song-like vocalizations have been studied in rodents [40, 124], hyraxes [76, 91] and bats [125]. In hyraxes, a quarter of dynamic vocal interactions are male-male countersinging exchanges [126] using a vocal element – “snort” – which is specifically utilized for countersinging and aims to advertise an individuals’ quality [127]. Bats participate in countersinging to mutually compare male rival performance to assert dominance or to jam the signal of the competitor [125].

In another example, male Alston's singing mice (*Scotinomys teguina*) engage in countersinging characterized by precise timing of song production in response to each other's vocalizations [124]. These interactions involve emission of trill-like songs, with song duration and note production rate reflecting individual fitness levels and correlating with female preference.

In songbirds, countersinging is a unique behavioral model, giving insight in aggressive [128] and sexual [129] behaviors, honest [130] and hierarchical signaling [131], communication networks [32], mechanisms of signal choice [132] and receiver physiology [133]. Countersinging encompasses several unique vocal behaviors, such as song alternating, song matching and song overlapping. In my thesis, I focus on overlapping interactions. Overlapped singing assumes that one bird starts singing before another bird has finished its song [36]. This way, the vocalizations of the second bird interfere with the vocal signals of the first bird and may affect the function of the first bird’s signal by masking. The intensity of the masking effect varies with several factors, including the temporal relationship between the songs, the acoustic characteristics of the songs, and the receiver's perceptual acuity [47]. Overlapping serves different signaling functions [48]. Initially, overlapping was considered as an aggressive signal [36, 69, 134-136], however, some recent studies cannot provide evidence for an aggressive function [48, 137, 138]. They suggest song overlap to be used in defensive signaling, or to signal male quality, or to provide information to eavesdroppers.

Birds that are overlapped during singing employ different strategies to avoid overlapping [47, 48]. Opponents can also manipulate their song intensities during male-male vocal interactions [139]. Within this form of social interaction, males exhibit significant individual differences in song amplitude [140]. This variability, in turn, has an effect on the mate selection process in females, with the latter exhibiting a preference for more intense vocalizations [141]. Amplitude modulation has been found in songs of common blackbirds (*Turdus merula*) [142] and nightingales [143].

It has been observed that birds adjust the syntax of their songs as a response to an opponent's song during countersinging interactions. Studies have provided evidence of this phenomenon in various avian species. For example, Cassin's vireos (*Vireo cassinii*) select matching syllable types in response to an opponent, considering their song syntax [132]. Nightingales, on the other hand, have been observed to alter the direction of song progression as a response to an opponent, allowing them to maintain a leadership position in the competitive vocal interaction [108]. Finally, house finches (*Haemorhous mexicanus*) have been found to increase within-song syntactical diversity in their countersinging behavior [89].

Neural circuits for song production: a dominant role of HVC in song timing and vocal patterning

The neural control of vocal communication behaviors is complex. It requires the ability to successfully plan and execute swift motor outputs based on available sensory information. Social interaction is an essential stimulus that drives changes in the neural activity in vocal motor brain areas [144-147].

Two distinct vocal motor networks exist in mammals, each serving specific functions in the production and modulation of vocalizations [148-150]. The primary vocal motor network is involved in generating stereotypical species-specific sounds and is predominantly located within the midbrain and brainstem regions. A pivotal component of this network is the periaqueductal gray (PAG), which acts as a core brain area responsible for the initiation and regulation of vocalizations [151, 152]. Notably, the PAG receives input from various brain regions, including the anterior cingulate cortex (ACC) [148], the hypothalamus, and the amygdala [153, 154]. Electrical or pharmaceutical stimulation of these areas can elicit vocalizations, however often reflecting changes in arousal levels rather than deliberate alterations in vocalization structure [155]. The voluntary control over vocalizations in mammals is mediated by a secondary system, often referred to as the "volitional control system" [156]. The primary vocal motor network is under cortical control by a secondary frontal/motor "volitional control system", that effectively enhances the flexibility of vocal production [156]. Empirical evidence elucidating the neural underpinnings of context-dependent modifications in competitive and cooperative vocal interactions among mammals has emerged from various sources. For instance, studies involving marmoset monkeys (*Callithrix jacchus*) engaged in turn-taking behavior have revealed increased activity within the ventral premotor cortex (vPMC) and prefrontal cortex (PFC) during instances of flexible vocal exchanges [157, 158]. Similarly, investigations into vocal interactions among non-primate mammals, such as Alston's singing mice, have identified a critical region within the orofacial motor cortex (OMC), which plays a pivotal role in coordinating turn-taking behavior [124].

The ability of songbirds to make context-dependent adjustments to song structure is based on their unique brain organization [9, 159-161]. Two interconnected neural circuits play a crucial role in controlling vocal communication: the auditory system and the vocal motor pathway (i.e. the song system, Figure 3A,B) [162]. The song system consists of interconnected telencephalic nuclei in the avian forebrain [162, 163], and is composed of two distinct anatomical and functional structures, namely the song motor pathway and the anterior forebrain pathway. The song motor pathway is essential for the initiation and production of vocalizations, while the anterior forebrain pathway is central for the sensorimotor learning and the refinement of vocalizations [164]. HVC (used as a proper name) is a key nucleus in the song system, being involved in both pathways [165]. HVC plays a major role in generating temporally patterned premotor commands for vocal production [166, 167] of syllables and syllable sequences [168, 169]. HVC functions as motor pattern generator for vocal emissions [166, 167, 170]. HVC neurons are also responsible for monitoring and integrating feedback between sensory and motor information during song communication [171]. HVC receives auditory input [172] from several auditory nuclei, such as NIf [nucleus interfacialis of the nidopallium, 173, 174], CM [caudal mesopallium, 164] and Uva (nucleus uvaeformis of the thalamus) [uvaeformis, 175].Within the motor pathway, HVC projects to RA (nucleus robustus of the arcopallium), which in turn, projects to a hypoglossal motor nucleus containing motor neurons that innervate the syrinx (Figure 3C) - the vocal organ of birds [164]. The syrinx contains two sound generators, which can produce sounds independently [176], or can interact in nonlinear ways to produce complex sounds [177]. The anterior forebrain pathway is involved in song learning and generating song variability [164, 178-182]. It indirectly connects HVC to RA through Area X in the basal ganglia. The anterior pathway also includes the thalamic nucleus DLM (medial portion of the dorsolateral thalamic nucleus), and ultimately LMAN (lateral magnocellular nucleus of the nidopallium) [164].

In an awake bird, HVC neurons are active only when the bird is singing [175, 183] and in anesthetized birds, HVC shows auditory activity with a preference for the bird's own song, but other auditory stimuli can also elicit responses in HVC [184]. The HVC-RA neurons, a subset of HVC neurons that project to RA, play a prominent role in the premotor activity that is closely linked to the temporal features of the song. Studies on the zebra finch have demonstrated that HVC-RA neurons fire at specific moments during the song phrase or motif, and the activity of these neurons collectively forms a clock-like pattern [166, 185]. However, whether the HVC clock model applies to all songbirds, and the extent to which other brain regions contribute to song timing, is still unknown. In the case of the canary (*Serinus canaria*), HVC activity appears to be more closely linked to the onset and offset of syllables, rather than operating like a clock [186, 187].

Several studies demonstrated that HVC plays a major role in the control of syllable sequencing [168, 169]. For example, cooling HVC resulted in changes to the song tempo and compromised the bird's ability to produce particular song sequences [188]. In the same species, firing activity of HVC-X neurons depended on the type of transition between syllables [189]. Recent studies on HVC activity in the domestic canary demonstrated that HVC encodes long-range sequence information [169].

HVC plays a role in facilitating the coordination of vocalizations between individuals during vocal interactions. Largely, HVC seemed to be influenced by the onset of conspecifics' vocal sounds. For example, during call exchanges in zebra fiches, the vocal output of the communication partner inhibits HVC activity in the focal bird [190, 191]. This suggested that HVC plays a major role in the precise timing of vocal onsets.

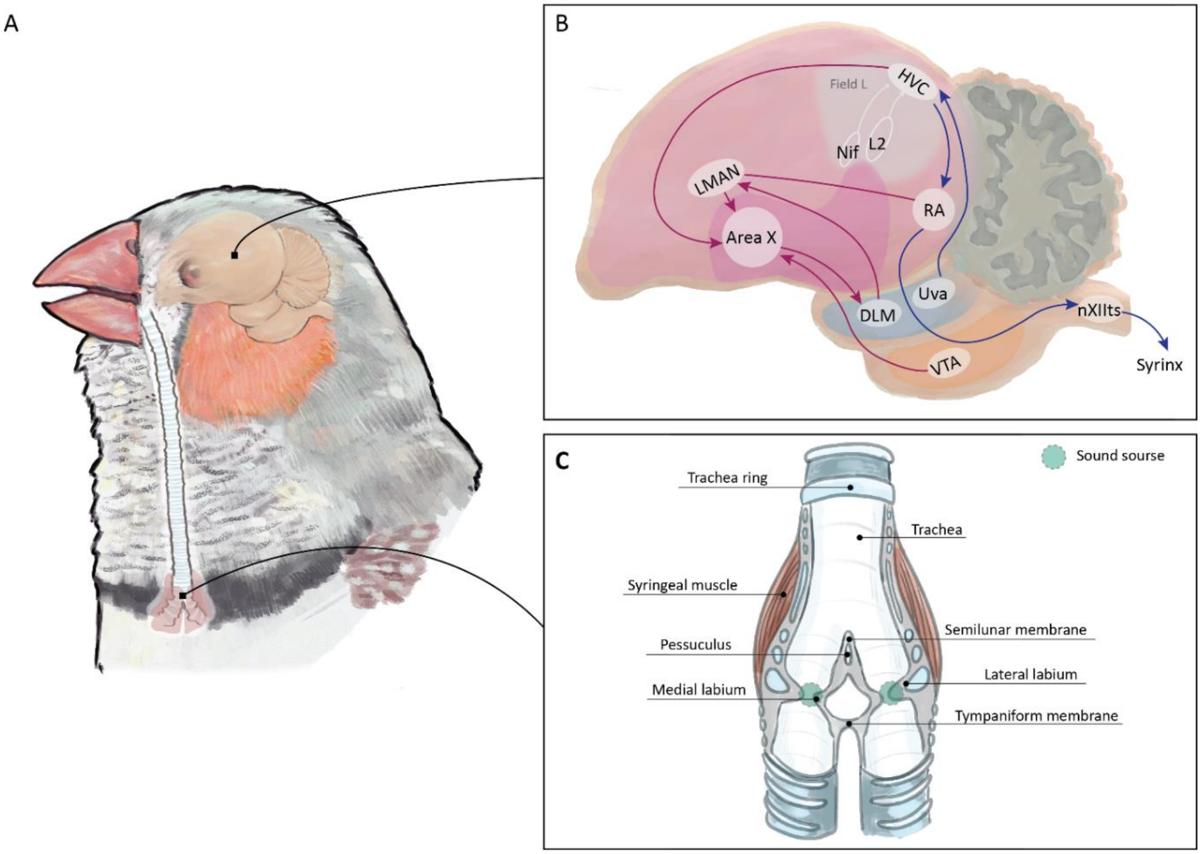

Figure 3. Schematics of the zebra finch brain and vocal organ, the syrinx. A. Overview of structures displayed in B and C. B. In the songbird brain, the song motor pathway (in dark blue) consists of the telencephalic nuclei HVC and RA, which project to neurons in the tracheosyringeal half of the hypoglossal nucleus (nXIIts), which in turn project to the vocal muscles in the syrinx. HVC receives inputs from the auditory system (Nif and Field L) and the thalamic nucleus Uva. The anterior forebrain pathway (in magenta) controls sensorimotor, vocal learning, and includes Area X and its inputs from the midbrain ventral tegmental area (VTA). HVC, used as a proper name; RA, robust nucleus of the arcopallium; LMAN, lateral part of the magnocellular nucleus of the nidopallium; DLM, dorsolateral part of the medial thalamus. C. Frontal view of the songbird vocal organ, syrinx.

Model systems

White-browed sparrow weaver (*Plocepasser mahali)*

The white-browed sparrow weaver (*Plocepasser mahali*) is a cooperatively breeding and duetting songbird [192]. It is a member of the family of sparrow weavers, *Ploceidae* [193]. White-browed sparrow weavers inhabit semi-arid parts of Africa, specifically areas covered by dry bush and acacia vegetation. White-browed sparrow weavers live in groups typically consisting of 2-12 birds, which include a dominant breeding pair and non-breeding subordinate birds of both sexes [194, 195]. Subordinate birds are typically family members, however also unrelated individuals can be part of a group. Subordinate birds assist with nest building activities and with raising the offspring of the breeding pair [192]. Usually, social groups of *P. mahali* occupy a territory with a permanent nesting/roosting site that is actively defended by all group members (Figure 4C). The group builds nests in a single tree or in several trees within immediate proximity. The roosting nest has two entrances to ensure the possibility of effective escape, while the breeding nest has only one entrance to prevent the eggs from falling out. White-browed sparrow weavers are sexually dimorphic [196-198], with male and female birds having different beak coloration, body size and behavior (Figure 4B). Male and female White-browed sparrow weavers differ in their dispersal patterns, with females tending to stay within their natal group, while males disperse to the territories of neighboring groups [197].

Prominent sexual dimorphism can be observed in the white-browed sparrow weaver vocal behavior [198, 199]. They possess a large vocal repertoire, which is not only sex- but also status-dependent. The white-browed sparrow weaver song repertoire comprises the solo song, performed by the dominant male, the duet songs of the dominant couple and the chorus songs of the entire group. In the summer months, the male white-browed sparrow weaver performs dawn solo songs exclusively during the early morning hours before the other group members engage in duetting and chorusing [192, 199, 200]. To produce solo songs, males use syllables that are distinctly different from syllables of duet songs [198]. While duet song syllables are built from two vocal elements, solo song syllables usually consist of only a single vocal element. It is argued that solo song is used for the inter-sexual communication, to signal an established breeding status, as well as for attraction of potential mates [198]. Solo song duration varies depending on the density of neighboring groups. The individual differences in solo song performances of different males influences their chances of winning territorial fights, and their success in mating [198]. Interestingly, the production of solo song and mating success is tied to the moon phases [201]. Subordinate males can also produce solo song, but with repertoire sizes smaller than in dominant males. It is hypothesized that subordinate males enlarge their syllable repertoire before transitioning to the dominant status [199].

In *P. mahali* groups, each group member can produce chorus song regardless of their social status or sex [202]. Birds participate in chorus song throughout the year, and chorus song is mostly associated with territory defense. Chorus song is commonly sung in the morning hours and in response to a threat or to an intrusion from unfamiliar conspecific or heterospecific birds [202, 203]. The rate of chorus singing is positively associated with the density of neighboring groups [200].

In addition to chorus songs, white-browed sparrow weavers sing duets all year round with a daily peak in song rate in the morning [200]. White-browed sparrow weavers perform duet songs for territorial defense and pair-bond maintenance [198]. Duets have a complex temporal and sequential pattern (Figure 4A). A duet bout is typically two to four seconds long and consists of a precisely timed string of syllables [198]. Partners perform antiphonal duets, there each partner can start duet with buzz syllable or with a group of introductory syllables [198]. In the subsequent part of the song, male syllables containing two strongly frequency-modulated elements, alternate with female, less frequency-modulated, whistle-like syllables with a harmonic structure. This pattern is repeated throughout the duet song. Duet repertoire sizes range from 45 to 61 syllable types. Similar to choruses, the rate of duet songs performed depends on the density of neighboring groups and increases in situations of invasions [204]. Thus, white-browed sparrow weavers have been observed participating in counter duetting at the territory boundaries. Duet usage for pair bond maintenance is facilitated by the long-term partnerships [205]. However, the acquisition of duet song in white-browed sparrow weavers has so far been observed exclusively in captive juvenile pairs [204].

The vocal behavior of white-browed sparrow weavers has attracted attention from the field of neuroscience, as scientists aim to examine the potential association between sex-specific vocalizations and variation in brain structure and function[199, 203, 204]. Additionally, research has sought to investigate how hierarchical status in this species is reflected in the brain [204]. The song production pathway in white-browed sparrow weavers shows sex differences in the cytoarchitecture of the premotor song nucleus HVC. The volume of HVC is two to three times larger in dominant males than in dominant females. In females, cell density in HVC is higher, however, total number of cells is 2.6 times higher in males than in females. Comparison between dominant and subordinate males demonstrated that the latter have smaller volumes of HVC and RA as well as a lower total number of HVC cells and a lower cell density in the surrounding neostriatum. Dominant females and subordinate males also differ in the volume of their HVC and their RA [199, 204]. Specifically, subordinate males have been found to exhibit a twofold larger size of these regions compared to their female subordinates.

In summary, white-browed sparrow weavers represent a valuable model organism for investigating the neural coding of vocal coordination, particularly for using electrophysiology recordings in a natural setting. This is due to a combination of practical and scientific factors. On a practical level, the site fidelity of white-browed sparrow weavers allows for reliable and repeated observation and recapture of individuals, while the sexual differences in appearance aid in identification. On the scientific level, previous studies on vocalization and brain anatomy provide a solid foundation to support electrophysiological and vocal recordings in this species.

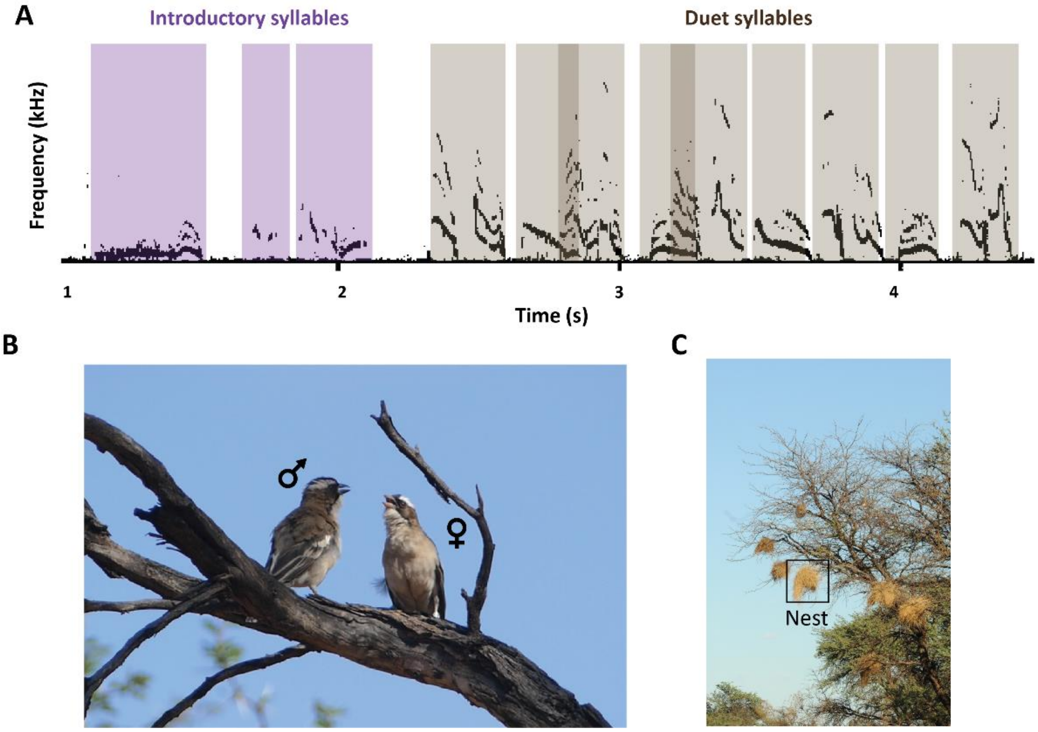

Figure 4 A. Spectrogram of a duet song produced by a pair of P. mahali. B. Pair of P. mahali performing a duet. C. Nest in a tree that marks the center of the group's territory.

Domestic canary (*Serinus canaria*)

The domestic canary is one of the most prominent songbird model species used for the behavioral and neuroanatomical investigation of vocal learning and vocal production due to its size, easy maintenance and complex songs. Canaries are widely utilized as model species due to their biology as an open-ended vocal learner, which indicates their ability to seasonally change their song structure [206]. This contrasts with closed-ended vocal learners, such as zebra finches, in which the song repertoire is fixed after a critical period during early development. During fall, canaries perform plastic song, which is more variable in syllable composition and syntax than the crystallized song they perform in spring [207, 208]. Performance of plastic song usually serves as training ground to produce courtship song before its utilization during the next breeding season. During the plastic song period, canaries change their song syllable composition through flexible imitations of tutor songs and innate song templates [207, 208]. During the breeding season, male canaries produce longer and louder songs than during the non-breeding season [207, 209]. Breeding songs (typically 5–15 s in duration) consist of a sequence of phrases (typically 500 ms – 3 s in duration), where phrases are constructed by the repetition of one type of syllable (typically 20 – 200 ms in duration) (Figure 5A)[210]. The adult male canary usually possesses a repertoire of 25–35 distinct syllable types [211]. During singing, male canaries produce sequences of rapid iterations of specific syllables. Moreover, they perform specific song elements called A-syllables or sexy syllables in breeding songs. A-syllables are strongly frequency-modulated vocal elements, composed of two notes with a wide frequency bandwidth of about 4 kHz [212-214]. Phrases constructed from A-syllables have a high syllable repetition rate, exceeding 15 syllables per second [212-214]. These syllables are generated by using both sides of the syrinx, and may serve as indicators of male fitness as they are energetically demanding to produce [215]. Female canaries prefer songs including a high number of A-syllables over songs with less A-syllables [212].

In canary song, higher-order context dependencies predict the transition between individual phrases: a specific phrase type sung depends on more than one (up to five) preceding phrase types within the song sequence [211]. The canaries have individual differences in song syntax, especially in sequences consisting of three or more phrases [216]. As an open-ended learner, canaries change their transition repertoire and demonstrate variability outside the breeding season [207].

During the breeding season, male domestic canaries engage in overlapping singing [217, 218]. These competitive vocal interactions have a significant biological impact as both females and males eavesdrop on overlapped songs to adjust their own behavior. Specifically, females use song overlaps as a means of evaluating the relative quality of potential mating partners [219], while males utilize these interactions to evaluate the perceived threat of rival males and to regulate their own vocalization rate in response [220]. Recent research on canary vocal competition has further highlighted that winners of overlapping interactions sing longer songs, and that the spectral characteristics are similar among rivals' songs [217]. Additionally, females have been observed to exhibit a preference for males that overlap the song of conspecifics over those whose songs are being overlapped [218, 219] and may even invest more resources in eggs fertilized by such males [221].

Canaries have been widely recognized as a fundamental model organism in the discipline of neuroscience [169, 209, 222-227]. They have been particularly useful in the study of the premotor song nuclei HVC. In canaries, HVC activity is associated with motor gestures, thus HVC projection neurons exhibit an increased level of activity, concentrated around syllable borders [187]. Under anesthesia, HVC in canaries shows auditory responses to both the bird's own song and conspecific songs [228]. Furthermore, HVC neurons in canaries have been shown to reflect long-range associations between specific syllable transitions [169]. In the canary brain, HVC undergoes seasonal changes in connectivity and in the number and rate of incorporated neurons [209, 223-225]. The canary HVC also demonstrates sexually specific differences: while HVC neurons in anesthetized males showed no sensitivity to A-phrases, A-phrases induced a decrease in spike rate in the female HVC [229].

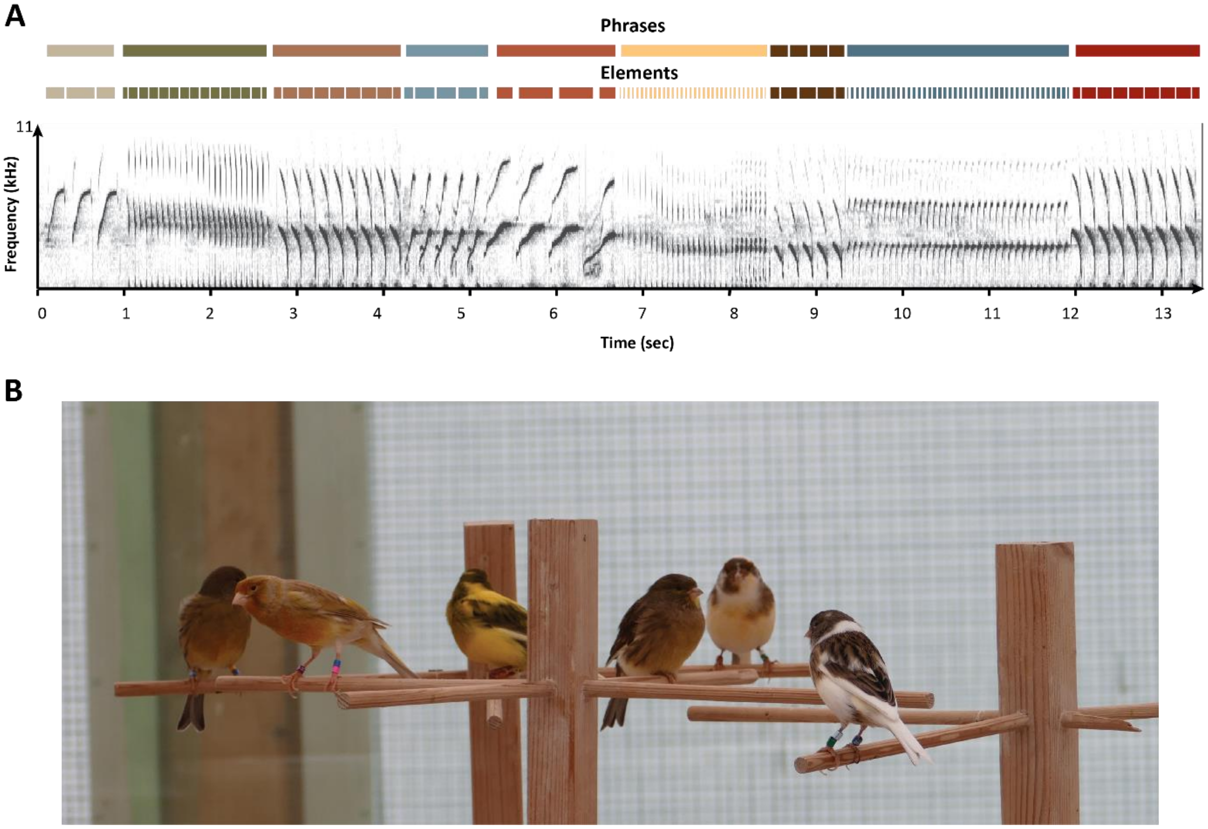

Figure 5 A. Spectrogram of canary song. B. Group of canaries in an aviary.

Methodological challenges of studing freely behaving birds

Vocal interactions between several conspecifics can happen concurrently. Usage of conventional recording methods in this scenario is challenging since it often suffers from the "cocktail party effect" and results in difficulties of detecting and separating individual vocal signals, while preserving the initial temporal relationship [230]. Researchers implemented different strategies to avoid this problem: keeping studied animals in different cages [146], using microphone arrays [231, 232], or performing playback experiments [233]. However, these methods can be insufficient when recording vocal interactions between naturally behaving free-living birds. Here, I took advantage of two in-house developed, radio-telemetric transmitters that allowed us not only to simultaneously record vocal activities of several freely behaving conspecific birds, but also to record their neural activity (Figure 6). These devices demonstrated great success in laboratory studies [234-236], but also allowed us to record dynamic vocal interactions in free-ranging, wild birds (Chapter 1 – Chapter 4). The transmitters send data continuously via radio frequencies to an antenna, allowing superior battery efficiency and reduced device weight when compared to data loggers. To record the individual vocal behavior and the neural activity in one bird in parallel, the bird was equipped with two types of transmitters. Both devices are very lightweight (microphone transmitter: 0.75 g, electrophysiology transmitter: 1 g) and allows to record from rather small-sized bird species without disturbing their natural behaviors. The transmitter technology allowed to perform synchronized vocal and neural recordings from different freely behaving individuals with high temporal resolution for several days (<15 days) [234, 236].

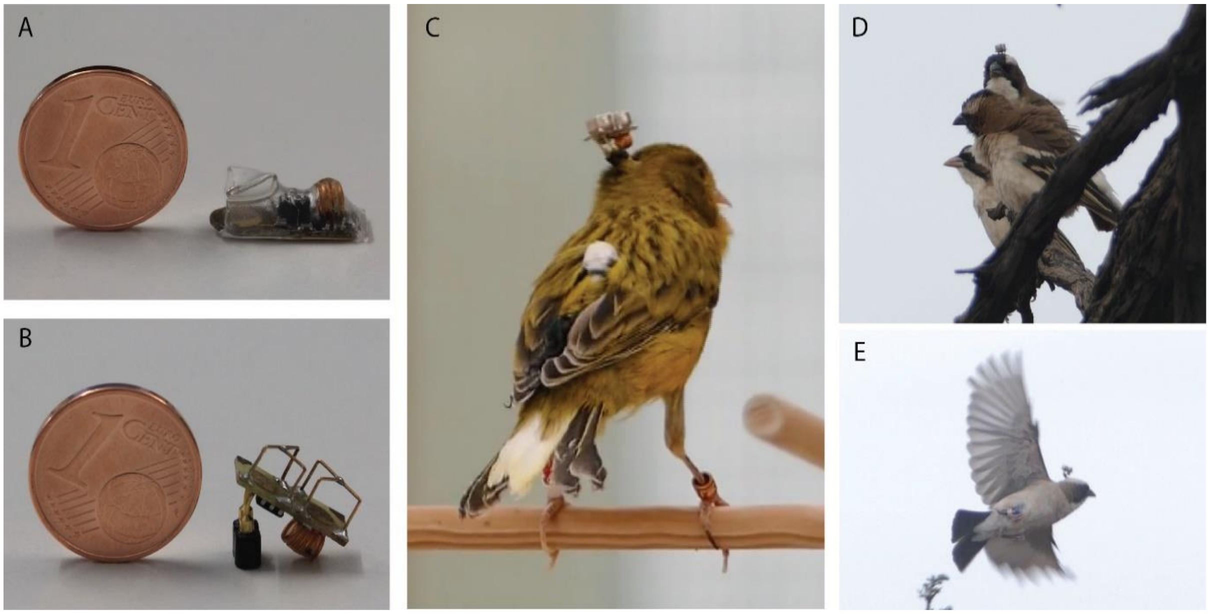

Figure 6 Experimental animal species equipped with radio-telemetric transmitters. A. Microphone transmitter, lateral view. B. Electrophysiology transmitter, lateral view. C. Male domestic canary with exposed microphone and electrophysiology transmitter. D. Male white-browed sparrow weaver perching with conspecifics, equipped with microphone (not visible) and electrophysiology transmitter. E. Female white-browed sparrow weaver in flight equipped with microphone (not visible) and electrophysiology transmitter. Photos in A and B were taken by Susanne Seltmann/MPIO.

Thesis outline

The present PhD thesis investigates the effects of social context on birdsong structure during dynamic vocal interactions and the underlying neural processes. The study focuses on specifically cooperative and competitive vocal interactions, and the underlying neural control of the sensory-motor brain nucleus HVC during these interactions. The primary focus of this thesis is to answer the following questions:

1. How do white-browed sparrow weavers arrange the vocal elements in their duet songs in relation to their duetting partner, in terms of both timing and spectral pattern?
2. What neurophysiological and anatomical adaptations in white-browed sparrow weavers allow precise and effective interindividual coordination of fast-paced vocal output?
3. How do conspecific vocalizations affect the neural control of vocal behavior in cooperative and competitive contexts?
4. How does song overlap during competitive vocal interactions affect syntax in songs of domestic canaries?

In **Chapter 1** of my thesis, I show that the motor-related activity in HVC is modulated in response to acoustic cues generated by the bird's duet partner. The partner's vocal output elicits a decrease in the burst rate of HVC neurons. Additionally, both partners demonstrate interindividually synchronized patterns of neural activity, which causes vocal production to alternate between duet partners in the ongoing song in a highly coordinated fashion.

In **Chapter 2**, I focused on the question of whether duetting partners mutually influence not only the timing but also the content of their vocal productions. Results revealed that white-browed sparrow weavers adhere to pair-specific answering rules to generate their highly complex duet songs. The sharing pattern of answering rules suggests that, in addition to pair-specific duetting rules, mated pairs use duetting rules that have been acquired prior to pair formation.

In **Chapter 3**, I investigated behavioral and anatomical adaptations that allow effective functioning of duet singing. Using synchronized vocal and neural recording techniques, as well as anatomical dissections of the white-browed sparrow weaver vocal organ, I demonstrated that white-browed sparrow weaver duet syllables can be categorized into four basic sex-specific categories according to their spectral pattern. The fixed ordering of syllable categories underlies the species-specific pattern of White-browed sparrow weaver duet songs. This study also revealed lateralization of premotor vocal control and morphological lateralization in the white-browed sparrow weaver syrinx as potential mechanisms for the generation of song syllables that differ in intonation.

In **Chapter 4**, I investigated whether participation in overlapping interactions altered birdsong syntax and HVC activity in domesticated canaries. The results demonstrated that the social context is associated with syntax variability, i.e. a bird increases the variability in its song to counter the masking of its song by the song of an opponent. Additionally, HVC activity was found to be higher during overlapping interactions than during production of solo songs, and social rank was found to influence the overall syntax structure of the canary song.

Chapter 1

Duets recorded in the wild reveal that interindividually coordinated motor control enables cooperative behavior

Abstract

Many organisms coordinate rhythmic motor actions with those of a partner to generate cooperative social behavior such as duet singing. The neural mechanisms that enable rhythmic interindividual coordination of motor actions are unknown. Here we investigate the neural basis of vocal duetting behavior by using an approach that enables simultaneous recordings of individual vocalizations and multiunit vocal premotor activity in songbird pairs ranging freely in their natural habitat. We find that in the duet-initiating bird, the onset of the partner's contribution to the duet triggers a change in rhythm in the periodic neural discharges that are exclusively locked to the initiating bird's own vocalizations. The resulting interindividually synchronized neural activity pattern elicits vocalizations that perfectly alternate between partners in the ongoing song. We suggest that rhythmic cooperative behavior requires exact interindividual coordination of premotor neural activity, which might be achieved by integration of sensory information originating from the interacting partner.

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Contributions:

S.H., L.T., A.T.M., and M.G. conceived the project; S.H., L.T., C.V., S.L., A.L., S.K., and A.T.M. contributed data and analyses; C.V., H.S., and M.A. provided technical support; and S.H., L.T., C.V., S.L., and A.T.M. wrote the manuscript with feedback from all authors.

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Duets recorded in the wild reveal that interindividually coordinated motor control enables cooperative behavior

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Many organisms coordinate rhythmic motor actions with those of a partner to generate cooperative social behavior such as duet singing. The neural mechanisms that enable rhythmic interindividual coordination of motor actions are unknown. Here we investigate the neural basis of vocal duetting behavior by using an approach that enables simultaneous recordings of individual vocalizations and multiunit vocal premotor activity in songbird pairs ranging freely in their natural habitat. We find that in the duet-initiating bird, the onset of the partner's contribution to the duet triggers a change in rhythm in the periodic neural discharges that are exclusively locked to the initiating bird's own vocalizations. The resulting interindividually synchronized neural activity pattern elicits vocalizations that perfectly alternate between partners in the ongoing song. We suggest that rhythmic cooperative behavior requires exact interindividual coordination of premotor neural activity, which might be achieved by integration of sensory information originating from the interacting partner.

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Avian duetting is a rare phenomenon, which is mainly found among bird species of the southern hemisphere. Duet songs are generally defined as overlapping bouts of sounds produced by both members of a pair[1]. Avian duets show a high diversity in complexity and in precision of coordination between the partners' vocal emissions. While in some bird species vocalizations from both duet partners temporally overlap to a variable degree[2–4], other birds produce vocal duets in which the partners' contributions alternate almost perfectly[5–7]. Growing evidence suggests that alternating vocalizations in avian duets are a direct result of the partners' effort to avoid signal overlap[2,8]. Although the degree of temporal coordination of vocal activity seems to be an important indication of the function of duetting, temporal properties of avian duets have previously rarely been quantified in detail.

A well-studied duetting songbird species is the white-browed sparrow-weaver, *Plocepasser mahali*, which is native to eastern and southern Africa. This cooperatively breeding species lives in mixed-sex groups consisting of a dominant breeding pair and up to eight subordinates[9,10]. All group members defend a year-round territory by producing highly coordinated duet and chorus songs throughout the day[11]. Duet songs of male and female *P. mahali* consist of introductory syllables sung by either of the partners and duet syllables emitted by both birds in a rapid but precisely timed fashion[12]. The temporal dynamics of male and female vocal productions during duetting are, however, unknown.

Another large gap in knowledge exists regarding the neural mechanisms mediating the precise interindividual coordination of vocalizations during duetting[13]. In birds, the production of song is controlled by an interconnected network of sensory and premotor brain nuclei, the song-control system[14]. Nucleus HVC represents a major relay station within this distributed, recurrent network[15], receives auditory information[16,17], and generates temporally patterned premotor commands for vocal production[18–20]. During singing, individual HVC neurons are active at specific time points in the song without any temporal relation to certain parameters of single song elements, such as syllable onset[21]. In anesthetized songbirds, HVC neurons show selective responses to auditory presentations of the bird's own vocalizations[22–24]. This pattern does not seem to apply to birds that are able to produce vocal duets. HVC neurons in anesthetized plain-tailed wrens (*Pheugopedius euophrys*), a songbird species known to sing well-coordinated duets, are not only responsive to the bird's own part of the duet but also to the partner's vocalizations. Most importantly, neurons in the wren's HVC respond strongest to presentations of the complete duet sequence[25]. Fortune et al.[25] suggest that the auditory information from both duet partners might be important for the precise coordination of vocalizations during duetting. This assumption can, however, only be tested by neural recordings in the HVC of actively duetting birds. In awake individuals of nonduetting songbird species, HVC activity is mainly premotor, and responses to auditory stimulation are suppressed during singing[26–28]. Only during nonsinging periods, HVC can be responsive to playbacks of the bird's own song[28]. From this knowledge, two alternative scenarios arise that could be present in HVC during duetting: (1) As in most nonduetting songbirds, HVC in actively duetting birds only shows premotor activity correlated to the bird's own vocal productions and is silent to auditory input, such as the partner's contributions to the duet. (2) In contrast to nonduetting songbirds, HVC neurons in duetting birds show premotor activity when the bird itself is vocalizing and auditory activity to the partner's vocalizations.

Investigating the neural mechanisms that underlie an animal's natural behavior is the fundamental aim of neuroethology[29]. Neurophysiological experiments are, however, usually conducted inside laboratories with caged animals limited in their ability to behave naturally[30]. Recent advances in the development of neurophysiological research methods have started to allow neural recordings in single laboratory animals behaving freely for a limited time within a confined outdoor area[31]. Here we present extracellular neural data that have been synchronously recorded for several days from pairs of socially interacting wild animals while they ranged completely free in their natural habitat. We exploited a self-developed radio-telemetric recording technique[32–34] to investigate the neural basis of duet singing. We show that vocalizations in pairs of wild *P. mahali* precisely alternate during duetting. As in nonduetting birds, the neural activity in HVC of *P. mahali* is exclusively premotor during singing. The auditory information generated by the duet partner, however, alters the temporal parameters of HVC activity in the duet-initiating bird and thus enables the birds to alternate their vocalizations. We therefore conclude that in *P. mahali*, the integration of auditory information originating from the interacting partner mediates the precise interindividual coordination of vocal motor programs, which is required to generate precisely coordinated duet songs.

Results

Duetting birds precisely alternate their vocalizations. Instead of directional microphones that are conventionally used to monitor acoustic behavior, we fitted miniature microphone transmitters[32] onto the backs of both partners of the *P. mahali* pairs (see "Methods"; Fig. 1; Supplementary Fig. 1c) and recorded the individual vocalizations of both partners during duetting in parallel. This allowed us to unambiguously assign each single vocalization to the one bird it was emitted from while preserving the precise temporal relationship between both partner's vocalizations. In contrast to studies that used a single microphone to record vocalizations of duetting birds, our onboard microphones provided data with a much higher temporal precision since changes in distance between the birds and the microphone were nonexistent.

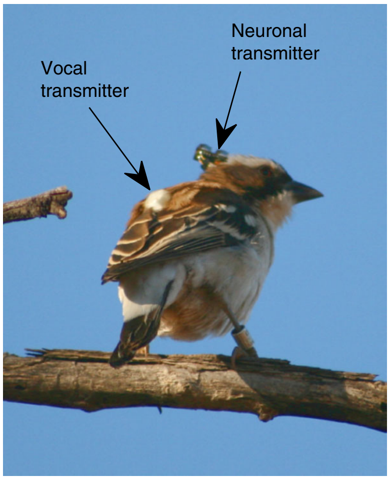

Fig. 1 Experimental animal species. Free-living male white-browed sparrow-weaver (*P. mahali*) perching in a tree in the South African Kalahari. The bird carries a vocal and a neuronal (see below) radio telemetric transmitter

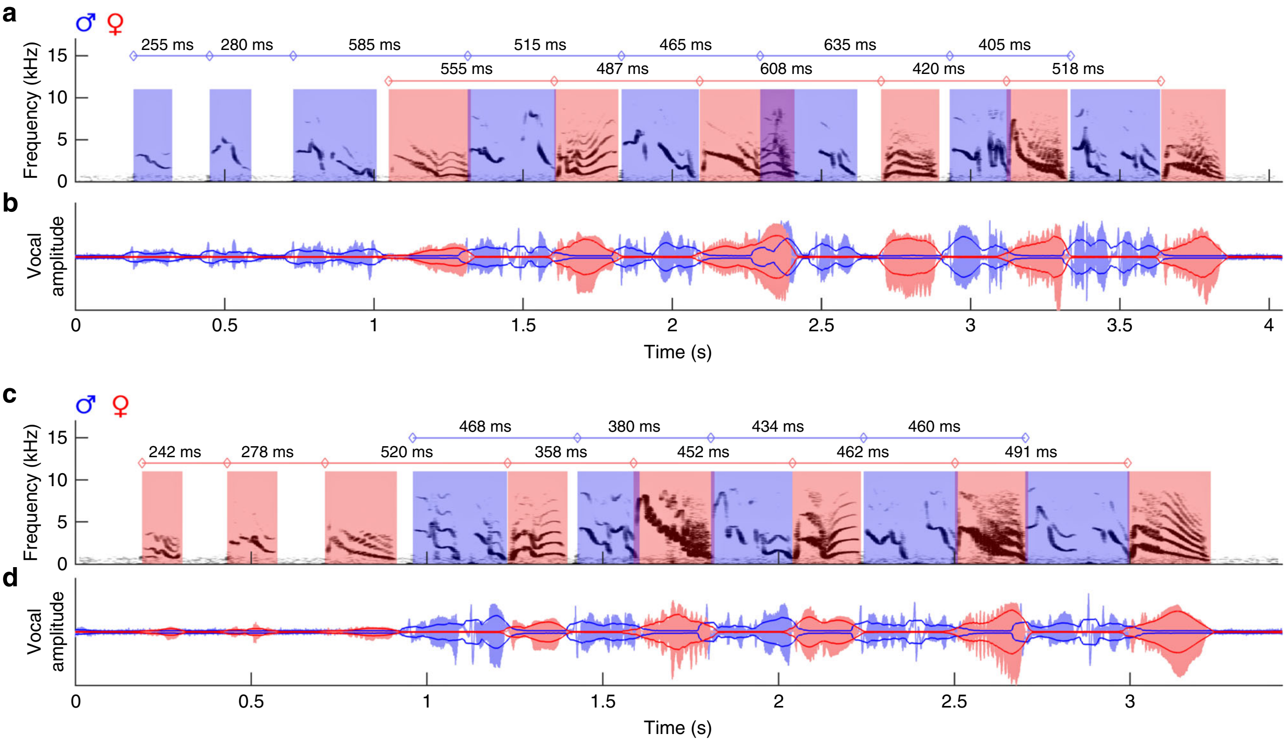

Fig. 2 Male and female syllables alternate precisely during duetting. The spectrogram (**a**, **c**) and amplitude waveform (**b**, **d**) of the combined male and female vocal traces are shown for exemplary duet bouts initiated by the male (**a**, **b**) or by the female (**c**, **d**) of Pair #4. Male signals are coded in blue and female signals are coded in red. Solid dark blue and dark red lines outline the root-mean-square envelope (see "Methods") of the amplitude waveforms. Onset-onset intervals of male and of female duet syllables are given by values above the spectrograms

In total, we analyzed 647 duet bouts produced by eight *P. mahali* pairs. In accordance with previous work[12], we found that duet bouts of *P. mahali* generally consisted of syllables sung by a single bird to initiate duetting, followed by male and female syllables that built the actual duet (see Fig. 2 for examples). As quantitative data were highly consistent among investigated pairs, in the following, we present quantitative data for the whole population of investigated birds but provide data from individual pairs in the Supplementary Information. The timing of vocalizations during duetting was strongly correlated between partners with maximal covariance of the root-mean-square (RMS) envelopes of male and female vocal signals at a median time shift of 250 ms (interquartile range: 193–377 ms; Fig. 3a, Supplementary Fig. 3). This indicates that the birds accurately synchronized their vocal output with a phase shift of approximately 250 ms, which conforms to the average duration of male and female duet syllables (see below, Fig. 3c, Supplementary Fig. 4) and is therefore a sign of the precise alternation (Supplementary Fig. 2) of male and female vocalizations.

Temporal fine structure of sparrow-weaver duet bouts. In 170 randomly chosen duet bouts produced by seven *P. mahali* pairs, we determined the onset and offset of each syllable to analyze the temporal fine structure of duet songs (see "Methods", Fig. 3b). Duet bouts were initiated by either sex, but male-initiated bouts (114 of 170) were more common. The partner joined the song of the duet-initiating bird after, on average, four duet-initiating syllables (range: two to nine). Duet bouts were terminated by male (in 86 of 170 bouts) or female birds (in 84 of 170 bouts).

While male duet syllables (646 of 684) generally consisted of a doublet of male song elements separated by a short (median: 31.6 ms) interval of silence, female duet syllables always consisted of a single female song element. During the initiating part of the duet prior to the second bird's song onset, male syllables were of significantly (one-sided Mann–Whitney U test, $p < 0.005$) shorter duration (median: 118 ms, interquartile range: 98–136 ms) than female syllables (median: 133 ms, interquartile range: 106–183 ms; Fig. 3c; Supplementary Fig. 4). In contrast, during the alternating part of the duet after the second bird's song onset, male syllables were of significantly (one-sided Mann–Whitney U test, $p < 0.005$) longer duration (median: 267 ms, interquartile range: 211–299 ms) than female syllables (median: 204 ms, interquartile range: 182–232 ms; Fig. 3c; Supplementary Fig. 4).

In accordance with the short duration of male duet-initiating syllables, these syllables were produced at significantly (one-sided Mann–Whitney U test, $p < 0.005$) higher vocal emission rates (median: 5.1 Hz, interquartile range: 3.99–6.24 Hz) than female duet-initiating syllables (median: 4.12 Hz, interquartile range: 3.52–5.46 Hz; Fig. 3d, Supplementary Fig. 5). Counterintuitively, even though male duet syllables were of longer duration than female duet syllables, both sexes produced duet syllables at an equal rate of ~2 Hz (male median: 2.09 Hz and interquartile range: 1.87–2.43 Hz; female median: 2.06 Hz and interquartile range: 1.82–2.41 Hz; Fig. 3d, Supplementary Fig. 5). Producing shorter syllables than males but at the same emission rate as males likely gives female birds the possibility to be slightly more variable in timing their vocalizations to fit them into the gaps between the male syllables. Support for this hypothesis was provided by the fact that the variance in female duet syllable emission rates was significantly larger (one-sided Ansari–Bradley test, $p < 0.05$) than the variance in male duet syllable emission rates. Additional evidence for the lack of flexibility in vocal timing in males was provided by the male vs. female difference in the frequency of extensive syllable overlaps. Duetting birds

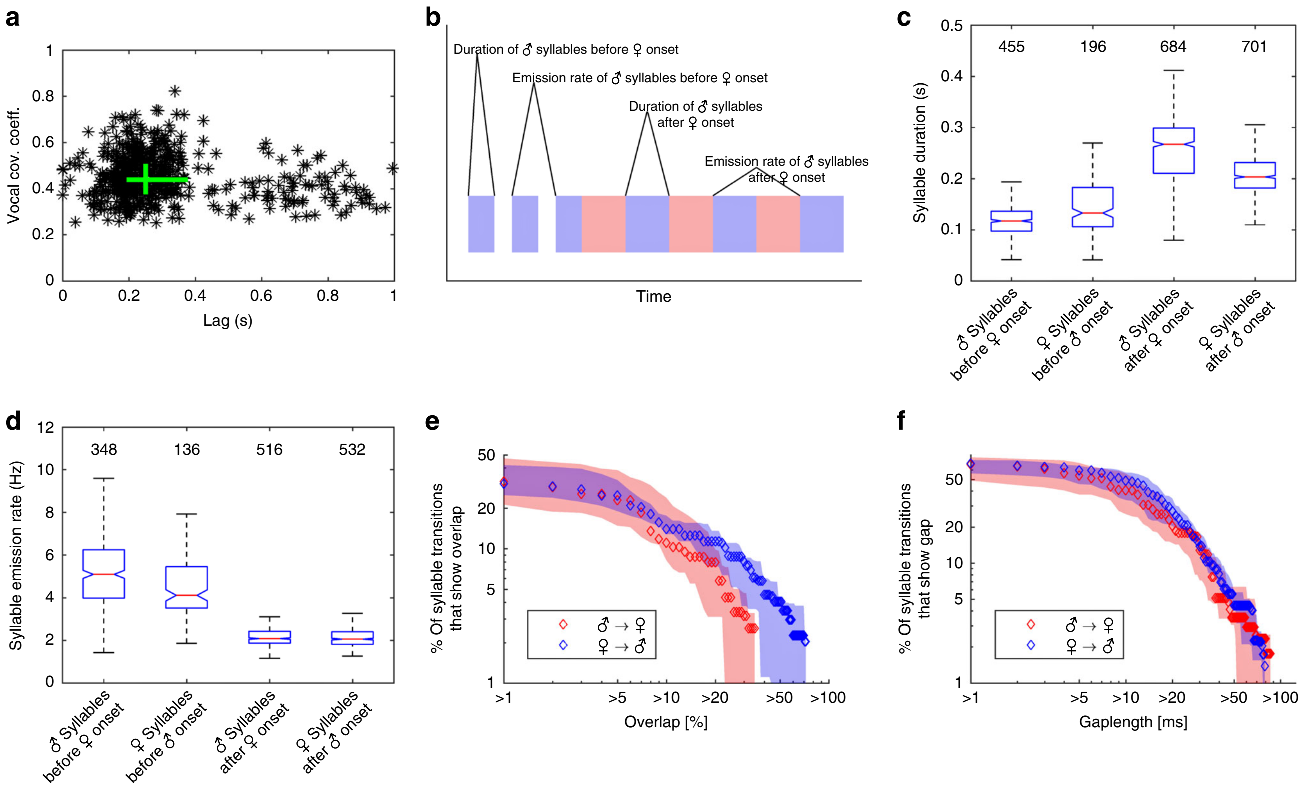

Fig. 3 Quantification of temporal properties of vocal activity during duetting. **a** Time lags of maximum cross-covariance between male and female vocal signals (root-mean-square envelopes) during 554 duet bouts produced by 8 bird pairs were clustered at approximately 250 ms. The green lines indicate interquartile ranges of lags and cross-covariance coefficients, and their intersection is at the medians of the distributions. **b** Cartoon of a male-initiated duet bout defining the temporal duet properties that are displayed in **c**, **d**. Blue and red rectangles represent male and female duet syllables, respectively. **c** The duration of syllables sung by a single bird before the second bird's song onset was significantly shorter than the duration of syllables sung in alternation after the second bird's song onset. Furthermore, male duet syllables were significantly longer in duration than female duet syllables. In the boxplot, the horizontal red line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The black whiskers extend to the most extreme data points not considered outliers (outliers are not shown). The extremes of the two notches of the box correspond to $y - 1.57(z - x)/\sqrt{n}$ and $y + 1.57(z - x)/\sqrt{n}$, where y is the median, x and z are the 25th and 75th percentiles, respectively, and n is the number of observations. Medians are significantly different at the 5% significance level if the boxes' notches do not overlap. **d** The emission rate of syllables sung by a single bird before the second bird's song onset was significantly higher than the emission rate of syllables sung in alternation after the second bird's song onset. Labeling of boxplots as in **c**. The median (seven pairs) percentage of duet syllable transitions that showed an overlap (**e**) or a gap (**f**) larger than the value on the x axis is marked by blue and red diamonds for female to male and male to female transitions, respectively. Shaded areas indicate the distributions' interquartile ranges

sometimes temporally mismatched vocalizations, which resulted in a partial overlap between consecutive male and female duet syllables (see Fig. 2a for an example). Syllable overlaps that were produced by male birds were significantly (two-sided Mann–Whitney U test, $p < 0.05$) larger (median: 11.4% and interquartile range: 5.6–27%) than syllable overlaps produced by female birds (median: 9.1% and interquartile range: 5.1–18.2%). In more detail, small overlaps of up to 7% of the duration of the preceding duet syllable were rather common in both sexes (present in 20–30% of all syllable transitions). Overlaps >20% of the duration of the preceding duet syllable were rare, but if present, they tended to be more often produced by male (in 3–11% of all female-to-male transitions) than by female birds (in 2–6% of all male-to-female transitions; Fig. 3e, Supplementary Fig. 6). Interestingly, female but not male duet syllables that were overlapped by the partner's following duet syllable were of significantly (two-sided Mann–Whitney U test, $p < 0.005$) longer duration (median: 214 ms and interquartile range: 192–260 ms) than duet syllables that were not overlapped (median: 199 ms and interquartile range: 180–223 ms). In contrast to overlaps, there was no difference in the frequency of gaps produced by male and female birds during duetting. In both the male to female and the female to male syllable transitions, short gaps between syllables were common, and long gaps were rare (Fig. 3f, Supplementary Fig. 7). Both large overlaps and long gaps occurred throughout the duet bout and were not restricted to the end of the bout, indicating that, even though the temporal pattern of the duet bout was altered by the mismatch, neither overlaps nor gaps resulted in a termination of the duet bout. Interestingly, only male birds compensated for overlaps by advancing the next duet syllable after being overlapped by a female. The median onset–onset interval of male but not of female duet syllables was significantly smaller (two-sided Mann–Whitney U test, $p < 0.005$) after an overlap compared to the intervals between non-overlapped duet syllables.

Mechanisms for vocal coordination between duetting birds. Above, we demonstrated that during duetting both partners of *P. mahali* pairs precisely coordinated their vocal emissions. The coordination of motor actions between individuals requires an external common event that defines the onset of coordinated behavior. In *P. mahali*, syllables sung solo by the duet-initiating bird before the second bird's song onset were produced at a median rate of 5.1 and 4.1 Hz by male and female birds, respectively (Fig. 3d; Supplementary Fig. 5). After the second bird's song onset, however, male and female duet syllables were produced at a significantly (two-sided Mann–Whitney *U* test, $p < 0.005$) lower rate (median emission rate: 2.1 Hz for males and females; Fig. 3d; Supplementary Fig. 5). This reduction by approximately half of the initial vocalization rate maintained the overall rate of male and female syllables in the alternating part of the duet bout constant at ~4 Hz. The sudden change in vocalization rate of the duet-initiating bird immediately after the partner joined the duet suggests that the auditory input generated by the partner's first duet syllable triggered the change in song rhythm in the duet-initiating bird. The bird that joined the duet immediately vocalized at a low rate of ~2 Hz. We therefore hypothesize that the song onset of the bird that joins the duet represents the common cue that defines the onset of vocal coordination in both birds.

Once initiated, the vocal coordination could either be maintained passively by each bird of a pair singing its own part of the duet with a temporally fixed pattern and without paying attention to the partner's vocalizations or actively by both birds fine-tuning the timing of their vocalizations to those of the partner. The first mechanism would be highly prone to external disturbances; for example, slight variations in the song rhythm of one bird would result in a breakdown of coordination. The latter mechanism would, however, allow the birds to compensate for small temporal irregularities such as syllable overlaps. We, therefore, suggest that *P. mahali* could actively maintain vocal coordination in the ongoing duet by one of the following three mechanisms: a bird locks the onset of its own duet syllable to (A) the onset of the partner's preceding duet syllable, (B) the offset of the partner's preceding duet syllable, or (C) the point in time when it has recognized the type of syllable the partner was currently vocalizing. In *P. mahali* of both sexes, we found the variance in partner-onset to own-onset latencies (male median latency: 219.2 ms and interquartile range: 187.9–258.5 ms; female median latency: 270.0 ms and interquartile range: 220.1–313.2 ms) to be significantly larger (one-sided Conover's Squared Ranks Test, $p < 0.005$) than partner-offset to own-onset latencies (male median latency: 15.6 ms and interquartile range: −11.3 to 40.4 ms; female median latency: 10.4 ms and interquartile range: −15.7 to 34.2 ms). Locking one's own syllable onset to the onset of the partner's preceding syllable should, however, result in onset–onset latencies that vary only minimally. Therefore, we exclude hypothesis A. Although the less variable offset-onset latencies between male and female *P. mahali* duet syllables would support hypothesis B, the fact that the birds sometimes produced syllable overlaps (see above) led us to exclude this hypothesis too. To lock its own syllable onset to the point in time when a bird has recognized the partner's preceding syllable type (i.e., hypothesis C) requires each bird to have knowledge about the duration of each syllable type in the partner's repertoire since latencies would depend on the duration of the partner's preceding syllable. Syllable overlaps would be possible in cases when the duration of the partner's preceding syllable exceeded the anticipated duration. After excluding two of the three proposed mechanisms, we suggest that to maintain vocal coordination during duetting, *P. mahali* adjust the time of syllable onset to the "expected" offset of the partner's preceding syllable (i.e., hypothesis C). One way to prove this hypothesis would be to perform syllable-type-specific analyses of *P. mahali* duets. Focusing on specific male–female syllable transitions eliminates variation in syllable duration and spectrotemporal composition and would therefore likely allow to determine the time point in the partner's syllable to which a bird locks its own syllable onset. Owing to the large vocal repertoire of *P. mahali*, this type of analysis would, however, require a dataset comprised of several hundreds of duet songs for each pair of birds and is therefore subject of future research.

Flexibility in vocal timing during duetting is limited. To test the degree of flexibility of vocal coordination in duetting *P. mahali*, we conducted playback experiments, which included presentations of manipulated duet bouts (see "Methods"). Since our dataset was too small to enable a quantitative analysis, we present here only exemplary data. In response to the playback, the birds were highly agitated and immediately started to duet with each other (Supplementary Movie 2). Sometimes, a single bird or both duet partners tried to duet with the playback. When we played back an unaltered version of the duet consisting of alternating male and female duet syllables with the normal rhythm, birds were able to follow the rhythm and produced their sex-specific syllables exactly at the same time as they were produced by the playback (Supplementary Fig. 8a). If the stimulus, however, consisted of only the male or female duet syllables with a two-fold increase or with a bisection of the length of intervals between sex-specific duet syllables (i.e., twice or half of the normal vocalization rate), the birds were no longer able to synchronize their vocal productions with the playback (Supplementary Fig. 8b, c). Although during duetting with a partner small irregularities in song rhythm could be compensated for (see above), the birds were not able to adjust their syllable emission rates to unnaturally large deviations from this rhythm while trying to duet with the playback. To determine the value of maximal deviation from the normal rhythm the birds would still be able to follow, a finer gradation of temporal manipulations of intervals between sex-specific duet syllables would be necessary.

It is generally assumed that rhythmic motor patterns are generated by the activity of central pattern generators (CPGs), which produce a rhythmically timed pattern of premotor neural activity[35,36] that can be regulated by sensory feedback[37,38]. Nucleus HVC has repeatedly been suggested to be a part of the CPG network that controls the timing of vocalizations during birdsong[20,21,39]. Although the production of duet syllables in male and female *P. mahali* followed a common rhythm, duet syllable emission rates were not completely stable but varied by approximately 50% of the median and could be adjusted to small irregularities produced by the partner. Thus we suggest that the rhythmical production of vocalizations during duet singing in *P. mahali* is controlled by a CPG network that can be naturally modulated by the social auditory input but only to a certain degree. This assumption has been supported by studies in nonduetting songbirds showing that perturbations of the CPG network controlling vocal production by brief electric stimulation of HVC[40] or by the presentation of altered auditory feedback[41] during singing results in altered song patterns.

HVC activity is locked to the bird's own duet syllables. Uncovering the high degree of precision with which vocal timing was coordinated during duetting in pairs of *P. mahali* raises the question of the neural substrates that enable the precise interindividual coordination of vocal productions in these birds. To find an answer to this question, we recorded extracellular neural activity in parallel with individual vocalizations during duetting in three wild *P. mahali* pairs in their natural habitat, the Kalahari savanna (see Supplementary Movies 1 and 2). In addition to the vocal transmitter, we equipped both birds of a pair with a radio transmitter that was connected to an electrode implanted in the bird's HVC (see "Methods"; Fig. 1; Supplementary Fig. 1a–c). This yielded perfectly synchronized recordings of the vocal and neural activity in both individuals of the pair, which cannot be

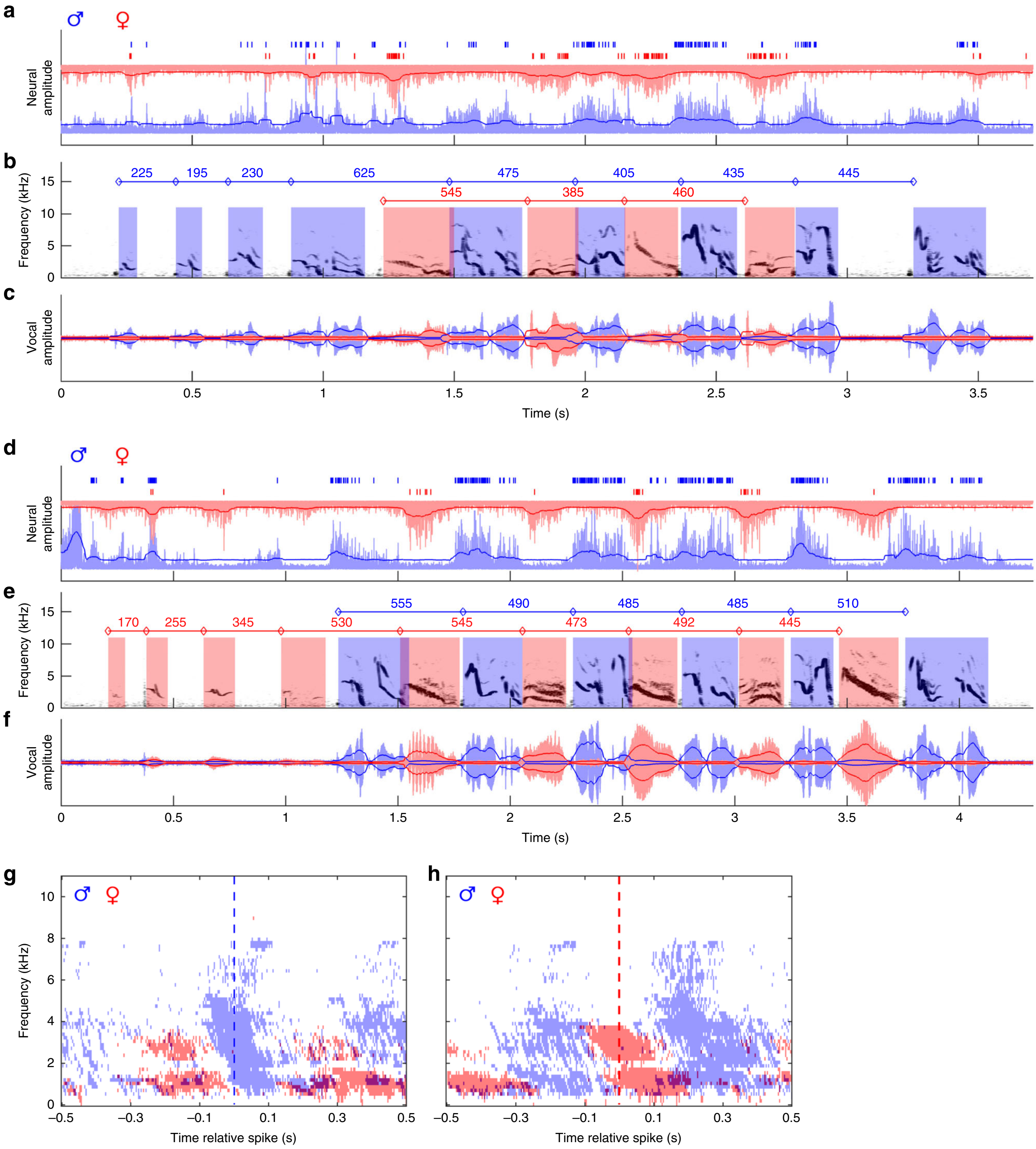

Fig. 4 Vocalizations are locked to bursts of premotor HVC activity in the singing bird. Throughout, male signals are coded in blue and female signals are coded in red. The filtered and rectified male and female neural traces (**a**, **d**) and the spectrogram (**b**, **e**) and amplitude waveform (**c**, **f**) of the combined male and female vocal traces are shown for exemplary duet bouts initiated by the male (**a**) or by the female (**d**) of Pair #5. Solid dark blue and dark red lines outline the root-mean-square envelope (see "Methods") of neural and vocal signals. Times of spike occurrence are indicated by short vertical lines above the neural traces. Onset–onset intervals of male and of female duet syllables are provided by values above the spectrograms. Please note the precise alternation of neural bursts between interacting males and females. Significant ($p < 0.01$, *t* test) activity in the averaged spectrogram (see "Methods") of male and female vocal signals within a window of 500 ms before and after the time of occurrence of 3000 male (**g**) and 3000 female (**h**) spikes that occurred during 46 duet bouts of Pair #5 is shown by clusters of blue and red time–frequency pixels. The dashed blue and red lines mark the time of occurrence of male and female spikes, respectively, used for generation of the averaged spectrogram

obtained when using conventional bioacoustic and neurophysiological recording techniques[32]. Most evident in the neural signal of each bird was the observation that, during duetting, multiunit activity was exclusively locked to the singing bird's own vocalizations. During the partner's duet syllables, activity in HVC was not increased (Fig. 4; Supplementary Fig. 9). The majority of spikes in all six birds occurred during the first half of a bird's own vocal emissions (Supplementary Fig. 10), which

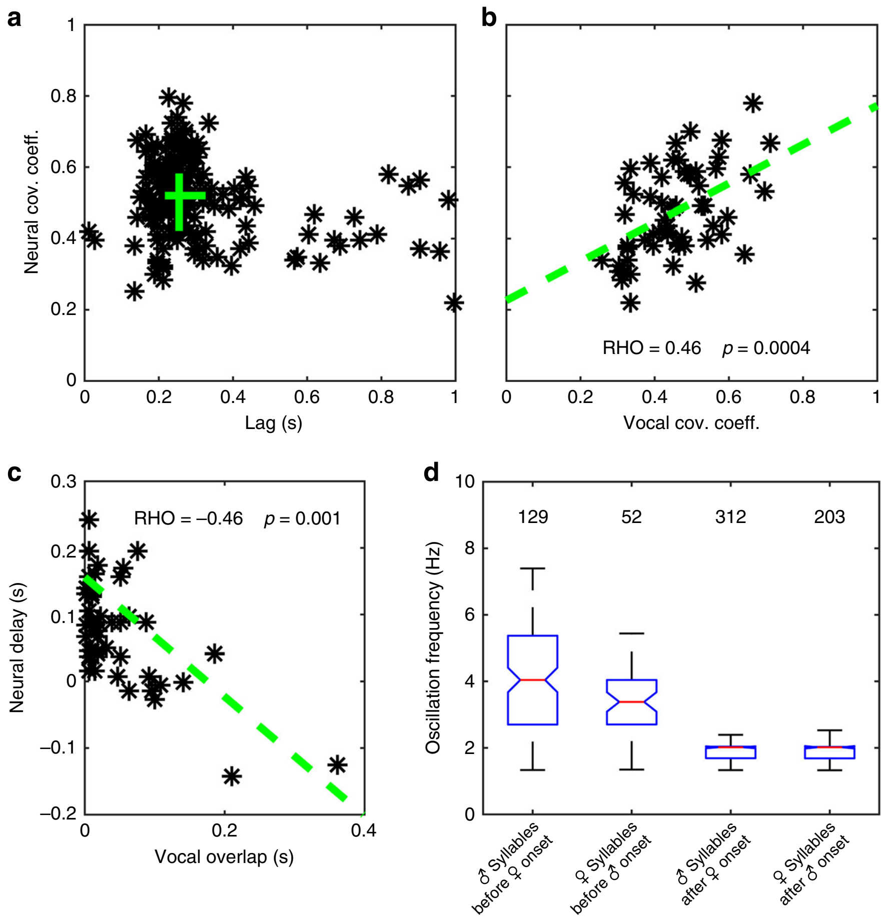

Fig. 5 Quantification of temporal properties of HVC premotor activity during duetting. **a** Time lags of maximum cross-covariance between male and female neural signals (root-mean-square envelopes) during 179 duet bouts produced by three bird pairs were clustered at approximately 250 ms. The green lines indicate interquartile ranges of lags and cross-covariance coefficients, and their intersection is at the medians of the distributions. **b** The covariance of neural signals was strongly correlated with the covariance of vocal signals. The dashed green line represents the regression line. $n = 56$ duet bouts of three bird pairs. **c** The degree of overlap in neural activity was strongly correlated with the degree of syllable overlap. The dashed green line represents the regression line. $n = 47$ overlaps produced by two bird pairs. **d** During emission of duet-initiating syllables, oscillation frequencies of the male and female neural signals were significantly higher ($p < 0.005$, Mann-Whitney *U* test) than during emission of alternating syllables during duetting. Labeling of boxplots as in Fig. 3c

indicates that the neural activity we recorded during duetting was mainly premotor and not auditory.

Although the partner's vocalizations did not result in auditory-evoked activity in HVC neurons, it might have been possible that this auditory input had modulating effects on premotor activity. We analyzed the spike rate during the production of duet syllables that were overlapped by the partner's duet syllable and found that HVC activity was not affected by overlaps. In both the male and female HVC, the median spike rate during overlapped syllables (male median: 23.5 and interquartile range: 8–36 spikes per syllable; female median: 12.5 and interquartile range: 5–25 spikes per syllable) did not differ from the median spike rate during nonoverlapped syllables (male median: 23 and interquartile range: 9–37.5 spikes per syllable; female median: 12.5 and interquartile range: 6–22 spikes per syllable) that preceded or succeeded the overlap. Note that the difference in the median spike rate between male and female birds was not significant (one-sided Mann–Whitney *U* test, $p > 0.01$).

Activity in the HVC is correlated between duet partners. As shown above for vocal signals, male and female neural signals were strongly correlated during the alternating part of duet bouts. The covariance of the RMS envelopes (see "Methods") of neural signals was maximal at a median time shift of 255 ms (interquartile range: 217–326 ms, Fig. 5a, Supplementary Fig. 11). Most importantly, during the alternating part of duet bouts, the degree of interindividual synchronization of neural activity was positively correlated (Spearman's Rho: 0.46, permutation test for large-sample approximations, $p < 0.001$) with the degree of interindividual synchronization of vocal activity (Fig. 5b, Supplementary Fig. 12). This may indicate that a precise synchronization of premotor neural activity between partners is required for an exactly timed duet performance. A similar relationship between neural activity and vocal behavior was observed during syllable overlaps. The degree of syllable overlap was significantly correlated (Spearman's Rho: −0.46, permutation test for large-sample approximations, $p < 0.005$; Fig. 5c, Supplementary Fig. 13) with the delay between the last spikes that occurred during the overlapped syllable and the first spikes that occurred during the overlapping syllable.

The partner's song onset affects the neural burst rate. Similar to the temporal pattern of the vocal behavior in *P. mahali*, we found a significant (two-sided Mann–Whitney *U* test, $p < 0.005$) decrease in oscillation frequency of HVC activity in the duet-initiating bird right after the partner had joined the duet. During the initiating part of the duet, bursts of multiunit activity in the neural signal of male and female duet-initiating birds occurred at a median rate of 4.0 and 3.4 Hz, respectively, whereas the median burst rate dropped to 2.0 Hz in both sexes immediately after the partner started contributing to the duet (Fig. 5d; Supplementary Fig. 14). This suggests that the auditory input generated by the partner's vocalizations influences the timing of vocal premotor activity in the singing bird.

Discussion

In contrast to conventional neurophysiological methods, our radio telemetric approach allows us to investigate the neural basis of an animal's behavior under completely natural conditions. To date, neurophysiological studies have been conducted with laboratory animals that spend their lives in cages. The behavior of caged animals, however, very likely differs from that of free-living conspecifics as the latter regularly face situations due to weather conditions, hunger, or predation, which would never be experienced by laboratory animals and which probably have a very large impact on an animal's natural behavior[30]. In addition, especially for animals such as *P. mahali* that live in groups with a sophisticated social structure, certain aspects of their behavioral ecology would be impossible to set up in a laboratory environment. Our technique now enables access to the neural activity of several group members in parallel, while each individual can behave naturally. This opens up a plenitude of possibilities to researchers who aim to study the neural substrates of complex social behaviors (e.g., collective movement or cooperative foraging) that can only be performed within a natural context.

Here we recorded individual vocalizations and premotor vocal activity in parallel from pairs of duetting birds to investigate how the precise interindividual coordination of motor activity during duet singing is neuronally controlled. Previous dual neuroimaging studies, performed in human subjects while they were engaged in rhythmic social interactions, showed that synchronized motor actions between subjects were accompanied by an overall synchronization of brain activity[42,43]. Our present study demonstrates that rhythmic social interactions that require temporal coordination are not just associated with a coherent oscillation of activity in large areas of both interacting partners' brains[43] but with an interindividually synchronized activation of small groups of neurons within the same brain nucleus in both partners. Our results strongly suggest a scenario in which the brains of two birds constitute a network that acts as a distributed circuit to organize the temporal pattern of vocal duets. We expect this to apply also to other rhythmic cooperative behaviors but experimental support provided by further dual-electrophysiological experiments in freely behaving animal models is required.

Our results show that, as in most nonduetting songbirds, during singing HVC activity in duetting birds is locked to each individual's own vocalizations. This strongly contrasts with what one would expect from the findings of a previous study in plain-tailed wrens. HVC neurons in anesthetized wrens differ in their activity pattern from that of most nonduetting songbirds by showing auditory-evoked activity not only to playbacks of the bird's own vocalizations but also to presentations of the partners song and even further increasing their activity when stimulated with a combination of both the male and female parts of this species' duet song[25]. HVC neurons in awake and actively duetting *P. mahali* were, however, responsive to neither the partner's vocalizations nor to playback of prerecorded duet bouts. It has been repeatedly shown that activity in the song-control system depends on the bird's behavioral state[44–46]. Under anesthesia and during sleep, HVC neurons usually show strong, consistent, and highly selective auditory responses to presentations of the bird's own vocalizations. In awake birds, however, if present, the strength of auditory responses in HVC is highly variable, and neurons are unselective for the bird's own song. McCasland and Konishi[47] reported for three different songbird species that auditory responses in HVC are completely suppressed by vocal motor activity during singing. More recently, Hamaguchi et al.[48] demonstrated that, while auditory input failed to alter intracellular activity of HVC neurons during singing, the same cells responded to auditory stimulation when the bird ceased to vocalize. This may indicate that, also in the awake condition, HVC processes auditory information, but during singing, the output of this processing is subthreshold. In duetting *P. mahali*, we observed that auditory information generated by the partner's syllables never elicited an auditory response but could trigger a change in vocalization rate in the duet-initiating bird. A similar observation has been made in duetting plain wrens (*Cantorchilus modestus zeledoni*), suggesting that vocal production in individuals who sing duets is controlled by autogenous and heterogeneous auditory feedback rather than by intrinsic fixed action patterns[49]. Further support for this hypothesis was provided by our finding that, during duetting, male *P. mahali* were able to compensate for syllable mismatches. This indicated that each bird listened to its own and the partner's vocalizations to fine-tune the timing of vocalizations. We therefore suggest that, during duetting, the auditory information from the partner's syllables is reaching HVC and is most likely used for the interindividual coordination of vocalizations in *P. mahali*. An alternative but less likely hypothesis is that, during duetting, HVC does not receive the "raw" auditory information generated by the partner but rather a premotor signal that is the result of auditory processing in downstream brain areas. Such a scenario has been demonstrated to exist in nonduetting songbirds to coordinate the premotor signals for song generated in the song-control system of both hemispheres in a way that the vocal organ is able to produce proper sounds[50].

In most bird species that produce vocal duets, the females are assumed to lead during duetting[2,51–53] (see ref. [4] for a contrary example). The leading role has been attributed to the bird of a pair that most frequently initiated duets[51–53], produced overlaps most often[2], determined the song rhythm[4], i.e., showed less behavioral adaptation, or produced vocalizations that elicited the stronger neural responses in the HVC[25]. Our study, however, provides support for the assumption that male birds lead during duetting: (1) Duets were most often initiated by male *P. mahali*. (2) Vocal emission rates during duetting were less flexible in males than in females, which may indicate that the male was determining the song rhythm. (3) Male *P. mahali* produced duet syllable overlaps more often than females. Although it is well established that the emergence of leader and follower roles enhances the quality of group performance[54], the factors that determine which partner adopts which role during joint actions are still not entirely clear. It has been suggested that the partner who maintains the rhythm becomes the leader and the partner who maintains the synchrony of the joint behavior becomes the follower, as maintaining synchrony requires greater adaptation[55]. Recently, a neural correlate of leader–follower distinctions has been described. Using dual-electroencephalogram measurements in two human participants who performed a synchronized tapping task, Konvalinka et al.[56] showed that leaders but not followers exhibited a reduction in alpha and low-beta oscillations over motor and frontal areas, which may have reflected the increase in prospective planning and control required for maintaining the rhythm of joint actions[57]. Additional studies such as ours, which measure brain activity synchronously in pairs or groups of individuals during natural social interactions, are, however, essential to further the understanding of the neural mechanisms that underlie the coordination of joint actions.

Methods

Animals and ethics approval. We studied a population of wild *P. mahali* near the village of Black Rock, Northern Cape, South Africa (27°7′S, 22°50′E) during February/March 2016 and November/December 2017. All investigated birds were color-banded for individual recognition. The birds were captured shortly after dusk (0800–1000 p.m.) inside their roosting nests. The sex was determined by bill color[58,59]. The male bird of all investigated pairs was likely the dominant male of the group, whereas the social status of female birds could not be determined reliably.

All bird-capture permits were obtained from the Northern Cape Department of Nature Conservation. All experiments described here complied with the relevant ethical regulations for animal testing and research and were approved by the Animal Ethics Committee of the University of Pretoria. To ensure that the birds would not be restricted in their natural behavior while carrying the transmitters, we tested all devices on captive *P. mahali* in Seewiesen, Germany, under permit 55.2-1-54-2532-175-2016 issued by the government of Upper Bavaria. After the initial experiments in Germany had proven that the birds only show short-term (up to a few hours) effects (e.g., less singing and less locomotion than usual) while habituating to carrying the transmitters, and other aspects of their normal behavior (e.g., flight performance, feeding, entering and exiting their nests) were not affected by the transmitters, we went to South Africa. In 2016, we only recorded the individual vocal behavior of wild *P. mahali* pairs with microphone transmitters. For these experiments, we received ethics approval from the University of Pretoria (permit: EC086-15). In 2017, we combined the vocal recordings with telemetric neural recordings. After paying special attention to pain management, to bird recapture, and to the possible effects of the transmitting devices on the birds' behavior, for this second set of experiments, the University of Pretoria granted us ethics approval (permit: EC026-17) bound to additional obligations (e.g., the presence of a state veterinarian during surgical interventions on the birds).

Vocal recordings. To monitor individual vocal activity in free-ranging *P. mahali*, we equipped both birds of wild pairs with on-board radio telemetric transmitters developed at the Max-Planck-Institute for Ornithology in Seewiesen, Germany. The lightweight vocal transmitter (0.6 g), which included a miniaturized microphone (FG23329, Knowles Electronics, USA), was covered by a thin silicon casing and fixed on the back of the bird (Fig. 1; Supplementary Fig. 1c) with cotton-covered rubber-band straps around both femurs and the abdomen[32]. The transmission range of the device averaged 50 m and the battery life 15 days. Carrying these microphone transmitters has only small and short-term habituation effects on the vocal and movement activity of songbirds[32]. For signal detection, a crossed Yagi antenna (Winkler Antennenbau, Germany) was placed below the nesting tree in the center of the birds' territory (Supplementary Fig. 1d). An antenna amplifier (TVS 14-00 axing, Goobay®, Germany) increased the antenna signal by 18 dB. The signal was split (BE 2-01 premium-line, Switzerland) and fed into up to eight communication receivers (AOR 8600, AOR Ltd., Japan), which were modified to handle 12 kHz audio bandwidth. The analog signals were digitized by an eight-channel audio A/D converter (M-Track Eight, M-Audio, USA; sampling rate: 22050 Hz) that was connected to a laptop computer (Supplementary Fig. 1e). All digitized signals were recorded in parallel as continuous audio files with a duration of 4 h using the multichannel software (16-bit, 22050 Hz; ASIO®, Germany). The recording set-up was placed in the trunk of a car ~30 m from the antenna (Supplementary Fig. 1f). For each pair, vocal recordings were continuously conducted over several consecutive days.

Neural recordings. Birds that were chosen to carry the additional neural transmitter (Fig. 1 and Supplementary Fig. 1c) received analgesic treatment (meloxicam 5 mg ml^{-1}, Metacam, Boehringer-Ingelheim, Germany; 0.2–0.5 mg kg^{-1}) prior to anesthetization with isoflurane inhalation (isoflurane 1.5–1.8% in 0.5 l O_2 min^{-1}, IsofluranCP, CP-pharma, Germany). The birds were wrapped in a thin gauze blanket and kept warm by warm water bags (Supplementary Fig. 1b). The skin of the head was treated with lidocaine liquid (lidocainhydrochlorid, Minocain, Bela-Pharm, Germany), plucked, and disinfected with alcohol swabs (Henry Schein, Germany). The skin was opened in rostro-caudal direction along the midline. A first craniotomy was performed to localize the bifurcation of the midsagittal sinus, which served as stereotaxic reference point, and a second craniotomy and a durotomy were performed over the HVC. Stereotaxic coordinates had been ascertained beforehand in captive animals. In all birds, the recording electrode was lowered into the HVC at a lateral distance of 2600 µm from the reference point using a piezo single-axis micromanipulator (SMX series, SENSAPEX, Sweden) that was mounted on a stereotaxic frame (WPI, USA). The reference electrode was inserted between the skull and dura mater. During electrode implantation, the recorded signal was amplified using an AC differential amplifier (DAM 80, WPI, USA), filtered between 200 and 10,000 Hz by a custom-made, dual state variable filter (Free University of Amsterdam, The Netherlands), digitized by a battery-operated USB audio interface (Duo-Capture SX, Roland, Germany) and monitored online by a laptop using a continuous update of the interspike interval of Schmitt-triggered spikes. Photographs of the set-up used to implant electrodes are shown in Supplementary Fig. 1a, b.

After detecting stable auditory-driven neural activity with a good signal-to-noise ratio, the electrode was fixed (at depths between 217 and 360 µm below brain surface) by gluing the connector pins of the reference and the recording electrode to the skull using dental cement (Tetric evoflow, Ivoclar Vivadent, Liechtenstein), and a self-made radio-telemetric transmitter (weight: 1.0 g) was connected to the pins[33,34]. The transmission range of the device covered >50 m, with a battery life of >7 days. The bandwidth of the transmitter's amplifier stage ranged between 10 and 11,000 Hz to enable recording of local field potentials as well as spiking activity. The transmitter was connected to a single parylene-coated tungsten electrode (FHC, USA; impedance: 2 MΩ) and a reference electrode (platinum wire with a diameter of 50 µm, Advent Research Materials, UK).

After the surgical intervention, the birds were allowed to recover in cloth cages until dawn. All birds recovered quickly from the treatment, and we were able to release them to their home territory a couple of hours after the surgical implantation of the electrode and mounting of the transmitters. This protocol was essential to our study since we had observed that removing birds from their group during the day could destroy the group's social structure. By releasing the birds soon after the treatment (shortly before sunset), we could guarantee that all treated birds readily reintegrated into their group and maintained their social position. Before release at civil twilight, we administered a second analgesic injection (Metacam 0.2–0.5 mg kg^{-1}, Boehringer-Ingelheim, Germany). Recording of neural and vocal activity (Supplementary Movies 1 and 2) started as soon as the birds were released. Whenever the birds were in the reception range of the antenna, the individual vocalizations and neural activity of both birds of a pair were telemetrically recorded in parallel for up to 4 days as described above. It is important to note that the position of the recording electrode was fixed in each bird. This means that we recorded for several days from one site in the HVC per bird.

Constant monitoring of the treated birds certified that they did not show any sign of discomfort or altered behavior due to the treatment. All but one female bird remained at their colony for the entire experiment. At the end of the experiments, the birds were re-trapped, and the transmitters were dismounted. Birds that carried neural transmitters were either released to their home territory ($n = 5$) or euthanized for brain sampling by an overdose of isoflurane after applying an electrolytic lesion at the recording site ($n = 6$). Only pairs of birds in which both neural transmitters provided stable signals over 2 consecutive days were euthanized since only data from those birds could be used for analysis. Electrolytic lesioning was performed by connecting both the reference and the recording electrode to the DAM 80 amplifier. Timing (6 s) and current (4.5 µA) of the lesions were adjusted according to Ter Maat et al.[34]. To verify electrode placement, dissected brains were transferred to 4% paraformaldehyde and transported to the Max-Planck-Institute for Ornithology. Here the brains were cryocut in 30 µm sections and stained with thionin. Supplementary Fig. 15 shows a sagittal brain section of a male *P. mahali* with a lesion in the HVC.

Acoustic playback. During the experiments in 2017, we presented playback of prerecorded duet songs to the experimental birds, for at least 30 min a day. The playback was broadcast by a battery powered active speaker (Mobile BA, ROLAND, Germany) that was placed in close vicinity to the roosting tree of the respective pair. We used four different duet song bouts in seven different versions: complete duet, male part only, male part only with prolonged gaps between syllables, male part only with shortened gaps between syllables, female part only, female part only with prolonged gaps and female part only with shortened gaps. All song versions were normalized to 3 dB of the maximum and bilaterally faded. Supplementary Movie 2 shows a pair of *P. mahali* duetting with the playback. In parallel with the vocal and neural signals of the birds that were equipped with transmitters, the playback signal was rerecorded by the use of a microphone transmitter located close to the speaker.

Sound analysis. The temporal fine structure of duet bouts for each studied pair of *P. mahali* was analyzed by different persons to avoid biasing the measurements. For each bird, all 4-h sound files were initially inspected audiovisually with the software Amadeus® or Audacity® to determine the time of duet bout occurrences. Based on Voigt et al.[12], a duet bout was defined as a sequence of introductory song syllables followed by male and female duet syllables. Each duet bout was surrounded by at least 1.5 s of silence and duet bouts temporally overlapped in the vocal signals of the male and female bird of a pair. The time point of the start of the first syllable and the end of the last syllable of each duet bout was noted. To obtain general timing properties, such as syllable duration and syllable interval, the start and the end of each female syllable and each male syllable element were measured in a subset of 15 to 20 duet bouts for each pair of birds. This approach follows the method used in Thorpe et al.[60] to define the reaction time in duetting pairs. Onsets and offsets of vocal elements were defined by a deviation of the vocal signal from baseline activity by 5%. The onset of the first syllable element and the offset of the second syllable element in the male duet syllables served as the onset and offset for male syllables, respectively.

In addition, a semiautomated method was used to quickly determine the onsets of female syllables and male syllable elements in all recorded duet songs. Sonograms from vocal signals were assembled from 512 point Fast Fourier Transforms (Intel

Libraries) using custom written software (R.F. Jansen; Delphi Pascal for Windows, and Andries Ter Maat; CodeWarrior and C++ for Mac OS X). Each sonogram describes a syllable or any other suprathreshold sound. To determine the average activity pattern in the vocal signals of each bird of a pair in relation to syllable occurrence in the vocal signal of one of the birds, syllable-triggered averaged spectrograms were generated for each bird using a reverse correlation technique. For this, the onset of each syllable in each duet bout was extracted from the data set. In accordance with a method described by Jenison et al.[61], for each syllable onset, the spectrogram (FFT length: 128, overlap: 50, Hamming window; MATLAB®, Mathworks, USA) of the vocal signal of each individual of a pair was calculated within a 500-ms segment preceding and succeeding the syllable onset. To evaluate the level of uncorrelated background activity, spectrograms were constructed from randomly drawn 1-s segments of each vocal signal at random positions within a period of 3 s before and 3 s after the syllable onset[62] and significant deviations from the background activity were determined by a pixelwise *t* test[63]. In the final display of the syllable-triggered average, significant ($p < 0.01$) pixels in the male and female vocal signals were indicated in blue and red, respectively. Each syllable-triggered average was based on at least 250 syllables that occurred during all duet bouts analyzed for a particular pair of birds. For each syllable-triggered average, the number of randomly drawn segments was equal to the number of syllable-triggered segments.

ITo determine the correlation between vocal signals of both duet partners, we measured the peak and the time lag at the peak of the cross-covariance function (MATLAB®) between the upper RMS envelopes (MATLAB®) of the signals for each duet bout. The envelopes were determined using a sliding window of a length of 1000 samples. During duetting, the male and the female bird often sat close together, which theoretically opened up the possibility that the microphone of the vocal transmitter of one bird picks up the vocal signal of the partner. However, since the receptive part of the microphone was directed toward the body surface of the bird it was mounted on, and the transmitter's plastic cover and the bird's feathers further shielded the microphone from external sound sources, the partner's vocalizations produced only negligible amplitude variations in the vocal traces, which were never detected by our sound analysis software and were averaged out in the RMS envelopes used for covariance analysis.

Neural analysis. ITo extract spike times from the continuous neural signals of each bird, each neural trace was initially bandpass filtered (200–4000 Hz, fourth order Butterworth) using the MATLAB® filtfilt function. Supplementary Fig. 16 shows examples of raw and filtered neural traces temporally aligned to vocal activity. A threshold for discriminating spikes from background activity was determined by averaging (MATLAB®) the mean plus three standard deviations of the neural signal during each duet bout within a 4-h sound file. The filtered neural signals were then fed into the software Spike2® (CED, UK), and spikes were discriminated from background activity by setting a threshold to the value calculated as described above (see Supplementary Fig. 16 for an example). The average signal-to-noise ratios (singing vs. non-singing) of filtered neural signals ranged between 3.5 and 9.6 dB, and average spike rates during singing and non-singing periods ranged from 25.0 to 113.3 spikes s^{-1} and from 5.7 to 35.1 spikes s^{-1}, respectively (see Supplementary Table 1). To examine the temporal relationship between the vocal and neural signals and to exclude the presence of artifacts, for each bird we plotted mean activity profiles of both signals aligned to syllable onset for all syllables produced within a 4-h recording period (see Supplementary Fig. 17 for examples).

To determine the averaged activity in the vocal signals of both partners in relation to spike occurrences in the neural signal of one partner, spike-triggered averaged spectrograms were generated for each bird. For each spike, the spectrogram (FFT length: 128, overlap: 50, Hamming window; MATLAB®, Mathworks, USA) of the vocal signal of each individual of a pair was calculated within a 500-ms segment preceding and succeeding the time of spike occurrence. To evaluate the level of uncorrelated background activity, spectrograms were constructed from randomly drawn 1-s segments of each vocal signal at random positions within a period of 3 s before and 3 s after spike occurrence[62] and significant deviations from the background activity were determined by a pixelwise *t* test[63]. In the final display of the spike-triggered average, significant ($p < 0.01$) pixels in the male and female vocal signal were indicated in blue and red, respectively. Each spike-triggered average was based on at least 400 spikes, and for each spike-triggered average, the number of randomly drawn segments was equal to the number of spike-triggered segments.

To determine the correlation between neural signals of both duet partners, we measured the peak and the time lag at the peak of the cross-covariance function (xcov, MATLAB®) between the upper RMS envelopes (envelope, MATLAB®) of the signals for each duet bout (see above). To measure the oscillation frequency of multiunit bursts in the neural signal of one bird before and after the song onset of the second bird, we calculated the autocovariance function (xcov, MATLAB®) between the upper RMS envelopes (MATLAB®) of the neural signal for both parts during each duet bout and performed a spectral analysis of the function by calculating the FFT (MATLAB®) with an input length that was the next power of two from the original signal length. The single-sided amplitude spectrum was plotted for the frequency range (1–10 Hz) that we expected to include the oscillation frequency of the signal, and the frequency at the peak of the spectrum was noted.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Owing to the large size of data files, they are not publicly available. A reporting summary for this article is available as a Supplementary Information file.

Code availability

All custom computer codes used to generate results that are reported in the paper are available from the corresponding author upon reasonable request.

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Author contributions

S.H., L.T., A.T.M., and M.G. conceived the project; S.H., L.T., C.V., S.L., A.L., S.K., and A. T.M. contributed data and analyses; C.V., H.S., and M.A. provided technical support; and S.H., L.T., C.V., S.L., and A.T.M. wrote the manuscript with feedback from all authors.

Additional information

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Supplementary Information

Duets recorded in the wild reveal that interindividually coordinated motor control enables cooperative behavior

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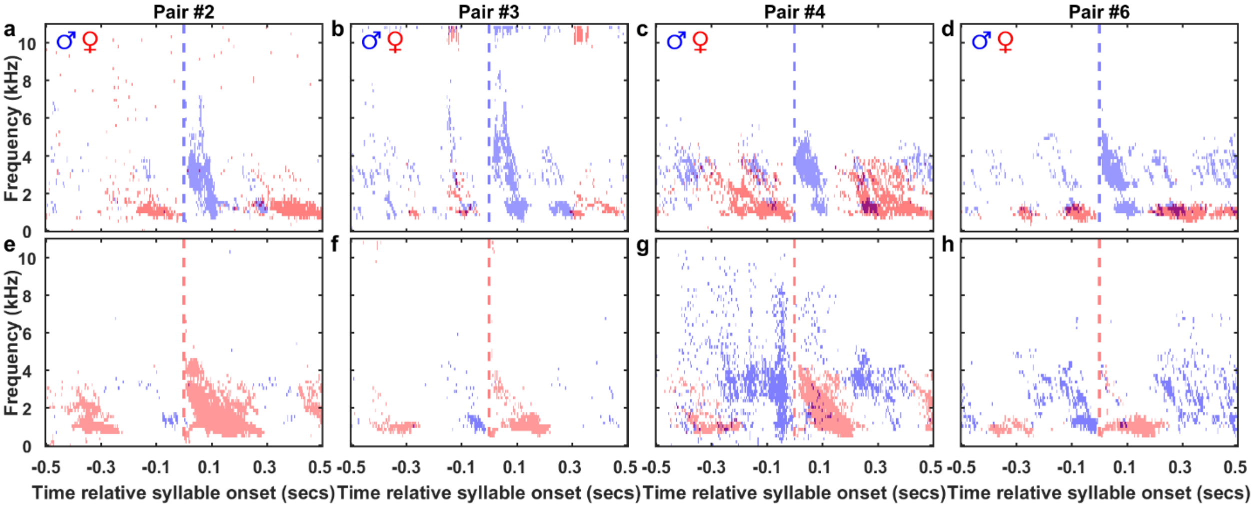
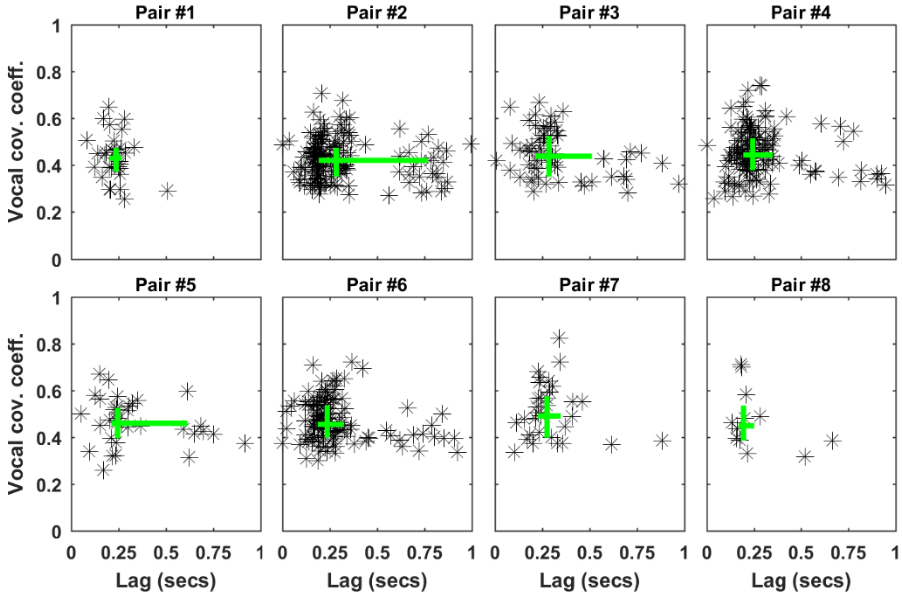
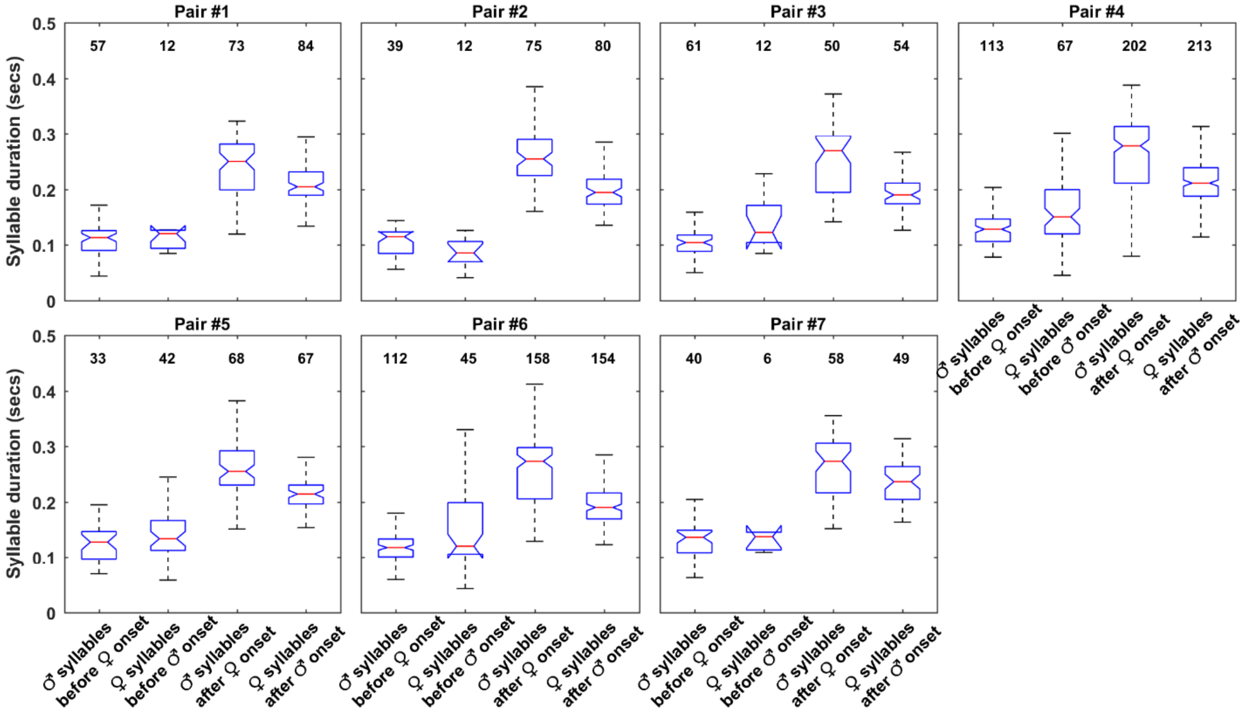
Supplementary Figure 1. Portable electrophysiology laboratory in the Kalahari.
(**a**) depicts the setup used for surgical implantation of electrodes in HVC. (**b**) shows an anesthetized bird fixed inside the stereotaxic device. In (**c**) a pair of *P. mahali* is shown, perching in a tree below their nest. The male bird on the right was equipped with a vocal transmitter on his back and with a neuronal transmitter on its head. (**d**) depicts the antenna used for signal reception, which was placed below a nesting tree. (**e**) shows the setup used for recording of individual vocal and neural signals, which was placed in the trunk of a car that was parked in ~30 m distance to the nesting tree (**f**).



Supplementary Figure 2. Syllable-triggered averaged spectrograms.
Significant (t-test, $p < 0.01$) activity in the averaged spectrogram (see Methods) of male (blue) and female (red) vocal signals within a window between 500 ms before to 500 ms after the onset of male (**a** - **d**) and female (**e** - **h**) duet syllables in duet bouts of three bird pairs is shown by clusters of blue and red time-frequency pixels. The dashed blue and red lines mark the onsets of male and female syllables, respectively, used for generation of the averaged spectrogram. Note the alternating pattern of clusters of red and blue pixels, which indicates that the occurrence of male and female syllables alternated within the duet. Averaged spectrograms of Pair #2, Pair #3, Pair #4 and Pair #6 are based on 1436 male and 1194 female syllables from 194 duet bouts, 421 male and 304 female syllables from 71 duet bouts, 893 male and 554 female syllables from 181 duet bouts and 710 male and 288 female syllables from 131 duet bouts, respectively. For the remaining pairs #1, #5, #7, #8 duet activity (32, 33, 38 and 12 duet bouts, respectively) was too low to generate significant frequency-time pixels in the syllable-triggered averaged spectrograms.

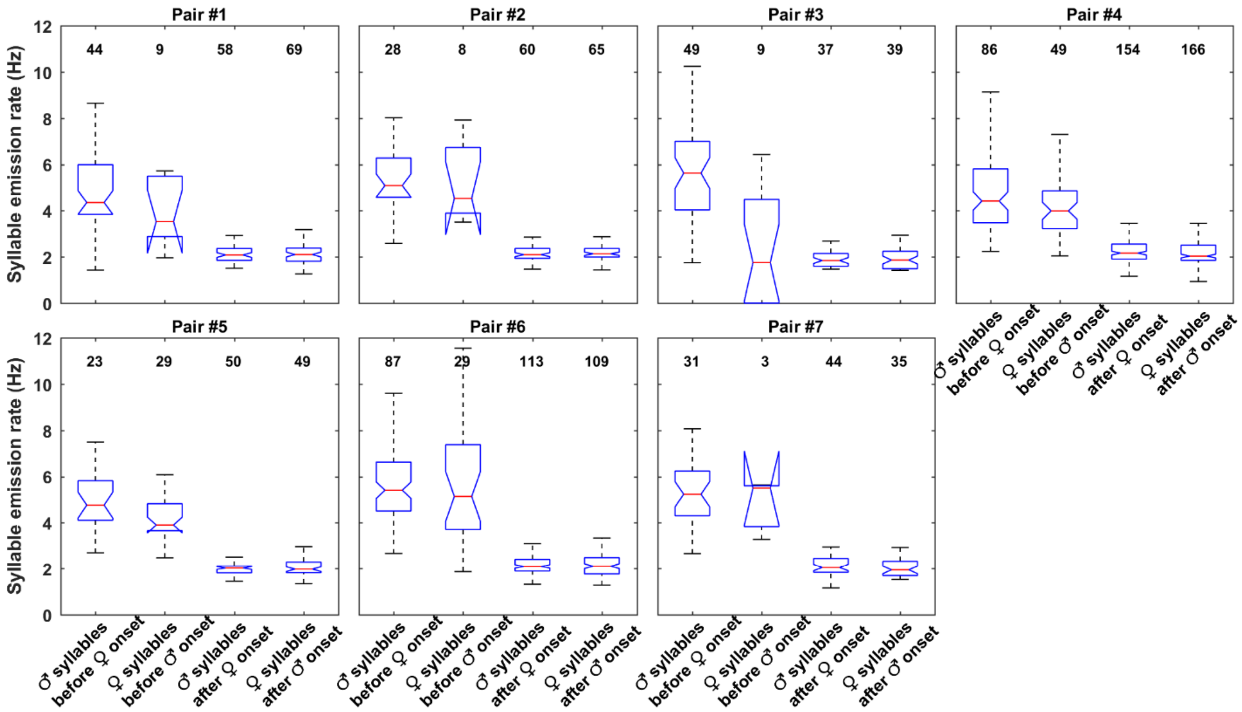


Supplementary Figure 3. Correlation of vocal activity during duetting.
Vocal signals of the male and the female bird of a pair were strongly correlated during duetting at a time shift of ~250 ms. This is indicated by the time lags of maximum cross-covariance between male and female vocal signals (RMS-envelopes) during duetting. The green lines indicate interquartile ranges of lags and cross-covariance coefficients, their intersection is at the medians of the distributions.



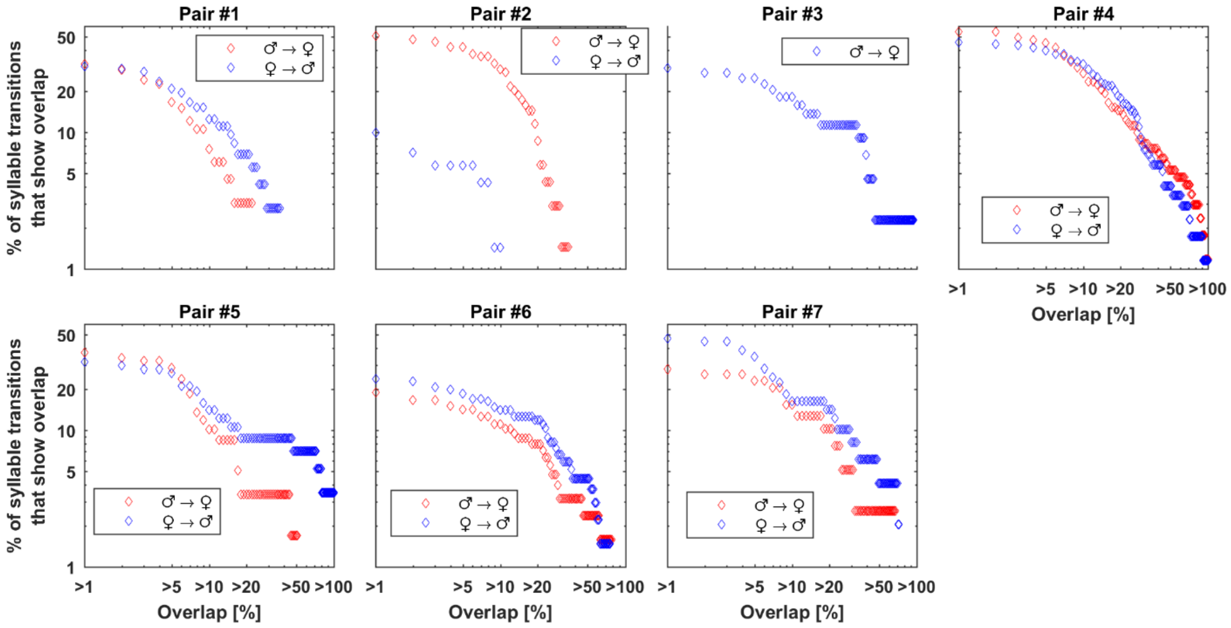
Supplementary Figure 4. Syllable durations.

The duration of syllables sung by a single bird before the second bird's song onset was significantly shorter than the duration of syllables sung in alternation after the second bird's song onset. During duetting, male syllables were significantly longer in duration than female syllables. Boxplots show the distribution of syllable durations for each bird of seven pairs. In the boxplot, the horizontal red line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The black whiskers extend to the most extreme data points not considered outliers (outliers are not shown). The extremes of the two notches of the box correspond to $y - 1.57(z - x)/\sqrt{n}$ and $y + 1.57(z - x)/\sqrt{n}$, where y is the median, x and z are the 25th and 75th percentiles, respectively, and n is the number of observations. Medians are significantly different at the 5% significance level if the boxes' notches do not overlap. The individual sample size is indicated above each box. The duration of syllables was measured in 15 to 20 duet bouts for each pair of birds. Pair #8 produced only 12 duet bouts during the time of recording and was therefore excluded from this analysis.



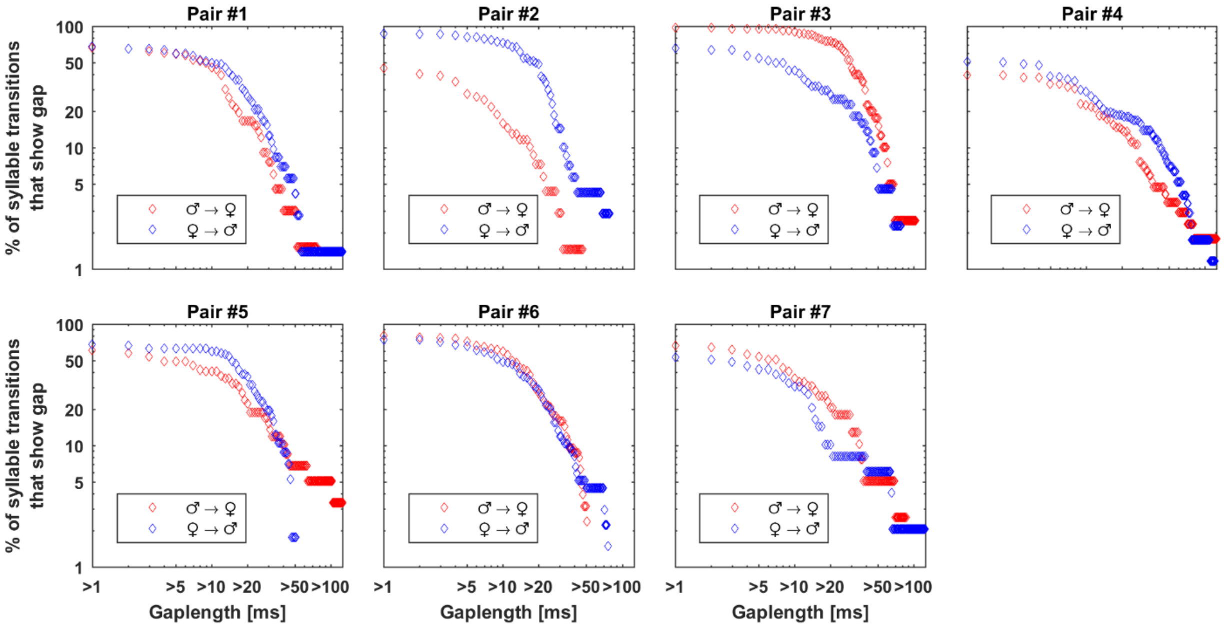
Supplementary Figure 5. Syllable emission rates.

The emission rate of syllables sung by a single bird before the second bird's song onset was significantly higher than the emission rate of syllables sung in alternation after the second bird's song onset. Please see Supplementary Figure 4 for details on box plot labeling. The individual sample size is indicated above each box. The emission rate of syllables was measured in 15 to 20 duet bouts for each pair. Pair #8 produced only 12 duet bouts during the time of recording and was therefore excluded from this analysis.

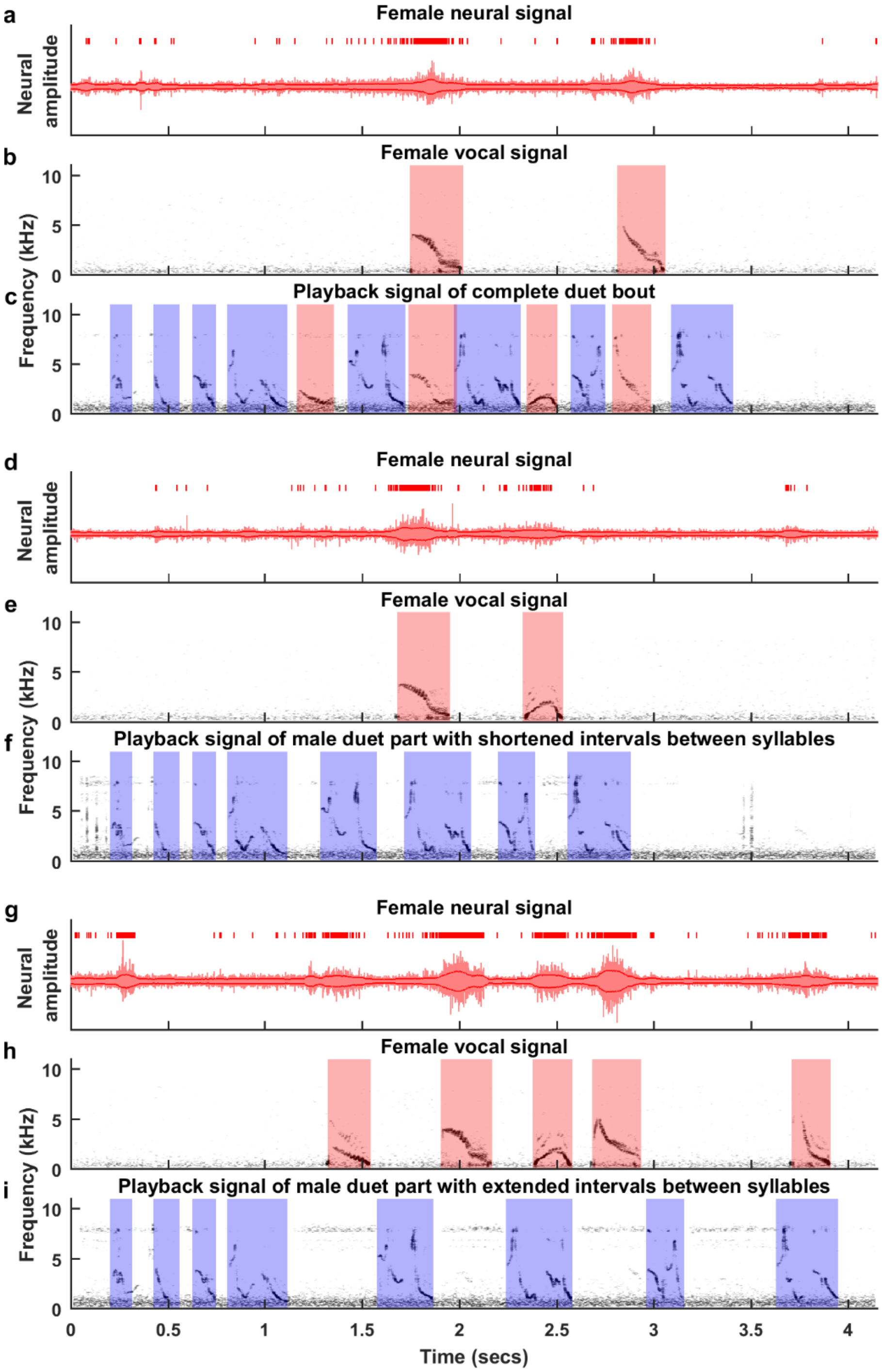


Supplementary Figure 6. Syllable overlaps.

The percentage of duet syllable transitions that showed an overlap larger than the value given on the x-axis is displayed for each bird of seven pairs. Female to male and male to female transitions are marked by blue and red diamonds, respectively. Note that the female bird of Pair #3 never produced syllable overlaps.

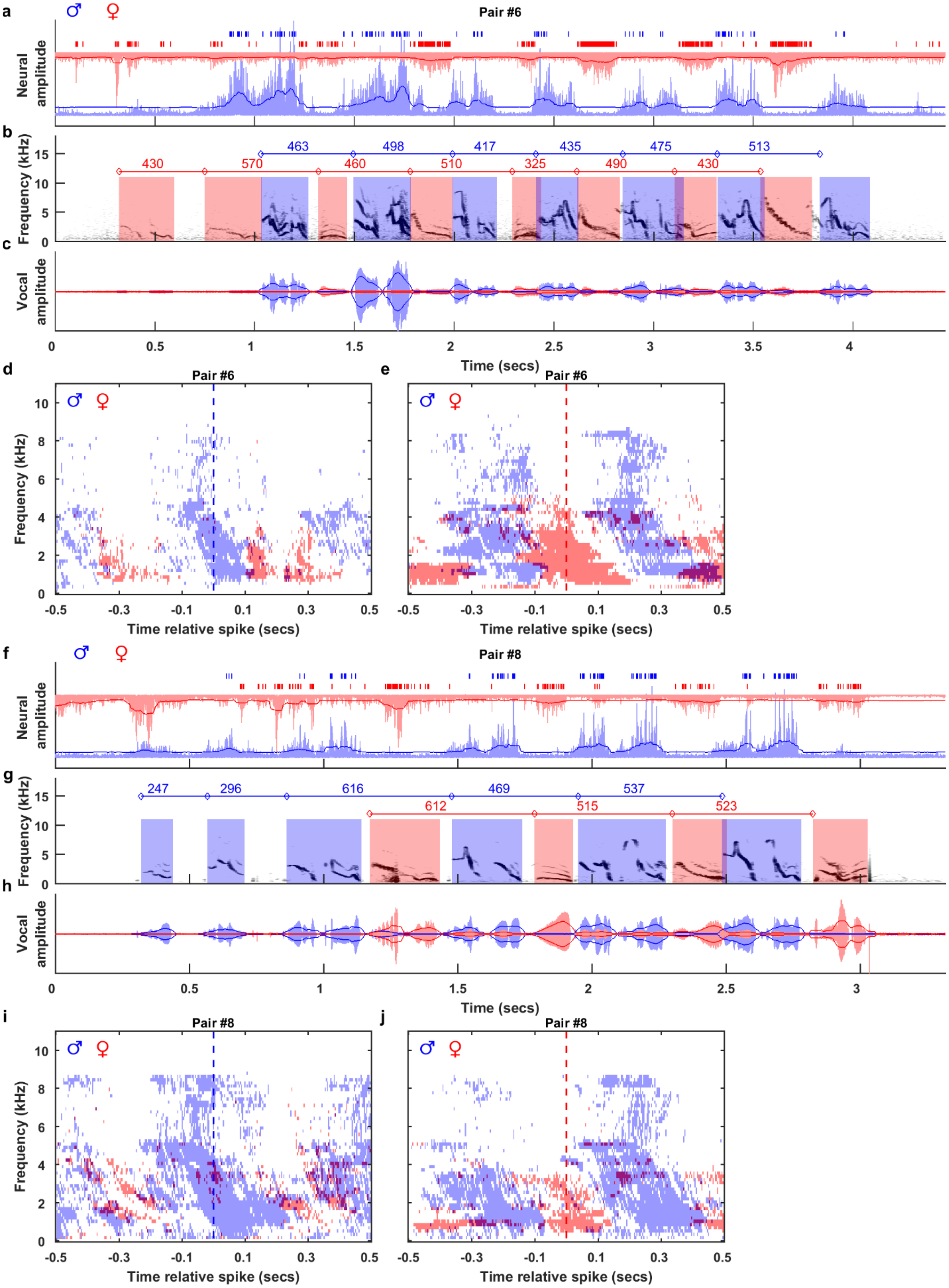


Supplementary Figure 7. Gaps between syllables.
The percentage of duet syllable transitions that showed a gap larger than the value given on the x-axis is displayed for each bird of seven pairs. Female to male and male to female transitions are marked by blue and red diamonds, respectively.

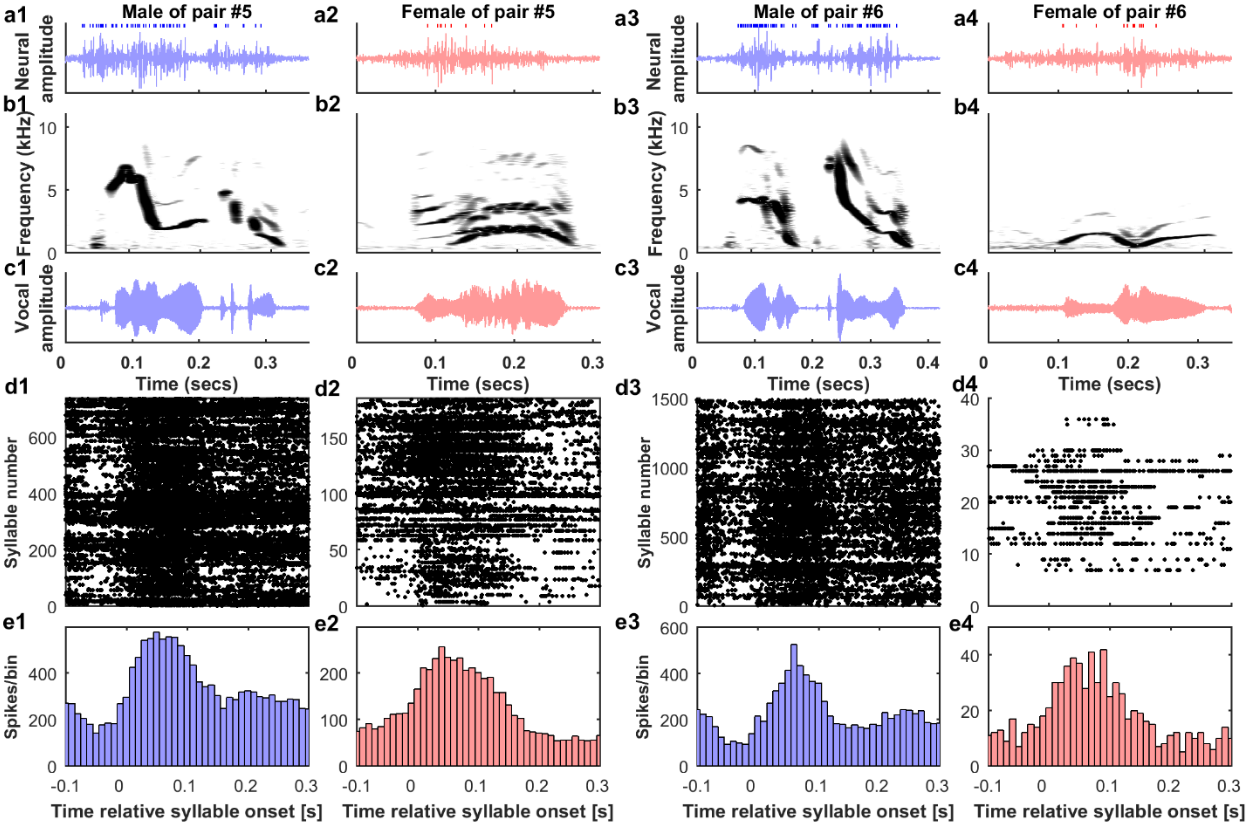


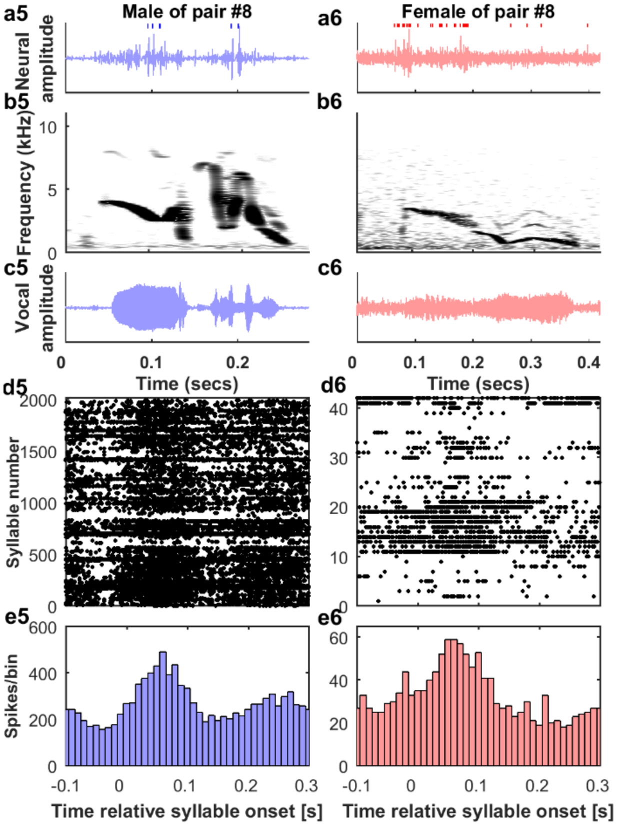
Supplementary Figure 8. Playback experiments.
White-browed sparrow-weavers were able to correctly duet with playbacks of its own duet bouts but was not able to correctly duet with playbacks of own duet bouts with altered temporal patterns. **a**) neural signal and (**b**) spectrogram of vocal signal of a female while duetting with the playback (spectrogram in **c**) of a complete duet bout. **d**) neural signal and (**e**) spectrogram of vocal signal of the same female as it tried to duet with the playback (spectrogram in **f**) of the male duet part with shortened intervals between male duet syllables. **g**) neural signal and (**h**) spectrogram of vocal

signal of the same female as it tried to duet with the playback (spectrogram in **i**) of the male duet part with extended intervals between male duet syllables. Please see the Methods section for details on playback experiments.

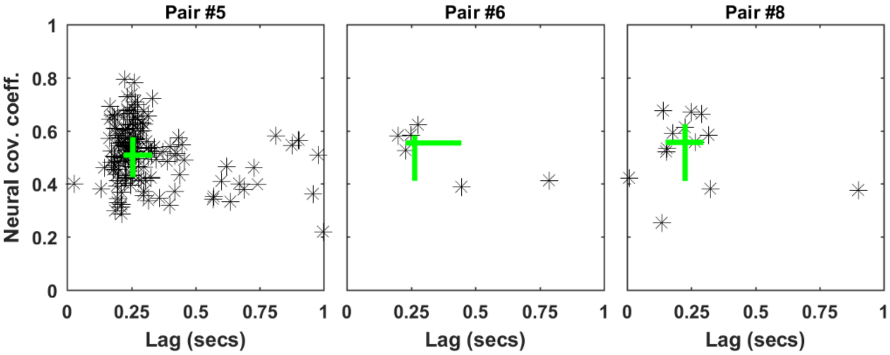

a
♂ ♀
Pair #6
Neural amplitude
b
Frequency (kHz)
15
10
5
0
463
498
417
435
475
513
430
570
460
510
325
490
430
c
Vocal amplitude
0
0.5
1
1.5
2
2.5
3
3.5
4
Time (secs)
d
Pair #6
♂ ♀
Frequency (kHz)
10
8
6
4
2
0
-0.5
-0.3
-0.1
0.1
0.3
0.5
Time relative spike (secs)
e
Pair #6
♂ ♀
-0.5
-0.3
-0.1
0.1
0.3
0.5
Time relative spike (secs)
f
♂ ♀
Pair #8
Neural amplitude
g
Frequency (kHz)
15
10
5
0
247
296
616
469
537
612
515
523
h
Vocal amplitude
0
0.5
1
1.5
2
2.5
3
Time (secs)
i
Pair #8
♂ ♀
Frequency (kHz)
10
8
6
4
2
0
-0.5
-0.3
-0.1
0.1
0.3
0.5
Time relative spike (secs)
j
Pair #8
♂ ♀
-0.5
-0.3
-0.1
0.1
0.3
0.5
Time relative spike (secs)

Supplementary Figure 9. Premotor activity in HVC during duetting.
Duet syllables were locked to bursts of premotor neural activity in the HVC of the singing bird, and neural burst alternated between interacting birds. The male and female neural traces (**a** and **f**), and the spectrogram (**b** and **g**) and time signal (**c** and **h**) of male and female vocal traces are shown for duet bouts initiated by the female of pair #6 (**a - c**) or by the male of pair #8 (**f - h**). Male and female syllables and male and female vocal time signals are indicated in blue and red, respectively. Solid dark blue and dark red lines outline the RMS-envelopes (see Methods) of male and female signals, respectively. Spike occurrences in the male and female neural traces are indicated by blue and red short vertical lines, respectively, above the neural signals. Onset-onset intervals male duet syllables and between female duet syllables are given by values above the spectrograms. Significant (t-test, $p < 0.01$) activity in the averaged spectrogram (see Methods) of male (blue) and female (red) vocal signals within a window between 500 ms before to 500 ms after the time of occurrence of male (**d** and **i**) and female (**e** and **j**) spikes in duet bouts of Pair #6 and Pair #8 is shown by clusters of blue and red time-frequency pixels. The dashed blue and red lines mark the time of occurrence of male and female spikes, respectively. Averaged spectrograms of Pair #6 and Pair #8 are based on 420 male and 439 female spikes from 16 duet bouts and 559 male and 411 female spikes from 12 duet bouts, respectively. The spike-triggered averaged spectrogram of Pair #5 is shown in Fig. 4.

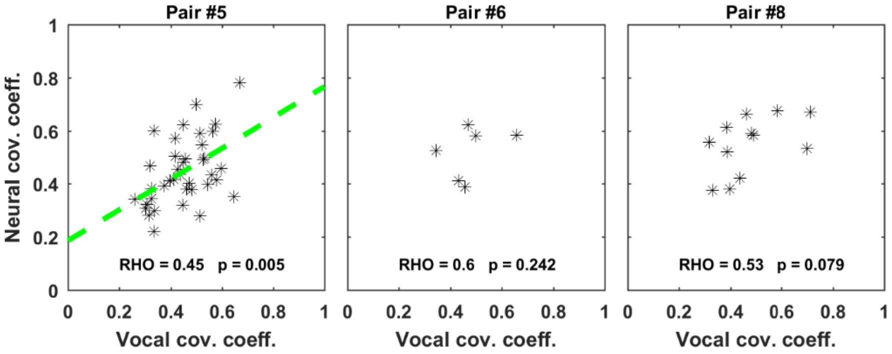

a1
Male of pair #5
a2
Female of pair #5
a3
Male of pair #6
a4
Female of pair #6
Neural amplitude
b1
b2
b3
b4
Frequency (kHz)
10
5
0
c1
c2
c3
c4
Vocal amplitude
0
0.1
0.2
0.3
0.4
Time (secs)
d1
d2
d3
d4
Syllable number
600
400
200
150
100
50
1500
1000
500
40
30
20
10
0
e1
e2
e3
e4
Spikes/bin
600
400
200
100
40
20
0
-0.1
0
0.1
0.2
0.3
Time relative syllable onset [s]


a5
Male of pair #8
a6
Female of pair #8
Neural amplitude
b5
b6
Frequency (kHz)
10
5
0
c5
c6
Vocal amplitude
0
0.1
0.2
0.3
0.4
Time (secs)
d5
d6
Syllable number
2000
1500
1000
500
40
30
20
10
0
e5
e6
Spikes/bin
600
400
200
60
40
20
0
-0.1
0
0.1
0.2
0.3
Time relative syllable onset [s]

Supplementary Figure 10. Syllable-triggered representation of neural data.
The filtered neural signal (**a1** – **a6**), spectrogram (**b1** – **b6**) and amplitude waveform (**c1** – **c6**) is shown for one male (blue) and one female (red) exemplary duet syllable for three pairs of *P. mahali*. Spike raster plots (**d1** – **d6**) and corresponding peri-stimulus time histograms (bin size = 0.01 secs, **e1** – **e6**) aligned to the onset of all duet elements within a four-hour recording are shown for the male (blue) and the female (red) bird of three *P. mahali* pairs to demonstrate the correlation between vocalization onset and spike occurrence.

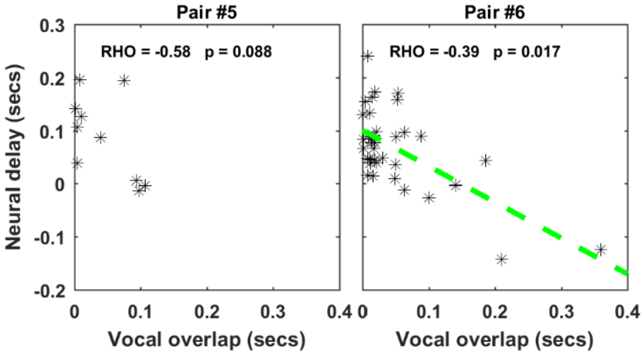


Supplementary Figure 11. Correlation between vocal and neural activity.
Neural signals of the male and the female bird of a pair were strongly correlated during duetting at a time shift of ~250 ms. The black asterisks mark the time lag when the cross-covariance function (see Methods) between the RMS-envelopes of the male and the female neural signal during the alternating part of duet bouts reached its maximum. The interquartile ranges of the covariance coefficients and the time lags are indicated by vertical and horizontal green lines, respectively, and the green lines' intersection marks the distributions' medians.



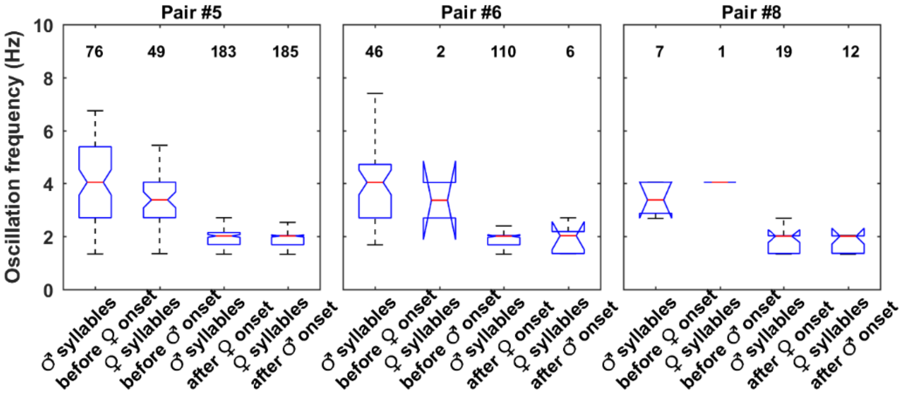
Supplementary Figure 12. The degree of neural synchronization between duet partners determined the degree of duet performance.

Black asterisks mark the coefficient of the cross-covariance function of the RMS-envelopes of male and female neural signals during duetting as a function of the coefficient of the cross-covariance function of the RMS-envelopes of male and female vocal signals during duetting. The green regression line demonstrates the positive correlation of the degree of synchronization between the male and female vocal signals and the degree of synchronization between the male and female neural signals. Spearman’s Rho and the significance level of correlation are indicated for each pair.



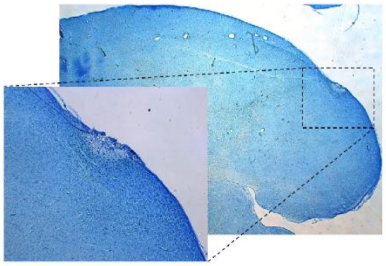
Supplementary Figure 13. The degree of overlap in neural activity was strongly correlated with the degree of syllable overlap.

The dashed green line represents the regression line. n = 10 overlaps produced by Pair #5 and n = 37 overlaps produced by Pair #6.

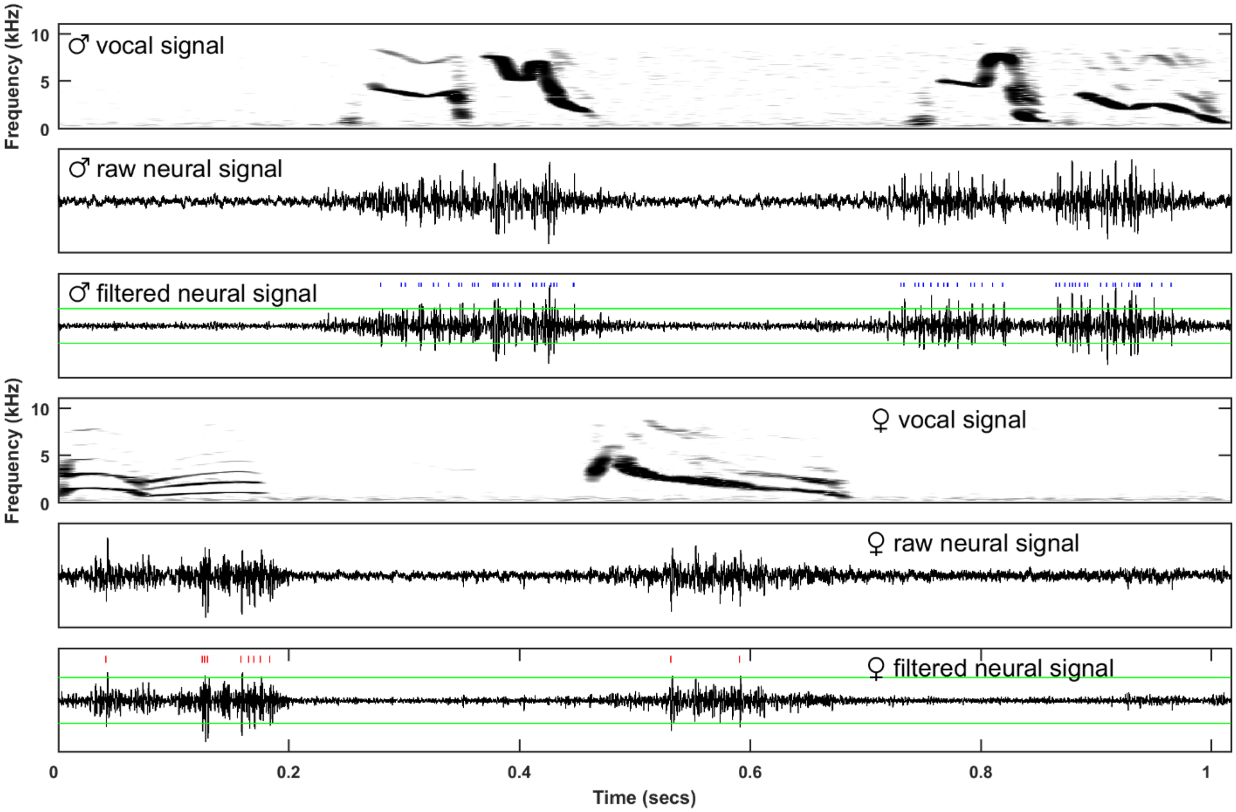


Supplementary Figure 14. Neural oscillation frequencies.

During emission of duet-initiating syllables, oscillation frequencies of male and female neural signals were significantly higher (Mann-Whitney U-test, $p < 0.005$) than during emission of alternating syllables during duetting. Boxplots show the distribution of oscillation frequencies for each bird of three pairs. Please see Supplementary Figure 4 for details on box plot labeling. The number of duet bouts for which the oscillation frequency of neural signals has been determined (see Methods) is indicated above each box.

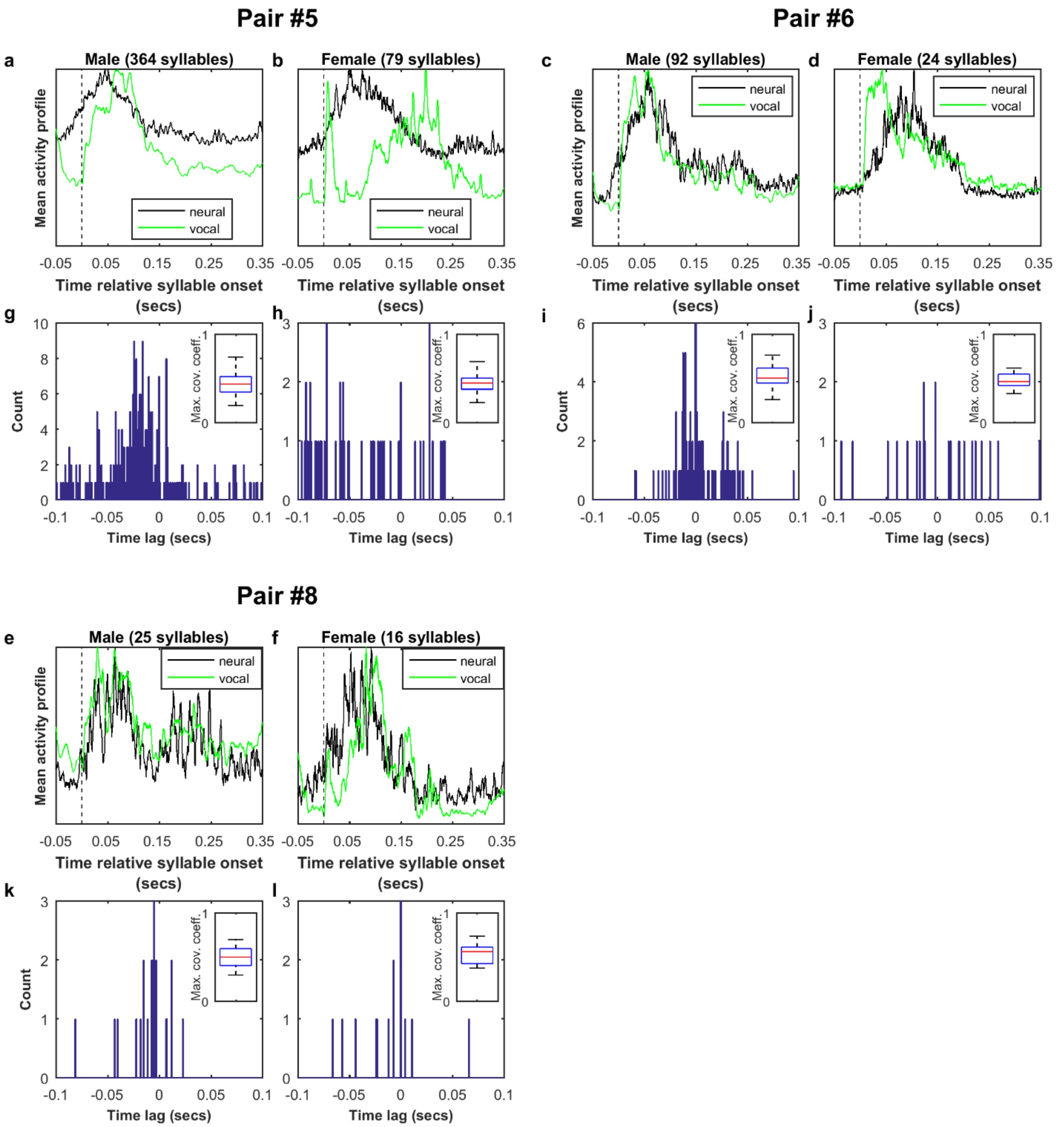


Supplementary Figure 15. Verification of recording sites.
Electrolytic lesions verified the correct positioning of recording electrodes. A Nissl-stained sagittal brain section (see Methods) containing a small lesion in HVC is shown for the male bird of Pair #6.



Supplementary Figure 16. Processing steps of neural data.

Cutout of temporally aligned male and female neural and vocal signals recorded from Pair #5 during duetting. From top to bottom: spectrogram of the male vocal signal, amplitude waveform of the raw male neural signal, amplitude waveform of the filtered male neural signal, spectrogram of the female vocal signal, amplitude waveform of the raw female neural signal and amplitude waveform of the filtered female neural signal. In the panels that show waveforms of filtered neural signals, the green horizontal lines indicate voltage thresholds used for spike discrimination, and the small blue and red vertical dashes mark spike occurrences in the male and female filtered neural signal, respectively.



Supplementary Figure 17. Correlation of activity profiles.

For each bird of each pair, the mean activity profile (normalized to maximum values, **a** - **f**) of filtered neural (black) and vocal (green) signals for the time period between 50 ms before to 350 ms after the onset of all duet syllables that were produced within a 4-hour period of recording. All signals have been rectified and smoothed with a 3-ms sliding window. The dashed vertical lines indicate syllable onsets. It is important to note that the vocal and the neural mean activity profiles were incongruent for each single bird. Histograms in **g** - **l** show distributions of time lags at which the cross-covariance between the neural and vocal signal during each syllable was maximal. Negative lags = neural signal leading. **Insets** show boxplots of maximum cross-covariance coefficients. Coefficients were normalized to the maximum of the auto-covariance function. Maximum coefficients were generally low, which indicates that the covariance between the vocal and the neural activity profiles was weak.

	Pair #5		Pair #6		Pair #8	
	male	**female**	**male**	**female**	**male**	**female**
Mean signal to noise ratio (dB)	5.2	5.1	8.4	6.2	9.6	3.5
Mean spike rate during singing (spikes s^{-1})	69.9	84.1	113.3	43.8	25.0	63.0
Mean spike rate during silence (spikes s^{-1})	19.8	27.6	35.1	15.8	6.5	5.7

Supplementary Table 1. Signal-to-noise ratios and spike rates of neural recordings.
Mean signal-to-noise ratios and spike rates calculated for duet syllables that were produced within one 4-hour period of recording and for the period of silence of equal duration that followed each syllable.

Chapter 2

The multifaceted vocal duets of white-browed sparrow weavers are based on complex duetting rules

Abstract

In some bird species, mated pairs sing duet songs for pair bond maintenance and joint territory defense. Duet partners often follow specific duetting rules, such as answering rules and duet codes, to construct their duet songs. Previous studies mainly concentrated on species that repeat particular phrase pairings during duetting. Our study aims to add to the comparative aspect of duetting research by investigating duet structure in songs of white-browed sparrow weavers Plocepasser mahali, a songbird species that does not repeat the same type of vocalization but switches between different types of vocal elements within one duet song. We used lightweight radio-telemetric microphone transmitters to record the vocal activity in both individuals of duetting pairs simultaneously. This technique enabled us not only to investigate duetting behavior in naturally behaving animals, but also to assign each vocalization to the respective individual while maintaining the precise temporal relationship between the vocalizations of both partners. Our data reveal that although white-browed sparrow weavers share a large part of their sex-specific vocal repertoires with same-sex birds of other pairs, duetting partners adhere to pair-specific answering rules to generate their highly complex duet songs. Moreover, partners can combine answering rules into consistent duet trains. Duet trains generally represent only the core of the duet songs, while at the beginning and the end of duet songs, partners arrange their vocalization types more flexibly. In addition to completely pair-specific duet trains, duet trains of white-browed sparrow weaver pairs can include subsequences that are shared between different pairs. The sharing pattern we find in this species suggests that in combination to pair-specific duetting rules, mated pairs use duetting rules that have been acquired prior to pair formation.

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Contributions:

Alena Lemazina: Data curation (lead); Formal analysis (lead); Investigation (equal); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). Lisa Trost: Conceptualization (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (supporting); Visualization (supporting); Writing – review and editing (equal). Manfred Gahr: Funding acquisition (lead); Resources (lead); Supervision (supporting); Writing – original draft (supporting). Susanne Hoffmann: Conceptualization (lead); Formal analysis (supporting); Investigation (equal); Supervision (lead); Visualization (supporting); Writing – original draft (supporting); Writing – review and editing (lead).

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Article

The multifaceted vocal duets of white-browed sparrow weavers are based on complex duetting rules

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In some bird species, mated pairs sing duet songs for pair bond maintenance and joint territory defense. Duet partners often follow specific duetting rules, such as answering rules and duet codes, to construct their duet songs. Previous studies mainly concentrated on species that repeat particular phrase pairings during duetting. Our study aims to add to the comparative aspect of duetting research by investigating duet structure in songs of white-browed sparrow weavers *Plocepasser mahali*, a songbird species that does not repeat the same type of vocalization but switches between different types of vocal elements within one duet song. We used lightweight radio-telemetric microphone transmitters to record the vocal activity in both individuals of duetting pairs simultaneously. This technique enabled us not only to investigate duetting behavior in naturally behaving animals, but also to assign each vocalization to the respective individual while maintaining the precise temporal relationship between the vocalizations of both partners. Our data reveal that although white-browed sparrow weavers share a large part of their sex-specific vocal repertoires with same-sex birds of other pairs, duetting partners adhere to pair-specific answering rules to generate their highly complex duet songs. Moreover, partners can combine answering rules into consistent duet trains. Duet trains generally represent only the core of the duet songs, while at the beginning and the end of duet songs, partners arrange their vocalization types more flexibly. In addition to completely pair-specific duet trains, duet trains of white-browed sparrow weaver pairs can include subsequences that are shared between different pairs. The sharing pattern we find in this species suggests that in combination to pair-specific duetting rules, mated pairs use duetting rules that have been acquired prior to pair formation.

Keywords: answering rule, birdsong, cooperative behavior, song syntax, vocal communication

Introduction

In some bird species, partners of a mated pair produce duet songs. Vocal duets are suggested to serve certain behavioral functions, such as pair bond maintenance or joint territory defense (reviewed by Hall 2009). Duet songs are defined as a pair-level

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singing performance, in which both partners sing alternating or overlapping song elements. The simplest form of duetting is a temporal overlap of song bouts produced by both partners without further fine scale coordination. Antiphonal duets, where the partners precisely coordinate their vocal outputs to avoid overlap of song bout elements, are considered a more complex form of duetting (Hall 2009). In addition to temporal complexity, several duetting species use sex-specific vocal repertoires to construct duet songs (Voigt et al. 2006, Hall 2009, Logue and Krupp 2016). Birds that possess both, antiphonal duets and sex-specific vocal repertoires, are considered to produce the most complex duets (Logue and Krupp 2016).

In some duetting species, the partners arrange vocalizations in nonrandom associations to form a duet code (Logue 2006). Duet codes consist of a set of answering rules, and each answering rule represents the link between a certain type of vocalization produced by one bird and a certain type of vocalization produced by the partner as an answer to the first bird's vocalization (Logue and Krupp 2016). For example, if the male syllable M is always followed by the female syllable F, the link between M and F is called an answering rule. A stable sequence of at least two syllable transitions that adhere to answering rules (e.g. M1–F1–M2) represents a duet train as defined by Brown and Lemon (1979).

Pair-specific duet codes are common in duetting birds. However, one individual can change its duet code several times during a lifetime, due to natural learning processes or re-mating (Rivera-Cáceres and Templeton 2019). Duet codes can, however, not only be pair-specific but can also be shared between different pairs of one species. One potential reason for high levels of repertoire and duet code sharing is the limited dispersal of offspring from the parental territory. Furthermore, duet code sharing could occur as a result of sexual selection, to mediate the intrasexual communication in both sexes (Hall 2009, Rivera-Cáceres and Templeton 2019, Quirós-Guerrero et al. 2020a).

Most duetting bird species use only one or two answering rules per duet bout and duet songs are constructed out of several repetitions of this answering rule (e.g. M1–F1–M1–F1, Hall 2009). Additional level of complexity is added to duet songs when partners switch answering rules within the duet (Hall 2009). Answering rule switching can occur in different ways: first, in the so-called repeat mode, where individuals repeat the same phrase type multiple times throughout the duet song before switching to a different one (e.g. M1–F1–M1–F1–M2–F2–M2–F2, Hall 2009). This type of singing is widespread among duetting birds and has been found in black-bellied wrens *Pheugopedius atrogulari* (Logue 2006), plain wrens *Thryothorus modestus zeledon* (Mann et al. 2003), rufous-and-white wrens *Thryophilus rufalbus* (Mennill and Vehrencamp 2005), eastern whipbirds *Psophodes olivaceu* (Rogers 2005) and plain-tailed wrens *Thryothorus euophry* (Mann et al. 2005). Second, in some species, duet songs show a high syllable and transition versatility, with birds switching answering rules multiple times throughout the duet (e.g. M1–F1–M2–F2–M3–F3, Hall 2009). This type of singing is less common and was only described for Australian magpies *Gymnorhina tibice* (Brown and Farabaugh 1991), white-crested laughing thrushes *Garrulax leucolophu* (Vencl and Soucek 1976) and African drongos *Dicrurus adsimili* (von Helversen and Wickler 1971). Sometimes, switching between different types of vocalizations does not affect the partner's response. For example, in antiphonal duets of yellow-naped amazons *Amazona ochrocephala*, the female responses are not associated with the male switching to a different call type (Wright and Dahlin 2007, Hall 2009). However, our interest lies in species, in which the switching of answering rules mid-duet by one bird results in a switch of answering rules in the partner. So far, it is largely unknown how the complexity of the duet coincides with the degree of mid-duet answering rule switching (Logue and Krupp 2016). To broaden the knowledge of duet song structure and duet song sharing in bird species with large syllable repertoires that frequently switch answering rules within their duet songs, we investigated duetting behavior in South African white-browed sparrow weavers.

The white-browed sparrow weaver is known as a duetting songbird species with a very large vocal repertoire, consisting of up to 60 different types of vocal syllables (Voigt et al. 2006). For comparison, the male and female vocal repertoires of plain wrens consist of only 35–45 and 25 phrase types (equivalent to syllable types in *P. mahali*), respectively (Mann et al. 2003), and male and female happy wrens *Pheugopedius felix* have a repertoire size of about 40 different phrase types (Templeton et al. 2013). Although the general song pattern and the syllable repertoire size of *P. mahali* is already described (Voigt et al. 2006), it is still unknown if this species adheres to answering rules. Due to the repeated usage of certain syllable type combinations, Voigt et al. (2006) considered the syllable order in *P. mahali* duet songs as nonrandom. Understanding the rules behind *P. mahali* duetting will broaden the scope of duetting research and could provide the framework for studying the syntactic aspects of duetting behavior.

Plocepasser mahali is a common inhabitant of eastern and southern Africa. Pairs of *P. mahali* sing antiphonal duets by precisely alternating their vocalizations. Typically, either the male or the female begins a duet with a single buzz call or with 2–9 introductory syllables. When the partner joins the duet, both birds sing duet syllables in alternation. Male duet syllables have a two-element doublet structure with a short gap between elements, and females produce whistle-like duet syllables (Hoffmann et al. 2019). A duet bout can be terminated by either partner (Hoffmann et al. 2019). Although coordination properties of duet songs in *P. mahali* indicate that partners simultaneously use mutual cues, the possibility that partners perform their duet contributions independently, still exists (Elie et al. 2019). It is further possible that only one sex follows a duet code and the other sex contributes syllable types randomly. It was shown previously that in *P. mahali* duets mainly the males determine the duet rhythm (Hoffmann et al. 2019). A duet code could hence exist in males, but pair-specific answering rules could be lacking.

Evidence of answering rules and duet code existence in this species would provide additional support for the idea of sensory-based tuning of duetting performances (Fortune et al. 2011, Hoffmann et al. 2019).

In our study, we recorded duet songs produced by six wild *P. mahali* pairs in South Africa. Our results show that parts of the vocal repertoire are shared between different pairs of *P. mahali*. The sharing levels differ between partners: females share a significantly higher proportion of syllable types than males. Our study further reveals the existence of answering rules in *P. mahali*, adhered to by both sexes. Even though answering rules can be shared between pairs of different colonies, pair-specificity in ordering of syllable types within the most commonly used duet trains exists. Most importantly, we found that a single answering rule was never used twice in one *P. mahali* duet song, but answering rules switched constantly, which contrasts the song structure in this species with the song structure of most other duetting bird species studied so far.

Methods

Animals and ethics approval

We studied a population of wild *Plocepasser mahali* near the village Black Rock, Northern Cape, South Africa (27°7′S, 22°50′E) during February/March 2016, November/December 2017 and January/February 2019. All birds of investigated colonies were color-banded for individual recognition. In one colony, we studied the duetting behavior of two pairs. After we had removed the male bird of Pair 2, we monitored the colony daily until a male from another colony entered and formed a new pair (Pair 3) with a different female of the original colony.

Vocal recordings

To monitor individual vocal activity in free-ranging *P. mahali*, we equipped both birds of wild pairs with on-board radio-telemetric transmitters developed at the Max Planck Inst. for Ornithology in Seewiesen, Germany (Gill et al. 2016). The birds were captured shortly after dusk (20:00–22:00 h) inside their roosting nests and the sex was determined by bill color (Earle 1983). The lightweight vocal transmitter (0.6 g), which included a miniaturized microphone (FG23329, Knowles Electronics, USA), was covered by a thin silicon casing and fixed on the back of the bird with cotton-covered rubber band straps around both femurs and the abdomen. All birds were released to their home territory at dusk in the following morning. The transmission range of the transmitter averaged 50 m and the battery life 15 days. Carrying these microphone transmitters has only weak and short-term suppressive effects on the vocal and movement activity of songbirds (Gill et al. 2016). For signal detection, a crossed Yagi antenna (Winkler Antennenbau, Germany) was placed below the nesting tree in the center of the colony's territory. An antenna amplifier (TVS 14-00 axing, Goobay, Germany) increased the antenna signal by 18 dB. The signal was split (BE 2-01 premium-line, Switzerland) and fed into up to eight communication receivers (AOR 8600, AOR Ltd., Japan), which were modified to handle 12 kHz audio bandwidth. The analog signals were digitized by an eight-channel audio A/D converter (M-Track Eight, M-Audio, USA; sampling rate: 22 050 Hz) that was connected to a laptop computer. All digitized signals were recorded in parallel as continuous sound files with a duration of four hours using multichannel software (16-bit, 22 050 Hz; ASIO, Germany). The recording setup was placed in the trunk of a car located at a distance of ~30 m from the antenna. For each pair of birds, vocal recordings were continuously conducted over several consecutive days.

Analysis of songs

To investigate duetting rules in *P. mahali*, we prepared sound files for a sequence analysis by mixing synchronous four-hour recordings from both partners of a pair into a single render using the software Audacity (ver. 2.3.2, Audacity Team, <www.audacityteam.org>). The mixed files were uploaded to a free available web-based software (KOE, ver. 5.4.1, <https://koe.io.ac.nz>, Fukazawa et al. 2020) and manually segmented into separate duet bouts.

Based on Voigt et al. (2006), a duet bout was defined as a sequence of introductory syllables followed by male and female duet syllables. Each duet bout was surrounded by at least 1.5 s of silence and duet bouts temporally overlapped in the sound files of the male and female bird of a pair. Subsequently, duet bouts were manually segmented into syllables. For male duet syllables, the onset of the first doublet element and the offset of the second doublet element served as duet syllable onset and offset, respectively.

We used an interactive ordination plot created by KOE as a basis for further manual audiovisual syllable classification. KOE automatically extracted various temporal and spectral syllable features, and used them to calculate an ordination between different syllables based on t-distributed stochastic neighbor embedding analysis (Fukuzawa et al. 2020). We audio-visually inspected resulting syllable clusters to classify them into syllable types.

Syllable types were included in the further analysis only if they occurred in the individual repertoire more than once. For sequence analysis, KOE discovered commonly-occurring transitions between syllables in a database using the cSPADE (constrained Sequential Pattern Discovery using Equivalence classes) algorithm (Zaki 2001). The credibility of sequence rules is asserted via the following factors: Support (proportion of songs in the database that contain a certain sequence at least once), Confidence (strength of association; the proportion of songs containing the syllable A that also contain the sequence A⇒B), and Lift (measure of the strength of the association relative to chance; ratio of the proportion of songs in which the transition A⇒B occurred versus the proportion of songs expected to contain the transition A⇒B by chance).

To determine answering rules in *P. mahali* duet songs, we filtered the database of syllable transitions provided by KOE for sequences with a chain length of two, which represented a single transition between a male and a female syllable. We included only transition types with a Lift value above two (probability of transition occurrence is two times higher than expected by chance) in the analysis of answering rules, even when a particular transition type occurred only once in the data set. We additionally validated all these transition types by using a Pearson's χ^2 test (Mann et al. 2003, Mennill and Vehrencamp 2005; R studio ver. 3) and counted only transition types with a p-value below 0.05 as adhering to a valid answering rule.

To determine duet trains in *P. mahali* duet songs, we filtered the database provided by KOE for sequences with a chain length of at least three syllables and a Support value larger than 0.06. At a Support value > 0.06, the song syntax of a pair was transforming from an entangled network to distinct directional sequences. According to Brown and Lemon (1979), a duet train is defined as a type of utterance, consisting of male and female components. A duet train in our study is a stable sequence of at least three syllables, which is produced by applying at least two consecutive answering rules (e.g. M1–F1–M2). Please note that a duet train was generated by lexicographically aligning syllables or syllable sequences that were found very frequently in different renditions of songs, and that therefore only the shortest duet trains could be observed as a whole in single duet bouts.

Repertoire sharing of male and female syllable types and answering rules was analyzed using the song sharing index $S = 2Ns/(2Ns + a + b)$, in which Ns is the number of syllable types that are shared between two individuals, and a and b are the numbers of unshared syllable types for each individual (McGregor and Krebs 1982). The index can assume values between zero and one, with zero indicating the lowest sharing level.

To analyze the sharing pattern of duet trains, we first determined if subsequences of a duet train were shared between different pairs. We used the R package qualV (Jachner et al. 2007) to find the longest common subsequence (LCS) for each combination of duet trains. The LCS had to include at least three consecutive syllables alternating between partners. We calculated the Levenshtein distance (LD, Levenshtein 1966) for all LCSs with the R package stringr (Wickham 2010). The LD was shown to be suitable to quantify the difference between animal acoustic sequences (Kershenbaum and Garland 2015) by comparing the position of elements between two strings and calculating the number of changes necessary to transform one string into the other. An LD of zero means that sequences are identical, and the larger the LD the larger the difference between sequences. In our analysis, LD values ranged between zero and two (median: one), but only subsequences of duet trains with LD values below or equal to one were considered to be shared. Consequently, subsequences that consisted of at least three consecutive syllables and showed an LD value larger than one were counted as unshared subsequences.

Results

We investigated the duetting behavior of six pairs in five *Plocepasser mahali* colonies. In one pair (Pair 4), the duetting behavior was studied in two consecutive observation periods (2016 and 2017). This pair can therefore be considered as a well-established pair. In another colony, we removed a male (Pair 2) at the end of the recordings. One week after removal of this male, a new pair (Pair 3) consisting of a foreign male and a different female of this colony was formed. In total, we analyzed 471 *P. mahali* duet songs. Voigt et al. (2006) demonstrated that a minimum of 30 duet bouts is sufficient to reveal the total syllable repertoire of a *P. mahali* pair. Therefore, the number of songs collected per pair in the present study (Table 1) can be considered adequate to categorize syllable types and describe individual syllable repertoires.

Syllable repertoire

In each pair, duet syllable repertoires were found to be sex-specific (Table 1), and only introductory syllable types were shared between partners. In total, we found 144 different male syllable types and 54 different female syllable types, with males having a significantly (Kruskal–Wallis test, p = 0.008) larger syllable repertoire (median number of syllable types: 62) than females (median number of syllable types: 38). While the female syllable repertoire only consisted of three groups of syllable types (buzz calls, introductory syllables and duet syllables), the male syllable repertoire consisted of four groups of syllable types (buzz calls, introductory syllables, doublet and triplet duet syllables). Both sexes' syllable repertoires included between two and seven introductory syllable types (median: five). Moreover, a male's syllable repertoire included between six and nine triplet syllable types (median: six). The previously undescribed triplet duet syllables are a sequence of three elements sung either at the beginning or at the end of the antiphonal part of the duet bout.

We found a high percentage of syllable type sharing between same-sex individuals of the six *P. mahali* pairs (Fig. 1). The sharing index for male syllable types (median: 0.54; interquartile range: 0.42–0.65; Table 2) was significantly smaller (Kruskal–Wallis, p < 0.001) than the sharing index for female syllable types (median: 0.81; interquartile range: 0.69–0.88; Table 2). This indicates that females shared a larger part of their syllable repertoire than males.

Table 1. Overview of sample sizes.

	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6
Songs	123	110	66	42	75	55
Male syllable types	66	60	64	45	68	46
Female syllable types	46	40	38	29	38	38
Answering rules	89	86	43	30	69	49
Duet trains	9	8	3	4	9	7
Shared subsequences	4	5	1	3	2	4
Unshared subsequences	3	3	2	2	6	3

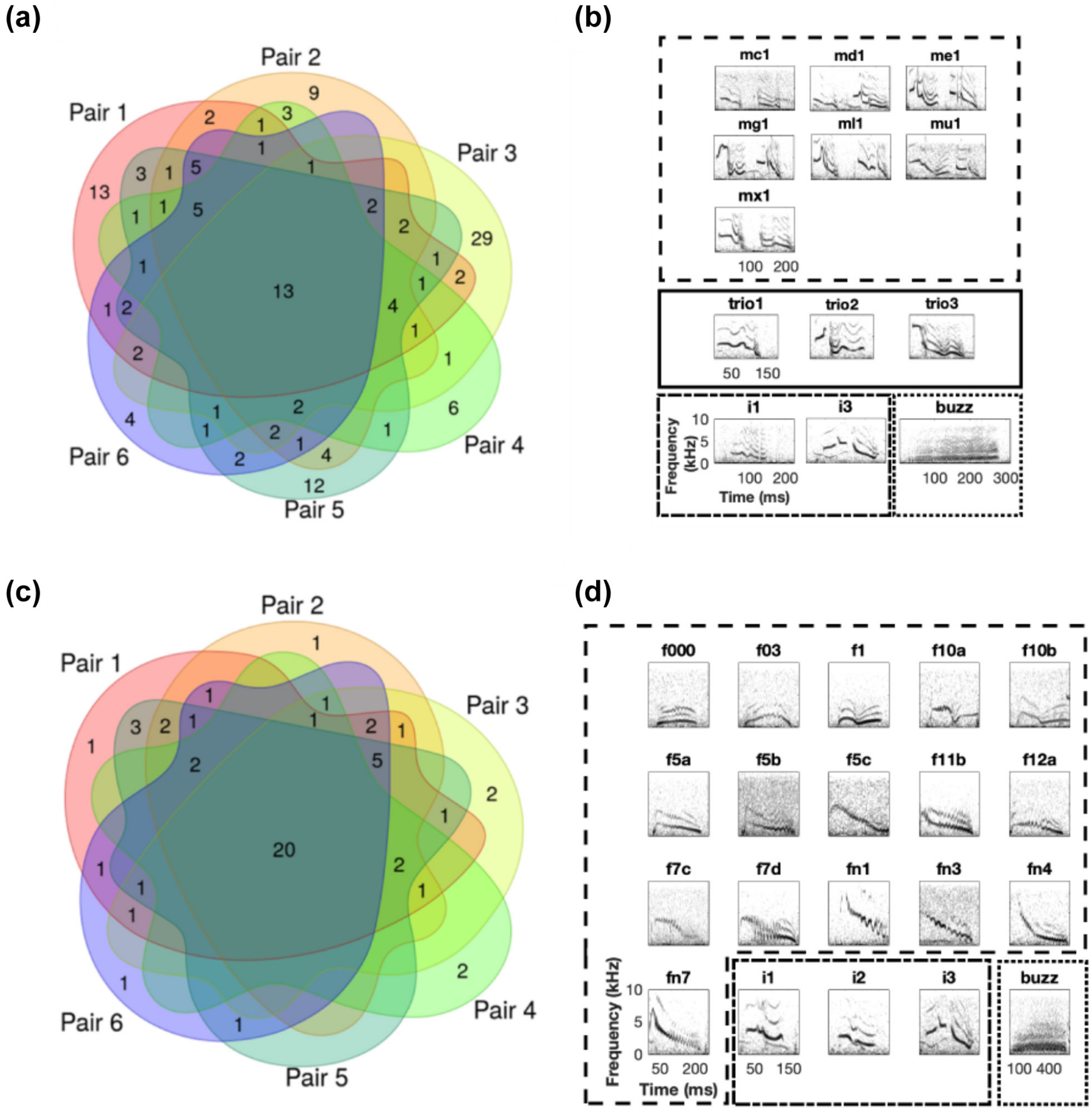

Figure 1. Repertoire sharing among males and females in *P. mahali*. (a) Venn diagram of shared syllable types in males. A core of 13 syllable types is shared between males of all pairs. (b) Spectrograms of examples of syllable types shared between all males. The shared male syllable repertoire consists of four syllable type groups: doublet duet syllables, trio duet syllables, introductory syllables and buzz calls. (c) Venn diagram of shared syllable types in females. A core of 20 syllable types is shared between females of all pairs. (d) Spectrograms of examples of syllable types shared between all females. The shared female syllable repertoire consists of three syllable type groups: duet syllables, introductory syllables and buzz calls.

Table 2. Sharing of syllable types between *P. mahali* pairs. Sharing indices for male and female syllable types between different pairs.

	Male					Female				
	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6
Pair 1	0.62	0.44	0.54	0.63	0.59	0.88	0.83	0.69	0.88	0.83
Pair 2		0.44	0.61	0.66	0.60		0.85	0.78	0.82	0.85
Pair 3			0.44	0.42	0.42			0.75	0.76	0.82
Pair 4				0.55	0.55				0.72	0.72
Pair 5					0.65					0.79

Answering rules and duet trains

In general, we observed a very high variability in syllable ordering in the duet songs of each investigated pair (Supporting information for examples of duet songs of Pair 4). Nevertheless, our analysis revealed the existence of answering rules and duet trains in duet songs of *P. mahali*. Both partners in all *P. mahali* pairs showed high syllable and transition versatility: neither syllables of one syllable type nor syllable transitions that adhered to a particular answering rule were repeated in a duet song.

Out of 424 observed transition types between male and female syllables, we could verify 254 significant answering rules in six pairs of *P. mahali*. Except for Pair 4, which showed only 30 different answering rules (Supporting information), the repertoire of answering rules was generally large in *P. mahali* pairs (median: 59, range: 30–89, Table 1). Answering rules were established by the bird that produced the second syllable in a sequence of two. Fifty-three percent of answering rules were established by females and 46% were established by males. The median confidence factor for all answering rules was 0.5 (range: 0.33–1.0). This indicates that the average probability that a two-syllable sequence adhered to a specific answering rule was 50%. Given the large syllable repertoire of *P. mahali* and, consequently, the high number of transition possibilities, an adherence probability of 50% is quite high. The strength of answering rule adherence was similar in both sexes in *P. mahali* (Mann–Whitney U-test, $p = 0.9$). While the median confidence factor for all answering rules established by female birds was 0.58, the median confidence factor for all answering rules established by male birds was 0.5.

In general, 11% of all syllables in a pair's repertoire are part of transitions that adhered to more than one answering rule (range: 0% in Pair 4–25% in Pair 5). In answering rules established by males, this ambiguity was less prevalent (one percent of answering rules) than in answering rules established by females (21% of answering rules). Due to the fact that the female syllable repertoire is much smaller than the male syllable repertoire, a female is required to use one specific syllable type to answer more than one male syllable type.

On average, a *P. mahali* pair shared 18.75 ± 6.63 answering rules with another pair. The sharing index (median: 0.20, range: 0.082–0.27) was lowest for the sharing of answering rules between Pair 3 and Pair 4, and highest for the sharing of answering rules between Pair 2 and Pair 4 (Table 3). Only one answering rule was shared between all six pairs (Fig. 2).

In total, we found 39 different duet train types in our dataset of *P. mahali* duet songs (Supporting information). On average, a *P. mahali* pair sang duet songs with 6.5 different duet train types. Pairs 1 and 5 showed the highest number (nine) of duet train types, while the newly formed Pair 3 had the lowest number (three) of duet train types. The median value of support for two-syllable sequences constructing the duet trains was 0.08 (range: 0.06–0.20). This indicates that all two-syllable sequences that adhered to answering rules in duet trains were on average observed in 8% of songs in our database.

Table 3. Indices for answering rule sharing between different pairs of *P. mahali*.

	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6
Pair 1	0.26	0.12	0.20	0.27	0.25
Pair 2		0.16	0.28	0.25	0.21
Pair 3			0.08	0.1	0.09
Pair 4				0.18	0.18
Pair 5					0.20

Pairs of *P. mahali* did not use all two-syllable sequences that adhered to answering rules to construct the duet trains. On average, a pair used 46% of its two-syllable sequences (range: 20% in Pair 3–67% in Pair 4) to form duet trains. Generally, a duet train is a composite of at least two syllable transitions that adhere to answering rules. The longest duet train (s21 in Pair 4, Supporting information) included 12 transitions, while the shortest duet trains (s5 in Pair 1, s13 in Pair 2, s20 in Pair 3, s23–s24 in Pair 4, s25 and s30–s33 in Pair 5 and s38–s39 in Pair 6, Supporting information) consisted of only two transitions. Across pairs, on average 4.23 transitions adhering to answering rules formed a duet train.

Duet trains consisted of shared and unshared subsequences. In total, we found five shared and 21 unshared subsequences in our set of duet trains (Supporting information, Fig. 3). All pairs had shared and unshared subsequences in their duet trains (Table 1). In general, pairs had more shared than unshared subsequences (mean ratio: 3.5:3.0), and none of the subsequences was shared between all pairs.

Discussion

Duetting rules in *Plocepasser mahali*

Our study of white-browed sparrow weaver duets provides a detailed description of this species' duetting behavior and the underlying duetting rules. We show that both male and female *P. mahali* have distinct duet syllable repertoires, and partners share only introductory syllable types with each other. The number of syllable types found in our study confirms the previous finding that both male and female *P. mahali* have a highly diverse syllable repertoire (Voigt et al. 2006). Here, we additionally found syllable repertoires in this species to be sex-specific, with females having the smaller repertoire.

The structure of *P. mahali* duet songs is characterized by several unique traits: First, both sexes of *P. mahali* show a high syllable and transition versatility in their duet songs. They do not repeat the same syllable type within duet song bouts and consecutive male–female syllable combinations consist of different syllable types. Second, *P. mahali* duet songs are highly variable. Besides the general pattern that male and female syllables are produced in alternation, it is challenging to find consistent patterns in the duet structure. A significant variability in syllable ordering between duet songs exists, even if songs are composed of the same syllable types.

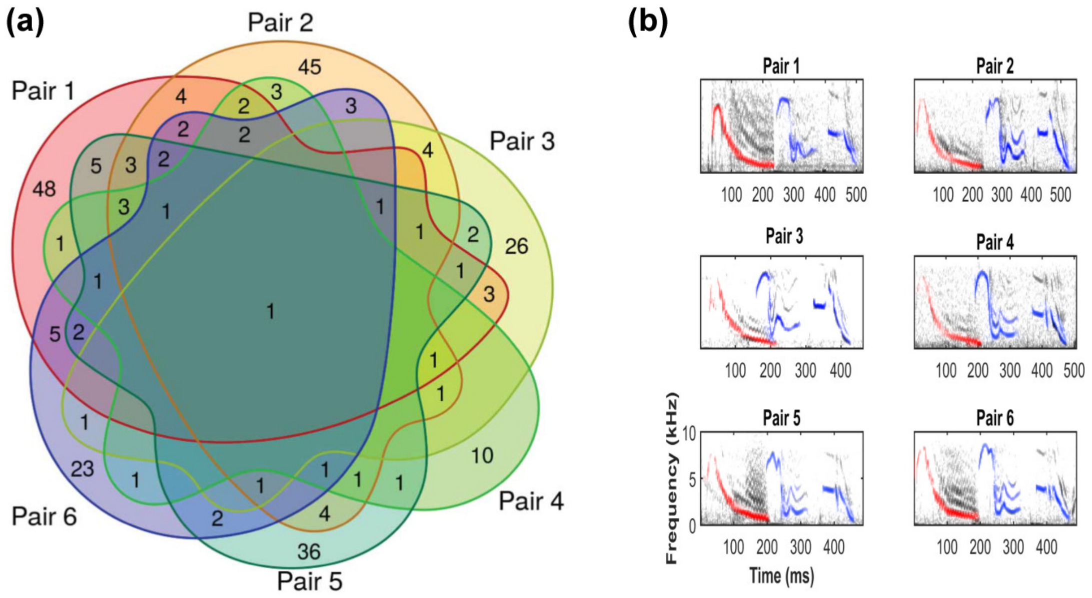

Figure 2. Answering rule sharing in *P. mahali*. (a) Venn diagram of answering rules shared between pairs. Only one answering rule is shared between all six *P. mahali* pairs. (b) Spectrograms of examples of the answering rule fn4–mg1 for all six pairs. Male and female syllables are highlighted in blue and red, respectively.

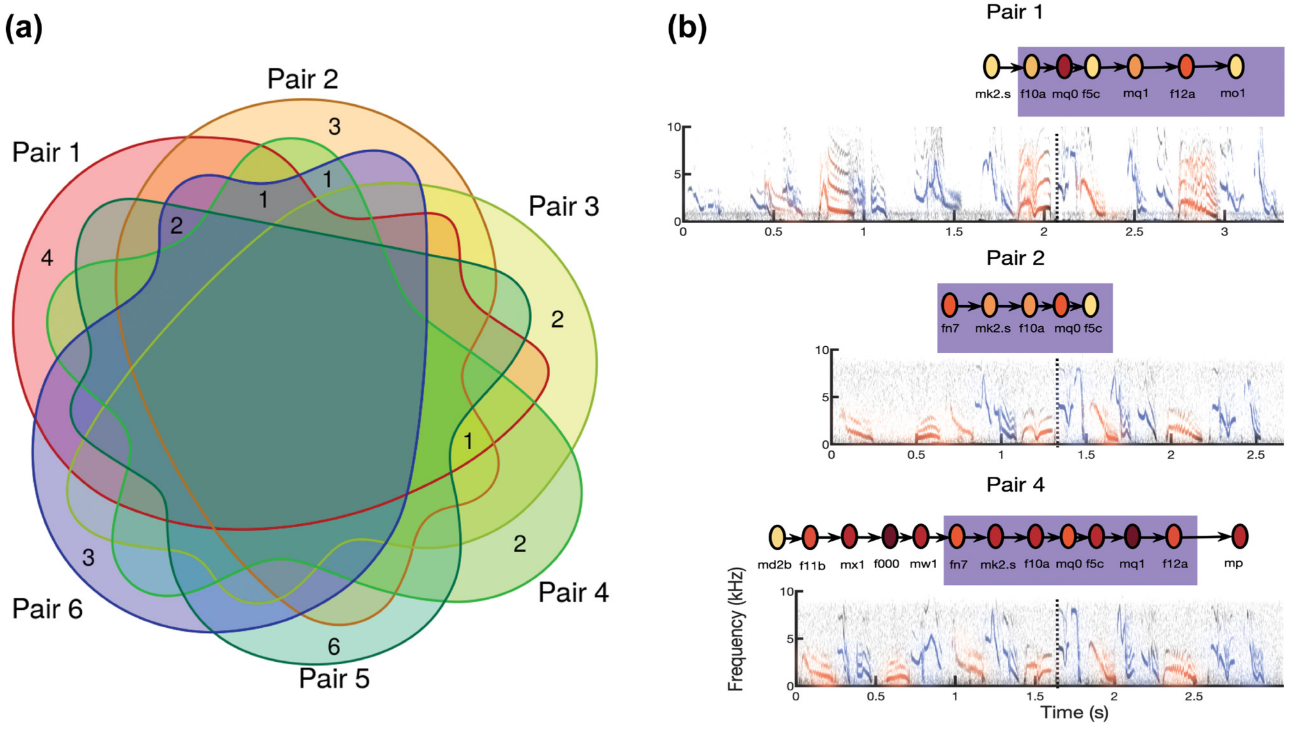

Figure 3. Duet train types contain shared subsequences in *P. mahali* pairs. (a) Venn diagram of shared subsequences. (b) Spectrograms of examples of duet trains with shared subsequences temporarily aligned by the time of occurrence of male syllable mq0. Male and female syllables are highlighted in blue and red, respectively. Syllables are represented by colored circles above each spectrogram. The circle's color indicates the syllable's commonness within the dataset with darker colors indicating higher commonness (equivalent to the value of support). The order of syllables is represented by the direction of arrows between the colored circles. The strength of the transition between two syllables (equivalent to the value of confidence) is indicated by the arrow's thickness. Violet highlights indicate shared subsequences.

For example, duet songs can start with or without introductory syllables, new syllable types can be incorporated and regularly used ones can be omitted. Such a high degree of variability in syllable composition and duet structure has not been described for duetting bird species so far. Comparable to the syllable type switching in *P. mahali*, Australian magpies switch between multiple phrase types within their duet songs (Brown and Farabaugh 1991), and white-crested laughing thrushes demonstrate variability by being able to switch to a random phrase type at the end of each answering rule cycle (Vencl and Soucek 1976), but still these species repeat phrase types in their songs. Among repeat mode singers, the species with a duet song structure most similar to the one in *P. mahali* is the plain-tailed wren. This species sings four-part duet songs, in which the two sex-specific parts that are produced in alternation are repeated throughout the duet song (M1–F1–M2–F2–M1–F1–M2–F2, Mann et al. 2005).

It is so far not known how species, which sing variable duets using multiple answering rules, exercise control over the fine-scale alignment of song elements in their duets. Repeat mode singing is suggested to facilitate adherence to answering rules (Brown and Lemon 1979, Hall 2009). The generation of song sequences with a variable structure is thought to be driven by adherence to individual answering rules, rather than adherence to the overall sequence structure (Logue and Krupp 2016). An alternative explanation would be that the phrase order in the sequence itself provides a cue for the duet coordination. For example, white-crested laughing thrushes sing rare phrase types at the end of duet songs, which possibly indicates the termination of the duet song (Vencl and Soucek 1976). However, more studies on species with variable duet song structure are necessary to fully understand how the syntax of duets in these species is controlled.

Distinguishing discrete duet types in duetting species, in which duets consist of variable sequences of phrase types, is usually not possible (Vencl and Soucek 1976, Brown and Farabaugh 1991, Dahlin and Benedict 2014, Logue and Krupp 2016). Therefore, we decided to describe the duet songs of *P. mahali* by determining the most frequently occurring syllable sequences (i.e. duet trains) for each pair of birds. This finally allowed us to access individual differences within the duet structure of studied pairs. Despite the high variability in syllable composition, we were able to find stable answering rules and pair-specific duet codes adhered to by both sexes of *P. mahali* during duetting. On average, a *P. mahali* pair had 59 different answering rules, an answering rule repertoire, which is large in comparison to other duetting bird species (Mann et al. 2003, 2005, Mennill and Vehrencamp 2005, Logue et al. 2007, Quirós-Guerrero et al. 2020a).

In all investigated *P. mahali* pairs, only a subset of the two-syllable sequences that adhered to answering rules was used to form duet trains. We suggest that the inclusion of only a small subset of the two-syllable sequences in the most frequent duet trains could be explained by repetitive answering rule learning. It has been shown that duetting bird species can learn multiple sets of answering rules throughout their life. Juveniles first learn duetting rules from their parents before they disperse, and then use the previously learned fixed syllable repertoire to create new answering rules upon pair formation in adulthood (Rivera-Cáceres et al. 2016, 2018, Rivera-Cáceres and Templeton 2019).

Both female and male subordinates in *P. mahali* colonies disperse very late from their natal groups (Harrison et al. 2013). It is, however, unclear whether answering rules in *P. mahali* are relearned after dispersal or after a change of partner. For *P. mahali*, it was shown that the syllable repertoire size gradually declines from the most inexperienced to the most experienced pair (Voigt et al. 2006). This suggests that during pair formation, both members build up a pair-specific answering rule repertoire through learning with the partner, and syllables that are not used frequently may be discarded after the learning period (Voigt et al. 2006). Our observations in the well-established Pair 4 support this hypothesis. On average, *P. mahali* pairs used 46% of their two-syllable sequences that adhered to answering rules in their duet trains. Compared to other pairs, Pair 4 had the lowest number of answering rules, but used the largest proportion of two-syllable sequences that adhered to answering rules (67%) to construct its duet trains. The frequent usage of only a few answering rules results in a high strength of transition between syllable types in the duet bouts of Pair 4 and consequently in a long stable duet train. The newly formed Pair 3 represents the other extreme of the continuum. Pair 3 used only 20% of the two-syllable sequences adhering to answering rules in their duet trains. We suggest that the low probability of incorporating two-syllable sequences adhering to these answering rules into duet trains in duets of Pair 3 was a result of the still ongoing process of establishing pair-specific answering rules. A similar behavior was found in newly formed pairs of canebrake wrens, where partners flexibly chose the reply to an unfamiliar syllable, and adhered less consistently to the duet code than established pairs (Marshall-Ball et al. 2006, Rivera-Cáceres et al. 2016).

Our results demonstrate that *P. mahali* pairs sing duet songs with at least three (range: three to nine) different duet trains. Many duetting species possess a repertoire of different duet types (Dahlin and Benedict 2014), and duets are known to be multifunctional, addressing simultaneously individuals within and between pairs (Hall 2009, Dahlin and Benedict 2014). It has been suggested that flexible duets are needed for signaling to multiple receivers in multiple contexts (Dahlin and Benedict 2014). Even though in *P. mahali* some evidence for a function of duet songs in both territory defense (Wingfield and Lewis 1993) and pair bond maintenance (Voigt et al. 2006) has been presented, further research is needed to describe the contexts under which different duet trains are produced, and consequently to issue a clear statement about the function of duetting in this species.

Song sharing in *Plocepasser mahali*

Syllable types, answering rules and subsequences of duet trains were shared between different *P. mahali* pairs. As in other duetting bird species (Vencl and Soucek 1976, Mann et al. 2005, Rivera-Cáceres et al. 2016, Quirós-Guerrero et al.

2020a), females of *P. mahali* shared a significantly higher number of syllable types with same-sex individuals of other pairs than males. The level of answering rule sharing can be positively correlated with the geographical distance between territories of conspecifics sharing answering rules (Quirós-Guerrero et al. 2020a), or with the dispersal pattern of the different sexes (Graham et al. 2017). As territories of all *P. mahali* pairs in the present study were located in close vicinity to each other, additional vocal recordings in *P. mahali* pairs with further distanced territories are necessary to test if geographical distance or dispersal patterns affect *P. mahali* repertoire sharing.

Although syllable type sharing was rather common between investigated *P. mahali* pairs, our results demonstrate that a pair of *P. mahali*, generally shares only a small amount of answering rules with other pairs. This indicates that different pairs use the same syllable types to generate two-syllable sequences that adhere to different answering rules. This sharing pattern appears to be common among duetting species (Rivera-Cáceres and Templeton 2019), and may facilitate learning of a pair-specific duet code after mating. Pair-specific answering rules, encoding a pair's identity, were found in several duetting species (Wiley and Wiley 1977, Rivera-Cáceres et al. 2016, Quirós-Guerrero et al. 2020a). Adherence to pair-specific answering rules could communicate reliably the location and identity of each bird to its mate, thus avoiding the possibility of misdirected aggression, and allowing the pair to coordinate and focus their efforts on intruding rivals (Farabaugh 1983, Logue 2006, 2007).

In addition to partner recognition, pair-specific answering rules could also be used in intra-pair recognition. Although in some duetting wrens duet codes did not facilitate neighbor recognition (Quirós-Guerrero et al. 2020b), *P. mahali*, at least to some degree, seems to be able to distinguish birds based on vocalizations. In experiments with simulated intrusions, birds reacted more aggressively to familiar neighbors than to unfamiliar conspecifics (York et al. 2019). In addition, our finding that there are no subsequences in *P. mahali* duet trains, which are shared between all pairs, indicates that there is no species-typical subsequence in this bird. These facts, together with the existence of pair-specific subsequences, may support the hypothesis that a pair's duet trains could encode pair identity.

Taken together, the overall variability in syllable order of duet songs, the large number of answering rules and the variety of duet trains, indicates that white-browed sparrow weavers possess one of the most complex duet songs known to date. Their intriguing system of aligning syllable transitions that adhere to answering rules to generate long sequences without repeating syllable transitions but constantly switching to syllable transitions adhering to different answering rules, distinguishes *P. mahali* from other duetting bird species studied so far, and makes them an interesting and promising additional model to study avian duetting behavior.

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Author contributions

Alena Lemazina: Data curation (lead); Formal analysis (lead); Investigation (equal); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Lisa Trost**: Conceptualization (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (supporting); Visualization (supporting); Writing – review and editing (equal). **Manfred Gahr**: Funding acquisition (lead); Resources (lead); Supervision (supporting); Writing – original draft (supporting). **Susanne Hoffmann**: Conceptualization (lead); Formal analysis (supporting); Investigation (equal); Supervision (lead); Visualization (supporting); Writing – original draft (supporting); Writing – review and editing (lead).

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Data availability statement

Data are available from the Dryad Digital Repository: <https://datadryad.org/stash/dataset/doi:10.5061/dryad.44j0zpcdt> (Lemasina et al. 2021).

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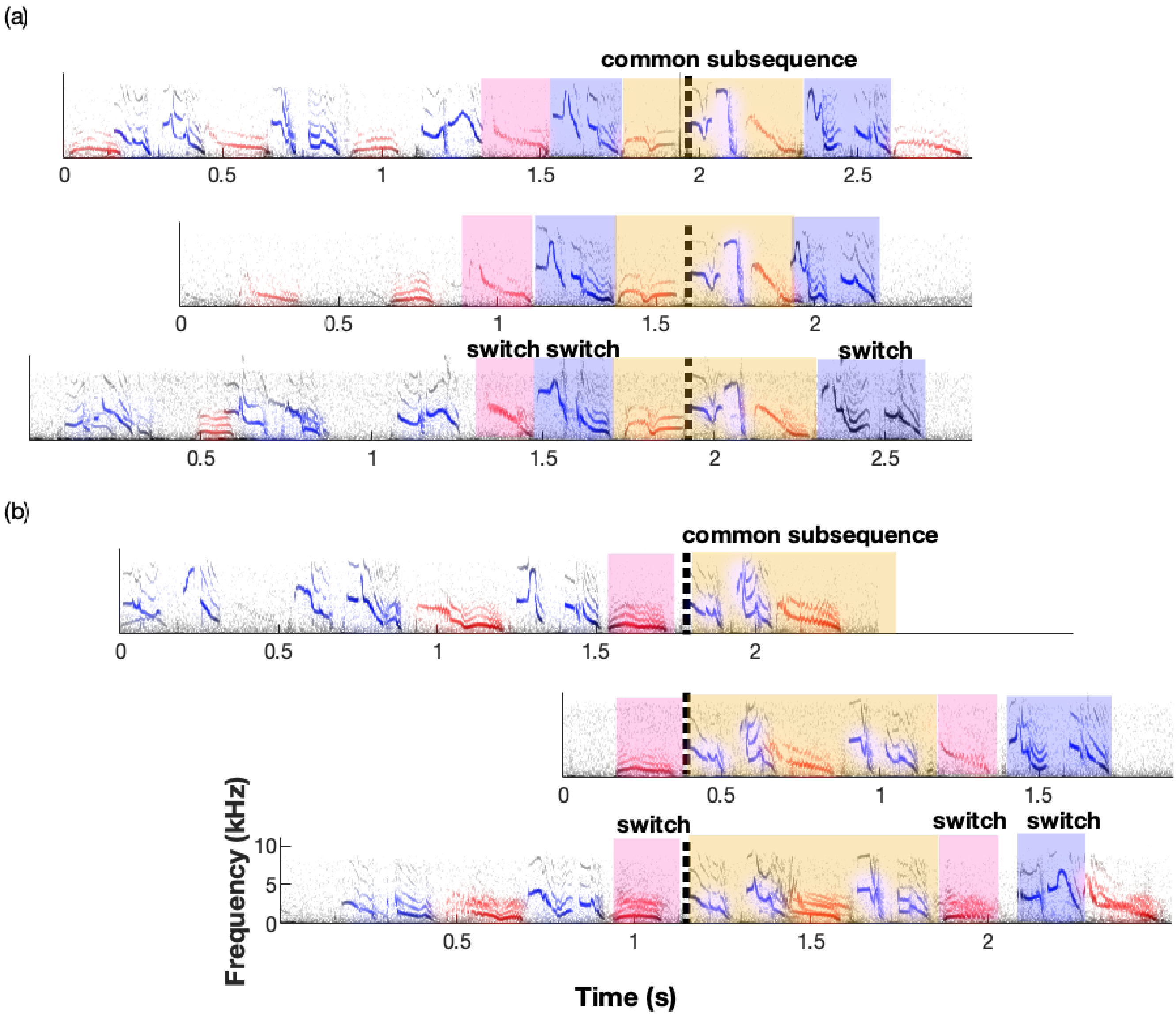
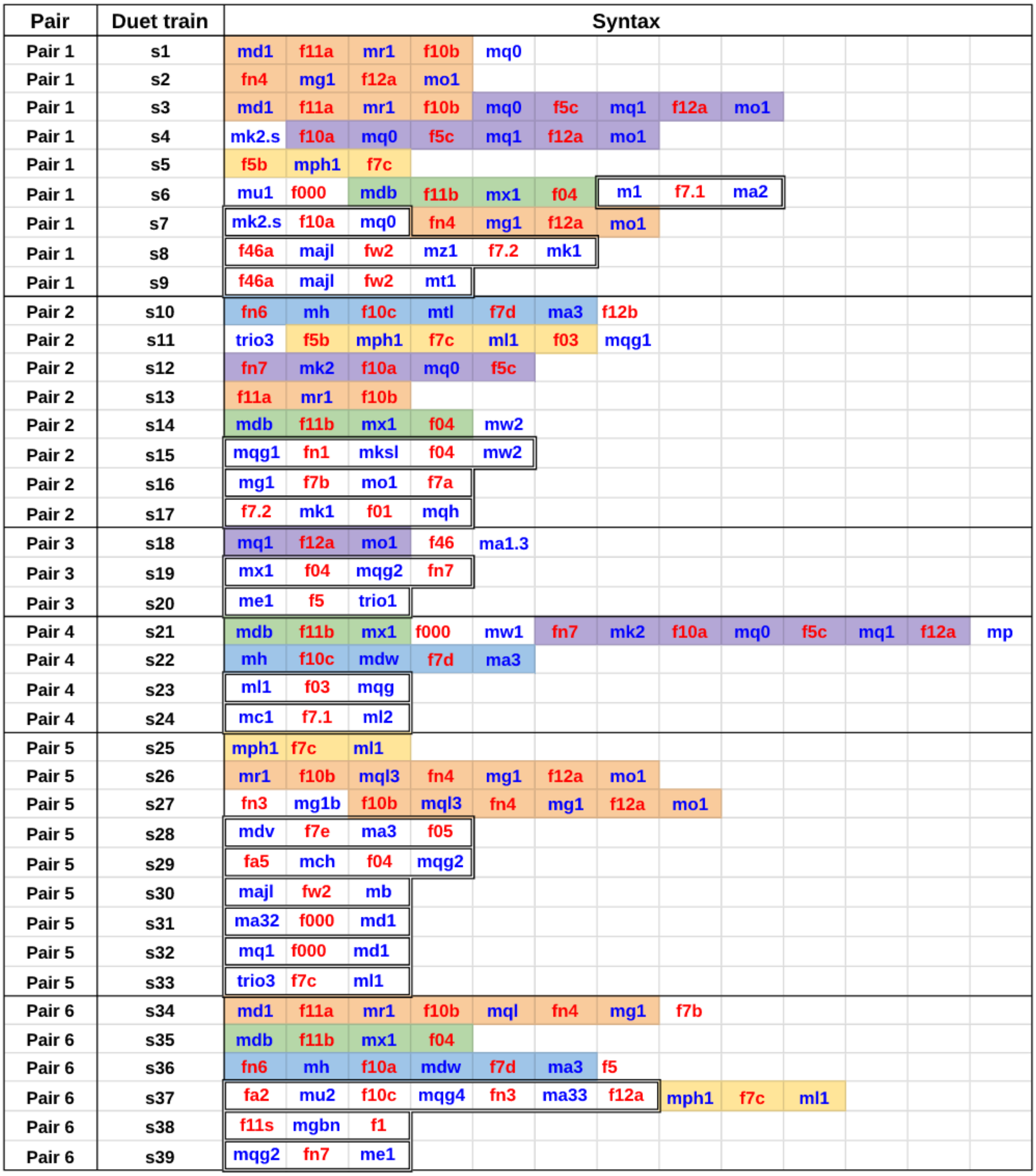


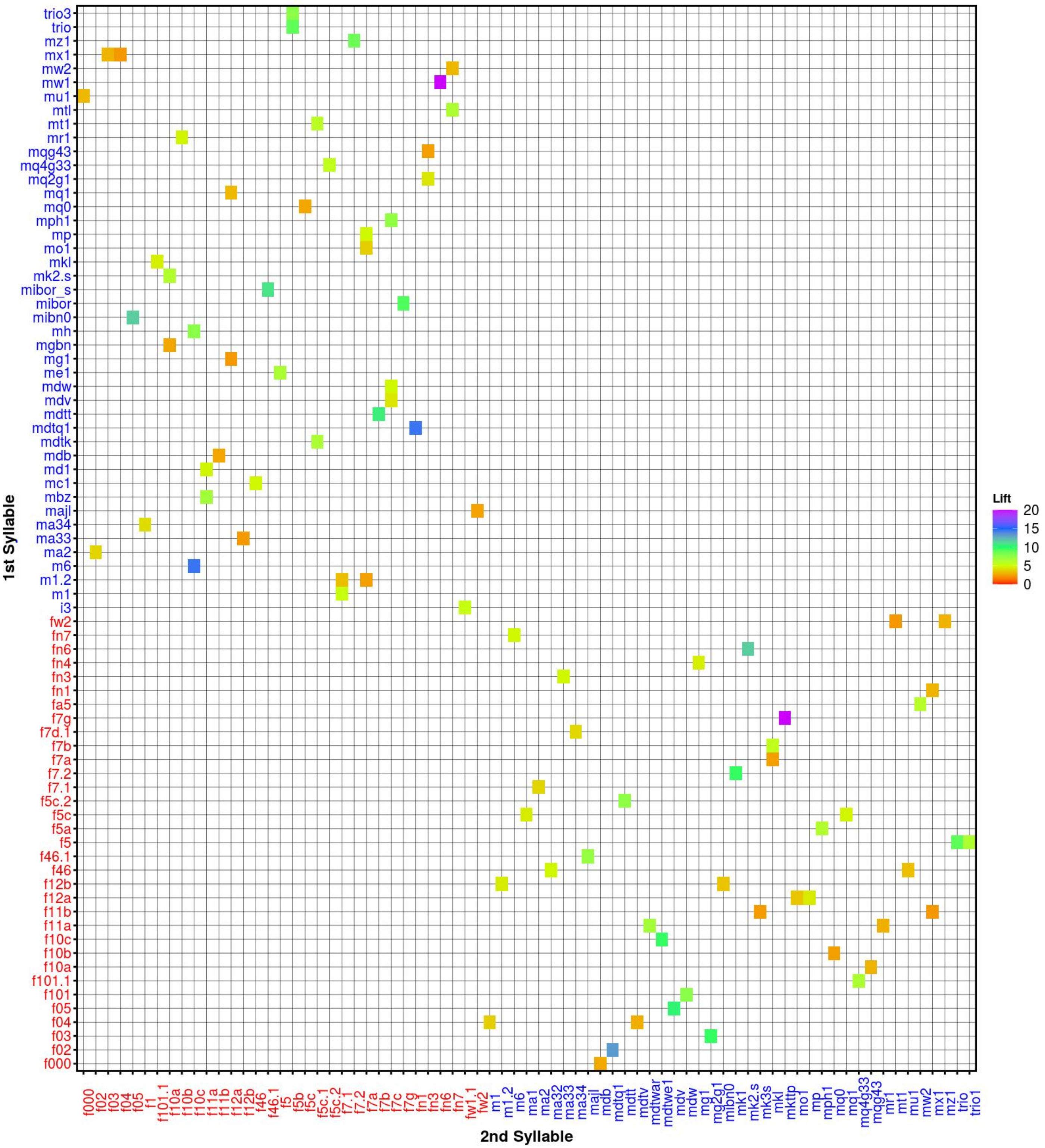
train subsequence in (a) and (b).

Supplementary figure 1. Syllable order in *P. mahali* duet songs is variable. Spectrograms of six duet songs of *Pair 4* are temporarily aligned by the time of occurrence of male syllable type *mq0* in **(a)**, and *mh* in **(b)**. Male and female syllables are highlighted in blue and red, respectively. While light-red and light-blue backgrounds in (a)

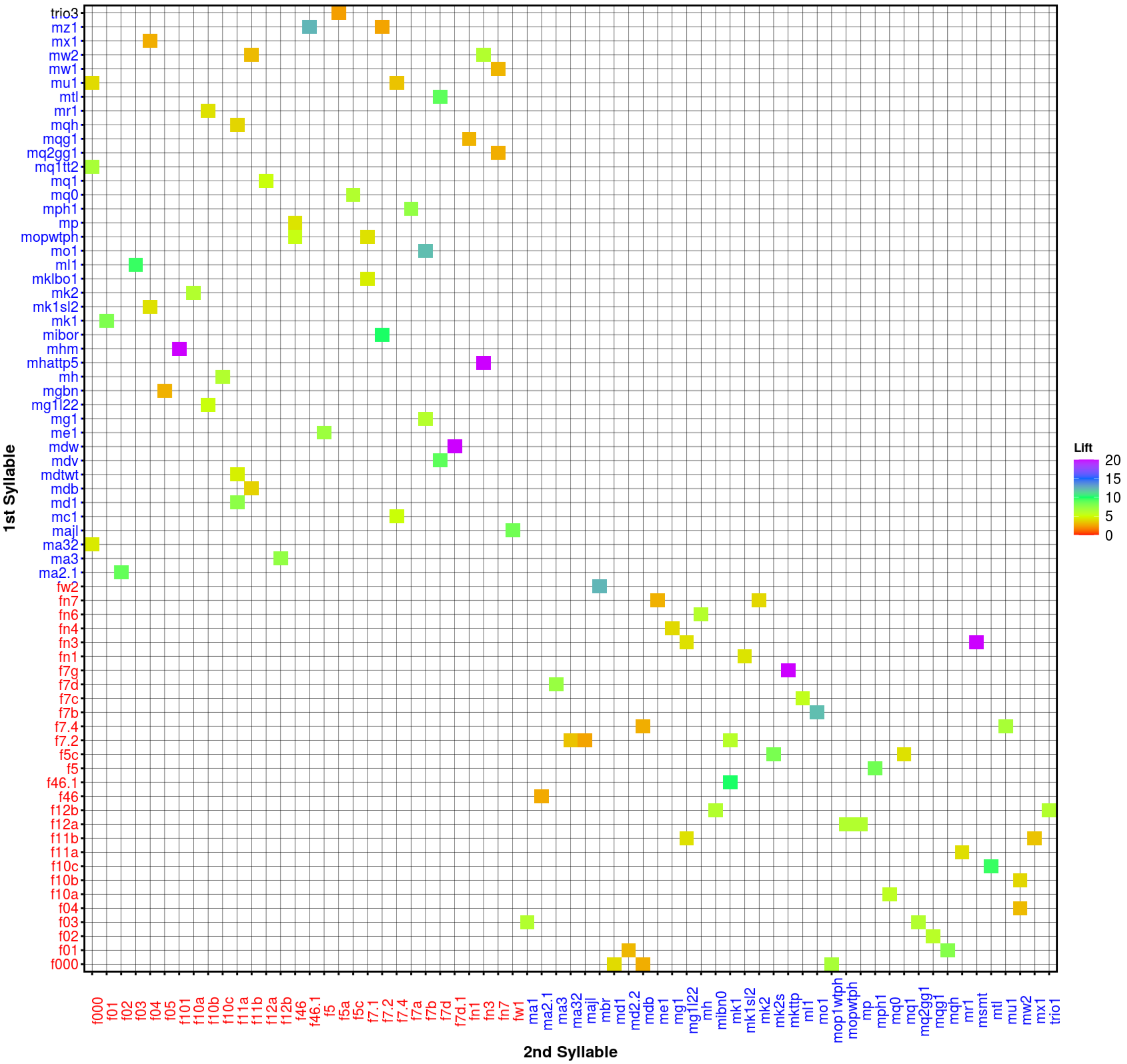


Pair	Duet train	Syntax												
Pair 1	s1	md1	f11a	mr1	f10b	mq0								
Pair 1	s2	fn4	mg1	f12a	mo1									
Pair 1	s3	md1	f11a	mr1	f10b	mq0	f5c	mq1	f12a	mo1				
Pair 1	s4	mk2.s	f10a	mq0	f5c	mq1	f12a	mo1						
Pair 1	s5	f5b	mph1	f7c										
Pair 1	s6	mu1	f000	mdb	f11b	mx1	f04	m1	f7.1	ma2				
Pair 1	s7	mk2.s	f10a	mq0	fn4	mg1	f12a	mo1						
Pair 1	s8	f46a	majl	fw2	mz1	f7.2	mk1							
Pair 1	s9	f46a	majl	fw2	mt1									
Pair 2	s10	fn6	mh	f10c	mtl	f7d	ma3	f12b						
Pair 2	s11	trio3	f5b	mph1	f7c	ml1	f03	mqg1						
Pair 2	s12	fn7	mk2	f10a	mq0	f5c								
Pair 2	s13	f11a	mr1	f10b										
Pair 2	s14	mdb	f11b	mx1	f04	mw2								
Pair 2	s15	mqg1	fn1	mksl	f04	mw2								
Pair 2	s16	mg1	f7b	mo1	f7a									
Pair 2	s17	f7.2	mk1	f01	mqh									
Pair 3	s18	mq1	f12a	mo1	f46	ma1.3								
Pair 3	s19	mx1	f04	mqg2	fn7									
Pair 3	s20	me1	f5	trio1										
Pair 4	s21	mdb	f11b	mx1	f000	mw1	fn7	mk2	f10a	mq0	f5c	mq1	f12a	mp
Pair 4	s22	mh	f10c	mdw	f7d	ma3								
Pair 4	s23	ml1	f03	mqg										
Pair 4	s24	mc1	f7.1	ml2										
Pair 5	s25	mph1	f7c	ml1										
Pair 5	s26	mr1	f10b	mql3	fn4	mg1	f12a	mo1						
Pair 5	s27	fn3	mg1b	f10b	mql3	fn4	mg1	f12a	mo1					
Pair 5	s28	mdv	f7e	ma3	f05									
Pair 5	s29	fa5	mch	f04	mqg2									
Pair 5	s30	majl	fw2	mb										
Pair 5	s31	ma32	f000	md1										
Pair 5	s32	mq1	f000	md1										
Pair 5	s33	trio3	f7c	ml1										
Pair 6	s34	md1	f11a	mr1	f10b	mql	fn4	mg1	f7b					
Pair 6	s35	mdb	f11b	mx1	f04									
Pair 6	s36	fn6	mh	f10a	mdw	f7d	ma3	f5						
Pair 6	s37	fa2	mu2	f10c	mqg4	fn3	ma33	f12a	mph1	f7c	ml1			
Pair 6	s38	f11s	mgbn	f1										
Pair 6	s39	mqg2	fn7	me1										

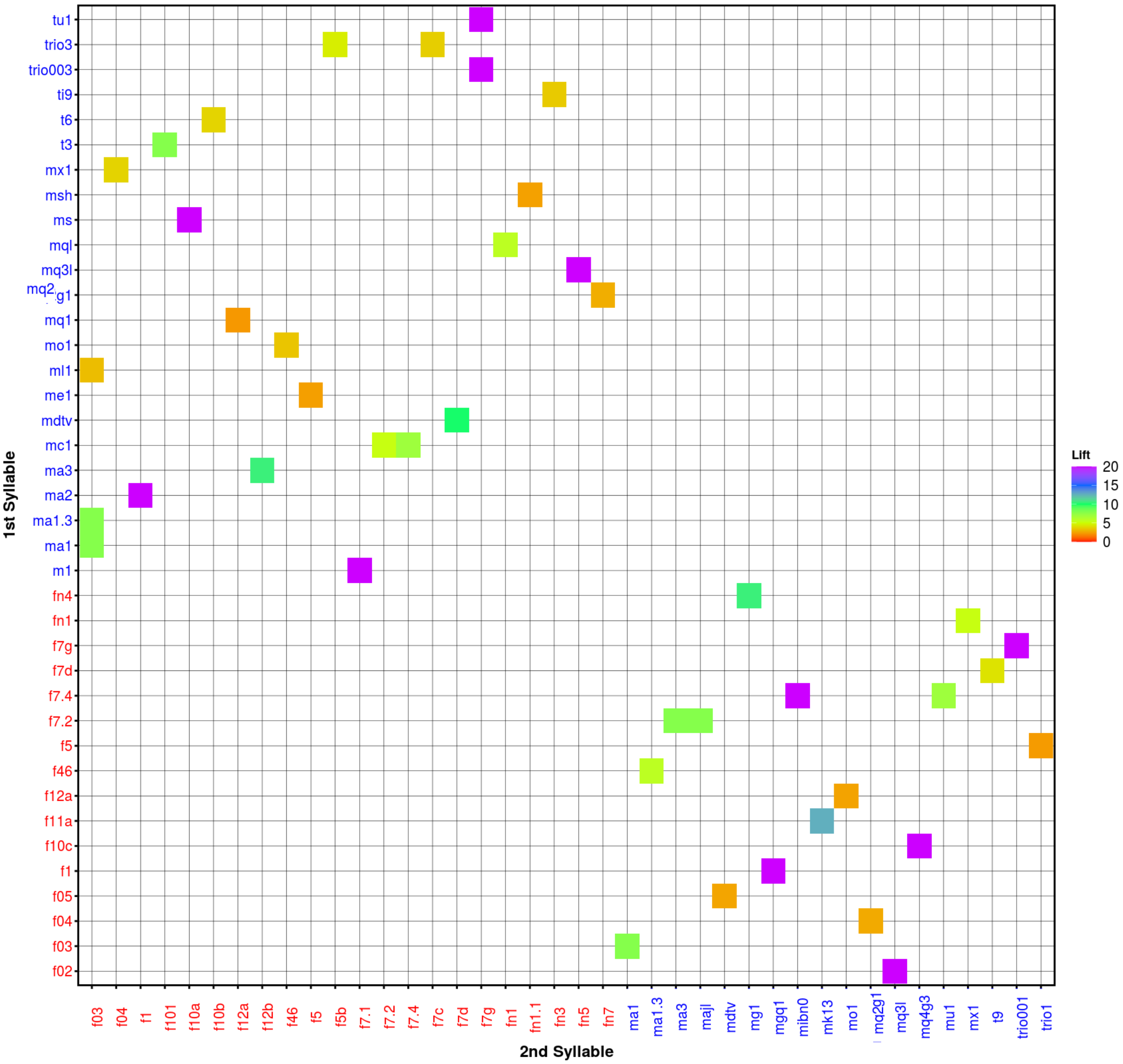
Supplementary figure 2. Duet train types found in pairs of *P. mahali.* Shared subsequences are color-coded in each duet train type. Unshared subsequences are indicated by a black frame. Male and female syllable types are highlighted in blue and red, respectively.



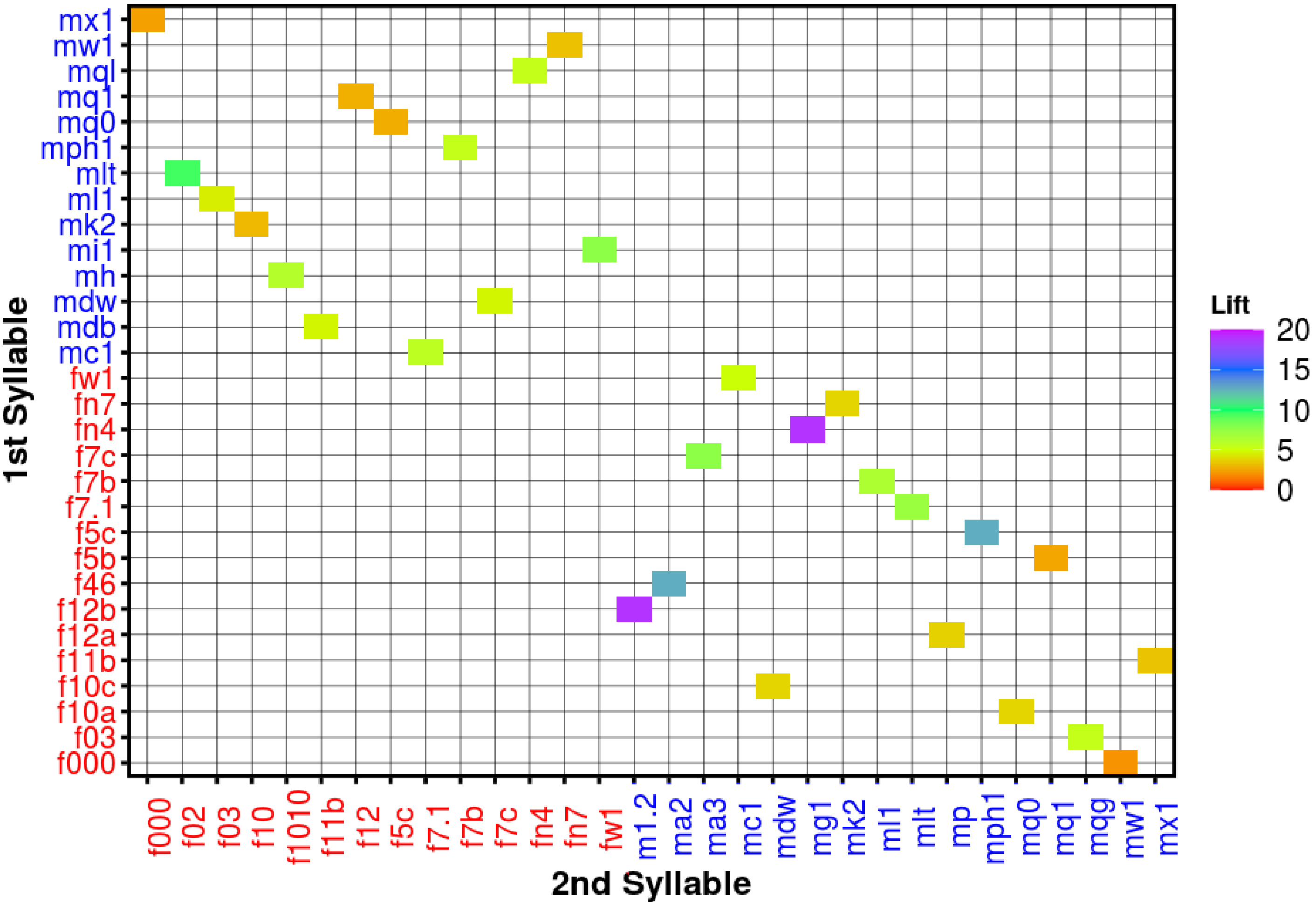
Supplementary figure 3. Matrix of answering rules observed in *Pair 1*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.



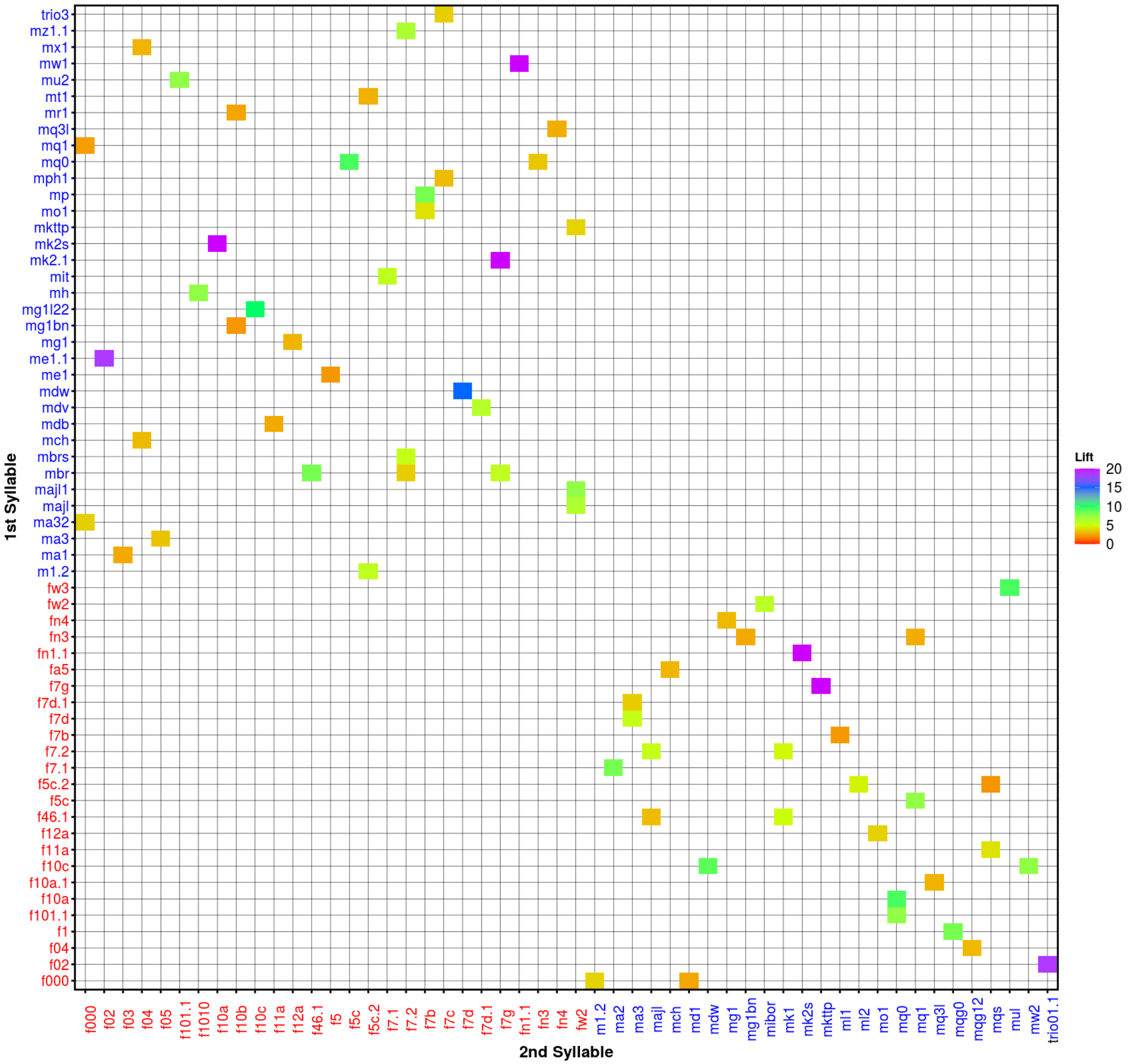
Supplementary figure 4. Matrix of answering rules observed in *Pair 2*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.



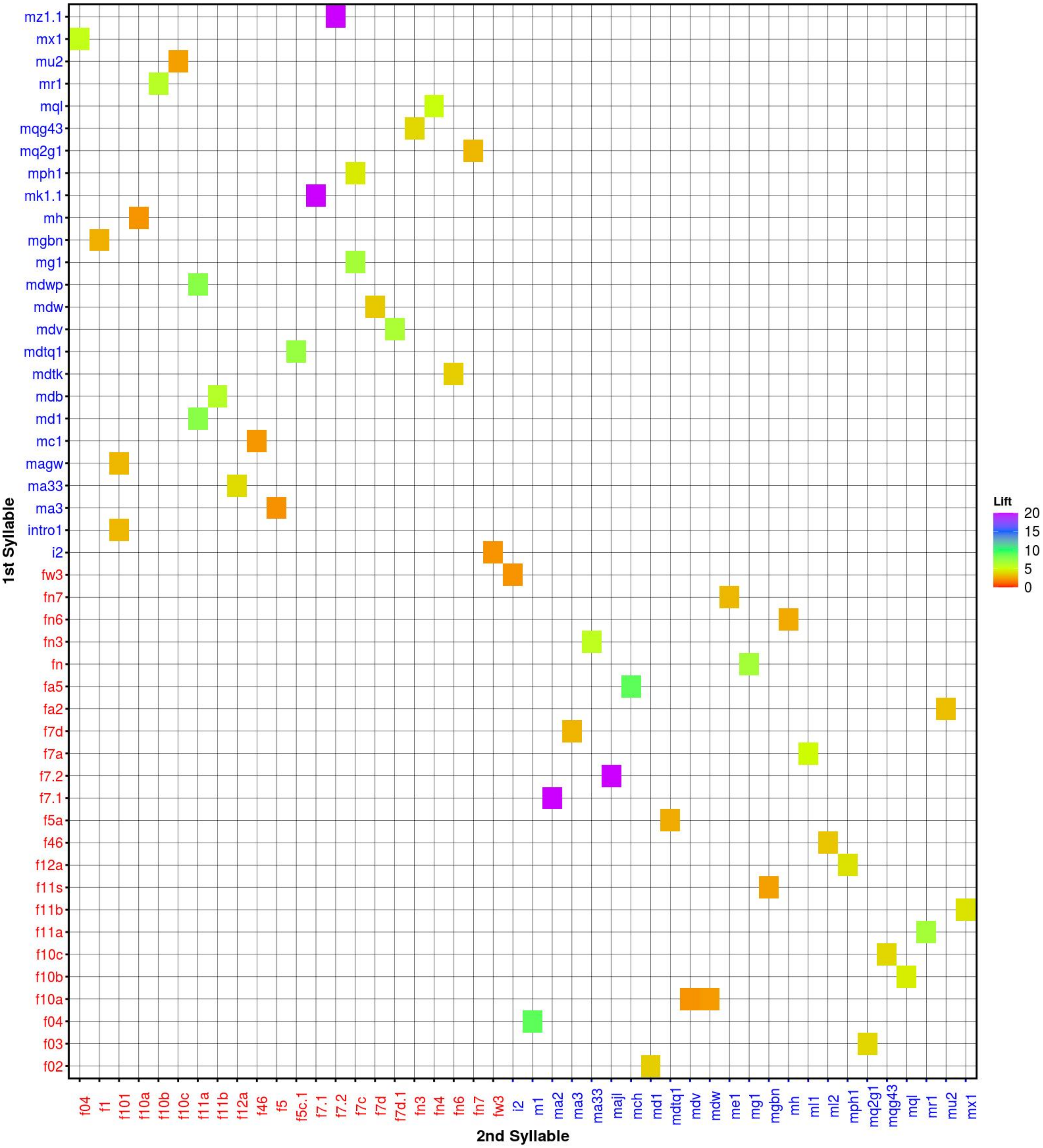
Supplementary figure 5. Matrix of answering rules observed in *Pair 3*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.



Supplementary figure 6. Matrix of answering rules observed in *Pair 4*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.



Supplementary figure 7. Matrix of answering rules observed in *Pair 5*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.



Supplementary figure 8. Matrix of answering rules observed in *Pair 6*. The value of *Lift* (i.e. measure of the strength of the answering rule adherence relative to chance) is color-coded. Male and female syllable types are highlighted in blue and red, respectively.

Chapter 3

Multilevel adaptations may facilitate prosodic projection in white-browed sparrow weaver duet songs

Abstract

Vocal turn-taking is widespread across taxa, ranging from antiphonal calls in insects to conversation in human speech. The precise mechanisms of this interactive behavior are, however, poorly understood. We investigate the spectral properties of song syllables in the duet songs of a songbird, the white-browed sparrow weaver (*Plocepasser mahali*), and classify the syllables into four categories based on their spectral pattern. Male duet syllables are classified as having a rising or falling frequency pattern, while female syllables are classified as being weakly or strongly frequency modulated. A Markov chain analysis demonstrates that the probability of occurrence of one syllable category in a *P. mahali* duet song depends on the syllable category of the preceding syllable. The transition probabilities between syllables of certain categories are significantly different from chance. We hypothesize that the spectral features of the four syllable categories function as prosodic cues for duet coordination. Using wireless radio-telemetric devices, we record extracellular, multiunit activity in the vocal premotor nucleus HVC of four male birds while they sing duet songs, and compare the neural activity between the two different male syllable categories. Our results show that HVC activity differs during the production of syllables of different categories. HVC is found to be active significantly earlier during the production of syllables with a low-frequency beginning and a high-frequency end (rising intonation) than during the production of syllables with a high-frequency beginning and a low-frequency end (falling intonation). These findings provide new insights into the neural mechanisms underlying syllable production in songbirds. In addition, we analyzed the microanatomy of the male *P. mahali* vocal organ, the syrinx, and found that the medial labia (ML), which in birds determines the fundamental frequency of a produced sound, have a two-layer structure. The left ML is significantly wider and contains a larger amount of collagen fibers than the right ML. Given that a ML consisting mainly of elastin fibers is likely able to oscillate at a lower range of frequencies than a stiffer ML which contains a considerable amount of collagen fibers, we suggest that the right side of the syrinx in male *P. mahali* is especially suited to produce the low-frequency parts of the male duet syllables. These findings provide anatomical evidence to support the hypothesis of lateralized song control in *P. mahali.*

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Contributions:

A.L., L.T. and S.H. conceptualized the project. A.L. led the data curation, conducted the investigation, formal analysis, and validation, and contributed to visualization. S.H. supervised the project and contributed to visualization and manuscript writing. L.T. was responsible for methodology, project administration, and provided supervision. M.G. provided funding, resources, and support in supervision. A.L. wrote the manuscript with the help of all authors.

Introduction

Prosody in vocal interactions

As a fascinating form of cooperative behavior, vocal turn-taking is widespread across taxa, ranging from antiphonal calls in insects to conversation in human speech [1]. Turn-taking requires participants to adjust the timing of their vocal production in response to acoustic cues received from a partner to ensure effective communication. In spoken conversation, speakers achieve successful coordination by using lexico-syntactic and prosodic cues to predict an upcoming change in turn. While syntax describes rules to arrange vocalization items into possible meaningful combinations, prosody uses acoustic properties of sounds, such as fundamental frequency, pitch, intensity, and duration, to describe the vocal output without relying on segmental features [3, 4]. During conversation, various prosodic features, such as low pitch or increased syllable length, signal the end of one conversation partner's turn to avoid gaps and overlaps between speakers [5]. Although prosodic features are independent of syntax, it has been shown that prosodic cues facilitate the acquisition of syntactic properties during language learning in humans via a phenomenon known as prosodic bootstrapping [6]. Prosodic bootstrapping is rooted in the robust correlation between prosodic structure and syntax, where prosodic cues as modifications in acoustic features (pitch, intensity, duration, and vowel quality) align with syntactic features [7]. Thus, infants can utilize prosodic cues as a means of organizing words into syntactic clauses [8] and to deduce the syntactic category of unfamiliar words [9]. Furthermore, prosodic cues have been found to aid in the identification of vocal patterns that are challenging to discern in artificially constructed languages [8]. Finally, prosodic and syntactic cues can co-occur during collaborative vocal behavior performed by two or more participants, such as duet and chorus singing in humans [10]. During duetting, the vocal elements produced by two individuals collaboratively form one syntactic unit by syntactically continuing each other's vocal outputs in respective turns. Such a behavior is known as prosodic projection, and creates a continued pitch contour across the vocal sounds produced by two individuals [Fig. 1A, 10].

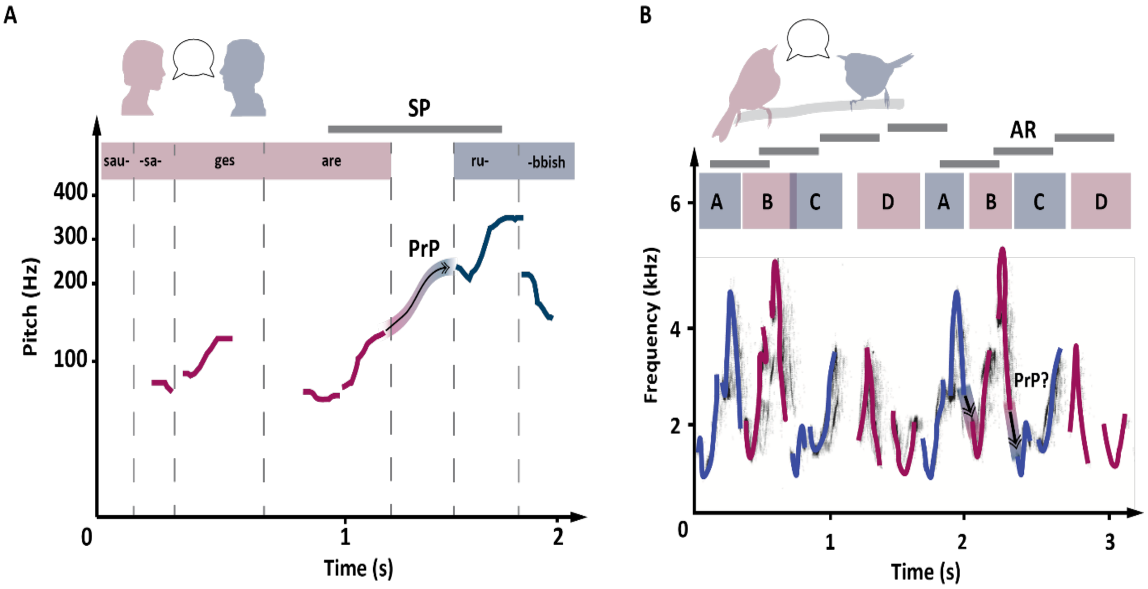

Figure 1 Prosodic cues during vocal interaction. *A. The pitch contour of a conversation between two speakers, modified from Reed [10]. B. The pitch contour of the male (blue) and female (red) contribution to a plain-tailed wren duet song, modified from Mann [2], Yip [25]. Each gray, horizontal line indicates a syntactic unit, a is called syntactic projection (SP) in (A) and answering rule (AR) in (B). The arrows between pitch contours indicate the direction of prosodic projection.*

As skilled vocal learners, certain songbird species represent an excellent model system to study various aspects of human language, such as linguistics or shared organizational patterns [4, 11]. Filippi [4] speculated that in humans, the ability to process linguistic prosody evolved from the ability to process prosodic cues for interactional coordination. Although prosodic features of human speech do not translate directly to birdsong, it has been demonstrated that birds use prosodic features for species-specific song learning. A study on cross-fostered zebra finches (*Taeniopygia guttata*) revealed that juvenile birds use inter-syllable silent gaps to learn the conspecific song [12]. In particular, the auditory cortex of the zebra finch includes a neuronal subpopulation that encodes the pauses between song syllables [12]. The coding of gaps between song syllables promotes social communication and conspecific vocal learning in situations with varying acoustic qualities [12]. During vocal interactions, birds can modulate temporal prosodic features [13] and can even group and discriminate sequences of human speech using prosodic cues [14]. Thus, European starlings (*Sturnus vulgaris*) coordinate temporal prosodic features of their vocal output based on social context [13], and zebra finches can categorize artificial vowel-like sounds using the fundamental frequency or pitch [15]. In comparison to the temporal coordination of sounds in birdsong, the importance of spectral prosody for vocal turn-taking in birds is still unknown. With operant conditioning methods, it has been demonstrated that birds can perceive prosodic properties in human speech, such as tempo or pitch modifications [16-19]. Despite the lack of direct studies on the usage of prosody in bird vocal interactions, one could argue that pitch matching in male-male countersinging vocal interactions could serve as an example of spectral prosody. Pitch matching occurs when an individual performs vocal units of different type but with the same fundamental frequencies as the opponent [20, 21].

Among the animal species that perform vocal turn-taking, birds that sing antiphonal duet songs exercise one of the most advanced form of this behavior [reviewed in 22, 23]. Antiphonal duet songs are defined as pair-level singing performances, in which both partners sing alternating song elements. In many species, duet coordination is assumed to rely on answering rules, which are nonrandom combinations of specific song elements (syllables or phrases) produced by the duet partners [24]. It has been speculated that prosodic cues paralleling the ones in human collaborative completions can be found in duet songs of plain-tailed wrens (*Pheugopedus euophrys*) [25]. Plain - tailed wrens are known for their complex four-part duet song, where both male and female birds alternate between two answering rules. In particular, Yip [25] notes that both duetting partners mutually continue each other’s pitch contour in 92% of cases (see Fig. 1B for an example).

Our model species, the white-browed sparrow weaver (*Plocepasser mahali*), demonstrates an even more complex antiphonal duet song, where each partner constantly switches between answering rules [26]. To successfully coordinate vocalization output between individuals in species with large syllable repertoires, the duet partners need to minimize the temporal and structural performance errors. In this chapter, I demonstrate that in addition to answering rules, syllables in *P. mahali* duet songs can be categorized according to spectral features into four universal categories. Moreover, the order of these categories follows a fixed pattern, which is repeated throughout the song. I hypothesize that both sexes of *P. mahali* use their respective syllable categories as prosodic cues for efficient duet coordination.

Vocal frequency control mechanisms and syrinx morphology

Songbirds possess a special vocal production organ, the syrinx [27], which is located at the caudal end of the trachea, where it divides into the two bronchi [27]. The syrinx contains two independent sound sources, one located within each individual bronchus, and sound is produced by generating airflow-driven vibrations of oscillating tissues, the medial labia (ML) in each sound source. Vibratory properties are determined by the labium size and tissue composition and by the relative orientation of elastic proteins (collagen, elastin) within the labium [28]. The positioning and tension of the labia within the airstream can be actively modulated by the syringeal musculature [29]. Muscle speed drives not only changes in sound frequency but also defines the maximal frequency modulation rate of sounds [30]. The interplay between the labium’s structural organization and the syringeal muscle activity determines the range of its oscillation frequencies, which in turn defines the fundamental frequency range of the produced sounds [28, 30]. In some species, the labia can differ in size and in their viscoelastic properties between the two syrinx halves. For example, in European starlings [31, 32], the left medial labium is significantly thicker than the right one. These morphological differences enable the two syrinx halves to generate different ranges of sound frequencies [32]. Aside of Australian magpies (*Gymnorhina tibicen*) and Bengalese finches [*Lonchura striata domestica*; 33], in most songbird species the right syringeal sound generator produces sounds of higher frequency than the left side of the syrinx [32, 34-36]. Although the precise correlation between labium morphology and vocal frequency range is still elusive, it has been suggested that an increase in morphological asymmetry between the two medial labia is correlated with an increase in vocal frequency range [28]. Moreover, the different arrangement of elastic proteins into multiple tissue layers also broadens the bandwidth of produced frequencies [28].

Previous studies indicated several constraints on the modulation of pitch during singing [27, 37-39]. For example, the frequency bandwidth of individual notes is limited when produced at high rates [40-42]. In another example, the motor constraint hypothesis suggests that descending pitch contours are predominant in birdsong [37]. Similarly, the sequential arrangement of prosodic vocal units defined by specific frequency modulation patterns can also face motor constraints. For instance, zebra finches position high frequency notes, produced during inhalation, in the middle of the song, between lower frequency notes typically produced during exhalation [43]. It is suggested that this technique enables the zebra finch to sustain an optimal breathing pattern throughout the whole song. The frequency shifts between subsequent syllables also seem to point towards limitation of vocal performance, in particular in neuromuscular control of the vocal apparatus [38]. In particular, acoustic recordings of skylark (*Alauda arvensis*) songs revealed that the duration of gaps between syllables positively correlated with the inter-syllable frequency shift, which indicates that this species can only produce large frequency jumps between syllables when there is sufficient time between syllables to adjust [38].

In this chapter, I provide insight into the possible production mechanisms of prosodic cues in *P. mahali* duet song. Since syllables in the male part of *P. mahali* duet songs consist of two sequentially arranged elements of different frequency ranges, we hypothesize that the syllables are generated by a successive contribution of each syringeal half. To see if the two halves of the syrinx in male *P. mahali* are adapted to produce sounds with different frequency ranges, I measured the size of the medial labium of each syringeal half and determined their elastin and collagen content. This allowed me to demonstrate that the syrinx in *P. mahali* males is indeed lateralized in its morphological structure.

Neural control of singing

The avian brain features an interconnected network of nuclei, the vocal control system, which controls vocal production [44]. One of the nuclei, HVC, plays a crucial role in the organization of individual vocal elements into sequential patterns, and alterations in vocal sequencing have been elicited by manipulation of HVC activity [45, 46]. For example, cooling HVC in Bengalese finches resulted in modified song tempo and impaired performance of distinct song sequences [47]. The vocal control systems in both brain hemispheres are anatomically symmetrical and unlike in the mammalian brain not linked by a callosum-type connection above the level of the midbrain [48, 49]. Thus, the control of the syringeal muscles by vocal premotor projection is ipsilateral, via the tracheosyringeal portion of the hypoglossal nerve [34, 48, 50, 51]. In zebra finches, in which the syrinx is not morphologically lateralized, the HVC in both hemispheres and consequently both halves of the syrinx are synchronously and equally active during vocal production [52].

Our results indicate that the anatomy of the medial labia in the syrinx of male *P. mahali* is asymmetrical. The lateralization of syrinx anatomy in male *P. mahali* suggests that low frequency sounds are produced with the right side of the syrinx and high frequency sounds with the left side. This, however, would require a sequential activity of the syrinx halves during the production of the two elements of the male duet syllables, which differ in frequency bandwidth. This in turn would require a sequential activation of the vocal control systems in the two hemispheres. We therefore hypothesize that song control is lateralized in male *P. mahali*. Our neural recordings from the right HVC support this hypothesis. In two out of three male *P. mahali*, we found the right HVC to be primarily active during the production of the low-frequency element of the male duet syllables. To present final proof for our hypothesis, activity from the left HVC in the brain of male *P. mahali* will have to be recorded during duetting within future experiments.

Material and Methods

For the present study, parts of the raw data from a previous study (Chapter 1) have been reanalyzed and combined with a recently collected set of anatomical data from 2021. All experimental methods that have already been used in the previous study will only be described briefly in the following but can be found in a more detailed form in [59].

Animals and ethics approval

All in-vivo experiments were conducted in a field-based setting near the village of Black Rock, Northern Cape, South Africa (27°7'S, 22°50'E) during November/December 2017. All investigated birds were color-banded for individual recognition. The birds were captured shortly after dusk (0800–1000 p.m.) inside their roosting nests. The sex was determined by beak color [53]. All bird-capture permits were obtained from the Northern Cape Department of Nature Conservation. All in-vivo experiments complied with the relevant ethical regulations for animal testing and research and were approved by the Animal Ethics Committee of the University of Pretoria (permit: EC026-17).

Vocal recordings

To monitor individual vocal activity in pairs of *P. mahali*, we equipped the male and the female bird of wild pairs with miniature radio-telemetric transmitters [54], developed at the Max Planck Institute for Ornithology in Seewiesen, Germany. The lightweight vocal transmitter (0.6 g), which included a miniaturized microphone (FG23329, Knowles Electronics, USA) and was covered by a thin silicon casing, was fixed on the back of the bird with cotton-covered rubber band straps around both femurs and the abdomen. The transmission range of the transmitter averaged 50 meters and the battery life was 10 days. Carrying these microphone transmitters has only weak and short-term suppressive effects on the vocal and movement activity of songbirds [54]. For signal detection, a crossed Yagi antenna (Winkler Antennenbau, Germany) was placed below the nesting tree. An antenna amplifier (TVS 14-00 axing, Goobay, Germany) increased the antenna signal by 18 dB. The signal was split (BE 2-01 premium-line, Switzerland) and fed into up to eight communication receivers (AOR 8600, AOR Ltd., Japan), which were modified to handle 12 kHz audio bandwidth. The analog signals were digitized by an eight-channel audio A/D converter (M-Track Eight, M-Audio, USA; sampling rate: 22050 Hz) that was connected to a laptop computer. All digitized signals were recorded in parallel as continuous sound files with a duration of four hours using multichannel software (16-bit, 22050 Hz; ASIO, Germany). The recording setup was placed in the trunk of a car located at a distance of about 30 m from the antenna. For each pair of birds, vocal recordings were continuously conducted over several consecutive days.

Neural recordings

In addition to the vocal transmitter, the birds were equipped with neural transmission devices. Under isoflurane anesthesia (1.5–1.8% isoflurane in 0.5 l O_2 min^{-1}, IsofluranCP, CP-pharma, Germany) and analgesic treatment (meloxicam 5.0 mg ml^{-1}, Metacam, Boehringer-Ingelheim, Germany; 0.2–0.5 mg kg^{-1}), a single parylene-coated tungsten electrode (FHC, USA; impedance: 2.0 MΩ) and a reference electrode (platinum wire with a diameter of 50 µm, Advent Research

Materials, UK) were surgically implanted in the bird's right HVC with the help of a piezo-driven single-axis micromanipulator (SMX series, SENSAPEX, Sweden) that was mounted on a stereotaxic frame (WPI, USA). The electrodes were fixed to the skull using dental cement (Tetric evoflow, Ivoclar Vivadent, Liechtenstein), and the neurophysiology transmitter (weight: 1.0 g) was subsequently connected to the electrodes' pins. The transmission range of the device was about 50 m, with a battery life of about seven days. After the surgical intervention, the birds were allowed to recover in cloth cages until dawn. All birds recovered quickly from the treatment, and we were able to release them to their home territory after mounting of the transmitters. Recording of neural and vocal activity started as soon as the birds were released. Whenever the birds were in the reception range of the antenna, the individual vocalizations and neural activity of both birds of a pair were telemetrically recorded synchronously for up to four days as described above. It is important to note that the position of the recording electrode was fixed in each bird. This means that we recorded for several days from one site in the right HVC of each bird. Constant monitoring of the treated birds certified that they did not show any sign of discomfort or altered behavior due to the treatment. At the end of the experiments, the birds were re-trapped, the transmitters were dismounted, and the birds were euthanized for brain sampling by an overdose of isoflurane.

Analysis of vocalization data

We prepared sound files for analysis by mixing synchronous four-hour recordings from both partners of a pair into a single render using the software Audacity (ver. 2.3.2, Audacity Team, www.audacityteam.org), and manually segmented them into separate duet bouts. Subsequently, we used Sound Analysis Pro 2011 to segment duet bouts into syllables, and to determine the contour of the mean frequency (mF) for each male and each female duet syllable in each bout. In previous studies, spectral contours were shown to effectively assist categorization of speech sequences into syntactically relevant units in humans [55-57] and non-human animals [58].

In *P. mahali*, male duet syllables generally consist of a doublet of song elements separated by a short interval of silence [59]. By visual inspection of sonograms of *P. mahali* duet songs, we realized that there are two major categories of male duet syllables, akin to universal categories found in other species. In one category, the first syllable element had a lower frequency content than the second element, and in the other category, the first element had a higher frequency content than the second syllable element. We investigated the spectral relationship between the two elements in more detail by calculating the difference between the maxima of the mF of both elements for each duet syllable following the equation: $2^{nd}mF_{max} - 1^{st}mF_{max}$. If the difference was positive, the syllable was classified as having a rising intonation (R-type), and respectively as having a falling intonation (F-type) in case of a negative difference (Fig. 2 A-B). Male song syllables that consisted only of a single element instead of a doublet structure, and male and female introductory syllables were grouped into the "solo syllable" (S) category.

Female *P. mahali* duet syllables were whistle-like sounds that always consisted of a single song element (Fig. 2 C-D). Female syllables seemed also to be classifiable into two major categories. One category includes syllables covering a large frequency bandwidth and the other category includes syllables of rather low spectral bandwidth. For each female, we calculated the mF bandwidth for each syllable and named the syllable strongly frequency modulated (SM-type) when its bandwidth was larger than the median bandwidth of all syllables of the female or weakly frequency modulated (WM-type) when its bandwidth was smaller than the median bandwidth of all syllables recorded in this female (Fig. 2 C-D).

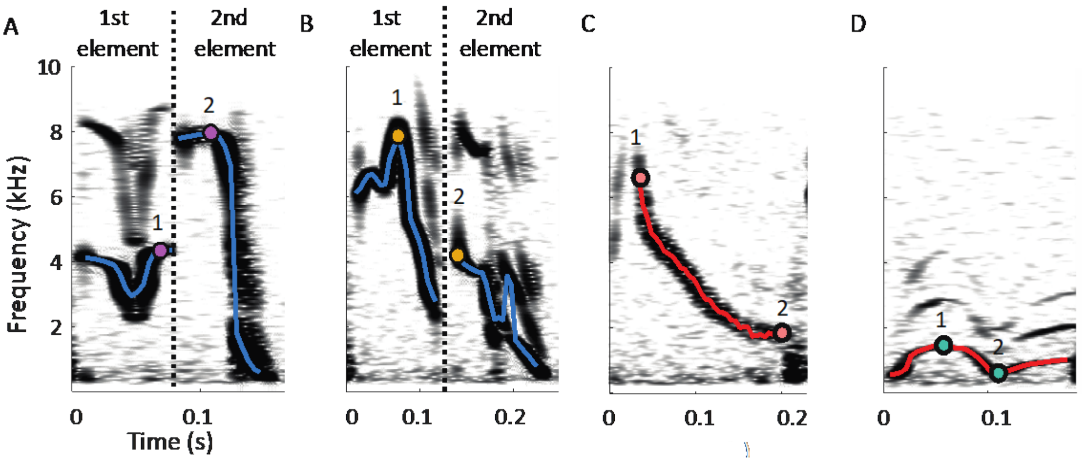

Figure 2 Determination of P. mahali syllable categories. *A. Spectrogram of an F-type male syllable. B. Spectrogram of an R-type male syllable. C. Spectrogram of a SM-type female syllable. D. Spectrogram of a WM-type female syllable. Blue lines indicate the contour of the mean frequency in male syllables, with orange and purple dots indicating the maximum of the mean frequency in both syllable elements of F- and R-type syllables, respectively. Red lines indicate the contour of the mean frequency in WM- and SM-type female syllables, with red and turquoise dots indicating the maximum and the minimum of the mean frequency in SM- and WM- type syllables, respectively.*

Stochastic modelling

To test whether *P. mahali* duet songs are constructed by following a specific order of syllable categories, we performed a Markov chain analysis using the R package *markovchain* [version 0.8.6, 60]. Based on earlier study [78], we defined a duet bout as a sequence of introductory syllables, followed by male and female duet syllables. For this part of the data analysis, we used only complete duet songs containing both an introductory and a duet component. For songs of three *P. mahali* pairs, we defined five syllable states (R-, F-, SM- and WM-type, and S), and for the songs of one pair, we defined only three states (R- and F-type, and S) due to the lack of vocal recordings from the female partner during duet song production. With the R package *markovchain*, we also tested if sequences constructed by randomly aligning syllables from the different categories can fit the Markov chain generated from given data by using a maximum likelihood estimator and determined if the given duet sequence is of first order. Subsequently, we generated a matrix of transition probabilities separately for each bird of a pair and tested whether the probability of transition to one syllable category depended on the category of the preceding syllable by performing a Chi-square goodness of fit test with the probability distributions.

Analysis of neural data

To extract spike times from the neural signals continuously recorded from each bird, each neural trace was initially bandpass filtered (200–4000 Hz, fourth-order Butterworth) using the MATLAB® filtfilt function. For each duet bout within a four-hour sound file, a threshold for discriminating spikes from background activity was determined by averaging (MATLAB®) the neural signal's mean plus three standard deviations for the time of bout duration. The filtered neural signals were then fed into the software Spike2® (CED, UK), and spikes were automatically discriminated from background activity by setting a threshold to the value calculated as described above. To examine the relationship between syllable categories and HVC activity, we aligned spike times to the onsets of each syllable of each male syllable category. To account for the difference in syllable duration, we normalized the times of spike occurrence to the average duration of male duet syllables (270 ms, [59]). To test for differences in timing of HVC activity between syllables of different categories, we calculated the median spike time relative to syllable onset within a time window of 200 ms before to 300 ms after syllable onset, and tested with a Wilcoxon signed rank test for significance. To visualize the average time-varying firing rate of HVC neurons, we plotted the Gaussian-smoothed peristimulus-time histogram.

Syrinx anatomy

Morphological measurements were taken from syrinxes of three male *P. mahali* different from the individuals used for neural and vocal data acquisition (Table 1). The isolated tissue was stored in 10% neutral buffered formalin for at least two days before each syrinx was transferred to 10% sucrose solution for cryoprotection. For sectioning on a freezing microtome, each syrinx was embedded in egg yolk containing 10% Glutaraldehyde. The complete organ was cut into 30 µm thick frontal cross-sections, which were mounted onto object slides, and adjacent sections were stained for either a general histological evaluation (H&E staining kit, Sigma Aldrich, ab245880) or to detect collagen fibers (Trichrome staining kit, Sigma Aldrich, ab150686). Micrographs were taken at 5x magnification from each section with a digital camera (Leica DFC480, Leica Microsystems GmbH, Germany) in combination with a Leica microscope (DM6000B, Leica Microsystems GmbH, Germany) and software (Leica Application Suite X (LASX), version 1.02, Leica Microsystems GmbH, Germany). In the micrographs taken from the H&E-stained sections, we measured the thickness of both the right and the left medial labium (ML) of each syrinx in reference to a known length using LASX software. First, the rostro-caudal lengths of both MLs were measured and subsequently, the MLs' widths were measured at the middle of their rostro-caudal extents. Measurements were taken in six sections equally distributed over the dorso-ventral extent of each labium. In each of these six sections, measurements were taken from 1-5 positions along the rostro-caudal extent of the ML separated by 200 microns.

To quantify the amount of collagen fibers in the right and left ML, we used the open-source image software ImageJ (version 1.53m) and an associated color deconvolution plugin (colordeconvolution2, Masson Trichrome method) based on the protocol described in Chen et al. (2017). In the Masson's trichrome-stained sections, collagen fibers are colored in blue, while elastin components are colored in black. We generated monochromatic (red, blue and green) copies of the micrographs of each section (10 measurements distributed over the rostro-caudal extent of the ML) via color deconvolution and estimated the amount of collagen and of elastin fibers in the blue and the red monochromatic image, respectively, by counting the number of color pixels within a region of interest (ROI). ROIs were defined by manually outlining the right and the left ML in the image. The threshold for each color component was set to 120-250 px.

Results

Vocal behavior

In total, we recorded 115 duet songs sung by four *P. mahali* pairs. In one pair (19 of the 115 songs), we were not able to record the female contribution to the duet due to technical failure of this bird's microphone transmitter but used the male song syllables for stochastic modelling (Table 1). It turned out that *P. mahali* duet syllables can be categorized into four universal categories based on their spectral pattern. Male duet syllables were classified either as having a rising (R) or a falling (F) pattern according to the relationship between the maximum mean frequency (mF_{max}) of the first and of the second syllable element. In 234 of 470 (49.7%) male duet syllables, the mF_{max} of the first syllable element was lower than the mF_{max} of the second syllable element. These syllables with a rising intonation were classified as R-type, while 236 of 470 (51.3%) male duet syllables with a falling intonation (i.e. mF_{max} of the first syllable element was higher than mF_{max} of the second syllable element) were classified as F-type syllables (see Fig. 2 A-B for examples). The median difference between mF_{max} of the two syllable elements was 2419.5 Hz and 2327.0 Hz in R- and F-type syllables, respectively (Fig. 3).

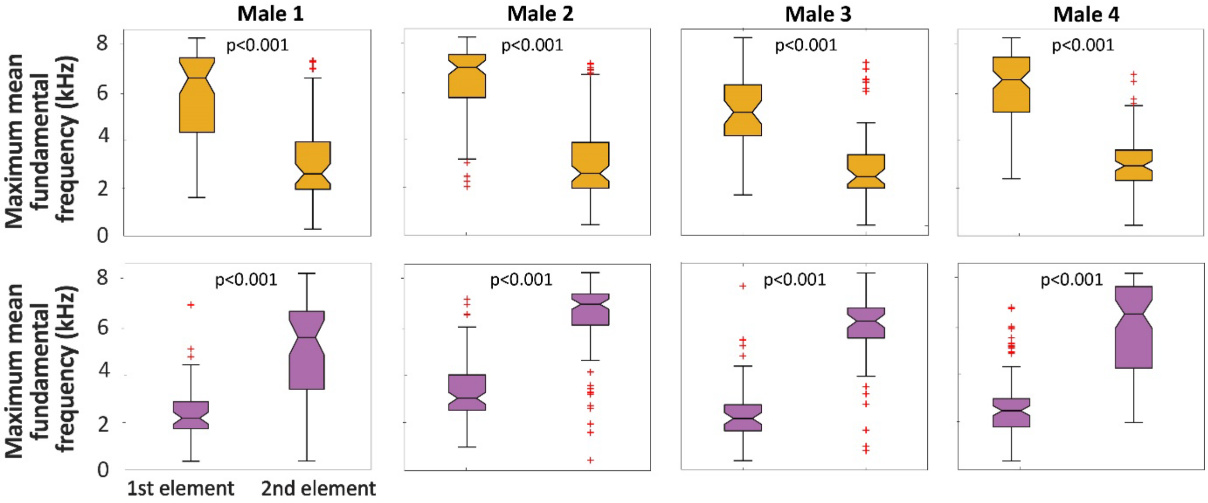

Figure 3 Distribution of mF_{max} in each element of male duet syllables. *In all males, the maximum mean fundamental frequency of the first syllable element was significantly larger than the maximum mean fundamental frequency of the second element in F-type syllables (orange) and significantly smaller in R-type syllables (lilac). In the boxplots, the horizontal line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The black whiskers extend to the most extreme data points without considering outliers. The extremes of the two notches of the box correspond to $y - 1.57(z - x)/\sqrt{n}$ and $y + 1.57(z - x)/\sqrt{n}$, where y is the median, x and z are the 25th and 75th percentiles, respectively, and n is the number of observations. The red plus signs indicate outliers. In all for birds, the difference in maximum mean fundamental frequency between the two syllable elements was significant (Wilcoxon test, $p<0.001$)) for both male syllable categories.*

Female duet syllables were classified either as being weakly (WM) or strongly (SM) frequency modulated. In SM-type syllables, the bandwidth of the syllable's mean frequency was higher than the median mean frequency bandwidth of all syllables recorded from a particular female, and in WM-type syllables, the bandwidth of the syllable's mean frequency was lower than the median mean frequency bandwidth. Median mean frequency bandwidth was 2630 Hz, 2920 Hz and 2750 Hz in Female # 2, Female # 3 and Female # 4, respectively. In each female bird, the median mean frequency bandwidth of WM-type syllables was significantly (Mann-Whitney U-test, $p < 0.001$) lower than the median mean frequency bandwidth of SM-type syllables (Fig. 4). In both sexes, the ratio between the number of syllables within the two syllable categories was 1:1. Male birds produced on average 59 F-type and 58.5 R-type syllables and female birds produced on average 45.0 WM-type and 47.6 SM-type syllables.

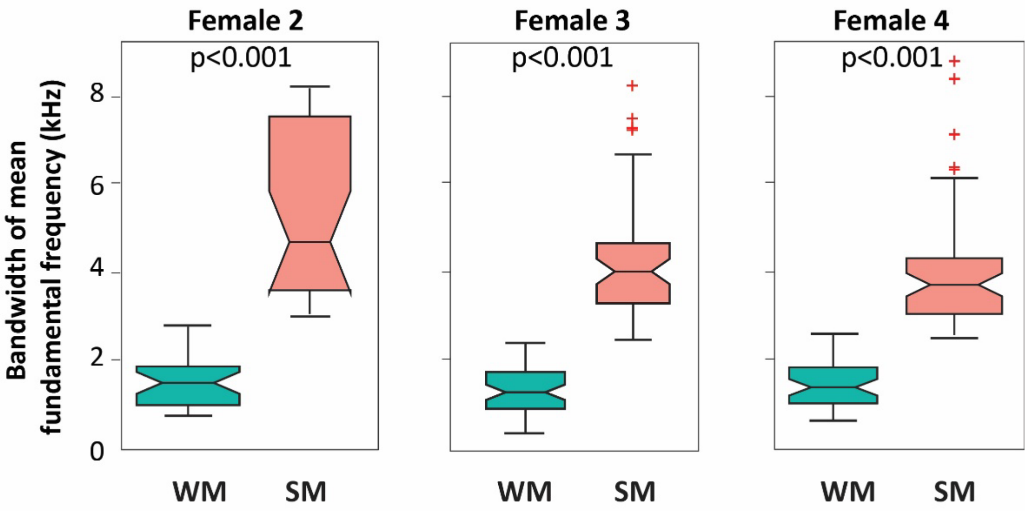

***Figure 4 Distribution of frequency bandwidth in female duet syllables**. In all females, the bandwidth of the mean fundamental frequency in SM-type syllables (pink) was significantly larger (Mann-Whitney U-test, $p < 0.001$) than in WM-type syllables (turquoise). Meaning of markers as in Fig. 3.*

Stochastic model

After assigning each song syllable to one of the five categories, (male: R and F; female: SM and WM; male and female: S) we conducted a Markov chain analysis, to test whether the probability of occurrence of one syllable category in a *P. mahali* duet song depends on the syllable category of the preceding syllable. We found that duet bouts of *P. mahali* can generally be described by a first order Markov chain, with each syllable category especially dependent on the immediately preceding syllable category (confidence level: 0.95). Female syllables of the WM-type and female syllables of the SM-type showed a probability greater than expected by chance to transition to male R-type and male F-type syllables, respectively, with a median transition probability of 58-67 % and 74-90 % respectively (Fig. 5 A). Male syllables of the R-type and male syllables of the F-type showed a probability greater than expected by chance to transition to female SM-type and female WM-type syllables, respectively, with a median transition probability of 42-76% and 42-72% respectively (Fig. 5 A). Male and female syllable categories were shown to alternate between each other, while S-type syllables of both sexes were shown to be the repetitive state. In summary, the alternating part of all duet songs of all pairs in which we recorded both male and female contributions to the duet followed a fixed pattern of syllable categories: An R-type male syllable was followed by an SM-type female syllable, which was followed by an F-type male syllable, which was followed by a WM-type female syllable (R->SM->F->WM). This sequence was repeated throughout the duet song (Fig. 5 B), and each syllable category could provide the first syllable following the initial cycling of introductory syllables. In Pair 1, the data on the female duet contribution was lacking. But nevertheless, the probability of F- and R-type syllables to alternate between each other was higher than the probability of these syllables to transition to S-type syllables or to repeat themselves (Fig. 5A).

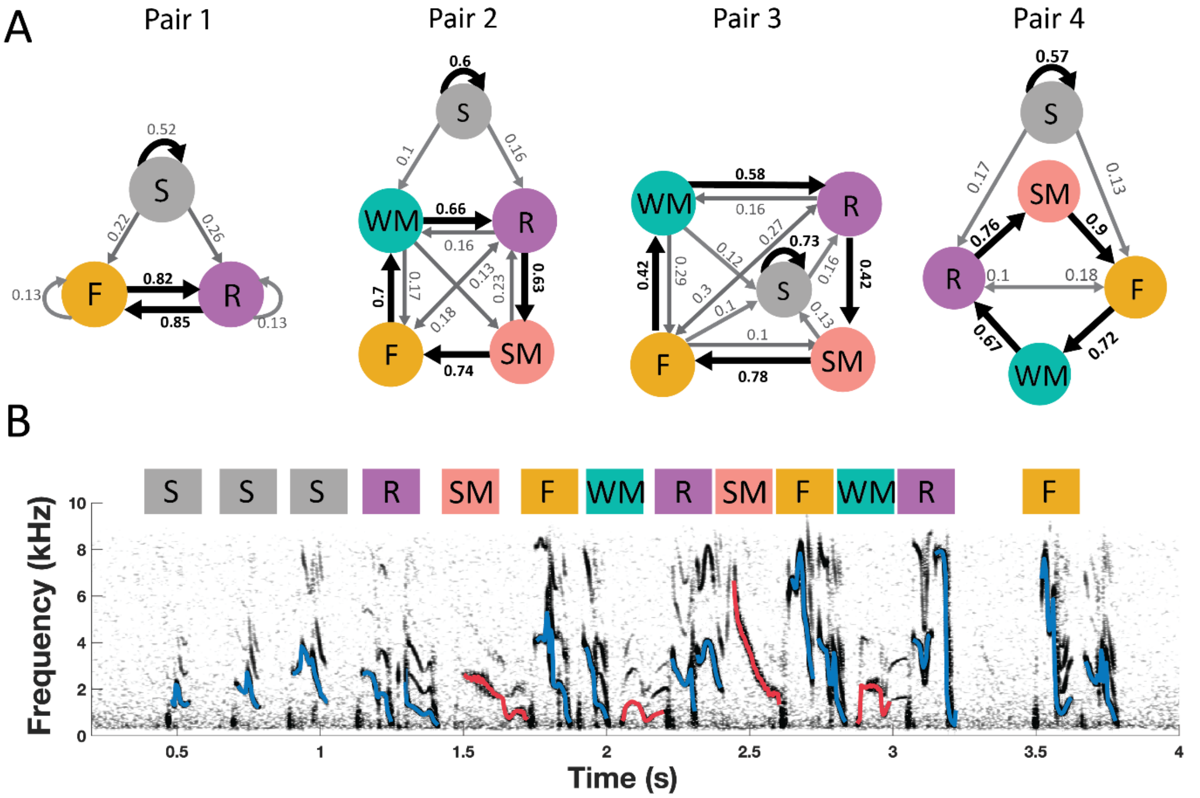

Figure 5. Syllable arrangement in white-browed sparrow weaver duet song. *A. Transition diagram of syllable categories for each pair of birds. The arrows represent the direction of transitions between syllables of different categories. The numbers indicate the transition probabilities. F: male syllables with falling intonation; SM: strongly modulated female syllables; WM: weakly modulated female syllables; R: male syllables with rising intonation; S: solo syllables. Transitions with probabilities below 0.1 are not shown. Due to the lack of female vocal data, the diagram of Pair 1 is based on male syllables only. B. Spectrogram of a P. mahali duet song. The contours of the mean fundamental frequency are indicated in blue and red, for male and female syllables, respectively. The syllable category is indicated above each syllable.*

Neurophysiology

To identify the contribution of HVC to the production of different duet syllables, also incomplete duet songs that lacked introductory syllables, and the male part of duet songs that were performed with females not carrying a microphone transmitter were used in addition to the complete duets used for the stochastic modelling. To uncover the neural mechanisms underlying the generation of song syllables of different syllable categories, we tested whether male HVC activity during singing differs between syllable categories. With wireless radio-telemetric devices (Fig. 6 A), we recorded extracellular multiunit activity in the right HVC of three male *P. mahali* (the neuronal recording of Male 1 did not yield utilizable data) during the production of 139 songs in total (average: 46.33 and interquartile range: 37-62 songs per male). We found HVC activity during the production of R-type syllables to differ from HVC activity during the production of F-type syllables (Fig. 6B - F). After aligning the times of spike occurrence to the syllable onsets, we found that in two out of three male birds the median spike time relative to syllable onset is significantly (Mann-Whitney U-test, $p<0.001$) higher in F-type syllables (Male 2: 0.151 s, Male 3: 0.11 s) than in R-type syllables (Male 2: 0.096 s, Male 3: 0.07). This indicates that in these birds HVC is active earlier during the production of R-type syllables than during the production of F-type syllables (Fig. 6, B-F). Male 1 did not show a significant (Mann–Whitney U test, $p > 0.05$) difference in spike timing between the production of R- and F-type syllables (Fig. 6 B-F).

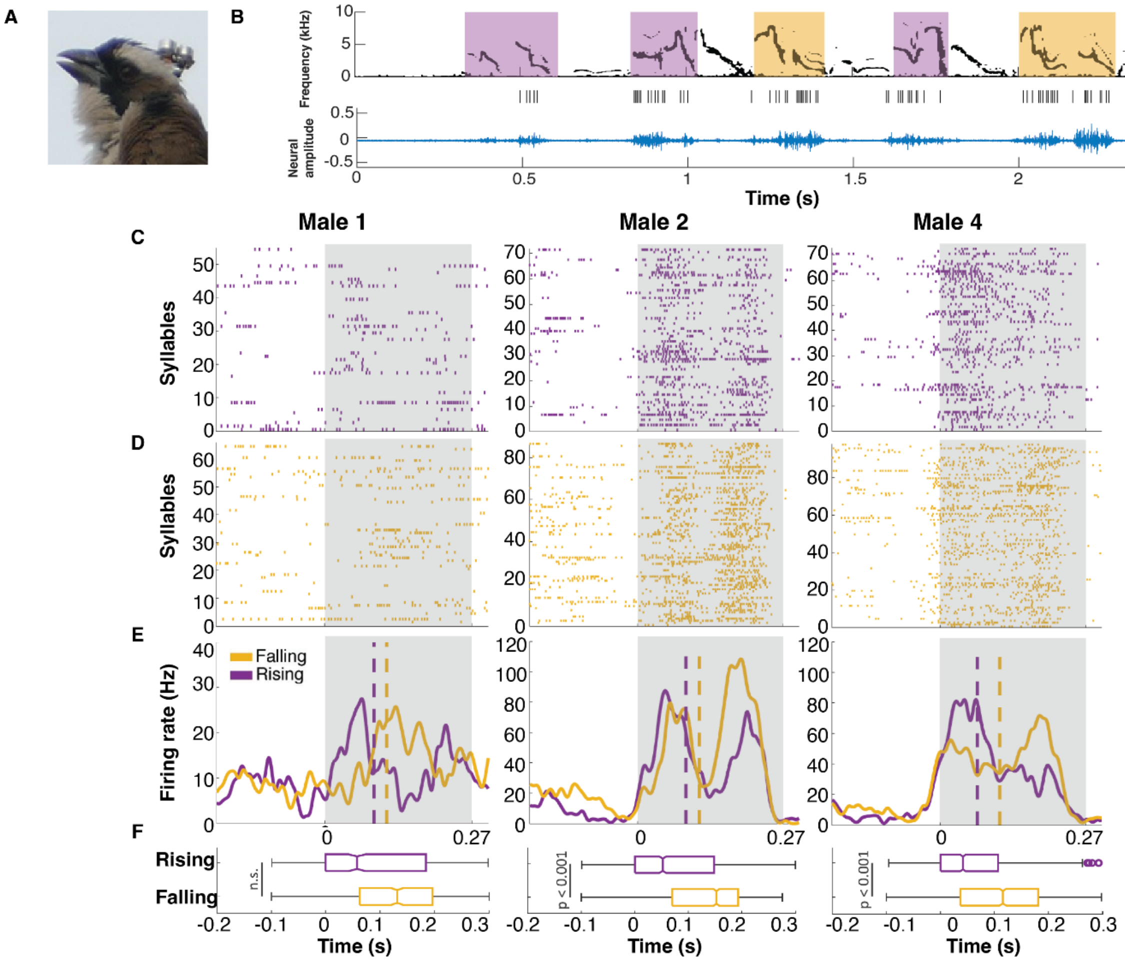

Figure 6 Neural activity underlying the production of male syllable categories. *A. White-browed sparrow weaver with electrophysiology transmitter. B. Spectrogram of a duet song produced by Pair 3. Male R-type and F-type syllables are indicated by lilac and yellow shading, respectively. The waveform of the filtered neural trace, which was recorded in the male's HVC during the production of the duet song is shown below. Spike times are indicated by vertical black lines above the waveform. C-D. Spike raster plots temporally aligned to syllable onsets. E. Spike histograms (bin size: 0.05 ms) temporally aligned to syllable onsets. The lilac and orange dashed vertical lines indicate the median spike time for R- and F-type syllables, respectively. The area shaded in gray indicates the median syllable duration. F. Boxplots of spike times relative to syllable onset for all R- and F-type syllables. P-values of a Mann–Whitney U test are indicated.*

Syrinx morphometry

To find anatomical evidence that could support our hypothesis that song control is lateralized in *P. mahali*, we investigated the micromorphology of three male *P. mahali* syrinxes. Given that the right HVC is mainly active during the production of the low-frequency element of duet song syllables (see above), and the right HVC controls the right side of the syrinx [61-63], we expected the right side of the syrinx to show some degree of specialization for the production of low-frequency sounds. We found that in general the medial labia (ML) in male *P. mahali* syrinxes have a two-layer structure. A deep layer with a prominent presence of elastin fibers is covered by a layer of loose connective tissue with lower elastin content but a much higher presence of collagen fibers (Fig. 7 A). This difference in composition of layers was especially apparent in the left ML. For each syrinx, we quantified the width of the right and the left ML, and the percentage of area composed of collagen and elastin fibers, respectively. We measured the labial width in serial frontal sections of the syrinx at six positions equally spaced from anterior to posterior to assess the asymmetry between the left and the right sound source for each individual. In all individuals, the left ML was of significantly (Wilcoxon signed rank test, $p < 0.001$) larger width (median: 180 µm, interquartile range: 160-280 µm) than the right ML (median: 100 µm, interquartile range: 80-150 µm) (Fig. 7 B). The content of elastin fibers did not differ significantly between the right and the left medial labia (Fig. 7 C, Wilcoxon signed rank test, $p > 0.05$). Interestingly, in all three syrinxes the left ML tended to contain a larger amount of collagen fibers (median: 94% of ROI, interquartile range: 62-98% of ROI) than the right medial labium (median: 75% of ROI, interquartile range: 60-81% of ROI) (Fig. 7 D). This trend was significant (Wilcoxon signed rank test, $p < 0.05$) in two of three syrinxes. Given that a rubber band-like ML consisting mainly of elastin fibers is likely able to oscillate at a lower range of frequencies than a stiffer ML containing a considerable amount of collagen fibers [28], we suggest that the right side of the syrinx in male *P. mahali* is indeed especially suited to produce the low-frequency parts of the male duet syllables, which would support our hypothesis of lateralization of song production in male birds of this species.

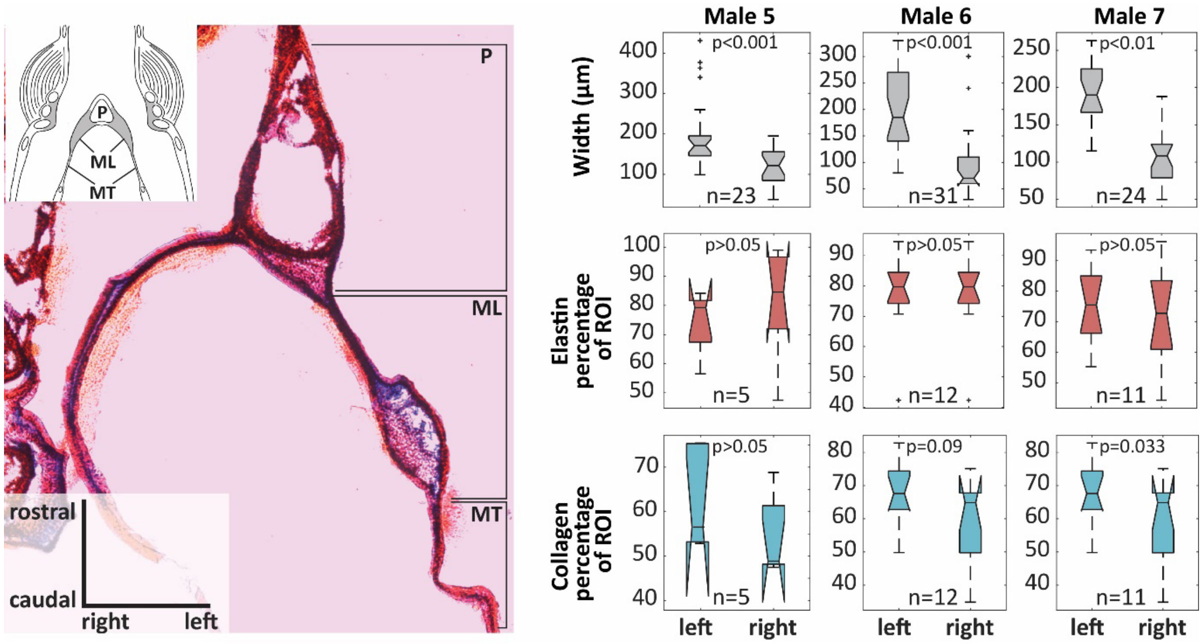

Figure 7. Syrinx anatomy in white-browed sparrow weavers. *A. Trichrome stain of a frontal section of the male syrinx. The inset shows a schematic of the syrinx. P, pessulus; ML, medial labium; MT, medial tympaniform membrane. Collagen fibers are stained in blue and elastin fibers are stained in black. B. Boxplots of ML width. In all three male syrinxes, the left ML is of significantly larger width than the right ML (Wilcoxon paired test). C. There is no difference in elastin fiber content between the left and the right ML (H&E staining) (Wilcoxon paired test). D. In two of three male syrinxes, the left ML contains a significantly larger amount of collagen fibers than the right ML (Trichrome staining). Meaning of markers as in Fig. 3. N indicates the number of sections analyzed.*

Discussion

In the present chapter, I investigate the spectral properties of song syllables in duet songs of *P. mahali*, the vocal premotor activity underlying the production of male syllables, and the anatomy of the male vocal organ. Mated pairs of *P. mahali* sing highly complex duet songs by combining male and female duet syllables of their extensive sex-specific repertoires in adherence to a large set of answering rules [26]. A previous study demonstrated that *P. mahali* duet songs have a rhythmic pattern, where the rate of vocal production differs between the initial part of the duet bout and the subsequent part in which partners produce song syllables in alternation [59]. The current data demonstrate that *P. mahali* duet syllables can be categorized into four basic sex-specific categories according to their spectral pattern. The fixed ordering of these syllable categories underlies the species-specific pattern of *P. mahali* duet songs. We discovered that the syllables of the two different male categories differed in their underlying neural activity pattern in HVC of the right hemisphere of *P. mahali* males. We further uncovered a morphological lateralization in the *P. mahali* syrinx as potential mechanisms for the generation of song syllables that differ in intonation.

Prosody in *P. mahali* duet songs

Previous research demonstrated that non-human animals possess the ability to utilize temporal prosodic cues to discriminate and categorize sequences of artificial sounds [4, 11, 64, 65]. For example, Java sparrows (*Padda oryzivora*) can use temporal prosodic patterns to differentiate between human sentences [66]. However, the existence of spectral prosodic cues in animal-generated vocal sequences and specifically vocal interactions, have not been demonstrated so far [11].

Here, we showed that in both sexes of *P. mahali*, the extensive syllable repertoire consists of discrete syllable categories that differ in their spectral properties. In both sexes of *P. mahali*, syllables can be grouped into two categories with specific spectral patterns: rising or falling intonation for male syllables, and weak or strong frequency modulation for female syllables. I suggest that the observed syllable categories provide spectral prosodic cues for duet coordination. The duet structure in *P. mahali* songs naturally recalls analogies to the structure of collaborative vocal interactions in human language. In human language, participants can use a prosodic orientation to coordinate collaborative vocal sequences [10, 55]. Prosodic orientation occurs when a speaker adjusts the prosodic structure of their vocal output to that of the preceding speaker (Fig. 1A). Several types of prosodic orientation exist. First, ‘prosodic complementation’, where the type of pitch contour of one speaker predicts the type of intonation contour of the next speaker [10]. Second, ‘prosodic continuation’, where the pitch contour produced by the first speaker is continued by the second speaker in the adjacent turn. Moreover, in human vocal turn-taking interactions, speakers function as collaborators and jointly produce vocal sequences by mutually linking prosodic and syntactic patterns to a partner’s turn [10, 55, 56]. Third, ‘prosodic orientation’, allows to jointly convey information, to display mutual understanding between participants and to signal the direction of vocal display progression to the partner in complex vocal interactions [10, 55]. We suggest that the fixed order of syllable categories in *P. mahali* duets could be comparable to ’prosodic complementation’ in human collaborative vocal interactions. Sequential similarities and differences between birdsong and human language have been discussed extensively [67-69]. It is impossible to discuss birdsong syntax, or more accurately, vocal unit sequencing, from an information transfer perspective since individual acoustic units in birdsong lack distinct semantic meaning. In human language, syntactic structure enhances the efficacy of information transfer while simultaneously reducing the costs associated with language production and comprehension [68]. Nevertheless, it is possible to address vocal unit processing costs and minimize the cost of vocal production, specifically motor production in birdsong. We suggest that the different syllable categories in *P. mahali* provide spectral cues to the duet partners, which may facilitate duet learning and duet coordination.

Universal categories of vocal production units have been found in multiple bird species with different levels of song structure complexity [70-73]. The existence of universal categories has been proposed as evidence for the existence of innate perceptual predispositions [71]. Another possibility is that syllable categories in *P. mahali* may potentially encode species-specific information, while the syllables themselves indicate individual and pair identity. For example, in the song of white-browed warblers (*Basileuterus leucoblepharus*), acoustic features, resistant to propagation changes, signal species identity while features that degrade quickly, encode individual identity [74].

We suggest that the syllable categories could contribute to the efficacy of duet learning in *P. mahali*. Sex-specific syllables of different categories could be learned during early development via a mechanism analogous to prosodic bootstrapping. Prosodic bootstrapping originally refers to acoustic cues in the speech stream that provide information of syntactic boundaries to infants, and facilitate language learning [75]. In duetting species, the acoustic features and the sequential patterning of vocal units in the song are learned from a tutor [76]. In canebrake wrens (*Cantorchilus zeledoni*), juveniles learn duet codes by singing with a mated pair of adults. And in bay wrens (*Cantorchilus nigricapillus*) juvenile birds are able to learn their sex-specific duet contributions only while being tutored by playbacks of two parents performing their respective contributions, but not by playbacks simulating a single parent performing both contributions simultaneously [77]. Future research could investigate if juvenile *P. mahali* learn from their parents not only specific syllables and answering rules but also the overall prosodic pattern of their species-specific duet song, which they could later (during adult duet learning) extrapolate to produce new syllable types. In Chapter 2, we showed that both sexes of *P. mahali* lack a universal duet syllable repertoire and thus a species-specific, stereotyped duet song is lacking. Instead, each individual produces a vocal repertoire that is shared to a variable degree with different conspecifics of the same sex. These findings suggest that *P. mahali* exhibits a flexible and variable vocal behavior, utilizing diverse acoustic elements to communicate with conspecifics rather than relying on a fixed set of stereotyped vocalizations. When a *P. mahali* disperses from its natal group to find a partner, it is likely not familiar with all syllable types and answering rules of the mate. Consequently, both individuals of the newly formed pair have to learn new duetting rules, to be able to produce well-honed duet songs together [78]. Although the ontogeny of pair-specific duet codes has not been studied in *P.mahali* in detail, research on canebrake wrens [76] and bay wrens [77] suggests how adult duet learning could potentially occur in *P. mahali*. For example, the canebrake wren employs a specific phrase repertoire to generate novel answering rules when establishing new pairs [79]. Therefore, duet code adherence is poor in the beginning until the phrase types of the new partner are integrated [79]. Concluding from this, the specific prosodic structure of the syllables in different syllable categories may support *P. mahali* in recognizing unknown syllable types produced by a new partner and accelerate the duet learning process. In many animal species, categorical perception enables receivers to assign a signal more consistently to a functional category and facilitates rapid decision-making in a critical context [80]. Categorization of song syllables has also been found in many bird species [70-73]. For example, the interactive value of syllable categories is particularly apparent in nightingales (*Luscinia megarhynchos*). Organizing syllables hierarchically into various categories allows nightingales to retrieve songs quickly in situations that demand a rapid vocal response during interactive countersinging [81]. We suggest that prosodic syllable categories support duet coordination in a similar manner. Choosing one specific syllable from the complete male syllable repertoire of approximately 60 different syllable types [26] to answer a specific female syllable is very likely more difficult and time consuming than using a syllable from the R-type category to answer a WM-type female syllable.

Interplay between central and peripheral vocal control mechanisms

Both sexes of *P. mahali* probably produce alternating syllable categories with a bias towards easier-to-produce transitions. Vocal patterns can indicate morphological or physiological constraints in the vocal production organ and in the respiratory system as well as the occurrence of motor biases due to the organization of central pattern generators for muscular and respiratory control [39]. In particular, canaries (*Serinus canaria*) and zebra finches produce songs that adhere to linguistic pattern independent of tutoring, thus suggesting that inherent biases in species-typical motor productions may cause the production of distinct vocal patterns [67]. In our study, we found anatomical differences between the right and the left side of the male *P. mahali* syrinx, which may build the basis for lateralization of sound production in this species. We show that the thickness of the left ML is increased in contrast to the right ML, and that the left ML contains a larger amount of collagen fibers, while both ML possess equal amounts of elastin fibers. In other songbird species, a thinner right medial labium, is believed to be more suitable to produce high frequency sounds [28, 32]. There is no evidence for this hypothesis in the white-browed sparrow weaver. However, the difference in elastin and collagen components makes each ML especially suited to produce sounds with a certain frequency bandwidth [28, 39]. Due to the higher collagen content, which can increase tissue stiffness, the left ML in the *P. mahali* syrinx may be able to resonate at higher frequencies and consequently produces higher-frequency sounds than the more elastic right ML.

The spectral features of vocalizations are regulated by the coordinated activation of respiratory and syringeal musculatures, which are controlled by motor neurons in the robust nucleus of the archopallium (RA) [82, 83]. This nucleus has two major projections, one to the ipsilateral hypoglossal nucleus (nXIIts), and the other to a series of interconnected vocal-respiratory nuclei in the ipsilateral brainstem. Song production in songbirds is governed by a distinct set of brain structures located within each hemisphere [82, 84]. However, these telencephalic nuclei lack interhemispheric connectivity [34, 50]. Despite this, song related neural activity is precisely synchronized across hemispheres via the utilization of shared inputs to the song control system originating from interhemispheric connections within the midbrain and hindbrain regions [48]. We hypothesized that the neural control of vocal output is lateralized in *P. mahali,* which is supported by the observed lateralization in the syrinx. Although RA is responsible for controlling the spectral structure of individual song syllables [45, 85], it acts in response to input from the HVC [83, 86]. Research has suggested that the HVC exhibits hemispheric asymmetry in its activity. In waterslager canaries, a canary strain exhibiting a pronounced functional bias towards the left side of the syrinx, song production was inhibited upon left HVC lesioning, while lesions of the right HVC had no effect [87]. In zebra finches, a songbird species with a symmetrical syrinx, a study including HVC lesions demonstrated that adult birds require an intact HVC in both hemispheres to produce song [52]. Despite HVC activity being highly synchronized over both hemispheres during singing, the lateralization of HVC functioning can occur by a “rapid switching” mechanism, a fast change of hierarchy between left and right HVC [52]. This mechanism is thought to be associated with a songbird’s capacity to control each half of the syrinx independently [33, 52].

Moreover, various experimental techniques, including the direct recording of HVC activity during song production, have highlighted the critical role of this nucleus in the sequencing of syllables [47, 88, 89] and the regulation of spectral syllable characteristics [90]. To gain a better understanding of HVC's involvement in the generation of vocal prosodic features, which requires both phonological and syntactic control, we analyzed neural data recorded from HVC in male *P. mahali* during the production of syllables of different categories. We found that HVC in the right hemisphere is active earlier during the production of R-type syllables than during the production of F-type syllables, which means that the right HVC is mainly active during the production of the low-frequency element of male duet syllables. In agreement with our assumption that the right side of the syrinx in male *P. mahali* is more suitable to produce low frequency sounds because it contains a low amount of collagen fibers, we find the right HVC activity being stronger associated with the production of the low frequency parts of duet syllables. This suggests that the left HVC in male *P. mahali* might be mainly active during the production of the high-frequency part of male duet syllables. Moreover, our data suggest a reciprocal activation of the HVCs of the two hemispheres during duetting. The identification of direct associations between patterns of neural activity in central brain regions and the movements generated by peripheral organs is challenging [91, 92]. The same fundamental frequency can be produced through different patterns of syringeal muscle activation in different syllabic contexts [93]. For example, in Bengalese finches, HVC demonstrated different levels of activity, depending on the position of the produced syllable in the song sequence [88]. Consequently, simultaneous recordings of song related activity in the left and the right HVC in male *P. mahali* will be necessary to finally confirm our hypothesis on interhemispheric switching of HVC activity in male *P. mahali* during duetting.

There are different theories about the neuronal mechanisms of sound generation in songbirds. For example, the gesture trajectory extrema (GTE) model describes the neural activity of HVC to be organized around motor gestures, which results in sparse neural firing in HVC neurons [94]. The production of vocal sounds involves specific motor gestures, such as manipulating air sac pressure and modulating tension in the syringeal labia to produce specific vocal components. HVC might control the motor gestures involved in sound production by spiking activity that occurs at the beginning and at the end of syllables, and correlates with the transitions between adjacent syllables. The differences in HVC activity that we demonstrated for the two male syllable categories in *P. mahali*, could well be attributed to variations in the underlying motor gestures used to generate syllables of these categories. Nevertheless, the GTE model is often opposed to the synfire chain model. This second model states that a chain of excitatory cells fires in succession throughout the duration of a song [86, 95]. Nevertheless, not every model seems to be applicable to every bird species. For instance, the HVC activity of zebra finches is most appropriately characterized by the chain model, whereas the GTE model is better suited to describe HVC activity in domestic canaries [96]. To understand what organization model underlies HVC activity during the production of prosodic categories, future studies will benefit from EMG recordings of syrinx muscles as well as recordings of air sac pressure traces in *P. mahali* during duet singing.

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Table 1. Overview of sample sizes

Bird	Stochastic model analysis	Neuronal analysis	Syrinx measurements
Male 1	19 songs, 38 R, 40 F syllables	37 songs, 57 R, 69 F syllables	
Male 2	32 songs, 73 R, 68 F syllables	61 songs, 72 R, 87 F syllables	
Male 3	31 songs, 56 R, 54 F syllables	No data	
Male 4	31 songs, 67 R, 74 F syllables	41 songs, 73 R, 98 F syllables	
Female 2	32 songs, 56 WM, 56 SM syllables		
Female 3	31 songs, 29 WM, 31 SM syllables		
Female 4	31 songs, 58 WM, 48 SM syllables		
Male 5			x
Male 6			x
Male 7			x

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Chapter 4

Competitive vocal interactions in male canaries: Context-dependent changes in song syntax and in vocal premotor activity

Abstract

Competitive vocal interactions have been studied in many bird species, mainly by recording vocal behavior in one bird that is presented with sound playback to simulate the conspecific competitor. The effects of natural competitive encounters between two birds on each bird's song syntax are not well studied. Here, we used a radio-telemetric method that enabled us to simultaneously record individual vocalizations and vocal premotor activity in male dyads of canaries (*Serinus canaria f. domestica*) during song competitions. Canary vocalization is composed of a series of distinct phrase types arranged in a sequence, which are constructed of repetitions of one syllable type. We found that a bird's social status can influence song structure: dominant males generally sang longer songs than subordinate birds. Furthermore, canaries modified the syntax of their songs when they were overlapped during singing by a conspecific. In contrast to song performed without competitor (solo song), overlapped song of the leading singer was characterized by an increased song complexity and duration. Birds that overlapped another bird's song did not change the global structure of their songs in comparison to their solo songs. Most interestingly, during the production of overlapping songs, activity in the vocal premotor nucleus HVC was stronger than during solo singing. These results highlight the importance of the social environment for bird song production, song syntax and vocal control.

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1 **Introduction**

Many songbird species engage in countersinging interactions with conspecifics [1, 2]. Countersinging serves as a fascinating behavioral model to study aggressive and sexual signaling [3-5], the underlying mechanisms of signal selection [6] and receiver physiology [7]. Countersinging requires precise, temporal coordination of vocal production with another individual [1, 2]. Here, we focused on a vocal behavior, where one bird is actively trying to interfere with the vocal output of a conspecific by overlapping the conspecific's song. Song overlapping requires that one bird starts singing before another singing bird has finished. This way, the vocalizations of the bird starting to sing later interfere with the vocal signals of the bird that started to sing first and may affect the function of the first bird's signal by acoustic masking. Overlapping vocal signals serve different signaling functions: they can be both aggressive and defensive signals [8, 9], they can signal male quality [10], or provide additional information to eavesdroppers [11].

So far, in studies on timing control during vocal interactions, experimental birds actively tried to avoid vocal interference. However, the mechanisms underlying the process of intentional song overlapping remain poorly understood. Songbirds can exercise rapid and flexible control of vocal timing due to a unique neural circuit in the forebrain – the "song control system" [12, 13]. Within this circuit, the vocal premotor nucleus HVC, which also receives auditory information, generates temporally patterned premotor commands for vocal production [14-16]. HVC plays a crucial role in timing of individual vocal units and their arrangement into sequences [17, 18]. For example, cooling HVC in Bengalese finches (*Lonchura striata*) resulted in changes to the song tempo and compromised the bird's ability to produce particular song sequences [19]. Recent studies on turn-taking call interactions demonstrated that HVC drastically influences a zebra finch's (*Taeniopygia guttata*) ability to control vocal response latencies in different social interactions [20, 21]. Blockage of inhibitory neural activity in HVC facilitated faster responses to conspecific vocalizations, which resulted in call overlapping [20, 22]. Further experiments demonstrated that auditory evoked inhibition in HVC suppresses premotor drive, which consequently reduces signal jamming [20, 23]. A study on timing control in duetting birds also suggests that coordination of HVC premotor activity between duet partners occurs through inhibition caused by auditory feedback [24]. In a different duetting bird species, the partner's vocal onset indeed triggered a change in rhythm in the periodic neural discharges of the duet-initiating bird [25].

Our study investigates the song modulation and its underlying neural mechanisms during countersinging interactions in the domestic canary (*Serinus canaria*). The canary is known for its high vocal plasticity. Songs consist of a sequence of different phrase types, which are constructed of repetitions of one syllable type [26]. During countersinging, canaries often perform overlapping interactions [27]. Here, the function of overlapping the song of a conspecific is to prevent the bird that starts to sing first from effectively conveying information to the receiver [1, 2]. In addition, female and male birds eavesdrop on overlapping interactions to guide their behavior. From overlapping songs, female canaries can evaluate relative quality of a possible mating partner [28, 29]. Females prefer males that produce song overlap over males whose songs are being overlapped [28, 29], and invest more yolk in eggs fertilized by males who sing songs that overlap other males' songs [30]. Male canaries use song overlaps to evaluate the relative threat of competitor birds and use overlapped singing to signal dominance [10]. In a study by [31], male canaries listened to playbacks simulating an individual that masked the song of its opponents by producing overlaps. The listening birds decreased their own vocalization rate in response to the playback of overlapped songs [10, 29]. Several studies suggest that canaries are able to perform moment-by-moment song adjustments during overlapping interactions. Male canaries can differentiate and selectively attend to a target conspecific vocalization even if the conspecific's song is masked by another conspecific's song [31]. Furthermore, when their song is masked by noise playback, canaries can swiftly adjust the song amplitude mid-phrase without pausing to sing [32]. Recent work on vocal competition in canaries demonstrated that the winner of a competition sings longer songs than its rival, but the spectral features of the rivals' songs remain similar [27].The structural changes in the song that underlie the modifications in song duration are so far not known. Furthermore, it is unclear if canaries alter song syntax during countersinging. We speculate that the difference in song duration, which is observed during countersinging interactions, can be explained by syntactic changes to the song. In male-male countersinging interactions, song syntax appears to play a role in several species. For instance, male Cassin's vireos (*Vireo cassinii*) choose matching syllable types while accounting for song syntax [6] and house finches (*Haemorhous mexicanus)* increase within-song syntactical diversity in countersinging interactions [33]. Interindividual differences in canary song syntax especially exist for sequences of three and more phrases [34]. As an open-ended learner, domesticated canaries change their repertoire of phrase types and phrase transitions seasonally during adulthood, which leads to a larger variability in the song outside the breeding season [35]. Social context also seems to affect the phrase transition repertoire since canaries that are co-housed for longer time periods, start to share song sequences [36]. Finally, higher-order context dependencies predict the transition between individual phrases in canary song: a certain phrase in a sequence depends on more than one (up to five) preceding phrases [37].

Our data demonstrate that canaries, participating in overlapping singing interactions, change the structure of their song in comparison to songs that were produced solo. Syntax modifications as a result of song overlapping, are an increase in number and length of phrases that consist of rapidly repeated syllables, which caused a general increase in song complexity and duration. Moreover, we demonstrated that vocal premotor activity changes depending on the social context.

1 Material and Methods

1.1 Subjects and housing

In this experiment, we recorded vocal activity from six adult male canaries (*S. canaria*), forming 22 male-male overlapping interaction dyads (see Table 2), and neural activity from two of the six birds. Two repetitions of the experiment were performed, one in May 2021 and one in June 2021. Male 1 to Male 4 participated in the first experimental cycle, and Male 5, 6, 3a and 4a in the second experimental cycle. Please note that Male 3 and Male 4 are the same individuals as Male 3a and Male 4a, respectively. Birds were randomly assigned to groups that were housed for the entire experiment in individual aviaries of 1m width x 1m height x 1m depth, which were located within a large outdoor aviary, allowing a natural day light cycle. Each group consisted of one female (to elicit singing behavior in the males) and two male canaries. In each experimental cycle, two groups were kept in neighboring aviaries, preventing visual but allowing acoustic contact between the birds (Figure 1A). The aviaries were equipped with branches and perches, sand baths and grit, and the birds had ad libitum access to water and food (seed mix, boiled eggs and greens) throughout the experiment. All experimental procedures were ethically approved by the government of Upper Bavaria (record number: ROB-55.2-

2532.Vet_02-20-47). All further animal husbandry or handling was conducted according to the directives 2010/63/EU of the European parliament and of the council of 22nd September 2010 on the protection of animals used for scientific purposes.

1.2 Song recordings

To monitor individual vocal activity, we equipped both male birds in each aviary with radio-telemetric transmitters developed at the Max Planck Institute for Ornithology in Seewiesen, Germany, [Figure 1B; 38, 39]. The lightweight (0.6 g) vocal transmitter, which included a miniaturized microphone (FG23329, Knowles Electronics, USA) and a battery (Activ Air 10, Duracell, Germany), was covered by a thin silicon casing and fixed on the back of the bird with cotton-covered rubber band straps around both femurs and the abdomen. The transmission range of the transmitter averaged 15 meters, and the battery life was 12 days. Carrying these microphone transmitters has only weak and short-term suppressive effects on songbirds' vocal and movement activity [38]. A crossed Yagi antenna (Winkler Antennenbau, Germany) was placed above the two aviaries for signal detection. An antenna amplifier (TVS 14-00 axing, Goobay, Germany) increased the antenna signal by 18 dB. The signal was split (BE 2-01 premium-line, Switzerland) and fed into communication receivers (AOR 8600, AOR Ltd., Japan), which were modified to handle 12 kHz audio bandwidth. The analog signals were digitized by an audio A/D converter (M-Track Eight, M-Audio, USA; sampling rate: 22050 Hz) connected to a computer. All digitized signals were recorded in parallel as continuous four-hour sound files using multichannel software (16-bit, 22050 Hz; ASIO, Germany). For each male-male dyad, vocal recordings were continuously conducted over several consecutive days.

1.3 Neural recordings

In addition to the vocal transmitter, one male bird in each of the two groups of the first experimental cycle were equipped with in-house manufactured neural transmitters [40; Figure 1B]. Under isoflurane anesthesia (1.5–1.8% isoflurane in 0.5 l O_2 min^{-1}, IsofluranCP, CP-pharma, Germany) and analgesic treatment (0.2–0.5 mg per kg bodyweight meloxicam 5.0 mg ml^{-1}, Metacam, Boehringer-Ingelheim, Germany), a single parylene-coated tungsten electrode (FHC, USA; impedance: 2.0 MΩ) was surgically implanted in the bird’s right HVC with the help of a SM-5 (Luigs and Neumann GmbH, Germany) remotely controlled micromanipulator. In addition, a reference electrode (platinum wire with a diameter of 50 µm, Advent Research Materials, UK) was inserted between the skull and the dura mater. The electrodes were fixed to the skull using dental cement (Tetric evoflow, Ivoclar Vivadent, Liechtenstein), and the neural transmitter (weight: 1.0 g) was subsequently connected to the electrodes' pins. The transmission range of the device was about 50 m, with a battery life of about seven days. All birds recovered quickly from the treatment and were released to their aviaries after mounting of the transmitters. Recording of neural and vocal activity started subsequently. The neural activity of the two male birds were telemetrically recorded for up to four days in parallel to their individual vocal activity as described above. Frequent monitoring of the treated birds certified that they did not show any sign of discomfort or altered behavior due to the treatment. At the end of the experiments, the birds were euthanized for brain sampling, after an electrolytic lesion for verification of the electrode's placement has been generated via the recording electrode.

1.4 Song analysis

Song overlapping is defined as a behavior in which an individual begins singing before its opponent has completed its song [2]. To analyze our vocalization data, songs were detected and cut out of the four-hour sound files with the software Audacity (ver. 2.3.2, Audacity Team, <www.audacityteam.org>). A song was defined as a continuous string of vocal emissions that was surrounded by at least 1.5 s of silence. All songs were subsequently classified into one of the following three categories (Figure 1C):

1. "Leading songs": Songs that were produced first in a song interaction, independent on how many individuals participated in the interaction.
2. "Trailing songs": Songs that overlapped the song of any other individual during a song interaction
3. "Solo songs": Songs performed by one individual, without interacting with other conspecifics.

Due to the birds' housing conditions in our study, it was possible for more than two birds to overlap their vocal productions. A male could interact vocally with its cage mate, and/or with the males in the neighboring aviary. In the case of choruses, where more than two birds interacted vocally, the songs of each bird in each possible dyadic combination of interacting males were analyzed independently, and only the very first song in the interaction was considered a leading song (Figure 1C). When the song interaction was very complex and the birds repeatedly overlapped each other's songs, each overlapping bird's song was considered a trailing song. We used the SONG (Song Overlap Null model Generator) package available in R (R Core Team 2020) to investigate if overlapped singing occurred intentionally or by chance [41]. SONG compares the observed rate of overlapping vocal interactions with an expected rate that is calculated via the generation of randomized performances. We used the "SampleGaps method" [41] to randomize instances of overlapping interactions by rearranging songs and placing them at random intervals while keeping the observed song length. The duration of overlapping songs in randomized interactions provides a null distribution for statistical comparison. A p-value ≤ 0.025 signals intentional overlapping, while a p-value ≥ 0.975 signals overlap avoidance.

1.4.1 Phrase clustering

Canary song is composed of phrases consisting of multiple repetitions of the same syllable type [26, 37]. To investigate the song syntax, we uploaded all songs to a web-based software (KOE, ver. 5.4.1, <https://koe.io.ac.nz>, [42]) and manually segmented them into phrases. We used an interactive ordination plot created by KOE as a basis for further manual audiovisual phrase classification. KOE automatically extracted various temporal and spectral phrase features to calculate an ordination between phrases based on t-distributed stochastic neighbor embedding analysis. We audio-visually inspected the resulting phrase clusters to classify them into phrase types. Subsequently, each song was automatically converted into a sequence of phrase types.

During the breeding season, canaries produce specific song syllables by using both sides of their vocal organ, the syrinx. These so-called “A-syllables” or “sexy syllables” are hypothesized to indicate a male’s fitness as they are difficult to produce [43]. A-syllables are composed of two vocal units spanning a wide frequency bandwidth of about four kHz. The phrases constructed from A-syllables have high syllable repetition rates that exceed 15 syllables per second [44, 45]. We used this value of syllable repetition rate to categorize phrase types into either regular phrases (syllable repetition rate <15 syllables per second) or F-phrases (syllable repetition rate >15 syllables per second), which included A-phrases, and which could potentially be attractive for females (Figure 1D).

1.4.2 Song sequence analysis

1.4.2.1 Global song network properties

To find and evaluate social context dependent differences in song behavior, we first calculated conventional measures of song complexity, such as the phrase repertoire size, the phrase transition repertoire size, the number of phrase transitions per song and the song duration. Moreover, we applied network analyses to describe context-dependent changes in song syntax. Network analysis proved to be a suitable method to visualize and assess the global transition structure of complex birdsongs [33, 46-48]. First, for each bird, we transformed all phrase type sequences off all recorded songs into an adjacency matrix [49]. The adjacency matrix reflected the number of times individual transitions occurred between two phrase types based on their order within the song. Then we used a Fisher exact test to validate the transitions between phrase types in each matrix and excluded insignificant ($p>0.05$) transitions. Finally, we generated networks for each social context from the resulting adjacency matrixes using the *igraph* package [50] in R. The produced networks are structured as directed weighted networks without loops (transitions occurred between the same phrase type). This network organization was chosen to determine the direction of a transition between distinct phrase types, and the probability of the transition to occur inside a song. In such networks, nodes represent phrase types, and edges represented first-order transitions from one phrase type to another. The rates of transitions between different phrase types is depicted by the thickness of arrows between the nodes. Afterwards, we compared each observed network to a random Erdös–Rényi network [46], which was constructed by permuting the same number of nodes. The nodes in the random network had the same average number of edges per node (i.e. Average degree) as the nodes in the observed network.

We extracted three network measurements, the network degree, the network density and the small-world index, which are commonly used to characterize song network structure [33, 46-48, 51, 52] and to evaluate the flexibility of transitions between vocal units:

1. The network degree reflects the diversity of phrase type transitions, where high degree values indicate that many phrase types connect to many other phrase types. The average network degree measures the total number of a node’s connections with preceding (in-degree) and subsequent (out-degree) nodes in the sequence.

2. The network density displays how strongly phrase types are connected in comparison to how strongly they could be connected. Higher density values reflect greater flexibility in the transitions between phrase types and thus a weaker connection. The network density measures the ratio of the number of observed phrase type transitions to the number of potentially occurring phrase type transitions.
3. The small-world index determines if the network matches properties of a small-world network. Small-world networks are characterized by nodes that are organized within highly connected groups, which have only few long-distance connections between them. Several animal repertoires follow small-world organizations, allowing efficient control over vocal production and information flow [46, 47, 52, 53]. The small-world network index (s) was calculated using the characteristic shortest path lengths (L) and the clustering coefficient (C) of the randomized and the observed network:

$$\boldsymbol{S} = \frac{\boldsymbol{C_{obs}/C_{rand}}}{\boldsymbol{L_{obs}/L_{rand}}}$$

The characteristic path length measures the average minimum number of transitions occurring between pairs of nodes in the network, while the clustering coefficient measures how strongly connected neighboring nodes are to each other. Networks with a small-world index above one can be recognized as small-world.

1.4.2.2 Phrase transition preferences

We analyzed context-dependent and interindividual levels of phrase type repertoire sharing. To access which phrase type transitions males prefer to use in different social contexts, we tested the similarity of phrase type transitions between individuals and between contexts according to Hedley, Logue [54]. We applied two Fisher exact tests to each adjacency matrix: The first test was used to determine significant phrase type transitions, and the second test was used to determine whether the number of phrase type transitions that were shared between different social contexts/different males was greater than expected by chance. According to Searcy [55], we named transitions that were shared between different social contexts as "consistently preferred transitions", and named transitions shared between different males as "mutually preferred transitions" after Hedley [54].

The sharing of phrase type and transition type repertoires between social contexts and between males was analyzed using the song sharing index:

$$I = 2Ns/(2Ns + a + b)$$

Ns is the number of phrase or transition types that are shared between two contexts or between two individuals, and a and b are the numbers of unshared phrase or transition types for each context or individual [56]. The index can assume values between zero and one, with zero indicating the lowest sharing level.

Additionally, we compared phrase type transitions in the overlapped and the nonoverlapped parts of leading songs with phrase type transitions in solo and in trailing songs to determine if leading songs have specific transitions at their beginning that can potentially induce countersinging interactions.

1.4.2.3 Local structural song properties

To determine the local changes in phrase type transitions in different social contexts, we employed an analysis of phrase transition patterns [46, 47, 52]. Transition 'patterns' are defined by the ratio between the number of incoming (indegree) and the number of outgoing (outdegree) transitions for each phrase type. Overall, four categories of transition patterns exist (Figure 1E):

1. "One-way": This type of deterministic pattern includes phrase types that transition from exactly one preceding phrase type to exactly one subsequent phrase type (indegree=1, outdegree=1).
2. "Bottleneck": This deterministic pattern includes phrase types that transition from many preceding phrase types to only few subsequent phrase types (indegree>outdegree).
3. ”Hourglass”: This non-deterministic pattern includes phrase types that transition from many preceding to many subsequent phrase types (indegree>1 and outdegree>1).
4. "Branch points": This non-deterministic pattern includes phrase types that transition from only a few preceding phrase types to many subsequent phrase types (indegree<outdegree).

After determining the transition patterns for phrase types that were shared between different social contexts, we calculated the percentage of phrase types that "rewire" – which means they change their transition pattern between social contexts.

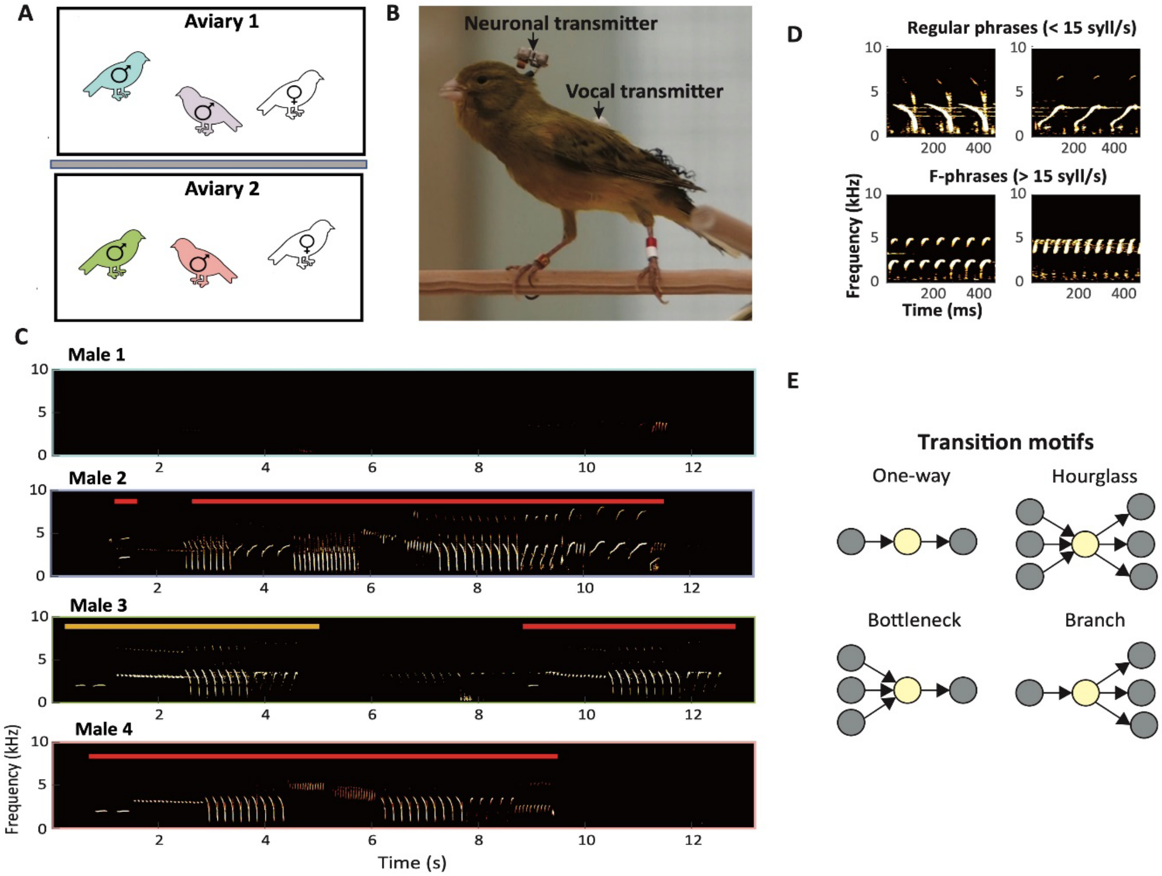


Figure 1. *Song behavior during overlapping interactions in canaries. (A) Schematic of the bird's housing arrangement. (B) Male canary carrying a vocal and a neural radio-telemetric transmitter. (C) Spectrograms of sound traces produced by four vocally interacting male canaries, simultaneously recorded with vocal transmitters. The leading song in this interaction is highlighted in orange, while trailing songs are highlighted in red. (D) Spectrograms of two examples each of phrase types belonging to the Regular phrase and the F-phrase category. (E) Schematic representation of the four types of phrase transitions patterns. Circles represent phrase types and arrows indicate phrase type transitions. Adapted from Sasahara et al. (2012).*

1.4.3 Statistical analysis

The values of network measurements can correlate with conventional measurements of song complexity such as phrase type repertoire size, and with overall sample size variables, such as number of recorded phrases. To estimate the interactions between the different measurements, we calculated Pearson's correlation coefficients with Bonferroni correction for multiple testing. Beforehand, we logarithmically transformed the following variables Network degree, Network density and Simple paths, to be normally distributed.

We constructed linear mixed models (LMMs) using *lme4* package [57, 58] in R to evaluate the effect of social context and social status on network-based and conventional measurements of song complexity. For each model, we included the identity of the bird and the housing condition as random factors, and the social context together with the social status as fixed factors. For the network measurements, we corrected all values for the number of nodes (repertoire size) and sample size (number of recorded phrases, number of recorded songs) and incorporated them as additional fixed factors based on the results of the Pearson's correlation. For conventional measurements of song complexity (phrase type transition repertoire, number of transitions per song), we also corrected for the number of nodes (repertoire size) and sample size (number of recorded songs). For significant models, we performed a post hoc Turkey test for multiple comparisons using the R package *multcomp* [59]. To compare differences in phrase and transition type repertoire sharing between different birds that sing in different contexts, we used the lme4 package, [57, 58] to generate LMMs in R. In the models, we included the identity of the bird as random factor, and the social context as fixed factor.

1.4.4 Analysis of neural data

To extract spike times from the continuously recorded neural signals, for each bird, each neural trace was initially bandpass filtered (200–4000 Hz, fourth-order Butterworth) using the MATLAB® filtfilt function. For each song within a four-hour sound file, a threshold for discriminating spikes from background activity was determined by calculating (MATLAB®) the mean of the neural signal for the time of song duration and adding five standard deviations. The filtered neural signals were then fed into the software Spike2® (CED, UK), and spikes were automatically discriminated from background activity by setting a threshold to the value calculated as described above. We then generated LMMs (*lme4* package, R) to test for statistical significance of the observed effect of social context on HVC firing rate, with social context as fixed factor and song and bird ID as random factors.

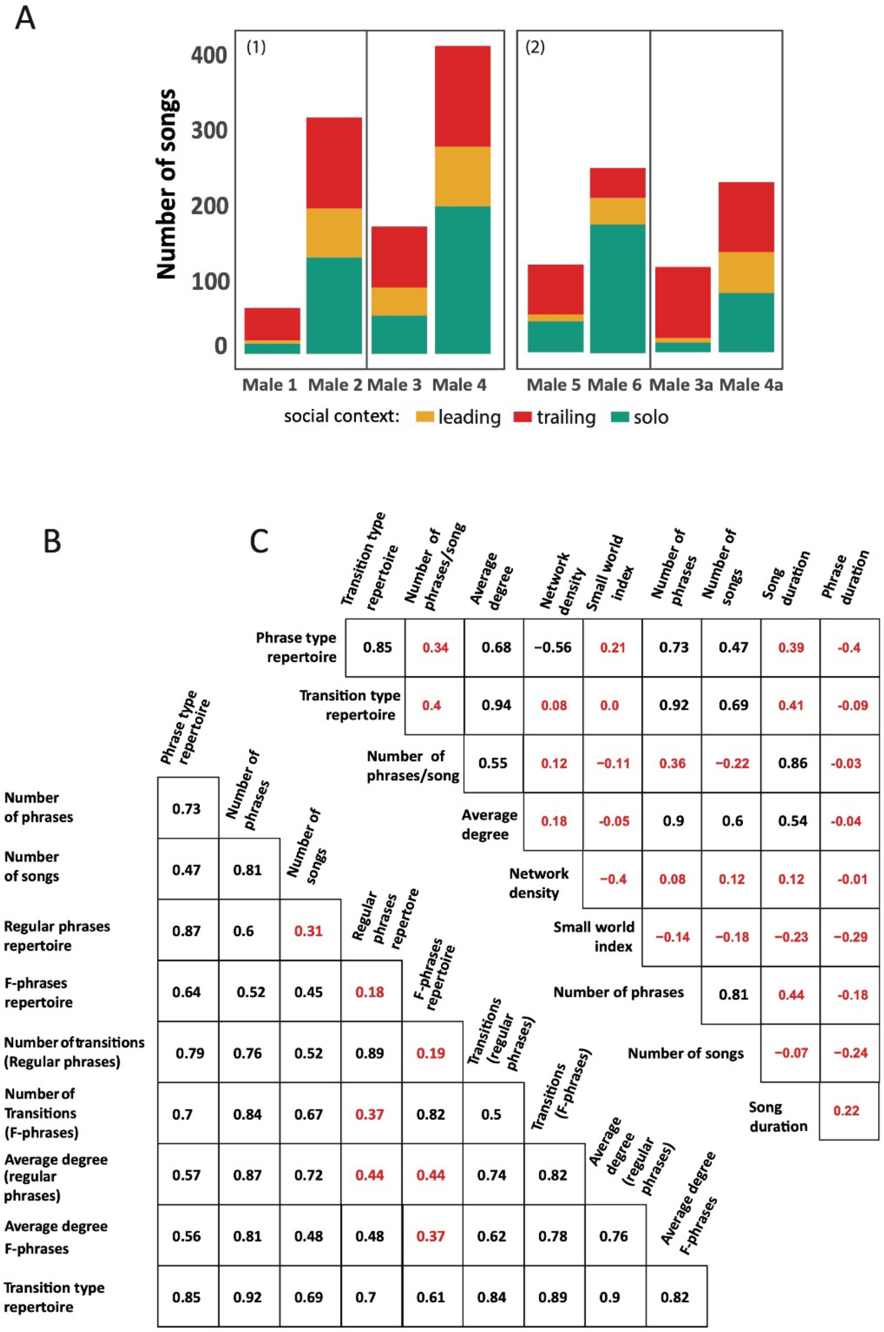
2 Results

In total, we recorded 38 hours of acoustic data from six different birds, forming 11 vocally interacting pairs in two experimental cycles (two individuals participated in both cycles). In the first cycle, Male 1 and Male 2 in one aviary, and Male 3 and Male 4 in the neighboring aviary) constructed six interactive dyads. In the second cycle, Male 5 and Male 6 in one aviary, and Male 3a and Male 4a (same individuals as Male 3 and Male 4) in the neighboring aviary also formed six interactive dyads. However, since interactions between Male 3 and Male 4 have already been measured in the first cycle, we excluded interactions between Male 3a and Male 4a from the analysis, leaving data from 11 interacting pairs for analysis. However, in an interaction, each male can assume one of two roles. The male can either produce leading song, which is overlapped by the song of the interaction partner, or the male can produce trailing song, which is overlapping the song of the interaction partner. Thus, overall, we analyzed data from 22 possible vocal interactions dynamics. After testing for the significance of overlapping interactions within dyads, we excluded data from dyads that did not show significant (i.e. unintentional) overlapping interactions from further song network analysis. Network analysis was performed for each individual and each social context by pooling all songs that were performed under a specific social context by one bird independent on the identity of the interaction partner. Male 3 and Male 3a, and Male 4 and Male 4a were analyzed as separate individuals in each experimental cycle, since they were participating in vocal interactions with different birds in each cycle (interactions between Male 3a and Male 4a were excluded from the analysis). We specified the individuality of Male 3 and Male 4 in the mixed model analysis to avoid statistical mistakes due to repeated measurements. All in all, we analyzed 1688 songs performed under different social contexts for all experimental birds (Table 1).

2.1 Overlapping interactions

The social group structure elicited competitive singing behavior in the male canaries within one aviary but also across the two neighboring aviaries. When housed together in dyads, male canaries establish a dominance-subordination relationship [60], where dominant males have higher singing rates than subordinates. The social status may potentially affect vocal behaviour in overlapping vocal interactions [61]. To determine the social status of each male in each aviary, we counted the overall number of songs performed by each individual, along with the number of songs belonging to each social category (Fig. 2A, Table 1). For both, the leading and the trailing social context, we counted all songs sung by one individual, independent on who was the interaction partner. We found that in each aviary, one male bird generally sung more frequently than the other male. According to the results of Boseret [60], the male that sung more often was assigned the status of the dominant male of the aviary, while the second male in the aviary was considered to be the subordinate individual.

Dominant males in this experiment used to overlap both the song of their subordinate male cage mate and the song of the subordinate male housed in the neighboring aviary. We assigned all vocal interactions to a specific male-male interaction dyad (Table 2). It is important to note that in each pair, males could interact in two directions: 1) Male A overlaps Male B, and 2) Male B overlaps Male A. Thus, each possible combination of two males represented two interaction dyads. Since we excluded songs sung during interactions between Male 3a and Male 4a, the following analysis was performed on 22 male-male interaction dyads. In 82% (18/22) of all possible one-directional male-male interaction dyads, overlapping interactions happened for a significantly longer duration than expected by chance and were therefore assumed to be performed intentionally (Table 2). While in the first experimental cycle, all males intentionally overlapped each other (see methods), in the second cycle of experiments, one subordinate male, Male 3a, unintentionally overlapped the dominant male (Male 6, SONG, p=0.07) and the subordinate male (Male 5, SONG, p=0.11) of the neighboring aviary. Furthermore, the dominant male, Male 6, unintentionally overlapped the subordinate male in the neighboring aviary (Male 3a, SONG, p=0.05), and the subordinate male, Male 5, unintentionally overlapped its dominant cage mate (Male 6, SONG, p=0.46). Consequently, we removed all songs sung during interactions between Male 3a and Male 6 and all songs of Male 5 that were overlapped by Male 3a or by Male 6 from further analysis on song syntax. Generally, dominant males participated in overlapping interactions significantly more often, than subordinate males (Two-way ANOVA, F (1,16) =7.72, p=0.013, N=22 interaction dyads).



B

	Phrase type repertoire	Number of phrases	Number of songs	Regular phrases repertoire	F-phrases repertoire	Transitions (regular phrases)	Transitions (F-phrases)	Average degree (regular phrases)	Average degree F-phrases
Number of phrases	0.73								
Number of songs	0.47	0.81							
Regular phrases repertoire	0.87	0.6	0.31						
F-phrases repertoire	0.64	0.52	0.45	0.18					
Number of transitions (Regular phrases)	0.79	0.76	0.52	0.89	0.19				
Number of Transitions (F-phrases)	0.7	0.84	0.67	0.37	0.82	0.5			
Average degree (regular phrases)	0.57	0.87	0.72	0.44	0.44	0.74	0.82		
Average degree F-phrases	0.56	0.81	0.48	0.48	0.37	0.62	0.78	0.76	
Transition type repertoire	0.85	0.92	0.69	0.7	0.61	0.84	0.89	0.9	0.82

C

	Transition type repertoire	Number of phrases/song	Average degree	Network density	Small world index	Number of phrases	Number of songs	Song duration	Phrase duration
Phrase type repertoire	0.85	0.34	0.68	−0.56	0.21	0.73	0.47	0.39	-0.4
Transition type repertoire		0.4	0.94	0.08	0.0	0.92	0.69	0.41	-0.09
Number of phrases/song			0.55	0.12	−0.11	0.36	−0.22	0.86	-0.03
Average degree				0.18	−0.05	0.9	0.6	0.54	-0.04
Network density					−0.4	0.08	0.12	0.12	-0.01
Small world index						−0.14	−0.18	−0.23	−0.29
Number of phrases							0.81	0.44	-0.18
Number of songs								−0.07	−0.24
Song duration									0.22

Figure 2. *(A) Independent of the identity of the interaction partner, the number of songs sung by each male canary differs between social contexts in both the first (1) and the second (2) experimental cycle. Leading songs are highlighted in orange, trailing songs are highlighted in red and solo songs are highlighted in green. (B) Color-coded Pearson correlation coefficients for correlations between sample size measurements, conventional measurements and network measurements for F- and Regular phrases. (C) Color-coded Pearson correlation coefficients for correlations between sample size measurements, conventional measurements and network measurements independent of the phrase type. red: not significant black: significant, p<0.05*

2.2 General patterns in song production and song sequence networks.

The songs of male canaries are composed of different phrase types, which can be ordered in multiple ways. On average, a male's phrase type repertoire consisted of 22±4.28 phrase types and 30.35±10.64 phrase transition types. The six birds sang on average 3.6±1.53 phrase types in one song (Table 1). These observations are consistent with previous studies on canary song structure and repertoire [26, 37].

To access the overall structure of song for each canary in each experimental cycle (N = 8 birds), we performed a network analysis on the song sequences that were sung in different social contexts, independent on the identity of the interaction partner. After comparing individual networks to a randomly generated Erdös–Rényi network, we excluded the following songs from the network analysis and consequently from the analysis of song complexity, because the sample size was too small to construct a network: all leading songs of Male 1, all leading songs of Male 5, and all leading and solo songs of Male 3a. However, we included these songs in the analysis on duration metrics (song duration, phrase duration, number of phrases per song), since these measurements were not directly affected by network measurements.

Overall, the structure of the canaries' song network can be described as follows: The average degree (i.e. the average number of transitions per phrase type) was 2.62±0.58. Most of the transition patterns in canary songs were deterministic (Two- way ANOVA, F (1, 34) = 52.04, $p < 0.001$; Eta2 (partial) = 0.60, 95% CI [0.42, 1.00], N=41 transition patterns), with the One-way pattern occurring 36% more often than other patterns (Two-way ANOVA, Pot hoc Turkey test, F (3, 68) = 52.37, $p < 0.001$; Eta2 (partial) = 0.70, 95% CI [0.60, 1.00]). Aside of Male 2, small-world indices for each bird had values of larger than one but smaller than three with an average of 1.4±0.66, indicating some degree of small-world topology. Therefore, some phrase types appeared in highly interconnected groups within song sequences. However, most phrase types were connected rather sparsely. The sparsity of the network can also be seen in the low value of the overall average network density of 0.06±0.01, which indicates that canaries performed only 6% of all theoretically possible transitions. The median weight of transitions was 0.2±0.08, and the median weight of half of the transitions ranged between 0.04 and 0.15.

A Pearson correlation analysis demonstrated that sample size metrics strongly and significantly affected several conventional song complexity metrics and network variables (Figure 2B, 2C). The size of phrase and transition type repertoires increased with the number of recorded songs (Pearson's correlation, phrase type repertoire size, R = 0.47, p=0.035; transition type repertoire size, R = 0.69, p<0.001, N = 20 observations) and with the overall number of recorded phrases (Pearson's correlation, phrase type repertoire size, R = 0.73, p<0.001; transition type repertoire size, R = 0.92, p<0.001, N = 20 observations). These observations parallel the finding of significant correlations between both F - and regular phrase type repertoire sizes and some sample size metrics (Pearson's correlation, F - phrase type repertoire size/ number of recorded songs, R = 0.45, p=0.045, N = 20 observations; regular phrase type repertoire size/ number of recorded phrases, R = 0.6, p=0.005, N = 20 observations) with the exception of an insignificant correlation between regular phrase type repertoire size and the number of recorded songs. While the average network degree correlated significantly and positively with the size of the phrase type repertoire (Pearson's correlation, R=0.68, p<0.001, N =20 observations) and with the size of the phrase transition type repertoire (Pearson's correlation, R=0.94, p<0.001, N = 20 observations , the network density correlated significantly and negatively with the size of the phrase type repertoire (Pearson's correlation, R=-0.48, p=0.031, N = 20 observations, while network density did not correlate with the size of the phrase transition type repertoire~~.~~The small-world index did not correlate with any conventional or network metrics (Figure 2C). Song duration correlated significantly and strongly with the number of phrases per song (Pearson's correlation, R=0.86, p<0.001, N = 20 observations) and correlated significantly with the average degree (Pearson's correlation, R=0.58, p=0.013, N = 20 observations), but not with the phrase duration.

2.3 Effects of social context and social status on song structure.

Independent of the identity of the interaction partner, the social context significantly influenced several conventional measures of syntax complexity (Figure 3A). The phrase transition type repertoire was significantly larger in leading songs than in trailing songs (LMM, estimate: +2.94, 95% CI [0.75, 5.13], t(10) = 2.99, p = 0.014; Std. beta = 0.27, 95% CI [0.07, 0.47], N = 8 birds). Post hoc Tukey contrast tests demonstrated that leading songs included 2.94±0.98 more phrase transition types than trailing songs (Post hoc Tukey test, estimate: 2.94±0.98, p =0.008, N = 8 birds), and 2.5±1 more transition types than solo songs (Post hoc Tukey test, estimate: 2.5±1, p =0.014, N = 8 birds). At the same time, trailing and solo songs did not differ in the size of their phrase transition type repertoire. The size of the phrase type repertoire did not differ between social contexts. Canaries utilized 1.36 ±0.55 more phrase types in leading songs than in trailing songs (Post hoc Tukey test, estimate: 1.36±0.55, p=0.042, N = 8 birds), but did not alter the number of phrase types between leading and solo songs. Canaries performed more phrases per song (LMM, estimate: +1.19, 95% CI [0.37, 2.01], t(17) = 3.07, p = 0.007; Std. beta = 0.63, 95% CI [0.2, 1.06], N = 8 birds) in leading songs, than in solo (Post hoc Tukey test, estimate: 1.01±0.38, p=0.019, N = 8 birds) and trailing (Post hoc Tukey test, estimate: 1.19±0.38, p=0.00635, N = 8 birds) songs. The number of phrase types did not differ between solo and trailing songs. The overall duration of leading songs was significantly (LMM, estimate: +1.37, 95% CI [0.59, 2.23], t(17) = 7.18, p = 0.004; Std. beta = 0.76, 95% CI [0.28, 1.24], N = 8 birds) longer than the duration of solo (Post hoc Tukey test, estimate: 1.45±0.4, p=0.002, N = 8 birds), which replicates the finding of Alcami [27]. The duration of leading songs was also longer than the duration of trailing (Post hoc Tukey test, estimate: 1.36±0.48, p=0.002, N = 8 birds) songs, but the duration of solo and trailing songs did not differ. However, the duration of phrases did not differ between leading, trailing and solo songs.

Among network measurements of syntax complexity, only the average network degree significantly varied between social contexts (LMM, estimate: +0.25, 95% CI [0.05, 0.46], t(10) = 2.80, p = 0.019; Std. beta = 0.43, 95% CI [0.09, 0.77], N = 8 birds), while network density did not. While leading songs had a higher average network degree than solo songs (Post hoc Tukey test, estimate: 0.26 ±0.9, p =0.016, N = 8 birds) and trailing songs (Post hoc Tukey test, estimate: 0.254±0.091, p =0.016, N = 8 birds), the average network degree did not differ between trailing and solo songs. Male canaries increased the average network degree of phrase types, and consequently the diversity of phrase type transitions in leading songs by 10 % in comparison to the average network degree in trailing and solo songs (mean and standard deviation: 2.62±0.6). The small-world index did not show any significant differences between songs sung in different social contexts.

The male's social status did neither influence the global song network properties nor the usage of F- and regular phrase types (Figure 3B). However, the social status strongly affected the duration of the song: subordinate males sang songs of significantly shorter duration (LMM, estimate: -1.85, 95% CI [-2.55, -1.15], t(17) = 4.87, p <0.001; Std. beta = -1.03, 95% CI [-1.42, -0.64], N = 8 birds) than dominant males. The subordinate males' song duration was 32 % shorter than the song duration of dominant males averaged across different social contexts (mean duration and standard deviation for dominant males: 5.66±1.3). A bird's status, however, did not affect the phrase duration and the number of phrases per song.

Next, we looked at the context-dependent utilization of F- and regular phrase types (Figure 3B). Regular phrase types showed no context-dependent changes, neither in repertoire size, nor in transition type repertoire size nor average degree. Similarly, the F-phrase type repertoire size and the average network degree of F- phrase types also did not differ significantly between social contexts. However, the overall number of transition types was significantly higher for F-phrase types within leading songs (LMM, estimate: 2.79, 95% CI [1.49, 4.10], t(10) = 4.78, p<0.001; Std. beta = 0.41, 95% CI [0.22, 0.60], N = 8 birds) than for F-phrase types within trailing (Post hoc Tukey test, estimate: 2.79±0.58, p<0.001, N = 8 birds) or solo (Post hoc Tukey test, estimate: -3.52±0.64, p<0.001, N = 8 birds) songs. The number of F-phrase type transitions in trailing songs did not differ from the number of F-phrase type transitions in solo song.

Although the network structure of solo and trailing songs did not differ in their global properties, we demonstrated local structural changes between songs sung in these two social contexts by tracking the changes in transition patterns of individual phrases. Interestingly, for 50±9.8% of phrase types (range: 33.0-66.6%), the transition pattern differed between solo and trailing songs (see Figure 4A for an example), while the overall number of deterministic and nondeterministic transition patterns and also the proportion of different transition patterns in the transition pattern repertoire did not differ between these two contexts.

Next, we looked at the existence of consistently preferred phrase and transition types in the canaries' songs. Although the social context did not affect the phrase type repertoire size, canaries used different types of phrase transitions depending on the social context (Figure 4B). Overall, we found a high percentage of phrase type sharing between leading, solo and trailing songs. On average, canaries used 90±10.2 % of phrase types but only 70±16 % of transition types in all social contexts (Figure 4B). Nevertheless, transition type usage did not differ significantly between social contexts. The birds used 66±17 % of phrase transition types in both solo and trailing songs, 73±7.0 % of phrase transition types in both solo and leading songs, and 77±5.0 % of phrase transition types in both leading and trailing songs. Importantly, the non-overlapped part of leading songs shared 93±2.0% of transition types with solo songs, which indicates that no structural differences occurred in the beginning of leading songs that could have elicited overlapping behavior in another bird.

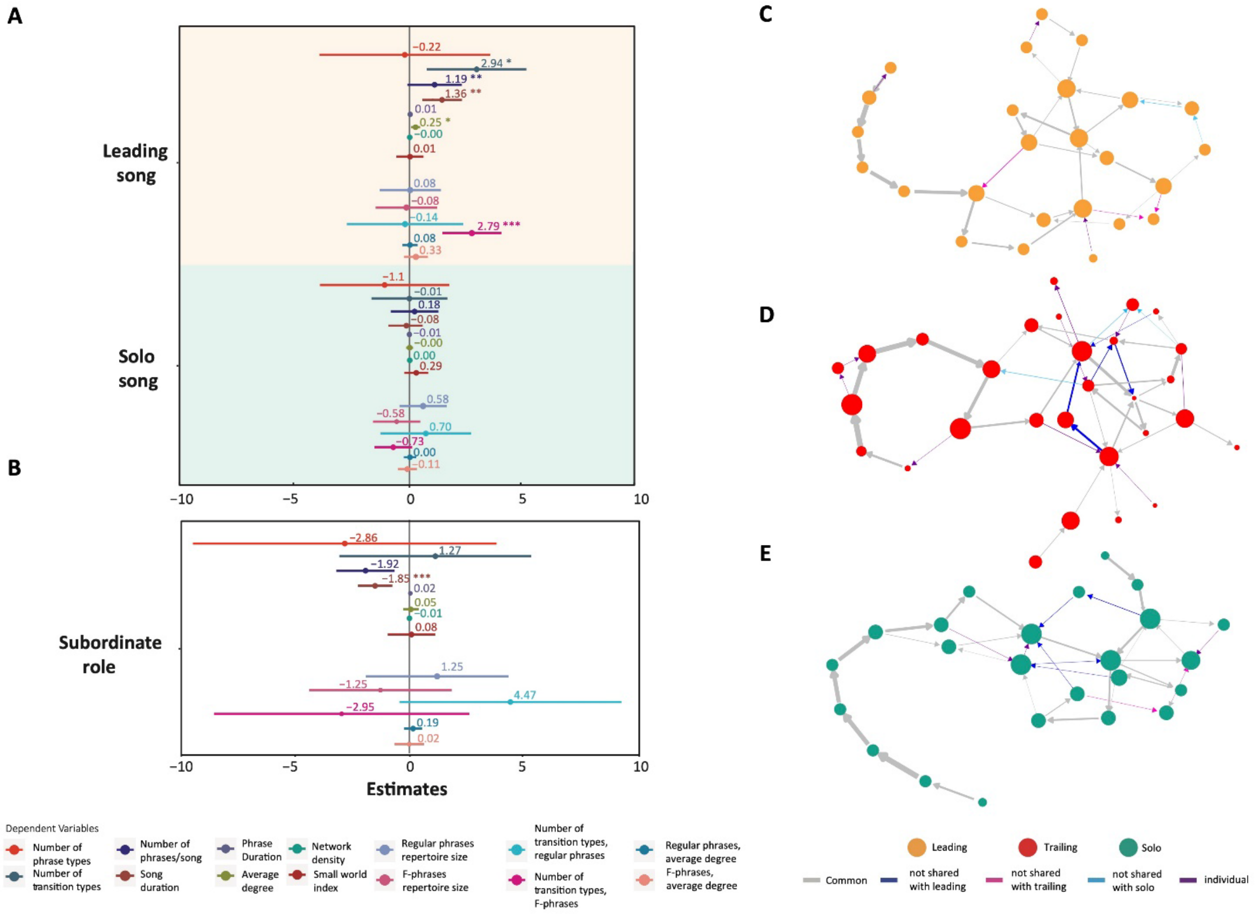


Figure 3. *Both the song's social context and the bird's social status affect the song structure. (A, B) Estimates of regression models illustrating variation in conventional syntax measurements and global network metrics for social context-dependent (A) and status-dependent (B) phrase type sequences. In (A), the trailing song context serves as a reference for the comparison with both the leading and the solo song context. In (B), the dominant role serves as a reference for the comparison with the subordinate role. (C, D, E) Representations of directed networks for leading (C), trailing (D) and solo (E) songs sung by Male 2 as an example. Phrase types, which are represented by colored dots serve as the nodes and the transitions between phrase types, which are represented by arrows, serve as directed weighted edges between nodes. Node size represents the number of occurrences of a particular phrase type. Arrow thickness represents the number of occurrences of a specific transition between two nodes.*

2.4 Context-dependent repertoire sharing between birds

Male canaries demonstrated evidence of “mutually” preferred phrase and transition types in each dyad composition. They shared significantly (Wilcoxon signed rank test, p=0.003, N = 8 birds) more phrase types (74.1±6.1%) than transition types (35.9±8.9%) between each other (see example in Figure 4C). The level of sharing did not change depending on the social context for both the phrase type repertoire and the phrase transition type repertoire. In solo songs, different individuals shared 74% of phrase types and 35% of transition types. In leading and trailing songs, different individuals shared 72% of phrase types and 32% of transition types (see example in Figure 4C).

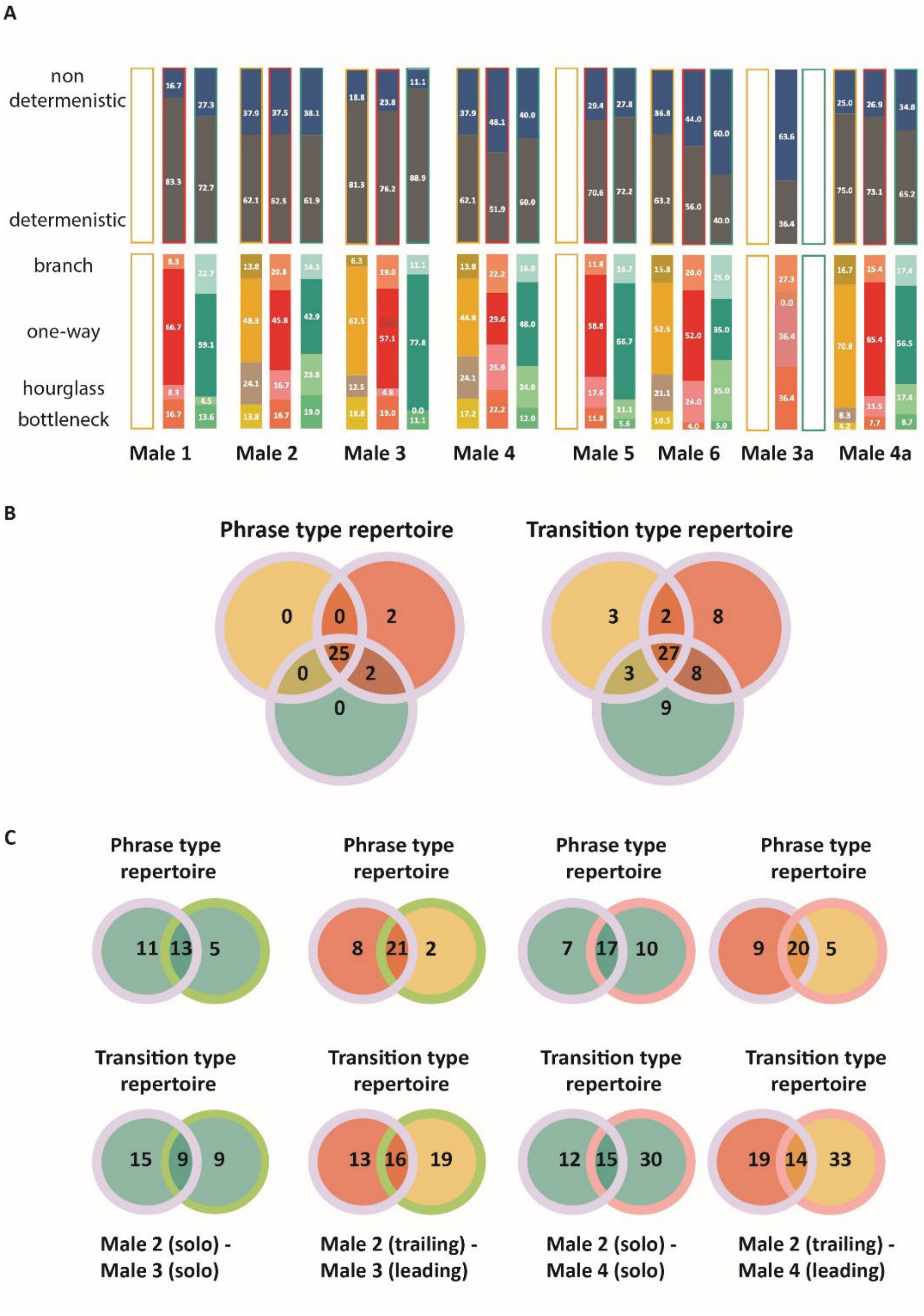


Figure 4. *Domestic canaries generally include more deterministic than non-deterministic transition types in their songs and share more transition types than phrase types between social contexts. Throughout the figure, leading songs are highlighted in orange, trailing songs are highlighted in red and solo songs are highlighted in turquoise. The top panel in (A) shows the distribution of deterministic (dark gray) and non-deterministic (dark blue) transition patterns sung by each experimental bird. The bottom panel in (A) depicts the distribution of branch, one-way, hourglass and bottleneck transition patterns (coded in colors of different hue). (B) Venn diagrams of phrase and transition types shared between different social contexts in songs of Male 2. (C) Venn diagrams of phrase and transition types shared between different social contexts and different individuals. As examples, sharing is shown for the male-male dyads Male 2 - Male 3, and Male 2 - Male 4. Light purple, light green, and pink rings indicate Male 2, Male 3 and Male 4, respectively.*

2.5 Effect of social context on vocal control

In two male canaries, Male 1 and Male 2, we recorded extracellular activity in HVC during singing (see Fig. 5A for an example). We restricted our analysis on neural data recorded during the production of solo and trailing songs because these two social contexts did not significantly alter the global song properties. The trailing songs were either part of overlapping interactions between Male 1 and Male 2, which were housed in one aviary, or between one of the birds and another male, Male 3 or Male 4, housed in the neighboring aviary. In total, we analyzed multiunit neural activity recorded during the production of 23 solo songs including 74 phrases, and 17 trailing songs including 55 phrases for Male 1, and during the production of 32 solo songs including 168 phrases, and 39 trailing songs including 151 phrases for Male 2. In agreement with previous work, we found that HVC in both males was generally active when the focal bird produced solo song, but not when its opponent produced solo song. This indicates that HVC shows premotor activity during production of own solo song but no auditory activity during listening to solo song produced by a conspecific. Surprisingly, in both birds HVC exhibited significantly (LMM; estimate: -5.97, 95% CI [-9.75, -2.20], t(444) = -3.11, p = 0.002; Std. beta = -0.57, 95% CI [-0.93, -0.21], N = 2 birds) lower activity when the focal bird sang solo songs (mean firing rate Male 1 and Male2: 7.43±5.4 and 13.01±13.5 spikes/second, respectively) than when it sang trailing songs (mean firing rate Male 1 and Male 2: 9.18±6.50 and 22.92±16.2 spikes/second, respectively, Figure 5). This may indicate that during song overlaps, the song of the overlapping bird affects HVC activity in the bird that is being overlapped.

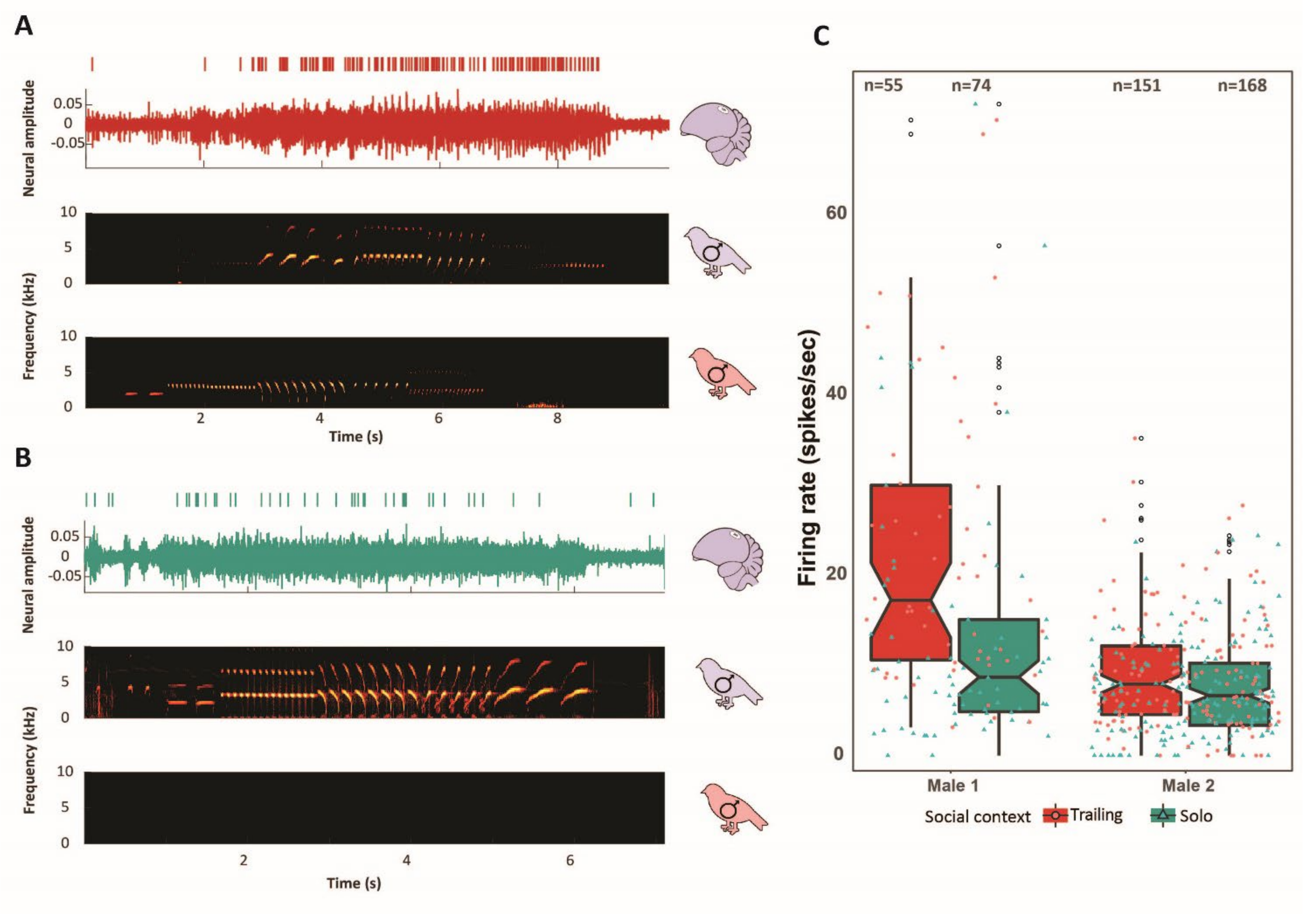


Figure 5. *HVC activity recorded during the production of trailing songs is stronger than during the production of solo songs. (A) Filtered neural signal (top panel) recorded in HVC of Male 2 during the production of trailing song. The time points of spike occurrences are indicated by short vertical lines above the neural trace. The middle and bottom panel show spectrograms of vocal activity recorded in parallel to the neural activity in Male 2 (middle panel) and Male 4 (bottom panel) during the performance of an overlapping interaction. (B) Filtered neural signal recorded in HVC of Male 2 during the production of solo song. The time points of spike occurrences are indicated by short vertical lines above the neural trace. The middle panel shows the spectrogram of vocal activity recorded in parallel to the neural activity in Male 2. The bottom panel shows that during the production of solo song by Male 2, Male 4 did not emit vocal signals. (C) HVC activity in Male 1 and Male 2 is stronger during the production of trailing songs (highlighted in red) than during the production of solo songs (highlighted in turquoise). In the boxplots, the horizontal black line indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The black whiskers extend to the most extreme data points not considered outliers, which are indicated by black circles. The extremes of the two notches of the box correspond to $y - 1.57(z - x)/\sqrt{n}$ and $y + 1.57(z - x)/\sqrt{n}$, where y is the median, x and z are the 25th and 75th percentiles, respectively, and n is the number of observations. Medians are significantly different at the 5% significance level if the boxes' notches do not overlap. The individual sample size is indicated above each box. Individual data points for trailing songs are shown by small red dots and of solo songs by small turquoise triangles.*

3 Discussion

The songs of domestic canaries as an example of exceptional seasonal vocal plasticity have attracted the attention of many researchers [13, 27, 28, 32, 37, 62-65]. The current study aimed to investigate the plasticity in canary songs as a result of countersinging behavior, which is essential for sexual selection and mating in this species. Specifically, we assessed whether the syntax complexity of song and the premotor vocal activity are influenced by vocal interactions between male canaries. This study is the first attempt to describe song syntax modifications performed by freely behaving birds that actively participate in overlapping countersinging. Despite the best efforts to study overlapping interactions by using playback experiments, it remains unknown whether changes in song structure occur in natural encounters [2]. This applies in particular for temporary overlapping vocal interactions. These competitive vocal displays are notoriously difficult to analyze because temporary overlapping vocal interactions appear to be a mix of vocal signals emitted by multiple individuals and captured in recordings conventionally performed by using a single directional microphone. Even though several studies have used alternative recording methods, such as microphone arrays or separation of vocal signals by distance, to record naturally occurring overlapping interaction, these solutions are not optimal for canaries in aviaries because rivals are often in close proximity during countersinging. As in the study of [27], the use of telemetric microphone transmitters in our study, solves this problem and provides clarity about which individual produced which vocal element during overlapping interactions.

The natural habitat of canaries often is structured by bushes and trees, and vocally interacting individuals might not always be able to see each other. Although we attempted to simulate such a natural condition by obscuring the visual contact between males in some of our male-male interaction dyads, it is still unclear whether countersinging vocal interactions follow the same behavioral pattern in wild canaries. Comparative studies performed with wild and with domesticated canaries would contribute to our knowledge of the function and evolution of countersinging behavior and the underlying neural control mechanisms. Songs of domesticated and wild canaries share similarities; however, they also differ in certain aspects crucial for countersinging. Wild canaries have a larger syllable repertoire and sing phrases with fewer syllable repetitions and with different levels of variability than their domesticated conspecifics [66, 67]. Moreover, wild female canaries are likely to eavesdrop on a particular singer for a relatively shorter time than domesticated females [67]. These differences may evoke a different implementation of syntax rules during countersinging interactions in wild individuals. The tools used here could be applied simultaneously to several freely behaving wild canaries to capture all intricacies of complex vocal interactions, as previously done for freely behaving duetting birds [25]. Although we did not address the effect of countersinging on long-range syntax rules here, our research contributes to establishing a natural social context for further research on the neural circuitry behind syntax control and on canary countersinging behavior as a model for studying vocal interactions.

3.1 Network analysis allows to investigate canary's song syntax

The implementation of network analysis allowed us to extract context-dependent syntax rules, despite the relatively small song sample size for some subordinate birds and social contexts. The average network degree of 2.7, which we observed in canary song sequences in our study, is consistent with the finding of other studies showing that long-range syntax rules can be explained by Markov processes. For example, Markowitz [37] also showed that in songs of domesticated canaries each phrase type typically transitions to only a maximum of three to four phrase types, and phrase types grouped into chunks do not significantly affect the transition probabilities in a song. Furthermore, we observed a low value of network density and a high percentage of deterministic one-way transition patterns that imply stability and a certain stereotypy of the canary song syntax. Aside from Male 2, songs of all males displayed some degree of small-world network structure, which suggests that canaries optimally navigate song progression through pre-determined phrase transitions, but simultaneously maintaining song flexibility by switching between transition types as required by the song's social context. The small-world index values of 0.46-2.59 that we observed in canary songs are comparable to values found in other songbird species with complex song syntax (California thrasher: 1.69-2.6 [68, 69], western tanager (*Piranga ludoviciana*): 2.1 [69], nightingale (*Luscinia megarhynchos*): 4.29 [47], Cassin's Vireo (*Vireo cassinii*): 4.7 [70]). The small-world architecture of canary song may reflect a "packaged" learning process, similar to nightingales [71].

3.2 Male canaries alter song structure during vocal interactions

In many bird species, females eavesdrop on male countersinging interactions to choose a breeding partner [2]. Females prefer, in a species-specific manner, males that perform songs at an elevated singing rate [72-74], with long song duration [74, 75], with high song complexity [33, 76, 77], and they prefer males that insert specific syllable types into their songs [45]. In domesticated canaries, countersinging and overlapped singing interactions between males have a biological effect: When exposed to overlapping song, females are more likely to engage in copulation behavior than they are when exposed to non-overlapping song [10, 28, 29, 45]. However, overlapping interactions present a challenge to male canaries: they need to convey information to the females, but simultaneously also need to mask the songs of their opponents. When being overlapped themselves, they need to develop a strategy to counteract the overlap and increase the attractiveness of their song. Our results demonstrate that male canaries employ two different strategies during overlapping interactions:

1) In comparison to solo songs, male canaries increase the syntax complexity of their songs in response to an overlapping opponent. Leading songs, which have been overlapped by the song of another bird, showed an increased number of transition types and an increased average network degree of their phrase types when compared to solo songs. Similar to our canaries, songs performed during countersinging interactions in house finches (*Carpodacus mexicanus*) are also higher in average network degree than songs that are sung solo [33]. These modifications, however, do not lead to the creation of completely distinct context dependent syntaxes but result in songs that are more variable in syllable order and syllable number [33, 78]. Our study suggests that the increase in the number of transition types in overlapped songs is likely due to an increase in the number of transitions between F-phrases. A previous study on overlapping song interactions in canaries, showed that the addition of A-phrases, which are characterized by a syllable repetition rate larger than 15 syllables/second, to artificial song playback cancelled the females' preferences for overlapping songs. We suggest that male canaries whose songs are overlapped by an opponent's song incorporate more F-phrases into their songs to enhance their attractiveness to females and thus to counter the sudden decrease in attractiveness of their songs due to overlapping. While increased song complexity could be beneficial for mating behavior by signaling male quality and by increasing attentiveness in female listeners, it might also increase the difficulty for females to evaluate and recognize songs that are subject to a constantly changing syntax [79]. We suggest that limited increase in song complexity serves as trade-off between song attractiveness enhancing and female perceptional abilities. Thus, the structure of the song of domesticated canaries seems to be tailored to facilitate perception and memorization in females.

2) Similar to the study of Alcami [27], we observed that male canaries that were overlapped during singing, increased the duration of their songs. We demonstrated that male canaries achieved longer songs by increasing the number of phrases in the song, and not by increasing the phrase duration. An increased song duration may be advantageous for counteracting acoustic masking by another male's song. Producing longer vocalizations is a widespread strategy to avoid acoustic masking or to increase the signal-to-noise ratio and [80-83]. Although we did not find differences in the overall phrase duration between social contexts in our study, there is evidence of phrase type dependent duration modifications in canary song. For example, the combination of altered song syntax and increased phrase duration is consistent with the previous observation that phrase duration in canary song can differ depending on the preceding phrase types [37]. Solo songs and trailing songs did not differ significantly in syntax complexity, usage of F- phrases or song duration. In European starlings (*Sturnus vulgaris*), another bird species that is known for its complex song structure, females prefer males that perform longer songs [84]. In this species, song duration has been shown to represent an honest indicator of high-quality fitness phenotypes [85]. However, trailing songs showed differences in syntax when compared to solo songs. In a large proportion of phrase types used both in solo songs and in trailing songs, the transition pattern changed when the bird performed these phrases in the trailing context. The purpose of these syntax changes in trailing song, however, is unclear. The overlapping bird may either change its syntax to diversify its song from the song of the leading bird, or to make it more similar to the song of the leader. In countersinging nightingales, interacting males prefer to actively diversify their song syntax from the opponent's song progression to take over the "vocal leadership" [47]. Alternatively, as suggested in general for overlapping songs of songbirds by Logue and Forstmeier [7], canaries might adjust the syntax of trailing song to the syntax of the leading bird's song to facilitate the comparison of their singing performances by females. For example, the song syntax of two canaries tends to become more similar after the birds have been housed together for a longer period of time [36]. Whether countersinging drives this change in song syntax, however, is not known. As an open-ended learner, the canary suits perfectly as a model to study the ontogeny of countersinging rules. Future experiments involving syntax rule tracking in groups of co-housed males over extended time periods may shed light on such open questions.

3.3 Effect of social status on singing behavior

By quantifying the singing rate of domesticated canaries, we found that one of two males housed within one aviary persistently assumed the dominant role, while the other male remained at a subordinate status. During song overlaps, the dominant males most often assumed the leader role, while the subordinate males rarely sang leading songs and mostly participated in overlapping interactions by producing trailing songs. Surprisingly, the bird’s social status did not affect any song network or conventional syntax complexity measurement. However, dominant males performed longer songs than subordinate birds. Overall, relative song duration can provide information for both male and female conspecific listeners. Female birds prefer longer songs, as it probably signals higher quality of the singer [86-88]. In particular, in the European starling, another open-ended learner, females are more attracted to males producing longer song [84], and song duration aligns positively with male fitness [85]. But in addition to females, also rivaling males could utilize song duration to evaluate the competitor’s fitness. Several songbird species perceive longer songs as indicator of aggressive intent and accordingly display stronger responses [89-92]. For example, male robins (*Erithacus rubecula*) responded more aggressively towards longer and overlapping songs of their opponents than towards shorter non-overlapping songs [93]. In our study, shorter songs of subordinate males induced lower rates of overlapping countersinging than longer songs. Thus, shorter songs could indicate lower effort and consequently lower threat value. This result agrees with our previous suggestion that song duration, but not song complexity, functions as an indicator of male fitness. This is additionally supported by the observation that the non-overlapped phrase transitions at the beginning of leading songs did not differ from the phrase transitions in solo songs.

3.4 Effect of countersinging on HVC activity

Here, we concentrated our analysis on differences in HVC activity between songs with similar level of song complexity: solo and trailing songs. In both experimental birds, HVC activity was higher during the production of trailing songs than during the production of solo songs. We suggest three possible explanations for the increased firing rate in HVC during the production of trailing songs: 1) Auditory input generated by the leading bird’s song to HVC of the overlapping bird may reduce the activity of inhibitory interneurons in this bird’s HVC, which allows the premotor projection neurons to fire more strongly. Experimentally blocking inhibition in the HVC of zebra finches (*Taeniopygia castanotis*) via activation of the GABAergic circuit resulted in much stronger premotor activity [20, 22]. GABAergic inhibition is also suggested to play a role in the coordination of vocal production in duetting plain-tailed wrens (*Pheugopedius euophrys*) [24]. Acoustic feedback generated by the duet partner's song may activate GABAergic circuits and increase inhibition in HVC of the focal bird, which may alter the timing within the vocal pattern-generating circuit and consequently prevents the duet partners from overlapping each other's vocalizations. In canary countersinging, however, signal jamming is the ultimate goal of the vocal interactions [10, 28]. We therefore suggest that opposite to duetting plain-tailed wrens, in canaries, acoustic feedback generated by another male's song may reduce GABAergic inhibition in HVC, which results in stronger premotor activity. 2) The increased HVC activity during the production of trailing song might be the result of altered control over the sequencing of individual vocal elements. In Bengalese finches, rare syllable transitions, encoded in the synaptic chain networks of HVC projection neurons, are selectively more likely to be produced in response to HVC stimulation [94]. In canaries, HVC has been shown to encode long-range order transitions between song phrases, and syntax changes can be driven by the alternating firing of neurons that encode song history [65]. 3) Excitatory activity of HVC interneurons in response to auditory input generated by the leading bird's song is causing the increase in activity in HVC of the bird that is producing trailing song. HVC contains different types of neurons: projection neurons that are active during the production of song and interneurons that are inactive during the production of song [95]. While HVC projection neurons in the HVC of awake zebra finches are unresponsive to acoustic stimulation [96], HVC interneurons can show excitatory activity when the bird is listening to sound playback [23]. When the multiunit signals recorded from HVC in our experiments were constructed from the combined activity of both projection neurons that are active during singing and interneurons that are activated by auditory input, the increase in spike rate during the production of trailing song compared to the spike rate during the production of solo song might be due to the summation of premotor activity of projection neurons and auditory activity of interneurons.

Male ID	Social context	Social status	Aviary	Total song duration (s)	Mean normilised weight	Number of analyzed songs	Number of analyzed phrases	Phrase type repertoire size	F-phrase repertoire size	Regular phrase repertoire size	Transition type repertoire size	F-phrase transition type repertoire size	Regular phrase transition type repertoire size	Mean number of phrase types per song	Mean phrase duration (s)	Mean average degree	Mean network density	Mean small world index	F-phrase mean average degree	Regular phrase mean average degree
Male 1	Leading	Subordinate	1	7.013	n.a.	4	33	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	8.25	0.85	n.a.	n.a.	n.a.	n.a.	n.a.
Male 1	Trailing	Subordinate	1	4.486	0.17	43	224	24	7	17	27	13	14	5.3	0.96	2.25	0.05	1.96	2.71	2.06
Male 1	Solo	Subordinate	1	4.025	0.33	29	188	22	6	16	25	9	16	6.5	1.08	2.27	0.05	1.94	1.67	2.5
Male 2	Leading	Dominant	1	7.661	0.18	65	466	25	6	19	35	16	19	7.16	1.01	2.8	0.06	0.46	2.83	2.79
Male 2	Trailing	Dominant	1	5.672	0.17	121	687	29	9	20	44	19	25	5,67	1.01	3.03	0.05	0.58	2.89	3.1
Male 2	Solo	Dominant	1	7.029	0.11	128	818	24	5	19	37	15	22	6,39	1.03	3.08	0.07	0.7	3.6	2.95
Male 3	Leading	Subordinate	2	3.555	0.33	37	153	23	9	14	25	15	10	4.28	1.07	2.17	0.05	1.83	2.33	2.07
Male 3	Trailing	Subordinate	2	3.349	0.11	81	344	21	7	14	26	14	12	4.24	0.8	2.47	0.061	1.85	2.57	2.43
Male 3	Solo	Subordinate	2	2.751	0.16	51	209	18	6	12	18	9	9	4.09	0.73	2	0.06	2.38	2	2
Male 4	Leading	Dominant	2	5.86	0.15	79	453	25	9	16	35	22	13	5.73	0.65	2.8	0.06	1.93	3.11	2.63
Male 4	Trailing	Dominant	2	4.872	0.16	134	702	29	11	18	45	26	19	5.23	0.79	3.1	0.05	1.53	3.18	3.06
Male 4	Solo	Dominant	2	4.553	0.09	196	875	27	10	17	47	29	18	4.46	0.92	3.48	0.06	1.81	3.5	
Male 5	Leading	Subordinate	3	6.119	n.a.	11	42	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.81	1.04	n.a.	n.a.	n.a.	n.a.	n.a.
Male 5	Trailing	Subordinate	3	3.85	0.22	62	213	17	4	13	20	8	12	3.43	1.33	2.35	0.07	1.84	2.25	2.38
Male 5	Solo	Subordinate	3	5.256	0.3	42	192	18	3	15	19	6	13	4.57	1.23	2.11	0.06	0.74	2.33	2.07
Male 6	Leading	Dominant	3	8.203	0.33	36	352	20	7	13	31	19	12	9.77	1.18	3.1	0.08	1.1	3.86	2.69
Male 6	Trailing	Dominant	3	6.539	0.2	42	313	19	9	10	24	18	6	7.45	0.89	2.52	0.07	1.17	2.67	2.4
Male 6	Solo	Dominant	3	4.202	0.135	179	1125	25	10	15	53	31	22	6.28	0.97	4.24	0.08	1.138	4.1	4.33
Male 3a	Leading	Subordinate	4	1.989	n.a.	10	23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.3	0.78	n.a.	n.a.	n.a.	n.a.	n.a.
Male 3a	Trailing	Subordinate	4	1.293	0.33	84	144	11	6	5	9	8	1	1.71	0.85	1.63	0.08	0	1.33	2
Male 3a	Solo	Subordinate	4	2.088	n.a.	11	26	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.36	0.81	n.a.	n.a.	n.a.	n.a.	n.a.
Male 4a	Leading	Dominant	4	5.032	0.39	62	376	23	9	14	28	17	11	6.06	0.89	2.43	0.05	1.38	2.78	2.21
Male 4a	Trailing	Dominant	4	4.422	0.38	90	441	24	9	15	29	17	12	4.9	0.89	2.33	0.05	1.06	2.33	2.33
Male 4a	Solo	Dominant	4	3.961	0.24	82	372	26	10	16	31	18	13	4.67	0.79	2.38	0.05	2.59	2.3	2.44

Table 1 Overview of the descriptive statistics for sample sizes, and conventional and network measurements for each bird and each social context. Songs performed by each bird under each social context were pooled independent of the identity of the interaction partner. Please note that leading songs of Male 1 and Male 5, and leading and solo songs of Male 3a have been excluded from the analysis of song complexity, because the sample size of collected songs was too small to generate the song network.

Experimental cycle	Production of overlapped song	Production of overlapping song	Observed	Expected	p-value	Overlapping pattern
1	Male 3	Male 4	25,51	0,37	0	Overlap
1	Male 2	Male 4	144,58	7,34	0	Overlap
1	Male 1	Male 4	19,86	1,46	0	Overlap
1	Male 4	Male 3	33,71	0,64	0	Overlap
1	Male 2	Male 3	27,92	0,96	0	Overlap
1	Male 1	Male 3	6,16	0,19	0,01	Overlap
1	Male 4	Male 2	182,91	6,11	0	Overlap
1	Male 3	Male 2	17,39	0,34	0	Overlap
1	Male 1	Male 2	26,52	1,66	0	Overlap
1	Male 4	Male 1	26,18	2,29	0	Overlap
1	Male 3	Male 1	2,27	0,01	0	Overlap
1	Male 2	Male 1	97,78	2,46	0	Overlap
2	Male 6	Male 4a	135,19	33,55	0	Overlap
2	Male 5	Male 4a	36,76	11,57	0	Overlap
2	Male 6	Male 3a	13,03	5,90	0,05	N.S.
2	Male 5	Male 3a	11,55	2,07	0	Overlap
2	Male 4a	Male 6	44,36	21,12	0,01	Overlap
2	Male 3a	Male 6	8,62	2,25	0,07	N.S.
2	Male 5	Male 6	14,36	14,72	0,46	N.S.
2	Male 4a	Male 5	41,15	5,92	0	Overlap
2	Male 3a	Male 5	3,48	0,66	0,11	N.S.
2	Male 6	Male 5	73,41	13,58	0	Overlap

Table 2 Overview of the statistics for overlapping vocal interactions in the two experimental cycles. Male-male overlapping vocal interactions are categorized as follows: intentional overlapping ($p \leq 0.025$), avoiding overlap ($p \geq 0.975$), or exhibiting no significant (unintentional) overlapping behavior ($0.025 \leq p \leq 0.975$).

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Discussion

General summary

Vocal interactions and the underlying neural control in songbirds in both cooperative and competitive social contexts are the focus of my thesis. The largest part of this dissertation (Chapters 1-3) is devoted to the investigation of the white-browed sparrow weaver (*Plocepasser mahali*), a songbird species that sings duet songs, which is a complex form of vocal cooperative behavior. I examined three interconnected aspects of duet singing in *P. mahali*. In **Chapter 1**, we investigated the synchronization of vocal timing and its motor control. We demonstrated that the premotor activity of the focal individual can be influenced by social auditory input from the partner, which changes the neuronal burst rate of premotor activity in parallel with shift in rhythm of vocal onset. In **Chapter 2**, we looked into the existence of rules governing the patterning of individual vocal outputs in *P. mahali* duets. We identified that both male and female white-browed sparrow weavers have distinct duet syllable repertoires and that both sexes adhere to certain answering rules while singing a duet. The white-browed sparrow weaver duet songs have a high syllable and transition versatility and a significant interindividual variability in syllable ordering. In **Chapter 3**, we investigated additional behavioral mechanisms that could aid vocal coordination, as well as potentially supporting neural and anatomical adaptations. Our research found that white-browed sparrow weaver duet syllables can be separated into four sex-specific categories based on spectral patterns. Moreover, the male's vocal organ, the syrinx, shows a lateral asymmetry in certain anatomical parameters, which might be a morphological adaptation to a specific frequency range in the vocalizations. We also found that the frequency pattern of different classes of male duet sounds is differently encoded in the vocal premotor brain nucleus HVC of the right hemisphere. In summary, my research has shed light on various crucial elements of white-browed sparrow weaver's vocal communication system. These findings have significant impact on our comprehension of vocal turn-taking communication dynamics in birds that interact cooperatively.

For the final project of my thesis (**Chapter 4**), I studied how participation in countersinging interaction affects song syntax in domesticated canaries (*Serinus canaria forma domestica*). Countersinging is considered a competitive vocal behavior. Domestic canaries modified the syntax of their songs by increasing complexity and duration when their song was overlapped by the song of a conspecific. A bird's social status also affected its song structure, with dominant males singing longer songs with more song elements than subordinates. Additionally, activity in the vocal premotor nucleus HVC was stronger during the production of overlapping songs than during the production of overlapped songs. These results highlight the importance of song shaping as a tool in the social ranking.

About a naturalistic and comparative approach to neuroscience

The importance of studying vocal behavior in naturalistic conditions appears as a recurrent theme throughout my thesis. By merging a state-of-the-art technique of individual vocal recording method with a wireless electrophysiology radio transmitter in freely behaving birds, I attempted to embed the research presented in this dissertation in the framework of naturalistic neuroscience. Naturalistic neuroscience seeks to understand the connection between brain and behavior of organisms as they naturally interact with their environment, rather than under artificial, controlled conditions [237-245]. This framework provides tools to combat persistent biases of classic ethology identified by Zilkha, Sofer [245], such as the individual bias, which is the focus when studying single animals in isolation rather than in social groups, or the experimental bias, which is the reliance on artificial laboratory settings and manipulations to study behavior. Here, I will discuss the findings presented in this thesis based on the (1) strengths and (2) limitations of the naturalistic neuroscience approach.

(1) The field of naturalistic neuroscience utilizes two main experimental design methodologies [237-245]. One is to perform experiments on organisms within their natural environments [239, 243, 246]. The behavior of animals kept in captivity likely varies significantly from that of their wild counterparts, as the latter are exposed to a variety of environmental factors such as weather conditions, abundance of resources and predation which would not be encountered by laboratory animals, and which may greatly influence an animal's natural behavior. The combination of behavioral observations in naturalistic conditions and neurophysiological methods has become increasingly prevalent in recent years, thanks to the development of advanced tools and techniques [239, 247, 248]. It has expanded the scope of research by providing access to a wider variety of unconventional animal models [240, 242, 249]. By studying patterns of variation across different species, researchers can gain a deeper understanding of the mechanisms that underlie brain function. As an example, the investigation of the neural representation of motor gestures in songbirds [187] with two competing hypotheses: a. the sequential activation of projection neurons during the production of song and b. neural activity driving the gesture trajectory extrema (GTEs), which are significant motor instances in the song. In the course of investigations, it turned out that the ubiquitous model zebra finch was unsuitable since GTEs occur so numerous in the zebra finch

song, that a clear distinction between the two hypotheses was not possible. The song behavior of canaries, however, with its different structural features offered the possibility to test both hypotheses. Furthermore, research has shown that in an unconventional bird species, the Plain-tailed wren (*Pheugopedius euophrys*), vocal output from other birds can elicit a response in the HVC [147]. In contrast, in the conventionally used zebra finch (*Taeniopygia guttata*), a response in the HVC to other birds' sounds is inhibited [227, 250-252]. The field of naturalistic neuroscience has also given rise to the development of the approach of studying model clades, or groups of species that share a common ancestor [239]. This approach allows researchers to study the evolution of the brain and behavior concurrently. For instance, the gastropod group *Euthyneura*, which independently evolved complex brains multiple times, has been a key subject in understanding synaptic plasticity and is a prominent clade in the study of neuroethology and central pattern generators [253-255]. Moreover, the broader diversity of animal model species could aid in bridging the gap between findings from animal experiments and their relevance to humans [238, 240, 242]. Although songbird species are currently the most prominent model for studying vocal production and vocal learning [164], there are notable structural differences between songbird brain and mammal brain [256]. Consequently, researchers are seeking for species whose brain anatomy is more similar to the human brain in order to establish a model organism for vocal learning. In addition to the conventional mammals species (bats), which have demonstrated some degree of vocal learning capacity in previous studies [257, 258], recent research has commenced to explore the potential of unconventional mammal species, such as grey seals (*Halichoerus grypus*) and naked mole-rats (*Heterocephalus glaber*) [259, 260].

The findings of the naturalistic neuroscience approach benefit greatly from stronger external validity, because data are collected in environments with a vast variety of biotic and abiotic sensory stimuli [237-245]. For example, a study on long-distance, thus more natural, flights in bats revealed different neuronal mechanisms for orientation compared to laboratory studies using short-distance flights [261, 262].

Moreover, naturalistic neuroscience can be used to study the neural mechanisms of group dynamics and social interactions, which can provide insight into how the brain processes social information. For example, Egyptian fruit bats (*Rousettus aegyptiacus*) seem to have a dedicated neural system for social group communication. Individual neurons in bat brains distinguished between vocalizations produced by themselves or others, as well as between specific individuals [263]. This research also revealed a relationship between social preferences and brain activity patterns within groups of bats by recording the bats' neural activity during social interactions and tracking the social and spatial arrangements within the group.

The second methodology in naturalistic neuroscience utilizes traditional laboratory model species within semi-naturalistic or fully naturalistic conditions, offering the potential for increased external validity and the ability to study the effects of a wider range of environmental variables on behaviors and physiology of the model species [264-266]. This approach benefits from a wide assortment of experimental techniques that have not yet been developed for less conventional species. Some laboratory animal species demonstrate different behavioral phenotypes than their wild conspecifics. For example, laboratory rats were observed to have increased levels of docility and tractability and decreased levels of neophobia and aggression in comparison to their wild counterparts [267]. Despite the transferability of fundamental neuroscience concepts across lab (*Rattus norvegicus domestica*) and wild (*Rattus norvegicus*) rat studies, significant differences in brain region size [268, 269] could hinder the application of findings in specific fields of research.

One of the notable examples of research on a classical model species in naturalistic conditions is the "barn" system developed by König, Lindholm [270], which houses a population of wild house mice (*Mus musculus domesticus*). This system allows for naturalistic movement and interactions both inside and outside the barn, which is safeguarded against common predators and provides the mice with ample resources such as food and shelter. Through this system, researchers have the ability to remotely gather extensive information on social interactions of a population of wild house mice. In this setting the brain gene expression differs depending on individual’s social network size [271].

(2) However, several limitations exist within the framework of a naturalistic approach. Aside from the logistical challenges of accessing experimental animals and establishing the experimental setup for neural recordings in the field, studying brain function in naturalistic settings can be challenging due to the complexity of the environment and the many variables that can affect brain function and behavior. As a result, it can be difficult to draw conclusions from the data. Another limitation of neuroscientific experiments during field research is the accessibility of the individuals. Thus, this type of research can only be conducted in animal species that exhibit a high degree of site-fidelity, remaining in a specific geographical location for the majority of the day and displaying a strong inclination to return to this location after being captured and subjected to experimental manipulation. Ethical considerations surrounding the recording of wild individuals and the disruption it causes to their established social

structures can also severely limit the studies [272]. The ethical debate surrounding the performance of experiments on wild animals' centers around the potential harm and stress caused to the animals, as well as the potential impact on their populations and ecosystems. Some argue that the benefits of such research, such as gaining a deeper understanding of wild animal behavior and ecology, outweigh the potential harm caused to individual animals. Others argue that the potential harm caused to wild animals is unjustifiable and that non-invasive methods, such as observational studies, should be used instead. Additionally, there are concerns about the potential impact of the disturbance caused by capturing and handling wild animals on their populations and on the ecosystem as a whole. Another ethical concern is that wild animals are not protected by the same laws and regulations that govern the treatment of laboratory animals, so there may be a lack of oversight and accountability for the welfare of wild animals used in research. Last but not least, currently the only extracellular electrophysiology technique applicable during field research, is multi-unit recording. However, this technique is limited. Recordings from a relatively small number of neurons at a time make it difficult to obtain a comprehensive view of the activity of the entire brain or even a brain circuit. Although in Chapter 1, HVC recordings were sufficient to understand the general pattern of HVC activity during duetting, in Chapters 3 and 4, I encountered the challenge of not being able to accurately identify the neurons from which recordings have been made, assuming that they were likely HVC interneurons [273].

The species used in this thesis reflect both advantages and disadvantages of naturalistic neuroscience approach. Vocalization of white-browed sparrow weavers demonstrate a combination of unique features, such as large repertoires (Chapter 2), complex syntactic song structure (Chapters 2 and 3), female song (Chapter 2) and vocal turn-taking behavior using learned vocalizations (Chapter 1). The white-browed sparrow weaver song characteristics render it a suitable model organism for investigating dynamic vocal communication and its demands on vocal behavior and neuronal regulation. Its use as a model organism also allows for the examination of comparative aspects in relation to more traditional model songbird species. For example, previous studies of the neural control of turn-taking vocal interactions have primarily focused on unlearned calls [190, 191]. The white-browed sparrow weaver allows to investigate this phenomenon using learned vocalizations. The social structure of white-browed sparrow weaver's colonies is also amenable to the study of intra- and interbrain activity patterns that facilitate social interactions, providing insight into the neural mechanisms underlying the coordination of joint actions. Furthermore, the presence of a consistent roosting sites in proximity to human settlements facilitates the observation of this species. The white-browed sparrow weaver is a sedentary and territorial bird species, that does not desert its territory when released after being captured and treated. Despite the general ability to observe and record the vocal behavior of white-browed sparrow weavers, the recordings were limited in duration and scope. Due to limitations in wireless signal transmission, recording of vocal and neural signals was possible only when the birds were close to the antenna placed at the birds' roosting site. This constraint prevented a comprehensive examination of the singing rate of white-browed sparrow weavers and the quantitative measurement of the use of specific types of duet trains.

For the 4th chapter of my thesis, I used semi-natural conditions to study the vocal interactions of domestic canaries in a competitive social setting. Since a long time, domestic canaries have been a valuable research model for vocal production and learning in neuroscience, providing a variety of tools and extensive data on their behavior and neural control. Unlike previous studies, which employed playback experiments [218, 220], I observed canaries in semi-natural aviary conditions, where the animals were able to interact freely. However, when working with domestic canaries, the problem of limited generalizability persists. The song of domestic canaries is significantly different from that of their wild counterparts, which means that the results could not be fully generalized to wild populations or settings. Future research with wild canaries would be useful to fully understand the nature of countersinging interactions in this species.

Furthermore, the success of my thesis projects was heavily dependent on the use of a naturalistic neuroscience framework, which in turn would not be possible without the wireless miniature microphone backpacks and neural transmitter devices developed at Max Planck Institute for Biological Intelligence [234-236]. These tools allowed me to study the vocal interactions in freely-behaving white-browed sparrow weavers and domestic canaries. As a result of this work, I have gained a deeper understanding of the complex and dynamic interactions between the brain and the social environment in the production and learning of vocal behavior.

Structure of white-browed sparrow weaver's song

White-browed sparrow weaver duet songs are a prime example of duet complexity. By observing this species' large syllable repertoire combined with its phrase-type switching behavior, one can easily see the origin of this notion. The large repertoire of sex-specific duet syllables in white-browed sparrow weavers can be grouped into two functionally distinctive hierarchical formations: duet trains and prosodic patterns. Duet trains represent subsets of sequentially associated answering rules, the occurrence of which is limited to a particular duet train type. The prosodic pattern is a broader categorization system. In Chapter 3, I found that white-browed sparrow weavers adhered to the specific prosodic structure of duet songs by following the fixed ordering of syllable categories, with both male and female consistently exchanging associated prosodic categories.

Numerous studies have compared the structure of human language and bird song [95, 102, 274-277]. As with human languages, the evolution of bird song favors learnability to simplify the task of memorizing and evaluating songs for conspecific listeners. In animal vocalization, syllable repertoire is analogous to phonological inventory, or systematic arrangement of distinct sounds [278]. The phonological inventory of human languages exhibits considerable variation in size, with larger communities generally possessing more extensive inventories [279, 280]. Despite this, languages that have developed in larger populations are lexically more uniform than languages that have emerged in smaller groups, for example, in sign languages [281, 282]. In larger social groups, there is a greater incentive to reduce linguistic variation and converge on a uniform lexical form, since members of a group need to facilitate effective communication based on mutual understanding [283, 284]. The hierarchical structure observed in the white-browed sparrow weaver song could follow the analogue pattern described for language in larger human communities. White-browed sparrow weaver species, performing and exchanging vocal output in cooperative breeding groups as well as outside communities of conspecifics, could be under pressure to reduce syllable variability through convergence to similar prosodic groups. As a result, we believe that the hierarchical structure of white-browed sparrow weaver duet song is designed to produce rapid and cooperative effort in a highly competitive environment.

Open questions on the white-browed sparrow weaver song behavior

White-browed sparrow weaver duet songs pose some more interesting questions on duet song structure and its underlying neural control. In the plain wren (*Thryothorus modestus zeledoni),* male introductory phrases are strongly associated with specific female A-phrases and subsequent male B-phrases [285], ensuring highly predictive duet progression. In white-browed sparrow weavers, introductory syllables are universal in all duet trains. Future research needs to determine if the first partner's response to introductory syllables determines the entire progression of the song. What kind of syllables serve as terminal points for a duet train? So far, it is unclear if duetting birds generally attend to syntax information during extra-pair interactions to recognize neighbors for example. The white-browed sparrow weaver uses a duet for intrapair communication and territory defense and reacts more aggressively to familiar neighbors than to unfamiliar conspecifics [286]. Duets are known to be multifunctional in many duetting species, but so far, there have been no studies assessing the context-dependent usage of different duet types. We can speculate that small-world syntactic structure facilitates navigation between different duet types in communication with neighboring pairs. Future research could investigate whether each duet train type encodes different types of information like, for example, a pair's identity. Another question is whether white-browed sparrow weavers could switch between performing shared and unshared duets to navigate territorial conflicts. For example, Washington song sparrows (*Melospiza melodia*) alternate between shared and unshared song types to escalate or de-escalate conflicts during countersinging interaction [287]. Alternatively, white-browed sparrow weavers could decode the level of duet coordination by accessing the variability of duet syntax. Precise coordination of avian duets serves as an honest signal of coalition strength in several duetting species [288]. Longer, less variable duet trains have been observed in established white-browed sparrow weaver pairs, which could potentially signal a higher level of duet coordination (Chapter 2).

Reliable roosting sites for white-browed sparrow weavers offer high research potential as field sites for studying both inter- and intracolonial vocal communication using microphone transmitters (Chapter 1-Chapter 3). There are still unresolved questions about neighbor-directed duet usage and the secondary acquisition of duetting rules. To further explore the latter, long-term microphone transmitter recordings can be utilized in combination with the monitoring of individual dispersal, similar to the approach taken in all-year monitoring research in Tswalu Kalahari Reserve, South Africa [197, 286, 289a, 290b]. This methodology can provide insight on the origin of new duetting partners and the transmission of duet songs between colonies.

In Chapter 3, despite the presence of different types of duet trains (consisting of different sets of male-female syllable combinations), we found that white-browed sparrow weavers’ duet songs generally follow a fixed ordering of syllable categories, which gives the song its specific prosodic structure, with both males and females consistently replying with the appropriate prosodic category. We interpret prosodic categories as underlying structures that reflect behavioral efficiency. However, it should be noted that prosodic patterns can also emerge randomly. Research has shown that songs that follow patterns of linguistic laws can occur both intentionally and randomly, depending on the species [98]. Future studies on emergence of hierarchical song structure in duetting species could further investigate the coevolution of social and communicative complexity. This idea is not new to duetting research, as a previous study of duet songs in the plain-tailed wrens suggested that group living could explain the evolution of duet complexity [62]. Among the wren family, the plain-tailed wren is the only species that performs phrase-type switching within a song bout, with each partner alternating between the production of two different components [62, 291]. Other duetting species that utilize phrase-type switching are also observed to be cooperative breeders, such as white-crested laughing thrushes [Garrulax leucolophu; 292] or Australian magpies [Gymnorhina tibice; 293, 294]. A study on duet form found that sex-specific syllables, a potential criteria for duet code complexity, were associated with extra-pair communication, which could be linked to higher social complexity in groups [295]. Future comparative studies would greatly benefit from following the reporting recommendations on duet form suggested by Dahlin and Benedict [295] and expanding upon them with descriptions of song syntax. As suggested by Logue and Krupp [113], the chosen characteristics should be described not only from the group perspective but from an individual perspective, since evolutionary drivers for song structure could be sex-specific. Vocal signals of male yellow-breasted boubous (*Laniarius atroflavus*), for example, are thought to have evolved for long-distance communication with females and rivals, whereas females use vocal output for within-pair communication [296]. In addition to the features used in the duet description of Dahlin and Benedict [295], I propose the following characteristics to describe duet structure to indicate different levels of syntactical complexity: adherence to duet code, duet form (cycle or phrase type switching), and grouping of vocal units in higher hierarchical categories. Similarly, I suggest using several social variables to capture different aspects of social complexity, such as pair or group living, average group size, short or long-term bonds, and the type of cooperative breeding.

In Chapter 3 I also describe the anatomical features of the syrinx in white-browed sparrow weaver males. However, the anatomical features of syrinxes in female white-browed sparrow weavers have not yet been looked at. Sexual dimorphism in songbird syrinxes has been observed in several species [297, 298], in particular in syrinx muscle mass, superfast fiber composition, and the structure of the medial labia, which was the focus of anatomical analysis in Chapter 3. In European starlings (*Sturnus vulgaris*), for example, males have size differences in the medial labia, whereas females show different lengths in the tympaniform membranes. [297]. However, the anatomical variation in the medial labia did not explain sex-specific differences in singing behavior or specialization in the production of a certain sound range in both starlings and other species [297]. But, sexual dimorphism in syrinx muscle mass allows an extended frequency range in sound production in starling males [297]. A sexual dimorphism in syrinx muscle fiber type composition has been found in zebra finches and Bengalese finches, species that show differences in vocal behavior between the sexes [298]. Interestingly, sexual dimorphism in syrinx muscle fiber type composition was not found in white-crowned sparrows (*Zonotrichia leucophrys*), where females can sing after testosterone injection [299].

In light of this, it is interesting to speculate if future studies could identify sex-specific anatomical differences in white-browed sparrow weavers. They have a clear sexual dimorphism in vocal behavior, such as a sex-specific duet syllable repertoire, and females do not typically perform solo songs (Chapter 2). Testosterone-treated females, exhibit comparable structures and syllabic compositions to those of males [300]. In some bird species, testosterone has been found to increase the overall syrinx weight [301] and the percentage of superfast fibers [302]. Up to now, there is no information on whether testosterone affects the production of duet singing. The fact that testosterone makes females sing solo songs suggests that there is no sex-specific difference in the syrinx muscle fiber type composition, similar to the findings in white-crowned sparrows [298]. A similar overall frequency range between female and male duet syllables (Chapter 3) could also indicate a lack of sexual dimorphism in syrinx muscle mass.

However, the solo song in testosterone-treated females had different temporal organization [300]. Females performed the solo song slower and with lower repetition rates than males. As it has been suggested that female birds may not exercise vocal motor control to a same degree as males, the question of which mechanism gives rise to the differences in temporal pattern between male solo song and testosterone-induced female solo song remains open [298]. With our current hypothesis that the female syrinx does not possess female-specific traits, the answer to this question could lie in the syrinx innervation or in higher structural levels of vocal motor control, such as RA or HVC.

Parallels in song syntax organization of two model species

In my thesis, I explored the variation in the pattern of vocal unit sequences in two bird species that both possess a diverse range of syllable repertoires, but live in very different social environments (Chapters 2 and 4). Regardless of the social context of vocal production, be it cooperative duetting or competitive interactions, the vocal repertoire seemed to be organized in a similar fashion, with closely interconnected groups of vocal units and more loosely connected units outside of these groups, referred to as "small-worldness" [303]. Small-world architecture provides regularity for highly irregular and complex sequences. Indeed, this song structure has been observed in many species recognized for their large repertoire of vocal units, such as California thrashers [Toxostoma redivivum; 303], Southern house wrens [Troglodytes aedon; 304] and nightingales [305]. In Chapter 2, I demonstrated that in duets of white-browed sparrow weavers, certain answering rules typically formed the core of the duet train whereas special, less common answering rules tend to occur at the beginning or the end of the duet songs, suggesting that sequential associations in the cores were stronger than in the marginal areas of the duet trains. I suggest that the duet song organization resembles the topology of small-worldness found in other species. A similar small-word organization was observed in domestic canary songs in competitive interactions (Chapter 4), suggesting that this is a common way for birds to organize their songs, regardless of whether the vocal production is cooperative or competitive. Additionally, both duet songs in white-browed sparrow weavers and competitive vocal interactions in canaries had a limited impact on the variability of transitions between vocal elements, resulting in a low number of rare transitions, with flexibility mostly occurring at certain branching positions in both social contexts (Chapter 2). It is suggested that small-world song organization ensures that information is encoded into an optimal number of song units while preserving flexible navigation between more randomized and more determined sequences [303]. Effective navigation through the song could be highly beneficial for vocal interaction because it allows quick retrieval of the appropriate song for a response. For example, nightingales take advantage of the small-world song structure in male-male vocal interaction by choosing song progression opposite to those of the opponent [305].

The following discussion highlights the need for further research to investigate the mechanisms of the ontogeny of countersinging rules, since it remains an area of uncertainty in the field of ornithology [47]. Canaries represent an optimal model organism for such research as they are known as open-ended learners [207, 209, 228, 306, 307]. Studies have shown that canaries have high levels of plasticity in their song structure during the non-breeding season, more rigid song structure during the breeding season [207-209]. The experimental approaches of studies on duet code learning could serve as an example for future research of countersinging vocal interactions. Studies on duetting canebrake wrens (*Cantorchilus zeledoni*), demonstrate that juveniles of this species first learn answering rules by singing with adult mated pairs and increase adherence to the code over time [308]. Moreover, the same species is shown to learn duet new duet code as adults, by forming new answering rules using a previously learned phrase repertoire.[309]. This paradigm could be applied to the study of countersinging interactions in canaries, specifically by addressing the following questions: Do juvenile canaries pay attention to vocal duels in adult conspecifics? Auditory feedback affects song features in canaries [228, 310]. Moreover, adult canaries have been shown to eavesdrop on conspecifics' vocal duels, resulting in differences in their singing rate depending on whether they observed the winner or the loser [220]. Does introducing new birds to an established social group of canaries during the plastic song period affect the syntax of countersinging vocalizations during the breeding period? It is known that syntax rules become more similar after canaries have been housed together for two years [228]. Does the variability of vocal sequences change when a canary is housed with a completely unfamiliar male during the breeding period after being housed with familiar birds during the non-breeding period? Answering these questions would provide important information about the development of countersinging rules in canaries.

Neural control of cooperative and competitive dynamic vocal interactions

The avian song system is an essential model for exploring the neural mechanisms underlying complex vocal behaviors [reviewed in 164]. Birds modify their singing behavior based on the presence of conspecifics [111], the conspecific's identity [220], and the social dynamics within their group [36]. Numerous studies have demonstrated that social context significantly influences the encoding and decoding of song within the song control system [160, 171, 311, 312]. These social factors affect both the neural representation of songs in the singer's brain [171, 312] and the perceptual processing in the listener's brain [312, 313].

Most studies on neural decoding and encoding of song have been conducted on zebra finches, which have been at the forefront of research, providing the first evidence of how social context influences song variability. Zebra finches perform directed, less variable songs in the presence of females [314], and detailed studies have revealed the structure of each nucleus in their song control system [reviewed in 164]. However, the song of the zebra finch is relatively rigid in structure and may not be comparable to the song of other bird species. For instance, females of European starling prefer more variable songs [315], indicating different aspects of song production and processing are relevant. Although most studied songbird species share a similar structure in their song systems, differences in vocal behaviors can lead to variations in the functioning of these systems. For example, there are debates regarding the nucleus HVC, which is the focus of this thesis. The investigation of the neural control of motor gestures in songbirds presents two competing hypotheses: a) the sequential activation of projection neurons during the production of song [166, 185, 316-318] and b) neural activity driving the gesture trajectory extrema (General Temporal Events, GTEs)[167, 187, 319], which are significant motor instances in the song. Studies reveal significant differences in HVC activity between canaries and zebra finches, particularly in how song sequences are encoded [187]. Canary songs feature long intervals between GTEs, especially in their distinctive whistle syllables, which exhibit extended segments of constant frequency. This suggests a more sparse and distinctive coding pattern in the HVC [187]. In contrast, zebra finch songs have shorter constant frequency segments with less pronounced separation of GTEs, resulting in a more continuous representation within the HVC [187]. This continuous representation does not subdivide into networks encoding individual vocal elements, reflecting the relatively rigid structure of zebra finch songs. Consequently, the canary's HVC displays more heterogeneous coding, making it a more suitable model for studying motor gestures' neural control, while the zebra finch's HVC indicates a more uniform and linear song structure. Further studies revealed that the entire song repertoire of the zebra finch is represented linearly and continuously within a sequence-sustained circuitry [320]. This organization does not subdivide into networks encoding individual vocal elements, despite their functional and developmental differences between vocal elements. While some researchers suggest that a similar network organization occurs in birds with more variable vocal sequences [320], it is possible that the linear and continuous representation of the song repertoire in the zebra finch's HVC reflects the rigidity of their song sequence. In contrast, species with more versatile syntax may reveal a subnetwork structure within the HVC. This thesis investigates canary and white-browed sparrow weaver songs, which have variable and highly versatile syntax with sequences of multiple syllable types that show different transition probabilities. In Chapter 2, it was found that white-browed sparrow weaver songs contain different subsequences consisting of different syllables. This song structure may be represented in the brain as distinct subnetworks, where the HVC is composed of multiple discrete sub circuits encoding individual song elements.

Moreover, we show in Chapter 2 and 4 that both canary and white-browed sparrow weaver can flexibly adjust their song syntax. The neural mechanisms behind flexible sequence adjustment have been studied most in Bengalese finches, a species with complex branching syntax shown to change song sequence depending on the audience or arbitrary visual cues [321]. In this species, increasing inhibition in the HVC increases the variability of syllable sequencing at branch points in the song, whereas increasing acetylcholine concentration in the HVC decreases this variability [322]. However, the neural circuits responsible for variability in song syntax in songbirds require further study, with potential involvement of other brain regions [323-326] in addition to HVC. HVC_X neurons' distinctive sparse bursting patterns are crucial for representing specific syllables' identities and managing syllable transitions, as shown in Bengalese finches [189]. Although the Bengalese finch experiment was a single-unit recording in contrast to our multi-unit recordings, I suggest that the differences in encoding distinct categories separated by spectral features in white-browed sparrow weavers' vocal elements could similarly depend on HVC_X neurons. One possible experiment would be to record HVC_X neurons while *P. mahali* performs duet songs.

Previous experiments on song behavior focused on courtship singing, with limited studies on competitive singing. In Chapter 4, it was demonstrated that during the production of trailing songs (songs produced to overlap the song of another bird), activity in the vocal premotor nucleus HVC was stronger than during solo singing. Unfortunately, the limited number of recorded birds does not allow us to disentangle the reasons behind this observed difference. I suggested several reasons for this observation: reduced inhibition for stronger premotor activity, altered sequencing of vocal elements, and excitatory interneurons' response to auditory input. However, another reason not previously specified was the neural activity of HVC_X neurons. In another example of competitive singing interaction, male swamp sparrows perform song matching by singing the same syllable as their rival [189]. During matched counter-singing, HVC_X neurons increase their activity both when a bird listens to and sings the matching song type, but these cells are silent when the bird hears or sings another song type [189]. Although we did not directly tackle the question of song matching, Chapter 4 showed that one studied male shared 70 % of his phrase types with other males during the production of trailing songs. It is possible that the increase in HVC activity we observed in canaries occurred during the production of matching elements, though this was not specifically investigated.

The dopaminergic system in songbirds, which includes the ventral tegmental area (VTA) and Area X, integrates both internal auditory feedback from correctly learned vocal performances [311, 327-330] and social feedback from conspecifics [331]. Experiments on zebra finches and Bengalese finches, using a reinforcement learning paradigm with white noise or distorted auditory feedback as stimuli, have shown that birds can maintain song performance by either avoiding noise disturbances or adjusting their vocal pitch to match the intended pitch, thus compensating for the manipulated feedback [311, 327-330]. Recordings of dopaminergic neurons in the VTA during song production in zebra finches under distorted auditory feedback conditions revealed modulated neuronal activity [328]. This modulation indicates that these neurons use auditory feedback to signal performance errors. Furthermore, it has been demonstrated that Area X specifically receives information on song performance errors, and this transmission is influenced by social context [331]. For example, when a male zebra finch receives ongoing auditory feedback from interrupting female calls during song production, the error signaling process is affected [331]. It is plausible that the observed differences in song variability among canary males during countersinging, as discussed in Chapter 4, may stem from perceived "errors" in vocal production. The auditory interference could prompt the bird to modify its song, leading to increased complexity as it attempts to correct or compensate for the perceived discrepancies caused by the overlapping vocalizations.

The previously mentioned nucleus LMAN also plays a role in socially modulated song variability [9, 111, 332]. Moreover, Chapter 4 demonstrated that the increase in song duration in countersinging canaries occurs due to an increase in the number of phrases per song, rather than phrase duration, and alters the transition probabilities for certain phrases. Although phrase and song duration, as well as song entropy in canary song during the breeding season, are not affected by LMAN lesions [207], other nuclei of the song system might influence song alterations in crystalized breeding song. Recent studies in Bengalese finches revealed that lesioning another nucleus (MMAN) showed increased sequencing variability and uncertainty at branch points [326], similar to the effects of LMAN lesions in canary in spring and autumn [207].

Visual feedback, in addition to auditory feedback, has been demonstrated to play a role during vocal performance. For example, adult Bengalese finches are able to rely on visual cues to alter song transition [321]. In another example, Area X is able to integrate visual information to shape vocal output during reinforcement learning in adult zebra finches [333]. Moreover, visual reinforcement has been shown to stimulate the learning of context-dependent modulation of syntax in the courtship songs of Bengalese finches [331]. However, all these prior examples concern the production of courtship song in courtship or neutral settings. It is yet to be studied how visual feedback influences song production in competitive settings. In Chapter 4, we demonstrated that song overlap occurred both in interactions between males housed in separate aviaries, where they could not see each other, and between canaries housed in the same aviary, where visual contact was possible. A missed opportunity in this study was to determine whether visual contact facilitates overlapping vocal interactions by analyzing the initiation of overlapping vocalizations between pairs with and without visual contact. Additionally, it would be valuable to investigate if differences in visual contact affect the functioning of HVC as well as other song system nuclei.

As documented in Chapter 1, In both male and female *P. mahali* HVC activity is only increased during the time of the bird's own vocal onset, and similar results have been observed in duetting plain-tailed wrens [312]. These results agree with studies on non-duetting songbird species, in which HVC activity is also purely premotor and no auditory activity is observed in awake and vocalizing birds [171, 175, 183]. However, in comparison to non-duetting species, in anesthetized wrens, HVC activity was strongest in response to playback of the species’ full duet song over other auditory stimuli [147]. Specifically, the auditory response to a complete duet song in HVC neurons was greater than the sum of individual responses to playbacks of the female and the male duet part presented alone. It has been suggested that duetting birds have the capacity to memorize both a bird's own song and the song of a conspecific that is necessary for social interactions [334]. For example, a recent study on vocal learning in duetting bay wrens suggested that juveniles learn the full duet song and reduce their vocal output to the sex-specific part over the time of subsequent development. Juveniles that were stereo-tutored (each sex-specific duet part was played back from a different speaker) showed more sex-specificity in early song production, while mono-tutored (both sex-specific duet parts were played back from a single speaker) birds showed no sex-specificity in song production [335].

Moreover, In Chapter 1, we explore the concept of inter-brain dynamics as a means of ensuring social coordination in animals. Our findings revealed a phase-shifted neural synchronization of vocal premotor activity in the brains of two birds during vocal production that alternated between the two individuals. Kingsbury and Hong [336] pointed out that other studies focusing on motor behaviors that alternate between two individuals, such as calling interactions in bats [42] and push-pull movements in mice [337], showed a qualitatively similar inter-brain activity in two interacting individuals. The difference in the observed synchronization pattern between our study (phase-shifted synchronization) and the studies on mammals (phase-aligned synchronization), however, could be attributed to the brain areas chosen for the neural recordings. HVC, the target in our study, is a premotor area, associated with vocal production. When two animals produce vocalizations in an alternating fashion, the underlying vocal premotor activity must consequently also alternate between the two individuals. The recordings in both mice and bats were performed in the medial prefrontal cortex, a brain region associated with social perception [338]. When two individuals perceive the same sensory stimulus, the sensory activity in the brain must consequently be phase-aligned. Inter-brain synchronization is widely attributed to humans performing many different types of cooperative behaviors [336]. The level of correlation between brains is a predictor of language comprehension ability [339], and people's ability to understand each other's language in the presence of background noise [340]. The phenomenon of interbrain synchronization has been observed to occur not only during cooperative interactions but also during competitive interactions. Furthermore, it is possible for the same brain region to exhibit interbrain synchronization across individuals in both competitive and cooperative contexts. For example, a study that focused on the neural basis of cooperation and competition in a two-person situation showed significant inter-brain neural synchronization in the right posterior superior temporal sulcus in both cooperation and competition conditions [341]. Consistent with this findings, in mice, inter-brain synchrony has been shown to predict the development of social dominance relationships, with dominant individuals causing greater influence on interbrain correlations than subordinates [337]., interbrain synchrony in competitive interactions in birds have not been studied before. The variety of competitive vocal interactions in birds highlights the need to investigate the effects of these interactions on inter-brain synchrony and its similarities and differences between cooperation and competition contexts.

In Chapter 3, I observed the white-browed sparrow weavers' syllables prosodic cues observed in human language. It is not yet clear if the observed prosodic syllable categories in *P. mahali* align with perceptual categories. During our experiments, we observed that P. mahali often tried to duet with the playback of their own duet songs. Future playback experiments could show whether the prosody-like structure of individual duet syllables play a role in duet coordination. In particular, playback experiments could be designed to test whether switches in the order of prosodic categories in a sequence disrupts duet performance. Experiments on swamp sparrows demonstrated that the birds assign different categories to song notes depending on the position of the note within a syllable [342]. To implement such an experiment, , it would be beneficial to employ a dynamic playback apparatus similar to the one utilized in previous investigations on the role of timing in zebra finch vocal communication [190]. By utilizing this technology, it would be possible to actively manipulate the presented stimuli while the bird is singing, and thereby modifying the expected prosodic category within a given song. A potential challenge associated with this experimental paradigm is the requirement for rapid and accurate categorization of syllabic elements in real-time. This could be overcome through the implementation of established techniques that facilitate swift and precise categorization of zebra finch syllables [343].

In addition to conducting behavioral experiments, an alternative approach to verifying the utilization of prosodic categories during duetting in *P. mahali* involves exploring the neural decoding within the auditory system. [344]. I suggest that in order to facilitate the integration of own and partner-produced vocalizations during duetting, the auditory system must be attuned to distinguish and respond to songs that are distinct from a bird's own repertoire. In this thesis, I also attempted to investigate the acoustic representation of duet singing in the auditory forebrain. I aimed to focus on the auditory function in the avian analogue of the mammalian auditory cortex, Field L. Field L is a part of the primary auditory pallium, which consists of the sub-regions L, L2a, L2b, L1, and L3 [164]. Region L2 neurons respond to a broader range of auditory stimuli, whereas Field L1 and L3 demonstrate more selective responses to the species-specific song. The selectivity of Field L1 and L3 neurons may be influenced by HVC through a motor-to-auditory pathway in the secondary auditory forebrain [345].

Experimental evidence from operant training, discrimination tests, and brain lesions gives support to the theory that HVC influences auditory discriminations in songbirds [346-348]. The objective of my study was to examine the differences in auditory responses to the sex-specific parts of duet songs in Field L.

Regrettably, after an initial field season, data acquisition for this study could not be continued due to the Covid-19 pandemic.

To tackle this question, one could focus on the caudomedial nidopallium (NCM) as the region that encodes vocal repertoire categories [349]. In NCM, I would expect that syllables belonging to one prosodic category could generate similar auditory response. In zebra finch auditory areas (Field L, NCM and CMM) neurons are indeed tuned towards those behaviorally defined call categories instead of towards acoustic properties

In Chapter 3, I suggested that the variance in premotor activity during duetting could be attributed to lateralized neuronal activity in HVC. It may be worth exploring in future research whether adaptations for duet coordination extend to lateralized auditory perception. For example, one could look at the lateralization patterns in the NCM towards a partner's vocal productions. As we suggested in Chapter 2, similar to other duetting bird species [308, 309, 335], white-browed sparrow weavers may relearn duetting rules as adults. In particular, the proper usage of acquired syllables might be developed to learn new answering rules with a new partner. Lateralized auditory processing was suggested as an adaptative mechanism to support the flexibility of vocal learning [350]. In male zebra finches, NCM demonstrates a lateralized pattern of activation, with right hemispheric activation correlating with song acquired from a first tutor and left hemispheric activation correlating with song learned from a second tutor [350]. Similar to the study of Olson (2016), one potential future avenue of research in *P. mahali* could involve examining the association between activation in the right auditory hemisphere and pre-existing answering rules, in comparison to the association between activation in the left auditory hemisphere and answering rules acquired during duet learning with a new partner.

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List of publications

1. **Lemazina, Alena**; Trost, Lisa; Gahr, Manfred; Hoffmann, Susanne. "The multifaceted vocal duets of white-browed sparrow weavers are based on complex duetting rules." Journal of Avian Biology, 52(9), 2021, doi: 10.1111/jav.02703.

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3. Popov, Vladimir; Supin, Alexander; Nechaev, Dmitry; **Lemazina, Alena**; Sysueva, Evgeniya. "Position of an acoustic window in a beluga whale: Computation based on auditory evoked potential latencies." The Journal of the Acoustical Society of America, 145(6): 3578-3585, 2019, doi: 10.1121/1.5111752.

4. Popov, Vladimir; Sysueva, Evgeniya; Nechaev, Dmitry; **Lemazina, Alena**; Supin, Alexander. "Auditory sensitivity to local stimulation of the head surface in a beluga whale (*Delphinapterus leucas*)." The Journal of the Acoustical Society of America, 140(2): 1218-1226, 2016 doi: 10.1121/1.4961014.

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Education

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Eidesstattliche Versicherung/Affidavit

Alena Lemazina

(Studierende / Student)

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation
Neural coding and control of vocalisation behavior in birds in different social contexts
selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation Neural coding and control of vocalisation behavior in birds in different social contexts is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München / Munich

07.03.2023

(Datum / Date)

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Declaration of author contributions

Chapter 1

S.H., L.T., A.T.M., and M.G. conceived the project; S.H., L.T., C.V., S.L., A.L., S.K., and A.T.M. contributed data and analyses; C.V., H.S., and M.A. provided technical support; and S.H., L.T., C.V., S.L., and A.T.M. wrote the manuscript with feedback from all authors.

Chapter 2

Alena Lemazina: Data curation (lead); Formal analysis (lead); Investigation (equal); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). Lisa Trost: Conceptualization (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (supporting); Visualization (supporting); Writing – review and editing (equal). Manfred Gahr: Funding acquisition (lead); Resources (lead); Supervision (supporting); Writing – original draft (supporting). Susanne Hoffmann: Conceptualization (lead); Formal analysis (supporting); Investigation (equal); Supervision (lead); Visualization (supporting); Writing – original draft (supporting); Writing – review and editing (lead).

Chapter 3

A.L., L.T. and S.H conceptualized the project. A.L. led the data curation, conducted the investigation, formal analysis, and validation, and contributed to visualization. S.H. supervised the project and contributed to visualization and manuscript writing. L.T. was responsible for methodology, project administration, and provided supervision. M.G. provided funding, resources, and support in supervision. A.L. wrote the manuscript with the help of all authors.

Chapter 4

Alena Lemazina: Data curation (lead); Formal analysis (lead); Investigation (equal); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). Lisa Trost: Conceptualization (equal); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (equal); Visualization (supporting); Writing – review and editing (equal). Birte Jung: Data curation (supporting); Formal analysis (supporting). Pepe Alcami: Methodology (supporting), Conceptualization (supporting). Manfred Gahr: Funding acquisition (lead); Resources (lead); Supervision (supporting); Writing – original draft (supporting). Susanne Hoffmann: Conceptualization (lead); Formal analysis (supporting); Investigation (equal); Supervision (lead); Visualization (supporting); Conceptualization (equal); Writing – original draft (supporting); Writing – review and editing (lead).

Signature of supervisor

Signature of doctoral candidate

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