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New tools for old questions: studying vocal communication
in the Zebra Finch (*Taeniopygia guttata*)

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1 INTRODUCTION

Vocal communication is a cardinal characteristic of our species and some bird species constitute important experimental models for the study of its evolution and its functions. In fact, vocal learning is very rare among mammals, as it has been observed only in cetaceans and some bats (Williams, 2004). The attention on Passerines vocalizations as a model for studying auditory vocal communication derives from its strong similarity with human speech (e.g. sensory motor learning (Brenowitz *et al.*, 2010) and obedience of syntactic rules (Abe & Watanabe, 2011)). The investigations on vocal learning and dynamics of vocal emission have concentrated on few species of birds and our study and most of research studies have been performed on Zebra Finch.

1.1 VOCAL COMMUNICATION

The study of vocal communication is not independent from the studies on the origin of the speech. At the beginning of the XIX century the dominant idea was that, among the unknown and unstudied languages of the people in remote corners of the world, a primitive form of language capable of unravel the origin of the speech from the animal vocal communication existed (Hockett, 1960). This language was obviously never found: none of the existing languages can be considered primitive and all the speeches possess the same level of syntactic standardized rules (Pinker 1994, Chomsky, 1965). Subsequently, more comparisons were made and more similarities were found between human and animal, particularly birds, vocal communicative systems, so that our highly developed cognitive skills started to be considered in an evolutionary continuum context (Doupe & Kuhl, 1999; Williams, 2004). Oscine birds, often highly vocal animals, have become an important model for the understanding of vocal communication. The importance of the vocal communication in social behavior of the birds has always been recognized. For instance Graig (1908) stated that pigeons social behavior is regulated by their extremely varied vocalizations and declared his impossibility to describe them precisely for their complexity. Nowadays we have the technical tools to quantify and characterize birds vocal emission. These tools allow individualized recording and a high capability of storing and processing the acoustic information. Hence it is now possible to set up experiments that consider the entire huge amount of bird's vocalization.

Vocal communication in Passeriformes involve songs and calls. On the basis of their structure we name “songs” the long and complex vocal emissions containing many different notes, and “calls” the shorter and simpler vocalizations. However, the distinction between them is not always sharp (Marler, 2004). The investigation on bird song has begun mainly with studies on its function. Evidences were found for the role of songs in establishment and maintenance of the territory and in mate attraction. In the 1950s systematic studies on the structure of the songs produced the description of different dialects and reported the existence of a critical period for song learning (e.g. Torphe, 1958, Marler, 1957). Further studies have described the phases of song learning, leading to important investigations on the neural bases of birds acoustic communication (e.g. Konishi 1965). Step by step it became widely accepted that the songs share fundamentals features with human speech (Marler, 1981), although vocal learning have probably evolved independently in birds and humans (Jarvis, 2004). The birdsong is a learned vocalisation, whereas there is little doubt that the acoustic structure of most birds calls is innate. The sensory-motor learning, shared with human speech acquisition (Brenowitz *et al.*, 2010), is the main reason why all the aspects of song production have received great attention. However, calls provide an opportunity to study the perceptual side of vocal communication, which is often neglected in the study of songs (Vicario 2004).

Currently we are in a stimulating moment for the study of songbirds vocal communication, as many fascinating features have been recently discovered. For instance it has been found that bird songs follow precise syntactic rules (Gentner *et al.*, 2006; Abe & Watanabe, 2011), that some calls are individually recognized (Vignal 2004,2007), that the social context strongly modify the behavioral output (Marler 2004a), that calling behavior have precise patterns and that this changes according to the environmental and social context (Andries Ter Maat and Lisa Trost, unpublished results; this work). Moreover new instruments, such as electrophysiology in freely behaving birds (Shergardus *et al.*, 2006), Zenk expression (Vignal *et al.*, 2007; Abe & Watanabe, 2011), backpack microphone (Andries Ter Maat and Lisa Trost, unpublished results), provide the possibility of a new approach to many open questions, hopefully allowing a deeper understanding of birds vocal communication system and of common constrains and basis that it shares with human speech.

1.2 STUDY MODEL

Zebra Finches (*Taeniopygia guttata*) is a highly vocal species often used to investigate vocal production, both song (e.g. Behavioral Neurobiology of Birdsong, Annals of the new york academy of sciences, 2004) and calls (e.g. Gahr & Beckers, 2009), at neurobiological and behavioral level (Vignal, 2004, Vicario, 2001, 2004, Marler 2004a). The Zebra Finch belongs to the estrildid finch family (Estrildidae) of the Order of Passeriformes and the Australian subspecies (*Taeniopygia guttata castanotis*) is the ancestor of the domesticated form commonly used in experiments (Zann, 1996), included this work thesis. In Australia this subspecies is distributed in most of the continent except for the tropical north and the southern coasts, living in different habitat and in all the principal climatic zones (Blakers *et al.*, 1984).

The Zebra Finch is a colonial species and forms monogamous pairs. Therefore it is presumed that the communication occurs mainly on two levels, one with the partner and one with the other members of the group (Zann, 1996; Elie *et al.*, 2011). In the majority of the cases the couple is inseparable in both breeding and non-breeding seasons and stable for more than one breeding season. Out of the breeding season zebra finches form groups with members from different breeding colonies (Zann, 1996). Zebra finches colonies are permanent in non-arid parts of the Australia, whereas are seasonal in desert regions. In our experiment, among other questions, we tried to find evidences that show these two level of communication, with the group members and with the mate, thanks to the investigation on the temporal association of calling.

Zebra Finches are an excellent model to study how the breeding phase affect vocal communication because they become immediately ready for breeding when nest material becomes available. The Zebra Finches feed almost exclusively on grass seeds as consequence time of breeding in wild populations is biased by abundance, accessibility and quality of grass seeds (reviewed in Zann, 1996). In some parts of their distribution the Zebra Finches are adapted to a desert environment where grass seeds are not a predictable resource, hence Zebra finches are adapted to exploit immediately the increasing of food supplies (opportunistic breeder). In captivity the presentation of nest material to a formed pair is an analog of the presence of food supply in nature and in fact it triggers breeding behavior. The presence of a suitable kind of nest material cause in a formed pair the immediate start of the breeding activities. It is known that wild Zebra Finches during the search of a nest site and during the whole breeding period modify their vocal repertoire (Zann, 1996; Elie

et al., 2010). In order to reproduce this repertoire modification in a more controlled situation, nest material was presented to the birds in the aviary: birds changed their repertoire and, described for the first time, also the types of calls used in temporal association.

1.3 SONG AND CALLS OF ZEBRA FINCHES

The typical zebra finch song begins with a variable number of introductory identical syllables comprising one or two notes, followed by a fixed sequence (sometime called motif) of complex syllables. The motifs are repeated into the songs usually in the same order and are often separated by introductory syllables or other simple "connecting" syllables often very similar to the other calls (Yu & Margoliash, 1996).

Different authors have given inconsistent descriptions of the zebra finches call repertoire (e.g. Zann 1996 and Marler 2004a). The main reasons behind the discordance are probably that Zebra finches have different types of calls and sometime the boundary between two call types is indistinct. In addition, the variability is increased by the fact that the repertoire can change in different context. As the present experiment aimed at investigating mainly the calls, I needed to define clearly the call repertoire of each bird. To do so, I made a my own classification that was principally based on Zann (1996). As reported in "Materials and methods", I have divided the calls in "contact calls", "breeding calls", "aggression calls" and the most studied learned "distance call". The proposed principal meaning of contact calls is to show the position of the bird so to maintain mate or group connection (Zann, 1996). Breeding calls are involved in the search of a suitable nest site (Zann, 1996) and maybe in bond maintenance and mate guarding (Elie *et al.*, 2011). Distance calls are used in different context, as communication without visual connection and in sudden changes of situation (Zann,1996).

1.4 INDIVIDUAL RECOGNITION BY SONGS AND CALLS

Individual recognition by vocal cues in birds has been mostly studied within the pair (Falls, 1982) and it was demonstrated in different species, also phylogenetically distant (e.g Davis 1986; Marzluff 1988). Vocal individual recognition in the Zebra finch was hypothesized by Immelman

(1965, 1969): he argued that mate recognition was necessary for zebra finches life style and that the inter-variability of songs might have supported individual recognition. In fact, Miller (1979) showed for the first time in this species the preference of the female for her mate's song without visual contact with the male. Both males and females Zebra finches are able of vocal individual recognition mediated by distance calls (Vignal *et al.*, 2004, 2007). Vignal and her groups demonstrated that Zebra Finches are able to recognize the distance calls of the mate and that the social context affects the rate of the response to the mate calling. Nevertheless, in the literature there are no reports assessing whether other kind of calls are individually recognizable. Robertson (1995) showed vocal mate recognition mediated by contact calls in silvereye, that similarly to zebra finch, maintain pair bonds outside the breeding season in foraging flocks. The results of our experiment shows that contact calls are individually directed, although it has not been tested which mechanism is involved in individual recognition. (My guess is with a combinations of information, in which acoustical cues are important and individually recognized). To unravel communication dynamics it would be important to understand if Zebra Finches are able to recognize just the mate or also individually the other members of the group.

1.5 AWARENESS IN THE USE OF CALLS

Marler (1996) started to give a modern perspective to two long standing old questions about bird calls: 1) "Can animals control vocalizations, or are they completely impulsive?", 2) "What is the relation between animal vocalizations and emotion?". Until about the 1990 the prevalent opinion can be resumed as follows: "obviously calls have meanings but animals have little control on them, they are involuntary and dominated by emotion" (Marler & Evans 1996). Using chicken as experimental model, Marler and colleagues have demonstrated the "audience effects" (Marler *et al.*, 1992; Marler & Evans 1996): the presence of others birds bias the vocalization emission. These earlier findings showing a certain degree of awareness in the calling emission were confirmed in other bird species (Marler 2004a). Also in zebra finches it has been shown that the emission of calls is affected by the social context (Vignal *et al.*, 2004, Elie *et al.*, 2011). However, the cited investigations have studied only specific calls, none have tested the entire repertoire, so that various types of calls might be affected by the social context in different ways. To explain the cognitive demand of the specific use of calls was suggested that the gregarious life of Zebra Finches might

have led to an high degree of awareness of the social relationship comparable to the one of some mammals such primates (Vignal *et al.*, 2004). In fact, zebra finches seem to be able to recognize individually other birds and utter calls specifically towards them, and they might be able to recognize the contextual employment of calls of other birds. We reported important evidences that different types of calls are controlled precisely and specifically. It was highlighted that calling associations could maintain the pair bond and might work in making aware the other members of the group of the existing social relationships. So far contact calls in zebra finches were considered unspecific and not individually directed (Zann, 1996). In contrast we have found that some calls are specifically directed towards an individual.

1.6 NEURAL CONTROL OF VOCAL COMMUNICATION

The neural basis of vocalizations have investigated at different levels in several species, although the most studied are the zebra finch and the canary. For instance the songbirds are an important general model to study behavioral sequences (Fee & Scharff, 2010). All the learned behaviors that are performed always in a particular sequence (e.g. recite the alphabet, sing a bird or human song) must be coded by a biophysical and neuronal circuit mechanisms shaped during learning and then maintained.

The main interest of neurobiological investigation of the vocal communication in Passeriformes has been on learned vocalizations, principally the song (Marler, 2004a).

In Oscines birds the generation of songs, i.e. vocal sequences of notes, is controlled by different premotor brain areas. In short, the network that permit the song production is composed by four fundamental stations. The organ by which vocalizations are directly modulated is the syrinx. Muscles of the syrinx are innervated by motor neurons of the hypoglossal nucleus (nXII_{ts}) (Wild 1993). The motor neurons receive synaptic inputs mainly from the forebrain nucleus Robust Nucleus of the Arcopallium (RA), which in turn receives the principal premotor input from another forebrain nucleus, the HVC (Fee *et al.*, 2004).

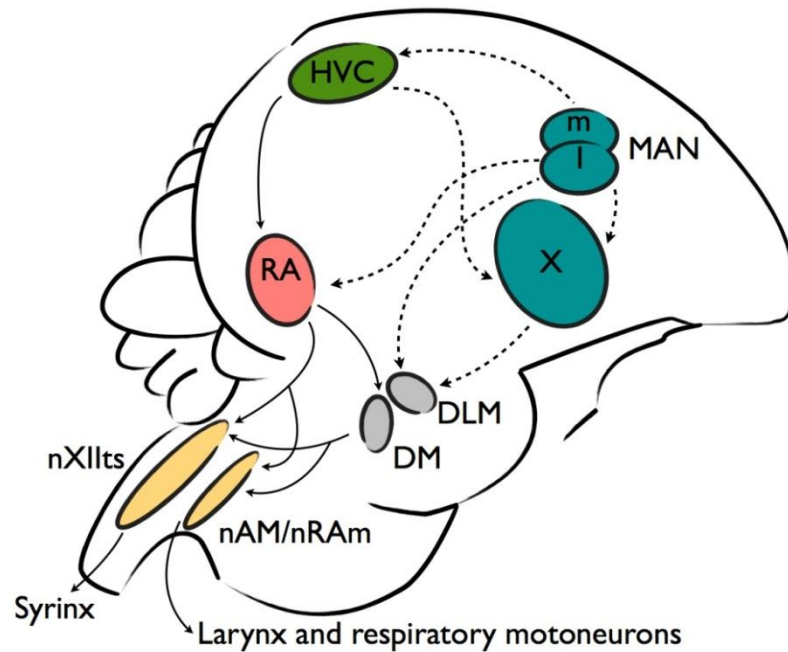


Figure A. Simplified side view of the song control system of the Oscine brain, front to right. HVC (green) projects to RA (pink). Both motor control nuclei are critical for the learning and the production of songs, while the nuclei of the anterior forebrain pathway (blue) are mainly related to song plasticity. Hypoglossal nucleus (nXIIIts) and respiratory nucleus (both yellow) directly innervate muscles that control song production and respiration. The central position of the robust nucleus of the arcopallium (RA) in the bird vocal system is highlighted.

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It seems that the circuitry under the production of learned and unlearned vocalizations is substantially different (Simpson & Vicario, 1990). Lesion experiments on HVC and RA abolished the learned features of the calls, but not vocal production. This observations led them to the conclusion that the RA is involved only in the production of learned vocalizations despite the function of the vocalizations was not tested. A similar lesion experiment on another Passeriformes,

the Brambling (*Fringilla montifringilla*), has given similar result: short unlearned calls were not affected by the lesion of the RA, while the learned ones have been modified.

The electrophysiological recording was performed in a vocal nucleus of the intermediate archistriatum (Reiner *et al.*,2004), the Robust Nucleus of the Arcopallium (RA). In the present work, we aimed at recording for the first time the Local field Potential from a vocal centre in a freely behaving bird. The choice of the RA for the electrophysiological recording has four main reasons. The first reason is that has been previously investigated in detail. The second reason is because this nucleus easily accessible for electrophysiology: the RA cells display a particular activation pattern making them well recognizable during the insertion of the electrodes. The third reason is that within the pathway controlling vocalization, the RA is the last telencephalic nucleus projecting to the brainstem vocal effectors, and besides is a target of the anterior forebrain pathway and of the HVC, which are implicated in song learning and production. The fourth reason is that the integration of behavioural data (e.g. different context situations; vocalization type) and the signal from RA might hopefully clarify the differential pathways controlling the learned and unlearned vocalizations (Simpson & Vicario 1990), and the different implication of the RA in directed and undirected songs in different context situations (Jarvis, 1998). A further interest for the study of RA lays in its involvement in perceptual processing (Vicario 2004). Hence recording from RA in freely behaving birds can hopefully clarify its role in the integration of multiple information.

Besides being controlled by the syrinx, the song production is modulated by a complex muscular interaction involving the respiratory muscles (Wild, 1997). Forebrain circuits controlling complex behaviour as singing must continuously interact with brainstem activities, as the respiration (Northcutt, 2002), although it is not known yet how this occurs (Andalman *et al.*, 2011). Moreover, also the RA projections are more complicated than described so far. In fact the RA projects also to regions of the lateral medulla, involved in the control of respiration, and to the dorsomedial nucleus of intercollicular complex in the midbrain (DM) (Wild 1997). All these connections, as the internal organization, have to be considered during the analysis of an electrophysiological signal.

Although an internal organization of the RA has been described, we still have only a partial knowledge about the functions of all the syringeal muscles, so that it is not possible to precisely identify the control activities of the parts of RA (Wild, 1997).

Nevertheless the knowledge on RA organization and projection suggest that RA makes two parallel contributions to vocal production: temporal patterning may be mediated by the dorsal, DM-

projecting region (and/or another output), whereas the organized projection to nXIIIts may enable the independent control of individual syringed muscles (Vicario, 1991).

Single unit recording from RA and from HVC have started to describe how the signal that control the syringe during singing is coded. Yu & Margoliash (1996) have described the activity of RA neurons during singing as associated with unique sub-syllabic acoustic event. It was found that, in contrast with the tonic discharge patterns of the HVC neurons, RA activity is characterized by trains of short burst of spikes separated by period of profound inhibition (Yu & Margoliash, 1996).

1.7 ELECTROPHYSIOLOGICAL RECORDING

To record from RA we have used a device specifically conceived for the Local Field Potential (LFP) recording. The Local Field Potential (LFP) is composed of low-frequency extracellular voltage fluctuations. These are thought to reflect synaptic potential and other slow electrical signals such as spike after potentials and voltage dependent membrane oscillation (Logothetis, 2003).

It has been often observed that the LFP is highly correlated with Multi Unit recording (Liu & Newsome, 2006). The LFP is composed by a lot of different cells, therefore its change of activity seems to reflect a coordinate post-synaptic activity of a large area (Destexhe *et al.*, 1999). Hence we expect to describe the change of activation of the RA through low frequency waves.

The LFP was chosen because it is more stable than a single unit recording, which results in longer recordings. A long period of clean signal could be exploited to study the development of some processes (e.g. the song learning). Single unit recordings allow almost only the study of instantaneous/short answers.

The possibility to record the electrophysiological signal from a telemetric device for a long period of time and to select, through the integration with other tools, the vocalizations that we want to analyse (e.g. by context, visual features and towards which individual was directed) will lead us into a deeper understanding on how the vocalization production is controlled.

The use of a telemetric device allows to record with less physical, and therefore behavioural, restrictions in comparison with the adoption of wires recording (Schregardus *et.al.*, 2006). As a multi approach project that evaluates behavioural output it is important that the devices used have as little bias as possible on the normal behaviour. It was shown that 1 day of recovery is enough to

allow the return at similar baseline levels of singing and hopping activities (Schregardus *et al.*, 2006).

To explain communication dynamics the possibility of integrate information from various tools must be considered. An holistic comprehension should use as much tools as possible; for instance it is now possible to aim to study a codified behavioral output with information from different levels: electrophysiological (instantaneous level), hormonal (from fast to life spam level), genetic level (from fast to life spam level).

1.8 PURPOSES AND LOGIC BEHIND THE SET UP

This work was a preliminary project in which some newly developed tools were tested (e.g. electrophysiological telemetric devices, backpack microphones, cross-correlation analysis). We used a multi-approach methods which includes many innovations on both hardware and software. The aim was to describe in detail the vocal communication of one species of songbirds: the Zebra Finches. Describing entirely the vocal communication of one species is obviously too ambitious; in fact only some features are highlighted by our work. Nevertheless vocal communication in this species is crucial for many activities, thus a lot of different topics were covered. To reach our purpose we tried to develop the integration of the output from different tools. Three types of information, temporally synchronized, were collected for our analysis: 1) bird vocalizations, 2) bird behavior, 3) neural signal. Thanks to these three types of information we have described different aspects of vocal communication under three different experimental conditions. The experimental treatments aimed at placing the birds in different social and environmental contexts in order to describe how they can influence vocal communication.

The main findings of this study were achieved by investigating on calls, and specifically on the temporal correlations of calling existing between two birds. The work of Andries Ter Maat and Lisa Trost, unpublished results (2011) pointed out precise relationships between the calling pattern of two birds; one goal of my project was to describe these associations of calls in different experimental conditions in order to infer their meanings. We tried to answer to two questions: 1) Which call types are used with temporal correlation? How does the temporal pattern of calls develop in different social/environmental situations?

To visualize whether calls emitted by a bird influence or are influenced by the vocal emission of another bird it is necessary to correlate temporally the calling behavior of the two birds. Hence from our recording we have extracted information about the type and the onset millisecond of each call emitted by each bird. The entire vocalization behavior of one bird for several days in different experimental conditions was temporally correlated with the entire vocalization behavior of the other birds around. The cross correlations analysis highlighted, if present, the calling relationship between two specific birds: a non-random probability to find answered\responding calls of one bird around the calls of the other bird. Our results demonstrate clearly for the first time that, at least a part of, unlearned contact calls are specifically directed towards an individual and that different combinations of associated calls have different meanings, so probably different functions. A variety of approaches has been used in this work; these tools, in expressly set up experiments, can be used to answer different specific questions, for instance: i) to define the call meaning, ii) whether contact calls are always individually directed, iii) whether contact calls are individually recognizable only by acoustic features, iiiii) if only with information from the calling pattern other members of a group of birds are aware of the presence of relationship between other birds. I will give some examples of possible detailed descriptions of vocal social interactions, and of integration of these information with electrophysiological recordings.

2 MATERIAL AND METHODS

2.1 ANIMALS AND EXPERIMENTAL PROCEDURE

Subjects

Four pairs of adult zebra finches (*Taenopigya guttata*) individually marked with color plastic rings were used in the experiments. Males and females were obtained from different breeding facilities to make sure that they were unknown to each other before the beginning of the experiments. The birds were kept and the experiments were carried out in accordance with the Government of Upper Bavaria (Az. 55.2-1 -54-231-25-09).

Experimental treatments

The audio, video and freely behaving electrophysiological recordings have been made on the birds kept for five days (occasionally the length of the period was shorter than 5 days due to technical problems) in each of three different experimental conditions, as follows.

- 1) At the beginning one male and one female were kept together into a sound box, isolated from other conspecifics. Inside the sound box usually the isolated male and female form a pair. The birds' vocalizations were sampled 8 hour per day, 4 hours in the morning and 4 hours in the evening, and contemporarily the electrophysiological recording was performed.
- 2) Then two pairs were placed together into a semi natural aviary. The presence of another pair in the aviary is supposed to modify the social context. The birds' vocalization and the electrophysiological signals were recorded all day long.
- 3) After a given time nest material and nests sites were introduced in the aviary. This environmental change triggers breeding behaviors. Sound and electrophysiological recording were done as in the previous condition.

Additionally, in the three experimental settings, the birds behavior was documented by video recording for one hour in the morning and one hour in the evening.

Pairs experience

The ideal condition for our experiment is that the birds were unknown to each other before the start of the recordings, because zebra finch pairs start to have temporal coordinated pattern of calls after less than 24 hour the partners have met (Andries Ter Maat and Lisa Trost, unpublished results). The temporal coordination of calls could imply individual recognition and pair bond (Andries Ter Maat and Lisa Trost, unpublished results, Elie *et al.*, 2010). Hence to know exactly from how long the birds were together before the start of the audio recording is important during the data analysis.

In the current experiments the pairs had different experience. In the first experiment one couple was together for more than 7 days before the audio recording started. In the other pair the audio recording, due to technical problems, started three days after partners' first encounter (individual recognition and pair bond present). In the second experiment the audio recording of both couples started exactly when birds were placed together (individual recognition and pair bond absent).

The data from the second experiment have been not analyzed yet. From here I will refer only on the first experiment if not differently specified.

2.2 EXPERIMENTAL SETTINGS

Sound box

The sound box consisted of a wooden cage of 60×30×40 cm; all sides and the top were closed with fine plastic gauze. Use of metal parts is avoided because they can interfere with reception of radio waves. The gauze is sufficiently transparent to allow video recording. Two wooden perches were present at different heights. The sound box could be divided into two compartments, by the same gauze used for the front. The division allows an easy catching of the birds and the isolated resting of males after the surgery. Every box is equipped with an internal antenna.

The light cycle was 14 h light and 10 h dark, from 8.00 to 22.00.

Food, millet seed mixture, egg food (hard-boiled hen's eggs, sprouted millet seed, wheat germ), and water were provided *ad libitum*.

Animals in the sound boxes are acoustically and visually isolated from surrounding environment.

Semi natural aviary

The two pairs were placed together in a specifically built aviary of 100×100×100 cm. The cage bottom was raised over the floor of 100 cm. The use of metal net on sides and top was necessarily, but the interference noise was at acceptable level when the telemetric device was tested injecting a pure tone (377 Hz, 50 .V) into the input stage of the device and placing it at different distances from the antenna.

Three wood perches were posed at different level on the same plane. The perches were placed circa at 25cm, 50cm and 75cm from the ground; dividing the cage in three equal portions. Food, same of sound boxes, grit and water were provided *ad libitum*. At middle perching level were provided a cuttlefish internal shell.

Nesting material was presented in the semi natural aviary; the aim was to trigger breeding attempts. Absence of nest sites prevents breeding activity (Zann 1996), hence nest material and sites were provided to prompt the pairs to start nesting. On the bottom of the aviary coco fibers and cotton strings (2cm) were offered as nesting material. On the middle perch level two pots were attached to the fence. Closed nest boxes were avoided to prevent injuries of the males that carried a transmitter on their head.

The light cycle was 15 h light and 10 h dark, from 7.00 to 22.00.

2.3 AUDIO

Sound transmitting

Transmitters equipped with microphones (Sparrow System, Fisher,III, USA) weighing 1.3 g, including battery, were mounted on the back of the animal. The backpack is custom-made for each bird. The backpack construction consisted of the transmitter rolled into gauze with a microphone facing inwards. Circa 19 cm silicone tubing (Detakta, 1.7 mm outer diameter) forms a ring. A second, 7 mm long silicon tubing (Dekata, 1.1mm) is stretched and pulled over the ring, separating it in two loops. The audio transmitter, protected with shrinkable tubing leaving a hole for the

microphone, is fixed on the narrow part between the loops with a cohesive elastic bandage (BSNmedical Elastomull©haft).

One loop is placed around the neck, and one around the tail base interconnected with 1.5 cm teflon tape. The posterior loop is placed taking care to remain rostral of the cloacal area. The knot sits over the furcula. (The method was developed by diploma student, Ebinger, Diplomarbeit LMU, 2002).

Sound recording

The AM-modulates radio signals were received using AOR8600 receivers.

The signal was processed in a 16 channel A/D converter (sound box room: M-Audio 1010, semi natural aviary room: Sonic Core A16 Ultra, Germany) operated at a sampling rate of 44100 Hz, and recorded with ASIO recording interface (Markus Kramer, MPIO Seewiesen).

Each recording channel was stored as .wav files of 4 h duration on the hard disk. From the hard disk the files were stored in different folders on the server.

Selectivity of recording

The wireless microphone placed on the back of the bird was mounted facing towards the animal's body, so to allow the selective recording of the bird's own vocalization. This method, contrary to general microphone recordings, avoided the overlap between calls.

Rarely the recordings included also vocalizations emitted by other birds. However during clustering processes they were readily recognizable due to their different intensity of the basal frequency. In rare doubtful cases the source of vocalizations was verified with direct synchronized comparison between recordings.

Sorting syllables

The sounds above a certain threshold of sound intensity set by the experimenter were extracted from the .wav files. These sounds were converted into sonograms assembled from 256 point fast fourier transformes (intel libraries)..

This procedure produces a large number of sonograms each of which is categorized as a song syllable, a call, or a supra-threshold sound. Each sonogram is saved separately and stored on the server.

The software written by Renè F. Jansen (Delphi Pascal for Windows) and Andries Ter Maat (CodeWarrior and XCode C++ for Mac OS X) called Sound Explorer was used to analyze the sonograms.

For each sonogram the following parameters were calculated: duration, mean frequency, mean frequency Standard Deviation (SD), mode frequency, mode frequency SD, first peak, first peak SD, zero crossing, maximum positive peak and minimum negative peak. These parameters were used for an automatic sorting and then subsequently the clustering was refined manually (see material & methods 2.4).

2.4 DEFINING THE REPERTOIRE

Once extracted from the recordings, the vocalizations were classified according to the criteria described in Zann (1996). A individualized repertoire was created for each bird. When a clusters did not match with Zann's classification different kind of clusters were described. I performed the clustering on the basis of the visual features of the sonograms. The consistency and repeatability of the clusters were not considered. The main type of clusters are described as follows.

Distance call (or Long call):

It is emitted in many different contexts (e.g. sudden danger/excitement, greeting, take-off).

The distance call is always individually structurally unique and it is emitted with high consistency in both sexes (Vignal *et al.*, 2004, 2007). Inter-individual variation of distance call characteristics is greater among males than females (Zann, 1984). The distance call of male zebra finch is a complex sound, as it is frequency modulated with several harmonics (Zann, 1984; Simpson & Vicario, 1990; Vignal *et al.*, 2007). The characteristics of the distance calls of the analyzed male birds were different from those previously described in literature (Zann 1996, Vignal *et al.*, 2007; Simpson & Vicario 1990), showing that there is a great inter-individual variability of this call type. Nevertheless each male bird emitted distant calls characterized by consistent shape and duration. Therefore the distant calls were readily recognizable from the other vocalizations. In females zebra finch it is possible to distinguish the distance calls from other vocalizations, as for example Stacks,

only on the basis of their duration. In fact, female's distance and Stack calls have similar sonogram shapes (Zann 1996). No clear differences between Stack and distance calls was found in our two females as well. By consequence, a length threshold was used to discriminate the two call types. The threshold was decided individually on the basis of the length of the call elicited by the presence of the experimenter nearby the birds, because the sudden experimenter's presence elicited danger/excitement reaction (pers. obs.). The threshold lengths were set to 60 ms (female A) and 75 ms (female B).

Stack call:

Its proposed function is to show the bird intention of taking-off or landing (Zann, 1996). It is characterized by a fundamental not modulated frequency and by a series of overtones forming Stack shaped sonograms (Zann 1996, Andries Ter Maat and Lisa Trost, unpublished results).

Tet call:

Zann (1996) suggested that this short and highly stereotyped call has the function to keep the birds in close contact. The fundamental frequency of Tet calls changes with time. In some studies (e.g. Elie *et al* 2010; Elie *et al* 2011) Stack and Tet calls are mixed up or lumped into one category. In my experiment the differences between these two call types were always evident.

Song Syllables:

Songs syllables differ in number and shape between individuals but they are stereotyped in each male. They are readily recognizable during clustering process with no degree of ambiguity. Every syllable type was set apart in different clusters.

Nest site calls, Kackle, Ark, Whine:

According to Zann (1996) these calls are used only in the nest proximity, or during the searching of a nest site. These calls are described by Zann as identical for male and female. They may intergrade, as the shapes of their sonograms can be blurred and difficult to be classified in some cases. The criteria used to discriminate them were length, frequency changes and number of harmonics.

Other calls:

Rarely zebra finches emit some types of vocalizations other than the most common described above. The types and the amount of these vocalizations are not standardized in the literature and often not mentioned. For instance Elie *et al.* (2011) distinguished only Tet, Distance calls and song syllables in 564 analyzed vocalizations. Zann, (1996), stated that other call types are emitted only in aggressive and breeding situations. In this study the precision of our recording allowed the recognition of other calls type. A new cluster was made every time that sonograms clearly differed from the most common ones. The main differences consist of different number of harmonics, of frequency peaks, and length.

2.5 AUDIO ANALYSYS

All the statistical analysis was performed with R.

After the manual clustering process the sorted calls and song syllables were saved in a .bin file.

The clustered vocalizations were visualized in Utilities, (Andries Ter Maat, CodeWarrior and XCode C++ for Mac OS X). Utilities is a multi-functional software allowing the extraction of qualitative and quantitative information about the organization of the syllables (e.g. order of song syllables, which is the cluster most commonly follows another cluster). This was used to distinguish visually vocalizations emitted by different animals.

Utilities was used to save a .macbs2 file of the clustered vocalizations; this file type contains only the information about the onset of syllables and the cluster to which the syllables belong to. The .macbs2 file is used in Spike (Andries Ter Maat, CodeWarrior and XCode C++ for Mac OS X), a program suitable for making cross-correlation diagrams. Spike was used to test all the possible combinations of calls of different or the same category between two birds, in order to detect temporal correlations: for example Stack calls of the male in relation to Stack calls, distance calls and Tet calls of the female.

For the relevant combinations a .txt file was saved containing information about the resulted cross correlation diagram.

2.6 ANALYSIS OF THE TEMPORAL PATTERN OF CALLING

On the basis of the onset of syllables emitted by different birds it is possible to determine, using “Spike”, their temporal association. Cross correlation histograms were used to determine cross correlation (Abeles, 1982). The onset times of the different vocalizations were used to shape cross-correlational density plots where the occurrences of various vocalizations of one animal were aligned to specific vocalization of another. As convention we set the female calls always at time 0. The length of the time window was two seconds before and two seconds after each female call. Then, we have set to one hundred the number of bins in which this time window is divided. Confidence limits were calculated using Poisson probabilities based on the baseline levels of the correlation determined between 4 and 2 seconds before the focal vocalizations. Hence it was assumed that calls emitted between 2 and 4 seconds after or before the other bird’s calls had a random distribution. In this work only combinations of vocalizations showing a significant temporal correlation are considered.

If available, eight or twelve hours of recording per day were analyzed: morning (7-12), afternoon (12-19) and evening (16-23). Given that temporal relations patterns between calls were consistent during the day, only the data collected in the morning are presented in this work.

To visualize the development of calling relations between the two members of the pair over a period of time longer than one day (e.g. 5 days of recording in a certain experimental condition), we have computed the average calling pattern by counting the percentage of calls occurring in each bin of the window. The mean and confidence limits for each time bin have been calculated.

2.7 VIDEO

Video recording

The video recording inside the sound boxes was done with a camera (Panasonic ® NV-GS400) connected to a videotape recorder (JVC ® RR-V10E). The video recorder was also connected to audio receivers, so that the video was synchronized with the sound. The video-audio recording was then digitalized in a .mov file, using Quicktime 7 player for Mac.

Video analysis

For each couple housed in the sound box two days of video recording were analyzed. When the couples were housed in the semi-natural aviary two hours (one hour between 8.00-10.00 and one hour between 19.00-21.00) of recording for each day of experiment were analyzed. The analysis of the video recordings allowed to record the bird's position in the environment and the onset of each behavioral pattern. The data were divided in four categories: social, individual, positional and feeding.

Social data include all interactions between individuals (behavior description based on Zann, 1996).

The behaviors considered were the following:

Allopreening: two birds cleaning each other's feathers.

Clumping: two birds perched side by side.

Greeting beak fence: two birds wiping each other's beak

Pecking/chasing: one bird chases or pecks another one. For this aggressive behavior it was reported if the action was active or passive.

Hopping: a sort of courtship dance where two birds exchange their position with fast and brief flight.

Distance: two birds do not interact.

Copulation.

Individual data include all the behaviors in which the subject is not interacting with another individual:

Exploring: the bird is moving on a perch or on the floor.

Perching: the bird is sitting.

Resting: the bird is crouched on a perch.

Cleaning: the bird is preening his own plumage.

In the natural aviary the video, due to the metallic fence, was of low quality so some behaviors were lumped in more large categories. For the individual behaviors were signed only activity or resting and for the social behaviors, allopreening, clumping and greeting beak fence were grouped together

under the name “clumping”. Furthermore, a new category was added: “closer”, the inter-individual distance between two birds of opposite sex is roughly less than a bird body size.

Feeding data include all foraging behaviours:

Feeding: dehusking seeds and eating activities.

Drinking.

Positional information data describe the position of the bird in the cage or aviary.

In the sound box there were six possible different positions: floor, first perch, second perch and the respective left or right side; in the it semi-natural aviary the possible positions were five: from the floor to the third perch and fence if the bird was clung to the metallic fence.

Video data analysis

Only qualitative examples of video analysis results are presented in this work. The examples will show some of the possible applications of the recorded information.

The starting point of each behavioral act was interpreted as an event and reported in a excel sheet on different categories, then behaviors of each categories were organized chronologically. Time\ behavior categories graphs were built, each line is the onset/offset of a particular behavior.

Ethograms of different birds can be tabulated so to permit fast qualitative comparison of social ethograms of different birds.

The ethograms resulting from these analyses can be synchronized, hence compared, with calls production. This classic approach is useful to gain more knowledge about meaning\function of calls. Now it could be used also to understand when correlate temporal pattern of calling take place.

2.8 ELECTROPHYSIOLOGY

Surgery

Surgeries were performed between 8:00 and 14:00.

Birds were placed on a warming operation table (38°C). Beak and head were fixed in a head holder to achieve an angle of 45° degrees. They were anesthetized using an isoflurane-vaporisator (flow: 350-450ml/min O₂ and 1% isoflurane, Isoflurane CP®). 10 minutes after was inducing the anesthesia, the plumage on back of head was plugged out and the skin was sanitized with 70% alcohol and treated with a local analgesic (Xylocain®, gel 2%).

The skin was opened with a pair of iris scissors.

It follows the opening of the first bony layer over the bifurcation of the sagittal sinus. The bifurcation represents the first zero-point from where thanks to suitable coordinates it was reached the proper position over RA. For successive recording of neurons two electrodes were used: a reference electrode and a recording one. The reference is situated between second layer and dura mater (first operation hatch); the recording is positioned over respective recording area.

To place the reference electrode (2 cm stainless steel, california fine wire) a tiny hole was made in the second bony layer of the first hatch. The stainless steel was pushed between bone of the second layer and dura mater and fixed with dental cement (Tetric EvoFlow, Ivoclar Vivadent).

Both electrode pins were stuck in a special electrode holder that ensures the correct distance between the electrode pins. With a digital manipulator the recording electrode was placed +2500 µm from the zero point on the x-axis and -1400µm on the y-axis. In this position a second opening was made. This time both layers and the dura mater were opened. To prepare the build-up of the telemetric plug a layer of resin based dental adhesive (Clearfil SE Bond, Kuraray Medical Inc.) was applied on the skull surface. Hardening was done with a UV-stick (Coltolux® led, Coltene). The recording electrode was brought in the second hatch, where both bony layers were opened. Using a digital manipulator, the electrode (FHC, Tungsten, diameter: shaft-25µm, tip < 1µm, impedance 1,9MΩ at 23°C) was driven in a depth of approximately 1800-1900 µm to reach RA.

To build a construction, that allows the attachment of a radio transmitter, the electrodes was embedded in dental cement (Tetric EvoFlow, Ivoclar Vivadent). Only the pins of both electrodes protruded from the dental cement. Sterile absorbable sutures (Resorba®, Mopylen®-blu,

polypropilen, 7/0 USP, 0.5 metric. HR 6, Germany) were used to close the skin around the plug construction. Treatment with an analgesic cream is applied afterwards.

After surgery, the birds were transferred to a recording cage (sound box). The male was separated physically but not visually (see materials & methods 2.2, sound box) from the mate for at least 4 hours to permit full recover (Schregardus *et al.*, 2006).

Telemetric Device

The telemetric device is the battery-powered apparatus designed for transmitting the electrophysiological signal recorded by the electrode to a receiver.

The telemetric devices is developed directly by a research team of the Max Planck Institute. It is described in Schregardus *et al.* (2006) as follow:

“The telemetric device consists of SMD components (surface mount device, type: 8-bump microchip scale package), mounted onto printed circuit board (type: FR4, 0.5 mm thickness). The print was etched under constant visual control, after which the miniature SMD components were soldered onto it by means of an infrared reflow oven. After soldering, the stocked circuit board was checked under a microscope. The device is attached to the bird’s head by connectors. Onto the electrodes male connectors were crimped. Onto the telemetric device two female connectors (AP 103d/G Assmann IC-pin) were soldered. These female connectors both supported the device and connected the print to the electrodes. Male connectors were made by cutting an AL-ST Assmann Icpin in half. A hole (\varnothing 0.13 mm, 2 mm deep) was drilled in the thin half. The tungsten recording electrode and the stainless steel reference electrode were each inserted into one such hole. The connectors were attached to the electrodes by crimping, resulting in good electrical and mechanical contact.”

In my experiment a vastly improved version suitable for the transmission of the low frequencies of the local field potential (developed by Hannes Sagunsky, MPIO) was used.

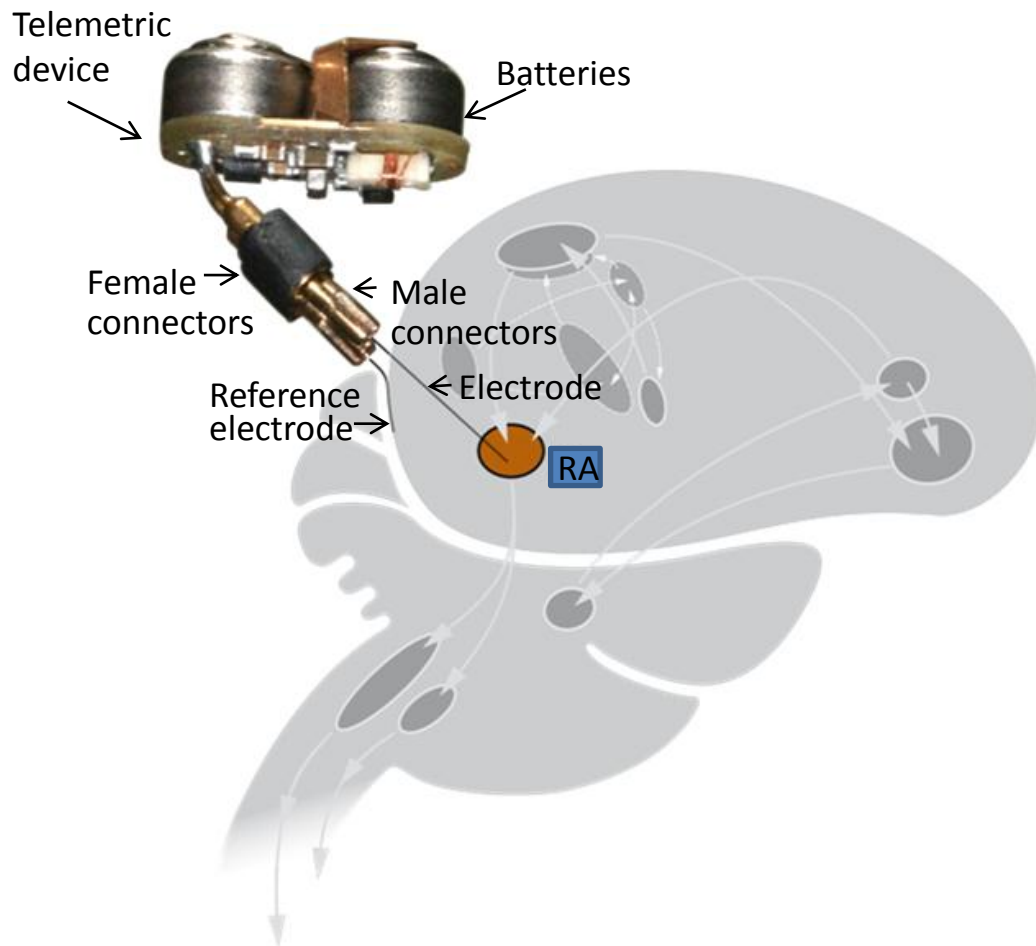


Figure B. Simplified side view of the songbird vocal control system, front to right. The song nucleus where the electrophysiological signal has been recorded (RA) is highlighted, together with the position of the reference electrode, situated between the second layer and the dura mater. The telemetric device is composed of transmitter and batteries, linked by the connectors to the electrodes.

Neural activity recording

For the FM-modulated electrophysiological signals we used AOR5000 receivers equipped with an AOR SDU5600 spectrum display unit that was used to tune the transmitted frequencies. The intermediate frequency bandwidth was set at 110 KHz. The signal was processed in an A/D converter (M-Audio 1010 in sound box room, Sonic Core A16 Ultra, Germany, in semi natural aviary room). The drift of the transmitting frequency was compensated using a program that compares the required frequency with the actual frequency and tunes the receiver-SDU combination accordingly (Markus Kramer, MPIO).

Analysis of electrophysiological signal

The recorded signal was low pass filtered with a cut-off frequency of 300 Hz and a gradient of 12 dB per octave.

The aim of the analysis is to find out whether the activity of the recorded nucleus change during the production of vocalizations (or during other events, e.g. female vocalizations). The analysis was performed with the software DRC, (Andries Ter Maat, CodeWarrior and XCode C++ for Mac OS X). The .macbs2 files (see materials & methods 2.5) contain the list of vocalization events with information about their onset and type. The .macbs2 file and the .wav file of the electrophysiological signal (these two files are synchronized) were loaded in DRC. DRC was used to cut fragments of the electrophysiological signal exactly where the occurrences of the cluster were. Then the average of all the data points across aligned traces was taken. Units are A/D conversion units. The experimenter can set a time window in which the obtained average signal before and after the vocalizations onset is visualized. If the nucleus activity changes during the production of vocalizations we expect a difference in the average electrophysiological signal before, during and after the vocalization onset.

To have a reliable description of the presumed change of RA activity the signal was then transformed in two ways. 1) On DRC the average resulted signal was transformed with a Root Mean Transformation (RMS). RMS is a statistical measure of the magnitude of a varying quantity.

$$X_i = \sqrt{(x_i - \mu_x)^2}$$

Where:

$$\mu_x = \sum_1^n x_i$$
$$i = 1 \dots n$$

With μ_x that is the mean of all the data points at that particular moment and x_i is the considered observation.

2) A spectrogram was computed from the calculated average signal, by using the R package “spectro”,. The consistency of the signal among the events is represented by the amplitude and therefore if the signal changes in a predictable way we expect to observe an increase in amplitude.

3 RESULTS

3.1 SAMPLING

For the couple A, about 160 hours of audio recording collected in 17 days for each bird were analyzed. For the couple B, 76 hours of audio recording collected in 8 days were analyzed. The total number of vocalizations (calls + song syllables) analyzed, all clustered and checked manually, was not counted precisely. Indicative vocalizations numbers are as following:

Male A: about 140.000

Female A: about 74.000

Male B: about 36.000

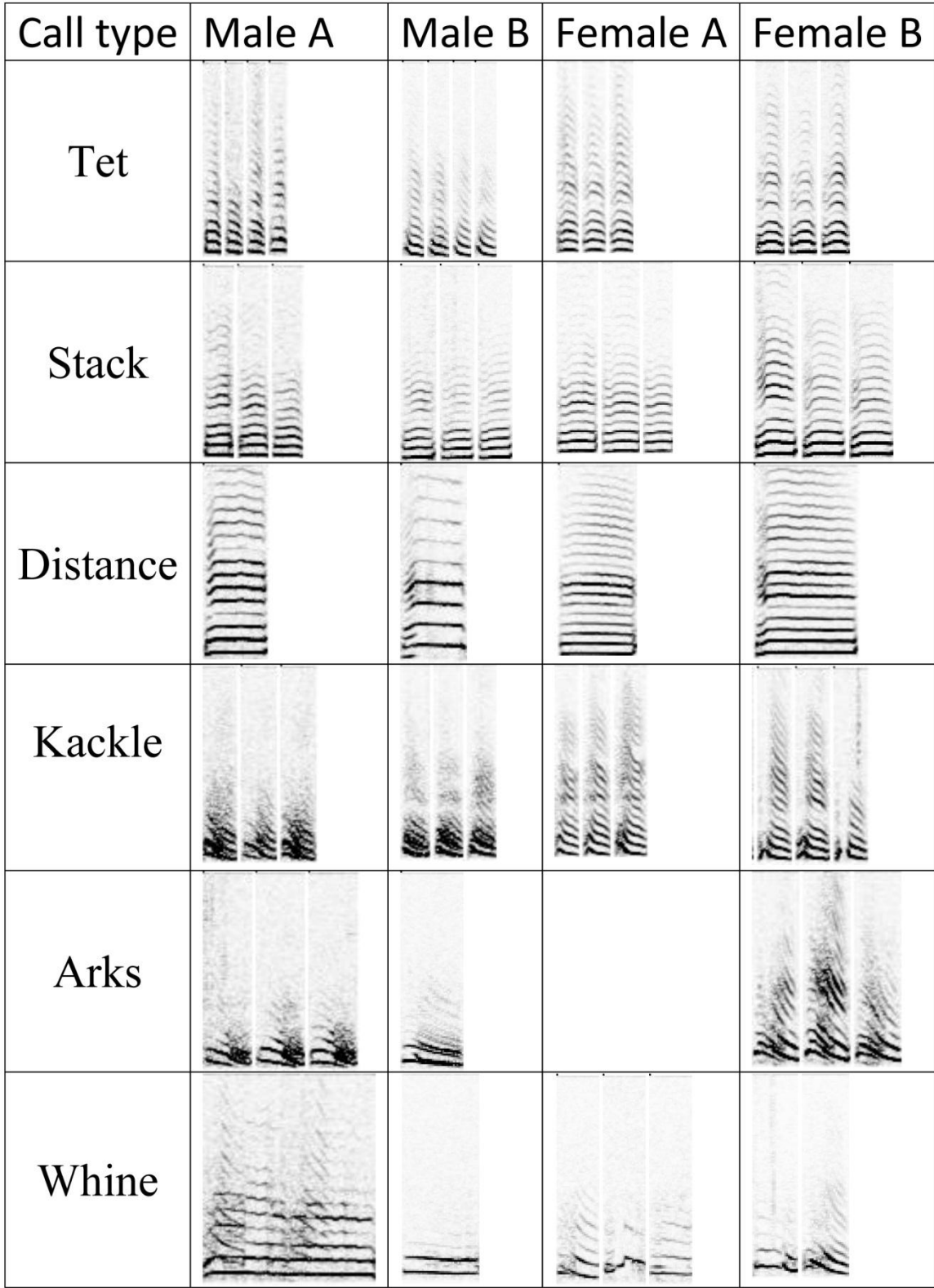
Female B: about 40.000

The pattern of the temporal relation of calling of the investigated call combinations was qualitative consistent along the day: morning, afternoon, evening (data not shown). Hence only the data collected in the morning are presented, if not differently indicated.

3.2 REPERTOIRE

On the basis of the visual characteristics of the sonograms we can distinguish different calls of every bird, as reported in figure 1. It was possible to identify similar call types in different birds and it emerged that each bird had a similar repertoire. Furthermore all main call types that Zann (1996) had described were found in this experiment.

Calls numbers were not precisely assessed but, it was found that Stack and Tet are the most common calls when the nest material was absent, while Kackle was the most common call when nest material was present. Some studies indicate the distance calls as the most common call (Vignal *et al.*, 2004, 2007; Elie *et al.*, 2011).. In contrast, in our experiment the distance calls were produced very frequently only when the experimenters were close to the aviary (pers. obs., data not shown); albeit their call definition was the same of our work.



50ms

Figure 1. Main call types of each bird. On the left there are the name used in the thesis. Sonograms 0-8 KHz.

3.3 TEMPORAL PATTERN OF CALLING. GENERAL

Previous studies (Andries Ter Maat and Lisa Trost, unpublished results) have shown that a new formed pair of zebra finch begins a temporally precise calling relationship within one day. Only some types of call have this temporal relations and different combinations of calls can have different patterns (Fig. 2). Precise temporal relationships are illustrated by an high, non-random probabilities to find a male call type before and/or after a female call type into a precise time gap. In other words, females calls are often emitted just after and/or before mate's calls.

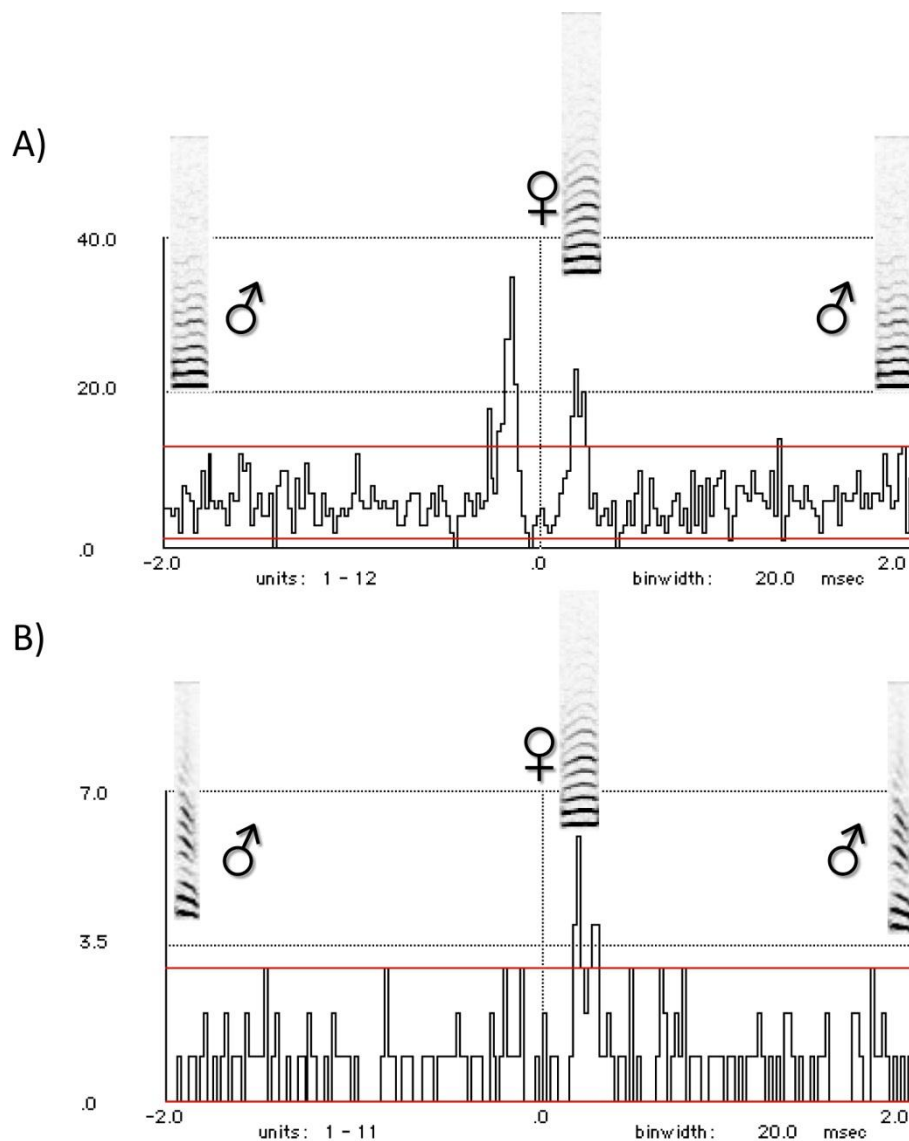


Figure 2. Temporal relationship of calling within a pair. Cross-correlation histograms show the temporal correlation between one male and one female call types within a given time window. Histograms were aligned on female vocalization. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s

after. The number of the male calls per bin are given on the y-axis. The graphs are referred to couple A. Total calls of four hours during the morning. Fourth day after the surgery (four days after surgery call total number has returned to pre-operation levels (Schregardus *et al.*, 2006)) A) Stacks calls of male and female, couple A . B) Male Tet calls and female's Stacks, the asymmetric right hump indicates that female Stack excites male Tet

The results of my work confirm those reported by Andries Ter Maat and Lisa Trost, unpublished results, that a pair of zebra finches shows a temporal patterned relations of calling. Two different examples of these temporal pattern of relations are reported in figure 2. The Stack-Stack correlation (Fig. 2A) is clearly symmetric and mutual: both the male and the female used this call as an answer. Differently, the Stack-Tet correlation was asymmetric (Fig. 2B): the male answered the female Stack calls by emitting a Tet call, but the other way around was extremely rare. The described call relations were consistent and fix for the entire sound box period (Fig. 3, 4 bottom).

3.4 TEMPORAL PATTERN OF CALLING. ENVIRONMENTAL AND SOCIAL INFLUENCES.

3.4.1 Couple A, Stack-Stack.

The Stack-Stack correlation in couple A was symmetrical and constant during the sound box period (Fig. 3 bottom): the female answered the male calls in a comparable number of times as the male did to the female calls. By contrast, when the pair was together with another pair in the semi-natural aviary this relation was asymmetric: the male was answering in a significant way to the female, but she was not replying with the same frequency (Fig. 3 middle). It is therefore clear that the patterns of the relation has changed when the pair was moved from the sound box context (one isolated pair in a small box) to the aviary context (2 pairs in a larger environment). Additionally, when nest material was added the Stack-Stack relationship returned to being symmetric for the first day (Fig.3 top), although this calling relation disappeared in the following days of the experiment. The reason of this change is inferred below and in discussion (4.4.1). To investigate the development of the calling relation that follows the presentation of the nest material in more detail, we have divided this experimental period into three different periods (Fig. 4).

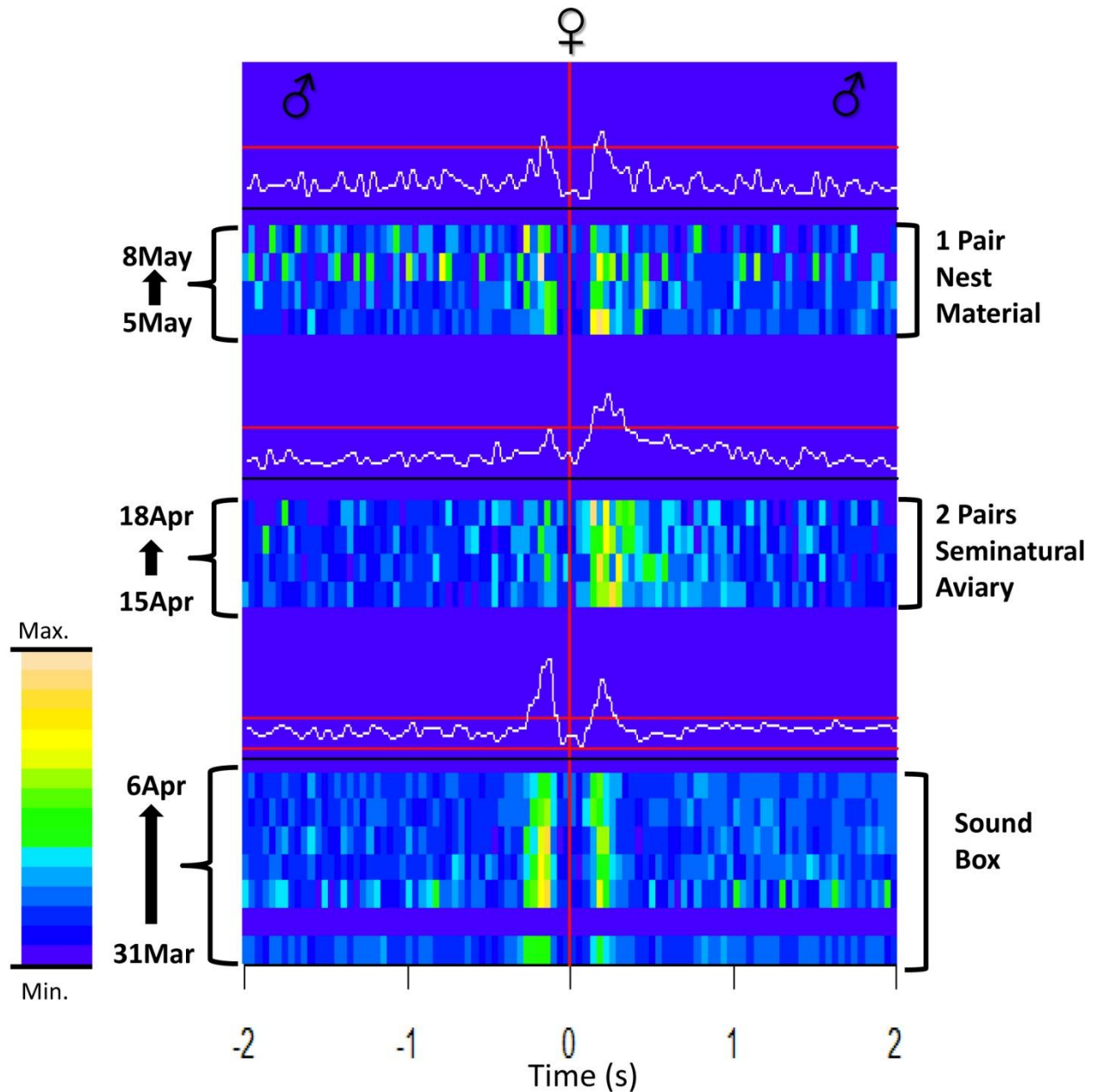


Figure 3. Temporal development of mutual Stack calling patterns of couple A in different experimental situations. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize four hours into one morning (6-12 a.m.). The color scale represents the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of the period. The 0.99 Poisson confidence limits (Abeles, 1982) are shown as horizontal red lines. The black line at the bottom of each graph is value 0. The pattern of the relation differed in the three experimental situations. The relation was roughly symmetrical in the sound box and, although weaker, when nest material was presented, in both situations this couple was alone. However, the male was answering with a Stack to the female Stack but rarely got a response, when they were housed together with another couple.

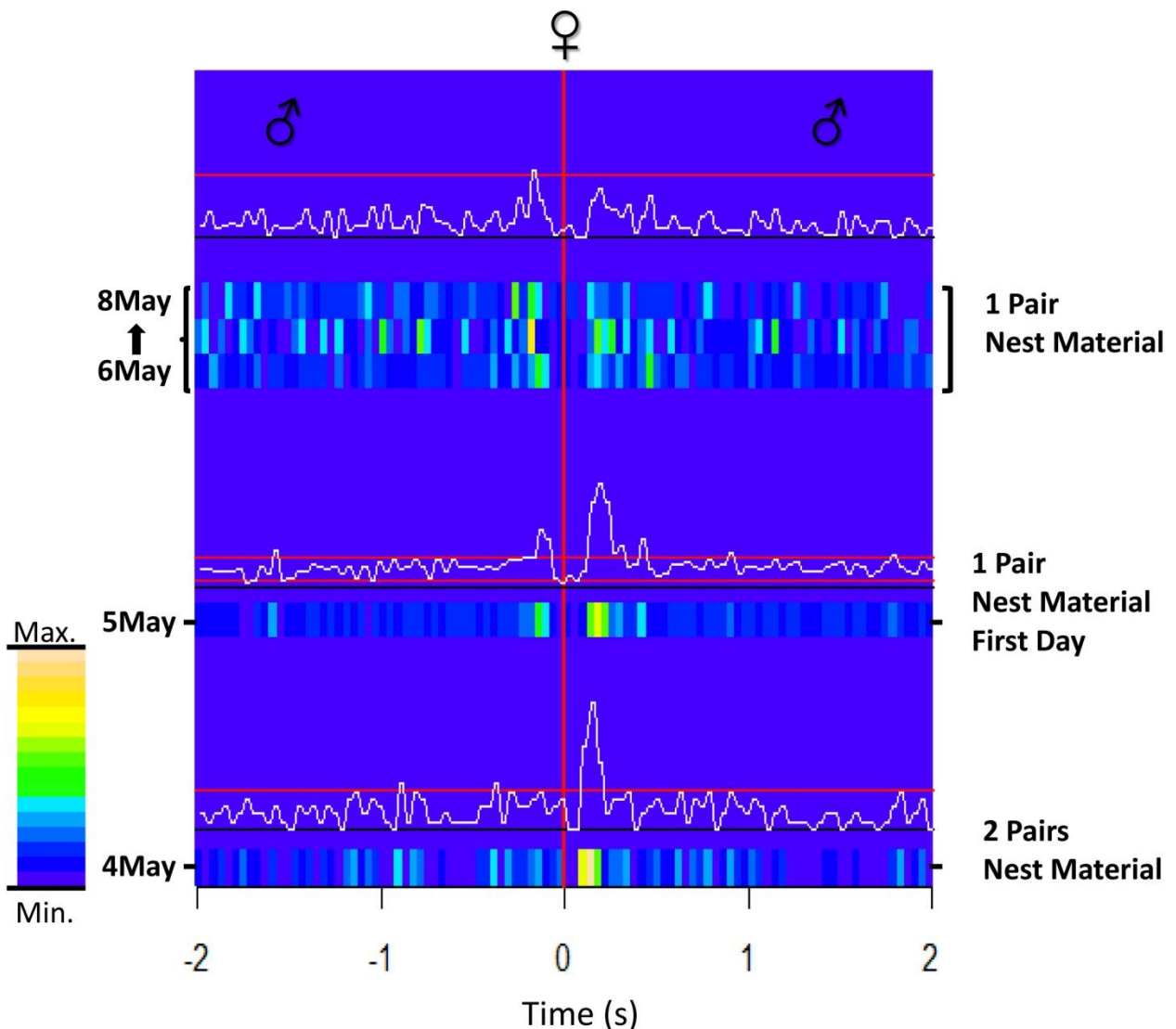


Figure 4. Development of mutual Stack calling patterns of couple A during nest material presence. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize a morning. The color scale represent the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of the whole period. The 0.99 Poisson confidence limits (Abeles, 1982) are shown as horizontal redlines. The black line at the bottom of each graph is value 0. The relation was completely asymmetrical when 4 birds were present, only the male was answering the female. The relation became stronger and more symmetrical after one couple was removed. During the following days this Stack-Stack relationship vanished.

The day in which the nest material was introduced in the aviary where the two pairs were housed together the call relation continued to be asymmetrical and very similar to that observed during the previous period without nest material. When on the following day (5th May, fig. 4 middle) the couple B was removed from the aviary the relation became more symmetric and stronger. Interestingly on the subsequent days the Stack-Stack relationship almost disappeared (6th -8th May, fig. 4 top), despite the fact that the bond of the pair appeared strong and the pair was observed to copulate and build a nest.

3.4.2 Couple A, Stack-Tet

The relation male Tet, female Stack has never been symmetrical (Fig. 5). The male answered frequently with a Tet to the female Stack, but the other way around was rare; in fact it never differed from the hypothesized Poisson random distribution. The overall pattern of this calling relationship did not change when the birds faced different social and/or environmental situations. In fact, in all the periods the average graphs have similar shapes (Fig. 5). The shapes are similar but the intensity of the call relationship was probably (not measured) not constant: when two couples were together in the aviary male calls have a more scattered distribution in the time window (Fig. 5 middle).

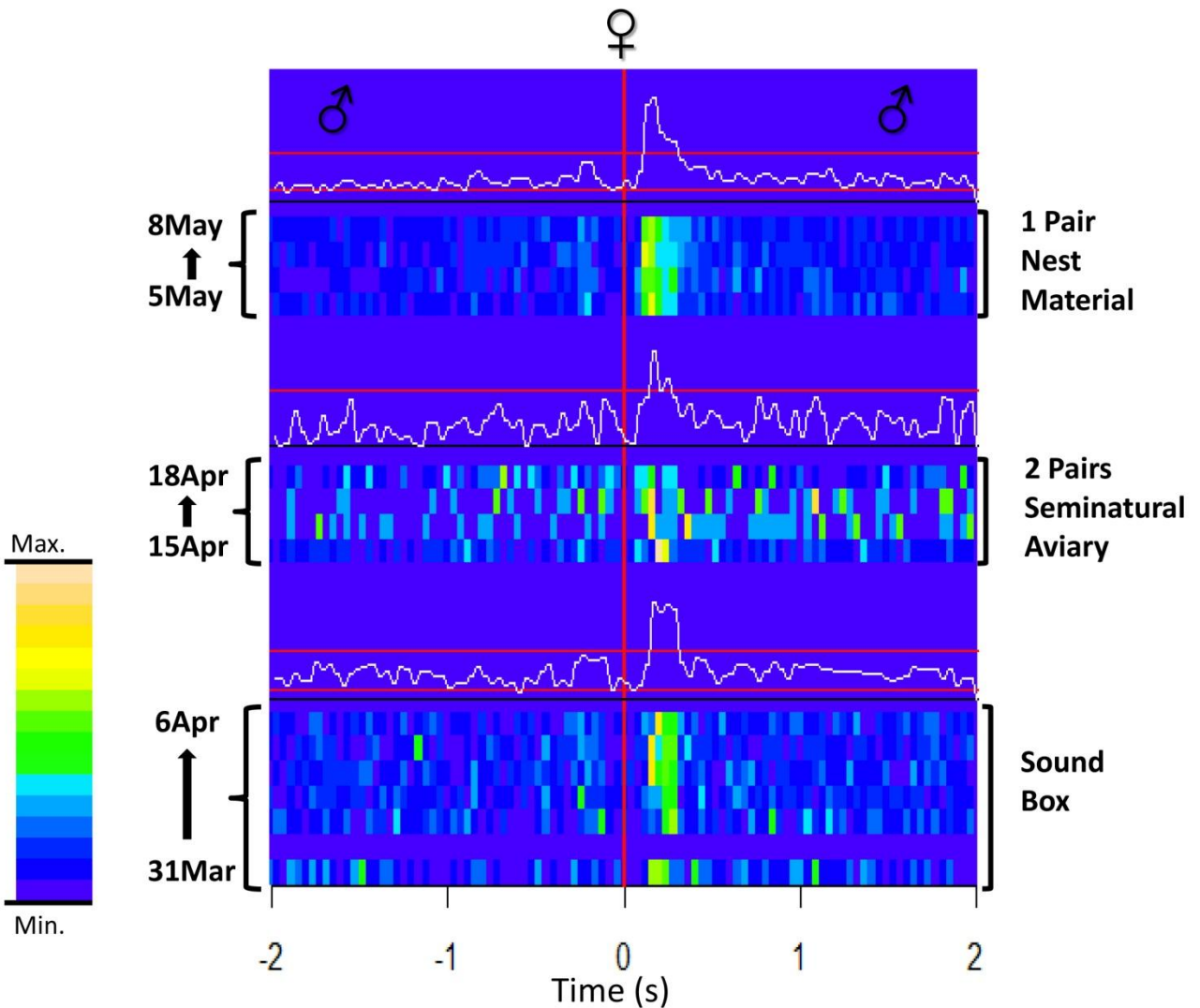


Figure 5. Temporal development of Stack-Tet calling patterns of couple A in different experimental situations. Female Stacks are plotted on time 0, male Tet counts are shown before and after female calls. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize a morning. The color scale represents the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation a graph is presented with the average counts of the period. The 0.99 Poisson confidence limits are shown as horizontal red lines (Abeles, 1982). The black line at the bottom of each graph is value 0. The relation remained asymmetrical in every situation, although the intensity is changing. The male was answering with Tets to female Stacks.

3.4.3 Couple B, Stack-Stack

Due to a technical problem only a few days of recording for this pair were analyzed. The Stack-Stack relation was relatively constant regardless of whether the pair was alone in the sound box or was housed with the other pair in the semi-natural aviary (Fig. 6). The male Stack occurred in response to the female Stack, but the female call back was never significant.

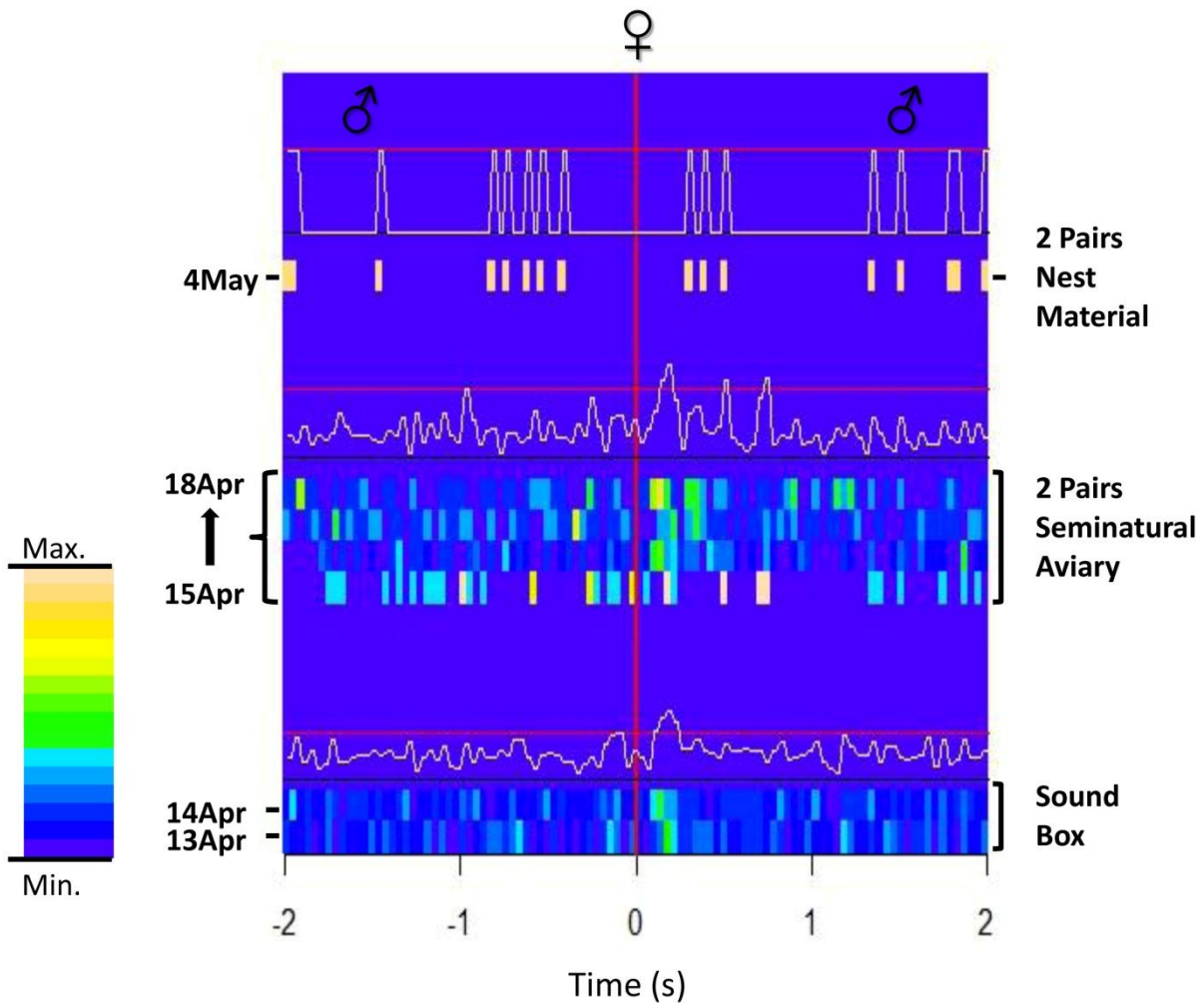


Figure 6. Development of Stack-Stack calling patterns of couple B in different experimental situations. Female Stacks are plotted on time 0, male Stacks are represented on the lateral windows. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarizes a morning. The color scale represents the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of the period. The 0.99 Poisson confidence limits (Abeles, 1982) are shown as horizontal red line. The black line at the bottom of each graph is value 0. This relation was never strong, somehow asymmetric, with the male answering the female, during sound box and semi-natural periods.

3.4.4 Couple B, Stack-Tet

This calling relationship was weak with a lot of scattered calls within the considered time window (Fig. 7). During the different experimental sessions changes in the Stack-Tet relation occurred. The association between these calls changed clearly between the treatments “sound box” and “semi-natural aviary”. Inside the sound box the male emitted Tets as answers to the female’s Stacks, while in the semi-natural aviary, when the two pairs were together, the pattern of this calling relation was inverted: female Stacks answered male Tet. When the nest material was provided (Fig. 7, top) very few male calls were emitted within the considered time window. This probably happened because male B rarely emitted calls, due to frequent attacks from the male A. These results differ greatly from what was observed in couple A.

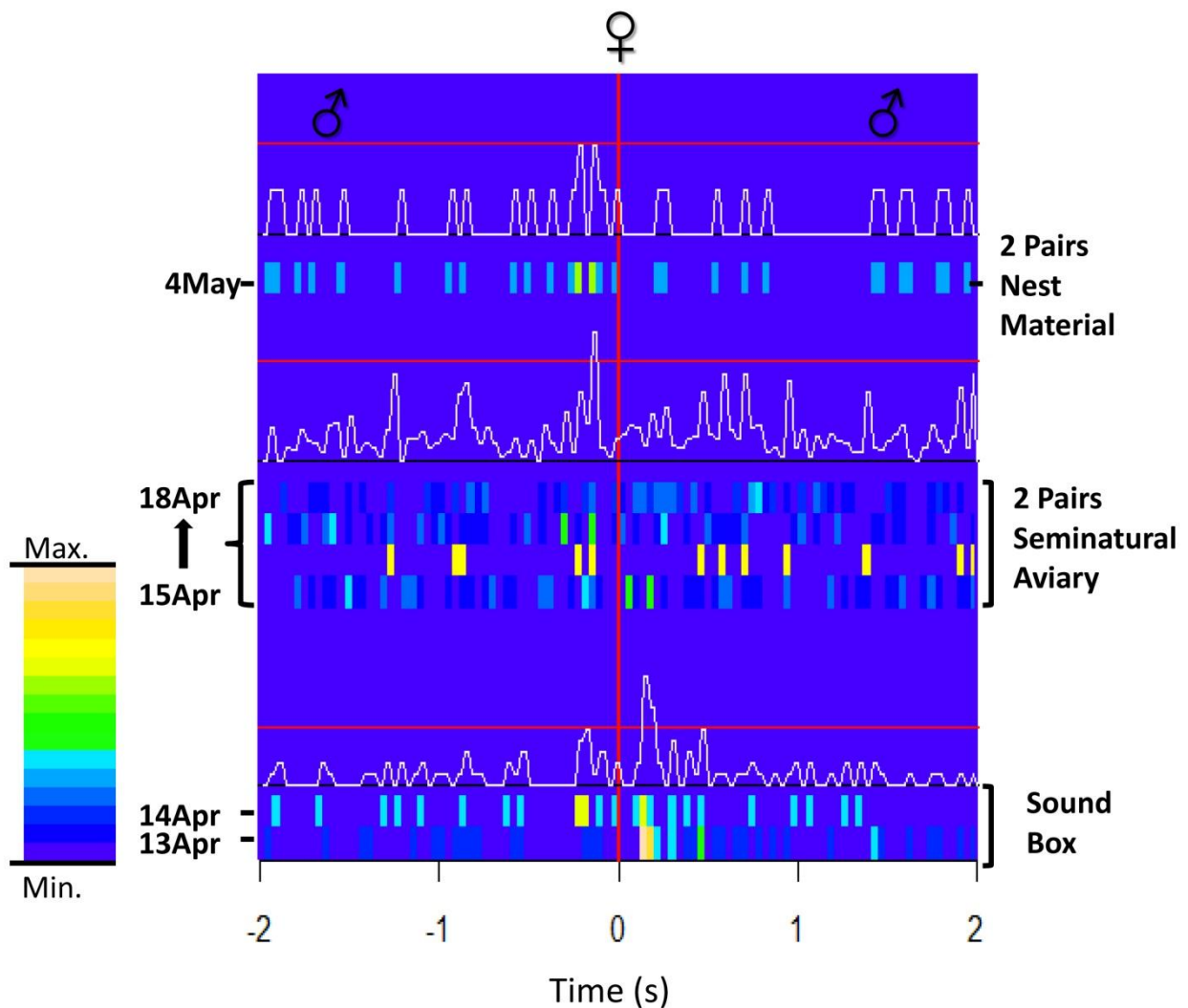


Figure 7. Development of Stack-Tet calling patterns of couple B in different experimental situations. Female Stacks are plotted on time 0, male Tets are represented on lateral windows. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize a morning. The color scale represent the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of whole period. The 0.99 Poisson confidence limits (Abeles, 1982) are shown as horizontal red line. The black line at the bottom of each graph is the value 0. An asymmetrical relation was present in the sound box, the male was answering to the female. No relations occurred in others experimental situations.

3.5 THE PATTERNS OF THE STACK-STACK CALLING RELATIONSHIPS DURING THE EXPERIMENT

The Stack-Stack association was significant in both couples during the sound box period, but it had a different pattern between the two pairs. In fact, in couple A the association was mutual while in couple B was asymmetrical, with only the male answering to the female (couple A fig. 3 bottom, couple B fig. 4 bottom). When the birds were placed together in the bigger aviary, the Stack-Stack relations in both couples became weak and asymmetrical, with only the males answering to the females. Starting from the third day a symmetrical calling relationship between the Stacks of the male A and the Stacks of the female B began. Interestingly, at around the same time male A started repeated attacks on male B, and to clump and to hop together with the female B (Figs. 13,14 and pers. obs.). A symmetrical Stack-Stack relation was present only between engaged pairs when the nest material was absent. When the couple A was left alone with the nest material (removal of couple B because there was too much aggression of male A towards male B) the first day, as if for reconciliation, a mutual Stack-Stack calling association was reestablished (Fig 4). However the following days the Stack-Stack correlation disappeared albeit the bond within the pair was strong as demonstrated by nest building and copulations. The, until that time, significant call correlation, Stack-Stack, disappeared as well between the couple male A female B during the only day in which they had nest material (Fig. 8 top). It seems that in the studied couples this calling correlation defined a non-breeding phase situations; in fact, during nest building this association disappeared although Stacks were still emitted.

3.6 THE PATTERNS OF THE STACK-TET CALLING RELATIONSHIPS DURING THE EXPERIMENT

The other investigated calling relation is between female's Stacks, male's Tets. This calling association was significant in both pairs while they were located inside the sound boxes, as in the new formed couple (male A with female B) when the birds where placed together in the semi-natural aviary. The Stack-Tet association was also renewed between the birds of couple A after that couple B was removed (Fig. 10, bottom right). The asymmetric call relation, female Stack answered by male Tet, was always present with the same pattern in all the identified couples, hence it is possible to hypothesize that only committed pairs use it. As described before for Stack - Stack there were a strong correlations between social behaviors and temporal patterned calling relations:

the engagement between male A and female B and this call relationship started the same day while it disappeared in couple B whose members started to be not engaged anymore.

3.7 TEMPORAL PATTERN OF CALLING. EXTRA PAIR CALLING RELATIONS.

Combinations of calls with a significant correlation were not found between birds of the same sex (data not shown); the only exception was the aggression calls emitted by male A against male B (Fig. 12 B). Some calling relations between birds of opposite sex are reported below.

The most interesting extra pair calling relation has developed between male A and female B (Fig. 8, 9). A symmetrical Stack-Stack relation (Fig. 8) was established between these birds. This relation started from the third day, as the cross correlations diagrams of the calls did not highlight significant difference from a random distribution before that day. During the day in which the nest material was present in the aviary this calling relationship changed, becoming weaker and asymmetrical (Fig. 8, top).

Also between male A Tets and female B Stacks there was an asymmetrical significant correlation (Fig. 9) with only the male that was answering to the female. Similar to what had happened for the Stack-Stack relation, the Stack - Tet relation started from the third day. This relation remained consistent, becoming more intense when the nest material was introduced in the aviary (Fig. 9, top).

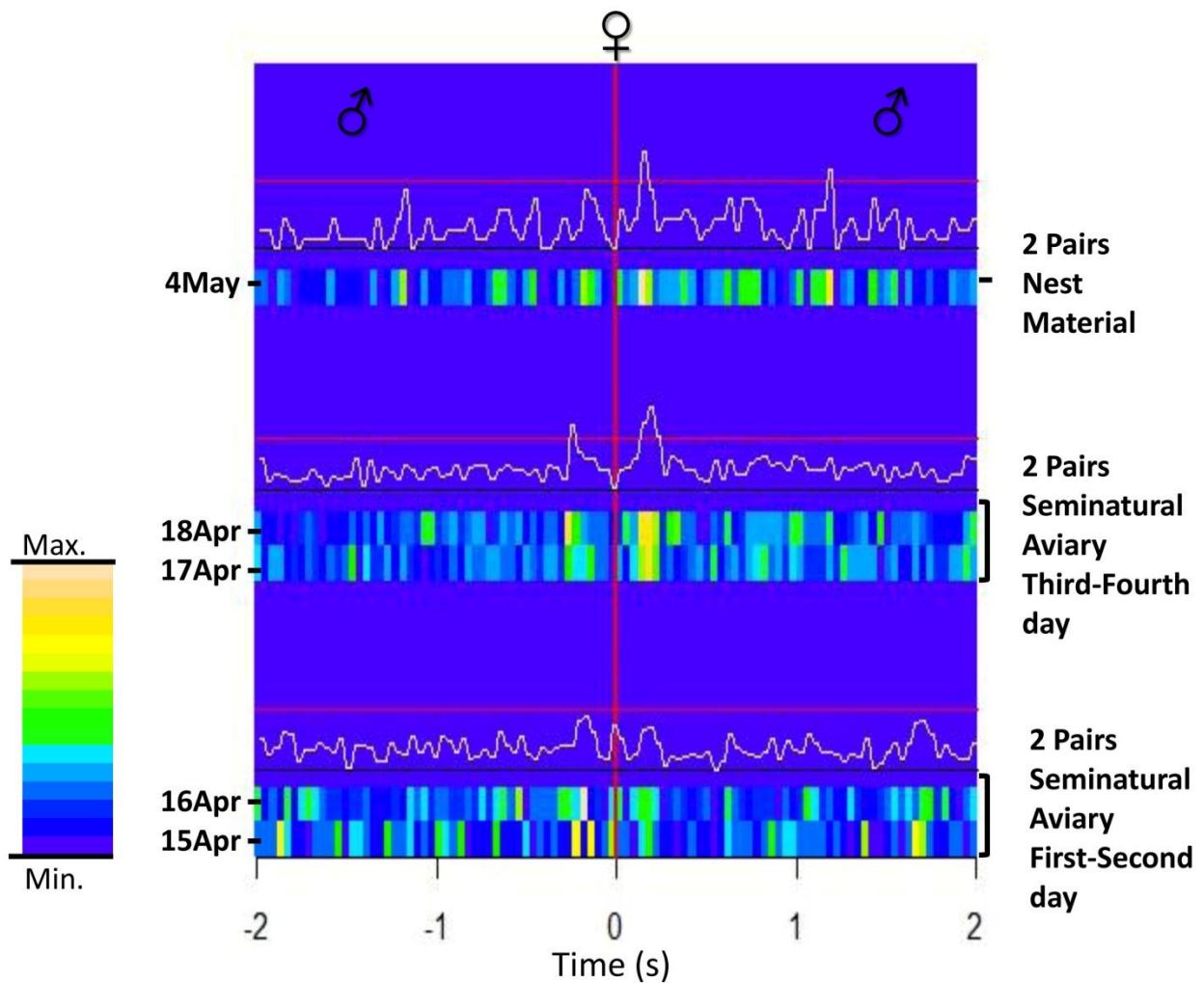


Figure 8. Development of Stack-Stack calling patterns of male A and female B in different experimental situations. Female Stacks are plotted on time 0, male Stacks are represented on the lateral windows. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize one morning. The color scale represent the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of the considered period. The 0.99 Poisson confidence limits are shown horizontal as horizontal red lines (Abeles, 1982). The black line at the bottom of each graph is the value 0. The period of the first experimental situation was divided to show the development of the relation. It took the couple two days to develop a symmetrically correlated calling pattern. When nest material was presented this relation almost disappeared.

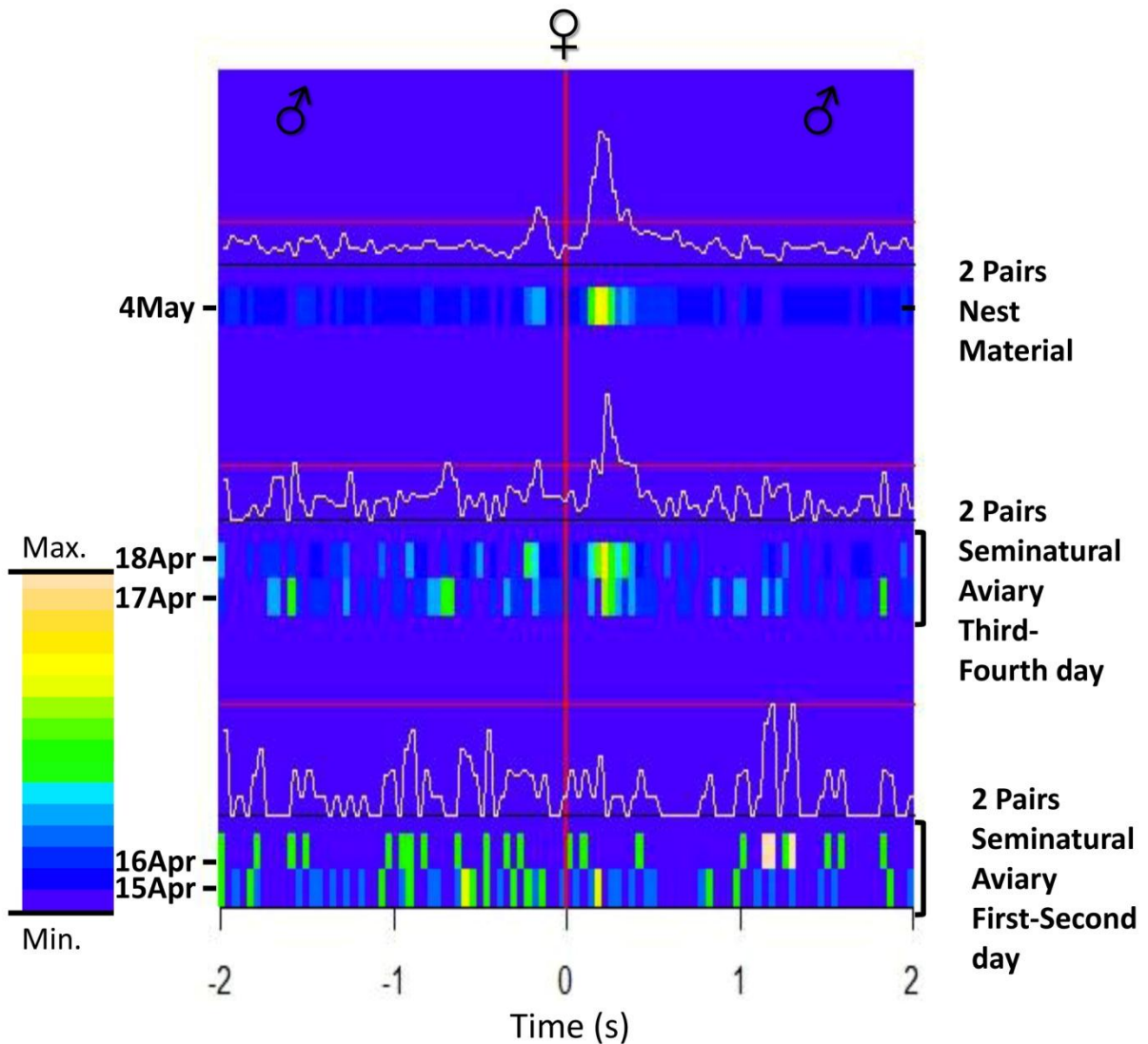


Figure 9. Development of Stack-Tet calling patterns of male A and female B in different experimental situations. Female Stacks are plotted on time 0, male Tets are represented on the lateral windows. The time window is 4 seconds, 2 s before the female call (time=0) and 2 s after. Each row summarize one morning. The color scale represent the normalized probability to find a call in a bin (see material and methods 2.6). After each experimental situation is presented a graph with the average situation of the period. The 0.99 Poisson confidence limits are shown as horizontal red lines (Abeles, 1982). The black line at the bottom of each graph is value 0 (no calls present). The period of the first experimental situation was divided to show the development of the relation. The first 2 days no relation was present, then an asymmetrical relation develop. When nest material was posed this relation became stronger.

Male A started to prefer female B at the third day in which the two couples were placed together in the semi-natural aviary. Subsequently they remained separated for 10 days, and then the two couples were placed again together in the semi-natural aviary. In this case male A and female B immediately formed a pair. That Zebra Finch tend to maintain the bond with the first mate that they have chosen was already described by Clayton (1988) and Silcox & Evans (1982). Two hypotheses can explain this behavior : i) memory of the other bird ii) presence of a particular behavioral or morphological aspect that drive the choice (whoever is the one who choose) always in the same direction.

3.8 TEMPORAL PATTERN OF CALLING. THE ANSWER IS A CHOICE.

The removal of the couple B from the aviary following the repeated attacks of the male A towards the male B caused an interesting behavioral change in the couple A (Fig. 10). On the day in which the nest material was provided and both couples were housed together in the aviary (4th May), the temporal relation of calling between female Stacks and male Tets occurred only between the Male A and the Female B (Fig. 10, top). This call relationship was asymmetrical with only the male answering the female calls. The following day the couple B was removed from the aviary, causing a dramatic change in the call relationship between the male A and the female A (Fig. 10, bottom). In fact, while the Tet-Stack calling relationship between female A and male A was absent in presence of the couple B, once the couple A was left alone in the aviary the male started to answer with Tets to the female Stacks.

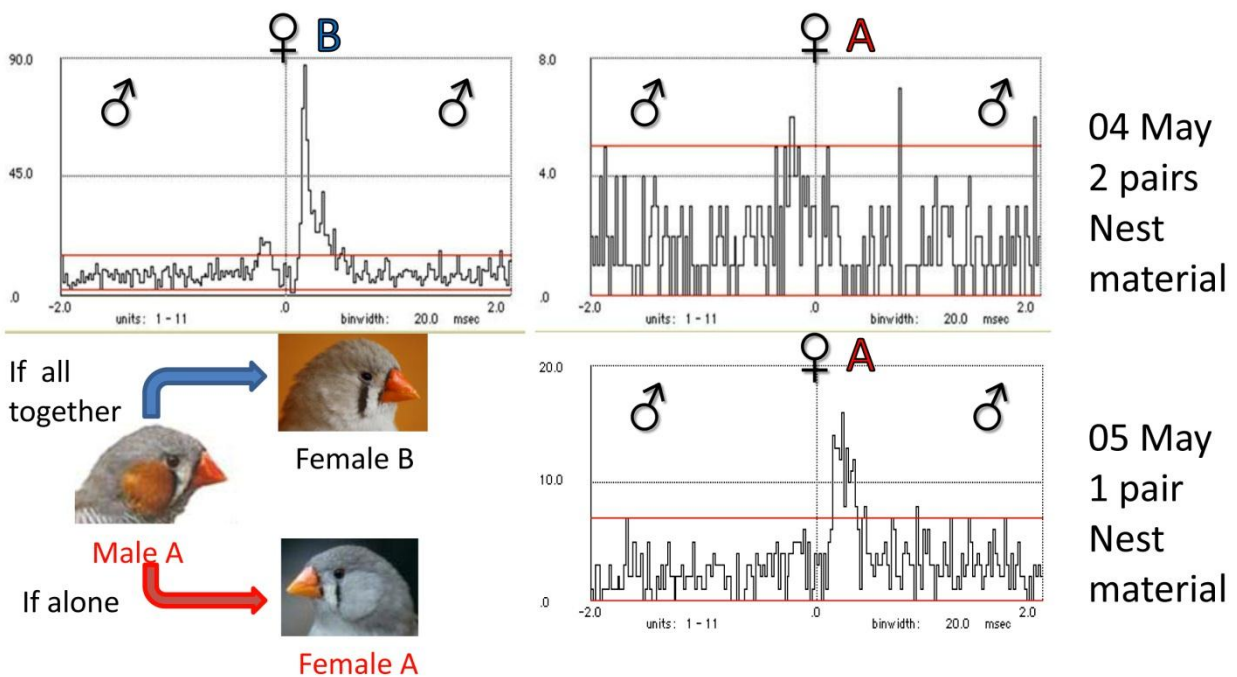


Figure 10. Relationship between Tet calls of male A and Stacks of the females A and B (females always at time 0) Even with his mate, female A, present, male A only answers the calls of female B (top left panel), and not those of female A (top right panel). After removal of female B, male A rebuilds a calling relationship with his former mate, female A.

3.9 TEMPORAL PATTERN OF CALLING. DEVELOPMENT OF NEW CALLS WITH A TEMPORAL RELATIONSHIP.

The presence of the nest material in the aviary modified the pairs' repertoire: male and female emitted a large number of the so called breeding calls, as Kackles, Arks and Whines. A few hours after the introduction of nest material in the aviary, a high number of breeding calls were emitted by all the birds present. Cross correlation diagrams highlighted a specific temporal correlation also between breeding calls (e.g. Fig. 11). However, the correlation diagrams between breeding calls were more blurred with some humps around the female's calls (female's calls are at time 0) in comparison to the contact calls, which were characterized by cross correlation graphs with a clear, sharply delineated shape with defined peaks after a precise temporal gaps. According to Zann (1996) the zebra finches' couples use the breeding calls when they are choosing an appropriate nest site. These calls were described (Zann 1996) as not sexually dimorphic, characterized by a continuum in shapes and lengths from short Kackles to long Whines. Our investigation showed that breeding calls are coordinately emitted, besides showing the characteristics already described by Zann (1996). However the precise succession and dynamics of their utterance remain to be investigated.

According to the literature the breeding calls are never emitted outside the breeding phase. We observed instead that Kackles, and more seldom Arks and Whines, occurred also in absence of the nest material and throughout the whole experiment. However outside the breeding phase the breeding calls were few and never emitted with a significant temporal relationship between birds (pers. obs. data not shown).

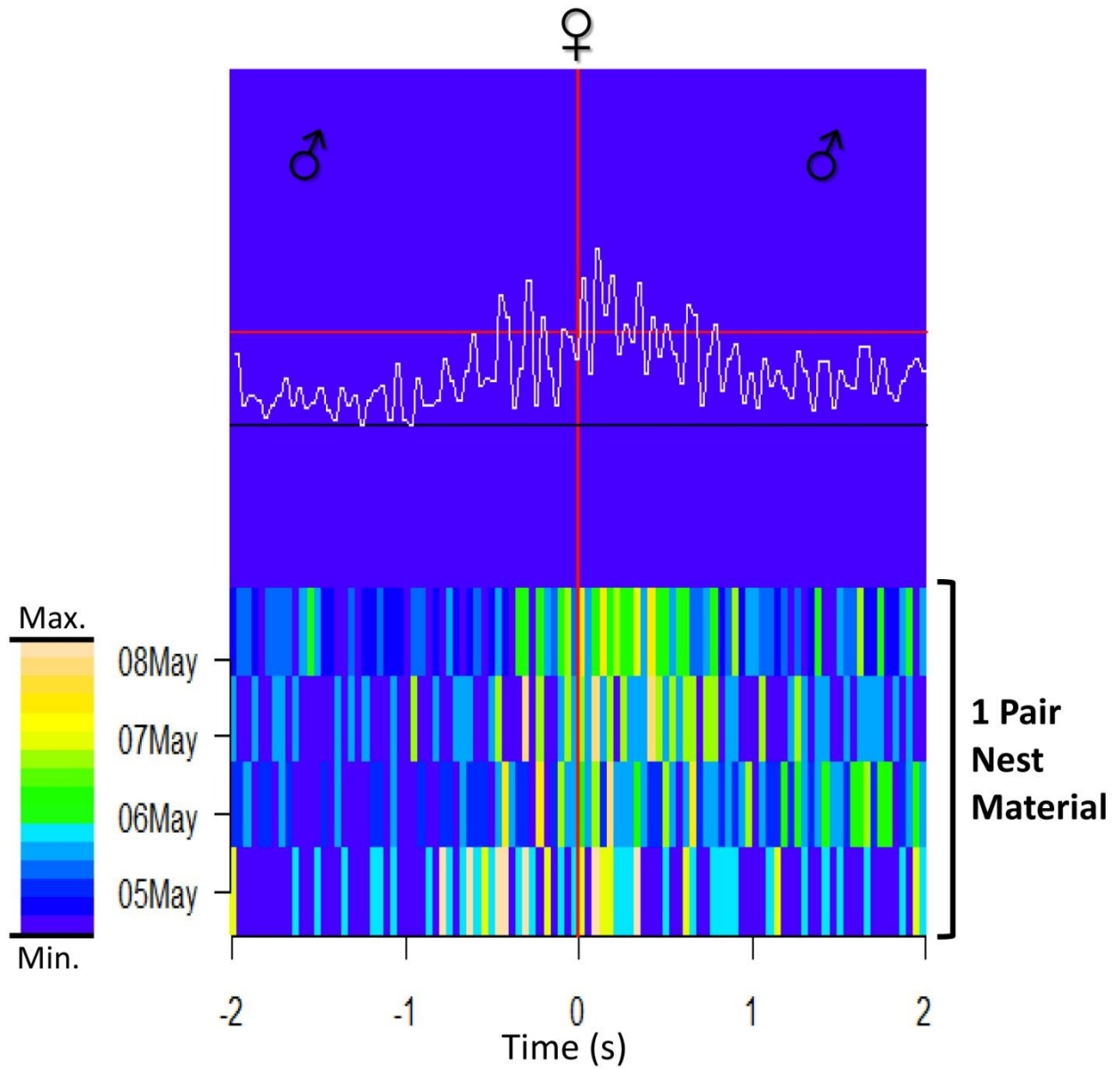


Figure 11. Temporal cross-correlation between Female Kackles and male Arks in couple A. Nest material was present. Clearly these two calls tend to be emitted at the same time.

3.10 TEMPORAL PATTERN OF CALLING. USE OF THE SAME TOOLS FOR FUNCTION\MEANING STUDIES OF CALLS

The cross correlation diagrams represent a powerful tool for visualizing the cause-effect relations. Examples of how a cross correlation diagram can help to understand the meaning and function of calls are given in Figure 12. For instance i) the female Whines were mostly occurred as a consequence of the male Stacks emitted during flight (Fig. 12 A); ii) the aggression calls are triggered by the other male vocalizations (Fig. 12 B): male A emitted aggression calls against the male B when the latter tried to communicate; iii) the contact call Stack is often emitted before takeoff (Fig. 12 C) probably to announce the movement intention to the other birds.

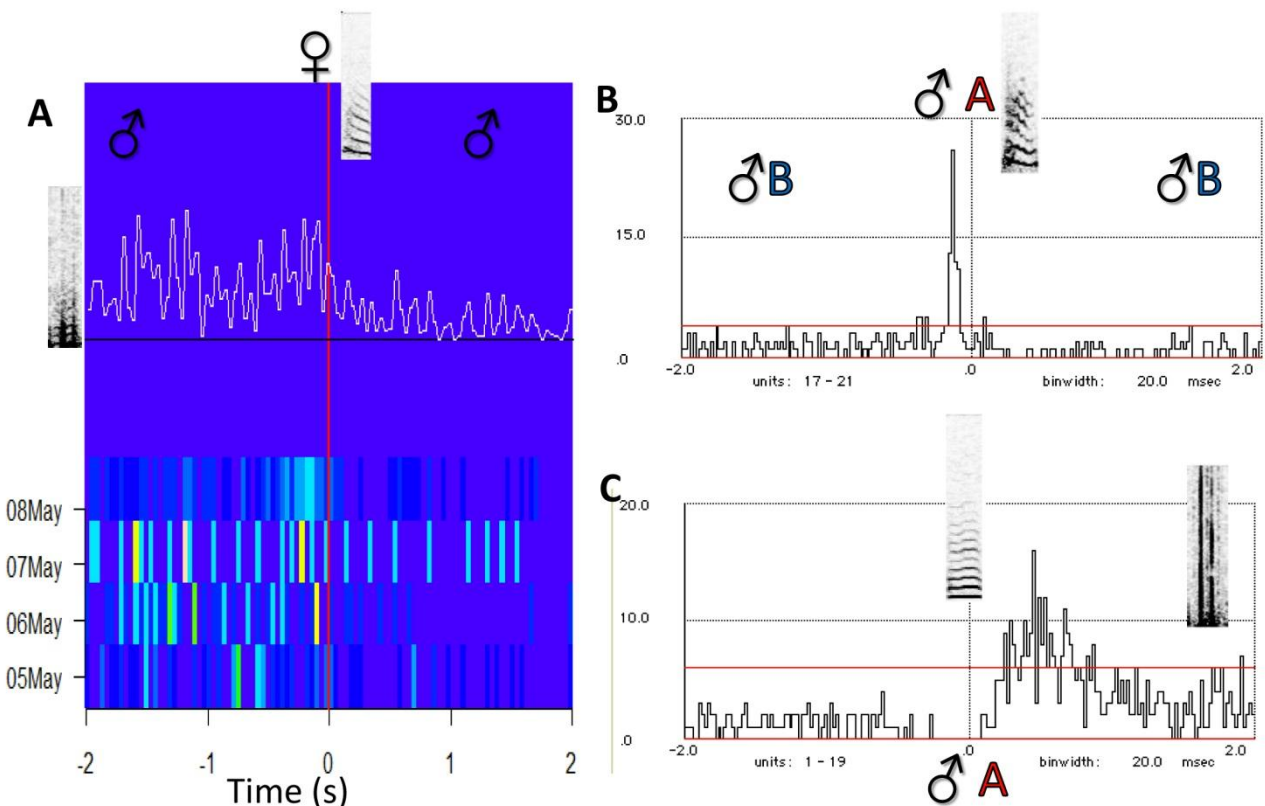


Figure 12. Examples of using temporal cross-correlation tools for studies on the function of calls. A) Flight Stacks of the male and female Whines. The female use Whines more often after the male's movements. B) Male A aggression calls are emitted just after male B Tet. C) Male A used Stacks just before takeoff, the sonogram on the right represents wing flapping.

3.11 ETHOLOGICAL OUTPUT

From the ethograms we extracted only qualitative information. Quantitative measurements were not done. The information obtained from the behavioral output are important to understand the context and the meaning of the observed calling relationship. For example the observation of the clumping behavior allowed us to understand the actual pairs composition. In fact clumping seems to reflect quite well the degree of engagement between two individuals (Zann, 1996; Silcox and Evans, 1982). However more data are necessary to state that this behavior alone is sufficient to indicate the presence of a monogamous relation. A sign of commitment between two birds is represented by hopping behavior, when it is performed by a male and a female together.

From the observation of the ethograms clearly emerged that on the second day of permanence in the semi-natural aviary two separated couples were present (Fig. 13). However we have observed some behaviors showing that an extra pair relationship started to be established. In fact, the female B hopped together with the male A and clumped between the two males A and B. After two more days (Fig. 14) it was clear that the relationships between the 4 individuals in the aviary had changed. Male B and female A were unpaired, whereas the male A and the female B clumped together most of the time indicating a developing pair bond.

An example of the relation between a call production and behavior is visualized in figure 15. Some example of indicative anecdotal observations are given: I) the male A never emitted calls during its singing activity; II) When clumping, the birds did not vocalize; III) Stack calls were frequently used during hopping; IV) The male A preferred to use higher perches and sang more often sitting on the highest perch.

16.04.2011, 19.00-20.00 Social Behaviour

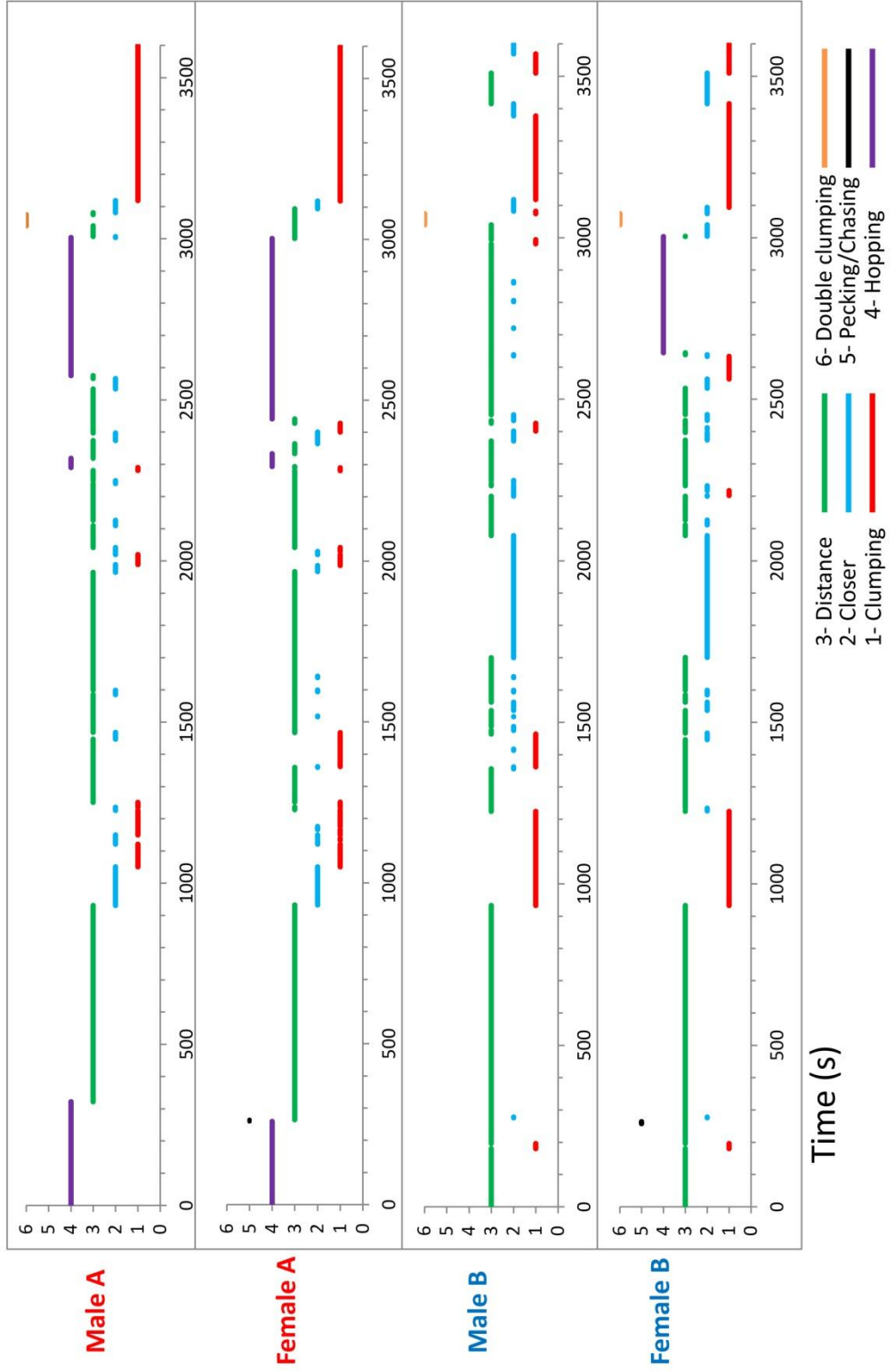


Figure 13. Ethograms of 4 birds together in aviary. Behaviors with possible social meaning are shown. The color and the vertical positions of the lines represent the behavior, the length represent the duration. It is possible to see pair composition. Birds that were clumping or hopping together are supposed to be a pair (Zann, 1996). Comparing different birds' behavior clearly shows that the situation was not stable, for instance female B clumped with Male B, but she also clumped with both male simultaneously and she was hopping with Male A.

18.04.2011, 19.30-20.30 Social Behaviour

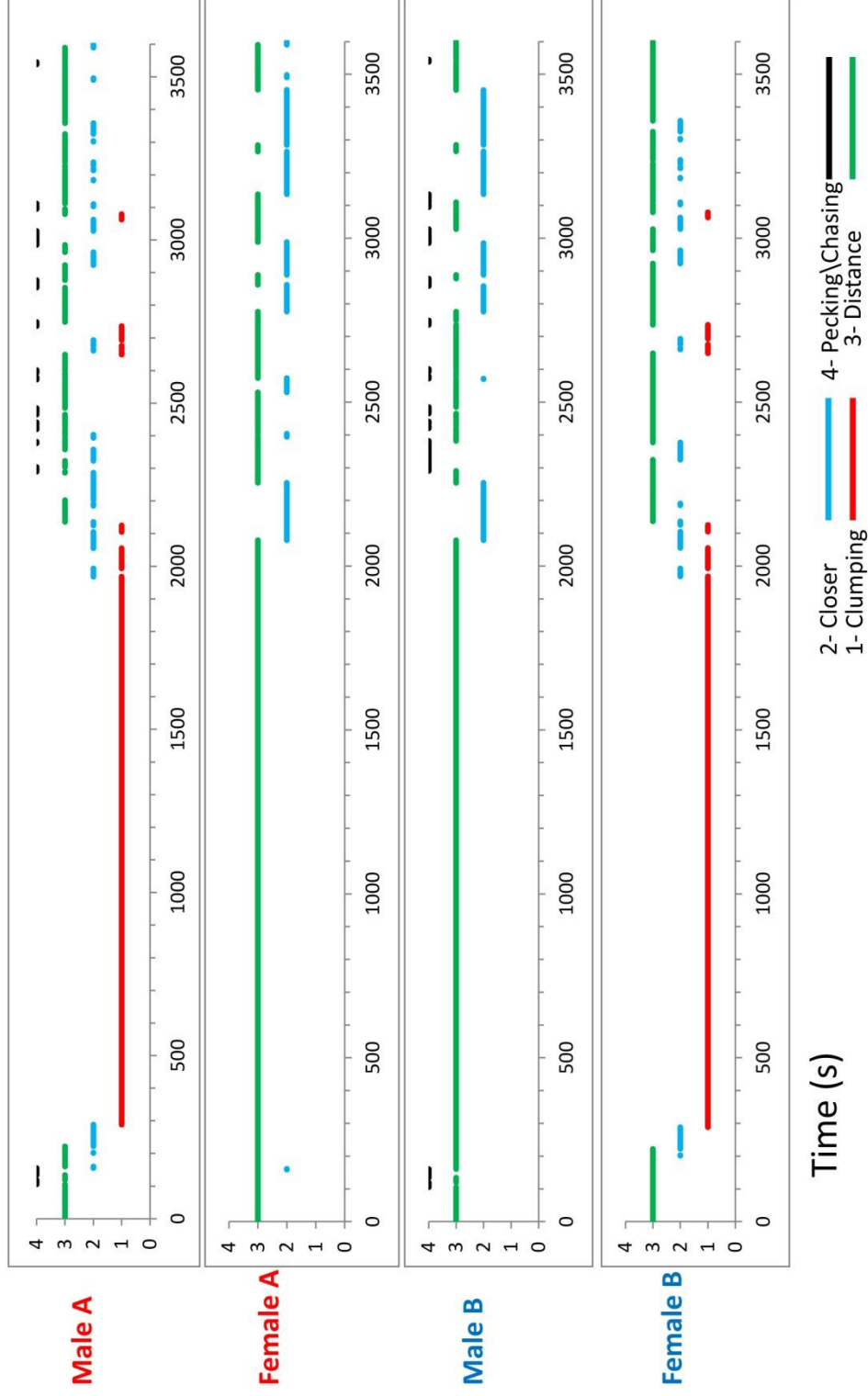


Figure 14. Same ethogram type as figure 13 but referred at a later date (2 days later than fig. 13). The comparison between different days show the development of birds' relationship. The development of a relationship between male A and female B is demonstrated by the fact that they were clumping together. Aggressive behaviors, like pecking and chasing, were always initiated from Male A toward Male B.

Male A Ethogram 16.04.2011 19:00-20:00

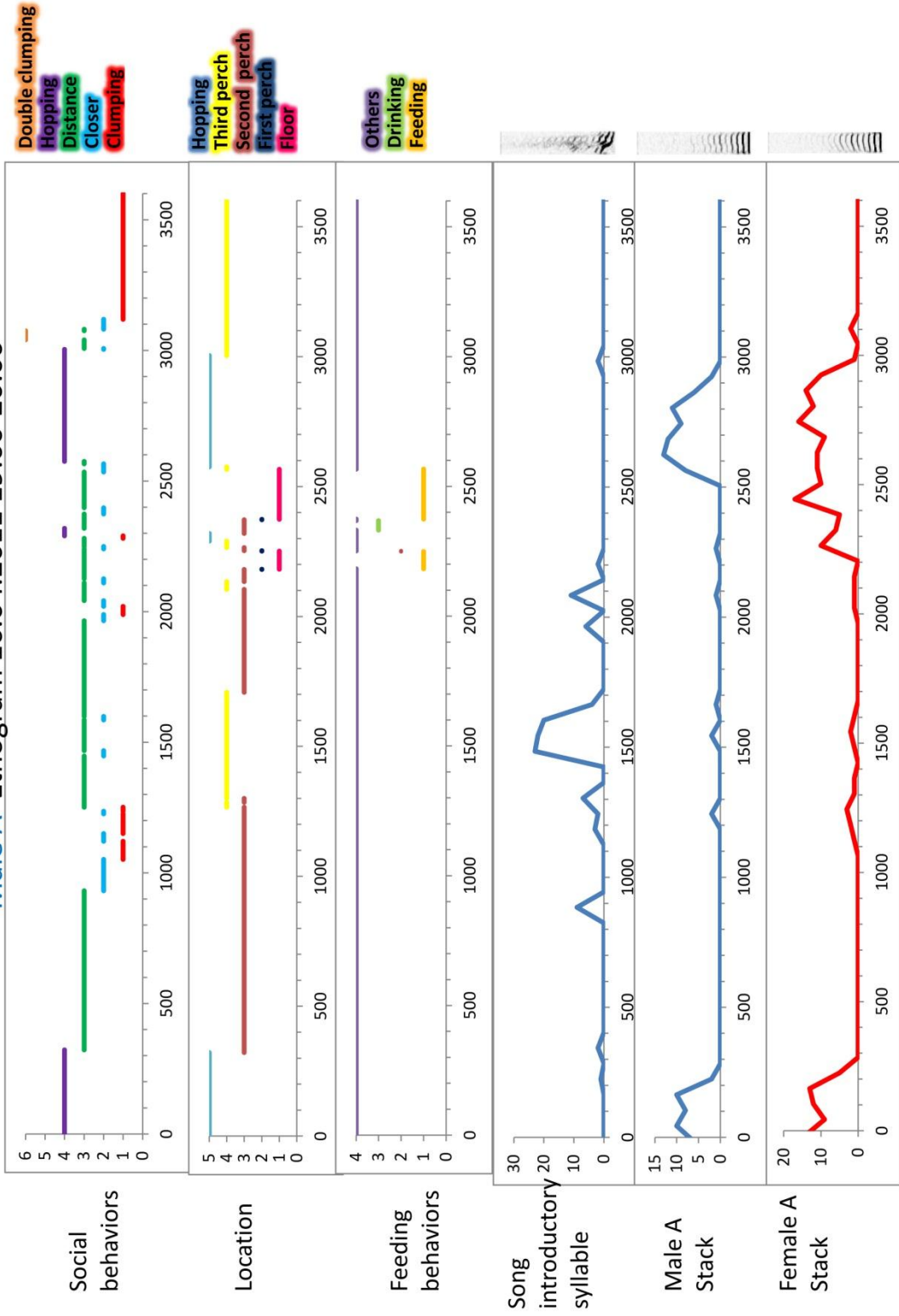


Figure 15. Combining behavior and vocalizations. Ethograms are all from Male A. Vocalizations sonograms are shown in the bottom three panels. This figure yields the following noticeable (qualitative) information:

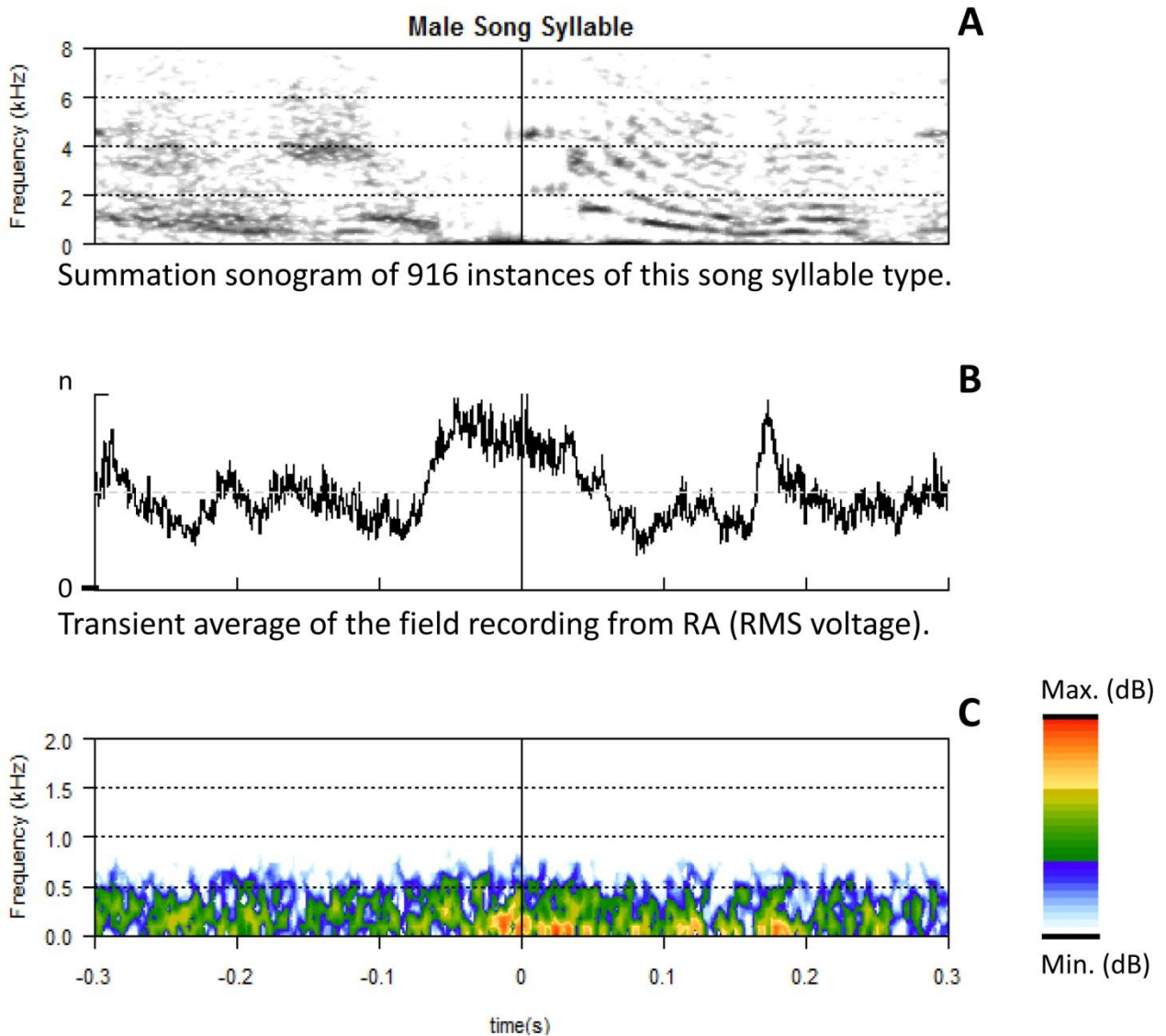
- I) The Male seems to sing only when his mate is not close.
- II) The male seems to sing more from high perch.
- III) Stacks call were emitted when the bird was hopping.
- IV) Intense calling periods (e.g. Stacks) happen at same time in male and female.
- V) The male is not calling or singing while clumping or feeding.

3.12 ELECTROPHYSIOLOGY

It has been possible to describe the electrophysiological signal only when the birds were in the sound box. In fact, in the semi-natural aviary possible of mutual interferences between the two transmitters made the signal unreliable. However, when the males were in the sound box it was possible record the change of the activity of the considered song nucleus, Robust Nucleus of the Arcopallium (Fig. 16). The spectrogram shows a higher amplitude synchronized with the vocalization production. The higher amplitude is a consequence of the repeatability of the wave shape. A repeatable wave shape gives a predictable average signal represented by the higher amplitude.

For each vocalization type, the Root Mean Square (RMS) transformation and the spectrogram of the average Local Field Potential (LFP) signal have a consistent and repeatable shape on different days (Figure 17 for song syllable male B, the data are not shown for the other vocalizations types and birds). The transformed wave shape was similar after 18 days. This result indicate that the LFP is more stable than the single unit and multi units recordings with a similar devices.

Possible mistakes, artifacts and also possible integrated uses with the other tools are analyzed in the discussion section.



Spectrogram of average signal of total production of this song syllable type.

Figure 16. Example of possible LFP signal description for a male A song syllable. It was recorded the 04 April during 4 hours in the morning, three day after the surgery (01 April morning). A) The Sonogram is the summation of 916 song syllable plotted on the same onset point. Call stimuli are recorded synchronously with the electrophysiological signal to verify correct alignment of both measurements. B) Root Mean Square (RMS) transformation, it is a possible visualization to describe the signal change. We are interested whether this pattern, that should represent the changing activity of the RA, remain consistent in different days\situations. C) Spectrogram of the summation of 916 time periods synchronized with the chosen song syllable production. Color represents the LFP amplitude. If the signal is consistent among events I would expect a higher amplitude.

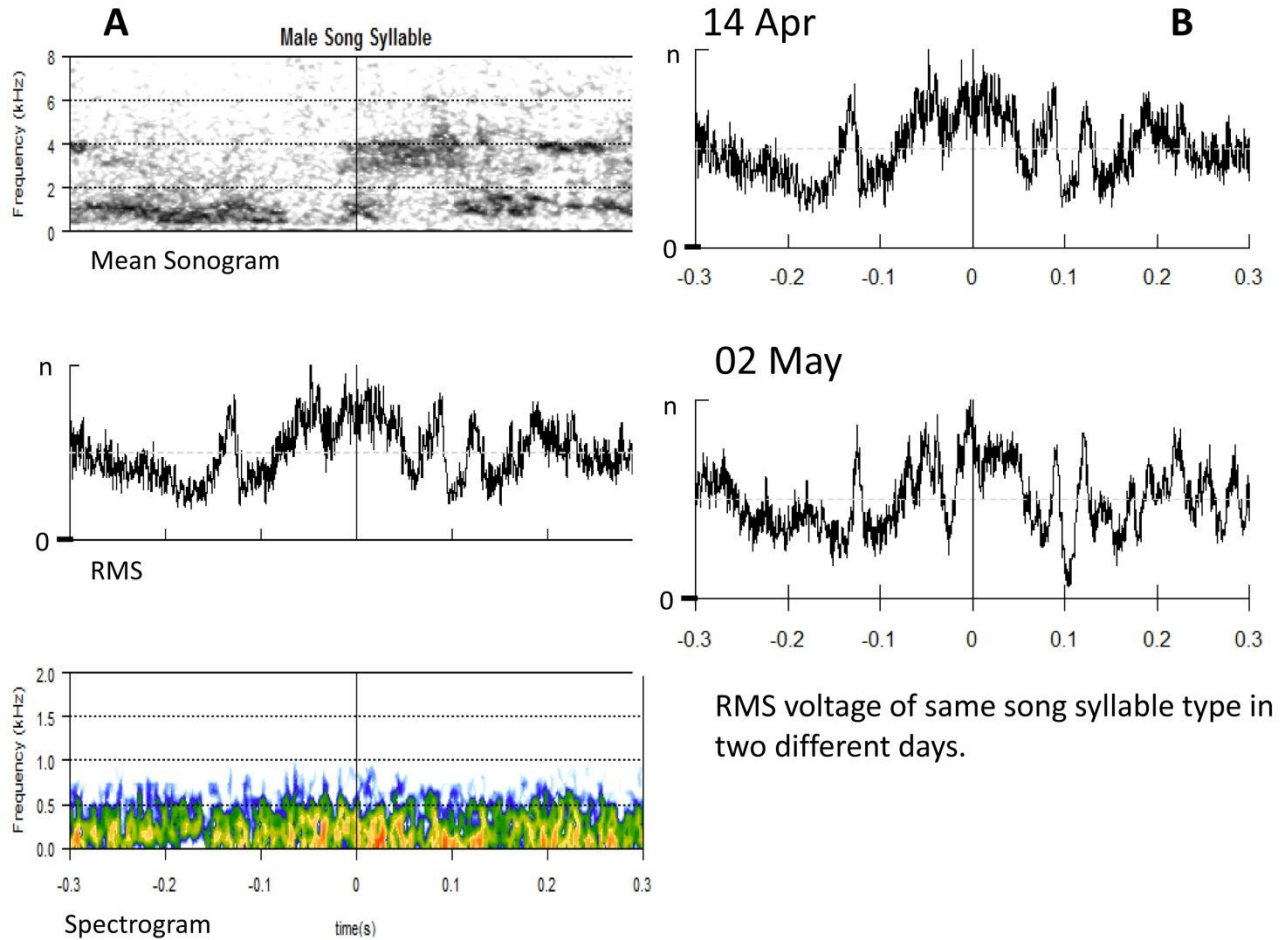


Figure 17. LFP description and electrophysiological signal lasting time of male B. A) Qualitative description of the low frequency composing the local field potential for a song syllable of male B. (Three days after the surgery). B) Consistency of the signal after 18 days: 14 April (316 occurrences), 02 May (807 occurrences), the bird was in the sound box. The transformed signal remains very similar even after a long period of time.

4...DISCUSSION

4.1 SAMPLING

This work is a preliminary study performed in order to find out the possibilities of the used techniques and improve the experimental set up. For this reason the number of birds was small and therefore not sufficient to achieve any conclusion about both the vocal behavior and the correlated neuronal response.

However, it has been possible to record the vocalizations emitted by an individual for the entire day during several days and it has been possible to process the huge amount of data in order to obtain and analyze the clusters. The applied methods allowed the recording and analysis of the complete individual daily vocalization emission with high precision. Nevertheless, data processing, particularly manual clustering, is very time consuming. As it was found that the temporal relations of calling do not change qualitatively within a day, further experiments can be based on limited portions of the day.

4.2 REPERTOIRE

A key step of this project was to define a repertoire of calls and song syllables in our experimental settings. In fact, the analysis of vocalizations depends on how the various types of calls are divided into clusters.

The repertoire compilation was based on visual characteristics of syllables and calls. In fact, we assumed that different sonograms shapes are perceived differently by the receiver birds. It has been demonstrated that call's physical characteristics are related to specific functions of the calls (Marler & Evans, 1996; Marler 2004b), therefore different sonogram shapes indicate different meanings of the calls. A further analysis will be necessary to provide a statistical assessment of the homogeneity of the clusters by comparing intra\inter clusters differences.

A detailed description of the Zebra finch call repertoire was first provided by Immelman (1965) and it was updated by Zann (1996) with more modern techniques. To define a call repertoire is difficult because of the variability of some kind of calls (pers. obs.; Zann, 1996; Marler, 2004b) and the more or less blurry boundaries between some call types which give way to multiple interpretations.

In fact, while Zann affirmed that Zebra finches have ten or eleven distinct calls in addition to the song, Marler P. (2004a) stated (based on Goodwin 1982, Güttinger & Nicolai, 1973) that zebra finches have less than eight types of calls. In this work we have observed all the types of calls described by Zann. In addition, we have observed some clusters showing a marked intra-variability, so that it was possible to further divide them in “sub clusters”. A detailed determination of Zebra finch calls names is still missing and this makes the comparison and the generalization of the calls analysis quite difficult.

Although I am not able to suggest standardization features that define the repertoire I propose to use both functional and visual characteristics. I have shown that clusters with different visual characteristics of the sonograms sometime are used differently by birds. This indicate that they must belong to different categories. However, not all the calls are emitted in the time window considered for the data analysis so it would be interesting to see if these calls are separable through functional role or to test if different calls might have the same meaning. Some of the used tools can help to define the important functional characteristics (see discussion 3.6).

In our study we have observed a high degree of repeatability of the defined call types in different birds. Moreover we have observed in our subjects all the main call types described by Zann (1996) for wild birds or birds caged during few generations. Therefore we have found that the Zebra Finch’s repertoire remained very similar even after many generations of captivity. However, some incongruence with Zann’s descriptions were pointed out. i) Stack, and not Tet, was frequently the most common contact call. ii) The breeding calls, Kackle, Ark and Whine were found in our birds also when nest sites and nest material were not present.

Investigations on the calls of the Zebra Finch are principally based on long\distance calls, which are learned in the case of the male (Vicario 2004; Vicario *et al.*, 2000; Gobes *et al.*, 2009). The description made by Vignal for both male (2007) and female (2004) distance calls is consistent with the features observed in our study. Distance calls are often described as the most frequently emitted calls (Vignal *et al.*, 2004, 2007; Elie *et al.*, 2011). Although a rigorous analysis has to be performed, at first glance the Tets and Stacks seemed to be the most emitted calls by the subjects of our study (pers. obs., data not shown) and I had the impression that the distance calls were more frequently produced mainly when a person was inside the experimental room (pers. obs., data not shown).

4.3 TEMPORAL PATTERN OF CALLING LEADS TO AN UNDERSTANDS ABOUT HOW BIRDS USE CALLS

The presence of a precise temporal pattern of calling between birds (Andries Ter Maat and Lisa Trost, unpublished results) was confirmed in our study: different combinations of call types had distinct patterns but not all combinations do show a patterned temporal relation. We can affirm that there is a temporal pattern of calling only when the cross correlation graphs show a significant association between two calls type. However the shape of the significant part of the cross correlation graphs is not the same for all the call combinations. For example, a precise hump shape, symmetrical or asymmetrical, of the cross correlation graphs was observed exclusively in contact calls (Tet-Stack). This particular shape was not found between the most studied distance calls or the breeding calls (data not shown for the distance calls, fig.11 for a combination of breeding calls). In fact, the male distance/breeding calls aggregate around 0 (female's distance calls) without a precise gap between call and answer, when plotted in a cross correlation histogram. The observed differences in shape of the cross correlation graphs might highlight differences on encoding and control of calls at neural level. Differences in neural pathways for learned and unlearned vocalizations were inferred by Simpson & Vicario (1990) with a study only on distance calls. Further investigations are needed to clarify the possible relation between the cross correlation graphs shapes and the neuronal control of vocalization production.

About the function of Tet calls Zann (1996) stated: “they are not directed at specific individuals and do not stimulate specific reply”. Therefore according to his view the Tet calls do not have any specific role in the communication between two particular individuals. In our work we have shown that the fraction of calls occurring in temporal calling relations are directed toward a particular individual and do stimulate a reply, which is specific both regarding the type and the temporal delay of the calls. In fact, it has been found that the male was able to decide towards which female to address his response: when the male was listening both females present in the active space of male's calls, he answered with Tet calls only to one of them (Fig. 10). This indicates that he was able to distinguish between the Stacks of the two females. The behavioral data showed that the male maintained a temporal relation of calling with the female with which he was engaged. Taking these evidences together we can state that the male is able of individual recognition of the females while calling, however whether the recognition is based on acoustic, visual, behavioral, olfactory cues or

on all these information together has to be experimentally demonstrated. Thanks to the backpack microphones recording we were always able to know which call was emitted by which female. However, although a test assessing the statistical difference between the Stacks of the two females was not performed, the two clusters were distinct and immediately recognizable at first glance (Fig 1).

For a monogamous but group living bird such as the Zebra Finch it is very unlikely that in a wild environment mates do not recognize each other by their voices (Vignal, 2004). Mate recognition through distance calls, both by males and females, has been recently demonstrated (Vignal, 2004, 2007). It has been shown that the distance calls contain cues that permit individual identity, therefore allowing mate recognition. However, it has been found that Zebra Finches are not able to recognize their own offspring by long calls (Rees H *et al.*, 2011). It would be very important for the study of zebra finch communication to know if the birds are able to recognize different individuals by contact calls. So far there is no published evidences whether contact calls of the Zebra Finches contain or do not contain identity information. However, Robertson (1995) has shown the first evidence of vocal mate recognition based on contact calls in a species that, like the Zebra finches, maintains pair bonds within mobile foraging flocks out of the breeding season (the Silvereye, *Zosterops lateralis*). Usually contact calls are more common than distance calls in wild bird, the latter are louder, easier to be detected by predators (Marler 2004b), so, in my opinion, it would be strange that only the louder calls are individually recognizable.

From what reported in figure 10 it seems that a Zebra finch can decide to answer to a particular individual and with which type of call. If these results will be confirmed, this is a good evidence for a cognitive process regulating the contact call emission.

In conclusion, on the basis of the temporal pattern of calling we have found that i) the contact calls are directed toward a specific individual, ii) there is a choice of what type of call will be used as response.

The data collected in this study and the technique we have developed opens up a new perspective for future experiments. For instance the strength of these calling relations could be studied looking at the percentage of answered calls. Furthermore it is known that vocalization emission it is not

constant during the day (Elie *et al.* 2011, pers. obs.) with bursts of vocalization followed by calm periods. The communication system of this species could be better understood by studying the dynamic of bursts and vocal pause.

With the cross correlation analysis it is possible to investigate only binary combinations of calling, but sometime the birds uttered more complex combinations (e.g. a sequence of male Tet – female Stack – male Tet). On the basis of our observations we have hypothesized that different call combinations have different functions (see also discussion 3.2). Further investigations and the development of tools that consider the possibility of more complex calls combinations are needed. It would be also important to compare our results with similar experiments on wild birds and on species of birds with a different social system, in fact cognitive demands of gregarious life could lead to an increase of communication abilities (Dunbar, 1998; Adolphs 2003; Vignal 2004).

4.4 TEMPORAL PATTERN OF CALLING. ENVIRONMENTAL AND SOCIAL INFLUENCES

It is well known that birds frequently call while other birds are calling (Lerch *et al.*, 2010; Gahr & Beckers, 2010; Marler, 2004b). However in this institute it has recently discovered that the calling emission of two birds has a precise temporal patterned relation (Andries Ter Maat and Lisa Trost, unpublished results). Our study provided evidence that this calling relations change according to different social\environmental situations. This suggests that a particular calling relation has a specific meaning\role\function.

Two call combinations, male and female Stacks and male Tet with female Stack, were consistent during the whole experiment. For this reason they have been investigated in detail. It has been demonstrated that different social and\or environmental conditions caused changes of temporal patterned calling: both calling correlations underwent drastic modifications during the experiment. The changes were due to both environmental and social influence: the Stack-Stack relation changed at the same time with the presence of nest material, that triggered breeding behavior; the Stack-Tet relation changed according to couple composition, demonstrated by ethological observations. The fact that different calling relations changed independently from each other following the same change in the experimental condition, suggest that different call associations have different

meanings. The temporal pattern of calling probably contain information about the relationship between two individuals which might be modified after changes in the environmental context.

The presence of others birds influences the dynamics of calling through the so called “audience effect” (Baltz & Clark, 1997; Evans & Evans, 1999; Marler 2004b). Vignal *et al.* (2004) and Elie *et al.* (2011) showed the importance of social context for the calling behavior in zebra finches, as the presence of other birds changed number, frequency and type of emitted calls. In our experiment we have confirmed that the social context influences the vocalization behavior and we have added new information about changes in calling associations. In fact, both the Stack-Tet and the Stack-Stack relationships of calling patterns were constant and repetitive when the pairs were isolated (Couple A figs. 3, 5 bottom, couple B figs. 6, 7 bottom), but they changed according to the social context.

When the birds were placed together inside the semi-natural aviary, two different changes in the experimental conditions occurred: a new aviary and a different social context. The effects of the two factors should be tested separately in a further experiment. Nevertheless, it is likely that the critical environmental change affecting the calling pattern was the one that have occurred at social level. In fact, modifications in calling relations were observed following changes in the social structure in the unchanged environmental condition (Figs. 8,9).

4.4.1 Stack-Stack & Stack-Tet: possible meanings.

It has been pointed out that the two considered calling relations have specific, different, meanings; however at this point of our research we can only hypothesize their functions and relevance.

Nest material triggered the breeding behavior, it is known that the breeding phase changes vocalizations repertoire in zebra finches (Zann, 1996), and Passeriformes in general.(Marler, 1956; Mundinger, 1970). Evidence was found for an influence of breeding on temporal relations of calling besides ones on the repertoire. In fact the disappearance of the Stack - Stack relation was in two cases contemporary with nest material presence and so nest building. The production of new calls types with a temporal calling relationships was associated with a loss of Stack-Stack (see results 3.5). It seems that, when mutual, the Stack-Stack calling correlation identifies a couple in a non-breeding phase. I do not have any hypothesis on the reasons of this behavior. Asymmetrical Stack-

Stack relations were also observed, both with male answering to the female and the other way around: so far we have not found clues regarding their meanings.

The asymmetric call relation, female Stack answered by male Tet, was always present with the same pattern in all the identified couples, hence, it is possible to hypothesize that only committed pairs use it. It is still unknown if this calling relation is informative only within the couple or also for the other member of the group. Further experiments can test the proposed meanings and start to investigate their functions and which birds are influenced by them.

In sum, there are strong evidences that these call relations have a social role, it is still unclear if only within the pair or also for the rest of the group. For the experimenter these vocal exchanges could provide information on existing relations between birds. To generalize these findings a bigger sample size is necessary, then it would be possible to try to answer different questions that arise on role and meaning of the call relations and concerning the degree of awareness of the birds.

4.5 TEMPORAL PATTERN OF CALLING. EXTRA PAIR CALLING RELATIONS.

The social relations of the Zebra finches can be divided in two types: the relations within a pair or between the members of a group and/or colony (Zann, 1996). Vocal communication is likely to be crucial at both level (Marler, 2004b, for a review). In our experiment extra pair calling relations were not present or they were significant only for brief periods (data not shown). Out of pairs formed by the experimenter, couple A and couple B, a new calling relation has begun only during the formation of the sole new established couple: the one between male A and female B. Thus, with our tools, evidences for call relations on a group level were not found; if some of the vocalizations are specifically directed towards other members of the group they are not highlighted by cross correlation analysis. It is possible that temporal patterned calling relations are only present within bonded pairs or that the conditions present in our experiment (e.g. social hierarchy) did not support this type of temporal patterns of calling.

Studies on wild zebra finches have revealed that the monogamy last for more than one breeding season and that the couples are inseparable all during the year (Zann, 1996). Thus it is necessary to carry on a continuous (vocal) contact: therefore, it is not surprising that association of calls are

maintained continuously only within the pair. Nevertheless it was shown that some of the calls, the ones used in a temporal pattern, are individually directed (see discussion 4.7) so further studies can try to understand if the other calls, although not answered, are individually directed or uttered for all the birds present in the active space.

4.6 TEMPORAL PATTERN OF CALLING. EFFECTS OF THE BREEDING PHASE

It is well known that during breeding phase the call repertoire changes in many species of Passeriformes (Marler, 2004a), and it happens also in Zebra finches (Zann, 1996). In our experiment we noticed that birds vocalizations changed in number, ratio (data not shown), and type (emission of the so called breeding calls) when breeding sites and nest material were available. We have demonstrated that also the temporal pattern of calling changed at the same time: the Stack-Stack relationship disappeared and new ones between breeding calls (Kackle, Arks and Whine) grew up. Elie *et al.* (2010) described the “duet possibility” at the nest site in wild zebra finches; the shown presence of a significant temporal correlation between breeding calls (Fig. 11) might support this hypothesis. Since these calls described by Zann (1996) are very similar to our descriptions, domestication has probably not changed birds’ use of breeding calls. Hence in our controlled situation we are able to describe the calls and their dynamics in more detail. Moreover the possibility to describe the duets in cage offers a chance to test functional and evolutionary sides of the phenomenon.

Another reason that makes the production of “new” calls interesting is because their hormonal and electrophysiological aspects can be investigated. Obviously the production of breeding calls come before and leads to physiological and neural activity modifications; it is interesting for the comprehension of the vocal control system to understand the mechanisms of these changes.

4.7 TEMPORAL PATTERN OF CALLING. USAGE OF THE SAME TOOLS FOR STUDIES ON THE FUNCTION\MEANING OF CALLS

As already pointed out the functional meaning of zebra finches calls can be refined, e.g. by standardizing their denominations. Some of the used tools are definitely ideal to study the meanings of calls: for example cross correlation graphs can detect cause-effect relations. As an example I have tried to clarify the meaning of the Stack calls: figure 12 C show that the bird's wing flapping is often subsequent after a Stack, so it possible to infer that the meaning is "I am taking off". This meaning is in agreement with the findings of Zann (1996). However, the available analysis could improve the knowledge on zebra finches' call meaning and function. For instance it would be possible to investigate if it is an invite to join the movement: with a cross-correlation analysis we can test the correlation with the wing flapping (flight) of the other birds, if someone join the call "I am taking off" we will see a significant correlation. Subsequently it is possible to corroborate one of the hypothesis correlating it with the behavioral output.

4.8 ETHOLOGY

Depending on the questions it is possible to analyze a combination of ethological and vocal information in many different ways. In the present work the results of the analysis of ethograms showed that the original pairs did not persist and was also showed one way to see during which behavior calls or songs are emitted. We have demonstrated an exchange of mates in beforehand established couples and that this event was attended by a change of the temporal pattern of calling between the interested birds. Thus I have suggested the meaning of the employed temporal relations of calling. The indication of a pair bond was deduced from the clumping behavior which has been shown to be indicative of a pair bond (Elie *et al.*, 2011, Zann 1996). However, non-engaged pairs may also clump (Clayton, 1988).

Comparing ethograms, behavioral output and vocalizations is a classical methods to find out meaning of calls (Marler, 2004b). The novel possibilities of our instrument are diverse, for instance: i) certain vocalization identity ii) possibility of contemporary electrophysiological recording. iii) precision of synchronization. In fig.15 a possible way to visualize the correlation between call production and behavior is shown. Further quantitative analysis will provide fundamentals information to assess the role of each call (the relevance to define a role has been discussed in 3.1).

It would be also possible to figure out information about during which behavior calls are used in a temporally correlated way. (because it is known that the one used after a precise time window are just a percentage of the total).

4.9 ELECTROPHYSIOLOGY

Although when the telemetric devices were tested in the semi-natural aviary they worked, when placed on birds the recorded electrophysiological signal was too noisy to be analyzed. It will be necessary to test which were the main reasons: a possible source of noise is the metal cage that could have interfered with wave transmission.

In the sound box the recording was of good quality and lasted for a long period. If the described change of activity was factual (see under) it is clear that Local Field Potential (LFP) is suitable to study ongoing process for a long period. In fact the described signal remained similar to itself after 18 days (Fig. 17).

The possibility of *in vivo* recording in a freely behaving bird offers a lot of different opportunities (Schregardus *et al.*, 2006). The calling system of birds is an interesting system to study neural processes because in short period different process like perception, cognitive integrative process and muscular answer take place (Vicario 2004). In male zebra finches RA is involved in production (Simpson & Vicario, 1990) and discrimination (Vicario *et al.*, 2000) of learned calls and song (Yu & Margoliash, 1996), but its role in the production of unlearned call it is not clear yet. Thanks to the electrophysiological telemetric devices it was achieved a description of the LFP in RA during vocalizations. The change in activity of the LFPs in RA were specific for specific calls or song syllables. This result was repeatable over the following days. These observations suggest a role of RA in both learned and unlearned calls. This finding is contrary to the conclusion of the lesion experiment made by Vicario 1990: he had hypothesized a role for RA only in learned vocalizations. The discussion on this point will not go further because during other analysis a possible artifact emerged (see under).

When different tools were integrated for the description of the electrophysiological signals some incongruences have arisen. These discordant elements will be briefly discussed. An apparent

change of activity of the RA of the male was detected also during female calling (data not shown), this change of the neural signal could suggest a perceptual role for the RA (already recognized by Vicario, 2004, Voss *et al.* 2007 though the latter was not able to differentiate between RA\RAcup). However it has been observed that the onset of the assumed change of activity was exactly synchronized with the onset of the sonogram of the calls. If an actual perceptual activity was present a delay of about 30-60 ms (Voss *et al.*, 2007) should have been expected. However our data are more consistent with an artifact. An interference between backpack microphone and electrophysiological device has been previously observed in our laboratories (Ter Maat pers. comm.). If there is an interference often it is possible visualize the acoustic sonogram on the electrophysiological recording, however in this case no direct evidences of these interference (pers. obs.). Since our experiment has shown that temporal relation of calling implies an answer within few milliseconds a pre-motor intention to answer could be another possible explanation for the synchronization between female vocalizations and the change of activation of the RA. To test for this possibility all the female answered calls within 300 ms were excluded for the analysis of the LFP but the result did not change (data not shown). Two hypothesis has been made to explain the observed data. 1) interferences: with the microphone signal, or with the vocalizations themselves, 2) inaccurate synchronization of neural and vocal signals.

Two experiments are suggested to solve the problem. 1) record LFP with the same device from another brain area for sure not implicated in vocalization/audition, 2) use an external microphone with the male alone\together .

This experience has shown one of many possibilities of combined use of different tools. The discrimination of answered and not answered vocalizations matched with electrophysiological recording could help studies on mechanisms behind individual recognition and choice of vocalize.

In my opinion this project helped to shed light on how social relationships are maintained in the zebra finch: it has opened more questions on how the involved calls and song syllables are i) controlled and how the social and environmental contexts ii) influence and iii) are influenced the\by the vocal communication.

5...ACKNOWLEDGEMENTS



Male A

Female A

Male B

Female B

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