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## Communicating about stress : modulation of vocalisations in the zebra finch

Emilie Perez

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Thesis presented by **Emilie PEREZ** for the degree of Doctor of Philosophy

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## **COMMUNICATING ABOUT STRESS: MODULATION OF VOCALISATIONS IN THE ZEBRA FINCH**



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## DECLARATION

This thesis is presented for the degree of Doctor of Philosophy at Macquarie University and Université Jean Monnet under a cotutelle arrangement.

I certify that the work in this thesis entitled “COMMUNICATING ABOUT STRESS : MODULATION OF VOCALISATIONS IN THE ZEBRA FINCH.” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University and Université Jean Monnet under a cotutelle arrangement.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by Macquarie University Ethics Review Committee, reference numbers: ARA 2010/053-5 ; ARA 2010/054-6.

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Student N° 42400031



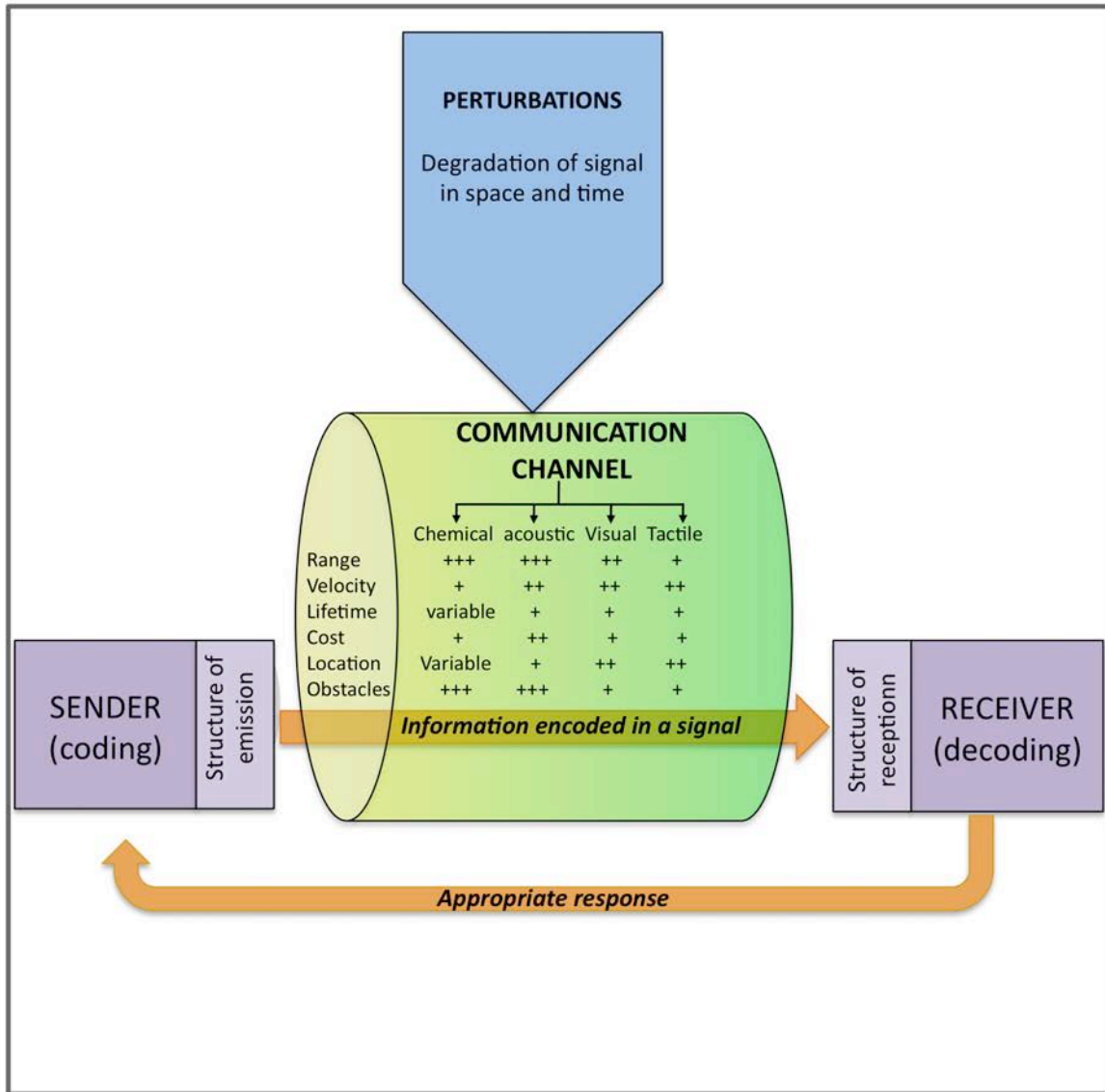
## GENERAL ABSTRACT

In social species, vocalisations convey information that participates in the maintenance and the survival of the group. While many studies were interested in stable information carried by vocal signals, like identity, fewer studies dealt with their potential role in informing about labile information such as the senders' emotional state. Stress is a good candidate for the study of the expression of emotions in animals, as it is directly measurable by the plasma levels of glucocorticoids. Stress is known to modify acoustic parameters of vocalisations in mammals, but few studies studied the process in birds, that also show complex social networks. The aim of this thesis is thus to determine how birds can vocally express their stress and to what extent corticosterone, the main stress hormone in birds, is implicated in this expression. I focused my research on the zebra finch (*Taenopygia guttata*), a gregarious Australian songbird that form lifelong pairbond and provides biparental care to its young. Using oral administration of exogenous corticosterone but also social stressful events, we show that both adult males and nestlings can express their stress through modulations of their calls' structure, suggesting that flexibility in birds' vocalisations is higher than previously expected. With a complete analysis of temporal and spectral parameters of calls, we show for the first time that stress evokes the emission of vocalisations with up-shifted frequency spectrum via a direct effect of corticosterone. Moreover, we show that females and parents are able to decode the information carried by stressed-induced calls of respectively their male partner and their young, as they exhibit modifications of behaviour in accordance with the context. The physical processes leading to the emission of stressed-induced vocalisations are discussed by applying the source-filter theory usually used in mammals. Adaptive values are also proposed, in regards with the zebra finch social network and predation risks for the caller.

This work gives new evidences about the expression of stress in birds, and proposes a comprehensive study, from the physiological signals involved in stress to the resulting modifications of communication behaviour for the sender, that leads to an adaptive response from the receivers.



# **GENERAL INTRODUCTION**



**Figure 1 : the communication loop**

A sender encodes an information thanks to specific structures. The resulting signal is transmitted in the environment. During transmission, the signal can suffer from modifications that can deteriorate the quality of the information. The receiver perceives the signal through specific structures, decodes it and extracts the information. If the message has been well transmitted and received, the receiver answers with an appropriate response.

Different communication channel can be used, each presenting particular properties in term of range of transmission, velocity to transmit, lifetime of the signal, cost to produce the signal, capacity to locate the sender by the receiver or a potential predators and capacity for the signal to cross obstacles.

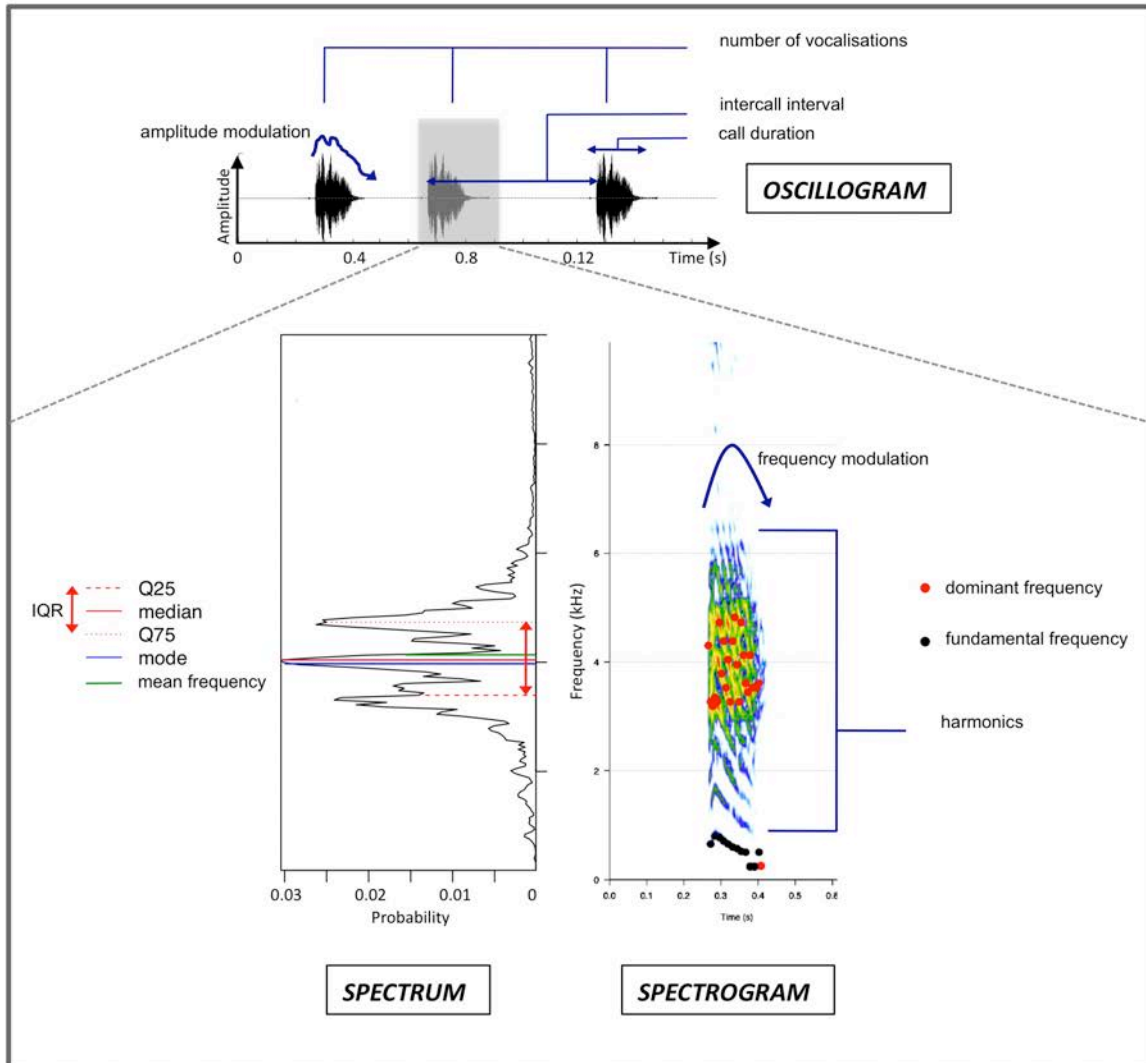
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## I. GENERAL INTRODUCTION

### I.1. The acoustic channel allows communication within a social group.

In social groups, communication between group-members is essential as it assures the establishment and the cohesion of the group. Communication is a process during which a sender encodes information into a signal that is transmitted to a receiver through the environment. The receiver receives the signal, then decodes it to recover the information and respond to the receiver (summarized in Rendall, Owren et al. 2009). Both sender and receiver present specific structures to respectively send and receive the signal.

The signal can be encoded in different ways, resulting in chemical, acoustic, visual or mechanical channels of communication. Depending on the species, more than one type of channel can be used resulting in multimodal communication (Partan and Marler 1999; Partan 2004; Gordon and Uetz 2011). This is the case for instance during courtship (Andrew 1961; Hebets and Uetz 1999; Partan, Yelda et al. 2005; Coleman 2009), or begging behaviour (Wright and Leonard 2002) during which visual, acoustic, chemical and sometimes tactile channels can be used at the same time. Each type of channel has its own properties in terms of propagation range, signal lifetime, velocity and energetic costs (**figure 1**). Thus, the use of a specific channel is determined not only by the individual capacities to emit and receive the signal, but also by the environmental specificities (propagation in the air or water, level of noise, presence of predators) and the type of information that is sent. Acoustic signals have the particularity to spread rapidly at short and long distances unlike visual signals, and do not leave any trace in the environment unlike chemical signals (Sparks 1984; Davies, Krebs et al. 2012). Acoustic communication is also costly because it can be obvious for predators, and because the information is impaired and even sometimes lost by deterioration during propagation in the environment (Wiley and Richards 1978). Despite these latter disadvantages, sound production has been described in many species throughout the animal kingdom, from arthropods (Alexander 1967; Rovner 1967; Bradbury and Vehrencamp 1998) to fish (Zelick, Mann et al. 1999; Bass and McKibben 2003; Bertucci, Attia et al. 2012), anurans (Ryan 1988; Gerhardt and Huber 2002), reptiles (Young 2003; Wang, Wang et al. 2007; Vergne, Pritz et al. 2009), birds (Nottebohm 1970; Klump and Shalter 1984) and mammals (Insley, Phillips et al. 2003; Reby and McComb 2003; Fitch, Hauser et al. 2005; Janik 2005; Feddersen-Petersen 2007; Portfors 2007). Acoustic communication is thus highly represented in social and gregarious species. The structure of acoustic signals is the result of a trade off for the sender: the signal needs to be informative, but its properties should also prevent deterioration during propagation



**Figure 2 : The three usual representations of sound and the main parameters measurable on complex vocalisations.**

A male zebra finch distance call was used as an example of complex vocalisation, i.e. a sound presenting a fundamental frequency and several harmonics.

Temporal, spectral and bi-dimensional parameters can be measured in complex calls.

Q25 : first quartile

Q75 : third quartile

IQR : interquartile range

and limit conspicuousness so as to avoid the cost of predation. The structure of acoustic signals thus represents a compromise between multiple constraints.

## **I.2. Vocalisations carry stable information about the sender.**

During the past decade, many studies decrypted what kind of information is carried by acoustic signals. Acoustic signals can present a variety of temporal and spectral parameters depending on their complexity, which are measurable and can potentially encode information (**figure 2**). They can carry stable information about the sender, i.e. information that is stable during the entire life of the individual. Many acoustic signals are species specific and thus encode the species identity of the individual, which is useful during mate choice and to avoid hybridization in sympatric species. In a variety of species, individuals can discriminate conspecifics from heterospecifics on vocalisations only (sulawesi tarsiers (Nietsch and Kopp 1998), gibbons (Mitani 1987), myiarchus flycatchers (Lanyon 1963), Darwin's finches (Ratcliffe and Grant 1985), zebra finches (Braaten and Reynolds 1999), european quails (Deregnaucourt and Guyomarc'h 2003), budgeridars, european starlings or canaries (Dooling, Brown et al. 1992)). The parameters encoding such information are varied among species: for instance, the whistles from five species of western north atlantic dolphins can be discriminated based on the maximum and minimum frequencies, the values of frequencies at the beginning and at the end of the vocalisation, the duration of the vocalisations and the number of inflexion points in the spectrum (Steiner 1981), while black-capped chickadees use the frequency modulation, the amplitude modulation, the frequency range but also the call rhythmicity and syntax for encoding (Charrier and Sturdy 2005). In a variety of taxa, sexual dimorphism is revealed in vocalisations, thus participating in the mate choice process. The mean fundamental frequency and the mean frequencies of the first three formants of vowel-like grunt vocalisations in baboons are lower in males than in females (Rendall, Owren et al. 2004), thus conspecifics have access to the sex of the caller based on this acoustic signal. Long calls of kittiwakes are sexually dimorphic, dimorphism being based on the value of the fundamental frequency (Aubin, Mathevon et al. 2007), and allow to discriminate potential sexual partners as kittiwakes have similar appearance. For the nocturnal and aquatic frog xenopus, carrier frequency and click rate represent sexually differentiated parameters that facilitate sexual meetings in dark, silt-filled ponds (Wetzel and Kelley 1983; Tobias, Barnard et al. 2004; Vignal and Kelley 2007). Finally, acoustic signals often encode the identity of the sender, especially in social species in which it is important to recognize group members and thus to discriminate close conspecifics such as the social partner, the parents or the young from other individuals. Early recognition based on vocal cues is established within 48h postpartum between ewes and their lamb (Sebe, Nowak et al. 2007), and goats show long-term recognition of the calls of their young (Briefer, de la Torre et al. 2012). In

capybara, the identity of the sender is carried by temporal parameters (duration and interval duration of the clicks, (Lacerda, Tokumaru et al. 2013)); a specific combination of six structural parameters characterizes the chucks of each individual in squirrel monkeys (Smith, Newman et al. 1982). Kittiwake's long calls are individualized thanks to a complex of temporal and frequency parameters of the signal (Aubin, Mathevon et al. 2007). Penguins calls carry not only information about the species (Waas 1988) but they are also highly individualised (Lengagne, Lauga et al. 1997; Jouventin, Aubin et al. 1999; Jouventin and Aubin 2002) and individual recognition based on calls only exists in several species of penguins (Derenne, Jouventin et al. 1979; Jouventin, Guillotin et al. 1979; Jouventin 1982; Speirs and Davis 1991). In penguins, kin recognition (mate and offspring) is either possible using the spectral profile and the pitch of the call (timbre analysis, for nesting penguins) or by using a more complex system involving a temporal decoding complemented by the beats generated by a two-voice system (non-nesting penguins, (Aubin, Jouventin et al. 2000). Thus, a huge range of acoustic parameters can encode stable information about the sender, with a coding-decoding system that is suitable with and dependent of the biotic and abiotic environmental context.

### **I.3. Vocalisations also carry labile information about the emitter.**

Labile information consists in information that is not stable over the individual lifespan. Individuals need to inform conspecifics about their reproductive status, their social status in the group, or their motivational and emotional states, so as to receive appropriate feedbacks such as a positive response from a sexually active individual or assistance from a kin in response to specific needs (starvation, isolation from the social group, presence of a predator). Labile information thus regulates social interactions. On top of informing about the identity of the sender, begging calls of young can also carry information on the general status of the young, another type of information that can vary over time. Begging calls intensity and begging calls rate are known as honest signals that inform parents about the level of hunger, the body condition and/or the specific needs in term of body temperature (Roper 1993; Mondloch 1995; Cotton, Kacelnik et al. 1996; Kilner and Johnstone 1997; Royle, Hartley et al. 2002; Anderson, Brunton et al. 2010) and result in a modification of parental feeding behaviour (Kitaysky, Wingfield et al. 2001). Calls of xenopus do not only carry information about the sex, but allow females to signal their reproductive state, with sexually receptive females vocalising with a rapid click rate that stimulates males courtship, while non receptive females emit clicks with a lower rate that suppress males sexual behaviour (Tobias, Viswanathan et al. 1998; Tobias, Barnard et al. 2004; Elliott and Kelley 2007). In these cases, the labile information is encoded in temporal parameters more than spectral parameters.

Spectral parameters can also encode labile information. In 1977, Morton developed the Motivation-structural rules based on empirical data from a huge variety of mammals and birds species (Morton 1977). The concept states that the structure of vocalisations can be predicted by the context of emission. Animals emit high frequency and pure tone signals in fearful or appeasing contexts while they use harsh and lower frequency signals in hostile context. Intermediate situations between hostility and fear or appeasement result in the emission of signals with intermediate structures. This hypothesis was validated in several species of mammals such as chimpanzees, dogs, white-faced capuchins or white-nosed macaques (Gouzoules and Gouzoules 2000; Siebert and Parr 2003; Yin and McCowan 2004; Pongracz, Molnàr et al. 2006; Gros-Louis, Perry et al. 2008; Lord, Feinstein et al. 2009; Taylor, Reby et al. 2009), but also in some bird species such as robewhites, yellow-headed blackbirds, black-capped chickadees or carolina chickadees (Nero 1963; Stokes 1967; Smith and Club 1972; Ficken, Ficken et al. 1978). Nevertheless, some studies did not verify the entire theory (Marler 1956; August and Anderson 1987). In 2010, Manser suggested to improve Morton's rules by proposing that variation between calls of different motivational state (friendly context vs hostile context in Morton study) might reflect different emotional valences (positive or negative valence), and variations within a specific call type reflect differences in arousal states (intensity from low to high, (Manser 2010; Briefer 2012). Thus, the structural-motivation rule proposed by Morton can be applied not only to the vocal repertoire of a species, but also to a specific type of acoustic signal within the repertoire, that can be modified depending on the level of arousal of the sender.

#### **I.4. The valence and the arousal of an emotion are reflected in vocalisations.**

Emotion is a particular type of labile information that has been only accepted and described in humans for decades, even though Darwin introduced in 1872 the possibility that animals might experience emotions (Darwin 1872). Emotions can be consensually defined as a short affective reaction to a specific stimulus that provokes a specific physiological state, which depends on the valence and the arousal of the experienced emotion (Arnold 1960; Kleinginna Jr and Kleinginna 1981; Briefer 2012). Basic emotions have been described, with a specific level of valence and arousal for each: seeking, rage, fear, stress, lust, care, panic and play are the main categories proposed in animals and humans (Gray 1987; Panksepp 2011). In social group living, expressing emotions allows to react to sudden situations, such as an imminent danger, the pain of a conspecific or the specific behavioural intentions of other individuals, and thus participates to the survival of the group (Keltner and Lerner 2010). Different physiological, physical and behavioural parameters indicate emotions in animals, such as heart rate, respiration rate, skin temperature, facial expressions, neuroendocrine activities, locomotor activity or body posture (Chevalier-Skolnikoff 1973; Briefer 2012).



Nevertheless, in social species that are highly vocal, modifications of acoustic signals might also participate in the expression of emotions, as they can inform quickly and at longer distances compared to facial expressions for instance. Acoustic signals could thus be the expression of a distinctive inner state of an animal that may occur spontaneously or as a result of an external event (Grandin 1998).

Many studies assessed the vocal expression of emotions in humans. Emotions such as stress, described as a negative emotion of high arousal, is known to provoke a modification of the speech through rhythm and intonation: the speech is usually higher pitched and sounds quicker, with at the same time a higher speech rate and shorter inter-vocalisation intervals (Scherer 1986; Murray and Arnott 1993; Bachorowski and Owren 1995; Banse and Scherer 1996; Keller, Bailly et al. 2001; Harrigan, Rosenthal et al. 2008). This phenomenon called affective prosody seems to inform about the emitter emotional state, and gives cues for potential receivers (Dellaert, Polzin et al. 1996; Buchanan, Lutz et al. 2000; Litman and Forbes 2003; Yacoub, Simske et al. 2003; Ververidis and Kotropoulos 2006).

In conformity with the modified motivation-structural theory of sound signals, several studies demonstrated that emotions are encoded through modifications of acoustic signals of many non-human mammals. Except the work of pioneers in affective neurosciences on positive emotions in mammals (Cabanac 1971; Berridge and Robinson 2003; Panksepp and Burgdorf 2003; Burgdorf and Panksepp 2006; Karpinski, Mazurkiewicz et al. 2012; Mellor 2012; Reimert, Bolhuis et al. 2013), most of the work on the acoustic expression of emotions in animals has been done on negative emotions (for an exhaustive list, see (Briefer 2012)). The main reason for this imbalance is that negative emotions have usually a higher arousal than positive emotions, making their expression more obvious and easier to study (Boissy, Manteuffel et al. 2007). As a consequence, more tools have been developed to study negative emotions, and most of the studies up to now focused on contexts such as isolation from the social group, separation from a close relative (social partner, infant), experience of pain or stress while handled, attack by a predator or agonistic interactions with conspecifics. Prosodic cues related to emotional state have been described so far in a large number of mammalian species, especially primates (Stanford 1998, for an exhaustive list, see (Boissy, Manteuffel et al. 2007; Briefer 2012)) but also non-primate mammals from highly domesticated species such as cattles, goats, pigs, sheeps, cats, dogs or horses (Weary and Fraser 1995; Schrader and Todt 1998; Rushen, Boissy et al. 1999; Watts and Stookey 2000; Thomas, Weary et al. 2001; Taylor, Reby et al. 2009; Von Borell, Baumgartner et al. 2009; Pond, Darre et al. 2010; Imfeld-Mueller, Van Wezemaal et al. 2011; Siebert, Langbein et al. 2011; Yeon, Kim et al. 2011), to wild species such as spotted hyenas, bottlenose dolphins, African elephants, rats or guinea pigs (Monticelli, Tokumaru et al. 2004; Soltis, Leong et al. 2005; Brudzynski 2007; Litvin, Blanchard et al.

2007; Theis, Greene et al. 2007; Esch, Sayigh et al. 2009; Soltis, Leighty et al. 2009; Soltis, Blowers et al. 2011).

A variety of parameters are involved in expressing emotions in mammals (**figure 3**), with the main parameters indicating arousal being the vocalisation rate, the contour and the range of the fundamental frequency, the amplitude contour, the distribution of energy, the frequency peak and the formant contour, as well as the inter-vocalisation interval (Briefer 2012).

### **I.5. There is a lack of information on vocal expression of emotions in avian species.**

While many bird species also show complex social groups and complex vocal repertoires, few studies clearly decrypted if and how bird species express their emotions through modifications of their vocalisations. The domesticated chicken has been the avian model in this field so far. Chicken vocalisations are context specific: The view of a predator elicits the emission of alarm calls (Karakashian, Gyger et al. 1988) while stressful situations elicit emission of distress calls (Marx, Leppelt et al. 2001). These vocalisations have thus been attributed to emotional states in the chicken. The distress call rate decreases in newly hatched chicks when experiencing warming or stimulated by the playback of clucking sounds of adults (Collias 1952), while the distress call rate increases when ambient temperature is lower than usual during the first week post hatching (Kaufman and Hinde 1961). The intensity of distress calls can also be modified by the social context: In young, the rate of distress calling at high intensities decreases significantly with increasing maternal contact (complete isolation vs vocal contact vs vocal and visual contact with the mother), while the rate of calling at lower intensities increases (Bermant 1963). Nevertheless, the domesticated chicken shows differences in fear responses to novelty compared to its wild ancestor, the red junglefowl (Schütz, Forkman et al. 2001), suggesting that domestication may have an impact on emotivity in chicken, and thus its acoustic expression.

Studies in domesticated chicken raise the idea that emotions are expressed through avian vocalisations. Some studies showed a change in the acoustic repertoire of chicken with an emotion of stress, as chicks use low energy call types in social context and shift to high energy call types (distress calls) while in isolation (Marx, Leppelt et al. 2001), but no study so far has demonstrated variations with emotions in spectral parameters within a given type of acoustic signal. The modifications of spectral parameters could be adaptive: by encoding stress without increasing the time spent vocalising or the call intensity, they could be less obvious to predators. It thus remains to decipher whether emotion in an avian species can modify not only call rate and repertoire use but also spectral properties of a given type of vocalisation.

| SPECIES   | TRIGGER                         | EMOTION LABEL         | VOCALISATION                 | EFFECT  |
|---|---------------------------------|-----------------------|------------------------------|---|
| Rat <i>Rattus norveicus</i> <sup>1</sup>                  | Threat (predator)               | Stress/fear           | 22 kHz vocalisations         | ↑ number  |
| Red Colobus monkey<br><i>Procolobus</i> sp <sup>2</sup>   | Threat (predator)               | Stress/fear           | Antipredator cries           | ↑ intensity   |
| Dolphins <i>Tursiops truncatus</i> <sup>3</sup>           | Separation from the calves      | Stress                | Whistles                     | ↑ maximum frequency<br>↓ interloop intervals                |
| Guinea pig pup <i>Cavia porcellus</i> <sup>4</sup>        | Separation from the mother      | Stress                | Distress whistles            | ↑ mean frequency<br>↓ number of harmonics                   |
| Female Elephant<br><i>Loxodonta africana</i> <sup>5</sup> | Dominance interaction           | Negative valence      | Rumbles                      | ↑ F0, ↑ F0 modulation, ↑ duration, ↑ first formant location |
| Female dwarf goats<br><i>Capra hircus</i> <sup>6</sup>    | Total isolation                 | Stress                | High bleats                  | ↓ IQR, ↓ entropy, ↑ peak frequency                          |
| juvenile hyenas <i>Crocuta crocuta</i> <sup>7</sup>       | Social excitement               | High arousal          | Whoops                       | ↑ maximum F0<br>↓ interwhoop interval                       |
| Domestic Pig<br><i>Sus scrofa domestica</i> <sup>8</sup>  | Anticipation of crossing a ramp | Negative valence/Fear | Screams and squeaks          | ↑ frequency of emission                                     |
| Dairy cattle<br><i>Bos primigenius</i> <sup>9</sup>       | Isolation                       | Stress                | High frequency vocalisations | ↑ frequency of emission                                     |

1. Litvin, Blanchard et al. 2007; 2. Stanford 1998; 3. Esch, Sayigh et al. 2009; 4. Monticelli, Tokumaru et al. 2004; 5. Soltis, Leighty et al. 2009; Soltis, Blowers et al. 2011; 6. Siebert, Langbein et al. 2011; 7. Theis, Greene et al. 2007; 8. (Taylor, Reby et al. 2009; Pond, Darre et al. 2010; Imfeld-Mueller, Van Wezemaal et al. 2011; 9- Rushen, Boissy et al. 1999.

**Figure 3: Some examples of the variety of the effect of emotions on vocalisations in mammals.**

↑ increase

↓ decrease

F0 fundamental frequency

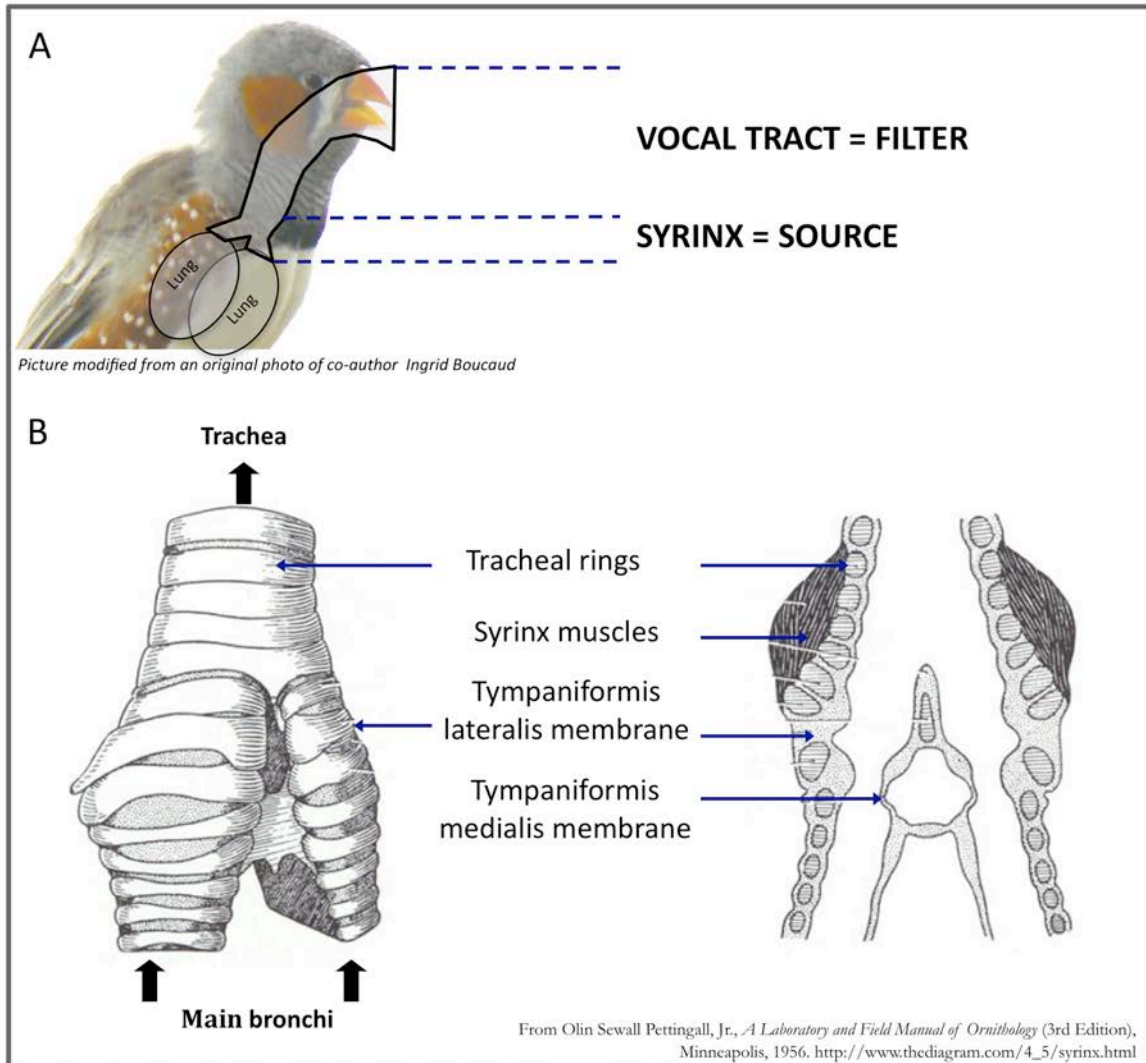
## **I.6. The modification of vocalisations' structure with emotions could be under neuroendocrine control.**

The physiological relationship between emotions and modifications of vocalisations has been studied in mammals: modifications of respiration, salivation and tension of a variety of muscles controlling vocal production and vocal tract were proposed as explanations for spectral and temporal modulations of vocal signals (Fant 1960; Scherer 1986; Titze 1994; Fitch and Hauser 1995; Keller, Bailly et al. 2001). These hypotheses can be applied to birds, as vocalisations are basically produced by a functionally similar apparatus (**figure 4**) (Suthers and Zollinger 2004). Some studies revealed that these physical transformations can possibly be under neuroendocrine control. The injection of chemicals that stimulate neuronal circuitries involved in emotions elicit the emission of vocalisations, such as apomorphine and corticotropin-releasing factor in chicks from chicken species (De Lanerolle and Youngren 1978; Zhang, Nakanishi et al. 2001), corticotropin-releasing hormone, anxiogenic peptides and cortisol in pigs (Salak-Johnson, McGlone et al. 1997; Parrott, Vellucci et al. 2000). In chickens and squirrel monkeys, electrical stimulation of brain area involved in emotions can also at the same time elicit the emission of vocalisations and reinforce the emotion that is supposed to normally evoke this type of vocalisation (Andrew 1969; Jürgens and Pratt 1979; Fichtel, Hammerschmidt et al. 2001; Manteuffel, Puppe et al. 2004). Nevertheless, the direct implication of such neuroendocrine signals in the modulation of the spectral properties of vocal signals has never been tested.

## **I.7. Stress appears as a key emotion in social species.**

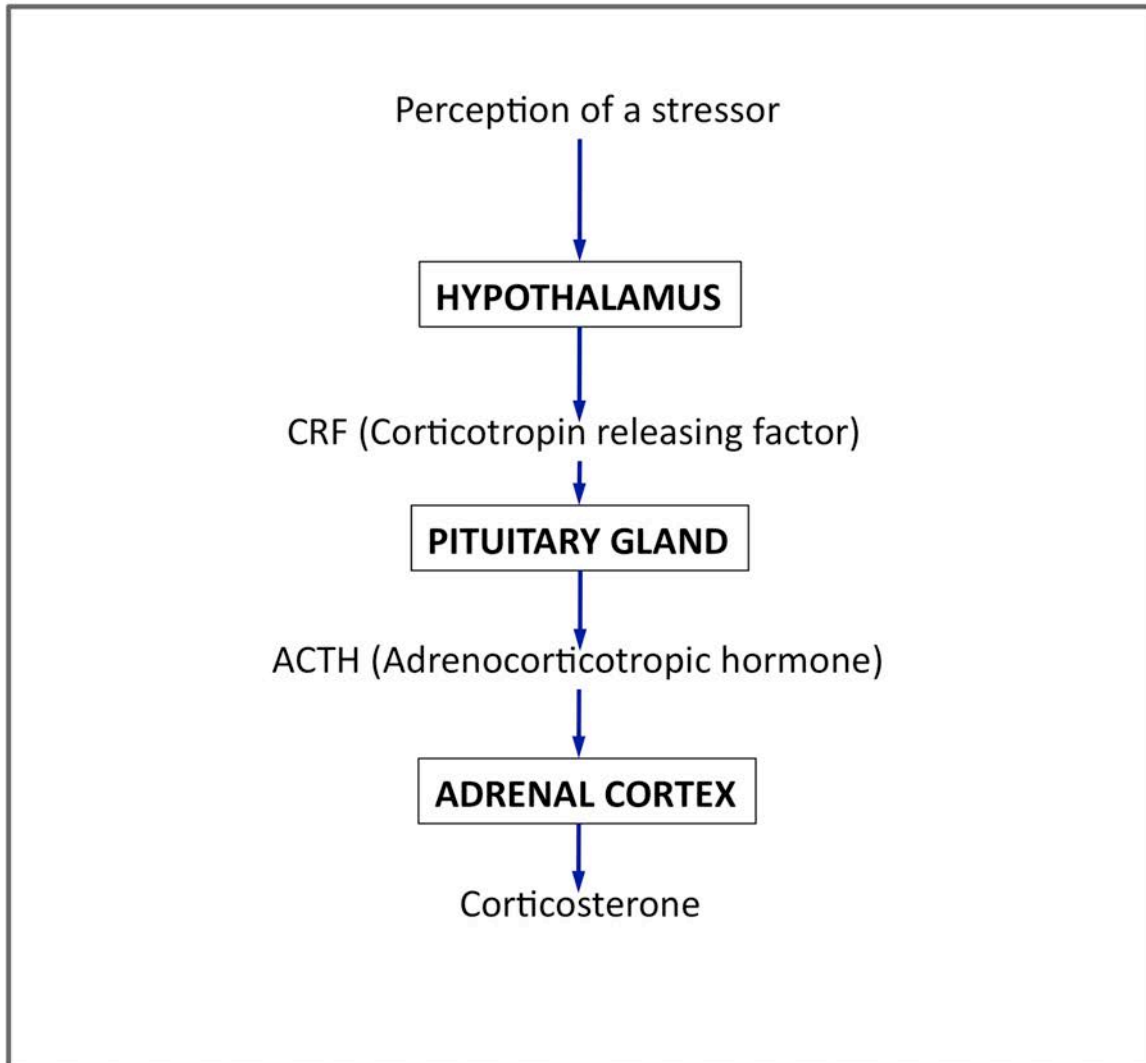
During its entire lifespan, one individual is exposed to multiple stressors. A stressor is an element that elicits the activation of the hypothalamo-pituitary-adrenal (HPA) axis and provokes the release of stress hormones i.e. glucocorticoids, mainly corticosterone in birds and cortisol in mammals (Harvey, Phillips et al. 1984; Creel 2001; Mostl and Palme 2002; Cockrem 2007; Cockrem 2013) (**figure 5**). Individuals can undergo an acute stress, which will occur when the stressor is new and/or applied on a short-term basis, while a chronic stress will be considered as caused by a recurrent stressor and/or a stressor that occurs on a long period of time. Acute stress fits with the definition of emotion proposed above, as it is a sudden reaction to a specific stimulus (here, a stressor) that provokes a specific physiological state (here, the elevation of stress hormones) depending on the valence and the arousal of the emotion experienced (here, negative valence of high arousal). Some studies have even integrated stress as belonging to the larger family of emotion of fear (LeDoux and Bemporad 1997; LeDoux 1998; Labar and Ledoux 2001; Rodrigues, LeDoux et al. 2009).

The stimulus involved in a reaction of stress, i.e. the nature of the stressor, can be intrinsic like the level of hunger or the modification of body temperature



**Figure 4 : Application of the source filter theory in songbirds.**

- A. Phenomena involved in vocal production in songbirds are highly complex, but as proposed for mammals, birds present a source of production of vocalisations, the syrinx and a filter; the vocal tract. After production, the vocalisation propagates in vocal tract before being emitted in the environment. The vocal tract can be considered as the filter; as its shape and length can be modified, which in return could potentially modify the acoustic parameters of the vocalisation emitted.
- B. Anatomy of the syrinx. Vocalisations are produced at expiration by vibration of the syringeal membranes. Different muscles can act to modify the syrinx performance and thus the acoustic parameters of the vocalisation.



**Figure 5 : the physiological response to a stressor in birds.**

A stress response is defined as the activation of the HPA axis by a stressor that provokes the secretion of corticosterone.

(Kitaysky, Kitaiskaia et al. 2001; Loiseau, Sorci et al. 2008; Fairhurst, Treen et al. 2012), which are known to modify plasma levels of stress hormones in birds. The stressor can also be an external stimulus: the sight of a predator, or handling by a human can elevate levels of glucocorticoids in many species (Cockrem and Silverin 2002; Cockrem 2007; Cockrem 2007; Miller, Vleck et al. 2009; Cockrem 2013). But among all the stressors experienced by an individual, the modification of social interactions appears to be the strongest stressor in social species (DeVries, Glasper et al. 2003). Isolation from the social group provokes a physiological stress in some mammals and bird species. In squirrel monkeys and rhesus monkeys, isolation from the mother modifies not only the call rate of the young but also elicits a raise of plasmatic cortisol (Levine, Johnson et al. 1985; Levine and Wiener 1988); pigs raised isolated from the social group show increases of plasmatic glucocorticoids, and increases in exploration and vocalisations (Ruis, te Brake et al. 2001). Isolation from the group members in european starlings increases plasmatic corticosterone and enhances motor activity (Apfelbeck and Raess 2008). In the zebra finch, separation from the partner elicits a raise in plasma levels of corticosterone, while reunion restores basal levels (Remage-Healey, Adkins-Regan et al. 2003). On top of the action of glucocorticoids on general activity as seen above, these hormones can also modify temporal parameters of vocalisation, e.g. they increase begging call rate in the house sparrow (Loiseau, Sorci et al. 2008) and the black-legged kittiwake (Kitaysky, Wingfield et al. 2001), or the latency to beg in the white-crowned sparrow (Wada and Breuner 2008). Corticosterone-implanted male sparrows *Melospiza melodia* show a longer latency to approach an opponant, and emit less territorial songs than non-implanted males (Wingfield and Silverin 1986). Glucocorticoids could thus be one of the main neuroendocrine signals that directly elicit the emission of modulated vocalisations while experiencing emotions. An increase in plasma corticosterone indicates when and to what degree a bird is experiencing stress, it is thus an interesting signal as it can be easily measured in plasma and feces and always reveals a basic emotion of stress (Cockrem 2007).

Studying vocal modulation during stress in a social bird will help to understand the chain reaction involved, from the stimulus eliciting an emotion to the modulation of vocal signal provoked via a neuroendocrine control. Chickens have been so far the main bird species used to study emission of vocalisations during stressful events, but studying the expression of stress in a monogamous species that shows strong attachment between partners could be a better model for the study of emotions in social species, as monogamous partners communicate intensively and could potentially signal their emotional state to their mate.

## **I.8. The zebra finch, a supermodel for the study of the communication of stress in social species.**

### ***I.8.1. The zebra finch's habitat: an unpredictable and stressful environment.***

The Australian zebra finch *Taeniopygia guttata castanotis* is a nomadic songbird that lives in the arid and semi-arid regions of Australia (**figure 6**). This habitat exposes the zebra finches to many environmental stressors. It is highly unpredictable as temperature can rise up to 47°C, or fall down to 7°C within the day (Stafford Smith and Morton 1990; Zann 1996) (personal observations from 2009 to 2012). Rainfalls are also unpredictable, rare but extremely important as they shape the landscape and provoke the proliferation of green grasses providing seeds eaten by the zebra finch (Davies 1977; Morton and Davies 1983; Zann and Straw 1984; Zann, Morton et al. 1995). Zebra finches are thus opportunistic reproducers that integrate environmental cues to time breeding, such as water availability, food availability, presence of green grasses, and photoperiod (Zann 1994; Perfito, Zann et al. 2007; Perfito, Kwong et al. 2008). They are able to breed several times in a year when conditions are optimized.

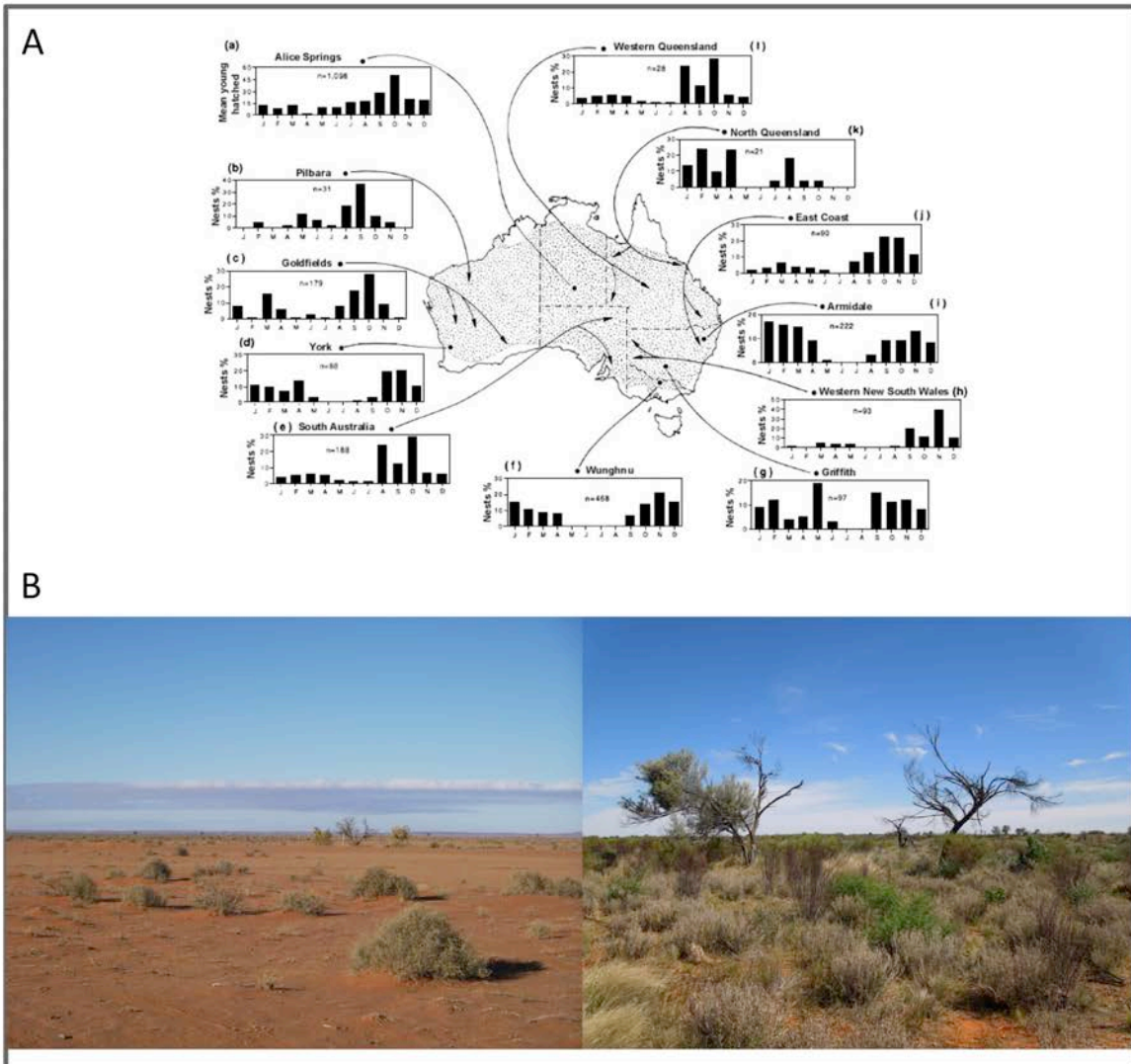
Zebra finches are vulnerable to predators, especially around water zones where they drink, social trees in which they socialize with the group, and around the nest when partners take turns to incubate the eggs or feed the nestlings. Raptors such as black kites *Milvus migrans*, brown goshawks *Accipiter fasciatus*, brown falcons *Palco berigora* or pied butcherbirds *Cracticus nigrogularis* have been reported to catch zebra finches in these areas (Evans, Collins et al. 1985; Zann 1996); personal observation 2009). Predation at the nest is also possible: Monitors have been seen to climb trees and try to eat eggs and nestlings in the nest (personal observation 2009). Snakes, rats and mice can also destroy the nest and eat the progeny. Other birds have been described to participate in predation at the nest, such as Australian magpies, the Australian ravens (Zann 1996) and more recently the chestnut-crowned babbler (personal observation 2011).

Thus, water restriction, food shortage, heat shocks and presence of predators are some of the main environmental stressors for the adult but also the young zebra finches.

### ***I.8.2. The zebra finch's pairbond: a strong partnership whose rupture provokes a physiological stress.***

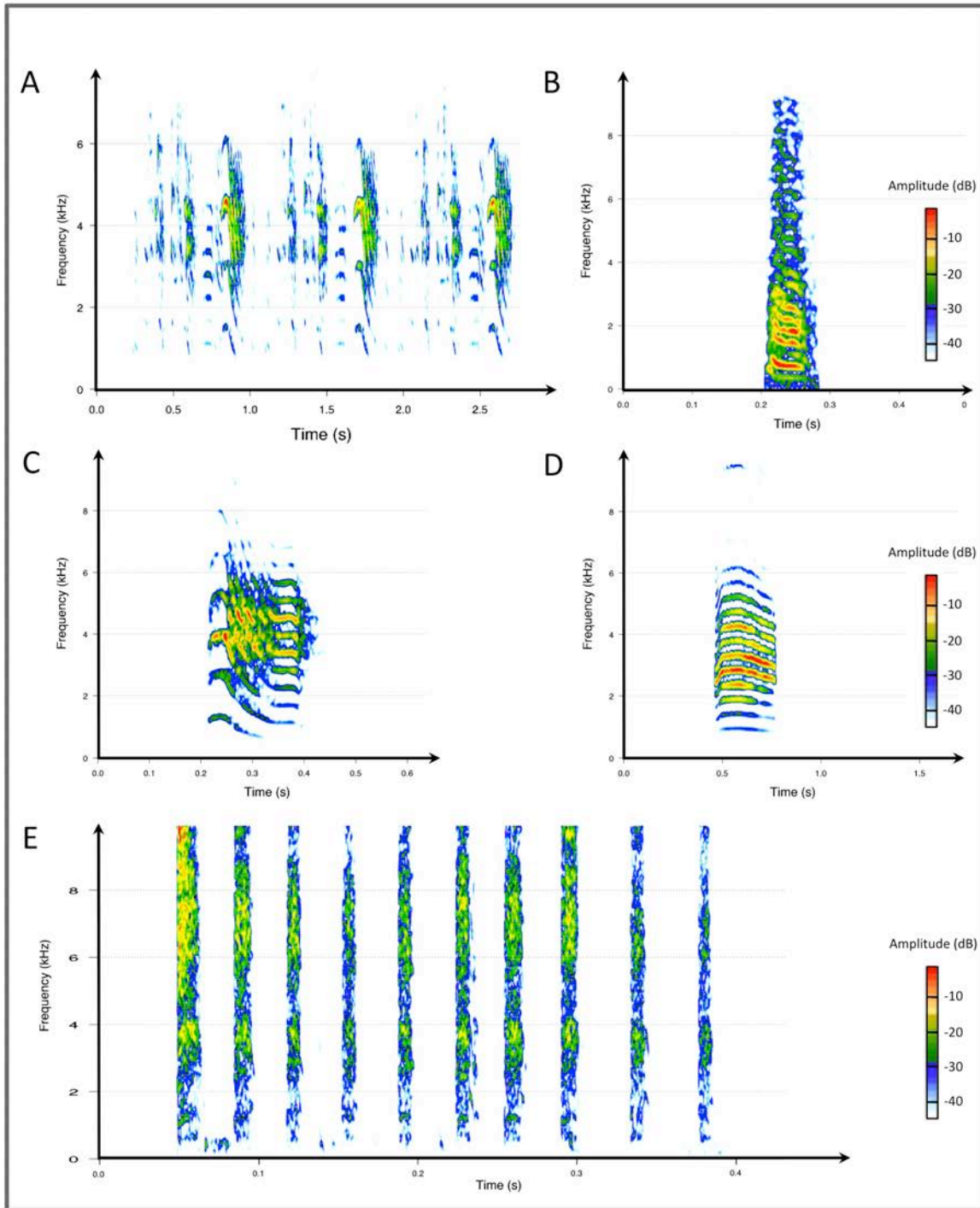
Zebra finches are gregarious birds that form groups of unstable size depending on the season. Giant flocks (more than 200 birds) are formed during events of migration. Smaller groups (5 to 20 birds) are usually seen during the breeding season: birds regroup around feeding and drinking areas, and pairs breed close to one another, forming a breeding colony (Zann 1996). During the breeding season, pairs can be seen accompanied by their young: fledglings usually follow their parents until they develop social interactions with young adults. Nevertheless, the core of the group is formed by





**Figure 6 : The zebra finch natural habitat.**

- A. Zebra finch distribution in the arid and the semi arid zone of Australia. Timing of breeding are shown at 12 locations (From Zann 1996). Zebra finches are opportunistic breeders that breed when conditions are optimized.
- B. Pictures from the semi-arid zone of Australia, Fowlers Gap, New South Wales. On the left, picture of the zone during a highly dry breeding season (2009). On the right, the same zone during a less dry breeding season (2011). Grass vegetation can drastically change depending on rainfalls.



**Figure 7 : Spectrograms of some of the main vocalisations in the zebra finch.**

- A. Zebra finch songs emitted only by males.
- B. Tet calls used by both sexes.
- C. Distance call from a male.
- D. Distance call from a female.
- E. Begging calls emitted in series by nestlings and fledglings.

the monogamous pairbond: Partners pair for life and stay together even outside of the breeding season. Extrapair copulations are extremely rare in the wild (less than 2%, (Griffith, Holleley et al. 2010), so is re-pairing, usually happening when a partner disappears from the colony presumably by dying (Immehnann 1966; Zann 1994). Partners exhibit specific behaviours that permit to distinguish them easily in the colony: they clump together on perches and perform mutual and exclusive allopreening. Partners also never aggress each other (Elie, Mathevon et al. 2011). Mates usually search a breeding spot, build the nest with dry herbs, incubate and feed nestlings together, with a very balanced involvement of both sexes during each process. Partners are also highly synchronized in their activities: during chicks feeding, they come together at the nest in 78% of the occurrences (Mariette and Griffith 2012), and they are usually found in very close contact while resting during the entire year, even out of the breeding season (Zann 1996). Partners only separate during incubation, one partner staying on the eggs when the other one is foraging (Zann 1996; Elie, Mariette et al. 2010). During incubation, birds can express a sentinel behaviour, during which one bird incubates inside the nest, while the other one stay outside at a close proximity and show vigilance behaviours against predators (Elie, Mariette et al. 2010; Mainwaring and Griffith 2013).

Laboratory studies (Ramage-Healey, Adkins-Regan et al. 2003) have also shown that separation from the partner provokes an acute stress, with a raise of plasma levels of corticosterone during separation, and a return to basal levels after reunion, suggesting that corticosterone could play a role in pairbond maintenance. Thus, the zebra finch pairbond is highly stable and strong. The social attachment and its neuroendocrine control described in the zebra finch is similar to what has been discovered in the prairie vole, the model used in mammals for the study of monogamy (Thomas and Birney 1979; DeVries, DeVries et al. 1995; Sue Carter, Courtney DeVries et al. 1995; Wang, Young et al. 1999; Aragona and Wang 2004; Young and Wang 2004; Bosch, Nair et al. 2008). The zebra finch is thus a good model for the study of neuroendocrine signals involved in pairbond establishment and maintenance in birds.

### ***1.8.3. The zebra finch's vocal repertoire: potential for emotional coding.***

Zebra finches are highly vocal. Only males sing, a signal restricted to the reproductive context, but both males and females use a large repertoire of calls during social interactions (**figure 7**), the call type being used depending on the context. The main calls are the distance calls that allow birds to keep contact at a long distance, and the softer 'tet' calls used during interactions at close distances. Partners exhibit a highly synchronised and discrete vocal duet at the nest (sentinel and meeting duets) during which they emit mainly short calls equivalent to tet calls but also another type of calls,

the whines (Elie, Mariette et al. 2010). Finally, nestlings and fledglings emit begging calls, noisy short calls emitted in series and expressed during parental feedings.

Zebra finch vocalisations are known to carry several pieces of information. The entire repertoire is formed of complex calls defined by a fundamental frequency modulated in time and the presence of associated harmonics (Zann and Straw 1984; Simpson and Vicario 1990). Distance calls are highly sexually dimorphic in their shape but also in their origin, as males learn their distance calls by imitation of an adult male tutors. As a consequence, male calls are more acoustically distinct between individuals than female calls (Zann and Straw 1984; Simpson and Vicario 1990; Forstmeier, Burger et al. 2009). Male and female calls elicit differential behavioural responses (Vicario, Naqvi et al. 2001), as well as differential brain responses from conspecifics (Gobes, Ter Haar et al. 2009), thus distance calls inform conspecifics about the sex of the emitter. The acoustic cues that are necessary to elicit a vocal response from a conspecific are not the same in male and female calls, the vocal response of zebra finches to female calls requiring the full harmonic structure of the call, whereas the frequency downsweep of male calls is necessary to evoke a vocal response (Vignal and Mathevon 2011). Both male and female distance calls have been shown to inform about the identity of the sender, helping birds to recognize their partners or their kin (Vignal, Mathevon et al. 2004; Vignal, Bouchut et al. 2008; Vignal, Mathevon et al. 2008). The identity of the sender is encoded in parameters that depend on the sex of the individual: the energy spectrum of the call and the frequency modulation of the final part of the call are highly individualised in males, and females use these specificities to identify the call of their mate (Vignal, Mathevon et al. 2008). Females distance calls encode individuality mainly through modulations of the fundamental frequency and the characteristics of the spectrum (Vignal, Mathevon et al. 2004). Fledglings are also able to recognize their parents based on their vocalisations as they preferentially respond to parental calls over other adult calls during playback experiments (Jacot, Reers et al. 2010; Mulard, Vignal et al. 2010). Finally, begging calls of nestlings are also individualized and allow parents to recognize their young (Levrero, Durand et al. 2009). Thus, vocal signals in zebra finches participate in the recognition of group members, and are highly important for social interactions and social bond maintenance (Miller 1979), especially between pairbond partners and between parents and chicks.

The coding-decoding processes of individual identity have been well decrypted in the zebra finch vocalisations, but nothing is known on the encoding of emotional state such as stress. As zebra finch undergo a variety of social and environmental stressors, it is also likely that conspecifics could answer to the expression of stress of individuals by modifying their own behaviour in an adaptive way depending on the potential danger. Finally, no specific alarm calls has yet been described in the zebra finch, thus it is likely that the distance calls of adults and maybe the begging calls of nestlings can carry this

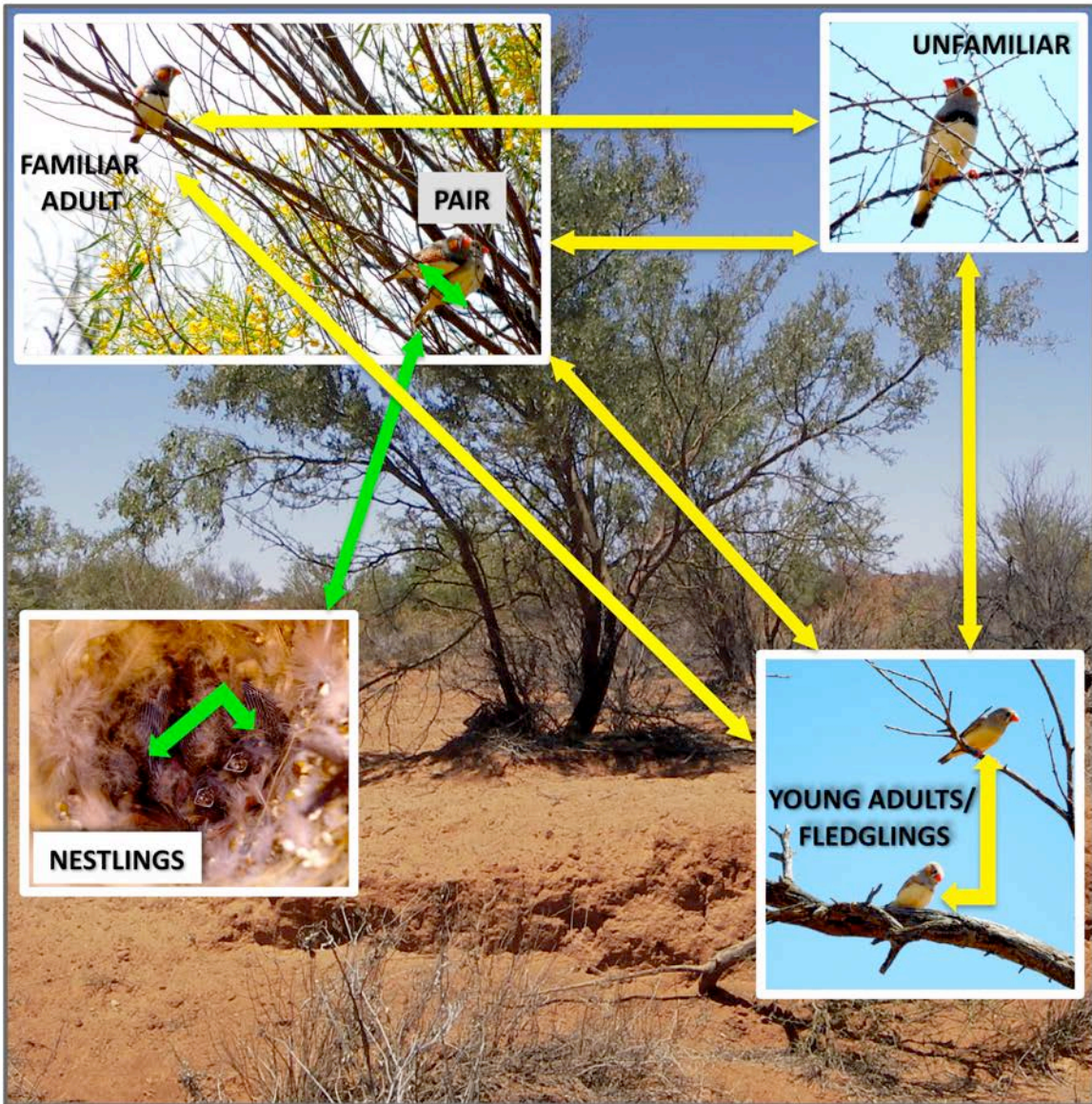


Figure 8 : Social interactions of the zebra finch's social network.



Interactions between kin



Interactions between non-kin

information. As these complex calls encode stable information about the individual such as sex or individual identity, the encoding of a labile information such as stress might be based on subtle modifications of their acoustic structure that do not alter the stable information. Considering also that one given information (i.e. sex or individuality) is not encoded by one but by several acoustic parameters, a redundancy allowing to maintain the information in case of deterioration of the signal, it is possible that the parameters encoding emotions such as stress could be some of the parameters bearing the stable information.

#### ***1.8.4. The social environment of the zebra finch: a complex social network.***

The social context has been shown to modify the vocal behaviour of male zebra finches in response to the calls of their female partners: males exhibit a preferential response to their female partner's calls only when placed in the presence of an audience composed of mated birds, while they do not show any differential response in the presence of either unmated males or an unmated pair (Vignal, Mathevon et al. 2004). Thus, male zebra finches are aware of the social relationships of their companions, and reveal their own relationship to them depending on the social context. This audience effect reveals that social interactions are very complex in the zebra finch and not only relevant within the pairbond but also between familiar conspecifics.

The social network of a given individual within a social group is shaped by the different types of social interactions experienced during its lifespan (**figure 8**). Both adults and young socially interact with other familiar birds, from kin (parents, siblings, progeny and mate) to familiar birds such as close neighbours (Reers, Forstmeier et al. 2013), but also eventually with newcomers during arrival of migrants in the colony or establishment of the group in a new location after migration. Thus, the level of familiarity between conspecifics seems highly important and could shape the way birds interact vocally, especially during the emission of labile information such as stress.

#### **1.9. Research objectives:**

This work aims at understanding the acoustic communication of emotions in the zebra finch by exploring the process through the three main social interactions known in the species: between adults of the social group (**chapter 1 and chapter 2**), between mates (**chapter 2 and chapter 3**) and between parents and nestlings (**chapter 4**). Studies interested in the expression of emotions in animals mainly focused on mammals, while avian species could be good models in this field of study, especially considering songbirds that show vocal learning processes very similar to human ones (Marler 1970). The overall purpose of this study is thus to understand the processes involved in the expression of a basic emotion, i.e. stress, in an avian species, from endocrine control by

corticosterone to behavioural modifications of both senders and receivers in the communication loop.

***1.9.1. Axis 1: Do male zebra finches encode their stress through their vocalisations?***

Zebra finches are gregarious birds, and familiar individuals from the same social group interact at feeding/drinking areas and in social trees. Because isolation has been reported to be a strong stressor in many social species and because separation from the partner in the zebra finch provokes a raise in plasma levels of corticosterone, we supposed that isolation of zebra finches from the social group could also be a strong stressor and thus provoke a raise of corticosterone. As zebra finches are highly vocal birds, we hypothesised that this emotional state could lead to the vocal expression of stress by the bird through the emission of modulated vocalisations. We focused on the distance calls, usually used during social interaction with the mate and familiar conspecifics when visual contact has been interrupted. They are complex vocalisations that carry stable information about the sender, from its sex to its identity, and are thus good candidates to also inform about the emotional state of the sender. To verify our hypotheses, we used a protocol of social isolation of unpaired males to check if breaking off social bonds with familiars could induce a social stress, as separation between mates does. Then, playback sessions of female calls on the isolated males allowed understanding if and how males can express their stress within vocalisations emitted in response to the playback. Neuroendocrine control of the emission of modulated vocalisations has been proposed in many studies, but direct implication of a specific physiological signal has never been proved, neither in mammals nor in birds. Thus, we also tried to understand whether corticosterone would drive isolation-induced modifications of zebra finch calls by feeding males with exogenous corticosterone before the playback session. Our results shown in **Chapter 1** present the first evidence of a direct implication of corticosterone in the emission of modulated vocalisations in the zebra finch and propose different possibilities for the biological values of these isolated-induced vocalisations.

***1.9.2. Axis 2: Do female zebra finches perceive the emotional value of control and stress-induced calls of males?***

If males can encode their stress through modifications of their distance calls, it is likely that possible receivers of these calls could decode the information and respond appropriately. Zebra finches have high hearing capacities (Okanoya and Dooling 1987) that allow them to discriminate fine acoustic modifications such as up-shift in the energy spectrum. If stress-induced vocalisations carry the emotional state of males, females could be able to perceive the modifications of parameters carrying this information. To

verify if stress-induced calls have a biological value, we used a separation protocol during which females isolated from their partner were stimulated by either the stressed calls or by the non-stressed calls of one male during different playback sessions. Because the level of familiarity of the sender could have an impact on female response, we used male calls from different saliences: partner calls, familiar male calls and unfamiliar male calls. Females were recorded and videotaped during the entire experiment to assess their vocal and locomotor behaviour. Because separation from the social partner is already known as a strong stressor and considering the high coordination of social partners in the zebra finch, we supposed that hearing the calls from different valences and levels of familiarity could have a specific impact on the female's physiological stress. We thus also measured plasma levels of corticosterone on females after each playback to check for physiological resonance, a cue of emotional contagion in animals. The results presented in **chapter 2** show that females discriminate between the control calls versus the stressed calls of their mate, and exhibit a behaviour related to an antipredator strategy. We verified that this effect was not due to a failure of the mate recognition system: analysis of male call structures and operant conditioning experiment on females show that stress-induced calls still carry the identity of the caller. Finally, females were more stressed after hearing the stressed-calls of their mate compared to control calls, suggesting the existence of physiological resonance between partners. This is the first evidence of emotional contagion based on acoustic cues only in birds, and raises questions about the existence of a form of empathy in avian species.

### **I.9.3. Axis 3: Do mates show an assortative mating by vocal and physiological profiles during a stressful event?**

Coordination between mates has been proposed as a key characteristic of the survival and the reproductive success of a pair. Coordination can be achieved via an assortative mating of individuals, leading to the association of compatible partners that are similar upon a combination of traits known to influence the reproductive success and the survival of individuals. Zebra finches form monogamous pairbonds that exhibit a highly coordinated and synchronised behaviour, especially around the nest. It is thus likely that assortative mating occurs in this species. Zebra finches are highly vocal, and partners can exchange fine labile information in their calls: males are able to express their stress via fine modulation of vocalisations and females decode the information and answer appropriately with a behaviour related to an antipredator strategy. Considering the importance of vocalisations in the interaction of the partners especially during stressful events, we hypothesised that partners could choose their mate based on their vocal but also physiological profile. Thus, to verify if an assortative mating exists on these criteria in the zebra finch, we performed a separation reunion protocole that elicits the emission of distance calls by partners. We also measured plasma levels of corticosterone after 30



minutes of separation and in control condition so as to check for assortative mating based on plasma levels of corticosterone. Our results do not reveal assortative mating based neither on physiological nor on vocal profile. Instead we show that partners adjust their behaviour while in acoustic contact. In **chapter 3**, we present how the history of the pair influences the vocal activities of birds, stronger pairs and pairs with breeding experience showing more intense vocal interactions. We finally discuss the hypothesis that assortative mating could not exist in the zebra finch and that adjustment of behaviours through familiarity is a plausible mechanism participating in coordination between mates.

#### **1.9.4. Axis 4: Can nestling zebra finches encode their stress through modulation of their begging calls and do parents decode the message and respond appropriately?**

Wild nestlings of zebra finches can suffer from early stress due to their variable and unpredictable habitat. They certainly need to express their stress and thus their specific needs to their parents so that adults can adjust their care in consequence. Nestlings exhibit a stereotypic begging behaviour that includes the emission of begging calls. Physiological stress is already known to elevate the rate and the intensity of begging calls in many bird species, a conspicuous signal that attracts predators but that has been supposed to inform parents about the young specific needs at anytime. Because several studies have discussed the contradiction between the conspicuousness of begging and the associated predation costs, we hypothesised that inconspicuous signals could also carry information about the nestlings' needs. Considering that male zebra finches express their stress through modulation of their vocalisations, we supposed that this flexibility could also be possible in nestlings. We thus mimicked a physiological stress by administering exogenous corticosterone in wild nestlings. To verify if corticosterone could have an impact on the acoustic parameters of the begging calls, we recorded these vocalisations before and after oral administration of corticosterone. Finally, to search for parental adjustment of behaviour, we monitored parental visits at the nest and foraging behaviours during the days following the experiment using transponders, and measured the nestlings' body condition at the end of the experiment. In **chapter 4**, we show that corticosterone provokes a modification of the frequency spectrum of begging calls similar to what has been found in adults. Parents perceive the message as they adjust their behaviour by visiting the nests and the feeding areas more often and for longer. These results suggest that even non-learned vocalisations can present a flexibility that encodes labile information. We also propose that emitting up-shifted vocalisations could be a universal signal for the expression of an emotion of stress.



# RESULTS

# CHAPTER 1

## **The acoustic expression of stress in a songbird: Does corticosterone drive isolation-induced modifications of zebra finch calls?**

Emilie C. Perez <sup>1</sup>, Julie E. Elie <sup>1</sup>, Christophe O. Soulage <sup>2</sup>, Hédi A. Soula <sup>1</sup>, Nicolas Mathevon <sup>1</sup>, Clémentine Vignal <sup>1</sup>

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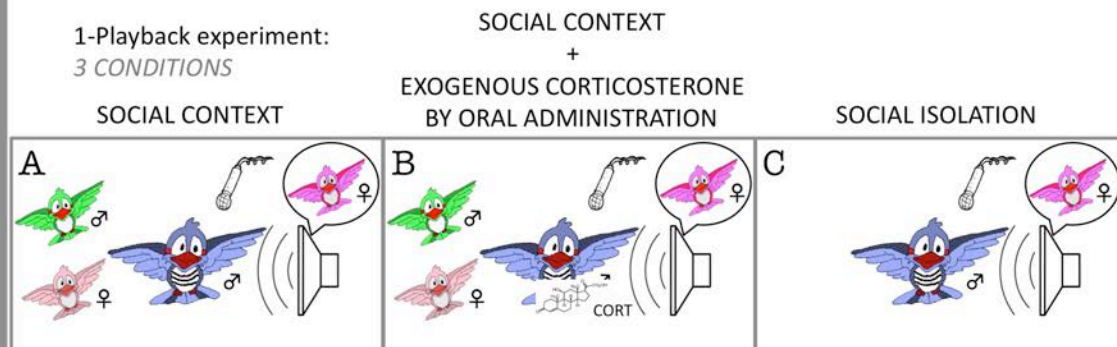
# SUMMARY CHAPTER 1

## Questions

Do male Zebra finches encode their emotion through their vocalisations during a stressful event?  
Is corticosterone, the main stress hormone in birds, implicated in this process?

## Methods

1-Playback experiment:  
3 CONDITIONS



2-Blood sampling and corticosterone assay

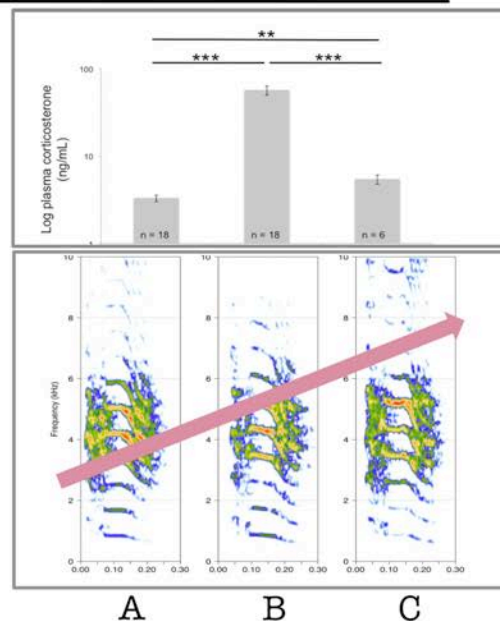
3-Call analysis

## Results

Social isolation → physiological stress  
*Elevation of plasma levels of corticosterone*

Social isolations → modulation of vocalisations  
*General decrease of vocal activity*  
*Vocalisations with up-shifted frequency spectrum*  
*Calls with longer duration*  
*Abolition of preference of response for stranger stimuli*

Corticosterone → similar effect to isolation  
*Lack of effect on call duration*  
*Frequency spectrum up-shift*



## Conclusions

Our results show for the first time the existence of an affective prosody in the Zebra finch. Males express their stress through modulations of their calls. These modifications are partly driven by corticosterone, but other signals could also act.



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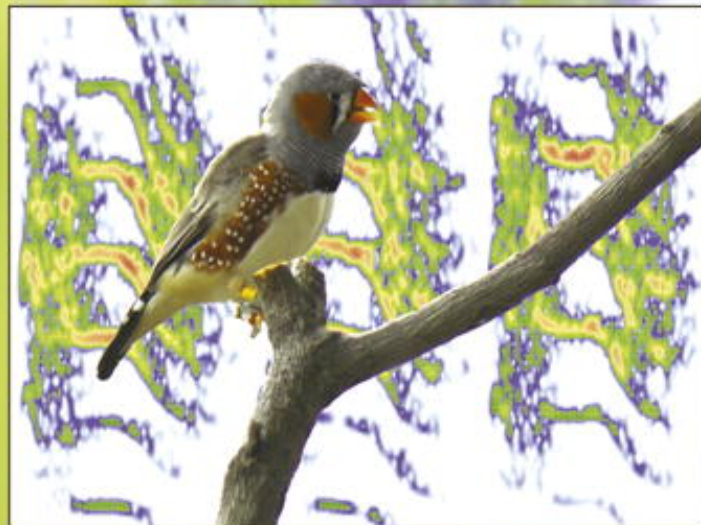
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## The acoustic expression of stress in a songbird: Does corticosterone drive isolation-induced modifications of zebra finch calls?

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### ABSTRACT

Animal vocalizations convey multiple pieces of information about the sender. Some of them are stable, such as identity or sex, but others are labile like the emotional or motivational state. Only a few studies have examined the acoustic expression of emotional state in non-human animals and related vocal cues to physiological parameters. In this paper, we examined the vocal expression of isolation-induced stress in a songbird, the zebra finch (*Taeniopygia guttata*). Although songbirds use acoustic communication extensively, nothing is known to date on how they might encode physiological states in their vocalizations. We tested the hypothesis that social isolation in zebra finches induces a rise of plasma corticosterone that modifies the vocal behavior. We monitored plasma corticosterone, as well as call rate and acoustic structure of calls of males in response to the playback of female calls of varied saliences (familiar versus stranger) in two situations: social isolation and social housing. Social isolation induced both a rise in plasma corticosterone, and a range of modifications in males' vocal behavior. Isolated birds showed a lower vocal activity, an abolition of the difference of response between the two stimuli, and evoked calls with longer duration and higher pitch. Because some of these effects were mimicked after oral administration of corticosterone in socially housed subjects, we conclude that corticosterone could be partly responsible for the isolation-related modifications of calls in male zebra finches. To our knowledge, this is the first demonstration of the direct implication of glucocorticoids in the modulation of the structure of vocal sounds.

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### Introduction

Animal vocalizations can convey information about context and events in the environment, as well as multiple pieces of information about the sender such as identity, physical characteristics, emotional or motivational state (Seyfarth and Cheney, 2003). Within a species, individuals often produce different context-specific types of calls that are characterized by different structures (amplitude, frequency, duration, etc). These calls generally carry stable characteristics related to the emitter's sex or individual identity, but also more instantaneous information such as the sender's motivation or physiological state. For instance, in the presence of a male, females of the South African clawed frog *Xenopus laevis* can produce two call types both consisting of repetitive click trains: a fast fertility call and a slower unreceptive call (Tobias et al., 1998). While these two call types differ in click rate, both are slower than male calls (Tobias et al., 1998). Thus, these calls encode stable information about the

sender's sex and social context, but also labile information about reproductive state (Tobias et al., 1998; Vignal and Kelley, 2007).

The emotional state of the emitter is an example of transient information conveyed in the vocalizations. Because of its importance in speech perception, several studies have focused on vocal expression of emotions in humans (Bachorowski, 1999; Bachorowski and Owren, 2003), and speech rhythm and intonation seem to be the main parameters affected by emotional state (Nygaard and Queen, 2008). In contrast, few studies have examined the acoustic expression of emotional state in non-human animals. One notable exception is the acoustic expression of distress during mother–young separation or during isolation from affiliated individuals in mammalian species. In infant rhesus monkeys, the isolation from the mother induces behavioral agitation and modification of the acoustic structure of vocalizations (Bayart et al., 1990; Levine et al., 1985). In several species of primates, isolation from the mother in young or separation from affiliated individuals in adults has been reported to elicit the emission of specific vocalizations named separation calls (Masataka and Symmes, 1986; Mendoza and Mason, 1986; Norcross and Newman, 1999; Scheumann et al., 2007). In rodents, isolated pups produce specific ultrasonic vocalizations that provoke a fast return of the mother to the nest (Ehret, 2005; Shair, 2007), suggesting that these separation calls convey information

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about the emotional distress of the young and promote physical reunion with the mother (D'Amato et al., 2005).

Physiological stress is a good candidate as proximate mechanism of the acoustic expression of separation distress. Indeed, the perturbation of social interactions is one of the most efficient stressors in animals (DeVries et al., 2003). Social isolation in particular provokes a rise of plasma glucocorticoids in both young and adults of many mammalian and avian species. In infant squirrel monkeys and rhesus monkeys, isolation from the mother elicits a rise of plasma cortisol (Levine and Wiener, 1988; Levine et al., 1985). In marmosets (Norcross and Newman, 1999) and titi monkeys (Mendoza and Mason, 1986), separation of adult pair-mates elevates cortisol levels. In pigs, young females isolated from the social group show an increase of plasma cortisol (Ruis et al., 2001). In European starlings, isolation from group members increases plasma corticosterone, the main stress hormone in birds (Apfelbeck and Raess, 2008). Thus, social isolation may be perceived as a strong stressor, triggering a hypothalamic–pituitary–adrenal (HPA) axis response through a glucocorticoid release. This physiological stress response could provoke the emission of modulated vocalizations that could be considered as the vocal expression of stress. To the best of our knowledge, this hypothesis has not yet been directly tested.

The present study aimed at investigating the vocal expression of stress and its control by glucocorticoids using a songbird as a model system. Although songbirds use acoustic communication extensively during social interactions, nothing is known to date on how they encode emotional and physiological states like stress in their vocalizations. Our study focused on a songbird model species, the zebra finch (*Taeniopygia guttata*). This gregarious bird forms monogamous life-long pair bonds, and is thus highly social (Zann, 1996). Perturbations of social interactions in this bird such as separation from the mate (Remage-Healey et al., 2003) or isolation (Banerjee and Adkins-Regan, 2011) are already suspected to provoke physiological stress but nothing is known on the related modifications of acoustic communication. We hypothesized that social isolation in zebra finches provokes a rise of plasma corticosterone that modifies the acoustic structure of calls and the call rate of emission. We monitored plasma corticosterone concentration, as well as call rate and acoustic structure of calls of males in response to the playback of female calls of varied saliences (familiar versus stranger) in two situations: social isolation and social housing. To test whether the vocal modifications induced by social isolation are triggered by a physiological stress, we investigated whether oral administration of corticosterone can mimic the effects of isolation in socially housed subjects.

## Materials and methods

### Subjects and housing conditions

Birds used for this study were zebra finches (*T. guttata*) bred in our colony (ENES lab, University of Saint-Etienne). Thirty male subjects were used to study the kinetics of circulating level of corticosterone after oral administration of exogenous hormone (Experiment 1). Eighteen male subjects were used in the playback experiment (Experiment 2). All male subjects ( $n = 48$ ) were housed in individual cages (dimensions  $24 \times 29 \times 39$  cm) for the duration of the experiments (light conditions: 14:10 h light:dark; temperature from 22 to 24 °C) to acclimate them to the experimental condition. Food (mixed seeds) and water were provided ad libitum. Birds had free access to a water pool for bathing and were also supplemented with fresh salad once a week.

For at least two weeks before the playback experiment, subjects shared the room with 10 breeding pairs (referred to as “audience birds” in the protocol; each breeding pair was housed in an individual cage) and six females (whose calls were used as familiar acoustic stimuli during playback experiments; each female was housed in an individual cage). Because all cages were in the same room allowing

visual and acoustic contacts between birds, all birds in the room can be considered as familiar to each other.

Six females unknown by the other birds were housed in individual cages and kept in a second room without any acoustic or visual contact with the subjects. Distance calls from these females were used as stranger acoustic stimuli during the playback experiment. The corticosterone baseline of our birds (see results, mean = 3.50 ng/mL) was not different from corticosterone baseline of birds living in aviaries of other studies (Remage-Healey et al., 2003, 3.80 ng/mL for pair housed birds, 3.50 ng/mL for group housed birds). Thus, we assumed that our housing conditions did not modify the baseline stress level and thus the basal behavior of our birds.

Experiments were performed under the authorization no. 42-218-0901-38 SV 09 (ENES Lab, Direction Départementale des Services Vétérinaires de la Loire) according to the guidelines laid down by the French Ministère de l'Agriculture (no. 87-848) and the E.U. Council Directive for the Care and Use of Laboratory Animals of November 24th, 1986 (86/609/EEC).

### Corticosterone manipulation

To non-invasively induce an acute increase of plasma corticosterone, a procedure of oral administration was used. The subjects were fed with 300 mg of seeds sprinkled either with corticosterone dissolved in peanut oil (CORT condition in Experiment 1 and 2) or peanut oil alone (Control condition in Experiment 1, NOCORT and ISOLATION conditions in Experiment 2). To elicit a huge increase of plasma corticosterone in few minutes (Spencer and Verhulst, 2007), a dose of 0.0125 mg of exogenous corticosterone was used (50  $\mu$ L, concentration: 0.25 mg/mL; Sigma Aldrich ref: 27840). Seeds were presented via a trap-door at the bottom of the experimental cage. The cage was inside an acoustically-isolated chamber (internal dimensions: 2.00 m H  $\times$  1.35 m W  $\times$  1.00 m D; Silence Box model, Tip-top Wood, Saint-Etienne, France). To make sure that subjects ate the entire dose of seeds in less than 5 min, any food was removed from the cage the day before the experiment (15 h  $30 \pm 30$  min before the experiment). To monitor when the subject started eating the seeds (denoted as  $t = 0$ ) and the time spent eating, the experimenter observed the bird through a one-way mirror and was thus outside the chamber.

### Blood sample collection and hormone assay

All blood samples (<150  $\mu$ L) were obtained by puncturing the alar vein with a 25-gauge needle. Blood was collected in heparinized tips, and plasma was separated under centrifugation (15 min, 3800  $\times$ g) and stored at  $-80$  °C until hormone assay. Blood samples were taken within 3 min so as to measure corticosterone baseline and not corticosterone due to handling (Wingfield et al., 1982). No bird was bled more than once per week to ensure that they had sufficiently replenished their blood volume between bleeds and HPA axis function had recovered from previous sampling (Romero and Reed, 2008).

Because circulating level of endogenous corticosterone shows circadian variations in many bird species with a peak just before the active period and a rapid decrease during the first hours of activity, all experiments were performed between 8 and 11 am (Remage-Healey and Romero, 2000; Tarlow et al., 2003).

Hormone assays were performed using the ELISA method (Kit Corticosterone EIA, Cayman, #5000561) according to the manufacturer's recommendations. All samples were run in duplicate in three different assays: all samples from Experiment 1 were run in one assay and samples from Experiment 2 in two other assays. The detection limit was 0.2 ng/mL, intra-assay coefficient of variation was 0.14, and inter-assay coefficient of variation was 0.09.

### Experimental setup and test procedures

#### Experiment 1: Study of the kinetics of circulating level of corticosterone after oral administration of exogenous hormone

To assess the effects of exogenous corticosterone administration on circulating levels of hormone over time, 30 males were used. The day before the experiment ( $15 \text{ h}30 \pm 30 \text{ min}$  before the experiment), the subject was placed without any food in an individual cage next to a pair of audience birds (placed in another individual cage). Both cages were inside an acoustically-isolated chamber. On the morning of the experiment, seeds with corticosterone (CORT condition,  $n = 13$ ) or without corticosterone (control condition,  $n = 13$ ) were presented to the subject via the trap-door. Blood was then collected either at 15 min ( $n = 4$  and  $n = 5$  for the control and the CORT conditions, respectively), or 25 min ( $n = 4$  for the control and the CORT condition) or 45 min ( $n = 5$  and  $n = 4$  for the control and the CORT conditions, respectively) after the subject started eating the seeds. Baseline corticosterone ( $t = 0$ ) was assessed using other subjects ( $n = 4$ ) placed in the same experimental conditions (placed without any food and with the presence of an audience) but without the presentation of the seeds before blood sampling.

#### Experiment 2: Playback experiments

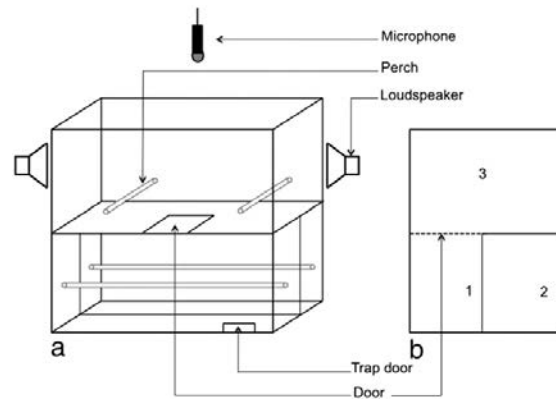
**Experimental groups.** Eighteen male subjects were used in playback experiments in three different conditions:

- NOCORT condition: subjects were placed with a pair of audience birds and received seeds with peanut oil.
- CORT condition: subjects were placed with a pair of audience birds and received seeds with corticosterone.
- ISOLATION condition: subjects were alone and received seeds with peanut oil.

Twelve males (group 1) experienced a NOCORT condition followed by a CORT condition a week later. 6 males (group 2) experienced a CORT, then a NOCORT and finally an ISOLATION condition, with one week between two conditions. Birds of group 2 were exposed to CORT and NOCORT conditions in the reverse order as group 1 so as to control for a potential order effect. Results did not differ between groups, so data were pooled and analyzed together.

**Repertoire choice for playback stimuli and analysis of the subject's vocal response.** While zebra finches have ten distinct calls in addition to the song (Zann 1996), we decided to focus our study on distance calls: first, these calls are usually used by sexual partners when they lose visual contact, and secondly, it has been demonstrated that distance calls carry the identity of the sender (Vignal et al. 2008, Forstmeier et al. 2009), supposedly allowing the birds to distinguish between calls from familiar females and calls from stranger females. Distance calls were thus supposed to have a stable and not easily alterable acoustic structure.

**Preparation of the subjects.** The day before the experiment (at least 12 h prior to the start of stimulus presentation), the subject was deprived of food in the experimental cage (Fig. 1) either in the presence of a pair of audience birds (CORT and NOCORT conditions) or alone (ISOLATION condition). The experimental cage was inside an acoustically-isolated chamber. Both the subject and the audience birds were placed in the lower part of the cage, but the subject was physically separated from the audience by a wire barrier that allowed acoustic and visual contact (Fig. 1). On the morning of the experiment, seeds were presented to the subject via the trap-door. Five minutes after the subject started eating the seeds, the experimenter opened the door between the lower part and the upper part of the experimental cage, so that only the subject was able to visit this part of the cage. It was important not to open the door between



**Fig. 1.** Experimental cage used in Experiment 2. a) Front view, b) Side view. The experimental cage is composed of three parts: the subject was placed in part 1. This area communicated with the upper part of the cage (part 3) when the door was open. The audience was placed in part 2. This area did not communicate with the other parts of the cage, so that only the subject could visit the upper part of the cage. The two lower parts of the cage were separated by wire mesh allowing visual and acoustic contacts between the subject and the audience but preventing physical contacts. A trap-door allowed the experimenter to give seeds to the subject.

the two parts of the cage before the subject started eating, so as to make sure that the bird would easily find the seeds and eat them quickly.

**Recording and preparation of acoustic stimuli.** Distance calls from six stranger and six familiar females were recorded in an acoustically isolated room. The day before the recording session, the female subject was placed in an individual cage in sight of another cage containing two females to provide a social context and to avoid social isolation. Recording started on the following morning. Subjects were stimulated with distance calls of males, played by a computer using Goldwave software (GoldWave Inc. St. John's, NL Canada) through an amplifier (Yamaha AX 396) connected to a loudspeaker (Audiopro Bravo Allroom sat E7140044). Evoked calls were recorded using a microphone (Sennheiser MD42) located 50 cm above the subject and connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz).

**Playbacks.** Fifteen minutes after the beginning of ingestion, a playback session started in the upper part of the cage. This delay was determined based on the results of Experiment 1. Each playback session consisted of four sets of five calls. Each set lasted five seconds (one call per second) and sets were separated by at least 1 min and 55 s. The next set started only if the subject remained silent for at least 10 s, considered as a criterion of basal activity level. Thus, a playback session lasted at least 8 min. Among the four sets, 2 sets contained different calls from one familiar female and 2 sets contained different calls from one stranger female. Sets were randomly played. The females used as stranger or familiar stimuli were never used in two different conditions for the same subject. So, the stranger/familiar stimuli used in one condition (e.g. NOCORT) were different from the stranger/familiar stimuli used in the following condition (e.g. CORT).

Calls were played by a computer using Goldwave software, through an amplifier (Yamaha AX 396) connected to two loudspeakers (Audiopro Bravo Allroom sat E7140044). Loudspeakers were placed on both sides of the experimental cage, so that sets of calls could be randomly played either on the right or on the left side of the cage. The intensity of calls given by audience birds placed in the test room was used as a sound intensity reference for the playback. The root mean square (RMS) sound pressure of the stimuli was set at half the RMS value of the audience's calls using Goldwave software, to mimic a distant bird.



Vocal response of the subjects was recorded during the entire session using a microphone (Sennheiser MD46) connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz).

At the end of the playback session, the subject was rapidly caught (less than 2 min after the end of the last playback set) and bled within 3 min after entering the acoustically-isolated chamber to measure the level of plasma corticosterone.

**Analysis of the vocal response.** For each playback test, the vocal response of the subject was assessed using two behavioral parameters:

- the latency to the first call (in seconds): the time between the start of the first call of a stimulus set and the first distance call emitted by the subject.
- the call density: the number of calls emitted by the subject during the first minute of each stimulus set was first quantified using Pratt software (Boersma, 2001). Since calling activity during one playback session was highly variable across individuals (mean number of calls =  $41 \pm 20$  (SD), min = 8, max = 81), normalization procedures were used. First, the number of calls emitted during each stimulus set was divided by the total number of calls emitted by the subject during the entire playback session (4 stimulus sets, 2 stranger and 2 familiar sets). This procedure produced a parameter called an allocation factor "AF STIM" that reflected the proportion of calling activity allocated to each stimulus set in a given playback session. Second, the number of calls emitted during one playback session was divided by the total number of calls emitted by the subject during the different playback sessions (2 sessions in group 1, 3 sessions in group 2). This procedure produced an allocation factor called "AF COND" that reflected the proportion of calling activity allocated to each condition (NOCORT versus CORT versus ISOLATION). Because birds underwent either 3 playback sessions (group 2) or 2 playback sessions (group 1), "AF COND" was scaled by a coefficient 2/3 in group 1 to allow for comparisons of all birds.

Distance calls used by the subjects in response to the stimuli were isolated using Pratt software and analyzed. Seven acoustic parameters were measured for each call. Using a home-made computer program, the call duration and the call mean frequency were quantified as follows:

- call duration (in seconds): the energy was first computed from the signal envelope and the peak maximum was used as an energy reference. The beginning and the end of the call were then defined as the points where the signal reached 10% of this reference before and after the peak maximum.
- call mean frequency (in Hertz): the mean frequency was first calculated between 0 and 6000 Hertz using the power spectrum of the signal (Fast-Fourier-Transform). A high-pitched call was characterized by a high mean frequency, whereas a low-pitched call showed a low mean frequency. An index of variation "I" was then calculated for each call as follows:  $I = FX - FM$  where FX is the mean frequency of the call analyzed, and FM is the average of the mean frequencies of the 10 lowest-pitched calls emitted by the subject during the whole experiment. This index thus represented a measure of the pitch variation relative to the average pitch of the subject's lower-pitched calls.

Using the Seewave package (Sueur et al., 2008) implemented in R software (R Development Core Team, 2007, [www.r-project.org](http://www.r-project.org)), we measured the following spectral parameters:

- the call dominant frequency (in Hertz) is the average over the duration of the call of the frequencies of highest amplitude (obtained via the 'dfreq' package function). The frequency of highest amplitude is first assessed on time bins using a window length of 512, and the series of values obtained over the duration of the call is then averaged.

- the call fundamental frequency (in Hertz) is the average over the duration of the call of the fundamental frequency values (these are tracks of the fundamental frequency through a short-term cepstral transform obtained via the 'fund' package function).
- the median, the mode and the inter-quartile range of the call spectrum (using 'specprop' package function).

#### Statistical analysis

All statistical tests were performed using R software. Normal distribution was tested using the Shapiro test and homoscedasticity was tested using the Bartlett test. When at least one of these two conditions was violated, non-parametric statistical tests were used.

In Experiment 1 (kinetic study), data were independent so the results were analyzed using t-tests.

In Experiment 2, hormone data were analyzed using a Linear Mixed-effects Model (LMM, using lme function of the R package nlme) with the condition (NOCORT, CORT or ISOLATION) as a fixed factor, and subject identity as a random factor. This allowed us to control for potential confounding effects of the number and order of conditions experienced by each subject. All post hoc tests were done using paired t-tests with Bonferroni correction.

Call density (AF STIM and AF COND) and fundamental frequency were all analyzed using LMM with the condition (NOCORT, CORT or ISOLATION) and the type of stimulus (familiar calls or stranger calls) as fixed factors, and subject identity and order of presentation of the stimulus as random factors. All post hoc tests were done using paired t-tests with Bonferroni correction.

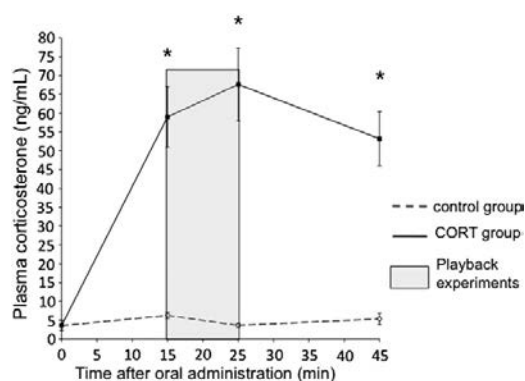
Latency to the first call, call duration, index of variation of call mean frequency, dominant frequency, median, mode and inter-quartile range values were analyzed using a Kruskal–Wallis ANOVA. Posthoc tests were done using Wilcoxon tests with Bonferroni correction.

Values displayed are means are given  $\pm$  SE. All significant differences are reported for  $P \leq 0.05$ .

## Results

### Experiment 1: Circulating levels of corticosterone after oral administration of exogenous hormone

Plasma corticosterone levels increased significantly following oral administration while levels of corticosterone in the control group did



**Fig. 2.** Effect of oral administration of exogenous hormone on plasma corticosterone levels: Kinetic study (Experiment 1).  $n = 13$  for both CORT group and control group. The zero time-point represents baseline corticosterone level ( $n = 4$ ). The gray rectangle highlights the choice of the beginning and the end of playback experiments (experiment 2). Data are presented as mean  $\pm$  SE. \*: significant differences between the two treatment groups at the  $P < 0.05$  level.

not change over time and remained at baseline with a mean value around 3.57 ng/mL  $\pm$  0.97 (t-tests, CORT group vs control group:  $P=0.008$  at 15 min,  $P=0.035$  at 25 min and  $P=0.018$  at 45 min, Fig. 2). Corticosterone levels peaked 15 min after oral administration and remained high until 45 min. Playback experiments (Experiment 2) were thus performed during this peak ranging from 15 to 25 min after oral administration – as shown with a gray time range on Fig. 2.

#### Experiment 2: Modifications of behavioral response to playback with elevation of circulating levels of corticosterone

##### Modification of circulating levels of corticosterone with experimental conditions

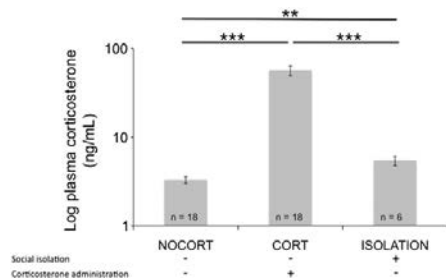
Plasma corticosterone was measured at the end of the playback session, i.e. 25 min after the ingestion of seeds. In line with the results obtained in Experiment 1, oral administration of exogenous corticosterone provoked a huge increase of circulating hormone reaching 1700% of basal values (LMM:  $F_{2,19}=40.80$ ,  $P<0.001$ , paired t-tests with Bonferroni correction: NOCORT versus CORT:  $P<0.001$ , ISOLATION versus CORT  $P<0.001$ ). Isolation also significantly increased circulating levels of corticosterone (164% of basal values) that did not reach the levels obtained in CORT condition (paired t-test with Bonferroni correction: ISOLATION versus NOCORT:  $P=0.002$ , Fig. 3).

##### Exogenous administration of corticosterone as well as isolation abolished the difference of response between the two stimuli

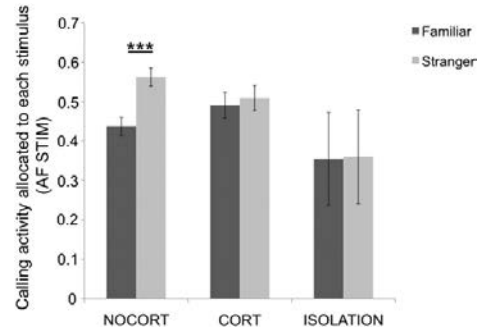
Whereas subjects in NOCORT condition showed higher calling activity in response to the “stranger” stimulus compared to the “familiar” stimulus (LMM:  $F_{5,63}=2.87$ ,  $P=0.02$ , paired t-test with Bonferroni correction:  $P<0.001$ , Fig. 4), ISOLATION and CORT conditions abolished this difference (paired t-tests with Bonferroni correction:  $P=0.98$  and  $P=0.69$  respectively). There was no significant difference in response latency in any of the three conditions (Kruskal–Wallis ANOVA:  $P=0.07$  for all comparisons).

##### Isolation, but not exogenous administration of corticosterone, decreased total calling activity

Subjects in ISOLATION condition showed a significantly lower total calling activity than subjects in experimental conditions with an audience (LMM:  $F_{2,23}=8.25$ ,  $P=0.002$ , paired t-tests with Bonferroni correction: ISOLATION versus NOCORT  $P=0.002$ , ISOLATION versus CORT  $P=0.05$ , Fig. 5). There was no significant difference between CORT and NOCORT conditions (paired t-test with Bonferroni correction:  $P=0.33$ ).



**Fig. 3.** Modifications of circulating level of corticosterone with experimental conditions (Experiment 2). Data are presented as mean  $\pm$  SE. \*\*:  $P=0.002$ ; \*\*\*:  $P<0.001$ . NOCORT ( $n=18$ ): subjects were placed with a pair of audience birds and received seeds with peanut oil; CORT ( $n=18$ ): subjects were placed with a pair of audience birds and received seeds with corticosterone; ISOLATION ( $n=6$ ): subjects were alone and received seeds with peanut oil. «+» and «-» indicate specific context for each condition: birds in social isolation (+) or in social context (-), administration of exogenous corticosterone (+) or no administration (-).



**Fig. 4.** Vocal activity allocated to each stimulus in response to the playback (Experiment 2). \*\*\*:  $P<0.001$ . Note the high variability of AF in ISOLATION condition that caused the sum of the 2 group means to be below 1.

##### Exogenous administration of corticosterone as well as isolation modified acoustic parameters of evoked calls

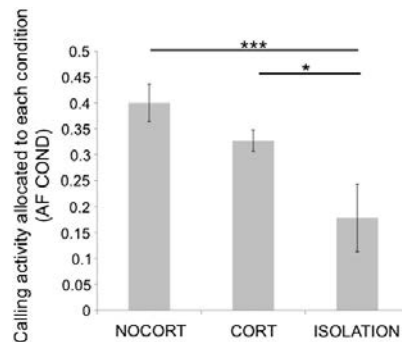
Subjects emitted higher-pitched and longer calls in ISOLATION than in the two other conditions (Table 1). Indeed, the index of variation of call's mean frequency and the duration showed significant differences between ISOLATION and the two other conditions (Tables 2 and 3). Albeit significant (see Tables 2 and 3), the differences in duration between conditions are probably too small (up to 6 ms) to be of biological importance. On the other hand, the index of variation of call's mean frequency not only showed significant differences but also amounted to easily discernable variation (Table 1).

In addition, the pitch of the calls in CORT condition can be described as intermediate between NOCORT and ISOLATION conditions since the dominant frequency, the mode and the median were all ordered decreasingly as ISOLATION > CORT > NOCORT (Tables 1, 2 and 3). These differences can be observed on a spectrogram of the calls (Fig. 6 and Supplementary sound file).

Calls in NOCORT condition showed a more broadband spectral composition than calls in CORT and ISOLATION. Indeed, the interquartile range (IQR) is significantly higher in NOCORT condition (Tables 1, 2 and 3).

The index of variation of call's mean frequency, the median and the mode of the spectrum as well as the fundamental frequency were all affected by the stimulus type: “stranger” stimuli evoked higher-pitched calls and calls of higher fundamental frequency than “familiar” stimuli (Tables 1, 2 and 3).

Finally, interactions between conditions and stimuli yielded no significant differences.



**Fig. 5.** Vocal activity allocated to each condition in response to the playback (Experiment 2). \*\*\*:  $P<0.001$ ; \*:  $P=0.05$ .

**Table 1**

Measure of seven acoustic parameters on the calls emitted during the playback experiment. Values are given as mean  $\pm$  SE. Duration is given in seconds, whereas other parameters are given in Hz. (Index = index of variation of call mean frequency; IQR = inter-quartile range).

|                       | Condition          |                   |                    | Stimulus           |                    |
|-----------------------|--------------------|-------------------|--------------------|--------------------|--------------------|
|                       | NOCORT             | CORT              | ISOLATION          | Familiar           | Stranger           |
| Duration              | 0.106 $\pm$ 1.6E-3 | 0.108 $\pm$ 2E-3  | 0.112 $\pm$ 2.2E-3 | 0.107 $\pm$ 1.6E-3 | 0.109 $\pm$ 1.5E-3 |
| Index                 | 259.6 $\pm$ 11.1   | 295.9 $\pm$ 13.0  | 380.9 $\pm$ 24.6   | 269.8 $\pm$ 11.7   | 309.0 $\pm$ 11.2   |
| Dominant frequency    | 3876.5 $\pm$ 20.6  | 4057.7 $\pm$ 13.5 | 4193.1 $\pm$ 19.1  | 3957.5 $\pm$ 18.9  | 4018.8 $\pm$ 15.3  |
| Fundamental frequency | 781.3 $\pm$ 6.6    | 773.6 $\pm$ 7.8   | 860.7 $\pm$ 10.7   | 774.9 $\pm$ 6.5    | 802.1 $\pm$ 6.7    |
| Median                | 4062.8 $\pm$ 12.4  | 4149.8 $\pm$ 11.1 | 4301.9 $\pm$ 18.1  | 4105.5 $\pm$ 12.5  | 4150.2 $\pm$ 10.5  |
| Mode                  | 4023.2 $\pm$ 27.2  | 4196.3 $\pm$ 28.3 | 4542.1 $\pm$ 41.9  | 4118.8 $\pm$ 27.8  | 4198.3 $\pm$ 25.8  |
| IQR                   | 1389.6 $\pm$ 16.9  | 1295.5 $\pm$ 13.6 | 1294.2 $\pm$ 19.4  | 1342.3 $\pm$ 15.2  | 1338.5 $\pm$ 13.5  |

## Discussion

In our experiment, social isolation induced in male zebra finches both an acute physiological stress, evidenced through a notable rise in plasma corticosterone concentration, and a range of modifications in their vocal behavior in response to playback. Indeed, isolated birds showed an overall decline of their vocal activity, an abolition of the difference of response between the two stimuli, and evoked calls with longer duration and higher pitch. Because some of these effects were mimicked after oral administration of corticosterone in social context (CORT group), we conclude that corticosterone could be partly responsible for the isolation-related modifications of vocal response to playback in male zebra finches.

### Implication of corticosterone in isolation-related modifications of vocal response to playback

To our knowledge, our evidence is the first demonstration of the direct effect of glucocorticoids in the modulation of the acoustic structure of vocal sounds on the short term. Indeed, natural elevations of glucocorticoids induced by both acute and chronic stress are known to modify or impair a huge range of behaviors such as locomotor activity (Breuner et al., 1998; Cox et al., 2011; Landys et al., 2006), maternal behavior (Saltzman and Abbott, 2009; Yamada et al., 2002) or aggressiveness (Meddle et al., 2002). However, very few studies have looked at the short-term impact of glucocorticoids on the vocal behavior of animals and apparently, no one has ever shown the implication of corticosterone in the modification of the fine structures of the acoustic signals such as spectral modifications. Indeed, previous studies have only dealt with modifications of temporal cues such as the rate of emission of vocalizations (Kitaysky et al., 2001), and long-term effects such as the impact of developmental stress on adult song structure (Buchanan et al., 2003, 2004; Spencer

et al., 2005). Inversely, modification of the finer structures of the vocal signals was observed in mammalian as well as avian species during stressful situations but the direct effect of glucocorticoids has not been previously demonstrated. For instance, female bottlenose dolphins with dependent calves produced whistles with higher maximum frequencies during capture–release sessions (Carter et al., 2009). Here we show for the first time that the isolation-induced modifications of the acoustic structure of vocal sounds are at least partly linked to an elevation in plasma corticosterone.

In the present experiment, oral administration of exogenous corticosterone in social condition provoked a huge rise of plasma corticosterone while isolation provoked a more moderate elevation compared to social condition. This range of variation is comparable to already described population variability in response to restraint stress: Evans et al. (2006) observed that peak corticosterone production showed considerable individual variation after 20 min holding in a cloth bag, a standardized capture/restraint technique. Indeed, corticosterone concentrations in their experiment ranged from 3.06 to 183.29 nmol/L, i.e. 1.06 to 63.5 ng/mL (Evans et al., 2006).

Nevertheless, our results show that the oral administration of exogenous corticosterone (CORT group) only partly mimicked the effect of social isolation (ISOLATION group). Several hypotheses can be envisaged. First, corticosterone has a non-monotonic dose-dependent effect on many behaviors: for instance, white-crowned sparrows that received an intermediate dose of exogenous corticosterone (10 ng/mL) show a higher motor activity than those that received a significantly higher dose (60 ng/mL) (Breuner et al., 1998). Such a non-monotonic effect of corticosterone could explain why the CORT condition did not totally mimic the ISOLATION condition.

Secondly, the audience could act as a social buffer on birds of the CORT group and could thus lessen the effect of the physiological stress. This buffering effect has been reported in rhesus and saïmiri monkeys, in which infants separated from their mother emit less vocalizations in the presence of conspecifics than when totally isolated (Levine and Wiener, 1988).

**Table 2**

Statistical tests on the seven acoustic parameters measured on the calls emitted during the playback experiment. Only significant effects are presented. Call duration, Index (= index of variation of call mean frequency), dominant frequency, median, mode and IQR (= inter-quartile range) values were analyzed using a Kruskal–Wallis ANOVA. Fundamental frequency values were analyzed using a linear Mixed-effects Model.

| Parameter          | Factors   | Chi-squared | df | P-value |
|--------------------|-----------|-------------|----|---------|
| Duration           | Condition | 14.52       | 2  | 0.0007  |
| Index              | Condition | 21.55       | 2  | <0.0001 |
|                    | Stimulus  | 6.53        | 1  | 0.0106  |
| Dominant frequency | Condition | 94.40       | 2  | <0.0001 |
|                    | Stimulus  | 95.41       | 2  | <0.0001 |
| Median             | Condition | 4.75        | 1  | 0.0292  |
|                    | Stimulus  | 79.72       | 2  | <0.0001 |
| Mode               | Condition | 5.94        | 1  | 0.0148  |
|                    | Stimulus  | 9.44        | 2  | 0.0089  |
| IQR                | Condition | 9.44        | 2  | 0.0089  |

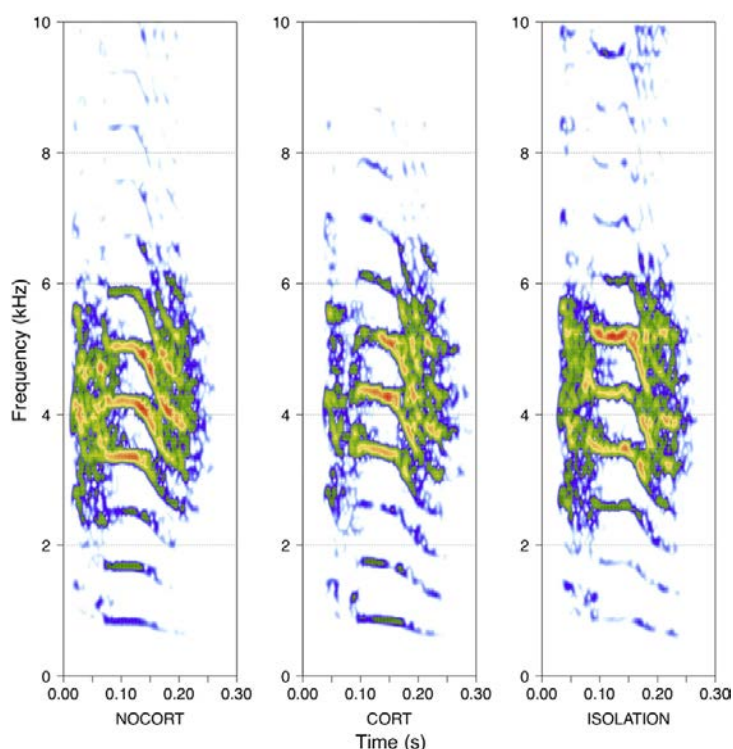
  

| Parameter             | Factors   | numDF | denDF | F-value | P-value |
|-----------------------|-----------|-------|-------|---------|---------|
| Fundamental frequency | Condition | 2     | 846   | 2.097   | 0.1234  |
|                       | Stimulus  | 1     | 846   | 7.493   | 0.0063  |

**Table 3**

Posthoc tests on the seven acoustic parameters measured on the calls emitted during the playback experiment. Posthoc tests were run only when there was a significant effect of the factor (condition and stimulus). Posthoc tests were done using Wilcoxon tests with Bonferroni correction for call duration, Index (= index of variation of call mean frequency), dominant frequency, median, mode and IQR (= inter-quartile range) values. Fundamental frequency values were analyzed using paired t-tests with Bonferroni correction.

|                       | Condition      |                     |                   | Stimulus             |
|-----------------------|----------------|---------------------|-------------------|----------------------|
|                       | NOCORT vs CORT | NOCORT vs ISOLATION | ISOLATION vs CORT | FAMILIAR vs STRANGER |
| Duration              | 1.0000         | 0.0010              | 0.0020            | –                    |
| Index                 | 0.1570         | <0.0001             | 0.0040            | 0.0110               |
| Dominant frequency    | <0.0001        | <0.0001             | <0.0001           | –                    |
| Fundamental frequency | –              | –                   | –                 | 0.0380               |
| Median                | <0.0001        | <0.0001             | <0.0001           | 0.0290               |
| Mode                  | 0.0001         | <0.0001             | <0.0001           | 0.0150               |
| IQR                   | 0.0210         | 0.0370              | 1.0000            | –                    |



**Fig. 6.** Spectrograms of the calls of the same subject in the three experimental conditions. Note that the intensity of the third harmonic (around 3.4 kHz) is ordered decreasingly as NOCORT > CORT > ISOLATION, whereas the intensity of the fifth harmonic (around 5 kHz) is ordered increasingly as NOCORT < CORT < ISOLATION. These variations are consistent with a pitch ordered increasingly as NOCORT < CORT < ISOLATION. Spectrograms are calculated using the Seewave package implemented in R software (overlap = 90, window length = 700, color scale = -40 dB - 0 dB). See Supplementary sound file.

Finally, others hormones such as catecholamines could contribute to the modulation of isolation-induced vocalizations. In rodent pups, the injection of a dopamine receptor agonist reduces the rate of emission of isolation-induced vocalizations (Muller et al., 2005). Nevertheless, this hypothesis remains to be tested in birds.

#### *Isolation-related modifications of zebra finch calls and their potential role in communication*

To the best of our knowledge, this is the first demonstration in a bird of a vocal flexibility induced by the social context. While it has been demonstrated that the presence of an audience as well as social isolation modifies the vocal activity of male zebra finches in response to playback stimuli (Vignat et al., 2004, 2005), here we show that the acoustic structure of calls is altered in response to the social context. Moreover, isolation-related modifications of the vocal response to playback were mimicked using an experimental increase of plasma corticosterone without any variation in the social context of the bird. Thus, physiological stress could be an underlying mechanism of the audience effect in zebra finches.

The behavioral modifications triggered by the elevation of plasma corticosterone could allow the birds to respond to the stressful situation. This function has been previously demonstrated in rodents, in which the emission of modulated ultrasonic calls by isolated pups elicits reunion with the mother (D'Amato et al., 2005). An equivalent function is highly possible in zebra finches, which use distance calls when social partners lose visual contact. The adjustment of behavior could then explain why stressed birds (CORT and ISOLATION groups) do not show any difference of response to either familiar or stranger stimuli, while

they do so when they are not stressed (NOCORT group). This loss of discrimination could reveal the urgent need to elicit physical reunion with other individuals. Another explanation could be that birds are no longer able to discriminate between stranger and familiar stimuli in stressful situations. This hypothesis remains to be explored.

Our results suggest that distance calls of male zebra finches are more flexible than previously thought and can be modified in relation to an increase in plasma corticosterone indicating that the bird is experiencing stress (Cockrem, 2007), that is to say in relation to the emotion of the emitter. Corticosterone is known to elevate basal metabolism and muscular activity. A recent study has also demonstrated that the mean frequency and the duration of vocalizations are directed by basal metabolism and muscular activity in many animals (Gillooly and Ophir, 2010). Thus, the effect of corticosterone on vocalizations could be mediated by its action on the muscular activity of the emitter. This leads to an important question: do modified vocalizations represent only a by-product of the bird's physiological stress, or do they carry information about the emotion of the emitter that could be perceived by conspecifics? Zebra finches are able to perceive fine changes in the spectral components of sounds (Dooling et al., 1995; Ohms et al., 2012; Weisman et al., 1998), so they could potentially discriminate stress-induced modifications in calls. Whether stress-induced modifications of calls have a role in zebra finches' communication remains a question to investigate. Moreover, none of the birds have ever emitted distance calls spontaneously during our experiment: consequently, we do not know if spontaneous calls are modified by stress in the same way as evoked calls and thus also carry information about the emotion of the emitter.

Modifications of duration and spectrum of vocalizations in relation to the emotional state of the emitter seem to represent a conserved effect in mammals and birds. In elephants, low-ranking females interacting with dominant individuals produce rumbles with increased and more variable fundamental frequency, as well as increased duration and amplitude (Soltis et al., 2009). In humans, stressful situations such as fear or anxiety modify not only the rhythm but also the mean fundamental frequency and the pitch of the speech (Banse and Scherer, 1996). In rodents, modifications in frequency modulations and call duration of isolation-calls correlate with the distress of the pup (Ehret, 2005; Hodgson et al., 2008). Finally, the calls of primates can also be modified by the emotional state of the emitter: for instance, separated infants of squirrel monkeys give longer calls at greater separation distances from their natal group members, and responding adults and juveniles similarly extend the length of their vocalizations (Masataka and Symmes, 1986). Our study demonstrates that birds are also able to modify their vocalizations in relation to their emotional state, using similar prosodic rules as mammals.

Supplementary materials related to this article can be found online at doi:10.1016/j.yhbeh.2012.02.004.

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# CHAPTER 2

## **Evidence for empathy in a songbird: emotional contagion between mates through vocal signals.**

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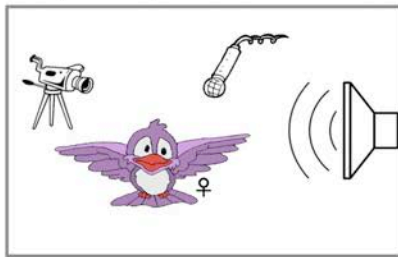
# SUMMARY CHAPTER 2

## Questions

Do female Zebra finches perceive the emotional value of control and stress-induced calls of males?

## Methods

1-Playback experiment on mated females

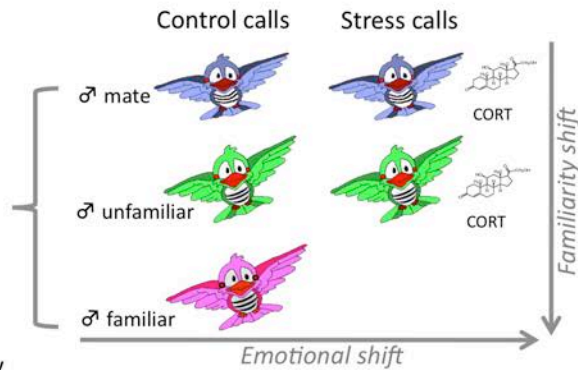


2-Blood sampling and corticosterone assay

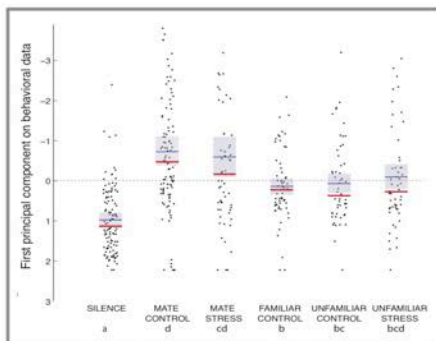
3-Behavioral scores

4- Male call analysis using DFA : individuality in stress-induced calls

5-GO-NOGO Conditioning paradigm on females using calls with different emotional values



## Results

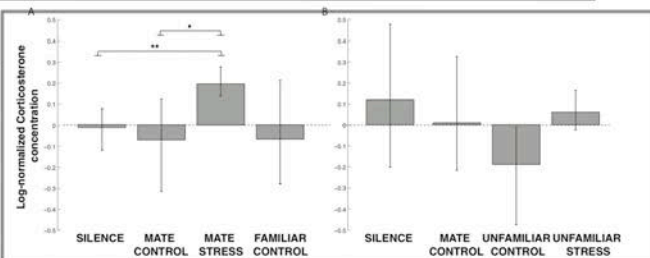


3-Females change their behaviour with The emotional value of their mate calls

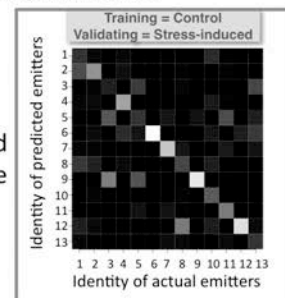
Vocal behaviour  
Vigilance behaviour

4-a DFA classifies control and stress-induced calls of one caller as belonging to the same individual Analyse based on 11 acoustic parameters

5- Females do the same in the GO-NOGO operant conditioning experiment



2-Females show a physiological resonance with their mate Rise in plasma levels of corticosterone after hearing stress calls of the mate, but No effect of other stimuli on the female's stress



## Conclusion

Female Zebra finches show an emotional contagion from their mate but not unfamiliar males. This difference of response is not due to a failure of the mate recognition system.. This is the first evidence of physiological resonance, a pre-requisite of empathy in birds based on acoustic signals only.



## **Summary**

Empathy - the capacity to share the emotions of another individual - could be a proximate basis for group-living and altruistic behaviors [1]. Empathy may be present through much of the evolutionary history of mammals [1-5] as witnessed in particular subtypes such as in emotional contagion, in which the affective state of an individual can generate the same affect in a conspecific. Achieved through acoustic [6], chemical [7] or visual cues [4], emotional contagion is more likely to occur with increasing familiarity or relatedness between protagonists [8,4] and may reflect a physiological resonance: in humans, cortisol response is contagious between observers and stressed speakers [9]. If empathy is shared among social vertebrates, the strong partnership of birds' monogamous pair bond is a likely candidate for emotional contagion. Here we show that the mate's stress-induced calls induce corticosterone responses and corresponding behavioral changes in female zebra finches. This emotional contagion depends on familiarity and is not due to failure of the call-based individual recognition system. Since the zebra finch is a widely used model organism with unique relevance to human vocal learning, this evidence of emotional contagion based on vocal cues opens the door for novel research opportunities in vocal signaling and perception of emotions in vertebrates.

## **Highlights**

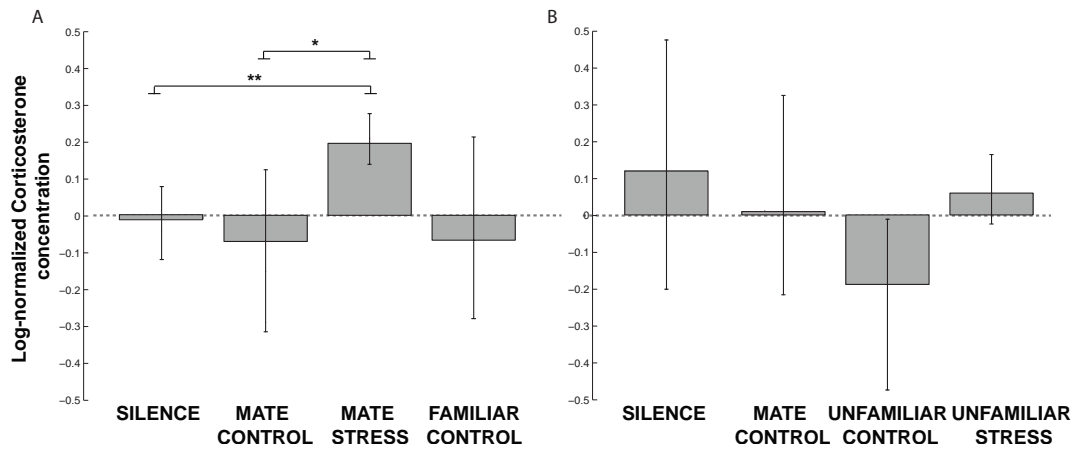
- Test of emotional contagion between pair bonded partners of a monogamous songbird
- Corticosterone response in females by exposure to mate's stress-induced calls
- Physiological resonance and related behavioral changes depend on call familiarity
- Persistence of call-based individual recognition with stress-induced calls

## Results and Discussion

In zebra finches, mates pair for life and are highly coordinated social partners [10,11], who collaborate in chick rearing, nest guarding and many other life-critical behaviors. Call-based individual recognition is used for pair bond maintenance [12-14]. Because male zebra finches can express their physiological stress through modifications of their vocalizations [15], we hypothesized that females can perceive the emotion in the calls of their mate.

We recorded male distance calls in control and stress-induced conditions and used them as playback stimuli on females. Distance calls are one of many calls in the vocal repertoire of the zebra finch [10]. Birds produced these calls to maintain contact with other birds when they are visually isolated, and upon reunion with their mate after separation [10]. Given their behavioral function, their emotional valence for the receiver in control situations is positive. Male control calls were recorded using a separation protocol in which mates were visually but not acoustically isolated. Male stress-induced calls were obtained with an oral administration of corticosterone before separation [15]. We previously showed that oral administration of corticosterone induces several modifications of behavior also observed in complete social isolation [15], a powerful stressor in zebra finches [16,17]. Calls of corticosterone-treated males show similar changes in acoustic structure as calls produced in social isolation and thus represent a controlled behavioral output of the male's stress.

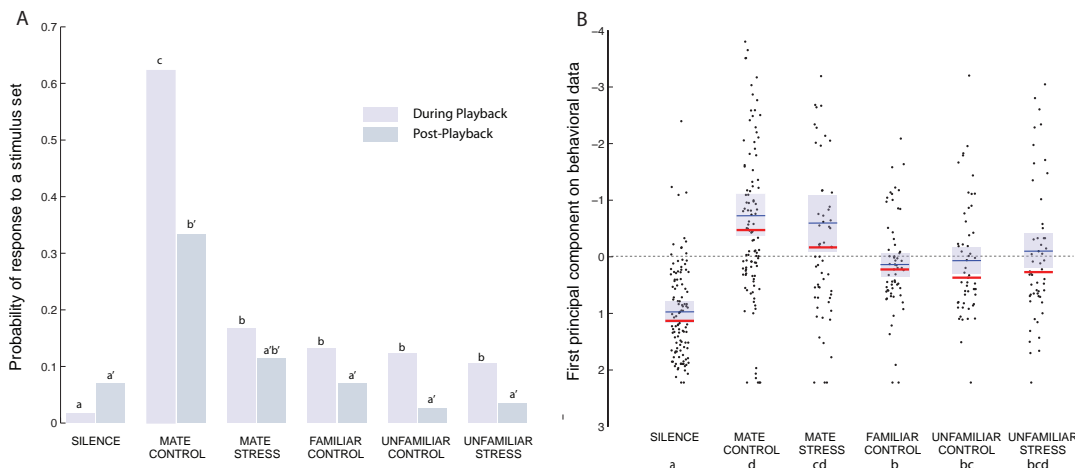
During a first set of playback sessions, females were stimulated with their mate's stress-induced calls (MATE STRESS), their mate's control calls (MATE CONTROL), control calls of another familiar male living in the same colony room (FAMILIAR CONTROL), or remained in silence. Repeating playback sessions in random order, all females were exposed to the four conditions. At the end of each playback session, blood was collected from the females for corticosterone assay. Females showed significantly higher plasma corticosterone concentrations after MATE STRESS than after SILENCE or MATE CONTROL (Figure 1A). Seven out of eight females had higher corticosterone concentrations after MATE STRESS than after MATE CONTROL. FAMILIAR CONTROL was not significantly different from the other conditions and showed high variability between individuals certainly because the real level of familiarity was difficult to control (Figure 1A). To know if this physiological resonance is restricted to affiliated individuals or if females are in general more stressed after hearing the stress-induced calls of any male, we investigated the effect of familiarity in a second set of playback sessions. In these sessions, females were stimulated with the stress-induced calls of an unfamiliar male (UNFAMILIAR STRESS), the control calls of another unfamiliar male (UNFAMILIAR CONTROL), their mate's control calls (MATE CONTROL), or remained in silence. Again, all females were exposed to these conditions in random order. None of these stimuli elicited significant differences in plasma corticosterone concentrations (Figure 1B).



**Figure 1. Effect of the stress-induced calls of male zebra finches on corticosterone plasma concentrations of their female partner.**

(A) First set of playback sessions ( $n = 8$ ), (B) second set of playback sessions ( $n = 9$ ). For display purpose, corticosterone plasma concentrations are normalized by dividing by individual means and log transformed. Original data ranged from 3.83 ng/mL to 126.36 ng/mL, with mean  $27.49 \pm 2.80$  ng/mL. Boxes are medians and error bars are interquartile-ranges (1<sup>st</sup> quartile-3<sup>rd</sup> quartile). Significance is based on non-parametric within subject tests (see Supplemental Information for full description of statistical analyses).

A) Females showed significantly higher plasma corticosterone concentrations after the playback of their mate's stress-induced calls than after the playback of silence or their mate's control calls (Friedman  $\chi^2 = 8.55$ ,  $df = 3$ ,  $P = 0.036$ , posthoc Wilcoxon signed rank test MATE STRESS vs SILENCE  $n = 8$  and  $V = 36$ ,  $P = 0.008$ , MATE STRESS vs MATE CONTROL  $n = 8$  and  $V = 3$ ,  $P = 0.039$ ). FAMILIAR CONTROL was not significantly different from the other conditions. B) None of the stimuli elicited differences in plasma corticosterone concentrations (Friedman  $\chi^2 = 3.2667$ ,  $df = 3$ ,  $P = 0.352$ ).



**Figure 2. Effect of the stress-induced calls of male zebra finches on the behavior of their female partner.**

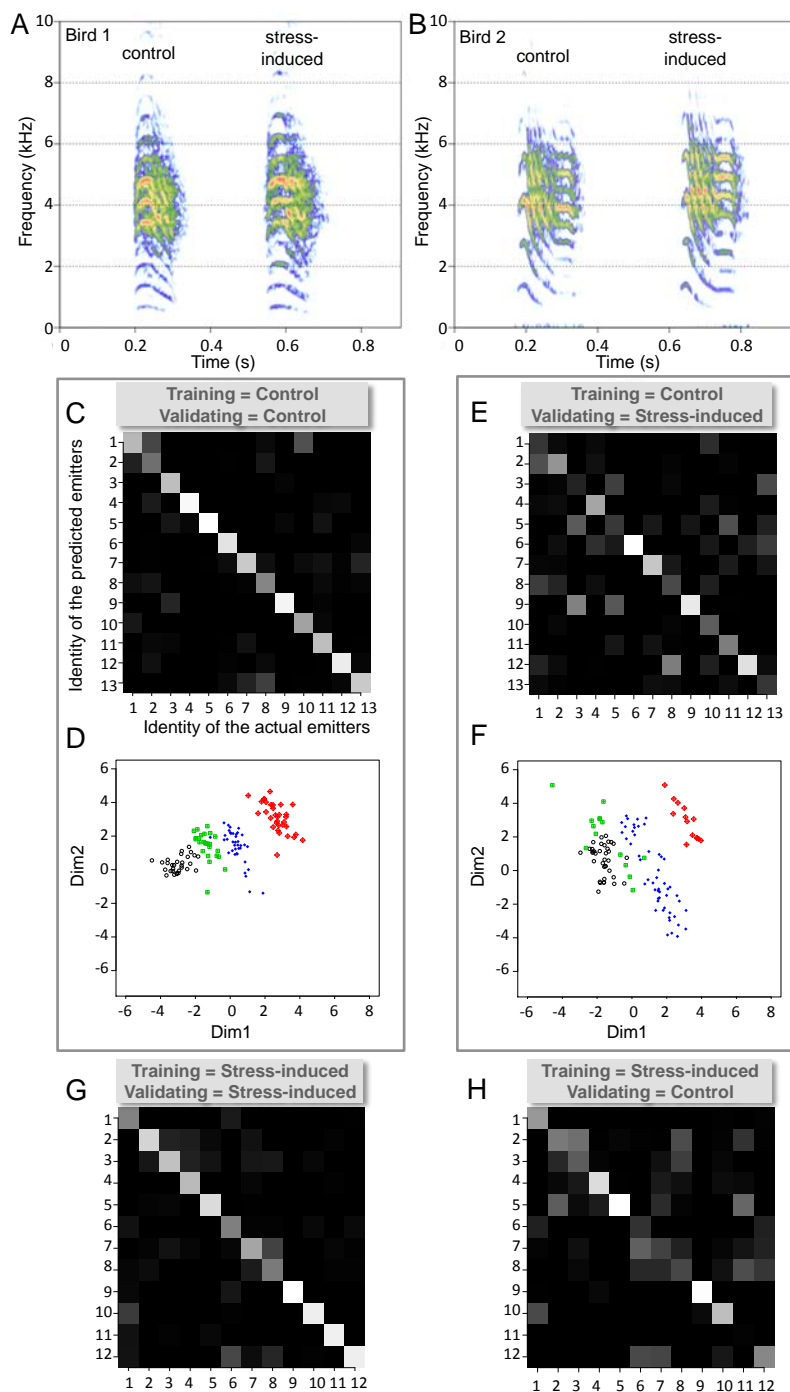
(A) Probability of vocal response to one stimulation. During each one-hour playback test, a female ( $n = 19$ ) was tested with six stimulations of 5s-playback, allowing a robust estimation of the probability of vocal response to a stimulus in each condition. During the playback periods (light bars), the highest probability of response was reached for the MATE CONTROL condition (all significant differences at  $P \leq 0.001$ , except  $P = 0.005$  MATE CONTROL vs MATE STRESS). After the playback periods (dark bars), the probability of response to MATE CONTROL was still the highest (all  $P \leq 0.008$ ) but did not differ from MATE STRESS anymore. Significance is based on multiple comparisons of means using Tukey contrasts for within subject design (see Supplemental Information for full description of statistical analyses).

(B) Index of behavioral response (first component of the PCA computed on four behavioral parameters, see text). Blue lines and boxes are mean  $\pm$  s.e.m, red lines are medians and points are individual data ( $n = 19$  females, six stimulations per test). Different letters indicate a significant difference between pairs at the  $P < 0.05$  level. MATE STRESS evoked intermediate responses between MATE CONTROL and other stimuli (all significant differences at  $P \leq 0.001$ , except  $P = 0.008$  MATE CONTROL vs UNFAMILIAR CONTROL,  $P = 0.04$  FAMILIAR CONTROL vs MATE STRESS). Significance is based on multiple comparisons of means using pairwise t-tests with Bonferroni correction (see Supplemental Information for full description of statistical analyses).

To further examine the particular effect of mate's stress-induced calls on females, we also investigated the behavioral responses to the playbacks. During the playback period of any stimulus, the probability of vocal activity was higher than during silence (Figure 2A). Thus, call playbacks were salient to females, but among them, MATE CONTROL had a significantly higher probability of response than any other stimulus. During the one-minute post-playback period, MATE CONTROL and MATE STRESS were the only stimuli to show vocal responses above basal levels (Figure 2A). The intermediate response level to MATE STRESS between MATE CONTROL and other stimuli was confirmed using an index of behavioral response obtained from a Principal Component Analysis (PCA). PCA is commonly used in behavioral analysis to eliminate redundancy caused by intercorrelation between variables and avoid a priori definition of meaningful parameters, which is particularly useful in playback experiments because subjects can display different combinations of behaviors to express the same functional response [18]. Here, the PCA was computed on four raw behavioral parameters affected by the playback (Extended Data Table 1): the number of calls and the number of vigilance behaviors (head-turns), both during playback periods and post-playback periods. The first principal component, which positively correlates with all four behavioral counts and explains 57% of the variance (Table S2), shows intermediate response levels to MATE STRESS between MATE CONTROL and other call stimuli (Figure 2B). The second principal component, which explains 23% of the variance (Table S2), discriminates all stimuli from SILENCE ( $P \leq 0.001$ ) except MATE STRESS ( $P = 0.11$ ). Taken together, behavioral results show that mate's stress-induced calls are not only less stimulating than mate's control calls but also inhibitory of some aspects of females' behavior, a response comparable to the common antipredator freezing and vigilance in birds [19].

Thus females show both a physiological stress response and behavioral changes after hearing the stress-induced calls of their mate compared to control calls. These effects differ from the response to the playback of stress-induced calls from unfamiliar individuals. These findings provide new evidence for the existence of emotional contagion between mates through acoustic cues. These results also suggest that the stressed response is not simply due to acoustical features that are characteristic of all stress-induced calls and that females are able to recognize the identity of the caller irrespective of his stress level. We further tested these conclusions in the analyses and experiments described below.

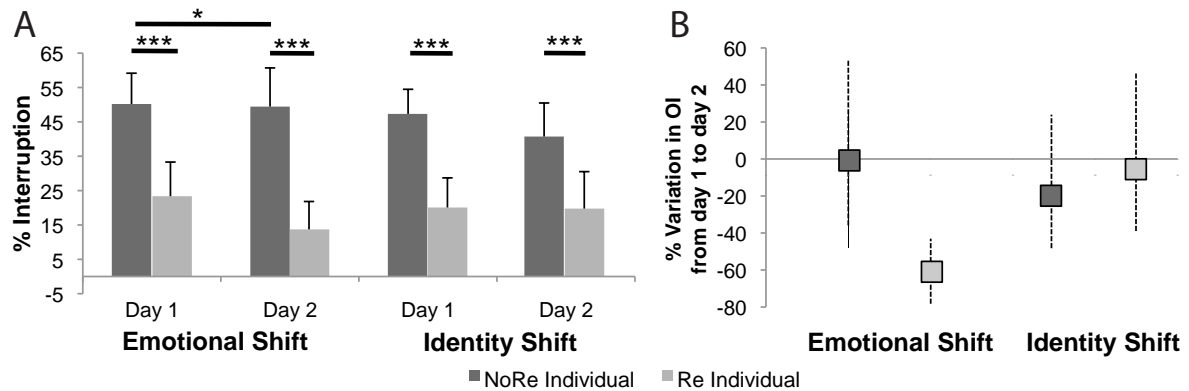
To further rule out the possibility that female birds react to acoustical features in the stress-induced calls that would be universally indicative of stress in vocalizations, such as increase energy in higher frequency bands, we tested whether any of 11 acoustic features of both control and stressed-induced male calls used as stimuli correlated with



**Figure 3. Analysis of the effect of stress on the individual signature of calls.**

(A) and (B) Spectrograms of the calls of two male birds in the two experimental conditions (control and stress). Note the clear individual signatures in both conditions. Note also that in the stress-induced call relative to the control call, the energy in the frequency band around 3 kHz is attenuated, whereas the energy in the frequency band between 4 and 5 kHz is enhanced. These variations are consistent with a shift towards higher frequencies in the spectral envelope of the stress-induced calls. Small differences in pitch can also be observed: in these examples more so for Bird 2 than for Bird 1. Spectrograms are calculated using the Seewave package implemented in R software (overlap = 90%, window length = 16 ms, color scale = 40 dB).

(C) to (F) Confusion matrices obtained from discriminant function analyses (DFA). Using a grey scale, cell (i, j) shows the conditional probability of guessing that the test calls came from individual j when in fact it was emitted by i. Results from four different DFAs are shown. (C) Classical DFA on control calls (classification success: 80.4%). (D) Plot of calls from four different birds (four colors) as a function of the first two discriminant functions obtained from the DFA (Dim1, Dim2). (E) DFA using control calls as a training set and stress-induced calls of the same individuals as a validating set (success: 45%). (F) Plot of stress-induced calls from the same four birds as in (D) as a function of the same Dim1 and Dim2. The acoustic signature of a given individual changes somewhat between control condition (D) and stress condition (F). (G) DFA on stress-induced calls (success rate: 80.1%). Note that success rates in (C) and (G) are similar. (H) DFA using stress-induced calls as a training set and control calls of the same individuals as a validating set (success: 50.6%).



**Figure 4. Recognition of male individuals by females based on control and stress-induced calls.**

Interruption behavior of females depending on the type of emitter (Re or NoRe) and the type of protocol (Identity Shift, i.e. tested with different pairs of individuals on day 1 and day 2,  $n = 3$ ; or Emotional Shift, i.e. tested with the same pair of individuals using stress-induced calls on day 2,  $n = 4$ ).

(A) Percentage of call sequences interrupted by females (mean  $\pm$  s.e.m),

(B) Percentage of change in the odds of Interrupting (OI) between day 1 and day 2 (value [5%-95%] confidence interval), depending on the type of emitter (Re or NoRe) and the type of protocol (Identity Shift, i.e. tested with different pairs of individuals on day 1 and day 2,  $n = 3$ ; or Emotional Shift, i.e. tested with same pair of individuals using stress-induced calls on day 2,  $n = 4$ ).

Females interrupted more the NoRe Individual than the Re Individual irrespective of the type of call used ( $\chi^2 = 159.4$ ,  $df = 1$ ,  $P < 0.001$ ), thus control and stress-induced calls were both individualized by females. Females undergoing the Emotional Shift protocol improved their performance on day2: they interrupted less the Re Individual (decrease of 61% in the OI) and kept interrupting the NoRe Individual at the same high level ( $\chi^2 = 6.32$ ,  $df = 1$ ,  $P = 0.012$ ). Thus, females tested with stress-induced call on day2 benefited from the learning that occurred on day 1 with the control calls from the same individuals.

the plasma corticosterone concentrations measured in females after playbacks. None of the bi-variate correlations were significantly different from zero ( $|r_{\min}| = 0.03$ ,  $|r_{\max}| = 0.16$ ), neither was the correlation with the optimal linear combination of these parameters obtained from a discriminant function analysis (DFA) estimated to separate stress-induced from control calls (see Supplemental Experimental Procedures). Thus, call structural variation does not act as a simple graded signal in which the calls of any stressed males provoke an aroused state in females.

To further explore the possibility that our results could be due to a failure of the mate recognition system, we investigated two different questions: How are the individual signatures present in male calls affected by stress? And, do females categorize control and stress-induced calls as belonging to the same individual?

We first used acoustical and statistical analyses of the calls, to investigate whether a potential deterioration of the individual signature in the stress-induced calls could lead to a failure of the mate recognition system (Figure 3A and 3B). Control calls and stress-induced calls are equally individualized: classical DFA based on 11 acoustic features identified significant acoustic differences between individuals in both sets separately (Figure 3C and 3G, success rates versus chance: control calls: 80.2% versus 7.7%; stress-induced calls: 79.8% versus 8.3%). Nevertheless, the individual signature shows a shift between conditions (Figure 3D and 3F): when a DFA was performed using control calls as training set and stress-induced calls of the same individuals as validating set, the success rate fell to 43.6% (Figure 3E). The same is true in the reverse analysis (50.8%, Figure 3H). The signature shift is confirmed by a DFA using control calls as training set and pooling control calls and stress-induced calls of the same individuals as validating set, showing intermediate success rate (68.4%). Thus, control and stress-induced calls both show clear individual signatures, but the signature of a given individual changes slightly between the two conditions.

Considering this signature shift, we tested whether stress-induced calls and control calls from the same individual would be categorized as calls from the same bird using a conditioning paradigm. In this paradigm, unpaired females discriminate between the calls of two individuals by interrupting the non-rewarded (NoRe) Individual and not interrupting the rewarded (Re) Individual (see Supplemental Experimental Procedures). Irrespective of the type of calls used (control or stress-induced), females interrupted more the NoRe than the Re Individual (Figure 4A, see also Figure S1). Thus, both types of calls could be individualized by females. We then examined whether females would benefit from having learned the identity cues on control calls when tested with stressed-induced calls of the same individuals. A first group of females was trained and tested on a first day using the control calls of one pair of individuals, and trained and tested on the following day with the same pair of individuals but using stress-induced calls (Emotional Shift protocol). The performance of these females was compared to another

group of females who were trained and tested on the second day with another pair of individuals using control calls (Identity Shift protocol). Our results supported the prediction that females undergoing the Emotional Shift would benefit from the training received in the first day and do better than females under the Identity Shift: on the second day, females under the Emotional Shift maintained a high rate of successes ( $49.4 \pm 22.5\%$  interruptions of NoRe) and decreased the percentage of errors compared to the first day ( $13.7 \pm 8.1\%$  interruptions of Re on day 2 versus  $23.4 \pm 9.9\%$  on day 1) (Figure 4A, see also Figure S1 and S2). This increase in performances from day 1 to day 2 translated in a decrease of 61% of the odds of interrupting the Re Individual (ratio of the probabilities of interrupting and not interrupting) (Figure 4B). On the contrary, females under the Identity Shift decreased the percentage of successes between day 1 and day 2 ( $47.32 \pm 7.2\%$  versus  $40.7 \pm 9.8\%$ ) and maintained the same level of errors (around 20%) (Figure 4A, see also Figure S1 and S2). This was measured by a decrease of 19.9% in the odds of interrupting the NoRe Individual (Figure 4B). Thus, although the acoustic signature of a given individual is changed between control and stress conditions, females can still categorize calls based on the identity of the bird.

As physiological stress was controlled both in the stimulus bird and in the subject, and physiological resonance resulted from acoustic cues only, our results demonstrate emotional contagion between affiliated birds such as mates. As often underlined in human studies of empathy [9], we can not rule out the possibility that this represents a self-oriented response to the partner's affect, with a potential motivation of reducing one's own distress. Male and female partners in zebra finches are nearly always associated and certainly undergo the same threats and risks. Females could learn that a stress for their mate is also, with high probability, a threat for themselves. The ability to share affective experience could correlate with the strength of the social bond, as shown in hens [21] and geese [22]. Since zebra finches form life long pair bonds, the age of the pair could predict the level of emotional contagion. Zebra finches could also be good candidates for the study of vocal buffering, in which an individual shows a better recovery from stressful experiences thanks to the vocalizations of a social partner [23,24].

The zebra finch is a widely used model system in biological sciences [25] and its significance is likely to further increase since the publication of its genome sequence [26]. The demonstration of emotional contagion in this bird offers new opportunities to study the neuronal substrates underlying the ability to detect and react to the emotional states of social partners.

## **Experimental Procedures**

All experiments were performed on adult domestic zebra finches (*Taeniopygia guttata*) bred in our colonies (ENES lab, University of Saint-Etienne; Theunissen Lab, University



of California, Berkeley) and were approved by the Animal Care and Use Committees of the respective institutions.

Male distance calls were recorded in control or stress-induced conditions and used as playback stimuli on their female mates. Male control calls were recorded using a separation protocol in which mates were visually but not acoustically isolated. Male stress-induced calls were obtained with an oral administration of corticosterone before separation as previously described [15].

Each female underwent four playback sessions in random order, separated at least by a week. The female was stimulated by only one stimulus type per session. During a first set of experiments, females ( $n = 10$ ) were stimulated by: MATE CONTROL, MATE STRESS, FAMILIAR CONTROL, or remained in SILENCE. In a second set of experiments, each female ( $n = 9$ ) underwent the playbacks of: MATE CONTROL, UNFAMILIAR STRESS, UNFAMILIAR CONTROL, or remained in SILENCE. In either playback sessions, females were stimulated every ten minutes for one hour (i.e. six times). Each stimulation consisted in a playback of ten distance calls from the same male individual recorded in one of the above conditions (mean rate: 2 calls per second). At the end of each playback session, blood samples ( $< 150 \mu\text{l}$ ) were obtained by puncturing the alar vein of the female within three minutes. Behavioral responses of the females to the playbacks were analyzed on video recordings by experts blind to the condition.

The categorization by females of control and stress-induced calls as belonging to the same individual was tested using an operant design, where the calls from one individual were infrequent (20%) and rewarded by seeds at the end of a 6 s playback of calls (Re Individual) while the other was frequent (80%) but not rewarded (NoRe Individual). After triggering the playback of a stimulus, the female could either wait to the end of the playback or interrupt it by pecking again. This interruption also immediately triggered the next playback. Because the females could only peck for a given period, they were encouraged to specifically interrupt the NoRe Individual to obtain more Re Individual stimuli and increase their access to seeds. Discrimination was quantified by measuring the relative differences in interruptions of the NoRe and Re Individual.

See Supplemental Information for details.

### **Supplemental Information**

Supplemental information includes four figures, four tables and detailed Supplemental Experimental Procedures.

### **Acknowledgments**

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## **Supplemental Experimental Procedures**

### **Subjects and housing conditions**

Subjects were 16 unpaired males, seven unpaired females and 27 pairs of adult domestic zebra finches (*Taeniopygia guttata*) bred in our colonies (ENES lab, University of Saint-Etienne; Theunissen Lab, University of California, Berkeley). Pairs were obtained as follows: males and females freely formed pair bonds in an aviary (dimensions: 200 x 200 x 300 cm) at least two months prior to the experiment 1. Identified pairs were then caught and kept in individual cages (dimensions: 24 x 29 x 39 cm) in the same room at least one month prior to the experiment. Unpaired females were kept in unisex cages (University of California, Berkeley, experiment 3) while unpaired males were kept alone in individual cages (University of Saint-Etienne, experiment 2).

Birds were maintained at a constant temperature of 22–24°C and with a 14:10 light–dark cycle or a 12:12 light-dark cycle (unpaired females only). Food and water were provided *ad libitum*, with a supplement of salad once a week. However, for the duration of the shaping and testing days in the operant chamber (experiment 3) the seven unpaired females were fasted: they were given daily only 1.5g of mixed seeds for finches per individual at the end of each day.

Experiments 1 and 2 were performed under the authorization no. 42-218-090-38SV09 (ENES Lab, Direction Départementale des Services Vétérinaires de la Loire) according to the guidelines laid down by the French Ministère de l'Agriculture (no. 87-848) and the E.U. Council Directive for the Care and Use of Laboratory Animals of November 24th, 1986 (86/609/EEC). The operant test (experiment 3) was approved by the Animal Care and Use Committee at the University of California at Berkeley.

### **Blood sample collection and hormone assay**

All blood samples (< 150 µl) were obtained by puncturing the alar vein with a 25-gauge needle within three minutes so as to measure baseline corticosterone concentration and not corticosterone release due to handling [27]. Blood was collected in heparinized tips, then plasma was separated under centrifugation (3800 *g*, 15 min) and stored at –20°C for subsequent hormone assay. No bird was bled more than once per week to ensure that they had sufficiently replenished their blood volume between bleeds and hypothalamic–pituitary–adrenal axis function had recovered from previous sampling [27]. Because circulating concentration of endogenous corticosterone shows circadian variations in many bird species with a peak just before the active period and a rapid decrease during the first hours of activity, all experiments were performed between 08:30 and 12:00, i.e. between 1.5 and 5 hours after daylight [28]. More precisely, blood samples were taken between 08:30 and 12:00 for the 8 pairs used to

determine the effect of isolation on circulating concentrations of corticosterone, and between 10:00 and 11:00 for the group of 19 females used for the playback experiment.

As previously described [15], hormone assays were performed using the ELISA method (Kit Corticosterone EIA, Cayman, #5000561) according to the manufacturer's recommendations. All samples were run in duplicate in four different assays. The detection limit was 0.2 ng/mL, intra-assay coefficient of variation was 0.058, and inter-assay coefficient of variation was 0.146.

### **Experiment 1: study of the perception by females of the emotional state of their mate using acoustic cues.**

#### Separation protocol

We developed a protocol to separate the partners of a pair without handling, avoiding unnecessary stress. The day before the experiment, the partners of one pair were moved to the experimental room and placed together in a home-made separation cage (dimensions: 28.5 cm x 23 cm x 35 cm), with food and water *ad libitum*. On the morning of the experiment, the partners were physically separated using two wood partitions placed in the middle of the experimental cage, which allowed separating the cage in two independent parts. Thus, the birds were separated without handling as allowed by the small size of the cage. One of the newly independent parts was then moved in a different room, so the partners were visually and acoustically isolated from each other. The partners of each pair used in this study were separated four times using this device.

#### Determination of the effect of isolation from the partner on circulating concentrations of corticosterone over time.

The effect of isolation from the partner on circulating concentrations of corticosterone was assessed on eight pairs (not used in the rest of the experiment) using the separation protocol detailed above. At the end of the separation period, the bird kept in the experimental room was caught for blood sampling before reunion with its partner. The separation period was defined according to four conditions, randomly assigned to each pair (each pair went through the four conditions):

- 1H: Separation during 1h
- 2H: Separation during 2h
- BASAL: no separation (baseline corticosterone concentration)
- CONTROL: no separation of the two parts of the cage, but wood partitions were placed so as to control for the effect of the manipulation on the stress of the birds.

Because corticosterone levels across birds did not follow a normal distribution, we used non-parametric statistics to test for significant differences. Differences across

groups were first examined using a Friedman test, the non-parametric equivalent of the repeated measures ANOVA. When the Friedman test showed significant differences across groups, we performed a post-hoc analysis for significant pairwise comparisons using Wilcoxon Signed Rank test. These statistical analyses were implemented in R software R (Development Core Team, [www.r-project.org](http://www.r-project.org), 2007). For displaying purposes, hormone data were normalized by dividing by the mean level obtained for each individual and log-transformed. Because we didn't find any effect of the sex of the birds, data from males and females were pooled.

Results show that plasma concentrations of corticosterone are the highest after 1 hour of separation (Figure S3). To standardize corticosterone concentrations of the females when starting the playback and avoid ascending or descending phases, we decided to perform the playback experiment after 1 hour of separation.

#### Recording of acoustic stimuli

19 pairs were used. Male calls were first recorded in varied contexts and then used as playback stimuli on females. Using the separation protocol detailed above, the female of the pair was moved to another room, and the male was then fed with either:

- seeds containing exogenous corticosterone (12.5 µg i.e. 50 µL of a 0.25 mg/mL solution; Sigma Aldrich ref: 27840) so as to provoke a physiological stress, and then record stress-induced distance calls (MATE STRESS, UNFAMILIAR STRESS) (protocol previously described<sup>15</sup>).

- seeds alone (control) so as to record regular distances calls (MATE CONTROL, FAMILIAR CONTROL, UNFAMILIAR CONTROL).

After ten minutes of separation, doors from the two rooms were opened, allowing the partners to hear but not see each other. This context generally provokes interactive calling between partners. Distance calls from the male calling its female were thus recorded during this visual separation phase (Microphone: Sennheiser MD42, recorder: Marantz PMD 670, sampling rate: 44.1 kHz) for at least 45 minutes.

#### Preparation of acoustic stimuli

Male distance calls were isolated and analyzed as previously described [15]. The mean frequency of calls was obtained from the power spectrum of the signal (Fast-Fourier-Transform). Two sets of calls were selected for each male:

- stress-induced calls (MATE STRESS, UNFAMILIAR STRESS): the ten higher-pitched calls emitted after exogenous corticosterone administration (Mean = 3970 ± 41 Hz).

- control calls (MATE CONTROL, FAMILIAR CONTROL, UNFAMILIAR CONTROL): the ten lower-pitched calls emitted in control condition (Mean = 3211 ± 49 Hz).

As previously demonstrated [15], stress-induced distance calls are higher-pitched than control distance calls. Extreme samples of this variation were chosen here to maximize the difference between playback conditions.

#### Analysis of acoustic stimuli for correlation with female playback-induced corticosterone concentrations

The distance calls selected as stimuli ( $n = 380$ ) were analyzed using the Seewave package ([rug.mnhn.fr/seewave/](http://rug.mnhn.fr/seewave/)) implemented in R software. Each call was first bandpass filtered (0.25-10 kHz, encompassing the range of maximum sensitivity of hearing in zebra finches). Then eleven acoustic parameters were measured for each call:

- the duration (in seconds) using 'timer' function, which computes the energy from the signal envelope and uses the peak maximum as an energy reference to define the start and the end of the call (threshold: 6%).

- the call dominant frequency (in Hertz) is the average over the duration of the call of the frequencies of highest amplitude (obtained via the 'dfreq' function). The frequency of highest amplitude is first assessed on time bins using a window length of 512 samples (11.6 ms) and the series of values obtained over the duration of the call is then averaged.

- the call fundamental frequency (in Hertz) is the average over the duration of the call of the fundamental frequency values (these are tracks of the fundamental frequency through a short-term cepstral transform obtained via the 'fund' function).

- eight parameters of the call spectrum (using 'specprop' function): the mean, the median, the mode, the first (25%) and third (75%) quartiles, the skewness, the kurtosis and the spectral flatness.

The correlation between each one of these acoustic parameters in male stimuli (average value for each male in a given condition) and the playback-induced corticosterone concentrations in females was assessed by calculating the Pearson's  $r$  correlation coefficient. Before analysis, corticosterone values were normalized by dividing by individual means and log transformed. To account for the multidimensional nature of the stimuli, we also computed the correlation with the optimal linear combination of the same acoustical parameters obtained from a discriminant function analysis (DFA) estimated to separate stressed from non-stressed calls (lda function, MASS package in R software, see details on this method in experiment 2).

#### Playback experiment

In a first set of playback sessions, each female ( $n = 10$ ) underwent four playback tests in random order, separated at least by a week. The female was stimulated by only one stimulus type per session:

- MATE CONTROL: the ten lower pitched calls of her mate.

- MATE STRESS: the ten higher pitched calls of her mate.
- FAMILIAR CONTROL: the ten lower pitched calls of a familiar male (the male of another pair, housed in the same colony room).
- SILENCE: no playback.

In a second set of playback sessions, each female ( $n = 9$ ) underwent also four playback tests in random order, separated at least by a week. The female was stimulated by only one stimulus type per session:

- MATE CONTROL: the ten lower pitched calls of her mate.
- UNFAMILIAR CONTROL: the ten lower pitched calls of an unfamiliar male (a male never housed in the same colony room as the subject).
- UNFAMILIAR STRESS: the ten higher pitched calls of another unfamiliar male (to avoid potential familiarity appearing between two successive experiments).
- SILENCE: no playback.

Using the separation protocol detailed above, the male of the pair was moved to another room. The female, alone in the experimental room for 5 minutes, was then stimulated every ten minutes during one hour (i.e. six times). Each stimulation was a playback of ten distance calls from the same male individual recorded in one of the above conditions (mean rate: 2 calls per second). Thus the total duration of the playback during the one-hour test was  $6 \times 5s$ , which represented 6 sets of 10 calls. Calls were played by a computer using Goldwave software (GoldWave Inc. St. John's, NL Canada) through an amplifier (Yamaha AX 396) connected to a loudspeaker (Audiopro Bravo Allroom sat E7140044).

At the end of the experiment (1 hour), the female was caught for blood sampling. Good quality blood sampling failed in two females of the first set of experiments, so the sample size of hormone data is  $n = 17$  ( $n = 8$  and  $n = 9$ ). Hormone data were analyzed as described above. Within subjects, non-parametric tests were applied to test for differences across all conditions (Friedman Test) as well as pair-wise differences (Wilcoxon Signed Rank test). On the figures, hormone data are normalized by dividing by the mean level obtained for each individual and log-transformed.

#### Analysis of the behavioral response

During the whole experiment, the behavior of the female was monitored using a webcam (Hercules® Webcam Deluxe) and a microphone (Sennheiser MD42) located 50 cm above the subject and connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz). The behaviors of the female were scored on video recordings of playback sessions by an observer blind to experimental conditions (TC). Videos were scored in random order regarding conditions and identity of the birds. Using Etholog software (created by Ottoni), four categories of behaviors were quantified:



- Vocal activity: two different types of vocalizations were emitted by the female during the experiment: distance calls normally used by zebra finches when they lose visual contact and short calls normally used during close interactions. These calls were pooled in one parameter of calling activity.

- Locomotor activity: two different activities were quantified: the jumps on the floor or on the perch and the flights or jumps with flapping wings. These parameters were not influenced by the treatments, so data are not presented.

- Preening activity: one event was scored every time the female cleaned her feathers. This parameter was not influenced by the treatments, so data are not presented.

- Vigilance: the number of head-turns was scored.

Each video was divided into three periods of time: a five-second playback period (repeated six times per video), following by a one-minute post-playback period (repeated six times), following by a 8min55s inter-playback period (repeated only five times). Each behavior was quantified both during playback and during post-playback periods. This yielded a total of 8 behavioral parameters.

Raw behavioral data were analyzed using a generalized linear mixed model (lmer) from the lme4 package implemented in R software. The effect of the playback condition (6 levels: SILENCE, MATE CONTROL, MATE STRESS, FAMILIAR CONTROL, UNFAMILIAR CONTROL, UNFAMILIAR STRESS) on the behavioral response of the female was assessed with the female identity (19 levels) and the playback set (2 levels) as random factors. As all raw behavioral parameters are counts in given time periods, the model was fitted using a poisson distribution and the log link function. Results of this analysis are presented in Extended Data Table 1 for the four behavioral parameters affected by the experimental conditions (vocal activity and vigilance behavior, both during and after the playback periods).

Vocal activity was also analyzed as a binary variable (0: no response, 1: response), disregarding the number of calls used in response. This allowed to quantify the probability of response to a stimulus set in each playback condition (during each one-hour test, a female was tested with six sets of 5s-playback). The model was then fitted using a binomial distribution and the logit link function. Multiple comparisons of means were performed on the models using the glht function from the mulcomp package using Tukey contrasts.

The four behavioral parameters affected by the experimental conditions (vocal activity and vigilance behavior, both during and after the playback periods) were used to compute a principal component analysis (PCA, function dudi.pca from the ade4 package in R software, computed on z-scored parameters). The PCA retained two components with eigen-values larger than one (Kaiser criterion), PC1 and PC2, explaining respectively 57% and 23% of the variance. These two principal components

were used in statistical analysis as index of response to the playback stimuli. Extended Data Table 2 shows the percentage of variance that is explained by the two principal components of the PCA, as well as the variable loadings on each component.

The behavioral index derived from the PCA, PC1 and PC2, were analyzed using a linear mixed model (lme) from the nlme package implemented in R software. The effect of the playback condition (SILENCE, MATE CONTROL, MATE STRESS, FAMILIAR CONTROL, UNFAMILIAR CONTROL, UNFAMILIAR STRESS) was assessed with the female identity as random factor. Results of this analysis are presented in Extended Data Table 3. Multiple comparisons of means were performed on the models using pairwise t-tests with Bonferroni correction.

## **Experiment 2: analysis of the variation with stress of individual signatures in calls**

### Acoustic analysis of male distance calls

The distance calls of 16 unpaired males naïve to the experimental conditions were recorded under control and stress conditions and analyzed as described in experiment 1.

### Statistical analysis of the variation with stress of individual signatures in male calls

The differences between calls from different individuals were analyzed using linear discriminant analysis or discriminant function analysis (DFA) implemented in R software (lda function, MASS package). Before multivariate analysis, the raw values of the twelve acoustical parameters were centered and normalized (i.e. transformed into z-scores) to insure correct weighting since the acoustical parameters had different units. The DFA is composed of two steps: in the first step a set of discriminant functions is obtained from a subset of the data (the training data set, i.e. different exemplars of the calls of multiple individuals); in the second step these functions are used to test classification on a validation set (i.e. new exemplars of calls of the same individuals). This cross-validation step gives a measure of the effect size (the percent correct of classification of the calls to the correct identity) and of the statistical significance by comparing the mean percent correct to chance. The mean percent correct is obtained by analyzing the percent correct assignment of 100 random selections of the training and validating data sets in the original data set.

To compare individual signatures in male calls between control and stress conditions, calls recorded from the same individuals in both conditions were used in several DFA. First, classical DFA were performed separately on either control calls or stress-induced calls. If stress impairs individual signatures, the success rates of these DFA should differ. Then, DFA were performed using one condition as training set and

the other as validating set (control calls as training set and stress calls of the same individuals as validating set, or the reverse situation). If stress modifies individual signatures in calls, the success rates of these DFA should be lower than the success of the above classical DFA. Finally, we performed a DFA using control calls as training set and pooling the calls of the same individuals recorded in both conditions as validating set. Again, if stress impairs individual signatures, the success rate of this DFA should be lower.

These five DFA were performed on slightly different subsets of the 16 males originally recorded, because a minimum of 10 calls per individual was required in the training set to ensure proper determination of the discriminant functions. Thus, the sample sizes were as follows:

- When the training set consisted of control calls: 642 male distance calls from 13 males ( $29.5 \pm 15.4$  calls per individual (min = 13; max = 75) in control condition and  $19.9 \pm 16.3$  calls per individual (min = 3; max = 52) in stress condition).

- When the training set consisted of stress-induced calls: 566 male distance calls from 12 males ( $22.2 \pm 11.6$  calls per individual (min = 5; max = 38) in control condition and  $25 \pm 14.3$  calls per individual (min = 11; max = 52) in stress condition).

Extended Data Table 4 shows the percentage of variance between individuals in control calls (a) or stress-induced calls (b) that are explained by the first four functions of the corresponding DFA, as well as the variable loadings on each function for the four most important factors.

### **Experiment 3: study of the recognition of male emitters by females based on control and stress-induced calls.**

In this third experiment, unpaired females had to peck a pad to induce the playback of calls from two previously unknown males. Calls from one of the individuals were infrequent (20%) and rewarded by seeds at the end of the sequences of calls (Re Individual) while calls from the second individual were probable (80%) but not rewarded (NoRe Individual). After triggering the playback of a stimulus, the female could either wait until the end of the playback (6 s) or interrupt it by pecking again to trigger another stimulus. Because the females could only peck for a given period, they were encouraged to specifically interrupt the NoRe Individual to obtain more Re Individual stimuli and, in this manner, increase their access to seeds during sessions. Discrimination was assessed by comparing the number of interruptions for the NoRe Individual to those obtained for the Re Individual. In contrast to other conditioning paradigms used to test discrimination capacities of birds [29,30], this paradigm avoids using punishment for incorrect responses. As a result, its rules were quickly acquired by females and learning could be achieved, with only a few hundreds of pecks within a total of 3 to 4 hours of training in the testing chamber (Figure S4). To motivate the subjects,

females were fasted at least 15 hours prior to the beginning of the experiment and were given only 1.5g of finch seeds at the end of each testing day.

#### Chamber apparatus

Fasting females were tested in a computer-assisted extra tall modular test chamber (Med Associates Inc., St Albans, VT 05478, USA; size: 30.5 cm x 24.1 cm x 29.2 cm) placed in a soundproof booth (Acoustic Systems). Acoustic stimuli were broadcasted by the computer using Matlab (MATLAB version 7.6. Natick, Massachusetts: The MathWorks Inc., R2008a.) connected to an amplifier (Technics, Matsushita Electronics, SA-EX140) and a loudspeaker (Bose Model 141) placed at 20 cm from the test chamber. The behavior of the bird was monitored both by the computer and by a webcam (Logitech) placed inside the soundproof booth.

#### Computer code and session procedure

A homemade code written in Matlab allowed the bird to run three sessions of 30 min each per day with a minimum inter-session delay of 90 min. The code detected the beginning of each session (when the bird pecked the key pad for the first time) and ended the session 30 min later. During each session, it monitored the pecking behavior of the bird by recording the status of the key pad every 0.1s and broadcasted a different 6 s sequence of calls each time the key pad was depressed by the bird. Interruption of stimuli was permitted: the bird could peck while the computer was playing a 6s stimulus and promptly get another stimulus before the end of the 6s. The probability to get a Re stimulus (rewarded by a 10s access to seeds) was set at 20%. Note that the reward was only accessible to the bird at the end of the Re stimulus and that interruption of the Re stimulus would cancel the access to the reward.

#### Acoustic stimuli

Each acoustic stimulus consisted of six band-pass filtered (0.25-12 kHz) distance calls of the same individual, randomly assigned within a 6s window. Before each trial, the computer was randomly constructing 80 Re stimuli and 320 NoRe stimuli using a call bank of 20 different calls per individual. Each stimulus was only played once during the session.

#### Shaping

Birds were shaped in two days. On the first day the female first had a period to acclimate to the cage and find the rewarding feeder. Once it had found the feeder and had three 10 s opportunities to eat, the female could run a long trial (1 to 3 hours) with the probability to get a Re stimulus set between 0.5 and 0.9 depending on the female motivation. During this first trial, the females learn two associations: 1) that pecking the

key pad triggers a vocalization, 2) that one of the two vocalizations is followed by a short access to the feeder. On the second day, three regular sessions of 30 min were conducted with a probability to get the Re stimulus of 0.2 (Figure S4). On this second day, the bird was expected to learn to interrupt the stimuli. A female was considered ready to run the experiment if it interrupted at least 20% of the NoRe stimuli and if the difference in percentage of interruption between NoRe and Re stimuli (NoRe – Re) was at least 20%. Stimuli used for shaping were two 6s sequences of songs recorded from 2 different males of our colony.

### Conditioning

Two protocols of conditioning were conducted: Identity Shift and Emotional Shift. In both protocols, females ( $n = 7$ ) run three sessions per day and were trained and tested on the first day with control distance calls of previously unknown males (four pairs of individuals were used for this experiment). On the second day, three females were re-trained and tested with control distance calls of new pairs of previously unknown males (Identity Shift protocol) while four females were tested with stress-induced distance calls emitted by the same males as the previous day (Emotional Shift protocol).

### Statistical analysis of the ability of female to individualize both control and stress-induced distance calls.

The effects of the type of stimulus (Re or NoRe) and of the type of distance calls (control or stress-induced) on the number of interruptions performed by females were first analyzed using logistic regression with the female identity nested in the day number (first, second) as random factors. Because stress-induced calls were only used on the second day of the experiment, we confirm the results of the previous regression by performing the same logistic regression using the data set restricted to values obtained for the second day and using only the female identity as a random factor. Results obtained were similar: the effect of the type of stimulus was highly significant in both regressions ( $P < 0.001$ ) and increases in odds of interrupting were of the same range (2.5 [1.9 – 3.2] for control calls and 7.1 [5.9 – 8.5] for stress-induced calls).

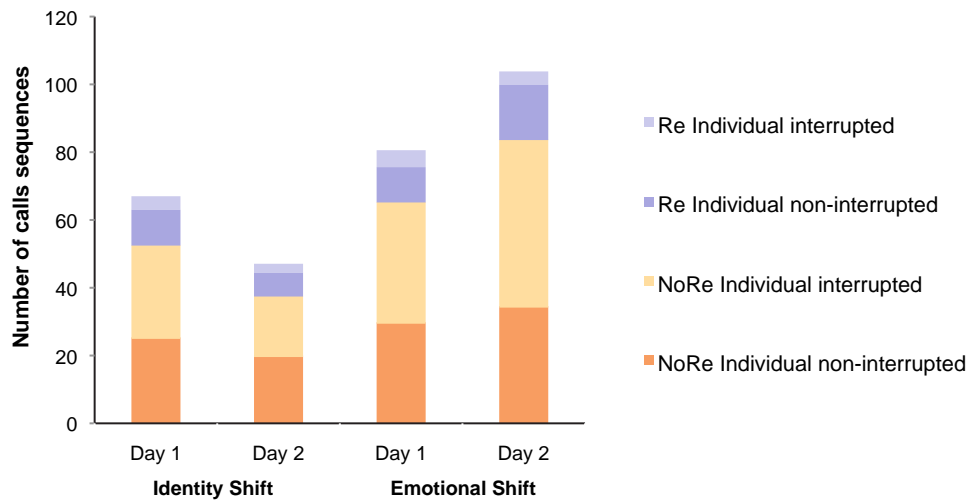
### Statistical analysis of the ability of females to categorize control and stress-induced calls from the same individual as calls from the same bird.

The effects of the type of stimulus (Re or NoRe), of the protocol (Identity Shift vs Emotional Shift) and of the day number (first, second) on the number of interruptions performed by females were analyzed using logistic regression with the female identity nested in the type of protocol and type of stimulus as random factors. Random factors

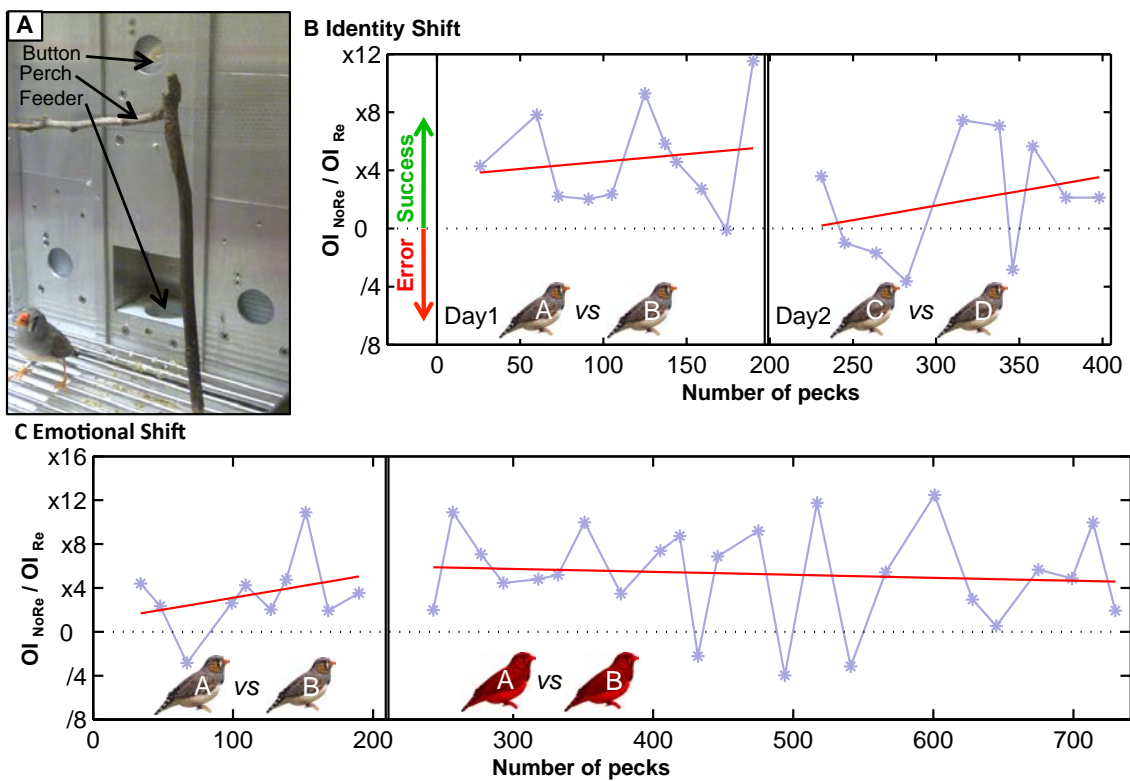
were chosen such as to take into account the different levels of motivation of females within each protocol and each day.

For all the regressions, effects were quantified by calculating the increase in odds of interrupting (the ratio of the probability of interrupting a stimulus and the probability of not interrupting). The statistical significance of each parameter was calculated using an anova on the logistic model. These statistical analyses were performed in R using the generalized linear mixed model (lmer) from the lme4 package and the anova (Anova) from the car package.

All values displayed are means $\pm$  s.e.m except variations in odds of interrupting which are given with their confidence intervals [5% - 95%]. All significant differences are reported for  $P\leq 0.05$ .



**Figure S1.** Number of sequences of calls that were interrupted or not interrupted by females depending on the type of individual (rewarded: Re, non-rewarded: NoRe) and the type of protocol (Identity Shift or Emotional Shift).



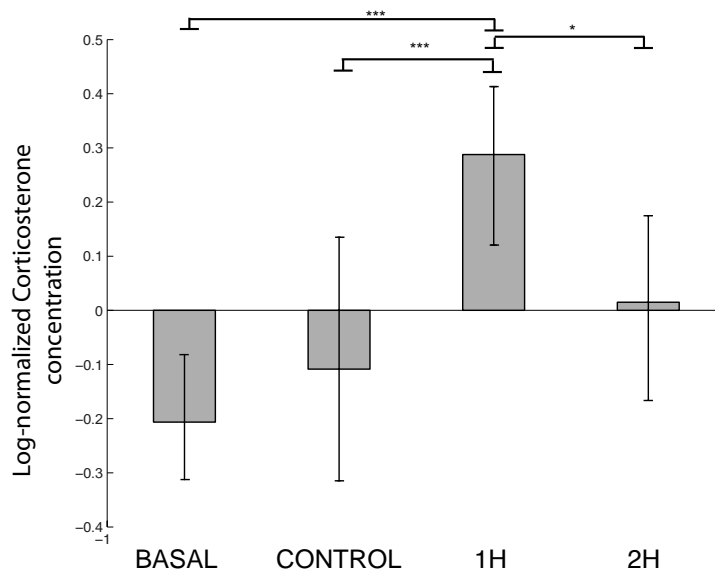
**Figure S2. Recognition of male emitters by females based on control and stress-induced calls.**

(A) Pecking test chamber.

(B) Performances of a subject under the Identity Shift protocol (i.e. tested with different pairs of individuals on day 1 and day 2).

(C) Performances of a subject under the Emotional Shift protocol (i.e. tested with the same pair of individuals using stress-induced calls on day 2).

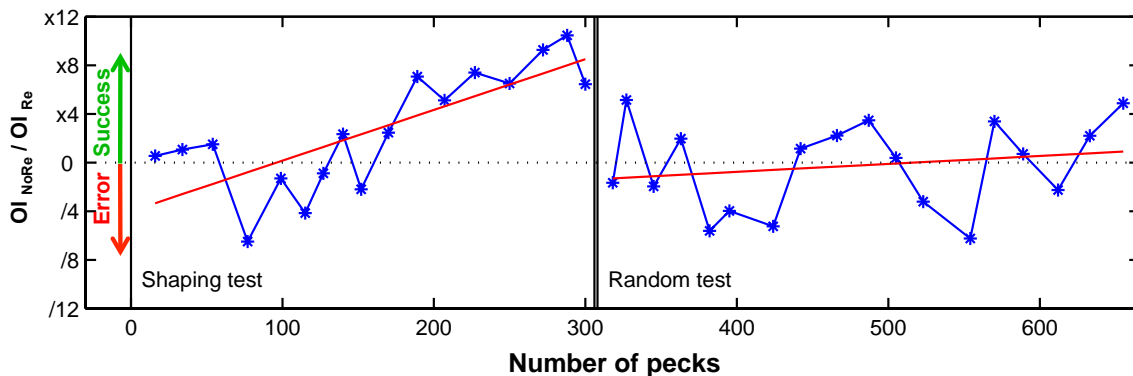
In (B-C), letters on birds indicate identity while their colors grey/red match the experimental condition control/stress. Performances are measured as an odds ratio: the ratio of the odds of interrupting the NoRe Individual ( $OI_{NoRe}$ ) and the odds of interrupting the Re Individual ( $OI_{Re}$ ).  $OI$  (the ratio of the odds for the Re stimuli and the odds for the NoRe stimuli) was obtained from responses to 4 Re stimuli and all the NoRe stimuli that occurred during the same time period (a varying number). This time window was chosen in order to get the minimum number of Re stimuli to estimate the  $OI$  and, in this manner, follow the time course of learning. To correct for the biases due to small numbers, the probabilities used in the calculation of the  $OI$  were estimated using the median unbiased estimate of the probabilities of interruptions for the Re and NoRe condition as proposed by Parzen et al. [20]. Red lines: linear regressions calculated on each daily data set.



**Figure S3. Effect of separation on the physiological stress of partners.**

For display purpose, corticosterone plasma concentrations are normalized by dividing by the individual means and log transformed. Original data ranged from 1.76 ng/mL to 29.63 ng/mL, with mean  $9.01 \pm 0.57$  ng/mL. Because there was no effect of the sex of the birds, data from males and females were pooled. Boxes are medians and error bars are interquartile-ranges (1<sup>st</sup> quartile-3<sup>rd</sup> quartile) for  $n = 8$  pairs i.e. 16 birds.

1H of separation significantly increased plasma corticosterone concentrations of the partners (Friedman  $\chi^2 = 13.15$ ,  $df = 3$ ,  $P = 0.0043$ , posthoc Wilcoxon signed rank test Control vs 1 hour  $n = 16$   $V = 9$ ,  $P = 0.001$ ). Plasma concentrations of corticosterone decreased after 2H of separation and returned to levels close to control levels (posthoc Wilcoxon signed rank test Control vs 2 hour  $n = 16$  and  $V = 50$ ,  $P = 0.37$ ). We therefore decided to perform the playback experiment during the hormonal peak, i.e. the first hour of separation.



**Figure S4. Performances of a subject under the Shaping protocol and the Random protocol in the pecking test chamber.**

The Shaping protocol is a learning phase where only two songs from two different individuals are used, one that matches the Re stimulus and the other that matches the NoRe stimulus. The Random protocol is a control test where the same vocalizations are used as the Re stimuli and the NoRe stimuli. Performances are measured as an odds ratio: the ratio of the odds of interrupting the NoRe Individual ( $OI_{NoRe}$ ) and the odds of interrupting the Re Individual ( $OI_{Re}$ ). OI were obtained from responses to 4 Re stimuli and all the NoRe stimuli that occurred during the same time period (a varying number). The probabilities used to calculate the OI were estimated using the median unbiased estimate of the probabilities of interruptions for the Re and NoRe conditions [20]. Red lines: linear regressions calculated on each daily data set.



# CHAPTER 3

## **Not similar but coordinated: Zebra finch mates show no similarity in response to a stressful event, but high coordination in post-stress vocal behaviours.**

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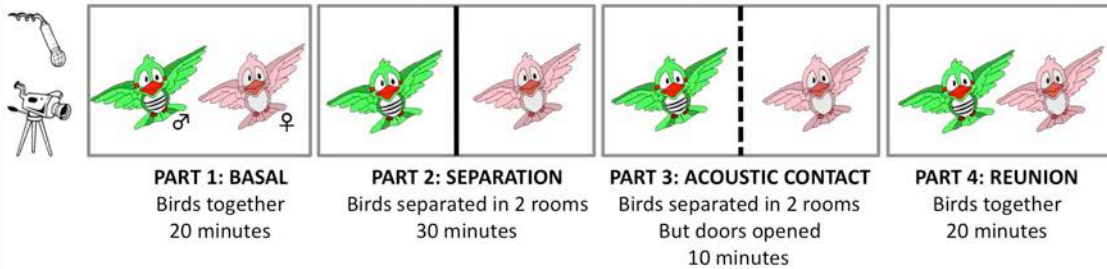
# SUMMARY CHAPTER 3

## Questions

Do mate show an assortative mating by vocal and physiological profile during a stressful event?

## Methods

### 1-Separation-reunion experiment



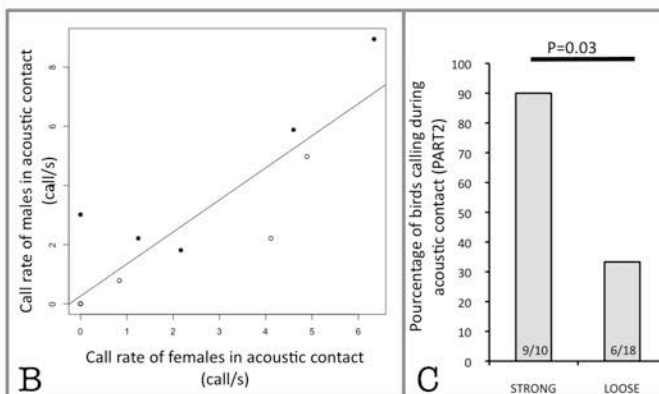
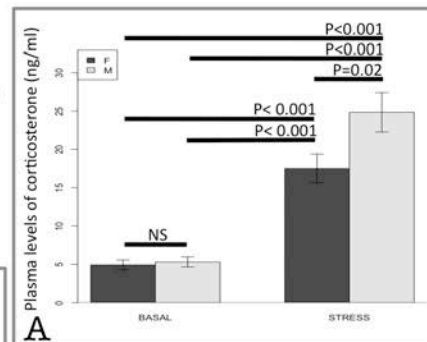
### 2-Blood sampling and corticosterone assay

### 3-Behaviour analysis

## Results

### 2-No assortative mating but Males more stressed than females

No correlation between the partners' levels of corticosterone  
Elevation of plasma levels of corticosterone in both sexes  
A- Males show higher levels of corticosterone after separation (A)



### 3-No assortative mating but vocal strategy depending on the pair history

Correlation of vocal behaviour between partners (B)  
Strong pairs and pairs that had a breeding experience interact more vocally ©  
coordinated vocal exchange with equal participation of both partners

## Conclusions

Our results couldn't show any physiological or vocal assortative mating. Instead, we discovered that partners adjust their behaviour while in acoustic contact, with a vocal activity that only depends on the pair history and couldn't be explained by plasma levels of corticosterone.

## **Abstract**

Coordination, an emerging property of interactions between mates, is primordial for the survival and reproductive success of the pair. Coordination is more likely to occur between compatible partners, who usually associate via assortative mating of individuals. Zebra finches are monogamous songbirds that form lifelong pairbonds. They exhibit highly coordinated behaviours especially at the nest. Because partners show coordinated vocal activity, we thus hypothesised that the vocal profile of individuals could be subject to assortative mating. Because corticosterone, the main stress hormone in birds, has a central role in pairbond maintenance and in vocal behaviour during stressful events, we also hypothesized that assortative mating could exist on hormonal profile.

We performed a separation-reunion protocol during which we recorded vocalisations and took blood samples for corticosterone assay. In isolation, vocal behaviour was not correlated between partners and could not predict behaviour during reunion. In contrast partners showed highly correlated vocal behaviours during reunion, and the history of the pair predicted their vocal activity: pairs exhibiting strong attachment and pairs who had breeding experience both emitted long, coordinated and balanced vocal interactions, while 2/3 of the pairs with loose relationships did not emit any calls during reunion. No similarity was found in the physiological profile of partners, and plasma level of corticosterone was not correlated to any of the behavioural parameters analysed. Instead, we found that males were more stressed than females after 30 minutes of separation.

Our results did not show any assortative mating in the zebra finch, neither on vocal nor on physiological profile of partners. We discuss the possibility that coordination in the zebra finch is achieved through a strong adjustment between partners behaviour, improved with familiarity between mates.

## INTRODUCTION

While individual traits usually drive probability of survival and breeding in a given environment, the success of a pair may also depend on properties emerging from interactions between mates, overriding the influence of intrinsic individual quality (Ens, Safriel et al. 1993; Ryan and Altmann 2001). In many bird species, coordination between partners seems to play an important role in survival and reproductive success of the pair (Spoon, Millam et al. 2006). Coordination has mainly been studied during foraging trips and at the nest during the breeding season (Davis 1988; Mariette and Griffith 2012; Massoni, Reboreda et al. 2012), especially in cooperative breeders and monogamous pairbonds, i.e. species whose nestlings' survival depends on the care of more than one individual. When predation risk is high, synchronized foraging behaviour could be more efficient for the survival of the pair (Krause and Ruxton 2002): partners can rely on each other for vigilance during feeding, and total time spent foraging at the same patch is reduced, decreasing the possibility of being seen by a potential predator. Partners of coordinated pairs visit the nest together, provisioning their young more efficiently by better estimating the young needs (McDonald, Kazem et al. 2007) and by reducing nestling competition (Shen, Chen et al. 2010). Synchronisation of nest visits also decreases the risk of attracting predators at the nest by reducing the overall activity around the nest (Fontaine and Martin 2006) but also the intensity of begging and/or the time spent begging by the nestlings (Eggers, Griesser et al. 2008; Peluc, Sillett et al. 2008; Raihani, Nelson-Flower et al. 2010).

Coordination and synchronization of the pair depend on how partners mesh together, i.e. on the compatibility of the partners. Compatibility is defined as a combination of traits found in both partners that leads to a high reproductive success of the pair. Thus, depending on the trait of interest, one individual can either mate with a similar partner leading to an assortative mating, or on the opposite, with a dissimilar but complementary partner, leading to disassortative mating (Gabriel and Black 2012). Monogamous animals mostly prefer partners with traits similar to their own phenotype (Burley 1983; Cooke and Davies 1983; Marsluff 1988; Pryke and Griffith 2007). In many bird species, highly compatible partners exhibit more coordinated behaviour and a better reproductive success (Coulson 1966; Coulson 1972; Spoon, Millam et al. 2006). Compatibility between cockatiel mates during the nonbreeding period, measured as the similarity in aggressive and affiliative behaviours, correlates positively with the pair's subsequent coordination of parental care and reproductive success (Spoon, Millam et al. 2006). In steller's jays, pairs' partners with similar exploration and risk-taking behaviours breed earlier in the season than dissimilar pairs (Pia O and Black 2012). Assortative pairs of great tits, in which both mates show fast-exploring profile or slow-exploring profile in a novel environment, produce more surviving offspring (Dingemanse and de Goede 2004) and offspring in better condition than disassortative

pairs (Both, Dingemanse et al. 2005). In mangellic penguins, successful breeders show assortative mating by body mass and pairs that are more similar in flipper length produce more young (Forero, Tella et al. 2001).

The assortative mating of a pair at a given time could be the result of a partner choice driven by specific traits, in which high quality mates are evaluated as individuals with self-similar traits (Ens, Safriel et al. 1993; Ryan and Altmann 2001; Dingemanse and de Goede 2004). But the similarity of the partners could also be the consequence of an adjustment of behaviour between the partners. Two birds could be different on a behavioural trait at the beginning of the relationship, and become more similar over time by adjustment of their behaviour. These two phenomena can also be associated: For instance in Budgerigars, mate choice is based on call similarity, but after pairing, the calls of the partners converge further in acoustic structure (Moravec, Striedter et al. 2006).

Many studies assessed the assortative mating of a pair using physical resemblance (Wiebe 2000; MacDougall and Montgomerie 2003; Huber, De Leon et al. 2007; Grant and Grant 2008), locomotor behaviour (Bearhop, Fiedler et al. 2005) or genetic similarities (Pryke 2010), while other characters central to the avian pairbond's biology could be similar between partners. While most bird species use vocalisations for pairbond communication and mate choice (Catchpole 1986; Searcy 1992; Andersson 1994; Hasselquist, Bensch et al. 1996; Searcy and Yasukawa 1996; Buchanan and Catchpole 1997; Reid, Arcese et al. 2004; Nowicki and Searcy 2005), no study to our knowledge has yet assessed the similarity between partners using vocal activity. Few studies showed call's structure matching between mates (Groth 1993; Moravec, Striedter et al. 2006; Smith, Sjoberg et al. 2012), but assortative mating based on individual propensity of calling could also be a way to reach synchronisation and coordination between partners. Vocalisations allow partners to exchange diverse pieces of information: they can convey information about the identity of the sender (Beer 1971; Marzluff 1988; Robertson 1996; Vignal, Mathevon et al. 2008; Hahn, Kryslar et al. 2013), but also about the mate social and dominance status (Thorpe, Hall-Craggs et al. 1972; Wickler and Seibt 1980; Hall 2000; Todt and Naguib 2000; Illes, Hall et al. 2006), its emotional state (Perez, Elie et al. 2012), its level of vigilance and alert in case of danger, especially predators (Krechmar 2003; Templeton, Greene et al. 2005; Colombelli-Negrel, Robertson et al. 2011). Partners use vocal interactions during key events (Marler and Slabbekoorn 2004), like courtship and maintenance of the pairbond (Black and Hulme 1996; Zann 1996), foraging behaviour (Gyger and Marler 1988; Evans and Marler 1994), or eggs brooding and nestlings feeding (Gorissen and Eens 2005). Some species even exhibit highly synchronised vocal duets (Farabaugh 1982; Hall 2004; Hall 2006; Benedict 2008; Hall 2009). Coordination of vocal exchange between partners is thus essential, and the vocal profile of the partner, i.e. individual propensity of vocalising,

could be subject to assortative mate choice, selecting a similar partner able to coordinate and synchronise its vocal behaviour more efficiently.

Some physiological parameters, such as immune status (Hamilton and Zuk 1982; Folstad and Karter 1992; Sheldon and Verhulst 1996; Andersson and Simmons 2006) or steroids levels (Owens and Short 1995; Zuk, Johnsen et al. 1995; Enstrom, Ketterson et al. 1997), are known to be subject to mate choice. Hormonal similarity has been previously shown in monogamous birds: greylag goose pairs with highly correlated levels of testosterone throughout the year show higher long-term reproductive success (Hirschenhauser, Mostl et al. 1999). Stress hormones also have a central role in the establishment and the maintenance of the pairbond: In both mammals and birds, plasma levels of stress hormone (i.e. glucocorticoids) decrease after pairing (Reburn and Wynne-Edwards 1999; Ginther, Ziegler et al. 2001; Angelier, Moe et al. 2007), and show a quick increase after separation from a social partner (Mendoza and Mason 1986; Jones and Williams 1992; Castro and Matt 1997; Norcross and Newman 1999; Ramage-Healey, Adkins-Regan et al. 2003). No study to our knowledge has reported assortative mating based on stress hormones' levels, which can also influence behaviour during stressful situations (Creel 2001) and thus survival or reproductive success. Partners with similar stress hormones' levels could be more coordinated and efficient to survive a stress.

Zebra finches are monogamous songbirds that form lifelong pairbonds; partners are inseparable even outside of the breeding season (Zann 1996). They keep a close relationship and spend most of their time together. Nest visits are synchronised between partners, and highly synchronised partners get better reproductive success (Mariette and Griffith 2012). The zebra finch is thus a good model species to study how coordination and synchronization are reached between partners of a pairbond. Despite what has been shown in other finches (Groth 1993; Christensen and Kleindorfer 2007; Pryke and Griffith 2007), no study to date found any cues of assortative mating in the zebra finch on physical, behavioural or physiological traits (Schielzeth, Burger et al. 2008).

Zebra finches use a large repertoire of calls during social interactions (Zann 1996). Male and female can recognize their mates using calls only (Vignal, Mathevon et al. 2004; Vignal, Mathevon et al. 2008; Elie, Mariette et al. 2010) and partners emit coordinated vocal duets at the nest during breeding that may help in maintaining the pairbond and coordinate brood care (Elie, Mariette et al. 2010). During foraging, mates keep constant acoustic contact even when visually separated (Zann 1996). Because acoustic signals like song are central to mate choice processes in the zebra finch (Collins, Hubbard et al. 1994; Forstmeier and Birkhead 2004; Spencer, Wimpenny et al. 2005; Riebel 2009) and considering that calls might participate in the zebra finch pairbond's coordination, we hypothesised that zebra finches could also base their mate choice on acoustic

production. In this case, a bird will be more likely to pair up with a partner with a self-similar vocal profile, talkative birds pairing together on one hand and less vocally productive birds on the other hand.

Corticosterone, the main stress hormone in birds, is also involved in the regulation of social bonds in the zebra finch: social isolation from familiar individuals provokes an elevation of plasma levels of corticosterone in male zebra finches (Perez, Elie et al. 2012), and corticosterone is directly involved in the emission of stress-induced vocalisations in isolated birds. The hormone also shows a quick increase in zebra finches separated from their partner, and a quick return to baseline after reunion (Ramage-Healey, Adkins-Regan et al. 2003). Chronic elevation of plasma corticosterone can also affect song quality of males (Spencer, Buchanan et al. 2003) while acute stress lower song rate (Lynn, Stamlis et al. 2010). Recent study proved that female zebra finches show a preference for males with low plasma levels of corticosterone (Roberts, Buchanan et al. 2007), suggesting that corticosterone profile is subject to mate choice in this species.

To evaluate assortative mating based on vocal and stress profiles, we measured vocal production and corticosterone response of partners during a separation-reunion experiment. The zebra finch pairbond is very strong even during the nonbreeding season: the partners stay together during all activities and throughout the year (Zann 1996), and separation from the partner is a stressful situation (Ramage-Healey, Adkins-Regan et al. 2003) that the birds actively try to terminate by calling and searching their mate (Zann 1996). We thus used a separation-reunion experiment to measure calling propensity as well as corticosterone response and evaluate similarity between mated partners.

Because individuals could pair up assortatively in the first place or adjust their behaviour and become more similar after pairing, we compared pairs with different experiences: (1) pairs that never bred together before (2) pairs that bred together at least once in the past 6 months. As social attachment is a strong factor influencing the mates physiology and behaviour (Ramage-Healey, Adkins-Regan et al. 2003), we also considered pairs that showed strong bond and compared them to pairs that showed looser bond.

Finally, some recent studies suggest that wild type and domestic strain of zebra finches do not exhibit the exact same behaviour, especially when it comes to reproduction (Gilby, Mainwaring et al. 2011). To verify this hypothesis, half of the pairs used in this experiment were wild-type zebra finches, while the other half were domestic birds.

## **METHODS**

### **Subjects and housing conditions**

Birds used for this study were zebra Finches (*Taeniopygia guttata*) kept in captivity in our aviaries. Half of the animals were domestic birds bred in our colony, the other half were wild-type birds. The domestic birds were the descendants of domesticated wild-type zebra finches originally obtained from aviculturists in the Sydney region in 2005, and had been bred in our facility for at least three generations (Rutstein, Brazill-Boast et al. 2007). The captive wild-type birds were either taken under licence from Sturt National Park (northwest New South Wales, Australia) in September 2007 using mist nets (Pariser, Mariette et al. 2010), or direct descendants of the previous ones.

Domestic and wild-type birds were housed separately in two outdoor aviaries (10 X 8 m and 2 m high), each containing from 30 to 50 birds. Each aviary was provided with ad libitum commercial finch seeds, water, cuttlefish, grit, sprouted seed, two heat lamps and nestboxes.

To select pairbonds for the study, birds were directly observed in the aviaries for three consecutive days during two hours so as to detect pairs using four specific behaviours (Zann 1996):

- Nestling rearing: Birds raising chicks together.
- Clumping: birds perching side by side in contact.
- Allopreening: one bird preening the feathers of the other one.
- Nest sharing: birds sharing the same nest.

Breeding activity in the two outdoor aviaries had been followed for a year prior to the experiment. This allowed to determine the age of the pairbonds in months, as well as previous reproductive success of the pair.

14 pairs (8 wild-type and 6 domestic) were caught in the aviaries and then housed in individual cages (1 pair per cage, height = 40 cm, width = 75 cm, depth = 47 cm) stored in the same rearing room for the duration of the experiment. Five of these 14 pairs (3 wild-type and 2 domestic) were formed by randomly putting together a male and a female in the same individual cage for one month prior to the experiment. One week before the beginning of the experiment, the effectiveness of these five pairbonds was checked using the same specific behaviours as above.

Rearing conditions in the room were the same as in aviaries (see above). The light conditions followed the natural cycle of daylight at this period of the year: the lights were successively switched on from 5:45 am to 6:00 am so as to mimic the sunrise, and they were successively switched off from 7:00 pm to 7:30 pm to mimic the sunset. The temperature in the rearing room and the experimental rooms stayed around 22°C for the duration of the experiment.



In order to avoid any supplementary stress to the birds that could affect the experiment, precautions were taken:

- Birds acclimated to the rearing room two weeks prior to the beginning of the experiment.
- Only one experimenter had access to the birds in the rearing room and in the experimental room during the duration of the experiment. The experimenter always wore the same white labcoat. Thus, stress from novelty was low.
- Food and water was distributed every day after the experiment (after 12:00) to avoid any interference of cage disturbance with corticosterone measurements.

### **Blood sample collection and hormone assay**

All blood samples (<150  $\mu$ l) were obtained by puncturing the alar vein with a 25-gauge needle. Blood was collected in heparinized tips, and plasma was separated under centrifugation (3800 G during 15 minutes) and stored at  $-20^{\circ}\text{C}$  for later hormone assay. Blood samples were taken within 3 min so as to measure corticosterone baseline and not corticosterone due to handling (Wingfield, Smith et al. 1982). No bird was bled more than once per week to ensure that they had sufficiently replenished their blood volume between bleeds and HPA axis function had recovered from previous sampling (Romero and Reed 2008).

Because circulating level of endogenous corticosterone shows circadian variations in many bird species with a peak just before the active period and a rapid decrease during the first hours of activity, all experiments were performed between 8 and 12 am (Ramage-Healey and Romero 2000; Tarlow, Hau et al. 2003).

Corticosterone concentrations were measured from duplicate 20  $\mu$ l plasma samples in one assay using direct radioimmunoassay (MP Biomedicals kit 07120002). The assay was run with 50% binding at 0.167 ng/ml, and the detection limit (for 20  $\mu$ l aliquots of extracted, diluted plasma) was 0.025 ng/ml.

### **Experimental setup and test procedures**

#### *Measurement of Baseline level of corticosterone*

To assess basal level of circulating corticosterone ("BASAL\_CORT"), birds were captured from previously undisturbed rearing room and bled in an adjacent room before going back to their cage. This procedure was carried out within 3 minutes of the light being turned off for capture. This protocol was repeated 3 times per morning at 8:00, 10:00 and 12:00 pm, thus 3 birds were captured and bled per day in the rearing room. Two hours between two blood sample events were necessary to allow plasma levels of corticosterone of all the birds to return to basal levels after disturbance of the room.

### *Separation-reunion protocol*

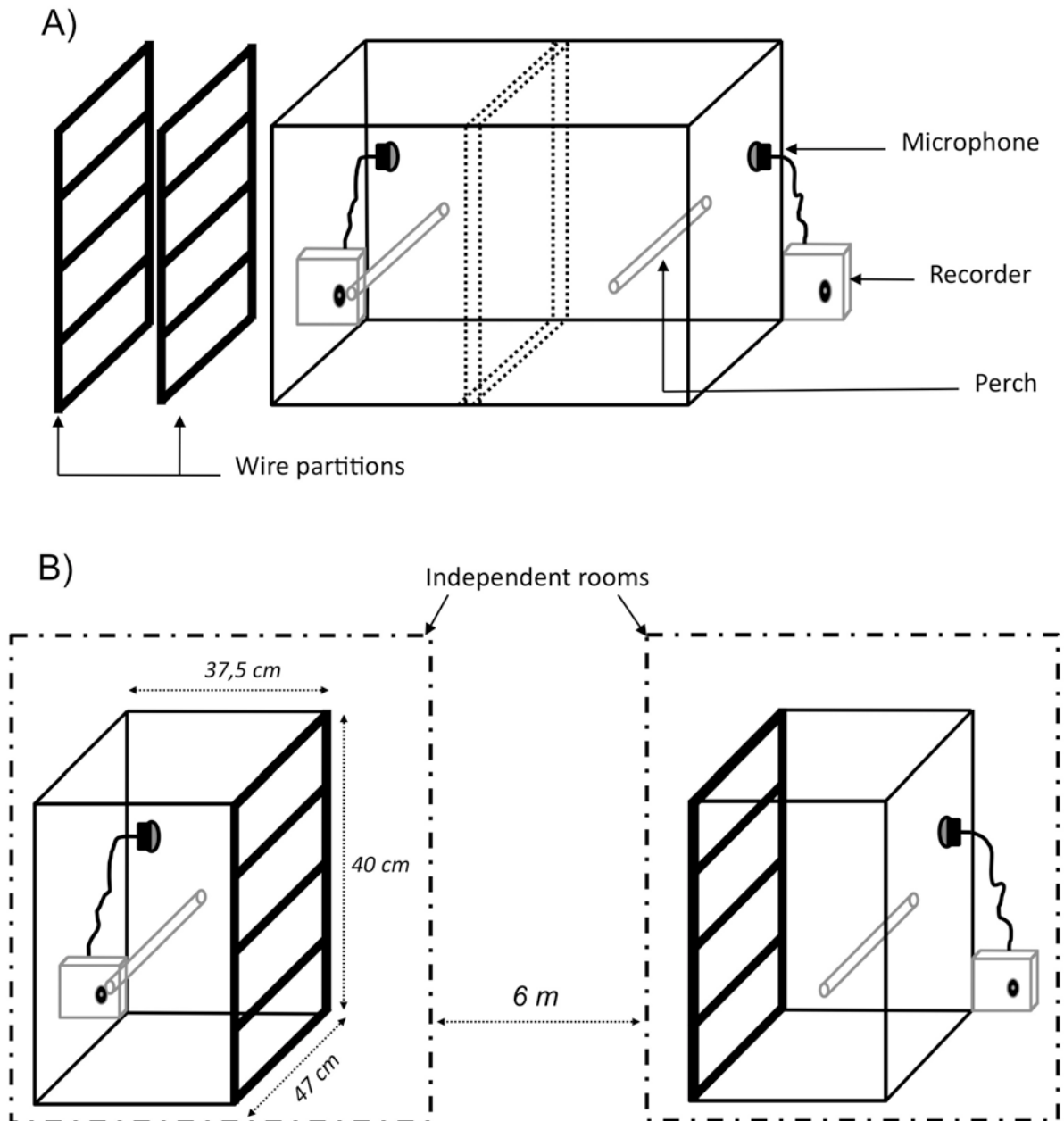
We developed a protocol to separate the partners of a pair without handling, avoiding unnecessary stress. The day before the experiment, the partners of a pair were moved from the rearing room to the experimental room and placed together in a separation cage (figure 1). The morning of the experiment, 2 webcams (logitech HD pro C910) and 2 microphones (AKG C 417 Clip-on Microphones, one per half cage) connected to a recorder (zoom H4n) were turned on to monitor the locomotor and vocal activities of the birds during the whole experiment. Two sessions were run in the same morning in two different experimental rooms, allowing us to record two pairs per day. The first session began at 8:00 am, the second session at 10:00 am.

Birds were first recorded without disturbance for 20 minutes together in the same cage (figure 1 “Pair” configuration) (STEP 1). Partners were then physically separated using two wire partitions placed in the middle of the experimental cage, which allowed separating the cage in two independent parts. Thus, the birds were separated without handling, known to provoke a raise in plasmatic levels of corticosterone within 5 minutes (wingfield 1982 and 1992). The newly independent parts were then moved in two independent rooms separated by 6 meters and two doors, so the partners were visually and acoustically isolated from each other (figure 1 “single” configuration). After 30 minutes of separation (STEP 2), the doors from the independent rooms were opened for 10 minutes, allowing an acoustic contact between the birds but preventing them from a visual contact (STEP 3). This situation was suitable for the exchange of distance calls between partners. Each bird was then placed back in the first experimental room so that partners will be in the same room again, for 20 minutes (STEP 4).

To measure the level of circulating corticosterone during a stress of separation (“STRESS\_CORT”), the birds were separated again in the two independent rooms (STEP 5). After 30 minutes of separation, blood samples were taken within 3 minutes and birds were placed back in the rearing room. The blood samples from the first pair were run at 9:50 am, the second at 11:50 am.

### *Randomization*

Half of the wild birds were recorded during a first session (from 8:00 to 9:50 am), the other half during a second session (from 10:00 to 11:50 am). The same precaution was used for the domestic birds. The order of blood sampling for assessing the baseline was also randomized: half of the females were bled for baseline before the male whereas the other half was bled after the male. Moreover, half of the birds were bled for baseline before the separation-reunion experiment, whereas the other half was bled for baseline after the separation-reunion experiment.



**Figure 1: Experimental cage.**

A) Separation cage in a « pair » configuration (STEP 1).

B) Separation cage in a « single » configuration (STEP 2).

The tested birds were placed in the experimental cage in the “pair configuration”, one day prior to the experiment. A) During the first part of the experiment (PART1, 20 minutes), the birds were free to interact. B) Partners were then separated by inserting wire partitions in the middle of the cage, so that one bird will stay in one side of the cage, while its partner will be on the other side. Birds were visually and acoustically isolated from each other (30 minutes, STEP 2) by moving each part of the cage in independent rooms. Doors from the two rooms were then opened after total separation, allowing the partners to hear but not see each other (10 minutes, STEP 3).

### **Analysis of affiliative behaviours and determination of the strength of the pair**

The behaviours of the pair during the first 20 minutes of the experiment (STEP 1) were scored on video recordings by an observer blind to the identity of the pair. Videos were scored in random order regarding identity of the pair. Using Etholog software (Ottoni 2000), five variables were quantified:

- Clumping: birds perched side by side in physical contact. This variable was considered as binary (Presence of the behavior = 1, absence = 0).
- Allopreening: one bird preened the feathers of the other one. This variable was considered as binary.
- Singing: songs emitted by the male toward the female (directed songs) or not (undirected songs, never observed). This variable was considered as binary.
- Proximity: time spent by the birds at close proximity (less than 6 cm), in seconds. Pairs received a score of 0 from 0 to 25% of time spent in proximity, 0.5 from 25 to 50% and 1 from 50 to 100%.
- Synchronisation: time spent by the partners in the same activity, in seconds. The activity of each bird was monitored and classified in 5 sub-types: affiliative behaviour (clumping and/or allopreening), preening (birds cleaning their own feathers), restoring (drinking and/or eating), jumping on the perch or on the floor, and resting (bird staying on a perch without any obvious activity). Then partners' activities were compared and the entire time spent doing the same activity was extracted. Pairs finally received a score of 0 from 0 to 33,3% of the time spent in synchrony, 0.5 from 33,3 to 66,6% and 1 from 66,6 to 100%=1.

The limits of the three score classes were different between the two previous variables so as to decrease the influence of the synchronization parameter. Because the number of categories of behaviour was small, the probability that some activities were synchronized between the two birds was high.

To then evaluate the strength of the pair, we computed a single composite index, the index of relationship strength (IRS): the five previous variables were used to compute a principal component analysis (PCA, function `dudi.pca` from the `ade4` package in R software, computed on z-scored parameters). The PCA retained two components with eigen-values larger than one (Kaiser criterion), PC1 and PC2, explaining respectively 44% and 29% of the variance (for variable loadings on each PC, see supplementary data). The first principal component (PC1) was used in statistical analysis as the index of relationship strength (IRS). PC1 showed a bimodal distribution with a gap around 0 (supplementary data). Thus, 0 was chosen as the limit to discriminate strong bonds ( $PC1 < 0$ ,  $n=5$  pairs with 3 wild-type and 2 domestic) from loose bonds ( $PC1 > 0$ ,  $n=9$  pairs with 5 wild-type and 4 domestic).

### **Analysis of vocal activity**

Calls emitted by the birds during complete isolation (STEP 2) and during acoustic contact at long distance (STEP 3) were first extracted from the original recordings using a custom-written program (PYTHON programming language, open-source libraries).

The program allowed determining 3 acoustic variables for each bird:

- The number of calls: NB\_CALL
- The time spent calling (in seconds): DUR\_CALL
- The call rate (in calls per second): RATE\_CALL as  $RATE\_CALL = NB\_CALL / (DUR\_CALL)$

These variables were used as such for correlation (see below), but were also combined to compute two PCA, one for data in isolation, one for data in acoustic contact. The PCA in isolation retained two components with eigen-values larger than one (Kaiser criterion), PC1 and PC2, explaining respectively 56% and 38% of the variance (for variable loadings on each PC, see supplementary data). The first principal component (PC1 in isolation) was used in statistical analysis. The PCA in acoustic contact retained one component with eigen-values larger than one (Kaiser criterion), and explained 83% of the variance (for variable loadings on each PC, see figure 5C). This first principal component (PC1 in visual contact) was used in statistical analysis.

Three other variables were then built:

- The proportion of female calls in the interaction (STEP 3) was calculated for each pair as the number of female calls divided per the total number of calls emitted during the interaction. If both partners participate equally to the interaction, the proportion of female calls is closed to 0.5.
- The talkativeness of each bird during acoustic contact (STEP 3) was assessed by building a binomial variable: 1 for birds that talked, 0 for birds that did not talk.
- The structure of the vocal sequences was described using the degree of alternation between partners, which is defined as the Number of Runs (NR) of elements in the sequence (i.e. the number of continuous series of calls of each partner) (Elie et al 2010). Alternating sequences between the male (M) and the female (F) yield high NR (e.g. MFMFMFMF, eight runs), while clumped sequences yield low NR (MMMMFFFF, two runs) and random sequences yield intermediate NR (MMMFFMFF, four runs). For each sequence, we computed the NR expected at random as the mean of the distribution of the NR obtained by 10 000 draws following a Bernoulli law and with the same number of elements for each partner as in the observed sequence. We then assessed the deviation of the NR (dev NR) in the observed sequence from the mean random NR by calculating:  $dev\ NR = (observed\ NR - mean\ random\ NR) / mean\ random\ NR$ . Positive values of this deviation parameter indicate alternated sequences (higher NR than expected at random).

## Statistical analysis

All statistics were performed using R software (Team 2005).

We had several pieces of information about the pairs that were used as factors in statistical analyses (see the repartition of the pairs in each category, see figure 2):

- The origin of the pair as WILD type or DOMESTIC birds.
- The reproductive background of the pair, as pairs who already had a CHICKS or who had NO CHICKS before.
- The age of the pairbond, as YOUNG pairs (paired for less than 3 months), and OLD pairs (paired for more than 3 months).
- The strength of the pair, as STRONG or LOOSE relationships (see Analysis of affiliative behaviours above).
- The experimental condition of blood sampling, used only to analyse plasma levels of corticosterone (CORT\_BASAL vs CORT\_STRESS)

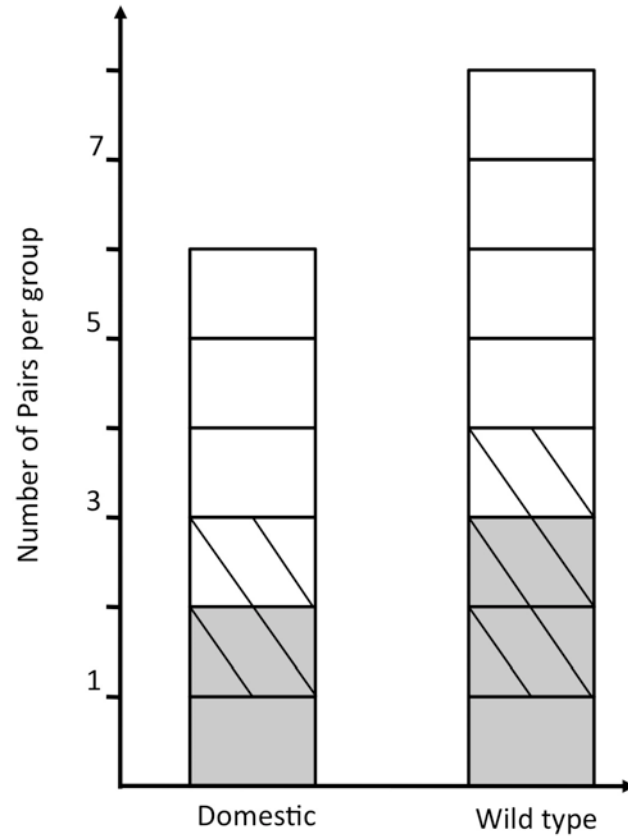
Correlations between variables were explored by fitting linear models (function `lm` from the `stats` package in R software).

Normality was tested using the Shapiro test and homoscedasticity was tested using the Bartlett test. When at least one of these two conditions was violated, models were fitted using adapted distribution (binomial).

All linear mixed models were originally performed with the origin, the reproductive background, the age of the pair, the strength of the bond, and the sex of the bird as fixed factors, with three exceptions: in the analysis of the proportion of female calls and the deviation of the NR, sex was not used as a factor, and in the analysis of plasma levels of corticosterone, the experimental condition was added as a factor. The identity of the pair was used as a random factor, except in the analysis of plasma levels of corticosterone in which we used the identity of the bird. To simplify the model, we followed a backward stepwise procedure where least significant terms were sequentially removed. Successive models were compared using the 'anova' function, and a factor was removed when  $p\text{-value} > 0.05$ .

Post hoc tests for linear mixed models with normal distribution were performed using Multiple Comparisons of Means with Tukey Contrasts, using the "glht" function from the "multcomp" package with the variable of interest as object of class "mcp". Post hoc tests for linear mixed models with bimodal distribution were performed using pairwise t tests.

Plasma levels of corticosterone, PC1 in isolation and PC1 in acoustic contact were normally distributed. Linear mixed models were performed ('lme' function from 'lme4' package).



**Figure 2: Composition of the experimental groups.**

One rectangle represents one pair.

Total number of pairs: 14

Total number of wild type pairs: 8

Total number of domestic pairs: 6

Total number of strong pairs (grey rectangle): 5

Total number of loose pairs (white rectangle): 9

Total number of pairs with previous reproductive success (hatched rectangle): 5

Total number of pairs without previous reproductive success (no hatch rectangle): 9

| VARIABLE 1                                | VARIABLE 2                              | SAMPLE SIZE | R <sup>2</sup> | P value |
|---|---|-------------|----------------|---------|
| STRESS_CORT female                        | STRESS_CORT male                        | 14          | -0.07          | 0.69    |
| STRESS_CORT female (STRONG group)         | STRESS_CORT male (STRONG group)         | 5           | -0.22          | 0.64    |
| STRESS_CORT female (LOOSE group)          | STRESS_CORT male (LOOSE group)          | 9           | -0.14          | 0.95    |
| STRESS_CORT female (WILD TYPE group)      | STRESS_CORT male (WILD TYPE group)      | 8           | -0.05          | 0.45    |
| STRESS_CORT female (DOMESTIC group)       | STRESS_CORT male (DOMESTIC group)       | 6           | -0.22          | 0.77    |
| STRESS_CORT female (group with CHICKS)    | STRESS_CORT male (group with CHICKS)    | 5           | 0.16           | 0.27    |
| STRESS_CORT female (group without CHICKS) | STRESS_CORT male (group without CHICKS) | 9           | -0.10          | 0.62    |
| BASAL_CORT female                         | BASAL_CORT male                         | 9           | -0.09          | 0.60    |
| BASAL_CORT female (STRONG group)          | BASAL_CORT male (STRONG group)          | 3           | X              | X       |
| BASAL_CORT female (LOOSE group)           | BASAL_CORT male (LOOSE group)           | 6           | 0.69           | 0.25    |
| BASAL_CORT female (WILD TYPE group)       | BASAL_CORT male (WILD TYPE group)       | 5           | -0.33          | 0.99    |
| BASAL_CORT female (DOMESTIC group)        | BASAL_CORT male (DOMESTIC group)        | 4           | X              | X       |
| BASAL_CORT female (group with CHICKS)     | BASAL_CORT male (group with CHICKS)     | 2           | X              | X       |
| BASAL_CORT female (group without CHICKS)  | BASAL_CORT male (group without CHICKS)  | 8           | -0.14          | 0.71    |

**Table1: results for linear regressions between plasma levels of corticosterone of partners.**

CORT : plasma levels of corticosterone

STRESS : condition after 30 min of separation.

BASAL : basal condition.

X : no results, sample size to small.

| VARIABLE 1                         | VARIABLE 2                       | SAMPLE SIZE | R <sup>2</sup> | P value          |
|------------------------------------|----------------------------------|-------------|----------------|------------------|
| NB_CALL female (PART2)             | NB_CALL male (PART2)             | 14          | - 0.07         | 0.77             |
| DUR_CALL female (PART2)            | DUR_CALL male (PART2)            | 14          | 0.05           | 0.21             |
| RATE_CALL female (PART2)           | RATE_CALL male (PART2)           | 14          | -0.10          | 0.95             |
| PC1_ISOLATION female (PART3)       | PC1_ISOLATION male (PART3)       | 14          | -0.04          | 0.47             |
| <b>NB_CALL female (PART3)</b>      | <b>NB_CALL male (PART3)</b>      | <b>14</b>   | <b>0.70</b>    | <b>&lt;0.001</b> |
| <b>DUR_CALL female (PART3)</b>     | <b>DUR_CALL male (PART3)</b>     | <b>14</b>   | <b>0.71</b>    | <b>&lt;0.001</b> |
| <b>RATE_CALL female (PART3)</b>    | <b>RATE_CALL male (PART3)</b>    | <b>14</b>   | <b>0.78</b>    | <b>&lt;0.001</b> |
| <b>PC1_ACOUSTIC female (PART3)</b> | <b>PC1_ACOUSTIC male (PART3)</b> | <b>14</b>   | <b>0.75</b>    | <b>&lt;0.001</b> |
| NB_CALL (PART2)                    | NB_CALL (PART3)                  | 28          | -0.02          | 0.55             |
| DUR_CALL (PART2)                   | DUR_CALL (PART3)                 | 28          | 0.09           | 0.06             |
| RATE_CALL (PART2)                  | RATE_CALL (PART3)                | 28          | 0.05           | 0.13             |
| PC1_ISOLATION (PART2)              | PC1_ACOUSTIC (PART3)             | 28          | -0.00          | 0.33             |

**Table2: results for linear regressions between acoustic profiles of partners.**

NB\_CALL : numbers of calls emitted.

DUR\_CALL : time spent vocalising.

RATE\_CALL : call rate.

PART2 : In separation, birds are physically and acoustically isolated from the partner.

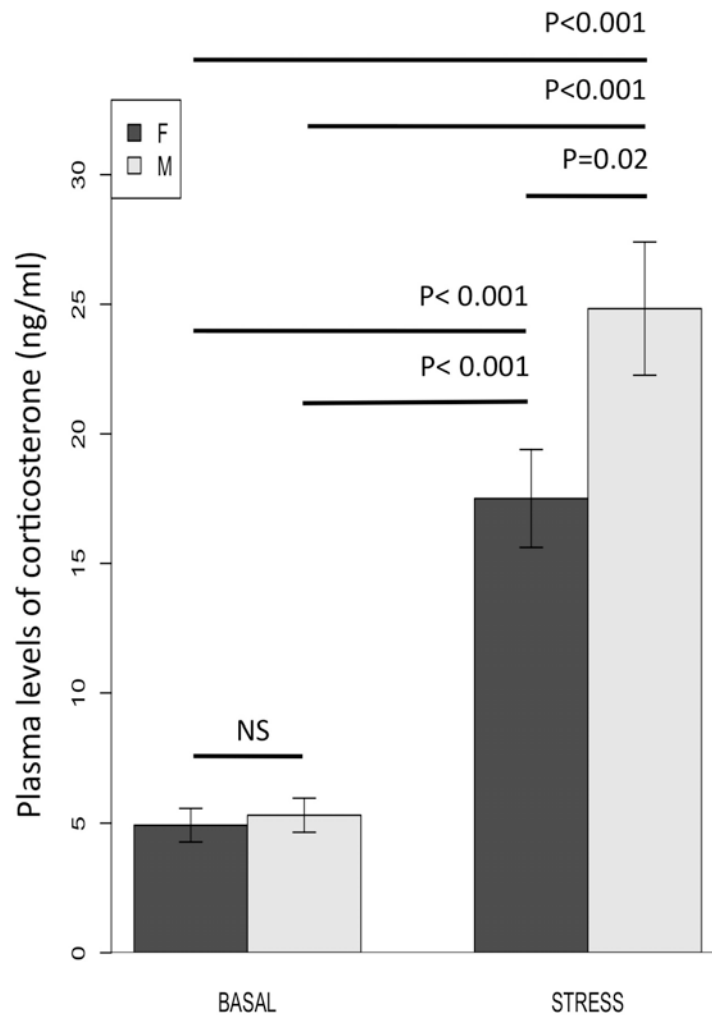
PART3 : in acoustic contact, birds can hear but not see their mate.

Lines 1 to 4 allow to verify the correlation of the partners' vocal profile while in isolation.

Lines 5 to 8 allow to verify the correlation of the partners' vocal profile while in acoustic contact.

Lines 9 to 12 allow to verify the correlation between the acoustic profile of one individual in isolation and its behaviour while in visual contact with the partner.





**Figure 3: Plasma levels of corticosterone: males are more stressed than females after separation.**

Data are presented as mean±SE, n=14 males and 14 females.

Males (n=14) were significantly more stressed than females (n=14) after 30 minutes of separation using the separation cage. Plasma level of corticosterone was not affected by any other fixed factor tested in the statistical analysis.

Talkativeness was analyzed using a General linear mixed models ('lmer' function from 'lme4' package) with binomial distribution. The proportion of female calls in the interaction during acoustic contact was normally distributed and thus analyzed using a General linear mixed models ('lme' function from 'lme4' package). A t test was used with comparison to 0.5, the theoretical value that described an equal participation of individuals in the interaction. The deviation of the NR was also compared to 0 using a t test, to verify if vocal interactions were more alternated than what is expected as random.

## **RESULTS**

### **No assortative mating based on physiological profile, but males more affected by isolation than females**

No correlation were found between male and female partners on physiological traits, i.e. basal plasma level of corticosterone and plasma level of corticosterone after separation (see Table 1 for correlation results per experimental groups).

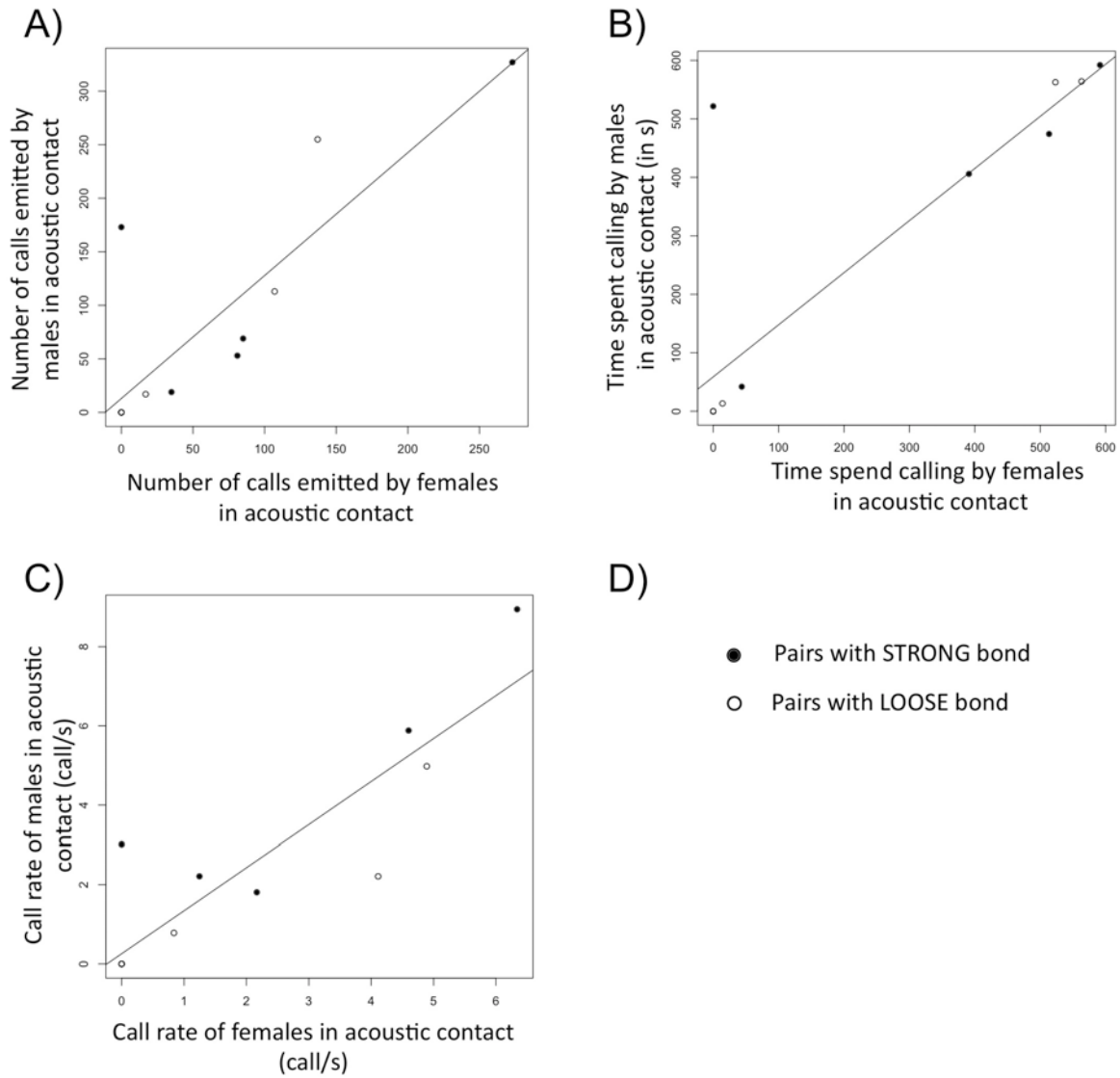
Plasma levels of corticosterone were not affected by the characteristics of the pair (strength of the bond, origin, age of the pair, productive background) but by the sex of the bird (lme  $F=5.61$ ,  $p=0.03$ ), the experimental condition of blood sampling (lme  $F=99.23956$ ,  $p < 0.001$ ) and the interaction between these two factors (lme  $F=4.66$ ,  $p=0.049$ ). Both males and females show a raise in plasma levels of corticosterone after isolation, but males show higher hormonal levels than females after isolation (Post hoc test  $p < 0.05$  except for Male\_BASAL vs Female\_BASAL where  $p=0.99$ , figure 3).

### **No assortative mating based on acoustic profile but coordinated interaction during acoustic contact with talkativeness depending on the pair status**

No correlation was found between male and female partners on acoustic traits in isolation (STEP 2), i.e. the number of calls, the time spent calling and the call rate (see Table 2 for correlation results), whereas strong correlations were found when partners were in acoustic contact (STEP 3, table 2 and figure 4), signing an acoustic interaction.

The vocal behaviour of one individual in isolation was not correlated with its behaviour while in acoustic contact with its partner (see Table 2 for correlation results); this was true for all categories of birds. Thus, the bird acoustic profile changed during acoustic contact with its partner.

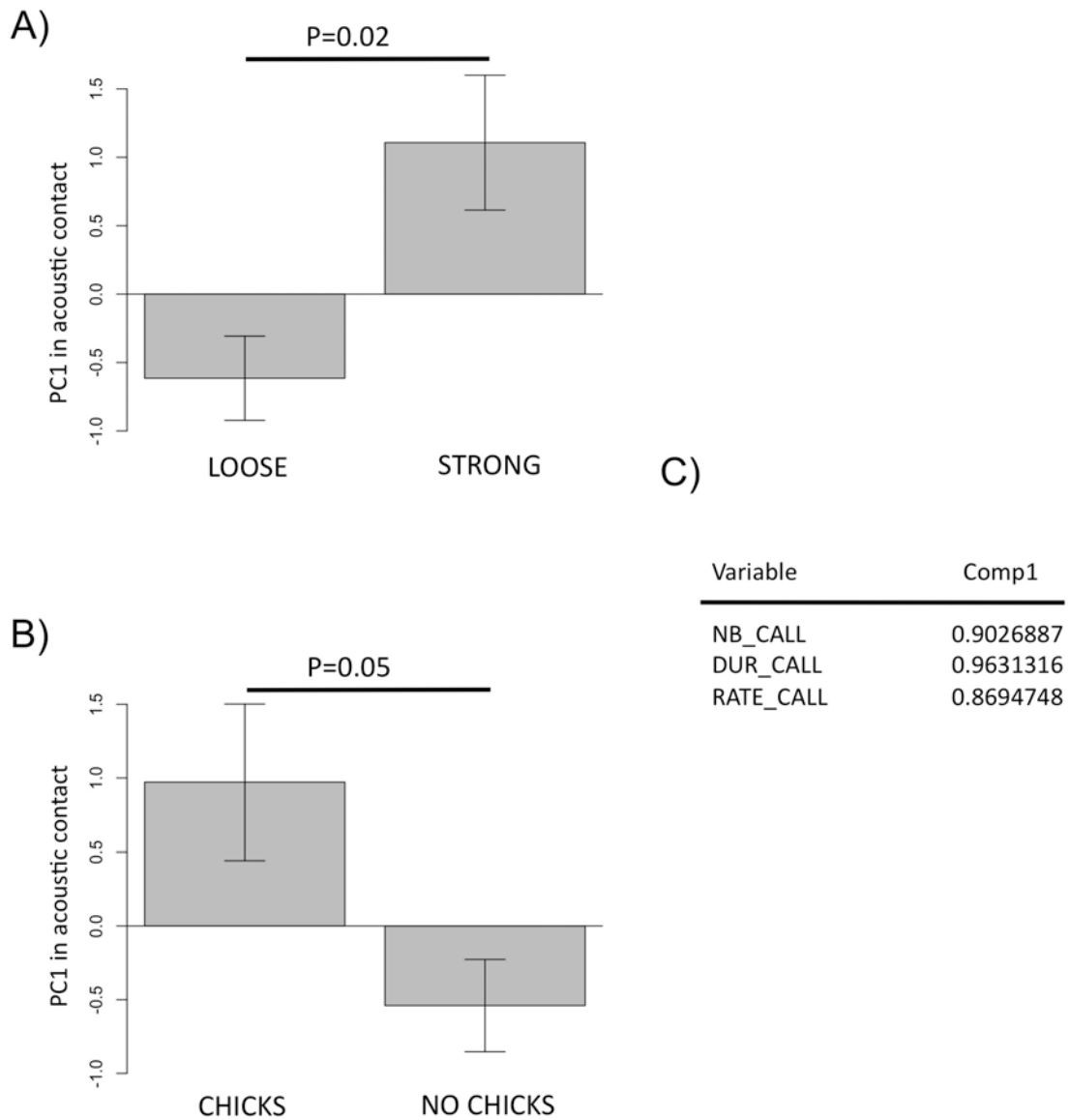
Acoustic parameters in isolation and in acoustic contact were affected neither by the sex of the bird, nor by the age of the pair nor by its origin (lme  $p > 0.05$  for all p-values on PC1 in isolation and PC1 in visual contact). But, acoustic parameters in acoustic contact depended on the pair history: pairs with a strong relationship called longer, emitted more calls with a higher call rate than pairs with loose bonds (figure 5, A, lme :  $F=4.28$   $p=0.05$  ; post hoc test: STRONG vs LOOSE,  $p=0.02$ ). Pairs that previously raised nestlings



**Figure 4: Partners equally participate in the interaction while in acoustic contact (STEP 3).**

Data are presented as mean±SE. n=14 for each correlation. The calling activities of the partners were correlated during acoustic contact:

- A) Number of calls.
- B) Time spent calling.
- C) Call rate.



**Figure 5: The acoustic behaviour while in acoustic contact depends on the history of the pair (STEP 3).**

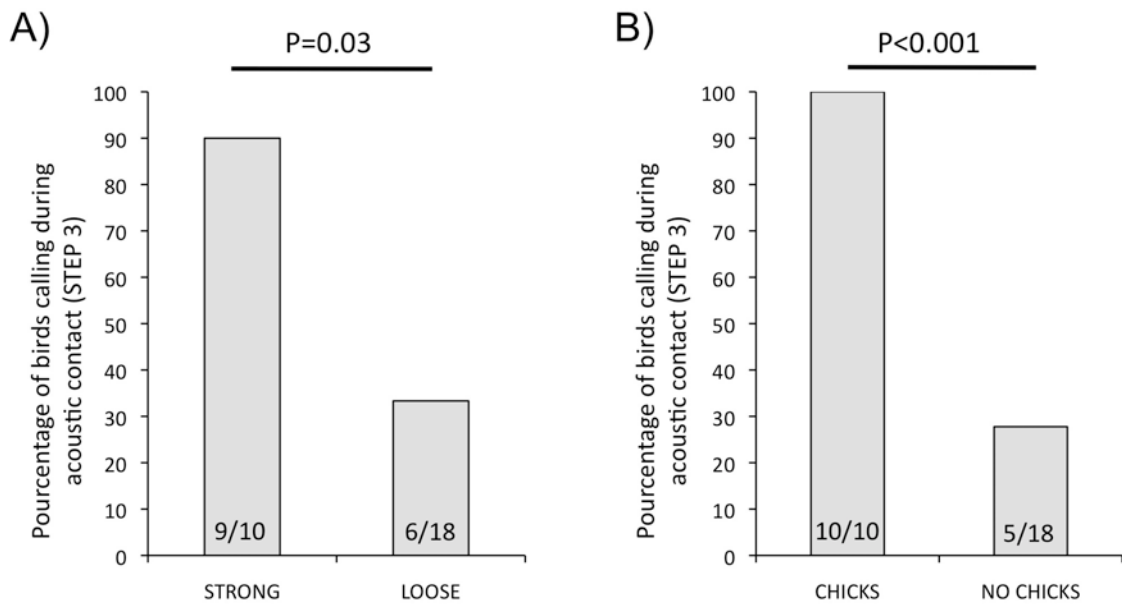
Data are presented as mean $\pm$ SE, n=14.

Birds called longer and emitted more calls with a higher call rate :

(A) in strong pairbonds versus loose pairbonds.

(B) in pairs that already had chicks versus pairs with no breeding experience.

(C) Contribution of each acoustic parameter to the first axis of the PCA.



**Figure 6: The level of talkativeness of the birds in acoustic contact depends on the history of the pair (STEP 3)**

(A) in strong pairbonds versus loose pairbonds.

(B) in pairs that already had chicks versus pairs with no breeding experience.

Numbers at the bottom of the columns give raw numbers of talkative birds in each group.

also show the same profile, compared to pairs that did not reproduce in the past (figure 5, B, lme :  $F=6.54$   $p=0.04$  post hoc test : CHICKS vs NO CHICKS,  $p=0.05$ ).

The talkativeness of the birds during acoustic contact (probability of emitting at least one call) was affected by the strength of the pair. 90% of the birds (9 out of 10 individuals, one pair with only the male calling) involved in a strong bond vocalised, while only 33% of the birds (6 out of 18 individuals) involved in a loose pair did so (STRONG vs LOOSE, Post hoc test:  $p<0.03$ , figure 6 A). The previous reproductive experience also affected the talkativeness of the birds: 100% of the birds (10 out of 10 individuals) that already had chicks called during acoustic contact, while only 28% of the birds (5 out of 10 individuals) did so in the group without previous nestlings (CHICKS vs NO CHICKS, Post hoc test:  $p<0.001$ , figure 6 B). The talkativeness of the partners was homogenous, as only one pair out of 14 showed a dissimilar profile with the male calling and the female staying quiet.

Finally, the relative participation of the partners to the acoustic interaction was balanced (t test with comparison to 0.5,  $p=0.5$ ) and was not affected by the strength of the bond, the origin, the age or the reproductive background of the pair (lme  $p>0.05$  for all p-values).

There was no correlation between plasma levels of corticosterone and any of the acoustic parameters analyzed (number of calls, time spent calling and call rate; in isolation or in acoustic contact), in any of the different type of pairs presented in the experiment (strength of the bond, origin, age of the pair, parental background).

## **DISCUSSION**

Our experiment revealed no assortative mating based on physiological profile in the zebra finch. Instead, we showed that males were more prone to stress compared to females. The plasma level of corticosterone of individuals could not predict the vocal behaviour of the birds and was not correlated to any of the parameters describing the pair status.

We also found no assortative mating between partners based on acoustic profile, as the vocal behaviours of mates were not correlated while birds were isolated. Nevertheless during acoustic contact, partners exhibited an adjusted vocal strategy that depended on the pair status: strong pairs and pairs that already bred before emitted numerous calls in a coordinated interaction with equal participation of both the male and the female, while loose pairs and pairs with no breeding experience stayed quieter.

Only eight out of the 14 pairs vocalised during the acoustic contact with their partner, and only one pair showed a dissimilar bond with the male calling while the female remained silent. This similarity of propensity of calling could sign an assortative mating,

as partners show the same vocal profile while in acoustic contact, with silent birds and talkative ones each being mated with self-similar partners. Nevertheless, the vocal profile of birds in isolation did not match the profile of their partner and could not predict the birds' behaviour while in acoustic contact with their mate. Thus, the similarity observed while in acoustic contact is better described as an adjustment of behaviour than an assortative mating. This adjustment of behaviour leading to a similarity of partners while interacting together has been described previously in some species: for instance, female black-bellied wrens choose to sing the song type similar to their mate while duetting, even if they can sing different types of song (Logue 2006).

Increasing experience and familiarity between mates allows a better reproductive success compared to new pairs (Gabriel and Black 2013), achieved through better coordination and synchrony based on a strong similarity in their phenotypical traits (Wickler 1976; Cooke, Bousfield et al. 1981; Choudhury and Black 1994). Thus, similarity increases with time and bond experience, with life-long pairbonds showing higher coordination and better reproductive success. Our results did not reveal any effect of the age of the pair on similarity of partners, but we did find that the pair previous experience had a strong effect on the partners vocal strategy. In our study, the strategy of interaction between mates appears different depending on the history of the bond: pairs that already bred together, and pairs that strongly expressed their social attachment through affiliative behaviours showed a higher propensity of calling, while loose pairs and pairs that did not breed before remained more silent.

In this study, the acoustic contact between partners occurred after a stress of separation, thus it represents a vocal reunion between mates in a non-reproductive context and could participate in the pairbond maintenance. This could explain why pairbonds with a loose relationship stay quieter, as they do not maintain the bond in a strong dynamic, compared to attached companions more motivated to maintain contact. This hypothesis is supported by previous results in our laboratory, showing that physical reunion with the partner elicit a long vocal exchange after separation, while physical reunion with a non mate does not elicit a full vocal exchange (Elie and Vignal 2009). In that case, staying quiet cannot be described as similarity between mates, but more as a lack of motivation to reconnect with the mate. On the other hand, all the pairs that already bred before responded to each other while in social contact. It is possible that experiencing a reproductive event allowed them to exhibit many vocal exchanges, especially during duets at the nest (Elie, Mariette et al. 2010). This could have led to a talkative and coordinated pair.

The vocal exchanges during acoustic contact are coordinated and well balanced between partners: the proportion of female and male calls in the exchange is statistically identical, the call rate and the time spent calling is the same between partners, and male

and female calls are not randomly ordered but highly alternated. This particularities fit with most of the essential characteristics used to define vocal duets (Farabaugh 1982; Wickler and Seibt 1982; Hall 2004). In this study, the vocal exchange between partners could thus be described as a duet-like exchange that participates in pairbond maintenance after a stress of separation. Our experimental procedure did not allow to find cues in the exchange that help understanding the information carried by this specific exchange, such as the level of stress of the birds, because none of the acoustic parameters were correlated with the plasma level of corticosterone. Nevertheless, it is important to keep in mind that pairs who did not breed before or who exhibit poor attachment failed to exchange vocalisations, thus did not show the same coordination and could not encode any important information in their vocalisations compared to strong pairs.

Our study did not show any assortative mating on the corticosterone profile of the birds. Instead, our results show that males were more affected by the stress provoked by the experiment than females. Other studies already reported sex differences in plasma levels of corticosterone in different bird species (Astheimer, Buttemer et al. 1994; Wasser, Bevis et al. 1997; Ninnes, Waas et al. 2010). Moreover, recent studies suggest that male birds are more subject to stress as they show stronger increase of corticosterone concentration after an acute stress (Schoech, Ketterson et al. 1999; Marin, Benavidez et al. 2002) or peptide injections (Madison, Jurkevich et al. 2008). Several evidences suggest that males react differently to stress compared to female in songbirds: In song sparrows, developmental stress has deleterious effect on females but no effect or advantageous effects in males in term of weight and standard metabolic rate as adult (Schmidt, MacDougall-Shackleton et al. 2012). A similar effect has been shown in the zebra finch (Verhulst, Holveck et al. 2006). These findings mean that males take advantage of stressful conditions compared to females while nestlings through sibling competition, suggesting that selection is not strong on males' plasma levels of corticosterone as it is for females. These effects of early stress could thus explain the difference of response to acute stress while adult.

Even if males showed a higher corticosterone concentration after a stress, it did not impact their vocal behaviour neither during isolation, nor during acoustic contact with the partner: males did not vocalise for longer or emit more calls than females. This contradicts previous hypotheses considering that the stronger individual signature of males calls would lead to asymmetric vocal interactions between mates, during which males vocalise more so as to be recognized and localized by their female partner (Zann 1996). Corticosterone is also known to lower the song rate (Lynn, Stimplis et al. 2010) but also the general vocal activity of males (Perez, Elie et al. 2012). Our results suggest that vocal interaction between mates after a stress of separation is not driven by the



physiological stress but by the strength of the pairbond. The adjustment of vocal behaviour and the differences in vocal strategy between strong and loose bonds seen in this study could be under the control of other peptides implicated in pairbond establishment and maintenance, such as vasotocin known to modify social behaviour in the zebra finch, especially in males (Goodson and Adkins-Regan 1999; Goodson, Lindberg et al. 2004).

We did not find any difference between sexes in basal levels of corticosterone, which is in contradiction with a recent study suggesting that females show higher basal levels of corticosterone than males in the zebra finch (Khan and Robert 2013). As strong evidences suggest that baseline and stress-induced levels of corticosterone positively covary in birds (Romero and Wingfield 1998; Schoech, Ketterson et al. 1999; Cockrem and Silverin 2002), our results are different from what could be predicted in the zebra finch. Nevertheless, in Khan et al.'s study, the pairing status of the subjects was not assessed and birds were housed in same sex aviaries 8 days prior to experiments, suggesting that they were not paired at the time of sampling. The difference in mating status in our experiment could explain that our results are different from what was expected previously.

Female zebra finches are already known to prefer males with low plasma levels of corticosterone (Roberts, Buchanan et al. 2007). Corticosterone has a strong impact on the fitness of an individual: levels of corticosterone impact the quality of the immune system, with chronic stress having immunosuppressive effects (Buchanan 2000; Sapolsky, Romero et al. 2000). High levels of corticosterone are also associated with poor body condition (Sockman and Schwabl 2001). Finally, chronic elevation of corticosterone can inhibit the reproductive system (Silverin 1998; Wingfield and Ramenofsky 1999). Because we did not find any assortative mating, our results thus suggest that in the zebra finch, mate choice based on physiological traits is driven by females who choose a male with the best quality (i.e. lower corticosterone titre) more than a similar partner.

Our study did not find any assortative mating in the zebra finch based on physiological and vocal profiles. To our knowledge, only two studies reported assortative mating in the zebra finch: one dealt with the preference for familiar kin, thus assortative mating based on genetic traits, but also potentially through self-reference phenotype traits such as physical traits, vocalisations, behavioural tendencies or physiological profile (Slater and Clements 1981). Nevertheless, females showed kin preference only when they were raised with their relatives, whereas no preference for kin was reported when females were raised in foster families (Schielzeth, Burger et al. 2008). The second study showed that individuals pair preferentially with birds that experienced the same rearing conditions as juveniles, but this process is not a mutual choice as only females prefer

males raised in the same condition while males do not show any preference (Holveck, Geberzahn et al. 2011).

Zebra finches form life long pairbond, show low levels of extra pair copulation (2% of the broods in the wild (Birkhead, Burke et al. 1990; Zann 1996), and partners stay together throughout all their activities even outside of the breeding season. Thus, zebra finches show one of the strictest social and reproductive monogamy in birds. Considering results on assortative mating in the zebra finch so far, it is likely that the process of pairbonding in this species is driven by mate choice based on objective qualities (i.e. testosterone levels, immune system strength, body condition) and not on similarity profile. The mate familiarity hypothesis suggests that individuals tend to breed again with partners they already bred with in order to increase their reproductive success (Black and Hulme 1996). This is the case in house sparrow where pairs that already bred together fledged more young than new pairs (Hatch and Westneat 2008). In geese, maintaining long-lasting pairbonds is correlated with the production of more young compared to geese changing partners more often (Black 2001). The performance of continuing pairs compared to new pairs could be a result of improving coordination between partners over the breeding attempts (Black 2001; Hatch and Westneat 2008). Thus, zebra finch partners could adjust their behaviour progressively after pairing, increasing their familiarity throughout reproductive events and improving their similarity and coordination. This process would be different in a less strictly monogamous species, where partners of a pair do not have many attempts of reproduction together, but only one breeding season. In this case, selecting a similar partner could be advantageous, allowing the pair to be quickly efficient in synchronising and coordinating behaviour, without any time-consuming adjustment.

Finally, none of the parameters tested in this study were affected by the origin of the bird, giving evidence that domestication has not modified the acoustical and physiological profiles of mated birds, at least during a stress of social separation. Thus, zebra finches from domesticated lines are good model systems for the study of pair bonding and pairbond maintenance in songbirds.

Overall, this study gives a new evidence of the absence of assortative mating in the zebra finch and shows that strong pairbonds show high coordination in post-stress vocal behaviours.

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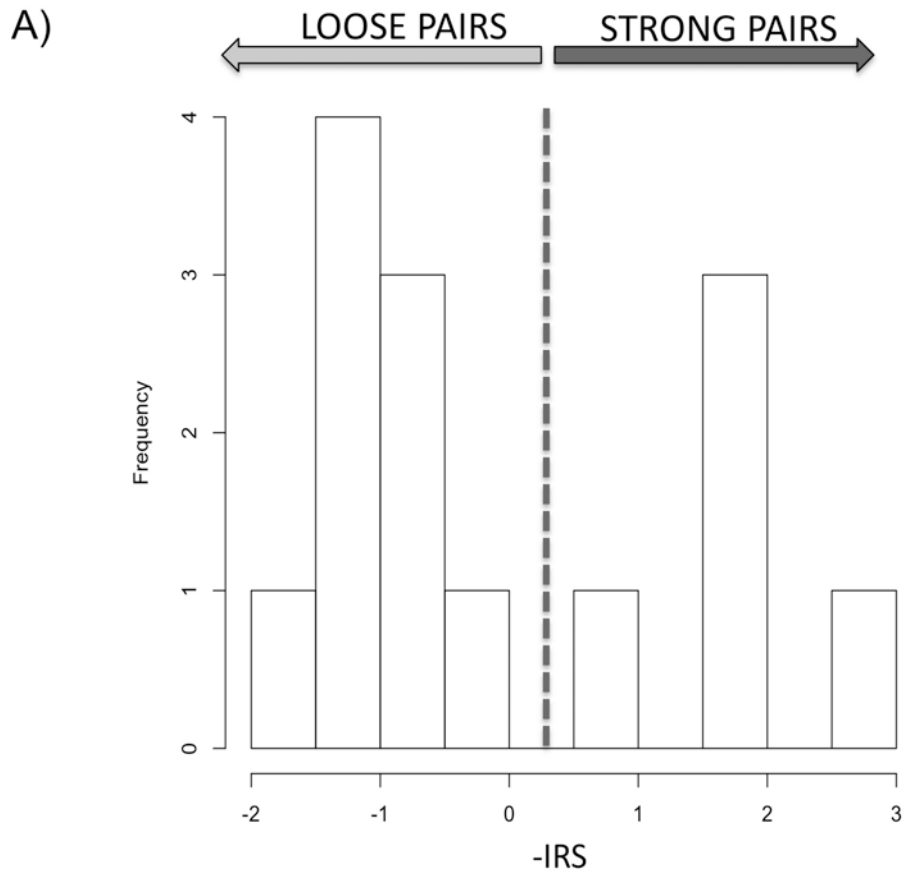
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B)

| Variable     | Comp1    | Comp2     |
|--------------|----------|-----------|
| SONG         | -0.28758 | 0.753368  |
| CLUMPING     | -0.91837 | -0.216093 |
| ALLOPREENING | -0.91753 | -0.077916 |
| SYNCHRONY    | -0.32513 | -0.751476 |
| PROXIMITY    | -0.57456 | 0.518001  |

C)

| Variable  | Comp1    | Comp2    |
|-----------|----------|----------|
| NB_CALL   | -0.83299 | -0.49853 |
| DUR_CALL  | -0.95367 | 0.15045  |
| RATE_CALL | -0.28835 | 0.94258  |

### Supplementary data

(A) Histogram of the index of relationship strength, i.e. PC1 obtained after computation of 5 affiliative variables. The bimodal distribution allowed to separate STRONG pairs from LOOSE pairs.

(B) Contribution of each affiliative behaviour to the first and second axes of the PCA.

(C) Contribution of each acoustic parameter to the first axis of the PCA in isolation.

# CHAPTER 4

## **Less free time for parents with young begging long and high: corticosterone-induced begging modifications affect parental nest visits and foraging.**

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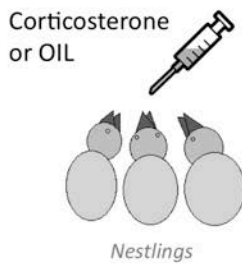
# SUMMARY CHAPTER 4

## Questions

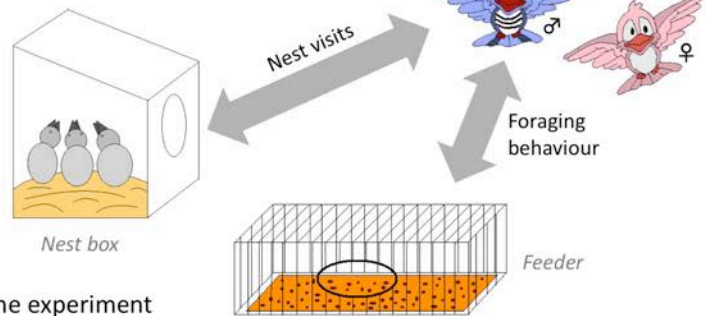
Do nestling Zebra finches encode their stress through modulation of their begging calls?  
Do parents decode the message and respond efficiently to the stress of their nestlings?

## Methods

1-Oral corticosterone treatment and recording of begging calls



2- Monitoring of parental behaviour (Pit-tag system)

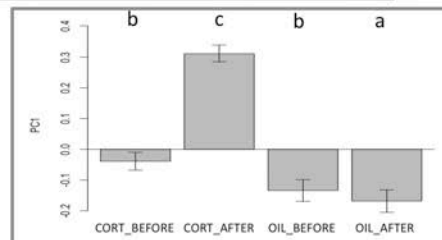


3-Chicks measurements after the experiment

## Results

1-CORT → begging calls with up-shifted frequency spectrum

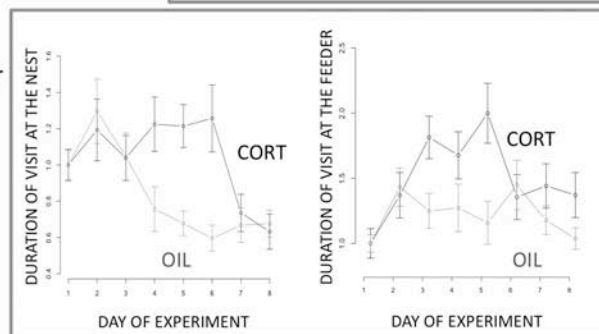
PCA on 9 parameters  
main loading factors : mean frequency (0.94), first quartile of the spectrum (0.94) dominant frequency (0.88).



And longer begging behaviour

2- CORT → modulation of parental behaviour

Longer visits at the nest  
Shorter intervals between 2 visits at the nest  
Longer visits at the feeder  
More visits at the feeders



3- CORT → Better weight gain for nestlings

But no effect on tarsus length

## Conclusions

Our results show that corticosterone modifies conspicuous (duration of begging behaviour) but also inconspicuous signals (acoustic structure of begging calls) of begging behaviour. Parents respond efficiently through an increase in their care of nestlings, which are consequently bigger than nestlings from the control group. Begging calls are thus more flexible than expected.

## **Abstract**

Because their needs vary in time, young birds have to communicate with their parents to receive the appropriate level of resources and care at any time. Nevertheless, the specific components of the signal driving parental feeding have not been completely discovered yet. In nestlings, food deprivation and need of warmth can induce a rise of circulating corticosterone that leads in some species to the emission of begging calls with higher call rate, a conspicuous signal for predators. While recent studies demonstrated that corticosterone provokes a modulation of the fine call structure in adult zebra finches (*Taeniopygia guttata*), we hypothesized that the fine acoustic structure of young begging calls could also vary according to stress level and might be a less conspicuous signal that modulates parental care.

We tested this hypothesis by performing oral administration of either exogenous corticosterone (CORT group) or peanut oil (OIL group) on wild zebra finch nestlings from 6 to 8 days old. We recorded their begging calls directly after administration and during the first parental visits. To verify the effect of our procedure on the parental behaviour, we monitored nest visits and foraging behaviour of parents during 6 days following the oral treatment, using a PIT-tag detection system. Our results show that corticosterone up-shifted the begging calls' spectrum, an effect similar to what has been shown in adults. Corticosterone also increased the amount of nestlings' begging. As a consequence of these modifications of both inconspicuous and conspicuous signals, parents spent more time in the nest and in feeders, and their chicks gained more weight. Begging calls thus show a corticosterone-driven flexibility, which informs parents about their nestlings' physiological state and allows them to give the appropriate care for the survival of the progeny.

## INTRODUCTION

In mammals as well as in birds, dependent young beg for food to adults (Godfray 1991). Many studies have tried to define the specific signal(s) sent by the young that drive parents to an appropriate level of care. The begging display has thus attracted considerable interest for studies on the evolution of animal communication systems.

Begging behaviour is an exuberant performance (Grodzinski and Lotem 2007; Kilner and Drummond 2007) expressed mainly during providers' visits (i.e. parents in most species, or affiliates from the social group in cooperative breeders) and during which the young perform a multimodal display, with visual and/or acoustic cues (Wright and Leonard 2002). Studies put particular effort on understanding the evolutionary origin of the intensity, multimodality and conspicuousness of this behaviour, as previous results showed that the offspring begging most vigorously are provided with more food than siblings begging less (Kilner and Johnstone 1997; Godfray and Johnstone 2000; Wright and Leonard 2002). Several theoretical frameworks have been tested: the parent-offspring communication system has usually been described as a signaller-receiver network with conflicts of interest over resource allocation (Trivers 1974; Godfray 1991; Parker, Royle et al. 2002). From this perspective, selection on offspring to manipulate parents to provide more food results in exaggerated begging displays (Godfray 1991). Other models predict that begging reliably reflects offspring needs, such as scramble-competition for food between siblings models (Trivers 1974; Godfray 1995) or honest signalling models (Royle, Hartley et al. 2002). The multimodality of the signal could be explained either considering the begging behaviour as the result of multiple signals correlated with multiple independent messages about the young's condition (multiple message hypothesis), or as the result of redundant signals of the same message, reducing the probability of errors in emitting and receiving the signal (redundant message hypothesis or backup hypothesis (Moller and Pomiankowski 1993; Johnstone 1996; Jacob, Rieucau et al. 2011)).

These theories have been specifically explored in altricial birds where providers are usually both parents, not only bringing food to the nest but also providing young with water and brooding (covering them to keep them warm or shielding them from the sun or rain) as hatchlings are unable to thermoregulate independently during the first days of life (King and Farner 1961; Radford, McCleery et al. 2001). Visual cues during begging behaviour can inform about the general condition of the nestlings and the nestlings' need, as they can be mediated by the level of hunger (Kilner and Davies 1998; Kedar, Yedvab et al. 2000). Nevertheless, the information carried by visual signals is sometimes paradoxical or not conservative among species (Kilner and Johnstone 1997). Acoustic cues on the other hand are a non-negligible part of the begging display, and also participate to the expression of the nestlings' needs (Iacovides and Evans 1998; Kilner, Noble et al. 1999; Marques, Vicente et al. 2009). The intensity of begging (call

rate, call duration) can signal the nestlings' body condition and/or level of hunger (Roper 1993; Mondloch 1995; Cotton, Kacelnik et al. 1996; Kilner and Johnstone 1997; Royle, Hartley et al. 2002; Anderson, Brunton et al. 2010) and can modify parents feeding behaviour (Kitaysky, Wingfield et al. 2001).

Several authors have underlined the apparent contradiction between the conspicuousness of begging and the associated predation costs, as intense begging strongly attracts predators (Leech and Leonard 1997; Dearborn 1999; Haskell 2002; McDonald, Wilson et al. 2009; Haff and Magrath 2011). However, very few studies have considered the inconspicuous component of begging behaviour as a possible signal in parent-offspring communication. Fine modifications of acoustic features could encode changes in offspring needs and potentially have lower predation cost than increasing begging intensity. A variety of acoustic parameters of the begging calls' structure are already known to be modulated by the level of hunger, body temperature or body condition (e.g. frequency modulations, (Leonard and Horn 2001; Gladbach et al. 2009; Marques, Vicente et al. 2009; Anderson, Brunton et al. 2010; Reers and Jacot 2011). Thus, considering begging behaviour as a display composed of conspicuous and inconspicuous signals could increase our understanding of the apparent paradox between begging behaviour design and associated predation risk. In the present paper, we study both conspicuous (call duration) and inconspicuous (begging call structure) characteristics of begging behaviour.

Many studies on begging display only manipulated the level of hunger of the chicks, while other intrinsic parameters such as the need for warmth can modulate begging behaviour. Moreover, these parameters are usually correlated with modifications in levels of corticosterone (Kitaysky, Kitaiskaia et al. 2001; Loiseau, Sorci et al. 2008; Fairhurst, Treen et al. 2012), the main stress hormone in bird. Modifications of corticosterone concentration are already known to have several impacts on begging behaviour (Smiseth, Scott et al. 2011) such as increasing the begging rate (Loiseau, Sorci et al. 2008) or the latency to beg (Wada and Breuner 2008). Considering these effects as well as the multiple effects of corticosterone in adults (facilitating foraging behavior, triggering irruptive migration, mobilizing stored energy resources to fuel increased locomotor activities, (Astheimer, Buttemer et al. 1992; Bray 1993; Wingfield and Ramenofsky 1997; Kitaysky, Kitaiskaia et al. 2001), and its particular role in energy mobilisation (Remage-Healey and Romero 2000; Kitaysky, Kitaiskaia et al. 2001; Norris 2006), we believe corticosterone could be the main physiological component adjusting begging behaviour to nestlings' needs. In the present paper, we study the effect of an oral administration of exogenous corticosterone on the begging behaviour of wild zebra finches.

If begging behaviour is an honest signal, it should reflect the young's real need and thus lead to an adjustment of parental care, and especially feeding behaviour (Redondo and

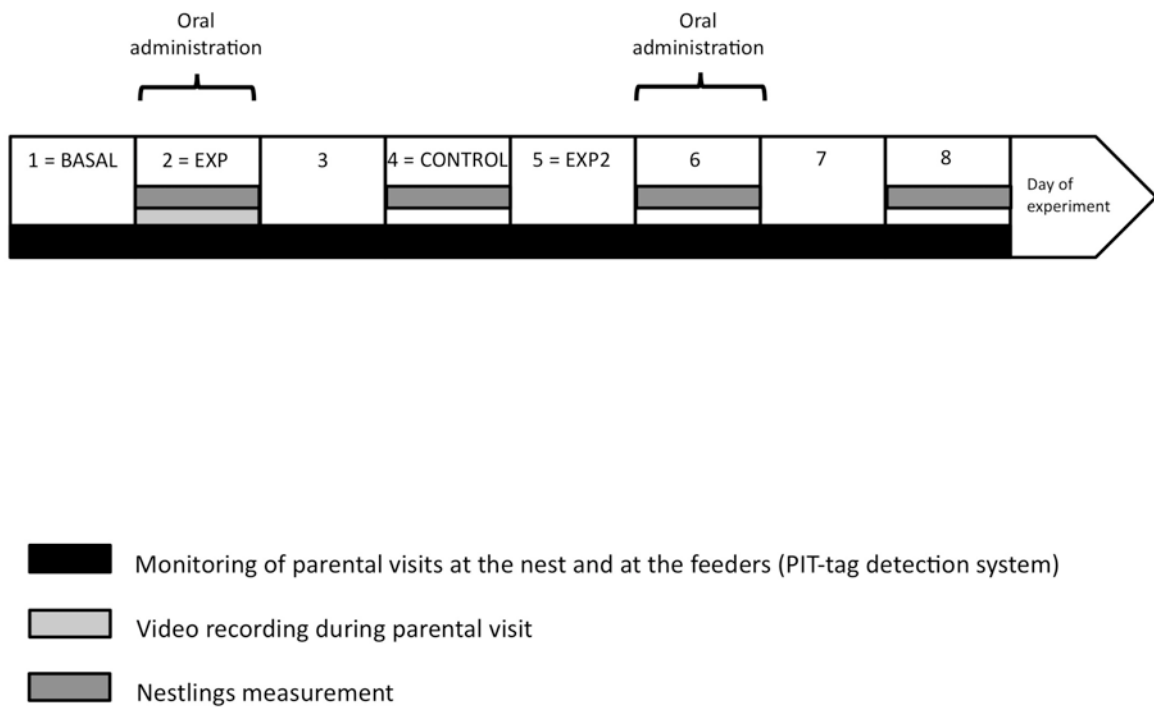
Castro 1992; Kilner and Johnstone 1997; Budden and Wright 2001; Royle, Hartley et al. 2002; Smiseth, Wright et al. 2008; Mock, Dugas et al. 2011). While some studies have showed that an increase in begging can affect the parental allocation of food (Redondo and Castro 1992; Cotton, Kacelnik et al. 1996; Ottosson, Bäckman et al. 1997; Granadeiro, Bolton et al. 2000; Leonard and Horn 2001), some studies reported no effect on parental care (Ricklefs 1992; Clark and Lee 1998). However, most studies evaluated the parental feeding response to begging behaviours but did not assess the parental foraging response, and thus failed to acknowledge that the ability of parents to respond to their offspring demand may vary with resource availability and the parents' ability to find food. If parental foraging activity is not measured, it is impossible to distinguish cases where parents do perceive and respond to a change in offspring demand from those where they perceive offspring needs but are unable to respond to it. Therefore, estimating parental foraging activity is essential to determine whether change in nestling begging calls influences parents' behaviour. In the present paper, we assess the consequences of begging modification on both the parents' nestling provisioning and foraging behaviours.

The zebra finch has been used for decades as a model for the study of sexual selection and reproductive investment (Griffith and Buchanan 2010). Parent-offspring communication at the nest and parental care has also been described and is now well documented (Zann 1996; Gilby, Mainwaring et al. 2011; Gilby, Mainwaring et al. 2011; Gilby, Sorato et al. 2012). Wild zebra finches live in Australian semi-arid zones, where predation risk is high and food availability is variable in space and time (Griffith, Pryke et al. 2008). This model species is thus a good candidate to study fine modifications in begging behaviour in a system where there is 1) a real trade off on begging behaviour of nestlings between soliciting for food and attracting predators, and 2) a strong food allocation choice for parents. Using oral administration of exogenous corticosterone in nestlings, we hypothesized that an elevation of corticosterone levels would (i) change the begging behaviour and the acoustic structure of the begging calls of supplemented offspring and (ii) lead to parental care adjustments. We predict that parents will adjust by modifying not only their feeding behaviour (visits to the nest and feeding during visits), but also their foraging activity to food patches. We therefore conducted a comprehensive study of begging behaviour modulation, from hormonal action to parental response.

## **METHODS**

### **Study site and subjects**

Data were collected over three months from September to November 2009, in a nest-box breeding colony of wild zebra finches at Fowlers Gap Arid Zone research station, western New South Wales, Australia (31 ° 05' S, 142 ° 42 ' E). Experiments were



**Figure 1: Overview and timeline of experimental procedure.**

Day 1 was used as a control for basal parental behaviour, as the nests were left undisturbed. Oral administration of either exogenous corticosterone dissolved in peanut ("CORT" group) or oil only ("OIL" group) was done on Day 2 and again on Day 5. Nestlings and parents behaviour were then followed by video recording directly after administration on Day 2. Nestlings were measured and weighted on Days 2, 4, 6 and 8 of the experiment. Parental visits were monitored at their nest and at the feeders placed in the area during the whole experiment.

approved by the Animal Ethics Committee of Macquarie University, NSW (Animal research Authority, authorization N° 2009/032 and 2007/038). Throughout the entire breeding season, birds were provided with commercial finch seed (e.g. canary seed and white millet) in feeders, placed in different areas within the colony. Each feeder consisted of a 50x30 cm seed tray in a wire finch holding cage (70x40x50 cm) on the ground, that birds accessed by walking through an 11x11 cm door. Empty nest-boxes were checked every three days, but once parents initiated nest building, the boxes were checked once daily. After hatching, chicks were identified by cutting the feather patches on their head using specific combination of hair cut, and the oldest chick in each brood was defined as RANK 1, the second as RANK 2, and so on. Thirty two active nest-boxes were used for this experiment, with a total of 96 nestlings, brood size varying from 1 to 5 chicks ( $3 \pm 1.27$ ).

### **Timeline of the experimental protocol**

For an overview of the timeline of the experimental procedure, see figure 1.

- Day 1= BASAL. Nests were kept undisturbed during one day. Results from this day served as baseline.
- Day 2: EXP. Oral administration of corticosterone to nestlings and audio recording of the begging calls (approximately 1h disturbance). Video recording during parental visits and nestlings measurement.
- Day 3: undisturbed.
- Day 4: nestlings measurement.
- Day 5: EXP2. Second oral administration of corticosterone to nestlings.
- Day 6: nestlings measurement.
- Day 7: undisturbed.
- Day 8: nestlings measurement.

### **Corticosterone administration and recording of nestling begging calls.**

To assess the effect of physiological stress on the begging behaviour of nestlings, we performed on Day 2 (see above) an oral administration of exogenous corticosterone dissolved in peanut oil (10  $\mu$ l, 2.5 $\mu$ g, C=0.25mg/mL, as in Spencer 2007) on chicks aged between 6 to 8 days post hatching (experimental group=CORT, n= 50 chicks in 17 nests). Nestlings from the control group received oral administration of peanut oil only (Control group=OIL, n=46 chicks in 15 nests). Nest-boxes were randomly assigned to the experimental treatment (OIL versus CORT), so all nestlings from the same nest received the same treatment.

On Day 2 (EXP, see figure 1 for timeline), the nest-box was moved for manipulation 50m away from its position and replaced with an empty nest-box with a closed entrance, so

that parents could see but not enter the nest. Nestlings stayed in their nest-box, which was taken away from the site of the nest to be recorded at two different times: BEFORE the oral administration of CORT/OIL, and exactly 10 minutes AFTER the administration. Thus, 4 CONDITIONS are defined to analyze the calls (CORT\_BEFORE, CORT\_AFTER, OIL\_BEFORE and OIL\_AFTER). We also defined the factor MOMENT composed with 2 levels, BEFORE and AFTER the oral administration. Before administration, the motivation of each chick to emit begging calls was evaluated by tapping on its beak with a red pencil, mimicking the parents' bill colour. As long as the chick did not emit begging calls in response to tapping, the brood was kept inside the nest-box away from parents so as to increase fasting duration and thus motivation. The time spent waiting for begging calls ranged from 5 to 80 min (mean 21.7 min  $\pm$  19.8 min); not long enough to elicit a significant increase in the level of plasmatic corticosterone (figure S1.B supplementary data). After this first recording, oral administration of corticosterone or oil was performed using a pipette equipped with a 10 $\mu$ L tip. A red pencil was used to tap the beak to elicit begging, and the pipette tip was quickly introduced in the open bill to release the fluid into the chick's throat. Nestlings were then kept in their nest-box, and recorded again exactly 10 minutes after oral administration, by again tapping the beak with a red pencil. Begging calls were recorded using a tie microphone (AKG C 417 Clip-on Microphones) located 10 cm above the subject and connected to a recorder (Marantz PMD 670, sampling rate: 44.1 kHz, PCM 16bit). After recording, nestlings were measured (weight and tarsus length, see details below) and replaced in their nest. The nest-box was then moved back to its original position.

A second administration of either corticosterone (CORT group) or oil (OIL group) was performed on Day 5 when nestlings were between 9 and 11 days post hatching to determine the effect of nestling stress on parental behaviour.

### **Analysis of begging calls**

Begging calls were first extracted from the original recordings of 30 nests using a custom-written program (PYTHON programming language, open-source libraries). Begging calls are emitted in bouts, which consist of a rapid succession of short syllables. In this study, we analyzed the information that could be extracted from single calls and did not analyze the structure of the bout. Calls were then analyzed using the Seewave package (Sueur et al., 2008) implemented in R software (R Development Core Team, 2007, [www.r-project.org](http://www.r-project.org)) so as to extract temporal, spectral and statistical parameters on each syllable. A PCA was then performed on the z-scores of 7 parameters of interest and statistics were performed on its first two first axis, accounting for 71% of the variance and showing an eigen-value above 1) (for a detailed description of the procedure, see supplementary material B.).



## **Brood and parental behaviour during nest visits**

Nestlings and parental behaviour was monitored in the nest after oral administration (Day 2=EXP) on 8 nests (n=3 for the CORT group, n=5 for the OIL group). A camera (colour CCD cameraHK-C3, Handykam, [www.handykam.com](http://www.handykam.com)) was attached to the nest-box lid pointing downward into the nest cup, and videos were recorded onto an external hard drive (Archos 605, 160 G memory) that allowed continuous filming for several hours (Gilby 2011). Cameras were placed in boxes at least 24 h prior to filming to allow birds to habituate to their presence. Videos were scored by an observer (PC) blind to the treatment and in a random order with respect to the treatment. Using Etholog software (Ottoni 2000), the following parameters were measured.

-Parental feeding behaviour was evaluated using the following parameters:

- \* Duration of the visit.
- \* Number of feedings, quantified by the heaving motion of the parent.
- \* Sex of the parent visiting (identified using the dimorphic plumage of adults).

A minimum of three parental feeding visits were analysed for each nest, with an overall range of 3–20 feeding bouts per nest.

- Nestlings' behaviour: because the presence of parents inside the nest masked part of the nestlings, we scored the brood behaviour and not the behaviour of each chick inside the nest individually.

- \* Latency to beg: time between the entrance of the parent and the first begging behaviour.
- \* Latency to emit begging calls: time between the entrance of the parent and the first begging call.
- \* Duration of begging behaviour in seconds: starts when the first chick begin to beg, and stops when the last chick stops begging.
- \* Duration of begging call emission in seconds: starts when the first chick emits begging calls, and stops when the last chick stops emitting begging calls.

The two last parameters were normalized by dividing by the duration of the visit, to avoid differences related to parental behaviour or brood size.

As these parameters of the nestlings' behaviour were correlated, a behavioural index was computed using a PCA on the four parameters. The first Axis of the PCA (accounting for 46 % of the variance and showing an eigen-value above 1) was then used for statistical analysis.

### **Parental behaviour monitoring using PIT-tag detection system**

A passive integrated transponder (PIT) tag detection system was used to quantify parental visits at the nest and at the feeders (Mariette, Pariser et al. 2011). Adults were caught in the morning at the nest with a nest trap when nestlings were between 4 and 6 days old. When possible, both the male and the female of the nest were fitted with a PIT-tag (11 X 2 mm, Trovan, UK) glued onto a plastic colour ring, and with an individually numbered metal band supplied by the Australian Bird and Bat Banding Scheme. Their nest-box was then replaced with an identical nest-box equipped with a PIT-tag detection system (LID-665 decoder, Trovan, UK) that automatically recorded the unique identification number of any tagged bird going through the nest entrance (with a visit detection probability 99%; (Mariette, Pariser et al. 2011)). Feeders placed in the area were also fitted with PIT-tag detection systems, allowing monitoring parental visits at the feeders. Parental visits at nest and feeders were monitored from Day 2 until Day 8 and quantified with the following parameters:

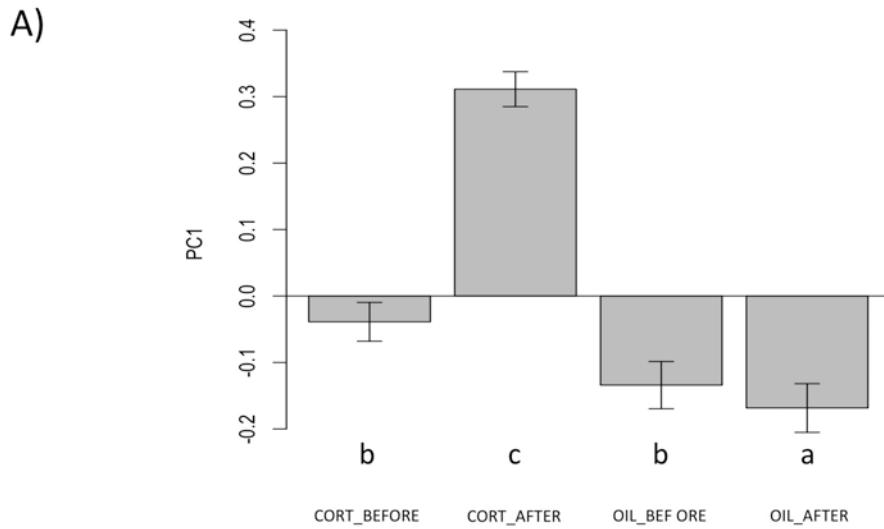
- \* Number of visits per day
- \* Duration of the visits in seconds
- \* Duration between visits in minutes (only for nest visits)

Data were normalized by dividing by the mean of the values of the parameter obtained on basal day for the same individual, i.e. Day 1. This allowed assessing the progression of parental visits over time. Data are presented for a total of 23 nests (n=11 nests for the CORT group, n=12 nests for the OIL group) : 5 nests with both parents equipped with pittags and 18 nests with only one parent equipped with a pittag (7 with only female, 11 with only male).

### **Nestling's growth measurements**

To monitor nestlings' growth, we measured tarsus length with a digital calliper ( $\pm 0.1$  mm) and mass with an electronic scale ( $\pm 0.01$  g) at Day 2, Day 4 and Day 6 and Day 8 of experiment.

The nestlings' body condition was assessed using the residuals of the linear regression between the tarsus length and the weight at Day 6 of experiment for 30 nests (Schulte-Hostedde, Zinner et al. 2005). The direct gains in weight and in tarsus length were also assessed for each nestling of 25 nests (n= 36 chicks in 12 nests for the CORT group, n=40 chicks in 13 nests for the OIL group) by subtracting for each parameter the value at Day 2 ( $8 \pm 1$  day-old, before oral administration) to the value at Day 6 ( $12 \pm 1$  day-old, after second oral administration), period during which the growth rate is still linear ((Skagen 1988; Martins 2004). Weight gain was log transformed to meet with normality and homoscedasticity criteria.



B)

|  | Comp1      | Comp2      |
|--|------------|------------|
| Mean   | 0.9380400  | -0.2011013 |
| Standard deviation of the mean               | -0.3281055 | -0.8698358 |
| Interquartile range                          | -0.1687237 | -0.9257082 |
| Dominant frequency                           | 0.8877265  | -0.1125325 |
| Fundamental frequency                        | -0.4497727 | 0.2879019  |
| Standard deviation of the dominant frequency | 0.3724777  | -0.4195402 |
| First quartile                               | 0.9449546  | 0.1404428  |

**Figure 2: Effect of oral administration of exogenous corticosterone on acoustic features of begging calls.**

A) Composite score of acoustic structure of the calls (Axis 1 of the PCA computed on seven acoustic parameters) "BEFORE" and "AFTER" administration of either corticosterone dissolved in peanut ("CORT") or oil only ("OIL"). Bars are mean  $\pm$  SE. Different letters indicate a significant difference between pairs at the  $P < 0.05$  level.  $n=5960$  calls for the CORT group,  $n=5780$  calls for the OIL group. Begging calls' spectrum is up-shifted in CORT chicks and down-shifted in OIL chicks after treatment. (Post hoc tests:  $p < 0.05$  for all comparisons except OIL\_BEFORE vs CORT\_BEFORE  $p=0.17$ ).

B) Contribution of each acoustic parameter to the first two Axes of the PCA.

## Statistical analysis

All statistics were performed using R software

Linear mixed models using “lmer” function from “lme4” package were fitted using the adapted distribution:

\*gaussian for the PCA resulting on nestling’s behaviour during parental visits, the PCA built on acoustic parameters of begging calls, the duration of visits of parents, and data of nestling’s growth.

\* poisson for the number of feeds during parental visits and the data from parental behaviour monitoring at the nest and at the feeder.

All linear mixed models were originally performed with the GROUP as fixed factors, with three exceptions: in the analysis of the parents’ behaviour monitored with PIT-tag, the DAY of the experiment was also used as a fixed factor, we added the RANK of the nestling for the data on nestlings’ weight and tarsus gain, and we also used the MOMENT as a fixed factor for the analysis of acoustic parameters. The identity of the nest, the brood size and the sex of the parent were used as a random factor, except in the analysis of residuals for which we also used the RANK of the nestling, and for the analysis of acoustic parameters for which we added the identity and the age of the nestling. To simplify the model, we followed a backward stepwise procedure where least significant terms were sequentially removed. Successive models were compared using the “anova” function, and a factor was removed when  $p\text{-value} > 0.05$ .

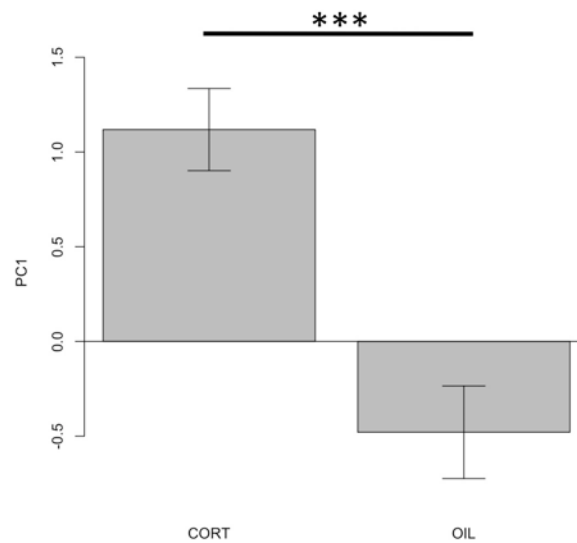
Post hoc tests for linear mixed models with normal distribution were performed using Multiple Comparisons of Means with Tukey Contrasts, using the “glht” function from the “multcomp” package with the variable of interest as object of class “mcp”.

As simplification lead to models with no random effects for the duration of parental visit at the nest and the PCA based on nestlings’ behaviour during parental visits, these data were analysed t-tests.

## RESULTS

The first axis of the PCA on acoustic parameters (figure 2B) accounted for 43% of the variance in calls. The main loading factors were the mean frequency (0.94), the first quartile of the spectrum (0.94) and the dominant frequency (0.88), so positive values of the first component signed calls with high-frequency spectral content (figure 2B). The first component was affected by the GROUP and the interaction between the GROUP and the MOMENT. Oral administration of corticosterone evoked higher-frequency begging calls than those recorded before the administration, while the oral administration of OIL evoked lower-frequency begging calls compared to baseline (Post hoc tests:  $p < 0.05$  for all comparisons except OIL\_BEFORE vs CORT\_BEFORE  $p = 0.17$ , figure 2A). thus begging calls’ spectrum is up-shifted in CORT chicks and down-shifted in control chicks

A)



B)

|                               | Comp1      | Comp2      |
|-------------------------------|------------|------------|
| Duration of begging behaviour | 0.8840289  | -0.2554011 |
| Duration of begging calls     | 0.8429469  | 0.3293466  |
| Latency to beg                | -0.4511382 | 0.6951700  |
| Latency to emit begging calls | -0.3554567 | -0.7364555 |

**Figure 3: Effect of oral administration of exogenous corticosterone on nestlings begging behaviour.**

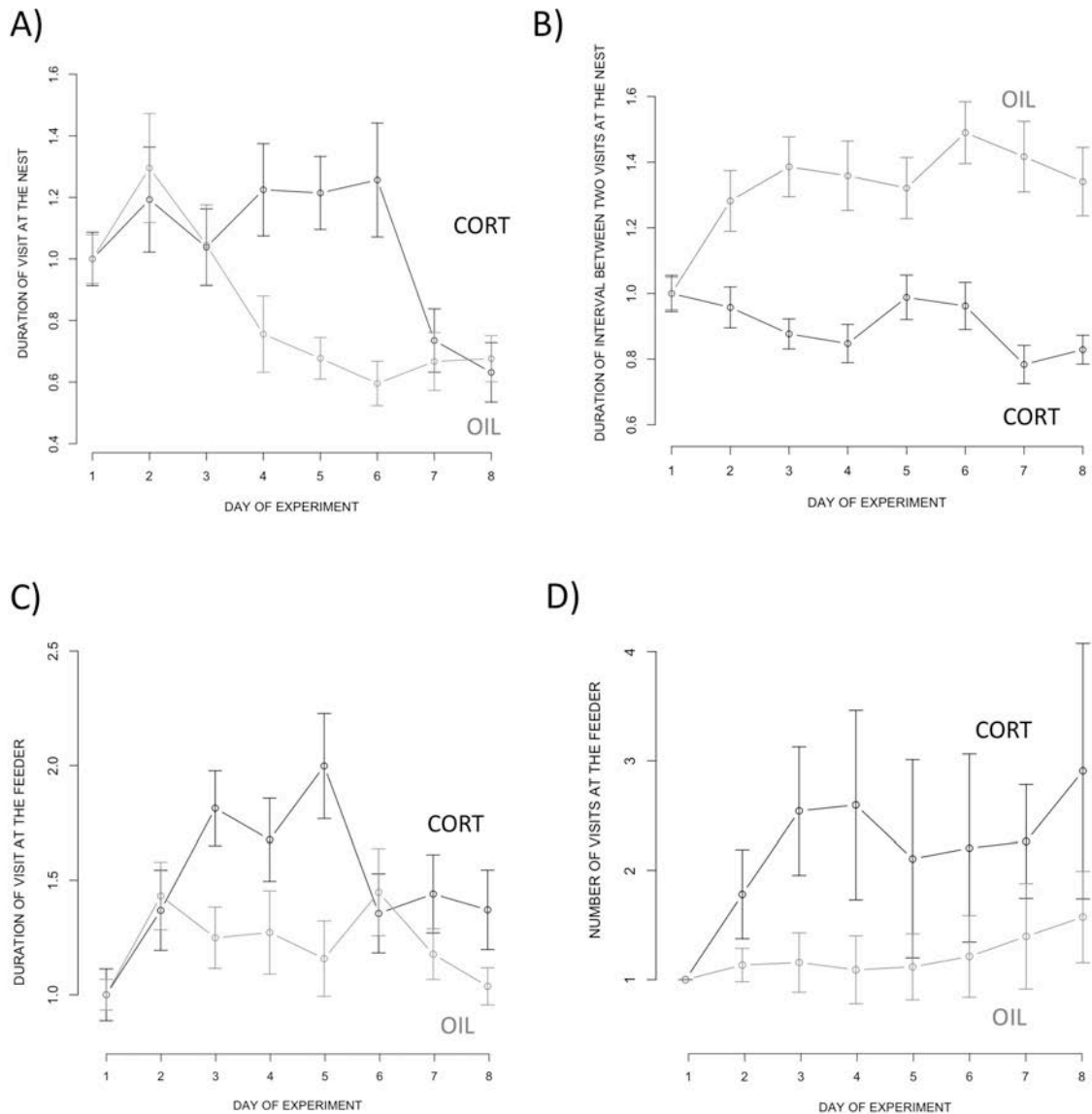
A) Composite score of begging behaviour of nestlings (Axis 1 of the PCA computed on four behavioural parameters), after administration of either corticosterone dissolved in peanut ("CORT") or oil only ("OIL"). Bars are mean  $\pm$  SE. n=3 nests for the CORT group, n=5 nests for the OIL group. CORT nestlings beg significantly more than OIL nestlings (CORT vs OIL : two sample t test with equal variance,  $p < 0.001$ ).

B) Contribution of each behavioural parameter to the first two Axes of the PCA.

The second axis of the PCA accounted for 28% of the variance. The main loading factors were the interquartile range (-0.92) and the standard deviation of the spectrum (-0.87), so negative values of the second component indicate calls with a more broadband spectral content. The second component was only affected by the MOMENT (BEFORE vs AFTER, Post hoc test:  $p < 0.001$ ), thus, both oral treatments provoked the emission of calls with a more broadband spectral content.

In the analysis of nestlings' behaviour during parental visit, the first axis of the PCA accounted for 46% of the variance (figure 3B). The main loading factors were the normalized duration of begging behaviour (0.88) and the normalized duration of begging call emission (0.84). CORT chicks begged for longer than control chicks (two sample t test, samples with equal variances, CORT vs OIL  $p < 0.001$ , figure 3A). The second axis of the PCA accounted for 30% of the variance. The main loading factors were the latency to emit begging calls (-0.74) and the latency to beg (0.69) (figure 3B). The second axis was not affected by the GROUP: the treatment did not modify the latency to start begging behaviours (Welch two sample t test, samples with unequal variances,  $p = 0.6$ ).

During visits, the number of feedings by parents was not affected by the GROUP (OIL vs CORT :  $p = 0.13$ ) neither was the duration of the visit (two sample t test, samples with equal variances,  $p = 0.12$ ). Thus parents from CORT chicks do not show any behavioural change during their first visits at the nest after nestlings' treatment. Nevertheless, parents of CORT chicks spent more time in the nest and in feeders than parents of OIL chicks for several days after treatment: The duration of the visit at the nest was affected by the Day (GLMM:  $z = -11.896$ ,  $p < 0.001$ ), and the interaction between the Day and the GROUP ( $z = -23.681$ ,  $p < 0.001$ ). While parents of the control group decreased progressively the duration of the visits after Day 3, parents of the CORT group decreased the duration of the visits only at Day 7 (figure 4A, for all post hoc comparisons, see figure S2). The duration of the interval between visits at the nest was affected by the Day (GLMM:  $z = 3.84$ ,  $p < 0.001$ ), and the interaction between the Day and the GROUP ( $z = 17.64$ ,  $p < 0.001$ ). While parents of the control group increased progressively the duration between visits after Day 3 (figure 4B and figure S3), parents of the CORT group did not modify this interval. The duration of the visit at the feeders was also affected by the Day (GLMM:  $z = -5.187$ ,  $p < 0.001$ ), and the interaction between the Day and the GROUP ( $z = 23.154$ ,  $p < 0.001$ ). Parents of the CORT group foraged for significantly longer in the feeders between Day 3 and Day 5 than parents of the OIL group (figure 4C and figure S4). The number of visits at the feeders was affected by the Day (GLMM:  $z = 22.048$ ,  $p < 0.001$ ), and the interaction between the Day and the GROUP ( $z = 18.561$ ,  $p < 0.001$ ), with parents of the CORT group increasing significantly the number of visits at



**Figure 4: Parental visits at the nest and at the feeders during the whole experiment.**

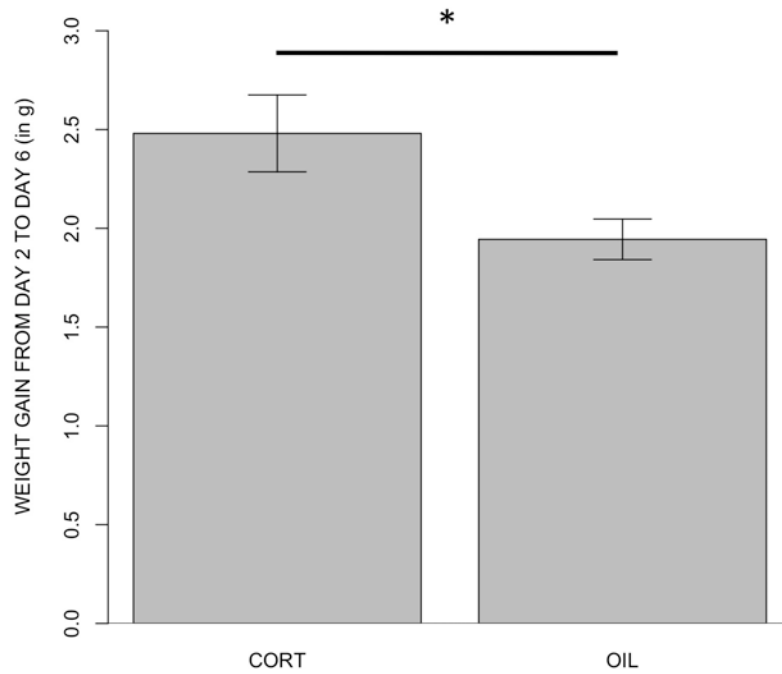
All data from Day 2 to Day 8 were normalised by dividing by baseline, i.e. Day 1. n=11 nests for the CORT group, n=12 nests for the OIL group.

A) Duration of visits at the nest. While parents of the OIL group decreased progressively the duration of the visits after Day 3, parents of the CORT group decreased the duration of the visits only at DAY 7 (see Fig.X for all post hoc comparisons).

B) Duration of interval between two visits at the nest. While parents of the OIL group increased progressively the duration between visits starting at DAY 3, parents of the CORT group did not modify this interval (see Fig.X for all post hoc comparisons).

C) Duration of visits at the feeders. Parents of the CORT group foraged significantly longer in the feeders between Day 3 and Day 5 than parents of the OIL group (see Fig.X for all post hoc comparisons).

D) Number of visits at the feeders. Parents of the CORT group increased significantly the number of visits at the feeders on Day 3.



**Figure 5: Effect of the treatment on nestlings weight gain.**

Weight gain is defined as the difference in weight (in g) between Day 6 and Day 2. Bars are mean  $\pm$  SE. n= 36 chicks in 12 nests for the CORT group, n=40 chicks in 13 nests for the OIL group. CORT nestlings gain significantly more weight between Day 2 and Day 6 than OIL nestlings (OIL vs CORT, Post hoc test: p=0.04).



the feeders on Day 3 (figure 4D and figure S5). Finally, the number of visits at the nest was not affected neither by the Day, nor by the GROUP nor by the interaction between the Day and the GROUP.

The nestling's body condition at Day 6 was not different between the two groups, but CORT chicks gained more weight than control chicks as weight gain between Day 2 and Day 6 was affected by the GROUP and the interaction between the GROUP and the RANK. (OIL vs CORT, all RANKS together, Post hoc test:  $p=0.041$ , Figure 5). This result was mainly due to nestlings from RANK1, the only ones to show a significant difference between CORT and OIL nestlings when analysis was performed on each RANK (OIL vs CORT from RANK 1, Post hoc test:  $p=0.018$ ,  $p>0.05$  for all other comparison in RANKS 2 from 5). On the other hand, CORT chicks did not gain more tarsus length than control chicks.

## **DISCUSSION**

As predicted, our results show that an oral administration of corticosterone change conspicuous signals (duration of visual and acoustic begging display) but also inconspicuous signals (acoustic structure of begging calls) of nestlings' begging behaviour. Whereas no immediate effect on parental feeding behaviour was measured (number of feedings or duration of the first visit at the nest), these changes in begging display led to modifications in patterns of parental visits at the nest as well as foraging activity lasting several days: parents of corticosterone-treated nestlings increased the time spent at the nest and at the feeders, having thus potentially less free time for other activities. Finally, four days after the first corticosterone administration, CORT chicks had gained more weight than control chicks.

### **Corticosterone treatment modifies conspicuous signals of begging behaviour**

The use of exogenous corticosterone to mimic a modification of the chicks' needs is relevant in this study as (i) dietary restriction can enhance plasma level of corticosterone in adults and nestlings (Kitaysky, Kitaiskaia et al. 2001), (ii) an experimental elevation of baseline corticosterone increases begging behaviour intensity (Kitaysky, Wingfield et al. 2001), while (iii) in high predation risk context, nestlings can show a decrease in basal plasma levels of corticosterone (Ibanez-Alamo, Chastel et al. 2011), as a possible mechanism to reduce begging activity and thus the risk of predation. Corticosterone appears as a central hormone involved in regulating begging behaviour with intrinsic but also environmental conditions.

In our study, corticosterone treatment enhanced conspicuous signals of begging, with CORT nestlings begging for longer, considering both begging movements and calls, than

OIL nestlings. This effect is strong, even with only 8 nests used to quantify these parameters, and this finding is congruent with many previous studies (Kitaysky, Wingfield et al. 2001; Loiseau, Sorci et al. 2008). Estimating whether this increase in begging duration enhanced the risk of predation (Leech and Leonard 1997; Briskie, Martin et al. 1999) was not possible in our population, as predation rate in the nest-boxes used in this study is very low compared to natural grass nests (2% of the nesting attempts (Griffith, Pryke et al. 2008) compared to 66% in natural nests (Zann 1994). Moreover, parents' behaviour can influence begging duration: zebra finch parents synchronise their nest visits in 78% of the cases, thus reducing by up to 50% the number of begging sequences emitted by the nestlings during a day (Mariette and Griffith 2012). We were rarely able to equip both parents of the nest with a PIT-tag in this study (5 out of 23 nests), thus our data set did not allow to assess a potential effect of the corticosterone treatment on parents' nest visit synchrony. One possible prediction is that parents could increase nest visit synchrony in response to an increase in begging conspicuousness due to nestlings' needs. It seems also possible that closed neighbours could synchronise their visits at the site and consequently reduce begging calls occurrence and thus predation risk at the site.

Begging call rate (number of begging syllables per time unit) is another conspicuous signal known to be modified by nestling's condition and need (Loiseau, Sorci et al. 2008). In this study, we did not analyse this parameter for two reasons: 1) during parental visits, it was not possible to separate out the individual vocalisations of each nestling, which often overlap, making it impossible for call rate calculation, 2) during the recordings of begging calls directly after oral administration of corticosterone, the experimenter activated several times the chick by ticking its bill so as to record enough calls for analysis. Thus, call rate here could be correlated with the action of ticking more than to the chicks' needs. The same argument holds true for call amplitude, another potential conspicuous parameter of begging calls.

### **Corticosterone treatment modifies also inconspicuous signals of begging behaviour**

Interestingly, inconspicuous signals are also modified by oral administration of corticosterone, as begging calls emitted by CORT nestlings showed an up-shifted spectrum. Corticosterone is already known to have effects on begging behaviour (Kitaysky, Wingfield et al. 2001) but our study is the first demonstration of its direct effect on acoustic parameters of begging calls. In a previous study (Perez, Elie et al. 2012), we already demonstrated that corticosterone impacts the acoustic structure of the Distance Calls of adult male zebra finches. Stressful situations can modify vocalisations in numerous mammalian species. In rats, social isolation is a strong social stress that increases plasma levels of corticosteroids (Grippe, Gerena et al. 2007) and

ultrasonic vocalisations emitted during social encounters show a higher peak frequency when individuals were first isolated instead of being in social context prior to encounter (Chabout, Serreau et al. 2012). Isolated rodent pups emit ultrasonic vocalisations modulated in frequency (Ehret 2005). Females bottlenose dolphins with dependent calves produce whistles with higher maximum frequencies during capture-release sessions (Esch, Sayigh et al. 2009). Stressful situations in humans modify the mean fundamental frequency and the pitch of the speech (Banse and Scherer 1996). In birds, alarm calls emitted on the sight of a predator show a convergent high-pitched structure (Marler 1955; Smith 1965), with peak frequency, frequency modulations and frequency bandwidth being strong predictors of the flying response of both conspecifics and heterospecifics (Wood, Sanderson et al. 2000; Fallow, Pitcher et al. 2013). Our results on a bird species together with these precedent findings in birds and mammals confirm the possibility of a universal signal of stress among Vertebrates, encoded in vocalisations with up-shifted frequency spectrum. This possibility of an universal signal of stress is congruent with studies in mammals in which emotions with negative valence and/or strong arousal tend to provoke the emission of vocalisations with up-shifted energy distribution among the frequency spectrum (stress being defined as a negative emotion of high arousal, Review (Briefer 2012)), and vocalisations emitted in fearful situations should be closed to high tonal sounds (Morton 1977).

How can corticosterone modulate acoustic parameters of calls in the zebra finch? The tracheobronchial syrinx of songbirds has several pairs of intrinsic muscles specialized for controlling particular aspects of sound production such as temporal and spectral properties of vocalisations (Suthers 2001). A recent study in house sparrow has shown that corticosterone receptors are present in muscle tissues in birds (Lattin, Waldron-Francis et al. 2012), thus corticosterone could have a direct effect on muscles controlling the syrinx. If birds vocalisations fit the source-filter theory applied in mammals (Fant 1960; Titze 1994; Mindlin and Laje 2005), a modification of the parameters of the syrinx should provoke a modification of the fundamental frequency, which is not the strongest effect of exogenous corticosterone in the present study and in a previous one (Perez, Elie et al. 2012). In passerine, begging behaviour of nestlings is a typical set of movements with neck stretched upwards and head raised with or without head bobbing, mouth opened, tongue wagging and emission of begging calls (Wright and Leonard 2002). Thus, the changes in body and head postures added to the action of corticosterone on vocal tract muscles could modify the filter, i.e. the length, the shape and the narrowness of the vocal tract, known to modify several acoustic parameters leading to a shift in energy distribution in the frequency spectrum (Briefer 2012). This hypothesis is also supported by a recent study in zebra finches showing that modification of the vocal tract by opening the beak emphasizes high frequencies above 5 kHz and over on song syllables (Ohms, Escudero et al. 2012).

**Parental response varies over time, showing changes in nest visits pattern and foraging activity over several days.**

In this study, parents showed no visible response to the changes in nestlings' begging behaviour during the first visits after treatment, as CORT chicks did not receive more feedings than OIL chicks. In a previous study, variation in begging behaviour strongly predicted the amount of food that individual nestlings received from their parents during a nest visit (Gilby, Mainwaring et al. 2011), but the parameters analysed were not related to the duration but to the strength of the begging behaviour (number of head and tongue movements, degrees of mouth opening and straightening of the head). It is possible that the two experimental groups of the present study did not differ in parameters reflecting begging strength, and thus did not differ in the amount of food received from the parents during the first visits after treatment. Nevertheless, parents in our study showed changes in behaviour in the days following the treatment. Parents of CORT chicks raised nests visits duration, shortened the intervals between visits, increasing the overall time spent in the nest. In parallel, parents changed their foraging behaviour by increasing both the number and the duration of the visits to the feeders. So, a change in offspring demand resulted in a change in parents' foraging activity as well as in nest visits pattern lasting several days. As a consequence, parents responded to a change in offspring demand by a modification of their time budget, decreasing their time free of activities related to offspring care. Wild zebra finches are usually seen bathing in water area and resting or socializing in so-called "social trees" when not around their nest (Zann 1996). By decreasing their free time, parents certainly reduced the amount of these activities. Thus, parental response is strong as they allocated more food and more time to their nestlings.

These modifications of parental behaviour seem effective in terms of parental care as CORT chicks gained more weight than OIL nestlings. In a previous study (Gilby, Mainwaring et al. 2011), the number of nest visits per hour as well as the time spent in the nest-box per hour were poor predictors of the amount of food received by the nestlings, which was best predicted by the number of individual feeds during the visits (which is not affected by the treatment in our study). If nest visit rate and duration have no impact on provisioning, the change in nest visits pattern observed in the present study could influence indirectly chicks' weight gain by improving brooding conditions (thermoregulation, hydration). Our study also show that parents not only modify their nest visits pattern in response to offspring needs but also their foraging activity at the feeders. Parents could vary the amount of seeds they take from the feeders (versus natural seeds) to provision their chicks. In a previous study on the same population, 68% of the seeds observed in the nestlings' crop visible through the skin were commercial seeds in nests around feeders (Mariette and Griffith 2012). Parents could thus adjust not only the amount of food but also food quality.

Our laboratory experiment (see Supplementary material) shows that the plasma concentration of corticosterone obtained by oral administration in our protocol is higher than the values induced by four hours of fasting: we thus created a strong effect on nestlings which may represent a supernormal stimulus for parents, and could explain why their response lasts several days.

### **What is the signal driving parents to an appropriate care?**

How to know which signal (i.e. conspicuous and/or inconspicuous signals) was effective in driving the response of the parents? Our study does not allow to discriminate between the effects of begging duration and begging calls' acoustic structure. zebra finches are able to perceive fine changes in the spectral components of sounds (Dooling, Brown et al. 1992; Dooling, Best et al. 1995; Sturdy, Phillmore et al. 1999; Ohms, Escudero et al. 2012), so they could potentially discriminate corticosterone-induced up-shifted begging calls. It is possible that parents have two types of response to begging behaviour modifications: a short-term response to higher intensity begging resulting in an immediate increase in food delivery (as shown by Gilby et al), and a medium-term response to more subtle modifications of begging calls' acoustic structure resulting in changes in foraging activity and nest visits pattern. This two-levels response would fit the "multiple message hypothesis" (Johnstone 1996), stating that begging behaviour is the result of multiple signals correlated with multiple independent messages coding for different types of needs and thus different types of expected parental care.

### **CORT Chicks are bigger but their growth could suffer from a direct action of exogenous corticosterone on their metabolism**

CORT chicks gained significantly more weight than OIL chicks, but the analysis of the residuals of the linear regression between tarsus length and weight did not show any difference in body condition. Nevertheless, this method is now known to lead to errors, especially in avian species in which the body size indicator (i.e. tarsus length) is 1) not always independent of the body mass, 2) not an accurate measure of overall structural size in several species (Green 2001). We also saw no difference in tarsus length between CORT and OIL chicks. In a previous study (Gilby, Sorato et al. 2012), chicks begging more strongly were more fed and gained more tarsus length. These differences were significant within the broods (the first hatched chicks were compared with the last hatched chicks from the same brood), while in our study, differences were tested between treatments, with varied brood sizes in each group and strong variation in growth rate between nests. Moreover, the experimental increase in begging in our study was not due to the nestling intrinsic condition as in Gilby et al.'s study but to the administration of exogenous corticosterone. Considering the effect of corticosterone in mobilizing energy reserves, no gain in body condition (or in tarsus length) could be

explained by a direct effect of corticosterone on nestlings' metabolism. The costs of begging have also been investigated in several studies: an increase in the intensity of begging has been reported to slow down growth (Kilner 2001; Rodriguez-Girones, Zuniga et al. 2001) or to increase energy expenditure (Harper 1986; Verhulst and Wiersma 1997; Weathers, Hodum et al. 1997). Thus, the potential gain in energy linked to the extra seeds received by CORT nestlings could have been partly used to keep on expressing a high intensity of begging forced by high level of exogenous plasmatic corticosterone.

We present in this paper a comprehensive study of begging behaviour in the zebra finch, from physiological cause of begging modifications to parental responses over time.

Our study confirms that begging signal sent by nestlings is complex, multimodal (visual, acoustic with duration of begging and modulation of acoustic parameters) highly flexible, and composed of conspicuous and inconspicuous signals that can be modified by the action of corticosterone. This finding could explain why other studies not interested in both types of signals did not get any strong responses from the parents.

More generally, we confirm here the capacity of birds to communicate labile information on their physiological state by modulating the acoustic structure of their vocalisations using similar cues as mammals, but our results extend this capacity to nestlings. Overall, our results bring new evidence for the understanding of parent-offspring communication, by showing that parents can respond to their offspring's needs by changing not only their behaviour at the nest but also their foraging pattern as well as their time budget.

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## **SUPPLEMENTARY MATERIAL**

### **A. Study of the effects of handling and fasting on nestlings' stress and choice of a corticosterone dose for oral administration**

To manipulate physiological stress in wild nestlings and generate a control group and an experimental group with different levels of plasma corticosterone, the treatment needed to provoke an increase in levels of corticosterone higher than those resulting from handling and from the fasting period. We thus tested on domestic zebra finches in the laboratory: the effect of handling and the effect of fasting duration on nestlings' plasma levels of corticosterone. Finally, we tested the effect of different doses of oral administration of corticosterone on nestlings' plasma levels of corticosterone. The results of these three experiments allowed us to choose the corticosterone dose for oral administration in the field experiment on wild zebra finches. Protocols were approved by the Animal Care and Use Committee of the University Jean Monnet, Saint Etienne, and experiments were performed under the authorization no. 42-218-0901-38 SV 09 (ENES Lab, Direction Départementale des Services Vétérinaires de la Loire) according to the guidelines laid down by the French Ministère de l'Agriculture (no. 87-848) and the E.U. Council Directive for the Care and Use of Laboratory Animals of November 24th, 1986 (86/609/EEC).

#### **Subjects**

75 nestlings of domestic zebra finches (*Taeniopygia guttata*) bred in our colony (ENES lab, University) were used as subjects. Parents of our subjects were pairs kept in two large aviaries (dimensions: 200 x 200 x 300 cm, balanced sex ratio) at least one month prior to the experiment. Aviaries were maintained at a constant temperature of 22–24°C with a 14:10 light–dark cycle and were provided with ad libitum food, water, nesting material, pools, and wooden nest-boxes (1 for 2 birds i.e. 1 per presumed pair). Nest-boxes were checked once daily between 8 and 9 a.m to monitor nest building, egg laying and chicks hatching.

#### **Handling and fasting protocol**

To evaluate the elevation of plasma corticosterone due to handling in our field protocol, nestlings were caught in the nest at different ages (Day 6, Day 9, Day 12 and Day 14 post hatching, n=4 in each group), handled in hands for 2 minutes, then put in a cloth bag during the following 8 minutes. Chicks were then caught again and bled within 3 minutes. Chicks from the control group (n=4, mixed ages) were directly caught in the

nest and bled within 3 minutes so as to measure basal levels of corticosterone. Data were log transformed to meet normality and homoscedasticity criteria.

Pairwise t tests with bonferroni correction were applied on log transformed data to compare the effect of handling on plasma levels of corticosterone at different ages. Handling significantly raised plasma levels of corticosterone at every ages (basal vs all ages:  $p < 0.001$ , figure S1.A.), but handling was a very strong stressor at 14 days of age (age 14 vs all other ages:  $p < 0.001$ ).

To assess the effect of fasting on plasma corticosterone, nestlings between 7 and 9 days of age were fasted by closing the entrance of the nest with a tissue, preventing the parents to enter and feed the chicks during this period. The nest was closed just after a parental visit to make sure that all the nestlings were fed before the start of the fasting period. Chicks were then caught at the nest at different times (0, 20, 60, 120, 180 or 240 minutes post fasting,  $n=3$  in each group) and bled within 3 minutes.

Data were log transformed to meet normality and homoscedasticity criteria. Pairwise t tests with bonferroni correction were applied on log transformed data to assess the effect of different durations of fasting on plasma levels of corticosterone. Fasting significantly enhanced plasma levels of corticosterone, with an increasing effect over time (figure S1.B.). The relative increase of plasma corticosterone after 20 minutes can be due to the action of the experimenter on the nest while installing the tissue at the entrance (shaking, loss of light, tapping sounds around the nest).

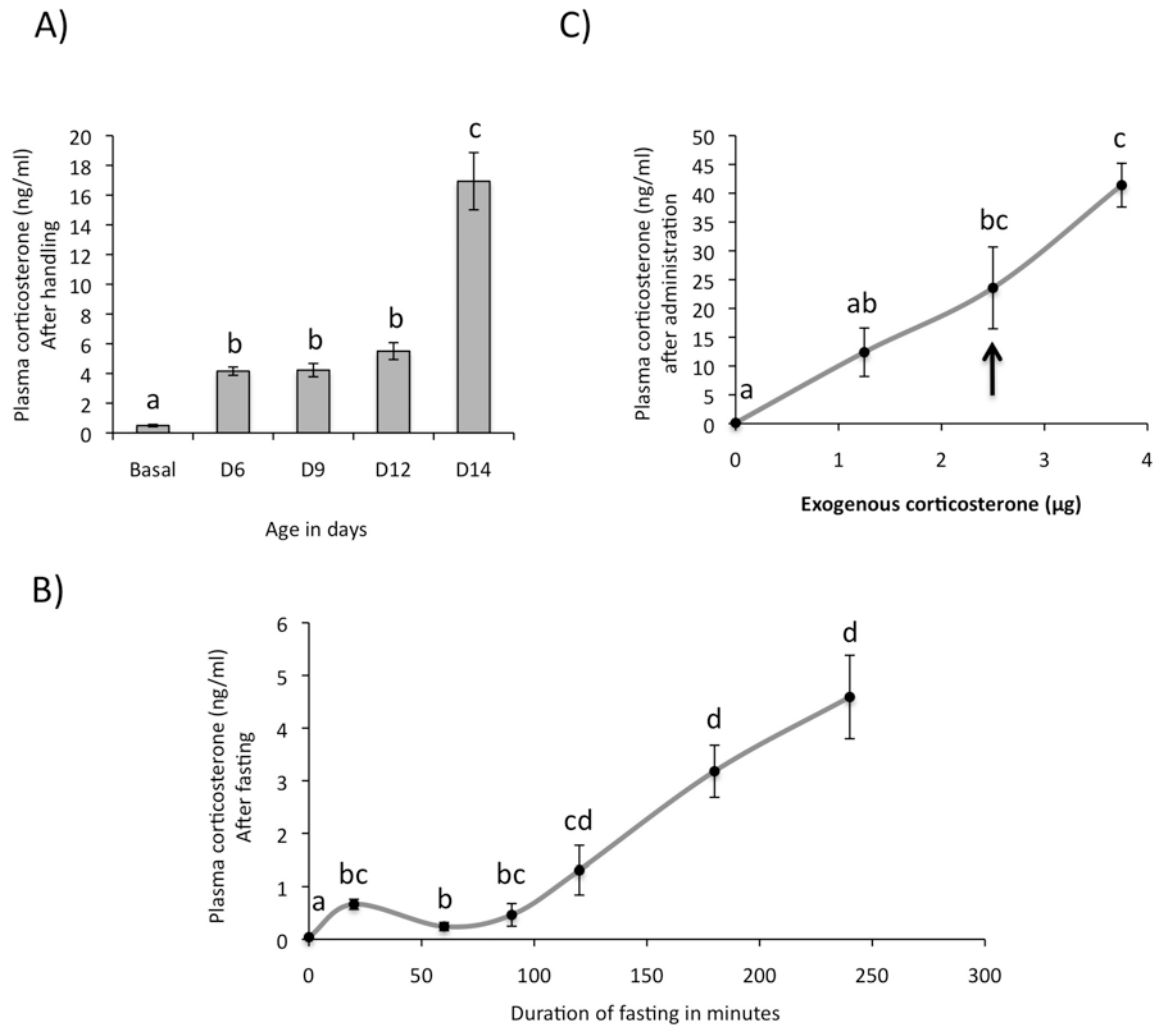
### **Corticosterone administration**

Following Spencer et al (Spencer and Verhulst 2007) domestic nestlings were fed with 1.25, 2.5 or 3.75  $\mu\text{g}$  of exogenous corticosterone dissolved in peanut oil (i.e. 5, 10 or 15  $\mu\text{l}$ , Concentration: 0.25 mg/ml) ( $n=3$  in each group). Nestlings were then caught 10 minutes after oral administration and bled within 3 minutes. Chicks from the control group ( $n=4$ ) received 10  $\mu\text{l}$  of peanut oil only.

Data were normally distributed and homoscedasticity was respected. Thus, pairwise t tests with bonferroni correction were used to compare plasma levels of corticosterone resulting from each dose of corticosterone administration. The treatment significantly increased plasma levels of corticosterone compared to basal (0g vs 2.5g :  $p=0.15$ , 0g vs 3.75g :  $p < 0.001$ , figure S1.C.), with the highest raise after administration of 3.75g of corticosterone (3.75g vs 1.25g :  $p=0.004$ ).

### **Final decision on corticosterone administration on wild nestlings**

To manipulate physiological stress in wild nestlings and generate a control group and an experimental group with different levels of plasma corticosterone, the treatment needed



**Figure S1: Effect of handling and fasting on nestlings' stress and choice of a corticosterone dose for oral administration.**

Data are presented with mean  $\pm$  SE. Different letters indicate a significant difference between pairs at the  $P < 0.05$  level.

A) Effect of handling on plasma concentration of corticosterone at different days of age. Handling significantly raised plasma concentration of corticosterone at every age (basal vs all age:  $p < 0.001$ ), but handling was a very strong stressor at 14 days of age (age 14 vs all other age:  $p < 0.001$ ).  $N=4$  subjects in each group.

B) Effect of different durations of fasting on plasma concentration of corticosterone. Fasting significantly enhanced plasma concentration of corticosterone, with an increasing effect over time ( $p < 0.05$  for all comparisons except 120min vs 20,90,180,240 min, 20 vs 60,90,180 min and 60min vs 90 min).  $N=3$  subjects in each group.

C) Effect of oral administration of exogenous corticosterone on plasma concentration of corticosterone. The treatment significantly increased plasma concentration of corticosterone compared to basal (0g vs 2.5g :  $p=0.15$ , 0g vs 3.75g :  $p < 0.001$ ), with the highest raise after administration of 3.75g of corticosterone (3.75g vs 1.25g :  $p=0.004$ ). The black arrow indicates the treatment chosen for the field experiment, i.e. administration of 2.5g of exogenous corticosterone.

to provoke an increase in levels of corticosterone higher than those resulting from handling and fasting, but within a range of variation relevant to the species. Administration of 2.5g was thus chosen as it increased plasma corticosterone to  $23.56 \text{ ng/ml} \pm 7.1$ , which was above a stress of handling and of 4h of fasting, but still within the normal range of physiological stress in zebra finch nestlings (figure S1.C.).

### **Blood sample collection and hormone assay**

All blood samples (<100  $\mu\text{L}$ ) were obtained by puncturing the alar vein with a 27-gauge needle. Blood was collected in heparinised tips, and plasma was separated under centrifugation (15 min, 3800xg) and stored at  $-80 \text{ }^\circ\text{C}$  until hormone assay. Blood samples were taken within 3 min (Wingfield, Smith et al. 1982). Nestlings were only bled once to avoid drastic drop in body temperature caused by insufficient blood volume that could potentially lead to quick death. Because circulating level of endogenous corticosterone shows circadian variations in many bird species with a peak just before the active period and a rapid decrease during the first hours of activity, all experiments were performed between 10:00 and 12:00 am (Remage-Healey and Romero 2000; Tarlow, Hau et al. 2003)(Remage-Healey and Romero, 2000; Tarlow et al., 2003).

As previously described (Perez, Elie et al. 2012), corticosterone assays were performed using the ELISA method (Kit Corticosterone EIA, Cayman, #5000561) according to the manufacturer's recommendations. All samples were run in duplicate in three different assays. The detection limit was 40 pg/mL, intra-assay coefficient of variation was 0.106, and inter-assay coefficient of variation was 0.162.

### **B. Acoustic analysis on calls**

13,682 calls from 89 chicks were originally extracted. Calls were then analyzed using the Seewave package (Sueur, Aubin et al. 2006) implemented in R software (R Development Core Team, 2007, [www.r-project.org](http://www.r-project.org)). Each call was first bandpass filtered (0.1-10 kHz) according to the zebra finch audiogram (Hashino & Okanoya 1989). Sixteen acoustic parameters were measured for each call:

- the duration (in seconds) using “timer” function, which computes the energy from the signal envelope and uses the peak maximum as an energy reference to define the start and the end of the call (threshold: 2%).

- the call dominant frequency (in Hertz) is the average over the duration of the call of the frequencies of highest amplitude (obtained via the “dfreq” function). The frequency of highest amplitude is first assessed on time bins using a window length of 11.6ms (512 samples) and the series of values obtained over the duration of the call is then averaged.

- the call fundamental frequency (in Hertz) is the average over the duration of the call of the fundamental frequency values (these are tracks of the fundamental frequency through a short-term cepstral transform obtained via the “fund” function).

- the standard deviations of the call dominant frequency and the fundamental frequency, as cues of frequency modulation within the call.

- 11 parameters of the call spectrum (using “specprop” function and a window length of 11.6ms): the mean, the standard deviation of the mean, the median, the mode, the first (25%) and third (75%) quartiles, the interquartile range, the skewness, the kurtosis, the spectral flatness and the spectral entropy.

For each acoustic parameter of the begging calls, distribution was evaluated using QQ plot and histogram shape: If the plot built with a normal distribution assumption showed a straight line, data are normally distributed. Square root transformation was applied for 3 parameters: the fundamental frequency, the standard deviation of the dominant frequency and the first quartile. Only the 9 parameters showing a normal distribution, after transformation if necessary, were kept for the next step: the duration, the dominant frequency, the fundamental frequency, the standard deviation of the dominant frequency, the standard deviation of the fundamental frequency, the mean, the standard deviation of the mean, the first quartile and the interquartile range.

11740 calls (86% of the initial data set, n=5960 calls for the CORT group, n=5780 calls for the OIL group) were then selected for statistical analysis by removing outliers: outliers were defined using a Principal Component Analysis (PCA) of 4 axes computed on the z-scores of the 9 previously selected parameters, using “dudi.pca” function from “ade4” package. Calls were defined as outliers if they were outside the range [mean-2SD, mean+2SD] on Axis1 and Axis2 of this PCA.

Acoustic parameters were first analyzed using univariate statistics so as to select the parameters affected by the experiment. General linear mixed models “lme” function from “nlme” package) were performed on each parameter with the CONDITION (4 levels: CORT\_BEFORE, CORT\_AFTER, OIL\_BEFORE and OIL\_AFTER) as a fixed effect and the identity of the nestling as a random effect.

All parameters affected by the CONDITION (p values<0.05) were kept for multivariate analysis, i.e 7 parameters: the dominant frequency, the fundamental frequency, the standard deviation of the dominant frequency, the mean, the standard deviation of the mean, the first quartile and the interquartile range.

A PCA was performed on the z-scores of the 7 parameters affected by the CONDITION. General linear mixed models “lmer” function from ‘lme4’ package) were finally performed on the first two first axis (accounting for 71% of the variance and showing an eigen-value above 1), with the GROUP (OIL or CORT) and the MOMENT of the recording (BEFORE or AFTER) and the CONDITION, i.e. the interaction between the GROUP and the MOMENT as fixed factors, and the identity of the nestling, the age of the nestling, the

identity of the nest, the brood size and the RANK of the nestling as random factors. To simplify the model, we followed a backward stepwise procedure where least significant terms were sequentially removed. Successive models were compared using the “anova” function, and a factor was removed when  $p\text{-value} > 0.05$ . The final model for Axis 1 used the MOMENT of the recording (BEFORE or AFTER) and the interaction between the GROUP and the MOMENT as fixed factors, and the identity of the nestling and the identity of the nest as random effects. Final model for Axis2 used only MOMENT as a fixed factor, brood size and identity of the nestling as random factors. Post hoc tests were performed using Multiple Comparisons of Means with Tukey Contrasts: “glht” function from “multcomp” package was used with the CONDITION, i.e. the interaction between the MOMENT and the GROUP, as object of class “mcp” for Axis1, and the MOMENT as object of class “mcp” for Axis 2.

### **C. Details on statistical analysis**

The number of feeds during parental visits was recorded as count data showing homoscedasticity, and they were analysed using a General linear mixed model with poisson distribution (“lmer” function from “lme4” package), with the GROUP (OIL vs CORT) as a fixed factor, the sex of the parent, the brood size and the identity of the nest as random factors. After simplification, the brood size was removed from the model. The sex of the parent was not defined as a fixed factor in the model as our data set rarely contained both parents from the same nest (5 out of 23 pairs), and thus did not allow us to properly compare potential differences between male and female behaviours.

The values of duration of the parental visit were log transformed to meet normality and homocedasticity criteria. This parameter was analysed using a General linear mixed model with normal distribution (“lmer” function from “lme4” package), with the GROUP (OIL vs CORT) as a fixed factor, the sex of the parent, the brood size and the identity of the nest as random factors. As none of the random factors had a significant effect on the duration of the visit at the nest, these data were finally analysed using a t-test.

The first Axis of the PCA (accounting for 46 % of the variance and showing an eigenvalue above 1) computed on nestlings’ behaviour during parental visits was analysed using a general linear mixed model with gaussian distribution (“lmer” function from “lme4” package), with the GROUP (OIL vs CORT) as a fixed factor, the sex of the parent, the brood size and the identity of the nest as random factors. We then simplified the model, but as none of the random factors had a significant effect on the first Axis of the PCA, these data were finally analysed using a t-test.

PIT-tag data related to parental visits at the nest and at the feeder resulted in counting data that respected homoscedasticity. They were thus analysed using General linear mixed models with poisson distribution (“lmer” function from “lme4” package), with the GROUP (OIL vs CORT) and the Day of the experiment (1 to 8) as fixed factors, the brood size and the identity of the nest as random factors. Post hoc tests consisted in pairwise Wilcoxon sum rank tests with the interaction between the Day and the GROUP as the predictor.

General linear mixed models (“lmer” function from “lme4” package) were finally performed on the residuals from correlation of nestlings’ growth measures with the GROUP (OIL or CORT) as a fixed factor, and the identity of the nest, the brood size and the RANK of the nestling as random factors. We simplified the model, and the final model used only the nest as a random factor with no fixed factor.

General linear mixed model (“lmer” function from “lme4” package) were then performed on the weight gain and the tarsus length gain with the GROUP (OIL or CORT) and the RANK of the nestling and the interaction between the GROUP and the RANK as fixed factors, and the identity of the nest and the brood size as random factors. After simplification, the final model for weight gain used the GROUP and the interaction between the GROUP and the RANK as fixed factors, and the identity of the nest as a random effect, while the final model for the tarsus length gain used only the identity of the nest as a random factor and no fixed factor. Post hoc tests for the weight gain consisted in multiple Comparisons of Means with Tukey Contrasts, using “glht” function from “multcomp” package with the interaction between the GROUP and the RANK as object of class “mcp”. Interaction between the GROUP and the RANK was then investigated by analysing results RANK per RANK with a General linear mixed model using the GROUP as a fixed factor, and the brood size and the identity of the nest as random factor. After simplification, final models consisted only in models with the identity of the nest as a random factor and no fixed factor, except for RANK 1 in which the model was simplified by using the GROUP as a fixed factor, and the identity of the nest as a random factor. Post hoc test for this last model consisted in multiple Comparisons of Means with Tukey Contrasts, using “glht” function from “multcomp” package with the GROUP as object of class “mcp”.

|       | CORT1  | OIL1  | CORT2 | OIL2   | CORT3  | OIL3  | CORT4 | OIL4 | CORT5 | OIL5 | CORT6 | OIL6 | CORT7 | OIL7 | CORT8 |
|-------|--------|-------|-------|--------|--------|-------|-------|------|-------|------|-------|------|-------|------|-------|
| OIL1  | 1      |       |       |        |        |       |       |      |       |      |       |      |       |      |       |
| CORT2 | 0.8    | 1     |       |        |        |       |       |      |       |      |       |      |       |      |       |
| OIL2  | 1      | 1     | 1     |        |        |       |       |      |       |      |       |      |       |      |       |
| CORT3 | 0.10   | 1     | 1     | 1      |        |       |       |      |       |      |       |      |       |      |       |
| OIL3  | 0.010  | 1     | 1     | 1      | 1      |       |       |      |       |      |       |      |       |      |       |
| CORT4 | 1      | 1     | 1     | 1      | 1      | 1     |       |      |       |      |       |      |       |      |       |
| OIL4  | 5e-07  | 0.05  | 0.07  | 0.09   | 0.72   | 1     | 0.15  |      |       |      |       |      |       |      |       |
| CORT5 | 1      | 1     | 1     | 1      | 1      | 1     | 1     | 0.02 |       |      |       |      |       |      |       |
| OIL5  | 6e-09  | 0.004 | 0.002 | 0.006  | 0.03   | 0.52  | 0.006 | 1    | 0.001 |      |       |      |       |      |       |
| CORT6 | 1      | 1     | 1     | 1      | 1      | 1     | 1     | 1    | 1     | 0.15 |       |      |       |      |       |
| OIL6  | 4e-12  | 5e-06 | 6e-06 | 6e-05  | 0.0001 | 0.01  | 2e-05 | 0.77 | 2e-06 | 1    | 0.004 |      |       |      |       |
| CORT7 | 9e-07  | 0.03  | 0.03  | 0.05   | 0.23   | 1     | 0.04  | 1    | 0.004 | 1    | 0.52  | 1    |       |      |       |
| OIL7  | 3e-12  | 2e-06 | 2e-06 | 2e-05  | 4e-05  | 0.003 | 9e-06 | 0.23 | 1e-06 | 1    | 0.002 | 1    | 1     |      |       |
| CORT8 | 9e-11  | 6e-06 | 3e-05 | 0.0001 | 0.0002 | 0.01  | 4e-05 | 0.49 | 7e-06 | 1    | 0.005 | 1    | 1     | 1    |       |
| OIL8  | 3.e-08 | 0.001 | 0.001 | 0.001  | 0.005  | 0.13  | 0.001 | 1    | 9e-05 | 1    | 0.03  | 1    | 1     | 1    | 1     |

**Figure S2: p-values of post-hoc tests on the duration of the visit at the nest per Day of experiment.**

CORT indicates data from the CORT group, OIL indicates data from the control GROUP. The number indicates the Day of the experiment.

|       | CORT1 | OIL1  | CORT2 | OIL2 | CORT3 | OIL3  | CORT4 | OIL4   | CORT5  | OIL5  | CORT6 | OIL6  | CORT7 | OIL7  | CORT8 |
|-------|-------|-------|-------|------|-------|-------|-------|--------|--------|-------|-------|-------|-------|-------|-------|
| OIL1  | 1     |       |       |      |       |       |       |        |        |       |       |       |       |       |       |
| CORT2 | 1     | 1     |       |      |       |       |       |        |        |       |       |       |       |       |       |
| OIL2  | 1     | 1     | 1     |      |       |       |       |        |        |       |       |       |       |       |       |
| CORT3 | 1     | 1     | 1     | 1    |       |       |       |        |        |       |       |       |       |       |       |
| OIL3  | 0.57  | 0.245 | 0.03  | 1    | 0.002 |       |       |        |        |       |       |       |       |       |       |
| CORT4 | 0.10  | 0.12  | 1     | 0.04 | 1     | 2e-05 |       |        |        |       |       |       |       |       |       |
| OIL4  | 1     | 1     | 0.09  | 1    | 0.008 | 1     | 3e-05 |        |        |       |       |       |       |       |       |
| CORT5 | 1     | 1     | 1     | 1    | 1     | 0.07  | 1     | 0.27   |        |       |       |       |       |       |       |
| OIL5  | 1     | 0.87  | 0.05  | 1    | 0.004 | 1     | 3e-05 | 1      | 0.23   |       |       |       |       |       |       |
| CORT6 | 1     | 1     | 1     | 1    | 1     | 0.10  | 1     | 0.42   | 1      | 0.27  |       |       |       |       |       |
| OIL6  | 0.003 | 0.001 | 4e-05 | 0.77 | 8e-07 | 1     | 6e-09 | 1      | 0.0003 | 1     | 0.001 |       |       |       |       |
| CORT7 | 0.11  | 0.11  | 1     | 0.04 | 1     | 1e-05 | 1     | 0.0001 | 1      | 5e-05 | 1     | 4e-09 |       |       |       |
| OIL7  | 1     | 0.5   | 0.06  | 1    | 0.004 | 1     | 2e-05 | 1      | 0.13   | 1     | 0.19  | 1     | 3e-05 |       |       |
| CORT8 | 1     | 1     | 1     | 0.75 | 1     | 0.001 | 1     | 0.007  | 1      | 0.002 | 1     | 5e-07 | 1     | 0.003 |       |
| OIL8  | 1     | 0.78  | 0.06  | 1    | 0.005 | 1     | 3e-05 | 1      | 0.23   | 1     | 0.29  | 1     | 7e-05 | 1     | 0.004 |

**Figure S3: p-values of post-hoc tests on the duration between visits at the nest per Day of experiment.**

CORT indicates data from the CORT group, OIL indicates data from the control GROUP. The number indicates the Day of the experiment.



|       | CORT1 | OIL1  | CORT2 | OIL2 | CORT3  | OIL3 | CORT4 | OIL4  | CORT5 | OIL5 | CORT6 | OIL6 | CORT7 | OIL7 | CORT8 |
|-------|-------|-------|-------|------|--------|------|-------|-------|-------|------|-------|------|-------|------|-------|
| OIL1  | 1     |       |       |      |        |      |       |       |       |      |       |      |       |      |       |
| CORT2 | 1     | 1     |       |      |        |      |       |       |       |      |       |      |       |      |       |
| OIL2  | 1     | 1     | 1     |      |        |      |       |       |       |      |       |      |       |      |       |
| CORT3 | 0.12  | 0.002 | 1     | 0.48 |        |      |       |       |       |      |       |      |       |      |       |
| OIL3  | 1     | 1     | 1     | 1    | 0.015  |      |       |       |       |      |       |      |       |      |       |
| CORT4 | 1     | 1     | 1     | 1    | 1      | 1    |       |       |       |      |       |      |       |      |       |
| OIL4  | 1     | 1     | 0.9   | 0.61 | 0.0001 | 1    | 0.03  |       |       |      |       |      |       |      |       |
| CORT5 | 0.61  | 0.05  | 1     | 1    | 1      | 0.11 | 1     | 0.002 |       |      |       |      |       |      |       |
| OIL5  | 1     | 1     | 1     | 1    | 0.0003 | 1    | 0.15  | 1     | 0.007 |      |       |      |       |      |       |
| CORT6 | 1     | 1     | 1     | 1    | 0.4    | 1    | 1     | 1     | 1     | 1    |       |      |       |      |       |
| OIL6  | 1     | 1     | 1     | 1    | 0.013  | 1    | 1     | 1     | 10.11 | 1    | 1     |      |       |      |       |
| CORT7 | 1     | 1     | 1     | 1    | 1      | 1    | 1     | 0.22  | 1     | 1    | 1     | 1    |       |      |       |
| OIL7  | 1     | 1     | 1     | 1    | 0.006  | 1    | 1     | 1     | 0.09  | 1    | 1     | 1    | 1     |      |       |
| CORT8 | 1     | 1     | 1     | 1    | 1      | 1    | 1     | 0.81  | 1     | 1    | 1     | 1    | 1     | 1    |       |
| OIL8  | 1     | 1     | 1     | 1    | 0.0001 | 1    | 0.27  | 1     | 0.006 | 1    | 1     | 1    | 1     | 1    | 1x    |

**Figure S4: p-values of post-hoc tests on the duration of the visit at the feeder per Day of experiment.**

CORT indicates data from the CORT group, OIL indicates data from the control GROUP. The number indicates the Day of the experiment.

|       | CORT1 | OIL1  | CORT2 | OIL2 | CORT3 | OIL3 | CORT4 | OIL4 | CORT5 | OIL5 | CORT6 | OIL6 | CORT7 | OIL7 | CORT8 |
|-------|-------|-------|-------|------|-------|------|-------|------|-------|------|-------|------|-------|------|-------|
| OIL1  | 1     |       |       |      |       |      |       |      |       |      |       |      |       |      |       |
| CORT2 | 0.21  | 0.27  |       |      |       |      |       |      |       |      |       |      |       |      |       |
| OIL2  | 1     | 1     | 1     |      |       |      |       |      |       |      |       |      |       |      |       |
| CORT3 | 0.005 | 0.008 | 1     | 0.58 |       |      |       |      |       |      |       |      |       |      |       |
| OIL3  | 1     | 1     | 1     | 1    | 0.72  |      |       |      |       |      |       |      |       |      |       |
| CORT4 | 1     | 1     | 1     | 1    | 1     | 1    |       |      |       |      |       |      |       |      |       |
| OIL4  | 1     | 1     | 1     | 1    | 0.27  | 1    | 1     |      |       |      |       |      |       |      |       |
| CORT5 | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    |       |      |       |      |       |      |       |
| OIL5  | 1     | 1     | 1     | 1    | 0.26  | 1    | 1     | 1    | 1     |      |       |      |       |      |       |
| CORT6 | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    |       |      |       |      |       |
| OIL6  | 1     | 1     | 1     | 1    | 0.37  | 1    | 1     | 1    | 1     | 1    | 1     |      |       |      |       |
| CORT7 | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    |       |      |       |
| OIL7  | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     |      |       |
| CORT8 | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    |       |
| OIL8  | 1     | 1     | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     | 1    | 1     |

**Figure S5: p-values of post-hoc tests on the number of visits at the feeder per Day of experiment.**

CORT indicates data from the CORT group, OIL indicates data from the control GROUP. The number indicates the Day of the experiment.



# **GENERAL DISCUSSION**

### III. GENERAL DISCUSSION

The present thesis work shows that zebra finches can encode their physiological state in their calls, through the emission of stress-induced vocalisations modulated in their spectral parameters (**chapters 1, 2 and 4**). It also shows for the first time the direct implication of corticosterone, the main stress hormone in birds, not only in temporal but also in spectral modifications of birds vocalisations (distances calls and begging calls) involved in expressing labile information such as an emotion of stress (**chapter 1 and 4**). Corticosterone, and more generally glucocorticosteroids, could be one of the neuroendocrine signals that drive the expression of emotions in animals. The spectral modifications revealed in stress-induced vocalisations are biologically relevant for potential receivers (females in **chapter 2**, parents in **chapter 4**), as they show adapted modifications in their behaviour after hearing corticosterone-induced vocalisations. We show here that zebra finch vocalisations are sufficient to inform the group about not only stable information i.e. sex and identity (Zann and Straw 1984; Simpson and Vicario 1990; Vicario, Naqvi et al. 2001; Vignal, Mathevon et al. 2004; Vignal, Mathevon et al. 2008; Forstmeier, Burger et al. 2009; Gobes, Ter Haar et al. 2009; Levrero, Durand et al. 2009; Vignal and Mathevon 2011) but also totally labile information such as an acute stress. The present thesis work proposes that corticosterone has a main role in modulating vocalisations, but data also suggest the implication of other neuroendocrine signals, as the level of stress experienced by an individual during separation from the partner cannot predict its vocal activity, while its life history (strong pairbond, breeding experience) is a more relevant predictor (**Chapter 3**).

#### **III.1. An acute stress is encoded through the emission of up-shifted vocalisations in adults and nestlings suggesting high flexibility in calls.**

After oral administration of exogenous corticosterone, both adults and chicks emit calls with higher mean frequency and higher dominant frequency. Both stress-induced begging calls in chicks and stress-induced distance calls in adults show a spectral structure that is up-shifted in higher frequency bands. Thus, both adult and young zebra finches show a flexibility of their calls. Many studies proposed that male calls (and song syllables) are more flexible than female's ones, because they are shaped by a learning process and are thus more individualised (Zann and Straw 1984; Simpson and Vicario 1990; Vicario, Naqvi et al. 2001; Forstmeier, Burger et al. 2009), while female calls are non-learned crystallized calls. As they were not designed to this aim, our experiments in **chapters 2 and 3** did not allow to record enough calls from females during isolation to verify if corticosterone can also affect female calls' structure. Complementary experiments are under process to answer this question. Nevertheless, the same

hypothesis of low call flexibility could have been formulated for nestlings, as begging calls are emitted from the first day post hatching, and no learning process has yet been shown at this age. Our results suggest that vocal flexibility in the zebra finch is not exclusive of learned vocalisations but is also possible for innate ones, and the process leading to flexibility is partially or totally independent of the vocal learning process.

Several studies conducted in amphibians, birds and mammals (Whitney and Krebs 1975; Aubin and Brémond 1989; Aubin and Bremond 1992; Brémond and Aubin 1992; Brenowitz and Rose 1994; Gerhardt 1994; Lengagne, Lauga et al. 2001; Charrier, Mathevon et al. 2002; Jouventin and Aubin 2002; Searby and Jouventin 2003) suggest that pitch and spectral parameters carry the identity of the signaller, while temporal parameters inform about motivation or reproductive status. We show in our study that spectral parameters can convey multiple information, from stable information such as the identity of the sender to labile information such as its emotional state. We also show that the modulation of the vocalisations carrying emotion is fine, as calls are still individualised, and thus the identity carried by the calls is not lost. Nevertheless, the individual signature is acoustically shifted, but females are able to recognize the identity of a given male on both control and stressed-induced vocalisations. Our results suggest that some of the parameters encoding individual identity are also used to encode emotion. Individual identity in male calls is carried by the frequency modulation of the downsweep part of the call, and emotion is encoded in modifications of the frequency spectrum. As individual identity is coded redundantly by several parameters in the calls, some of them could be less important for individual recognition by conspecifics, and could thus be modified to carry labile information without losing the individuality in the calls. The use of modified signals in playback experiments could help understanding this process (Vignal and Mathevon 2011).

The study of the neural processes underlying the vocal expression of stress in males is currently in progress and will hopefully lead to a better understanding of how corticosterone can provoke a modulation of the spectral parameters of calls in the zebra finch. The protocol from [chapter 1](#) has recently been adapted for neurobiological experiments. We used 36 males that were stimulated by female calls in three different contexts: 12 males were completely isolated, 12 males were in the presence of an audience, and 12 males were with an audience but received an oral administration of exogenous corticosterone before the playback. We focused our investigation on specific brain areas: (i) the so-called social behaviour network, a group of structures involved in the social behaviour of birds, and involving the neurotransmitter AVT (arginine vasotocine), also implicated in the release of corticosterone in plasma. (ii) the NCM (caudomedial nidopallium), a secondary auditory area in birds, involved in the neural treatment of acoustic stimuli of different salience. Using immunohistochemistry on brain tissues, we studied the general activity of the structures by monitoring the

expression of an immediate early gene (c-Fos), and the nature of the neurons involved by labelling AVT. The data will bring new evidence on the possible action of corticosterone in the modulation of neural process that could lead to the expression of stress-induced calls.

### **III.2. The source filter theory can be applied to the zebra finch vocalisations.**

Our results are convergent with studies in humans and non-human mammals (for a review see (Briefer 2012)), showing up-shifted vocalisations while experiencing an emotion of negative valence and high arousal, i.e. fear or stress. Our results, together with previous studies in birds and mammals, rise the possibility of a universal signal of stress among vertebrates, encoded in vocalizations with up-shifted frequency spectrum. This hypothesis matches the Motivation-Structural Rules of Morton (Morton 1977) modified by Manser and suggesting that variations of acoustic parameters of a given type of calls reflect differences in arousal states (Manser 2010; Briefer 2012), thus different emotional states. How can a physiological stress provoke the emission of up-shifted vocalisations? If our hypothesis of a convergent up-shifted signal is true, the processes involved in mammals and birds might be similar.

The source filter theory of vocal production proposed in mammals (Fant 1960; Titze 1994; Mindlin and Laje 2005; Taylor and Reby 2010) suggests that vocal signals are created by vibrations of a source, i.e. the vocal folds, and are then filtered in the vocal tract. The value of the fundamental frequency of the final signal is predicted by the performance of the vocal folds, while the energy distribution in the frequency spectrum depends on the shape, the length and the tension of the vocal tract. The generation of vocal signals in birds follows the same pattern: the source consists in the vibration of tympaniform membranes in the syrinx, and the vocal tract is formed by the trachea and the beak. Our results did not show a strong effect of corticosterone on the fundamental frequency, whereas the energy distribution in the frequency spectrum was significantly up-shifted. Thus, if the source filter theory is applicable to birds, we are searching for an effect of corticosterone on the vocal tract more than an effect on the syrinx itself (Elemans, Muller et al. 2009).

Birds can modify their vocal tract mainly through movements of their beak, tongue and hyoid, modifying the volume of the oropharyngeal-esophageal cavity, glottal movements and tracheal length changes (Riede, Schilling et al. 2013). The spectral properties of their vocalisations can thus be modified using different strategies: (i) by changing the trachea length using the tracheolateralis muscles, (ii) by using the glottis to change the extent to which the trachea is occluded, (iii) with tongue movements (iv) by changing the degree of beak opening that can modify the effective length of the vocal tract (Nowicki and Marler 1988; Patterson and Pepperberg 1994; Fletcher and Tarnopolsky

1999). These models predict that a closed beak will produce lower-frequency vocal tract resonances, whereas an open beak will provoke the emission of higher-frequency vocal tract resonances (Nowicki and Marler 1988), as verified by experimental studies in three sparrow species (Westneat, Long et al. 1993; Podos, Sherer et al. 1995). A recent study on the zebra finch has shown that opening the beak during singing accentuates high frequencies of song syllables above 5 kHz (Ohms, Snelderwaard et al. 2010). Considering that several song syllables are structurally close to distance calls, we could extend this property to distance calls. Unfortunately, our video recordings from **chapter 1** did not allow us to evaluate the body position of the isolated males while vocalising.

Begging behaviour of nestlings is a typical set of movements with neck stretched upwards and head raised with or without head bobbing, mouth opening, tongue wagging and emission of begging calls (Zann 1996). Thus, nestlings have numerous possibilities to modify their effective vocal tract length. In **chapter 4**, we could not measure the body position during begging, but we did so on videos when nestlings emitted spontaneous begging calls outside of a parental visit. We defined different levels of begging behaviour describing the chick's motivation to beg : level 1 with bill open but neck not stretched, level 2 with bill open and neck stretched, and level 3 with bill open, neck stretched and wing flapping. We did not find any difference in begging level between chicks that received corticosterone and control chicks. Nevertheless, we have no data on begging body position during parental visits. This remains to be investigated using cameras in the nest placed in a position that will allow to see nestlings while parents are in the nest, which was not possible in our experiment because of the camera size.

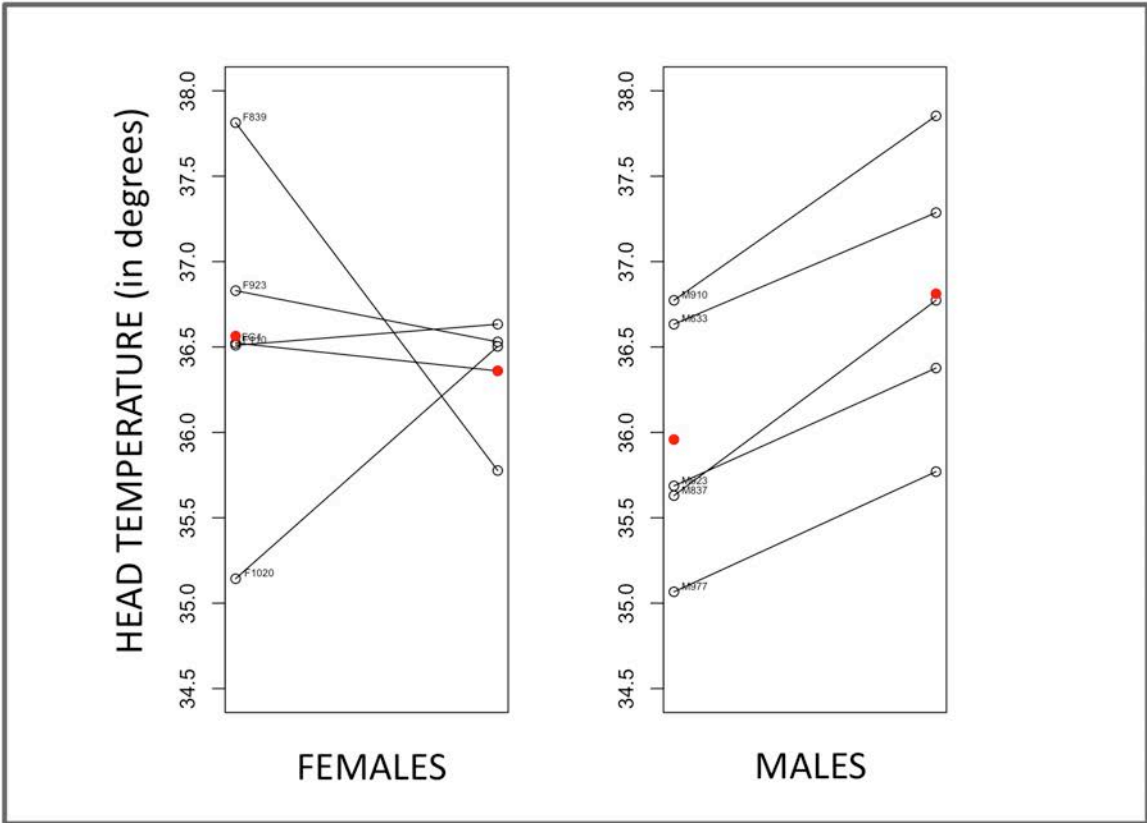
Recent studies interested in how songbirds cope with anthropogenic noise proposed that a higher pitched song is adaptive to face low-frequency urban noises (Fernandez-Juricic, Poston et al. 2005; Slabbekoorn and den Boer-Visser 2006; Gross, Pasinelli et al. 2010; Nemeth and Brumm 2010; Potvin, Parris et al. 2011). Some studies has shown that this high frequency shift in song is not only due to a change in syllable type, but also to a modification of the frequency of a given syllable type (Bermudez-Cuamatzin, Rios-Chelen et al. 2009; Bermudez-Cuamatzin, Rios-Chelen et al. 2011). Pitch and amplitude are often considered independently, but recent work found a positive correlation between pitch and amplitude of vocalisations (Cardoso and Atwell 2012; Zollinger, Podos et al. 2012; Nemeth, Pieretti et al. 2013). Thus, singing at a higher frequency could be a by-product of singing louder, because high-frequency song elements can be easily produced at higher amplitudes (Brumm and Zollinger 2011). In **chapter 1**, we chose not to measure the amplitude of the signal as microphones were fixed in the cage while birds could move close or far from the microphone and change their head orientation, making the amplitude of the signal dependent on the distance between the bird and the microphone and its position. Portative microphones similar to the light-weight

“telemike” that has been used in bats could help measuring precisely amplitude in zebra finch stress-induced vocalisations and understand the implication of this phenomenon in vocalisations with up-shifted frequency spectrum (Hiryu, Shiori et al. 2008). The contraction of muscles (thoracic and abdominal) forces air from air sacs through the bronchi and the syrinx, and these forces can modulate air pressure and consequently the amplitude of the signal. Corticosterone might act not only on the length and shape of the vocal tract, but also on muscles driving respiratory movements during vocal behaviour, which could lead to the emission of, higher pitched calls with higher amplitude.

How can corticosterone influence the vocal tract length and thus provoke a change in the spectral parameters of the calls? Corticosterone is known to modify the locomotor behaviour of birds (Breuner, Greenberg et al. 1998; Landys, Ramenofsky et al. 2006; Lohmus, Sundstrom et al. 2006). A recent study in house sparrow showed that corticosterone receptors are present in muscles tissues (Lattin, Waldron-Francis et al. 2012). Considering also that the vocal flexibility induced by exogenous corticosterone does not involve any vocal learning process, it is possible that corticosterone has a direct effect on muscles acting on the modification of the vocal tract length such as tongue muscles, muscles involved in neck stretching and beak opening, or epiglottis shape.

### **III.3. Corticosterone is a good candidate for driving expression of emotion, but other signals and/or processes could also be involved.**

Our results also suggest that corticosterone might not be the only signal implicated in the emission of modified vocalisations. In [chapter 1](#), isolated males vocalised less and emit calls with longer duration compared to social condition, and these effects were not mimicked by oral administration of corticosterone. Furthermore, males emitted higher pitched calls in response to the calls of a stranger female compared to a familiar female during the same playback session, thus with the same circulating level of corticosterone. Other hormones implicated in stress response could be implicated here: While glucocorticoids (corticosterone and cortisol) are secreted within 3 minutes after disturbance of the animal, epinephrine and norepinephrine are released within fractions of seconds upon activation of the sympathetic nervous system (von Holst 1998; Palme, Rettenbacher et al. 2005; Perry and Capaldo 2011). They also have a shorter half-life between 1 and 30 seconds. For this reason, we could not evaluate levels of catecholamines in plasma samples that we used for corticosterone assay. Catecholamines are also unstable in urine, which make them very difficult to follow. The definition of stress and stress responses have been debated for decades, but Palme in 2005 proposed a consensual definition: two main axes are implicated in a stress response, namely the hypothalamo-pituitary-adrenocortical (HPA) axis involved in glucocorticoids release, and the sympatho-adrenal (SA) axis implicated in catecholamines release. The type, intensity, unpredictability, temporal dynamics and



**Figure 9 : Preliminary results of head temperature as indicator of stress in the zebra finch.**

Results show that males have a higher head temperature after handling in comparison with females. This finding suggests that males are more affected by a stress of handling compared to females. Red dots show mean values, black and white dots show individual values. Labels indicated the bird's identity.



perception of stressors modulate the participation of each axis and result in a large variety of stress responses (Palme, Rettenbacher et al. 2005). Catecholamines have many physiological effects that include modification of heart rate and blood pressure, elevation of body temperature and specific action on smooth muscles (in mammals, (Büllbring and Tomita 1987)). Stress responses in birds have recently been investigated using an indirect measure of catecholamine release by assessing the heart rate as indicator of stress (Cabanac and Guillemette 2001). If and how catecholamines could affect vocalisations after a stress could be further investigated. These results anyway suggest the necessity of developing different ways of measuring stress in animals to assess their emotional state. We thus performed a preliminary study to verify if temperature is a good indicator of stress in the zebra finch, as proposed for other avian species (Cabanac and Aizawa 2000; Cabanac and Guillemette 2001). Mated birds were separated from their partner and exposed to a handling restraint protocol. Eye temperature was then measured using an infrared camera before and after the restraint protocol. First results support our findings from **chapter 3**, with males showing a higher elevation of eye temperature after the handling protocol compared to females (**figure 9**). These results are promising because they allow a less invasive measurement of stress in the zebra finch.

In **chapter 3**, the plasma levels of corticosterone of isolated birds could not predict their vocal behaviour during the experiment, and even if males showed higher levels of corticosterone in comparison to females, there was no difference in vocal behaviour between the sexes. Only parameters describing the history of the pair could predict the vocal behaviour of partners during vocal interaction (but not in isolation), with strong pairs and pairs with previous breeding experience being more talkative. One explanation could be that the social context has a strong influence on the behaviour of the birds, and the context of reunion with the partner after separation could override the physiological effects of corticosterone. Partners could adjust their vocal behaviour in stressful situations, with the degree of adjustment being correlated with the nature and the strength of their relationship, and not with the level of stress experienced by the individuals. This effect could be strong enough to override the effect of corticosterone. The adjustment of vocal behaviour between partners, and the differences in vocal strategy between strong and loose bonds could be controlled by other peptides implicated in pairbond establishment and maintenance, such as vasotocin known to modify social behaviour in the zebra finch, especially in males (Goodson and Adkins-Regan 1999; Goodson, Lindberg et al. 2004).

#### **III.4. Corticosterone elicits the emission of inconspicuous and conspicuous signals.**

Our results show that an emotion of stress provokes a modification of the fine acoustic structure of the calls in the zebra finch, creating vocalisations with up-shifted frequency

spectrum. Sending information to conspecifics using up-shifted vocalisations, when the sender is either an adult (**Chapter 1**) or a nestling (**Chapter 4**), is less conspicuous for potential predators than emitting louder calls (Briskie, Martin et al. 1999), calling at a higher call rate or with a longer duration (Leech and Leonard 1997). The effect of corticosterone on vocalisations in the zebra finch appears adaptive, but it is possible that this response to stressors (e.g. predators) has evolved as a by-product of the physiological effect of stress hormones on vocal signals. Nevertheless, using exogenous corticosterone, we also provoked more conspicuous signals: in **chapter 1**, stressed males answered strongly to both familiar and stranger female calls while they only answered strongly to the stranger calls in social context, and in **chapter 4** nestlings begged longer after oral administration of corticosterone. Thus, the effect of corticosterone is complex, and could lead to both conspicuous and inconspicuous signals. The effect of corticosterone could be dose-dependent, the impact on conspicuous and inconspicuous signals depending on the plasma concentration of the hormone. During this thesis work, we decided to elevate basal plasma levels of corticosterone so as to analyse its effect. A complementary approach could involve the suppression of corticosterone action, either by removing adrenal glands so as to prevent the release of corticosterone in blood, or by targeting corticosterone receptors in specific areas to avoid cells to react to elevation of plasma levels of the hormone. This could help separating the specific effects of corticosterone from other neuroendocrine effects not related to the activation of the HPA axis.

### **III.5. Adult zebra finches show strong bonds with familiars from their social group.**

The isolation of unmated males from their social group is enough to provoke an elevation of plasma levels of corticosterone, similar to what has been shown in the zebra finch when mates are separated (Ramage-Healey, Adkins-Regan et al. 2003). The effect of an isolation from the social group on levels of stress has been mainly described in mammals, with few examples in birds such as the european starling (Apfelbeck and Raess 2008). One recent study in the zebra finch showed that separation from the flock resulted on an elevation of plasma levels of corticosterone 10 minutes but not 30 minutes after isolation from the group (Banerjee and Adkins-Regan 2011). In our study (**chapter 1**), we show that even after one night of isolation, plasma levels of corticosterone in isolated males did not return to baseline. This effect cannot be due to the following playback in our experiment as results from three undisturbed males also show a raise of plasma levels of corticosterone after a night of isolation from the group (data not shown). In Banerjee et al.'s experiment, birds are moved from the aviary to the isolation room using a protocol during which the bird is hand-captured and then released. Hand capture could explain the corticosterone peak at 10 minutes, as plasma levels of corticosterone rise in 3 minutes after a stress (Wingfield, Smith et al. 1982).

Thus, the possible elevation after 30 minutes of isolation, less important, did not show up with statistical analyses.

The elevation of plasma levels of corticosterone with isolation from the social group suggests that familiar individuals share social bonds. The social network of one individual is complex, as the bonds formed with familiars of the group are strong enough to trigger a physiological stress when broken off.

During our experiments, we could not evaluate the level of familiarity between our animals, as they were placed in individual cages (alone for unpaired males in [chapter 1](#), with the partner if mated in [chapter 2](#)). Nevertheless, some playbacks from familiar females in [chapter 1](#) elicited more responses than expected in some specific males. Even if we tried to avoid the establishment of different levels of familiarity by moving the cages every three days so that one individual was never close to the same neighbour during the entire experiment, it is likely that specific relationships were established anyhow. In Ramage et al.'s experiment, the elevation of corticosterone provoked by separation from the mate is reduced if partners are placed with familiar conspecifics during separation (Ramage-Healey, Adkins-Regan et al. 2003). This buffering effect of familiars on the distress of a conspecific, mainly described in mammals (Porter, Nowak et al. 1995; Gust, Gordon et al. 1996; Porter, Nowak et al. 1997; Rukstalis and French 2005; Kikusui, Winslow et al. 2006) also suggests the possibility that birds can develop strong bonds with familiars. This has also been observed on wild free living zebra finches in Fowlers Gap (NSW, Australia), where several observations of small groups led to the conclusion that partners usually join the same close group members during feeding/drinking events, and are more likely to be found in the same social trees together (personal observation). This association of individuals not involved in a sexual bond has been observed in the zebra finch, with same-sex pairs (Elie, Mathevon et al. 2011), and in other avian species: in european starling, same-sex birds associate out of the breeding season and exhibit strong affiliative behaviours (Hausberger and Black 1991; Hausberger, Richard-Yris et al. 1995; Henry and Hausberger 2001) ; corvids are known to cooperate and form social alliances (Emery, Seed et al. 2007; Seed, Clayton et al. 2007; Seed, Emery et al. 2009), and raven social partners sharing a valuable relationship but not involved in a pairbond are less likely to aggress each other and show cues of reconciliation after such event (Fraser and Bugnyar 2011). These preferential affiliative associations outside of the pairbond in birds could be considered as basis of social group formation and maintenance. The pairbond is the core of zebra finches' groups, but the cohesion of the social group could be maintained by affiliative bonds between familiars.

### III.6. Distance calls as alarm calls in the zebra finch.

There was previous evidence that a strong emotion could provoke the emission of up-shifted vocalisations in birds. Alarm calls, a specific call used to inform the group about potential danger, show high-pitched structure after the sight of a predator in many species (Marler 1955; Smith 1965). In this case, the dominant frequency, frequency modulations and frequency bandwidth are usually good predictors of the response of conspecifics (Wood, Sanderson et al. 2000; Fallow, Pitcher et al. 2013). No specific alarm calls have been described in the zebra finch. Considering that distance calls are the call type that adults use when they lose visual contact and when they prepare to take off, it is likely that zebra finch also use this type of call as alarm calls (Zann 1996). Moreover, unmated males in [chapter 1](#) showed a stronger response to the calls of a stranger female compared to the calls of a familiar female only in social context, but not in stress context (isolation and social context with exogenous corticosterone), during which they used up-shifted vocalisations to answer strongly to both call categories. Thus, the stress context elicits not only a modification of the fine structure of the calls, but also an abolition of the preference for the stranger stimuli. One explanation to this effect could be that stressed birds are not anymore able to discriminate between familiar and stranger females based on their calls only. This hypothesis could be tested using a go-nogo operant design. As proposed above, the exploration of the activity of the NCM, secondary auditory area in birds involved in the neural treatment of acoustic stimuli of different saliences, will also help to conclude on this hypothesis. Another explanation could be that the stress context influences the vocal behaviour of the bird. Males are already known to show different responses to the calls of their partner depending on the social context during the playback experiment (Vignal, Mathevon et al. 2004). In the present work, a physiological stress could elicit a disinhibition of the bird's vocal behaviour that leads to exhibiting the same type of responses for both familiar and stranger female calls.

Thus, distance calls encode multiple pieces of information. Our results show that even if the calls structure is up-shifted, the individuality encoded in the call remains. This specificity means that receivers of the signal potentially know which bird is emitting stress-induced vocalisations. It is thus possible that, knowing the caller, the receiver might respond differently to the stress-induced calls depending on its relationship with the caller. In [chapter 2](#), we have shown that females modify their behaviour to the stress-induced call of their mate, thus a familiar individual, while they do not do so when they hear the stress-induced calls of a stranger. The structure of distance calls varies considerably between individuals (Vignal, Mathevon et al. 2004; Vignal, Mathevon et al. 2008; Forstmeier, Burger et al. 2009), and one individual can present a regular pitch that is higher than the regular pitch of another individual, so the control pitch of one individual can be in the range of the stress-induced pitch of another bird. One

explanation of our results would thus be that a receiver of stress-induced calls needs to know beforehand the regular calls of the sender, so as to use it as a reference and compare stress-induced calls with this reference. In this case, stranger regular calls are not known by the receiver, and their stress-induced calls cannot be considered as such, and thus do not elicit any specific response.

### **III.7. Stress-induced vocalisations elicit efficient responses from group members.**

Our results show that the receivers of stress-induced vocalisations exhibit a relevant response to the sender. Parents of stressed nestlings, thus of nestlings that could suffer from starvation or need for warmth (Kitaysky, Wingfield et al. 2001; Loiseau, Sorci et al. 2008; Fairhurst, Treen et al. 2012) responded by foraging more and spending more time in the nest, thus potentially searching for more food, feeding and brooding the chicks more. We could not evaluate which parental response would be elicited either by the emission of up-shifted begging calls or begging calls with longer duration. Nevertheless, corticosterone appears as a central hormone involved in regulating begging behaviour in nestlings, eliciting the emission of a modified signal that is perceived by parents and provokes an efficient modification of the parental feeding and foraging patterns.

In **chapter 2**, females responded to the stress-induced calls of their partner by showing a modification in their behaviour with two dimensions: (i) a modification of their vigilance related to an anti-predator behaviour, that could fit a situation in which they are informed about a imminent danger such as a predator, (ii) a modification in their vocal behaviour to maintain contact with the mate and potentially initiate physical reunion. In this experiment, females also showed an elevation of their own plasma levels of corticosterone while hearing the stress-induced calls of their partner. This effect could not be due to the failure of the mate recognition system as, in the go-nogo operant design, females categorize control and stress-induced calls as belonging to the same emitter, and stressed calls of familiar males do not elicit higher levels of corticosterone in females. Thus, our results suggest an emotional contagion between partners, a process in which the emotional state of an individual activates the same state in nearby conspecifics (Panksepp 2011). This physiological resonance, a premise of empathy, could control the female specific response to the stress-induced calls of its partner and not to other individuals, and thus participates in the maintenance of the pair bond. Empathy in birds has been previously proposed (Edgar, Lowe et al. 2011), but we provide the first evidence of the phenomenon based on acoustic cues only. Our results from **chapter 3** did not show any assortative mating based on physiological profile but suggest that an behavioural adjustment drives coordination and thus synchrony between mates. In **chapter 2**, the physiological resonance between mates is another type of adjustment that will presumably enhance the synchrony of mates in their activities after a stressful event.

For a communication system to evolve, both the sender and the receiver must derive benefits from the signal (Zahavi 1991). In our experiments, the direct benefit for the parent receivers (**chapter 4**) is to enhance their reproductive success, i.e. to ameliorate their fitness by ensuring the survival of the progeny. Females (**chapter 2** and **chapter 3**) can benefit from a direct effect on their survival by being aware of a potential danger, but they can also efficiently respond to their partner, and potentially increase the pair's survival. It is now recognized that the evolution of an acoustic communication system occurs not only in the context of individual senders and receivers, but also among a network of senders and receivers (McGregor and Dabelsteen 1996). In **chapter 4**, siblings are potential receivers of the stress-induced vocalisations of their brothers and sisters, informing them of their siblings' needs, which could participate in a global regulation of siblings competition in the nest as previously shown in barn owls (Dreiss, Ruppli et al. 2013) or barn swallows (Romano, Boncoraglio et al. 2013). Other adult pairs could benefit from hearing stress-induced beggings by getting information about food availability of potential breeding spots (Horn and Leonard 2005): a poor patch means that parents will potentially have difficulties to satisfy their chicks, which will consequently emit stress-induced begging calls because starvation elevates plasma levels of corticosterone (**chapter 4**). Eavesdropping could thus help other adults to choose if the place is a good choice for breeding or not. Finally, stress-induced calls from unmated (**chapter 1**) or mated males (**chapter 2**) could inform any bird in the social group about an imminent danger, especially if distance calls are used as alarm calls in this species. We know from **chapter 2** that females respond to the stress-induced calls of their mates but not to the stress-induced calls of a stranger bird, but we still do not know if females would react to the stress-induced calls of a familiar male, i.e. a male from the same social group. This question remains to be explored.

All these benefits impact on specific interactions of the zebra finch social network, i.e. pairbond, parents-offspring, siblings-siblings and potentially familiar-familiar interactions. This could overall participate in the maintenance of the social group over time, as it impacts on different types of valuable social interactions. Acoustic signals and physiological signals such as corticosterone involved in the modulation of social relationships are thus central parameters in the evolution of group living in vocal species.

### **III.8 Conclusion and perspectives.**

The present work proposes corticosterone as a key hormone that drives the expression of emotion in birds through fine modifications of the acoustic structure of the calls of both adults and nestlings. We show that these fine modulations elicit adaptive responses from the receivers and could overall participate in the survival of individuals, but also in

the maintenance of strong bonds such as the pairbond. Using a multidisciplinary approach from physiology to acoustics, this work brings a better understanding of the processes involved in vocal flexibility in birds, and suggest that non-learned vocal signals are highly flexible and inform senders about multiple pieces of information. Further investigations are needed to understand how corticosterone can provoke the emission of modulated vocalisations in both adults and nestlings.

The present work supports previous studies revealing that the pairbond in the zebra finch is extremely strong (Zann 1996; Ramage-Healey, Adkins-Regan et al. 2003; Elie, Mathevon et al. 2011) and is associated with complex processes, as we were able to describe emotional contagion based on vocal signals only, a potential basis of empathy. Stress can be quantified by measuring plasma levels of corticosterone, but it is not always possible to take blood samples, especially in wild animals and young nestlings that could suffer from invasive blood sampling. Our findings show that stress-induced calls could be good indicators of the distress of a bird and the stress of an individual could be evaluated by measuring acoustic parameters on its vocalisations. Acoustic cues of emotions in birds could thus be used as indicators of animal welfare without using any invasive process, and represent a good alternative to blood sampling. Other indicators could be potentially interesting: the preliminary study using an infrared camera to evaluate the temperature of a bird during a stressful situation show congruent conclusions with our results from **chapter 3** and could thus be a good non invasive indicator of stress in this species.

The zebra finch is a gregarious species that relies on vocalisations to communicate with group members. Nevertheless, other channels of communication should be considered to get a more complete understanding of the expression of emotions in this species. Recent studies have showed that bird species present active olfactive bulbs able to discriminate between relevant olfactory signals (Mabayo, Okumura et al. 1996; Jones and Roper 1997): for instance, zebra finch fledglings recognize their nest based on its odour (Caspers and Krause 2011), great tits elaborate a bouquet of plant odours in their nest and use it as cues for frequency of nest maintenance (Petit, Hossaert-McKey et al. 2002) and petrels are capable of olfactory partner recognition (Bonadonna and Nevitt 2004; Bonadonna, Caro et al. 2009). The olfactory channel could thus be a good candidate for the study of emotions in birds. My post-doctorate project will investigate emotions in chickens, and especially how olfactory signals can generate and/or modulate emotions of different valences in young adults confronted to a novel environment. This project following my thesis work will bring new evidence on the multimodal expression of emotions in bird and will allow to improve our knowledge about the complexity of birds' cognitive abilities.



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**TITLE: COMMUNICATING ABOUT STRESS: MODULATION OF VOCALISATIONS IN THE ZEBRA FINCH.**

In social species, vocalisations convey information that participates in the maintenance and the survival of the group. While many studies were interested in stable information carried by vocal signals, like identity, fewer studies dealt with their potential role in informing about labile information such as the senders' emotional state. Stress is a good candidate for the study of the expression of emotions in animals, as it is directly measurable by the plasma levels of glucocorticoids. Stress is known to modify acoustic parameters of vocalisations in mammals, but few studies studied the process in birds, that also show complex social networks. The aim of this thesis is thus to determine how birds can vocally express their stress and to what extent corticosterone, the main stress hormone in birds, is implicated in this expression. I focused my research on the zebra finch (*Taenopygia guttata*), a gregarious Australian songbird that form lifelong pairbond and provides biparental care to its young. Using oral administration of exogenous corticosterone but also social stressful events, we show that both adult males and nestlings can express their stress through modulations of their calls' structure, suggesting that flexibility in birds' vocalisations is higher than previously expected. With a complete analysis of temporal and spectral parameters of calls, we show for the first time that stress evokes the emission of vocalisations with up-shifted frequency spectrum via a direct effect of corticosterone. Moreover, we show that females and parents are able to decode the information carried by stressed-induced calls of respectively their male partner and their young, as they exhibit modifications of behaviour in accordance with the context. The physical processes leading to the emission of stressed-induced vocalisations are discussed by applying the source-filter theory usually used in mammals. Adaptive values are also proposed, in regards with the zebra finch social network and predation risks for the caller.

This work gives new evidences about the expression of stress in birds, and proposes a comprehensive study, from the physiological signals involved in stress to the resulting modifications of communication behaviour for the sender, that leads to an adaptive response from the receivers.

**Key words: acoustic communication, begging call, call structure, corticosterone, distance call, emotion, emotional state, pairbond, stress, vocalisation, zebra finch.**

**TITRE : COMMUNIQUER SON STRESS : MODULATION DES VOCALISATIONS CHEZ LE DIAMANT MANDARIN**

Chez les espèces sociales, les vocalisations transmettent des informations qui participent au maintien et à la survie du groupe. Alors que de nombreuses études se sont intéressées aux informations stables portées par les vocalisations telles que l'identité, peu d'études se sont interrogées sur le rôle des signaux vocaux dans la transmission des informations plus labiles, telles que l'état émotionnel de l'émetteur. Le stress est un bon candidat pour l'étude de l'expression des émotions chez les animaux, puisqu'il est directement mesurable via un dosage de la concentration plasmatique en glucocorticoïdes. Le stress est connu pour modifier les paramètres acoustiques des vocalisations chez les mammifères, mais peu d'études ont traité ce processus chez les oiseaux, qui présentent pourtant des réseaux sociaux complexes. Le but de cette thèse est de déterminer de quelle manière les oiseaux expriment vocalement leur stress, et d'évaluer dans quelle mesure la corticostérone, hormone de stress principale chez l'oiseau, est impliquée dans le phénomène. Je me suis intéressée au Diamant mandarin (*Taenopygia guttata*), un oiseau chanteur australien au comportement grégaire qui forme des liens d'appariements à vie et prodigue des soins biparentaux à sa progéniture. En administrant de la corticostérone exogène à des oiseaux et en utilisant également des événements sociaux stressants, nous montrons que les mâles adultes et les poussins expriment leur stress à travers l'émission de cris modifiés dans leur structure, ce qui suggère que la flexibilité des cris chez les oiseaux est plus importante que l'avaient montré des études précédentes. Par une analyse complète des paramètres temporels et spectraux des cris, nous montrons pour la première fois que le stress, par un effet direct de la corticostérone, déclenche l'émission de vocalisations présentant un spectre de fréquence déplacé vers les hautes fréquences. De plus, les receveurs du signal (respectivement les partenaires femelles et les parents) semblent capables de décoder l'information portée par ces cris de stress car ils modifient leur comportement en conséquence. Les processus physiques impliqués dans l'émission de cris modulés par le stress sont également discutés en appliquant la théorie « source-filtre » généralement utilisée chez les mammifères. Enfin, les valeurs adaptatives de ces cris sont également envisagées, en rapport avec le réseau social du Diamant mandarin et les risques de prédation encourus par l'émetteur du signal.

Ce travail apporte de nouvelles preuves sur l'expression du stress chez les oiseaux, et propose une étude complète, des signaux physiologiques impliqués dans le stress aux modifications de comportement de l'émetteur, qui déclenchent une réponse adaptative des receveurs du signal.

**Mots clés : communication acoustique, corticostérone, cris de distance, cris de quémante, Diamant mandarin, émotion, état émotionnel, lien d'appariement, stress, structure des cris, vocalisations.**

**Discipline:** Ethologie, Biologie, Physiologie, Bioacoustique.

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