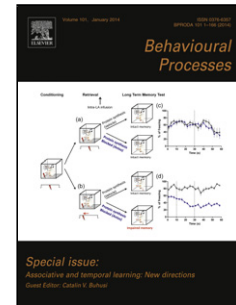


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Territorial responses to song components in a suboscine, the vermilion flycatcher**Running title:** Territorial responses to song components

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Highlights

- Vermilion flycatcher songs consist of a first part (variable in number of elements) and a second part (with four elements)
- Males respond to the first part alone, to the second part alone, and to complete songs
- Both the first part and second part are important during territorial competition
- Males call more when exposed to the first part than to the second part
- The first part of the song seems to play a special role in territorial competition

Abstract

Vermilion flycatchers songs are composed of two acoustically different parts: a first part (FP) containing a variable number of introductory elements, and a second part (SP) composed of four elements. Previous work suggests that the FP is important for territorial competition and that it conveys information on threat level. By exposing free-living males to playbacks of complete songs (CS's), FP's and SP's, we evaluated the relative contribution of each song part in males' territorial responses. Males called in response to all three treatments, suggesting each song component is important for territorial competition. Males' call response did not differ toward CS's and FP's, and toward CS's and SP's (although a non-significant tendency was found for SP's to elicit a weaker response than CS's), but it was greater toward FP's than toward SP's. These results, coupled with previous work, further support the idea that the FP plays a special role during territorial competition and may give information on level of threat. We further discuss our result in terms of mechanistic and functional hypotheses.

Key words: suboscine, vermilion flycatcher, territorial competition, bird song,

Pyrocephalus rubinus

1. Introduction

Many animals rely on vocal signals to establish and maintain social relations (Naguib, 2005); vocal signaling is necessary in a broad range of contexts including mate selection, pair-bond maintenance, territory defense, and parent-offspring communication (Bradbury and Vehrencamp, 2011). In birds songs have been extensively studied in the past and we now have a reasonable understanding of their function and evolution. While bird song may play an inter-specific role (e.g. Doutrelant et al. 2000), its main functions are recognized as intra-specific (mate attraction and territorial defense, Catchpole and Slater, 2008).

Catchpole (1982) proposed that in passerine birds, long, complex and variable songs evolved through inter-sexual selection, while short, simple and stereotyped songs evolved through intra-sexual selection. This model was based in studies addressing the function of songs in oscine passerines, who differ from their sister taxa (the suboscine passerines) in a number of ways. One such way is song ontogeny: while song learning has been accepted as the mechanism by which oscine birds acquire their songs (Kroodsma, 1982), innate production of songs seems a more general mechanism for suboscines (e.g. Kroodsma, 1984, 1985, 1989; Kroodsma and Konishi, 1991; Toughton et al 2014; but see Kroodsma, 2013;

Trainer et al. 2002, 2013; Fitzsimons et al. 2008). Song learning has been related to song variation (Kroodsma, 1982) and suboscines are generally thought to produce simpler and less variable songs than oscines (e.g. Moseley and Willey, 2013). Limited variation in song may reduce the scope for some discrimination processes in suboscines (Tobias and Seddon, 2009), prompting the question of how songs in this group function during inter- and intra-sexual interactions. This question remains far from answered because studies on the social function of suboscine songs are scarce.

Most studies on suboscine songs have been descriptive and comparative (see below). Some of these studies have acknowledged that despite the innate nature of song development in this group, there is, sometimes subtle, variation in acoustic attributes among individuals (Fitzsimmons et al. 2008; Lovell and Lein, 2004a; Moseley and Wiley, 2013; Clark and Leung, 2011; Foote et al. 2013). This variation sometimes enables discrimination between neighbors and strangers (Westcott, 1997; Lovell and Lein, 2004b), between mates and strangers (Seddon and Tobias, 2010), and between different neighbors (Lovell and Lein, 2005); intra-specific song variation can also be expressed as geographical variation in some suboscines (Lovell and Lein, 2013), but not in others (Foote et al. 2012). At an inter-specific level, it has been shown that closely related suboscine species show sufficient variation in song to be distinguished (Isler et al., 1998), allowing species discrimination (Seddon and Tobias, 2010). Species discrimination has been proposed as an important mechanism promoting the evolution of complex songs in tyrant suboscines (Wiley, 2005), enabling reproductive isolation, and ultimately being one important factor promoting speciation in some suboscines (antbirds, *Thamnophilidae*, Seddon, 2005). The pioneering work by Smith (1988) and by Smith and Smith (1992, 1996a, 1996b) on the

social function of songs in suboscines showed that species that sing different song forms provide information on the probability to interact with others by singing different proportions of these song forms or by uttering a particular song form.

In suboscine species like the vermilion flycatcher (*Pyrocephalus rubinus*), which produces only one song type while perching (Smith, 1970; Ríos Chelén et al. 2005), variation both among and within males can be achieved in other ways, such as by changing the number of introductory elements in the song (Ríos Chelén et al. 2005, Ríos-Chelén et al. 2013). Vermilion flycatcher songs are composed of two acoustically distinct parts: the first part composed of a variable number of introductory elements (mean \pm SE = 5.3 ± 1), and the second part with four elements: two middle elements, one high frequency element, and a terminal element (Fig. 1; Ríos Chelén et al. 2005). Ríos Chelén et al. (2005), showed that, from a number of measured acoustic attributes, the number of introductory elements exhibited the highest coefficient of variation among males, while the duration of the terminal element showed the highest repeatability (indicating an attribute that varied little within males). Ríos-Chelén and colleagues also found that vermilion flycatchers show vocal flexibility regarding the first part (Fig. 2): the number of introductory elements slightly increases after the onset of nest construction (Ríos Chelén et al. 2005) and as sunrise approaches (Ríos-Chelén et al. 2013). After the commencement of nest construction males respond more strongly (i.e. fly more from perch to perch) to songs with longer first parts (songs with more introductory elements) than to songs with shorter first parts (Ríos-Chelén and Macías Garcia, 2007), and thus the authors suggested that the first part of the song plays an important role during territorial defense and that longer songs may be perceived as more threatening than shorter ones. However, the study by Ríos-Chelén and

Macías Garcia (2007) exposed territorial males to complete songs and it is not clear whether the first part alone is sufficient to elicit a territorial response. If males respond as strongly to the first part as to the complete song, this would suggest that the first part alone could play an important role for territorial competition encoding relevant information for this task; on the other hand, if the first part elicits a weaker response than the complete song, this would suggest that the first part encodes incomplete information for territorial competition. Additionally, if the second part alone elicits a response as strong as the first part and the complete song this would suggest that the second part and the first part encode redundant information for territorial competition.

To evaluate the relative salience of each song part, we exposed male vermilion flycatchers to playbacks of complete songs, first parts only, and second parts only, and compared their response across treatments. We were also interested in evaluating whether the stronger response toward songs with longer first parts (see above, Ríos Chelén and Macías Garcia, 2007) is dependent upon hearing the complete song or whether males respond strongly to longer first parts on their own. If males respond strongly to longer first parts, this would suggest that males perceive longer first parts as more threatening.

2. Material and methods

2.1 Study site and subjects

This experiment was carried out at the forest of San Diego Metepec (19°17.97N, 98°14.60W), in the city of Tlaxcala, Mexico, during the breeding season from April 23rd to May 27th 2015, and during the morning from 08:00 to 12:00 hrs. This habitat has already

been described in Ríos Chelén et al. (2005). For the experiment, we used free-living and unmarked vermilion flycatcher males (for sample size see “experimental design and preparation of treatments”, below). Males were identified by their territory occupancy, provided that their territories were previously mapped. This seems to be an effective way of identifying males as they are territorial and defend their territories by singing and calling throughout the breeding season; two marked males have each been seen to occupy the same territory for four consecutive years and males commonly use the same song perches during the dawn chorus, as was assessed with banded individuals (AAR-CH pers. obs., Ríos Chelén et al. 2005). Additionally, each male was exposed to the three trials in one single morning.

2.2 Experimental design and preparation of treatments

We placed a speaker (Mineroff SME-AFS), connected with a 10m cable to a digital audio player (SONY, NWD-B103F), inside the focal male’s territory; the speaker was attached to a tree at a height of around 1m and facing towards the focal male. From the digital audio player location (10m away from the speaker), the observer started the trial; from this point the observer registered the behavior of the focal male. The sound files were played at a sound pressure level of 65-70dB(A) (re 20 μ Pa); this sound amplitude has proved effective for eliciting territorial responses in vermilion flycatchers (Ríos-Chelén and Macías Garcia, 2007). Fifteen males were exposed to three different treatments (see below). To prepare the treatments, we first selected high-quality songs recorded at the same site in 2001 using a Sennheiser ME66 microphone connected to a Marantz PMD221 cassette recorder

(sampling rate 22050 Hz, Ríos Chelén et al. 2005), and split each song into its first and second parts (Fig. 3a).

Each male was exposed to three different playback treatments that came from the same song: Complete Song (CS), consisting of complete songs (control); First Part (FP), made up of first parts only; and Second Part (SP), composed of second parts only. To avoid pseudo-replication, we used a song from a different individual (recorded in 2001) to prepare the treatments and to test each focal male. Thus, we used songs recorded from 15 males to test the 15 males. To control for a possible order effect, the order of treatments was such that the probability of each male being exposed to the CS, FP, and SP treatments as a first, second or third treatment was the same. To take into account the natural variation in number of introductory elements (IE's, Ríos Chelén et al. 2005), and to test for a possible effect of this variable in males' responses, one third of the males (5 males) were tested with treatments obtained from 4IE's songs, one third with treatments obtained from 5IE's songs, and one third from 6IE's songs. Vermillion flycatchers sing in song bouts at dawn (Rivera-Cáceres et al. 2011), and also but more sporadically during the day (AARCH pers. obs.); that is, in groups of songs. To simulate the singing behavior of vermilion flycatchers, each treatment (CS, FP, SP) was prepared by forming a one-minute recording containing five bouts, each bout containing four repetitions of CS's, FP's, or SP's (Fig. 3b). This bout size falls within the natural range of song bout sizes (Rivera-Cáceres et al. 2011). This means that each treatment was played at a rate of 20 CS's (or FP's or SP's) /min; this rate is within the natural singing rate of vermilion flycatchers at dawn (Rivera-Cáceres et al. 2011). Intra-bout CS (or FP or SP) rate and inter-bout periods were kept constant across treatments. The mean (\pm SE) period between songs in a bout was as follow. For CS's, 0.903

s (± 0.004), FP's 1.217 s (± 0.008), SP's 1.621 s (± 0.039). Following Ríos-Chelén and Macías Garcia (2007), we waited at least 10 min (range: 10-20 min) between treatments to allow birds to return to basal levels of activity. Post-hoc analysis indicates that this interval is generally sufficient: males' call response toward the CS treatment did not differ if they heard the treatment in the first place, in the second place, or in the third place (ANOVA, $F(2, 12) = 1.57$, $p = 0.247$). However, males' flight response toward the CS treatment differed depending upon the order in which this treatment was applied (ANOVA, $F(2, 12) = 6.93$, $p = 0.010$); males flew from perch to perch less when they heard the CS treatment in the third place than in the first place (Post-hoc Holm-Sidak method, $p = 0.029$) or second place (Post-hoc Holm-Sidak method, $p = 0.004$). Males' flight response toward the CS treatment did not differ when they heard this treatment in the first place compared to the second place (Post-hoc Holm-Sidak method, $p = 0.253$). The order of the FP treatment had no effect on birds' call response (ANOVA, $F(2, 12) = 0.36$, $p = 0.702$) and flight response (ANOVA, $F(2, 12) = 0.28$, $p = 0.755$). Similarly, call response and flight response did not differ with the order of the SP treatment (ANOVA, call response, $F(2, 12) = 1.70$, $p = 0.22$; flight response, $F(2, 12) = 0.90$, $p = 0.43$).

Each trial was composed of three different periods: a pre-playback minute of silence (control), followed by a playback minute (where the playback treatment was broadcast, Fig. 3b), and a post-playback minute of silence. This experimental design has proved effective for evaluating behavioral discrimination of acoustic stimuli in the studied species (Ríos-Chelén and Macías Garcia, 2007).

2.3 Behavioral measures

During each of the pre-playback, playback, and post-playback minutes we registered the number of calls and flights (from perch to perch) of the focal male. We then obtained call and flight rates in these minutes (for more details see statistical analyses, below). Calls and flights (to chase intruders, or in our case to presumably search for it) are the most common response behaviors elicited by natural and simulated intruders after the dawn chorus (AARCH pers.obs.; Ríos-Chelén and Macías Garcia, 2007).

2.4 Statistical analyses

When residuals from statistical models (see below) conformed to a normal distribution (Kolmogorov-Smirnov, $P < 0.05$) we used parametric tests; otherwise non-parametric tests were used.

We first performed within-male comparisons. We evaluated whether males responded to our CS's, FP's, and SP's treatments. To do so we obtained activity rates; that is, call rate (number of calls per min) and flight rate (number of songs per min) for each of the three different periods (i.e. pre-playback, playback, and post-playback minutes). We then combined these activity rates for the playback and post-playback periods into a single playback+post-playback period to compare it with the pre-playback period. We used the Wilcoxon test for related samples (Kolmogorov-Smirnov, $P < 0.05$) or the paired t-test (Kolmogorov-Smirnov, $P > 0.05$) to compare the call rate and flight rate during the pre-playback period with the playback+post-playback period. To evaluate whether males responded differently or similarly to the treatments (CS, FP, SP), we obtained differences in activity rate (e.g. call rate) for each treatment. To this end, we subtracted the activity rate

during the pre-playback period from the activity rate during the playback+post-playback period (i.e. activity rate in (playback+post-playback) – (activity rate in pre-playback)). This difference in response (from now on “call response” and “flight response”) can be seen as the intensity in response relative to the basal activity rate (during the pre-playback minute) of the focal male. Because each male was exposed to the CS, FP, and SP treatments, this allowed us to compare the response of the same male with itself to these treatments. To do so we used a repeated measures General Linear Model in which we introduced the treatment (CS, FP, SP) as an intra-subject independent variable, and the number of introductory elements as a between-subjects factor. This allowed us to compare the response of each individual with itself during the three different treatments and, at the same time, to determine whether the number of introductory elements had an effect among males’ responses. Call and flight responses were introduced as dependent variables.

Depending on the statistical test, we report the following scores of effect size. For repeated measures General Linear Models we report Partial Eta Squared (partial η^2 , Lakens, 2013), for paired t-tests we report Cohen’s d scores for related samples (Cohen’s d_z , Lakens, 2013), and for Wilcoxon Signed Rank tests we report PS_{dep} (with ties discarded, Grissom and Kim, 2012); for completeness we also report Cohen’s d_z scores for Wilcoxon Signed Rank tests.

Post-hoc tests were carried out with the Post-hoc Holm- Sidak method. Analyses were done with SPSS version 10.0.1 and Sigma Plot version 2.03. All tests were two-tailed.

3. Results

Individuals responded to CS's (paired t-test: calls, $t(14) = -4.82$, $p < 0.001$, Cohen's $d_z = -1.24$; Wilcoxon Signed Rank test, flights, $Z = 3.08$, $W = 78$, $p < 0.001$, Cohen's $d_z = -1.17$, $PS_{dep} = 1$), FP's (calls, $t(14) = -6.52$, $p < 0.001$, Cohen's $d_z = -1.68$; flights, $t(14) = -3.074$, $p = 0.008$, Cohen's $d_z = -0.79$), and SP's (calls, $Z = 3.074$, $W = 78$, $p < 0.001$, Cohen's $d_z = -0.84$, $PS_{dep} = 1$; flights, $t(14) = -3.151$, $p = 0.007$, Cohen's $d_z = -0.81$), by increasing their basal activity (Fig. 4).

Males' call response differed between the CS, FP, and SP treatments (repeated measures General linear Model: Wilks' $\lambda = 0.511$, $F(2, 11) = 5.25$, $p = 0.025$, partial $\eta^2 = 0.48$). Males' call response was greater when they heard the FP's than the SP's (Post-hoc Holm- Sidak method, $p = 0.006$, Fig. 5). Call response did not differ between CS's and FP's (Post-hoc Holm- Sidak method, $p = 0.337$) and there was a non-significant trend for males to call less in response to SP's than to CS's (Post-hoc Holm- Sidak method $p = 0.053$, Fig. 5). On the other hand, there was no effect of treatments on flight response (repeated measures General Linear Model: Wilks' $\lambda = 0.847$, $F(2, 11) = 0.99$, $p = 0.400$).

Our repeated measures General Linear Models, where we introduced number of IE's as a between-subject factor variable, showed no evidence that number of IE's had an effect on call response among males (test of between subjects factor, $F(2) = 1.31$, $p = 0.305$), or on flight response (test of between subjects factor, $F(2) = 1.66$, $p = 0.231$) either.

4. Discussion

Male vermilion flycatchers called and flew as response to all three treatments (CS, FP, and SP), suggesting that each song part encodes information relevant for territorial competition

in this species. The call and flight responses toward the CS were similar to that toward each of the FP and SP, suggesting that each of the FP and SP are sufficient to elicit complete calling and flying territorial responses from territorial males and that each song part are important during territorial competition.

The fact that males flew less when they heard the CS treatment in the third place than in the first or second place (see Experimental design and preparation of treatments, above) suggests males habituated to the playback. Given this order effect, it is possible that the effect of CS's on flight response was underestimated. However, this effect was observed only when considering flights as a response, and thus our conclusions that stem from our main significant result (i.e. a stronger call response toward FP's than toward SP's, see below) do not change. The period that we left between treatments was 10-20 min; future playback experiments with this species could probably benefit by leaving at least 20 min between treatments to reduce the probability of habituation.

We found that the SP elicited a weaker call response than the FP. Vermilion flycatchers may employ a syntactical rule by which the SP needs to be preceded by the FP to elicit a territorial response comparable to that elicited by the FP. Alternatively, because the FP is composed of more elements than the SP it could be that vermilion flycatchers are more stimulated when hearing the FP than the SP because the former represents a stimulus with more energy. These ideas would have been more strongly supported if we had found that SP's elicit a significantly lower territorial response than CS's.

The idea that the FP is a section of the song involved in conveying level of threat is in accordance with a previous study that suggested that males perceive longer songs (with more introductory elements) as more threatening than shorter songs (Ríos-Chelén and

Macías Garcia, 2007). This result was not replicated in the current study, however. A number of differences between both studies may account for the different results. First, the Ríos-Chelén and Macías Garcia (2007)'s study compared within male responses toward songs with different number of introductory elements, the present study compared among male responses; among-male variation in behavior may account for less clear-cut results in the present study. Second, the 2007's study considered variation in behavior with the breeding period (before vs. after nest construction), while the present study did not, which could have obscured our results. While the intra-male comparisons were based in 15 males (Ríos-Chelén and Macías Garcia, 2007), our among-male comparisons were based in 5 males only (i.e. five males were exposed to treatments coming from songs with 4IE, five to treatments coming from songs with 5IE, and five to treatments based in songs with 6IE, this study) which could have resulted in a low statistical power to find a significant result.

Finally, the study by Ríos Chelén and Macías Garcia (2007) compared males' responses toward a difference of 4 IE's between treatments (3 vs. 7 and 4 vs. 8 IE's); we evaluated a difference of only 1 or 2 IE's (i.e. we used 4, 5 and 6 IE's in our playbacks). It is possible that the relatively small variation in number of IE's in our treatments was not sufficient to elicit a differential response in males. Overall, our data is in line with the idea that both the FP and SP play a role in territorial competition (this study), and that variation, with a large enough difference in number of IE's, is perceived as a signal that conveys information about threat level (this study, Ríos Chelén and Macías Garcia, 2007). A previous study determined that the mean number (\pm SE) of IE's is 5.3 ± 1 songs (Ríos Chelén et al. 2005) and that the number of IE's is, among different song attributes, the song parameter with the greatest among-male coefficient of variation (18%, Ríos Chelén et al.

2005), enabling the potential to encode information (e.g. threat level, male quality), that varies among males, with the FP.

Our results are similar to results in oscines showing that some song components elicit stronger territorial responses than others. For instance, Soha and Whaling (2002), showed that Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*) mount a similar response to the trill portion of their songs as to the complete song, but the response to the whistle and buzz parts are comparatively weaker than the response to the trill. This result coupled with the fact that trill length varies within males led Soha and Whaling (2002) to suggest that the trill "may carry most of the territorial signal" and give information on motivation. Our results more closely resembles the results by Thompson and Baker (1993), with another population of Nuttall's white-crowned sparrows, that showed that the introductory whistle elicit a stronger territorial response than other song components located later in the song. It is important to note, however, that the Thompson and Baker (1993)'s study used phrase substitution rather than presenting isolated phrases; thus our experimental approach was more similar to Soha and Whaling (2002)'s, who used isolated phrases. The results of Soha and Whaling (2002) and of Thompson and Baker (1993), coupled with ours, show that songs in oscines and suboscines may be partitioned to convey specific information.

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Figures legends

Fig. 1. Spectrogram showing a vermilion flycatcher song. Vermilion flycatcher songs are composed of a first part, composed of a variable number of introductory elements (IE), and a second part with four elements: two middle elements (ME), one high frequency element (HF), and a terminal element (T). The figure also shows the length of the T. This song has 4 introductory elements.

Fig. 2. Three spectrograms of vermilion flycatcher songs varying in number of introductory elements (IE's). There is among-male and within male variation in the number of introductory elements. A song with 4 IE's (a), with 5 IE's (b), and with 6 IE's (c).

Fig. 3. Preparation of treatments and playback minute. a) a Complete song (CS) was split into its first part (FP) and second part (SP) to prepare the playback treatments. b) Each male was exposed to a 1 min playback treatment of CS's, FP's, and SP's. Each playback minute consisted of five bouts composed of 4 stimuli units (CS, FP or SP) each. The upper panel in b shows a playback minute of CS's, the middle panel shows a playback minute of FP's only, and the lower panel a playback minute of SP's only. The pre-playback and post-playback minutes are not shown.

Fig. 4. Call (upper panel) and flight (lower panel) rates of vermilion flycatchers during the pre-playback minute (pre) and the playback+post-playback minutes (PB). Males increased calling and flight rates during the PB relative to the pre when they were exposed to playback of complete songs (CS), first parts (FP) only, and second parts (SP) only. Each broken line represents a single male. Note that even though we used 15 males for these analyses, there seem to be less than 15 lines in each graph because some of them are overlapping. Boxplots are shown to aid interpretation. Number 3 on the bottom right plot indicates that there were 3 males with a value of 2. ** $P < 0.001$, * $P < 0.01$.

Fig. 5. Male vermilion flycatcher call response toward playback of complete songs (CS), first parts of songs (FP), and second parts of songs (SP). Males increased calling rate more when they heard the FP than the SP. Call response is obtained by subtracting call rate during the pre-playback minute from that during the playback+post-playback minutes; thus, it represents an increase in call rate between both periods. Each broken line represents a male. Boxplots are shown to aid interpretation. * $P < 0.01$.

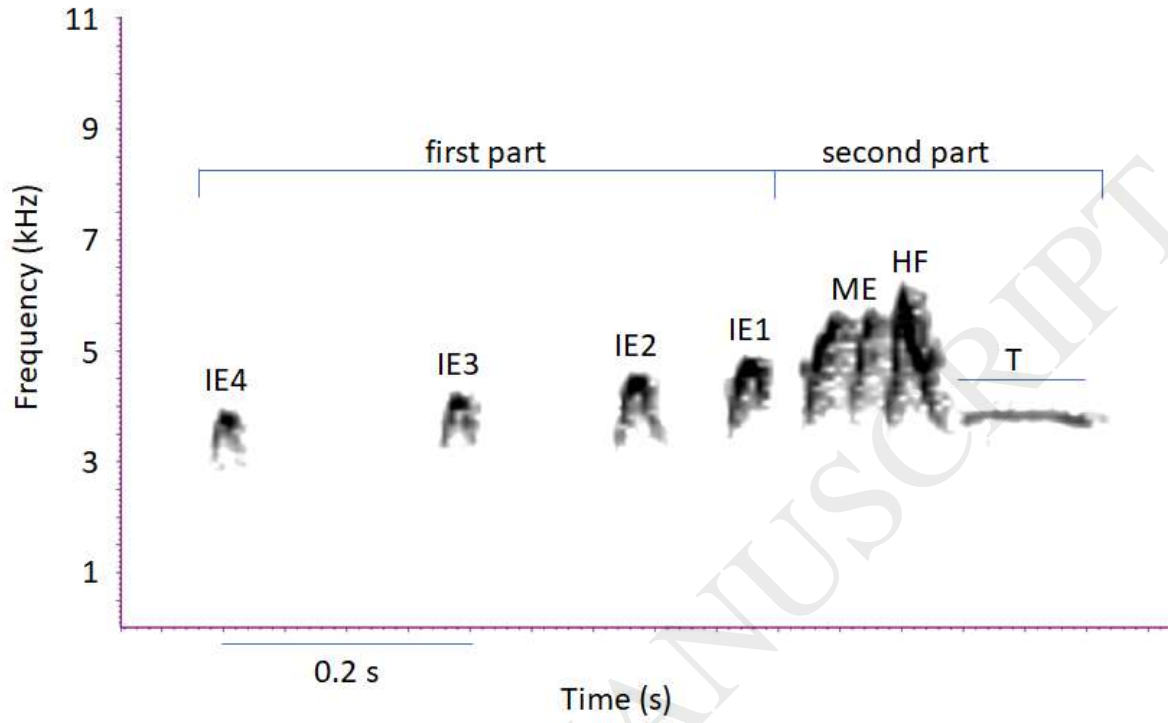


Fig. 1

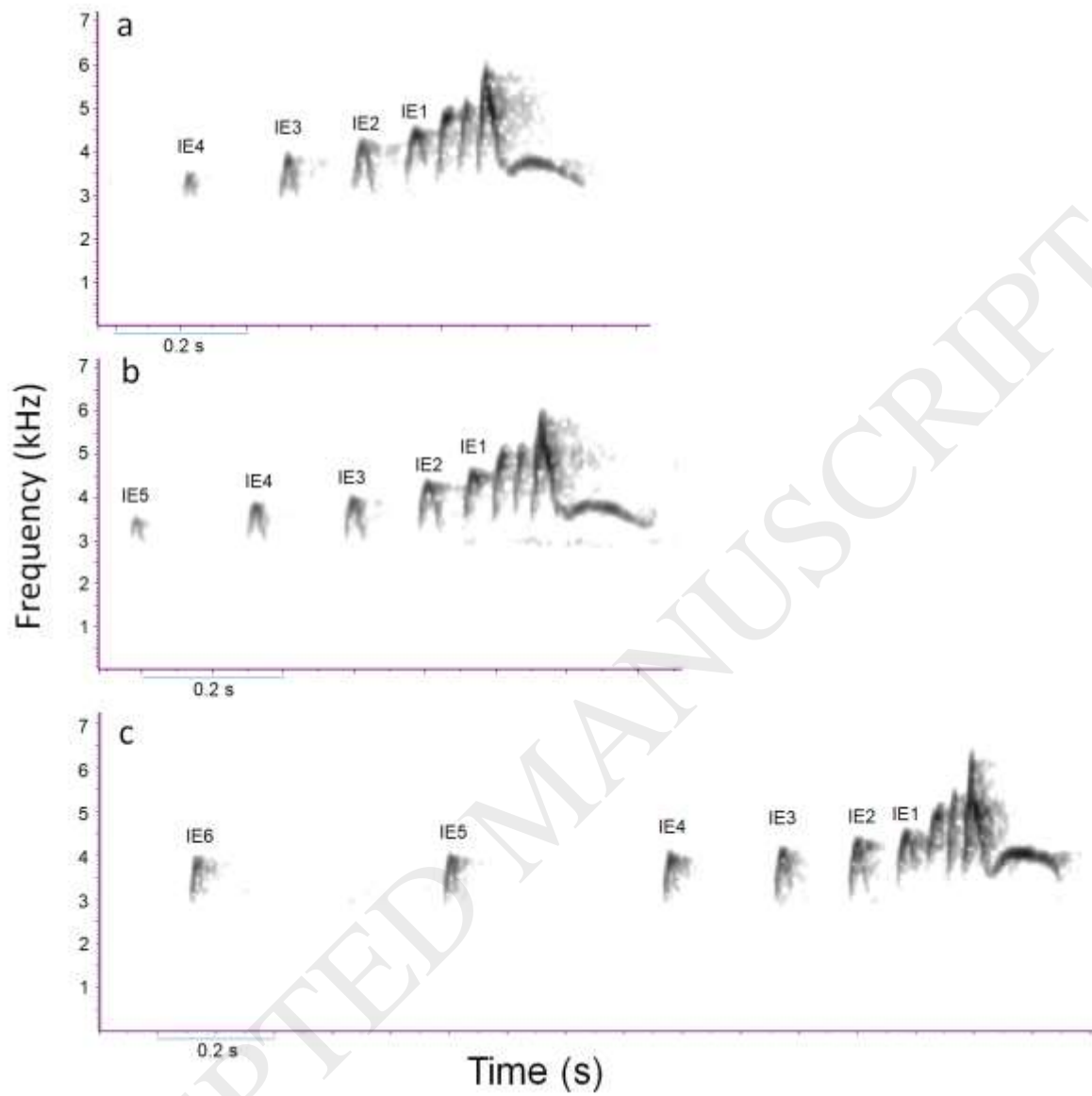


Fig 2

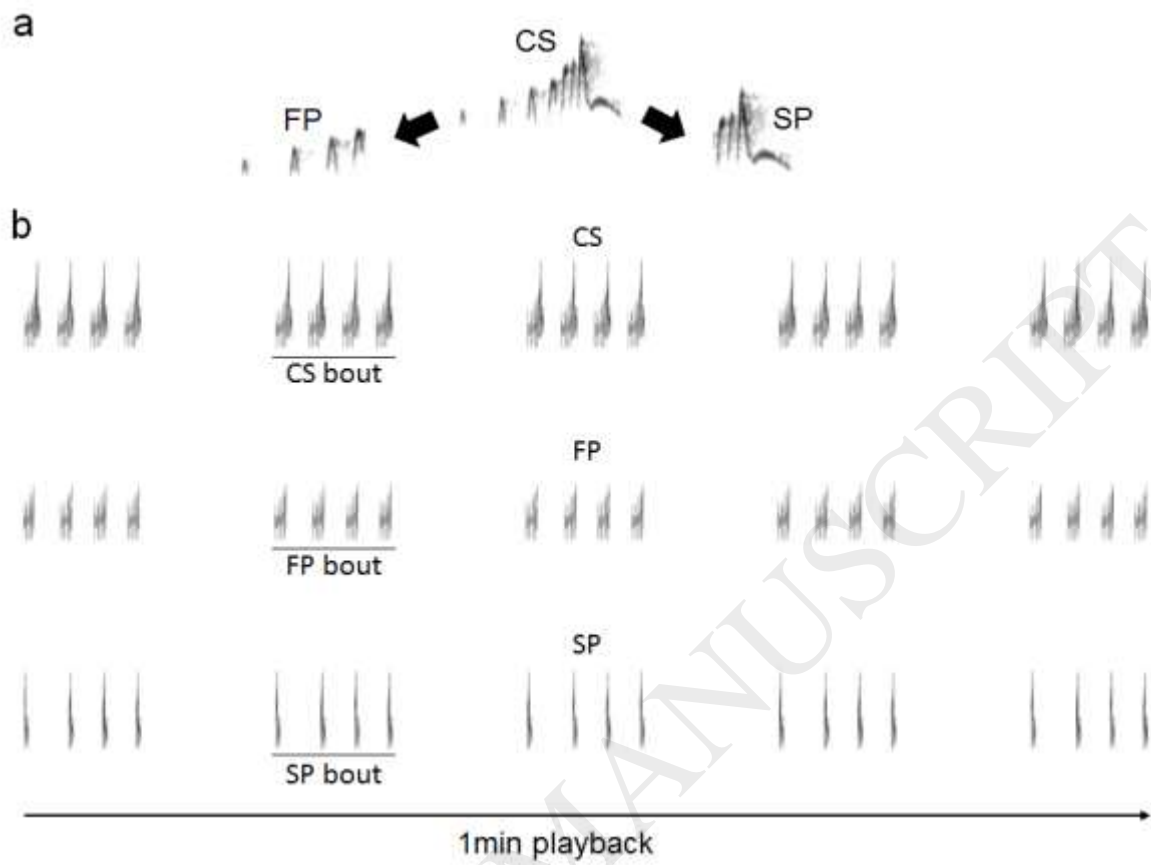


Fig. 3

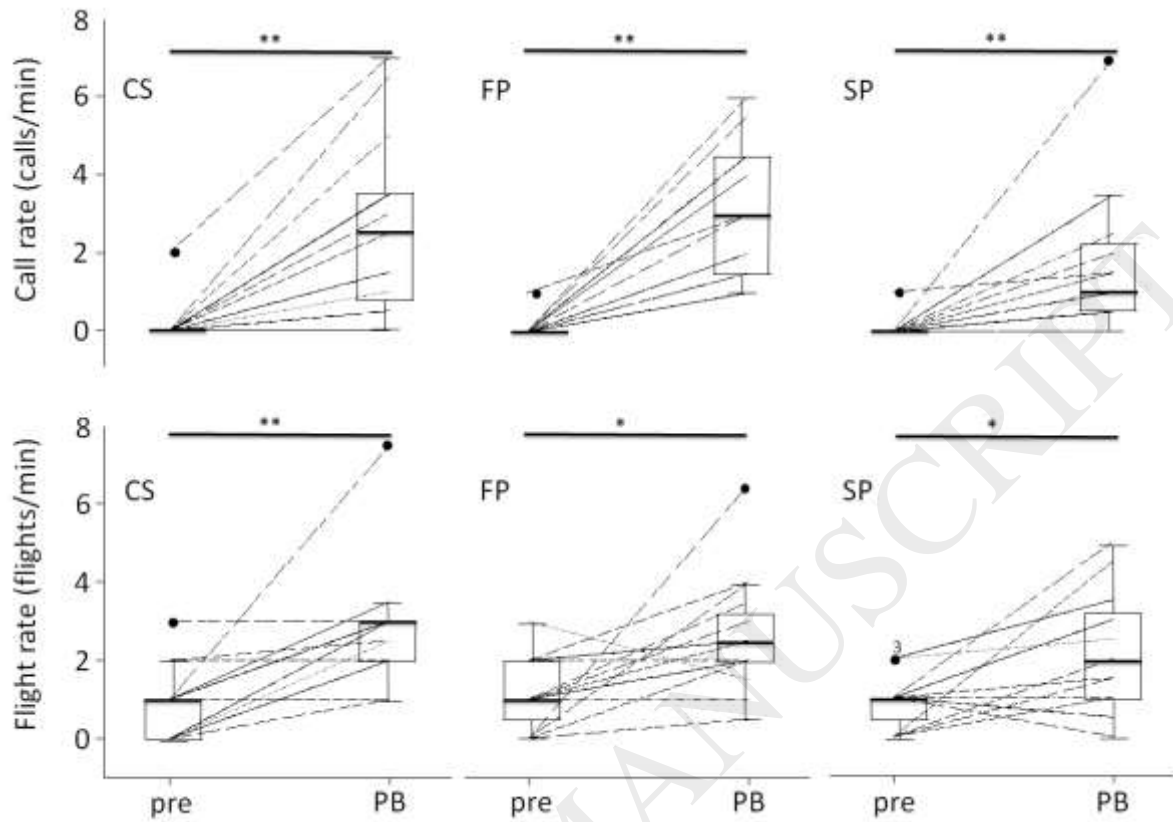


Fig. 4

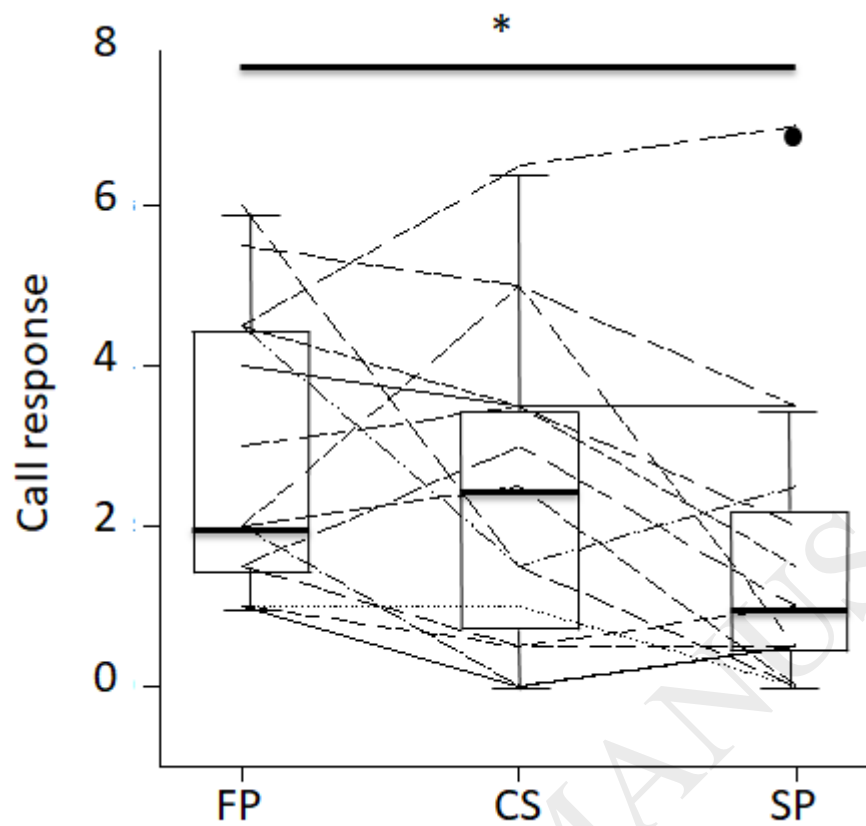


Fig. 5