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DOI: <https://doi.org/10.1016/j.anbehav.2021.05.018>

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ZORA URL: <https://doi.org/10.5167/uzh-220974>

Journal Article

Published Version

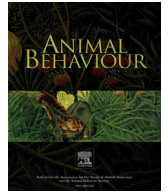


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Originally published at:

Bliard, Louis; Qvarnström, Anna; Wheatcroft, David (2021). The role of introductory alarm calls for song discrimination in *Ficedula* flycatchers. *Animal Behaviour*, 177:241-251.

DOI: <https://doi.org/10.1016/j.anbehav.2021.05.018>



The role of introductory alarm calls for song discrimination in *Ficedula* flycatchers

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ARTICLE INFO

Article history:

Received 31 August 2020

Initial acceptance 2 November 2020

Final acceptance 16 April 2021

Available online 16 June 2021

MS. number: 20-00664R

Keywords:

begging call

innate

nestling

playback

signal convergence

song learning

Assortative mating depends on species distinctiveness in mating traits and preferences, which can be challenging to maintain when traits and/or preferences are learned. This is because learning may cause individuals to copy heterospecific signals. Juvenile songbirds possess innate sensory biases favouring them to learn and to prefer conspecific songs, but the effectiveness of these biases relies on consistent and sufficient differences between the songs produced by different species. However, mating signals, including learned songs, sometimes converge in sympatry, and the species-specific cues that individuals use to shape their preferences are often unknown. In *Ficedula* flycatchers, a stereotyped and highly species-specific alarm call is often incorporated as the first syllable of their songs. However, where the two species co-occur, pied flycatchers, *Ficedula hypoleuca*, learn to incorporate the introductory calls of the closely related collared flycatcher, *Ficedula albicollis*, into their songs. In this study, we investigated the role of introductory alarm calls for song discrimination in collared flycatchers, using playback experiments of both manipulated and unmanipulated songs on adults and nestlings within the hybrid zone of Öland, Sweden. We predicted that the introductory alarm call would be sufficient to trigger song responses, such that adults and nestlings would respond similarly to song phrases including the call, whether it is followed by conspecific or heterospecific notes. Our results provide evidence that the introductory alarm call is sufficient to trigger song discrimination in nestlings, but not in adult males, potentially due to their greater experience with songs and, therefore, subtler discrimination. Altogether, this study highlights the often-overlooked importance of calls within or associated with songs.

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Species assortative mating depends on species distinctiveness in mating traits and associated preferences (Coyne & Orr, 2004; Price, 2008). Despite the importance of maintaining species distinctiveness, there is a growing number of examples of convergence in mating signals when closely related species come into secondary contact. Convergence may arise due to adaptive benefits, such as when mating signals are also used in interspecific male–male competition (e.g. agonistic character displacement; Grether, Losin, Anderson, & Okamoto, 2009; Tobias et al., 2014), or when signals are learned, as a by-product of mistaken copying (Helb, Dowsett-Lemaire, Bergmann, & Conrads, 1985). No matter the cause, signal convergence has the potential to weaken species assortative mating. When signals converge, we know comparatively little about

how species-specific preferences are maintained (Seddon & Tobias, 2010). Here, we investigated the role of species-specific acoustic cues for song discrimination in two closely related species that display asymmetric signal convergence in sympatry.

Among bird species, song is a common secondary sexual trait, mostly used by males which are the primary singers in many temperate species (Catchpole & Slater, 2008). Songs are usually involved in mitigating intrasexual interactions, such as territory defence against competing males, as well as in intersexual interactions (Catchpole & Slater, 2008; Krebs, Ashcroft, & Webber, 1978). In the latter, song is often used as a sexual display for female attraction. It is thought from numerous studies to reliably indicate male quality or condition (Gil & Gahr, 2002; Lampe & Espmark, 1994; Lampe & Saetre, 1995), thus ultimately influencing female choice (Catchpole, 1987; Reid et al., 2004). In oscine birds, song is an innate behaviour with a learned component. Juvenile males and females develop, respectively, their song and song

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preferences in part through imprinting on the songs of surrounding conspecifics. For instance, Mennill et al. (2018) demonstrated through playback experiments that wild Savannah sparrows, *Passerculus sandwichensis*, learn their songs by listening to tutors present in their environment. However, learning from surrounding individuals in nature can sometimes lead to inappropriate tutoring if individuals imprint partly on heterospecifics. This often results in individuals developing mixed songs that include heterospecific notes (Haavie et al., 2004; Helb et al., 1985). This has also been shown extensively through cross-fostering experiments, in which birds often imprint on their foster parents' songs (Clayton, 1989; Eriksen, Lampe, & Slagsvold, 2009).

Despite songs having a learned component in oscine birds, there is a growing body of evidence since the early work of Thorpe (1958) that birds possess an auditory bias for conspecific songs early in life (reviewed by Wheatcroft & Qvarnström, 2015). It is thought to focus learning onto the song of their own species, or at least songs that do not depart too far from typical conspecific songs (Lachlan & Feldman, 2003). For instance, zebra finches, *Taeniopygia guttata*, nestlings removed from their parents at a young age and reared in isolation respond more strongly to zebra finch songs than to starling, *Sturnus vulgaris*, songs (Braaten & Reynolds, 1999). Similarly, nestlings of canary, *Serinus canaria*, breeds artificially selected for low-pitch songs display a reduced hearing sensitivity to high-pitch sounds and are thus more likely to discriminate in favour of low-pitch sounds and to later develop songs resembling that of their own breed (Wright, Brittan-Powell, Dooling, & Mundinger, 2004). In addition, both white-crowned sparrows, *Zonotrichia leucophrys*, and golden-crowned sparrows, *Zonotrichia atricapilla*, have been shown to respond to conspecific songs early in life (Nelson & Marler, 1993; Shizuka, 2014). These studies demonstrate that young birds express auditory biases, which in some cases lead to discrimination in favour of conspecific or breed-specific songs.

Sensory biases for conspecific songs necessarily imply that there are sufficient, consistent acoustic differences between the songs produced by different species. These song features are likely to be conserved within species (Lachlan, Verhagen, Peters, & Cate, 2010) and variable among species (some have been reviewed by Wheatcroft & Qvarnström, 2015). They can involve species-specific syntax (e.g. ordering of the notes within the song or species-specific tempo) or species-specific notes. Regarding the former, previous studies have suggested that syntax could be involved in the song preferences of chaffinches, *Fringilla coelebs*, and nightingales, *Luscinia megarhynchos* (Hultsch & Todt, 1989; Lachlan et al., 2013). In addition, the ordering of syllables can drive the responsiveness of receivers in some species (Briefer, Rybak, & Aubin, 2013; Holland, Dabelsteen, & Paris, 2000), but not in others (Beletsky, Chao, & Smith, 1980; Nowicki, Searcy, Hughes, & Podols, 2001). Alternatively, in some species, song discrimination only depends on the presence of species-specific notes. Examples include the trill of red-winged blackbirds, *Agelaius phoeniceus* (Beletsky et al., 1980) or the introductory whistle of white-crowned sparrows and golden-crowned sparrows (Hudson & Shizuka, 2017; Soha & Marler, 2000). Another intriguing example is provided by brown-headed cowbirds, *Molothrus ater*, whose young are entirely raised by heterospecific parents. Song learning in juvenile cowbirds is triggered by the production of an innately recognized chattering call in close temporal proximity of songs (Louder et al., 2019).

In this study, we investigated the importance of species-specific song features for discrimination (i.e. species-specific cue) in the collared flycatcher, *Ficedula albicollis*. Collared flycatcher nestlings and adults respond more strongly to conspecific songs, including when they co-occur with closely related pied flycatchers, *Ficedula*

hypoleuca (Qvarnström, Haavie, ; Wheatcroft & Qvarnström, 2017a, 2017b). However, the song features that underlie discrimination are unknown. Male collared flycatchers often include a species-specific alarm call in the song, almost always placed as the initial syllable of the song phrase (Haavie et al., 2004; Wallin, 1987). This behaviour seems to be conserved to some degree across populations (Haavie et al., 2004). The acoustic form of calls produced by *Ficedula* flycatcher species, including collared flycatchers, in alarm contexts is species-specific and consistent across individuals (Gelter, 1987). Moreover, recognition of these alarm calls is also thought to be innate, as has been shown in other songbird species (Davies, Madden, & Butchart, 2004), or at least learned very early in life. Wheatcroft (2015) suggested that nestling flycatchers discriminate in favour of their own species' alarm calls to some degree. Therefore, we might expect that the presence of a species-specific introductory alarm call in *Ficedula* flycatcher songs should be an important feature underlying song discrimination. One challenge to the idea that collared flycatcher nestlings use the introductory alarm call as a species discrimination cue is that it is, paradoxically, not species specific. In populations in which pied and collared flycatchers co-occur, pied flycatchers often include the collared flycatcher call as the initial syllable in their own songs during the production of so-called 'mixed songs' (Haavie et al., 2004). To clarify, pied flycatchers are not known to produce the collared flycatcher alarm call in an alarm context but can learn and produce it as a song element. These mixed songs, which may comprise up to 65% of pied flycatcher songs, increase the responsiveness of male collared flycatchers and have been hypothesized to increase the attractiveness of pied flycatcher songs to collared flycatcher females (Qvarnström et al., 2006). Thus, previous work suggests that the call may be an important feature for song discrimination in collared flycatchers, but one that risks having collared flycatchers responding to mixed-singing pied flycatchers in populations where both species co-occur.

In this study, we first tested the importance of the introductory alarm call for song discrimination, by comparing the responses of nestling and adult male collared flycatchers to playbacks of conspecific and heterospecific songs that were manipulated to include the introductory alarm call. We evaluated the hypothesis that the introductory alarm call alone drives song responses and species discrimination. Nestling collared flycatchers respond relatively weakly to unmanipulated heterospecific songs. Therefore, when we exposed nestling collared flycatchers to manipulated song phrases starting with the alarm call followed by either conspecific song notes (i.e. collared flycatcher song) or heterospecific song notes (i.e. pied flycatcher song), we predicted that they should respond similarly to both treatments due to the inclusion of the call. Likewise, since adult collared flycatcher males respond similarly to conspecific and 'mixed' heterospecific songs, many of which include the alarm call, we predicted that they should respond similarly to both playback treatments. Although equally strong responses to both playback treatments for nestlings and/or adults would suggest that the call drives responses given large enough sample sizes, not detecting a difference does not imply lack of a true difference. To support the conclusion that alarm call incorporation influences responses, we re-evaluated the behavioural responses of collared flycatcher nestlings to unmanipulated collared flycatcher songs from Wheatcroft and Qvarnström (2017a) by including an additional explanatory variable: the natural incorporation of calls into the songs being played back. We predicted that nestlings should respond more strongly to songs naturally including calls. This prediction and the analysis were developed subsequently to the present study's playbacks, but we note that the experiments from Wheatcroft and Qvarnström (2017a) are

entirely independent and were not designed to test this prediction.

METHODS

Study System

Collared and pied flycatchers are sexually dimorphic oscine bird species. Males of both species have a black-and-white breeding plumage. They differ most obviously by the presence or absence of a large white collar. They are easily distinguishable from females which are brownish in both species, even though collared flycatcher females tend to be slightly paler. Both species breed throughout large regions of Europe. They tend to have mostly distinct breeding ranges, with pied flycatchers breeding throughout Spain and northern parts of Europe, while collared flycatchers are more restricted to southeastern Europe. Despite largely allopatric breeding ranges, both species co-occur in Central Europe, where they tend to occupy different altitudinal breeding niches (Grinkov, Palko, & Sternberg, 2019). In addition to this historical range of overlap, recent sympatric populations of collared and pied flycatchers are present on the Swedish islands of Gotland and Öland in the Baltic sea. The colonization of these islands by collared flycatchers is thought to have happened recently, during the 19th century (Qvarnström, Wiley, Svedin, & Vallin, 2009). Following colonization, the number of collared flycatchers increased on these islands, where they tend to outcompete and have slowly replaced pied flycatchers from preferred breeding habitats (Rybinski et al., 2016). Despite marked differences in male plumage and song characteristics, the species sometimes hybridize in the secondary contact zone (Qvarnström, Rice, & Ellegren, 2010). Both species are long-distance migrants wintering in sub-Saharan Africa; however, their wintering ranges are mostly allopatric (Billerman, Keeney, Rodewald, & Schulenberg, 2020). Both species arrive on their breeding grounds at the study site throughout April, when they begin to nest in natural cavities as well as in artificial nestboxes. During the breeding season, song is an important communication feature for males of both species. It is used mainly as a sexual display towards females, and also in male–male competition over territories (Alatalo, Lundberg, & Bjorklund, 1982; Eriksson & Wallin, 1986). The two species produce complex songs and possess large note repertoires, but their songs largely differ. Pied flycatchers tend to produce shorter notes at a lower pitch and at a faster tempo (Gelter, 1987). Interestingly, both species often introduce a species-specific alarm call as the introductory syllable of their songs, particularly in collared flycatchers (Haavie et al., 2004; Wallin, 1987).

Artificially Manipulated Playbacks

The song recordings of both collared and pied flycatchers ($N = 10$ males for each species) were obtained on Öland between 2014 and 2016 using a digital audio recorder (TASCAM DR-40, TEAC America Inc.) and a condenser microphone (Sennheiser ME66, GmbH & Co. KG), except for two song files that were recorded on Öland in 2002. All sound files were recorded in 44.1 kHz PCM WAVE format. The alarm call recordings of collared flycatchers ($N = 15$ males) were also obtained on Öland. Spectrograms were viewed in Raven pro v.1.5 (The Cornell Lab of Ornithology, 2014) in a Hann window using a frequency grid of 512 samples with 86.1 Hz spacing and a time grid with 256 samples with 50% overlap. A single observer (D.W.) removed nontarget sound, such as other avian vocalizations and low-frequency background noise, using Raven's built-in Filter Out function to construct playback files containing 8–12 song phrases/min, each separated by 4 s of white noise.

White noise was inserted so that the sound pressure level of the playback did not change drastically when song phrases started and stopped. The white noise was of similar amplitude to the background noise during the song phrases. Then, another observer (L.B.) used these previous files to prepare 10 playback files for both experimental treatments. The first treatment consisted of a collared flycatcher alarm call placed before a collared flycatcher song phrase (Fig. 1a). The second treatment consisted of a collared flycatcher alarm call followed by a pied flycatcher song phrase (Fig. 1b). The alarm calls were taken from an alarm context (recorded while displaying a stuffed sparrowhawk, *Accipiter nisus*, see Wheatcroft, Gallego-Abenza, & Qvarnström, 2016) and were placed at a custom time interval before the song phrases. This custom time interval of 0.24 s (± 0.14 SD) had been estimated based on the mean time between the introductory alarm call and the following note in 122 song phrases recorded from a sample of 26 individual collared flycatchers. When naturally occurring alarm calls were already present at the beginning of some song phrases, we replaced them by the alarm calls taken from an alarm context. All alarm calls used within each artificially modified playback file were unique calls from the same individual. To reduce pseudoreplication, 10 of the 15 individual males whose calls were used to create artificially modified playbacks were used in one playback file only, while the remaining five males were used in two playback files (one of each experimental treatment). The playback trials on adult males and nestlings of collared flycatcher using these manipulated playbacks were conducted between May and June 2019 on Öland. Playbacks were done using a 'SME-AFS Amplified Field Speaker' (Saul Mincroff Electronics Inc., Elmont, NY, U.S.A.), at a loudness of approximately 80 dB when measured 1 m away using a decibel meter on C-frequency weighting with fast response time (Radio shack model 3300099). We used all 10 recordings for each treatment in both playback experiments.

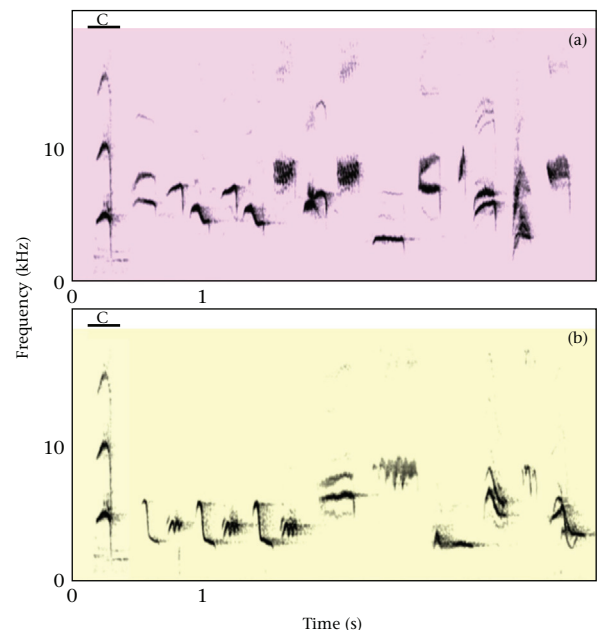


Figure 1. Sound spectrograms of calls and songs played back to adult male and juvenile collared flycatchers. The collared flycatcher alarm call is indicated by 'c'. (a) Collared flycatcher alarm call followed by a collared flycatcher song phrase. (b) Collared flycatcher alarm call followed by a pied flycatcher song phrase.

Nestling Song Discrimination

We exposed 12-day-old pied flycatcher nestlings to playbacks of different sound recordings. All the nestlings of a clutch were temporarily removed from their nest and placed on a layer of moss inside an experimental nestbox. Each nestling was marked with symbols on the head using correction fluid, to enable individual recognition of the nestlings in video recordings. The nestbox was set up at least 200 m from the closest known flycatcher nest to avoid hearing adult flycatchers singing or alarm calling. An 'Eco Extreme Speaker' (Grace Digital, Poway, CA, U.S.A.) was positioned approximately 1 m from the nestbox and connected to a smartphone. We waited a minimum of 5 min after the nestlings were placed inside the experimental nestbox to start a trial. After each trial, nestlings were individually ringed and weighed, and then brought back to their natal nestbox. The whole trial was watched via a live video feed and video recorded using a digital video recorder (PV-1000, Lawmate International, Taipei, Taiwan). Subsequently, the video recording of each experimental trial was analysed by a single observer. We noted the number of times each nestling begged, opened its gape, looked up and shifted its position inside the experimental nestbox.

During the breeding seasons from 2013 to 2016, both pied flycatcher and collared flycatcher nestlings were exposed to conspecific and heterospecific songs, respectively, to assess whether these species discriminated in favour of conspecific songs over heterospecific songs (see [Wheatcroft & Qvarnström, 2017a](#) for further details). This previously published study showed that nestlings discriminate in favour of conspecific song but did not directly test whether the alarm call per se triggered discrimination. To test whether the natural occurrence of introductory alarm calls within conspecific songs influenced the behavioural response of collared flycatcher nestlings, we therefore revisited the data used in [Wheatcroft and Qvarnström \(2017a\)](#), but here only focusing on the trials where collared flycatcher nestlings ($N = 235$, from 45 nests) were exposed to recordings of collared flycatcher songs. Each collared flycatcher nestling was exposed to 1 min of silence, followed by collared flycatcher songs played back for 1 min. These playbacks comprised an average of 8.6 song phrases. Since song phrases of collared flycatchers do not always include an introductory alarm call, a single observer (D.W.) visually inspected all the spectrograms of the song recordings that were played back and judged, blind to the response and identity of the nestlings, whether each song phrase started with an alarm call or not. We did this to assess whether the proportion of song phrases starting with an alarm call during a given playback had an influence on the strength of the nestling response. Song phrases naturally starting with a call and song phrases lacking this call did not differ substantially in their acoustic form (see [Appendix I](#)). Therefore, the only significant difference among these unmanipulated song phrases of collared flycatchers was the presence, or the lack thereof, of an introductory call.

During the 2019 breeding season (12–21 June 2019), we exposed collared flycatcher nestlings to manipulated songs, and the experiment proceeded as follows. After 1 min of silence, we played back one of two treatments: collared flycatcher songs, each preceded by a collared flycatcher call ([Fig. 1a](#)), or pied flycatcher songs, each preceded by a collared flycatcher call ([Fig. 1b](#)). Each playback lasted 1 min. Then, a minute of silence was observed again, followed by a recording from the other treatment. Thus, each collared flycatcher nestling was exposed to the two different treatments, alternating the order between each trial. This playback experiment was performed on 222 nestlings from 45 nests.

Adult Male Song Discrimination

The playback experiment on adults was performed at the beginning of the breeding season (3–16 May 2019) when song activity is high and when males are defending their territory fiercely ([Qvarnström et al., 2006](#)). This experiment was thus mimicking a territorial intrusion by another male flycatcher. Each responding male ($N = 24$) was used only once. A trial consisted of a 5 min period of silence where only the speaker was set up and a wooden collared flycatcher dummy was mounted on the nestbox. During the initial silence period, we ensured that males did not respond to the dummy itself to be sure that they were not responding to cues other than the following playback. The dummy was used to make this experiment comparable to earlier studies ([Qvarnström et al., 2006](#)). This was followed by one of the two experimental treatments for a duration of 10 min (except for three trials per treatment where the playback duration was only 5 min, but the inclusion or exclusion of these trials from analyses did not substantially change the results). During the 10 min of the playback, we recorded the behavioural response of the focal male, noting for every minute whether the male was alarm calling, whether it was wing flicking and its minimal perching distance to the speaker.

Ethical Note

Ethical permission for this work was provided by the Swedish animal ethical permission board (Linköpings djuretiska nämnd Dnr 18-15). During the experiment, the nestlings of a clutch were temporarily removed from their nest. Each nest was tested only once. We plugged the nestbox hole while removing the nestlings to ensure that parents would not notice their absence and thus abandon them. After the experiment, the nestlings were then brought back to their natal nestbox, no later than 30 min after being initially removed. No nests were subsequently abandoned, and parents resumed feeding soon after the nestlings were returned ([Wheatcroft, 2015; Wheatcroft & Qvarnström, 2017a](#)).

Statistical Analyses

We evaluated the effect of the different playbacks on the behavioural response of nestlings and adults using Bayesian hierarchical models. For the analysis of nestling responses to unmanipulated conspecific song phrases naturally preceded by the alarm call or not, we used the number of begs during the playback as a dependent variable, as it has been shown to be a reliable proxy to assess song discrimination ([Wheatcroft & Qvarnström, 2017a](#)). Because these count data were both zero-inflated and overdispersed (zero inflation index of 0.91 and dispersion index of 22.7; [Blasco-Moreno, Pérez-Casany, Puig, Morante, & Castells, 2019; Puig & Valero, 2006](#)), we used a zero-inflated negative binomial model (ZINB), which is a mixture model of a negative binomial regression with a Bernoulli trial to account for the zero-inflated nature of the data. We preferred a ZINB model to a hurdle model (which would use a zero-truncated negative binomial likelihood, thus forcing each zero to be a structural zero) to account for the zero inflation, because in our experiment no nestling response could either mean that the nestling was unable to respond to stimuli (structural zero) or that the nestling did not discriminate in favour of a given treatment (true zero). The fixed effects on the linear component of the count data on the log scale (but not on the logit scale of the zero-inflation component) were the nestling mass and their begging activity before the playback. We included as a covariate the proportion of song phrases starting with an introductory call. All the continuous variables included in the model were mean centred

and scaled. A nestbox identity random intercept and a playback identity random intercept were also added (see [Supplementary Material](#) for the code of the model). The posterior predictive P value of 0.61 did not indicate a lack of fit of the model to the data. Posterior predictive P values are computed by comparing some metrics of the data simulated under the model to metrics of the actual data set that is analysed using that model. Values close to 0 or 1 indicate a lack of fit while values close to 0.5 indicate a good fit (Gelman, Meng, & Stern, 1996; Kéry & Schaub, 2012).

For the behavioural responses of nestlings to manipulated playbacks, we used a hierarchical model similar to the one previously described (ZINB), since these count data were both zero-inflated and overdispersed (zero inflation index of 0.88 and dispersion index of 17.2; Blasco-Moreno, Pérez-Casany, Puig, Morante, & Castells, 2019; Puig & Valero, 2006). The mass and number of begs during the rest period were added as fixed effects on the linear component of the count data on the log scale (but not on the logit scale of the zero-inflation component), and both were mean centred and scaled before analyses. The experimental treatment was also included as a covariate. Random intercepts of ring number nested in nestbox identity were included, as well as a playback identity random intercept (see [Supplementary Material](#) for the code of the model). The posterior predictive P value of 0.55 indicated a good fit of the model to the data.

For the responses of adult males, we combined whether the male was alarm calling during a minute of the playback, whether it was wing flicking and whether it came in the vicinity of the speaker (within 10 m, as this was the maximum distance that could be accurately estimated in any given vegetation cover) into a single graded behavioural response (ranging from 0 if none of these behaviours were performed within a given minute of the playback to 3 if all three kinds of behaviour were performed within a minute of the playback) that was used as the dependent variable. Owing to the ordered nature of the dependent variable, we used an ordinal mixed-effect logistic regression (see [Supplementary Material](#) for the code of the model). The treatment was added as a fixed effect, while the nestbox identity (i.e. male identity) was used as a random intercept during the modelling procedure. The posterior predictive P value of 0.43 indicated a good fit of the model to the data. We also analysed individually the three behaviours that were combined into the graded behavioural response, yielding similar results ([Appendix II](#)).

All the hierarchical models were specified in the BUGS language (Kéry, 2010). Analysis of these models was performed in a Bayesian framework, using Markov chain Monte Carlo simulation with Gibbs sampling in the software JAGS (Plummer, 2003) and the R package R2jags (Su & Masanao Yajima, 2015) was used to call JAGS and to export the result into the software R v.3.5.1 (R Core Team, 2020). All the models ran on three chains, saving 1000 posterior samples per chain. For the models assessing the importance of the alarm call for nestling discrimination, the simulations ran for 500 000 iterations, sampling every 250 iterations after a burn-in of 250 000 iterations. The model focusing on adult behavioural responses to artificially modified playbacks was analysed using the simulation of 200 000 iterations, discarding the first 100 000 and sampling every 100 iterations. In all the models, we used uniform prior distributions for the variance parameters of the random effects. The zero-inflation coefficients of the nestling behaviour analyses were also defined with uniform prior distributions. We used vague priors, with a normal distribution of mean 0 and precision 0.001 for the coefficient's covariates on the log scale or logit scale, of all parameters included in the models and thought to influence the behavioural response of the nestlings and adults during the

playbacks. For all models, convergence and mixing of the three chains were assessed visually as well as using the Gelman–Rubin diagnostic (Gelman & Rubin, 1992), and potential scale reduction factors were all inferior to 1.01. Posterior predictive P values were all estimated using sum of square discrepancies (Gelman et al., 1996; Kéry & Schaub, 2012).

RESULTS

Nestling Song Discrimination

We found that the proportion of song phrases naturally starting with an alarm call during a given unmanipulated playback of collared flycatcher song had a clear effect on the begging response of collared flycatcher nestlings. They begged more intensely as the proportion of song phrases starting with a call increased ([Table 1](#), [Fig. 2](#); positive effect estimated from the posterior distribution in 98.1% of the cases).

When collared flycatcher nestlings were exposed to artificially manipulated song phrases, we did not detect any clear differences

Table 1

Posterior distributions of the model parameters from the analysis of nestling begging calls from Wheatcroft and Qvarnström's (2017) data

Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Intercept	−0.94	−2.22	0.11
Fixed effects			
Resting behaviour	0.74	0.34	1.21
Condition	−1.22	−2.10	−0.44
Proportion of song phrase with introductory call	1.03	0.05	2.10
Random effects			
Nestbox variance	5.04	1.73	11.47
Playback ID variance	1.53	0.00	6.72

Results of the Bayesian hierarchical model exploring the effect of the proportion of collared flycatcher song phrases starting with an alarm call on the begging response of the collared flycatcher nestlings ($N = 235$ nestlings from 45 nests, from the data of Wheatcroft & Qvarnström (2017)). Estimates and effect sizes presented are on the log scale. Posterior predictive P value = 0.61. CI: credible interval.

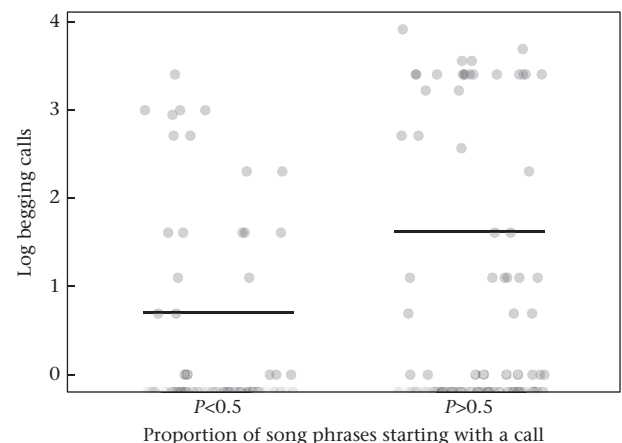


Figure 2. Begging response (log transformed for display purposes only) of the collared flycatcher nestlings to collared flycatcher songs, depending on the proportion of song phrases naturally starting with a call. The bold horizontal lines represent the log of the mean begging call response (based on the raw data). Each circle represents the log begging call for an individual nestling within a minute of playback. The proportion of song phrases starting with a call was made categorical for display purposes only.

Table 2
Posterior distributions of the model parameters from the analysis of nestling begging calls in response to the playbacks

Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Begging calls			
Intercept	0.91	−0.82	2.52
Fixed effects			
Resting behaviour	0.57	0.23	1.25
Condition	−0.47	−0.89	−0.11
'Collared call – Pied song' ¹	0.33	−0.66	1.50
Random effects			
Nestbox variance	0.75	0.00	6.30
Nestling variance	0.12	0.00	0.77
Playback ID variance	0.84	0.07	2.58

Results of the Bayesian hierarchical model exploring the effect of the two experimental treatments on the begging response of the collared flycatcher nestlings ($N = 222$ nestlings from 45 nests). Estimates and effect sizes presented are on the log scale. Posterior predictive P value = 0.55. CI: credible interval.

¹ Reference category is 'Collared call – Collared song'.

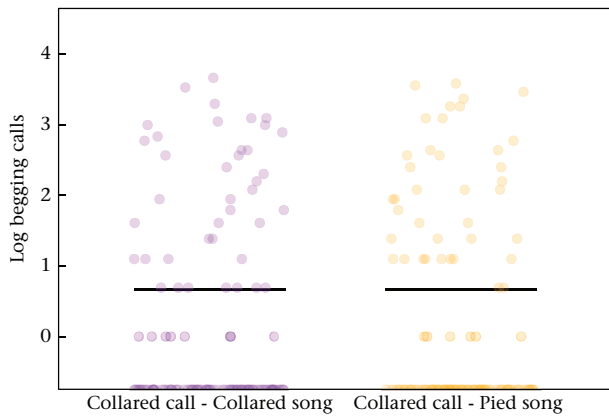


Figure 3. Begging response (log transformed for display purposes only) of the collared flycatcher nestlings during the playback, depending on the experimental treatment. The bold horizontal lines represent the log of the mean begging call response for each treatment (based on the raw data). Each circle represents the log begging call for an individual nestling within a minute of playback.

in their behavioural responses to the two experimental treatments. The nestlings did not beg differently in response to playbacks with the sequence 'collared flycatcher call – pied flycatcher song' in comparison to the treatment 'collared flycatcher call – collared flycatcher song' (Table 2, Fig. 3).

Adult Male Song Discrimination

Adult collared flycatchers tended to respond less strongly to their species alarm call followed by a pied flycatcher song than when followed by a collared flycatcher song (Table 3, Fig. 4; lower behavioural response estimated from the posterior distribution in 88.6% of the cases). However, the variance associated with this trend is large, and caution is needed in the interpretation of this result.

DISCUSSION

Our results, from a combination of field experiments with both manipulated and unmanipulated playbacks on collared flycatcher nestlings and adult males, suggest that the incorporation of introductory alarm calls is sufficient in itself to drive responses in nestlings. However, call incorporation alone does not seem to be the

Table 3
Posterior distributions of the model parameters from the analysis of adult graded behavioural response in response to the playbacks

Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Graded behavioural response			
Intercepts			
Intercept 0 1	−0.42	−2.01	1.20
Intercept 1 2	0.77	−0.79	2.38
Intercept 2 3	2.01	0.40	3.67
Fixed effect			
'Collared call – Pied song' ¹	−1.31	−3.59	0.88
Random effect			
Individual male variance	6.42	2.41	14.94

Results of the ordinal Bayesian hierarchical model exploring the effect of the three experimental treatments on the graded behavioural response of the collared flycatcher males for each minute of the playback ($N = 24$ playbacks, including 18 playbacks of 10 min and six playbacks of 5 min). Estimates and effect sizes presented are on the logit scale. Posterior predictive P value = 0.43. CI: credible interval.

¹ Reference category is 'Collared call – Collared song'.

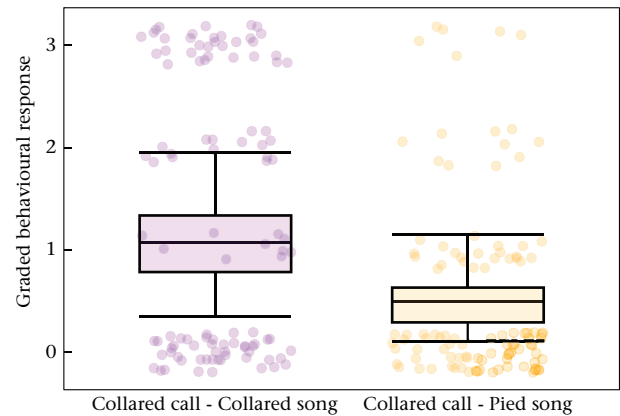


Figure 4. Graded behavioural response of collared flycatcher males for each minute of the playback, depending on the experimental treatment. The box plot indicates the mean response, as well as the 50% and 95% credible intervals, as predicted by the hierarchical model. Each circle represents the graded response for an individual adult male within a minute of playback. A small amount of random variation was added for display only to avoid overlapping points.

only driver of responses to simulated territorial intrusion in adult males. Here, we discuss the potential role of introductory alarm calls in early song discrimination, and why adult males may be expected to base their behavioural responses on more information contained in the song than conveyed by the introductory alarm call alone.

Ficedula flycatchers are thought to learn their songs and song preferences both by imprinting on their social father and through oblique imprinting on neighbouring males (Eriksen, Slagsvold, & Lampe, 2011; Labra & Lampe, 2018). However, there is also a genetic component underlying species differences in song discrimination at the onset of the putative imprinting phase (Wheatcroft & Qvarnström,). Thus, some song features should mechanistically drive the sensory bias allowing for early song discrimination. Our field experiment using artificially modified playbacks in combination with an analysis of an earlier playback experiment from a different angle highlight the importance of the introductory alarm call for song recognition at the nestling stage in collared flycatchers. As future songs and song preferences are partly shaped by learning, flycatcher nestlings must imprint on some individuals. However, we do not yet know when this occurs. Indeed, it has been shown that flycatcher males sing only sporadically when rearing nestlings

(Espmark & Lampe, 1993; Pärt, 1991), thus offering limited imprinting opportunities, even though little vocal input might be needed for imprinting (Deshpande, Pirlepesov, & Lints, 2014). However, flycatcher nestlings are often exposed to alarm calls of their parents during the rearing stage, as they often produce this note while approaching the nest during provisioning (Wheatcroft, 2015). Therefore, if nestlings respond innately to the call, as is the case in many songbirds (Davies et al., 2004; Louder et al., 2019), and possibly in flycatchers as well, or, alternatively, if nestlings imprint on the alarm call itself, it could potentially make them more alert upon hearing it, and therefore more likely to both respond to and imprint on the songs that include it later in life.

The incorporation of this introductory call into songs could possibly influence the receiver's response by increasing its attention to the following song phrase. This hypothesis is supported by studies on fairy-wren species, wherein males have been seen singing mostly after hearing the call of predators (Greig & Pruett-Jones, 2010; Langmore & Mulder, 1992). More specifically, splendid fairy-wrens, *Malurus splendens*, often sing after hearing grey butcherbirds, *Cracticus torquatus*, vocalizations, thus forming a vocal duet which increases the attentiveness of the females (Greig & Pruett-Jones, 2010), hence ultimately increasing the salience of the signal. The incorporation of calls in songs is not exclusive to flycatchers. For instance, zebra finches have been shown to include unmodified calls as the first and last syllable of their song phrases (Lachlan, van Heijningen, ter Haar, & ten Cate, 2016; Price, 1979; Suzuki, Griesser, & Wheatcroft, 2019; Zann, 1993). It has therefore been hypothesized that their song originated and evolved through the incorporation of calls (Zann, 1993). However, even though some hypotheses have been proposed for why calls are incorporated into songs in some species, this call incorporation remains poorly understood and mostly overlooked.

The introductory alarm call might not be the only song feature that nestlings use to trigger discrimination, but it seems sufficient to trigger discrimination to conspecific songs in collared flycatcher nestlings. Accordingly, another study on golden-crowned sparrows (Hudson & Shizuka, 2017) found similar results on the importance of introductory notes for song discrimination. Some features present in the song itself might also be used by nestlings to allow discrimination. However, unlike alarm calls that are most often highly conserved notes, song is a labile trait evolving at a fast rate due to both genetic changes and cultural evolution (Catchpole & Slater, 2008; Verzijden et al., 2012). It can result in significant song differences between populations of the same species separated by only small geographical distances (Podos, 2007), or between current and ancestral populations separated by only a short period of time on an evolutionary scale (Derryberry, 2011). Thus, due to the high evolvability and the high variability of songs within species, it might be possible that notes within songs are less likely candidates than alarm calls to allow an innate early song discrimination such as that of *Ficedula* flycatchers (Wheatcroft & Qvarnström,). However, Lipshutz, Overcast, Hickerson, Brumfield, and Derryberry (2017) found the acoustic divergence of the whistle note of white-crowned sparrows to be associated with the genetic distance between individuals, thus highlighting that cues important for population recognition are not always strongly conserved across populations. Therefore, it could be possible that other consistent acoustic features in the song play a role in early song discrimination.

Despite the low sample size of our playback experiment on adult males, our results suggest that the introductory call of collared flycatchers itself might not be sufficient to trigger responses at the adult stage. In a previous study using a similar experimental set-up, Qvarnström et al. (2006) showed that collared flycatcher males respond similarly to collared flycatcher songs or mixed-singing

pied flycatchers. This contrasts with our own results, as both the songs of mixed-singing pied flycatchers and our experimental treatment (collared flycatcher call followed by pied flycatcher song) share the inclusion of the introductory alarm call, while differing in other song features or notes present in the song. Therefore, taking the results of these two studies together into consideration, this tends to provide evidence that the responses of collared flycatcher males to mixed pied flycatcher songs are based on song features other than the call alone. This is likely due to the continuous learning of flycatchers throughout their lifetime, as greater exposure to conspecific songs might enable a more subtle discrimination of the song; thus adult flycatchers are not only using the highly conserved introductory alarm call as a cue.

The lower response of adult collared flycatcher males to pied flycatcher songs in the presence of the introductory call could be hypothesized to result from an asymmetry in population sizes between the two species, from adaptive learning in males or from selection against responding to pied flycatcher songs. In the Baltic islands' hybrid zones wherein pied flycatchers were originally present, collared flycatchers have colonized the island and rapidly outnumbered pied flycatchers due to their higher competitive abilities (Kulma, Low, Bensch, & Qvarnström; Rybinski et al., 2016; Vallin, Rice, Arnsten, Kulma, & Qvarnström, 2012), resulting in strongly skewed densities in favour of collared flycatchers (Alatalo, Gustafsson, & Lundberg, 1982). Therefore, we could expect that collared flycatchers have only seldom encountered and heard pied flycatcher songs. It might be possible that natural mixed songs are close enough to collared flycatcher songs to trigger a response without previous exposure, while pure pied flycatcher songs (with or without the collared flycatcher introductory call) would differ more, thus triggering lowered behavioural responses of collared flycatcher males (de Kort & ten Cate, 2001). Another possibility is that the lower response could result from adaptive learning in males, whereby they learn to fine-tune their aggressive responses in accordance with past experienced variation in fighting abilities among rival males associated with the corresponding variation in song features of these rivals. Male pied flycatchers have a lower fighting ability than collared flycatchers (Vallin et al., 2012). Therefore, collared flycatchers should perceive a singing male as a greater threat if collared flycatcher notes follow the introductory alarm call. Alternatively, the lower response to pied flycatcher songs despite the presence of the alarm call observed in males may reflect a correlative response to selection acting on females favouring increased discrimination at the adult stage. In females, selection strongly favours discrimination against responding to pied flycatcher songs due to the associated costs of heterospecific mating and hybridization (Qvarnström et al., 2006; Wheatcroft & Qvarnström, 2017b), thus ultimately contributing to prezygotic isolation.

The convergence of competitive signals between closely related species, even if adaptive in the context of competition between rival males (Kirschel, Seddon, & Tobias, 2019; Tobias et al., 2014), has the potential to weaken assortative mating (Lipshutz, 2018). In the case of collared flycatchers, it is known to increase the probability of cross-species mating (Qvarnström et al., 2006). Despite this potential to weaken species boundaries, species discrimination seems to be maintained alongside song convergence in some cases (Seddon & Tobias, 2010). However, the latter example of species discrimination despite song convergence is in antbirds, which are subsocial birds with innate songs. Freeman, Montgomery, and Schluter (2017) found that subsocial birds have greater song discrimination in sympatry with sister species than social birds, highlighting a 'downside of learning'. This is likely a result of song learners having greater within-population variation in song traits. It is therefore possible that incorporating innate notes within the

learned songs of oscine birds, as is the case in collared flycatchers, could have evolved to counter this ‘downside of learning’, ultimately strengthening species discrimination.

In conclusion, this study provides evidence that the introductory alarm call of collared flycatchers may be sufficient to trigger song discrimination in collared flycatcher nestlings but the aggressive responses of adult males seem guided by additional information conveyed by conspecific songs. Further studies are needed to assess whether other song features that differ between collared flycatcher and closely related species could also be involved in allowing song discrimination. More importantly, we argue that future studies must consider the importance of alarm calls within or associated with songs, which has so far been largely overlooked.

Data Availability

All data and scripts necessary to reproduce the findings and the figures from this article are available at <https://doi.org/10.5281/zenodo.4607842> (Bliard, Qvarnström, & Wheatcroft, 2021).

Acknowledgments

We are thankful to Matthieu Paquet for comments on the methods and statistical advice. We thank Selvino de Kort and two anonymous referees for comments and suggestions that helped improve this manuscript. A.Q. was funded by a Vetenskapsrådet grant ‘VR 2016-05138’.

Supplementary Material

Supplementary material associated with this article can be found online at <https://doi.org/10.1016/j.anbehav.2021.05.018>.

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Appendix I. Acoustic comparison of songs with and without calls

To determine whether songs starting with calls differ acoustically from those not starting with calls, we compared the acoustic features of songs used in the playback experiment of [Wheatcroft and Qvarnström \(2017\)](#). Altogether, 142 songs from 27 different individuals were included. These 142 songs included 56 songs starting with a call and 86 songs not starting with a call; 14 of the individuals analysed produced both types of song. Songs were measured by a single author (D.W.) using Luscinia software ([Lachlan, 2007](#)). Each song recording was imported, and song phrases were measured separately. Spectrograms were visualized using a Gaussian windowing function with the following settings: 12 kHz maximum frequency, 5 ms frame length, 220 spectrograph points, 80% spectrograph overlap, 50 dB dynamic range, 30% dereverberation and 50 ms of dereverberation range. For each song phrase, we measured individual elements as continuous sound traces. Elements were grouped into syllables if their sound traces were overlapping in time or the gap between them was less than 30 ms.

We compared individual songs using Luscinia's built-in dynamic time-warping (DTW) algorithm, which searches for an optimal alignment of each pair of songs and uses this alignment to calculate a dissimilarity matrix ([Lachlan, 2007](#)). We performed DTW using

Table A1
Variance in acoustic features explained by each NMDS component.

PC	Cumulative % explained	Eigenvalue
1	68.18738	28.0724
2	85.21238	19.7769
3	93.29334	8.510218
4	96.36732	4.520368
5	98.23054	4.315351

the following acoustic feature weightings: time (5), mean frequency (1), mean frequency change (1) and normalized mean frequency (1). All other measures were weighted by their standard deviation. We used a compression factor of 0.001, a minimum element length of 25 samples, time SD weighting of 1, a maximum warp of 25%, and compared syllables by stitching elements. All other options were selected. The resulting dissimilarity matrix was then converted to five Euclidean dimensions through nonmetric multidimensional scaling. The resulting principal component scores were used to compare noncall syllables across songs.

To compare the acoustic features between song types, we used linear mixed models implemented in the R package lme4 (Bates et al., 2015). Syllable level principal component scores were included as the independent variable and whether the song started with a call or not was included as a fixed effect. Individual was included as a random effect.

The stress value from a five-dimensional nonmetric multidimensional scaling (NMDS) analysis on syllables was 0.006271, indicating an excellent preservation of distances among syllables. The first three principal components preserved more than 90% of the variation in distances (Table A1). There was no evidence that syllables found in songs with calls differed from those found in songs without calls for any of the five PCs ($P > 0.177$).

Appendix II. Additional results for the playback experiment on adult males

In the Methods and Results given above, male behavioural responses to playbacks were analysed using an ordinal model, presenting a graded behavioural response, that was constructed by combining three different behaviours for each minute of the playback: whether the individual was heard alarm calling, whether it was seen wing flicking and whether it was seen perching within 10 m of the speaker. Here we analysed these three behaviours separately, and obtained similar results, which are concordant with the model presented in the main text. We analysed the data using Bayesian hierarchical models that included a nestbox identity random effect (to account for the nonindependence of the minutes of recording within a trial, as each trial was 10 min, but the response variable was computed for each minute of the trial). The

Table A2
Posterior distributions of the model parameters from the analysis of adult minimum distance in response to the playbacks

Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Intercept	21.75	14.54	28.91
Fixed effect			
'Collared call – Pied song' ¹	9.20	–0.56	19.19
Random effect			
Individual male variance	142.29	69.35	277.21

Results of the Bayesian hierarchical model exploring the effect of the three experimental treatments on the minimum perching distance of the male to the speaker for each minute of the playback ($N = 24$ playbacks, including 18 playbacks of 10 min and six playbacks of 5 min). Posterior predictive p -value = 0.51. CI: credible interval.

¹ Reference category is 'Collared call – Collared song'.

experimental treatment was included as a covariate in each of the models. The model having the minimal perching distance (continuous variable, value set to 40 m if the bird was not seen) as a dependent variable was analysed without any link function on the linear component (Table A2, Fig. A1). The models that had either the wing flick (yes/no; Table A3, Fig. A2) or the alarm (yes/no; Table A4, Fig. A3) as a response variable were analysed using a binomial model with a logit link. Ideally, a truncated normal likelihood (truncated at 0 and 40) should have been used, but this

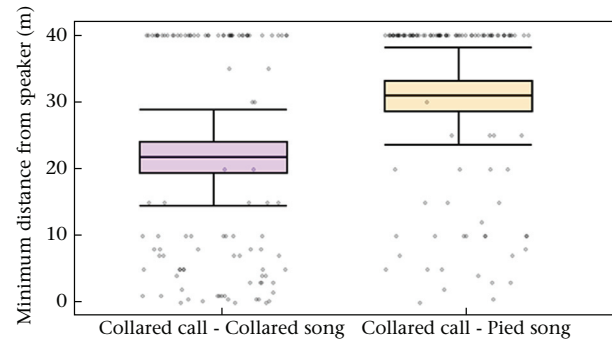


Figure A1. Minimum perching distance between the male and the speaker for each minute of the playback, depending on the experimental treatment. The box plot indicates the mean response, as well as the 50% and 95% credible intervals, as predicted by the hierarchical model. The dots represent the raw data.

Table A3
Posterior distributions of the model parameters from the analysis of adult wing flicks in response to the playbacks

Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Intercept	–0.43	–2.22	1.14
Fixed effect			
'Collared call – Pied song' ¹	–1.95	–4.51	0.47
Random effect			
Individual male variance	7.12	2.25	17.99

Results of the Bayesian hierarchical model exploring the effect of the three experimental treatments on the probability of wing flicking by the male for each minute of the playback ($N = 24$ playbacks, including 18 playbacks of 10 min and six playbacks of 5 min). Estimates presented are on the logit scale. Posterior predictive P value = 0.36. CI: credible interval.

¹ Reference category is 'Collared call – Collared song'.

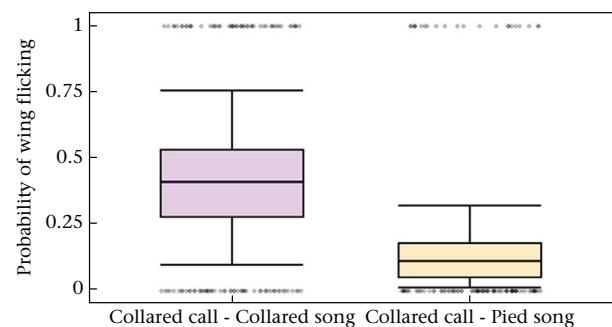


Figure A2. Probability of wing flicking by the male for each minute of the playback, depending on the experimental treatment. The box plot indicates the mean response, as well as the 50% and 95% credible intervals, as predicted by the hierarchical model. The dots represent the raw data.

Table A4

Posterior distributions of the model parameters from the analysis of adult alarm calls in response to the playbacks

Probability of alarm calling			
Predictor variables	Estimate	95% CI	
		Lower 2.5% CI	Upper 2.5% CI
Intercept	−0.78	−2.09	0.39
Fixed effect			
'Collared call – Pied song' ¹	−1.18	−3.02	0.51
Random effect			
Individual male variance	3.43	0.93	9.25

Results of the Bayesian hierarchical model exploring the effect of the two experimental treatments on the probability of alarm calling by the male for each minute of the playback ($N = 24$ playbacks, including 18 playbacks of 10 min and six playbacks of 5 min). Estimates presented are on the logit scale. Posterior predictive P value = 0.41. CI: credible interval.

¹ Reference category is 'Collared call – Collared song'.

model suffered from computational issues (lack of convergence and lack of fit), as it is computationally expensive and thus would likely require more data. Posterior predictive P values of the models were estimated using sum of square discrepancy.

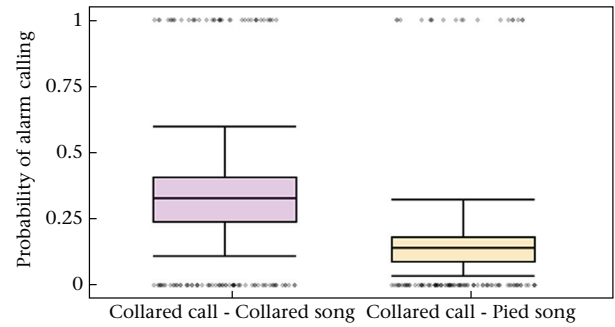


Figure A3. Probability of alarm calling by the male for each minute of the playback, depending on the experimental treatment. The box plot indicates the mean response, as well as the 50% and 95% credible intervals, as predicted by the hierarchical model. The dots represent the raw data.