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Does song overlap signal aggressiveness? An experimental study with repeated measures in free-ranging great tits

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Vocal communication is often used to signal willingness to escalate into a physical fight during territorial conflicts. In songbirds, starting to sing when an opponent already sings (song overlapping) has been suggested to signal aggressive intent (willingness to escalate). We used a multiyear data set to test whether song overlapping predicts aggressiveness in great tits, *Parus major*. Territorial males were subjected twice to a simulated territorial intrusion when their mate was in the egg-laying phase, and twice when she was incubating. Males were presented with a taxidermic mount and a noninteractive playback of a conspecific song near their nestbox. The experiment was conducted over 3 consecutive years, resulting in repeated measures for males that bred across multiple years. The estimated minimum approach distance to the intruder, a repeatable and heritable trait that predicts the likelihood of physical attack, was used as a measure of aggression. We determined the duration of song overlapping by the focal male relative to values expected by chance. Against expectations, we found that birds that overlapped were less (rather than more) aggressive. In addition, variance partitioning demonstrated that this link resulted from a within-individual effect: when birds became less aggressive from one observation to the next, they also overlapped more. There was no among-individual effect: individuals that were on average more aggressive did not, on average, overlap either more or less than others. Our results thus imply that song overlapping is linked to aggression but opposite to expectations, and not among individuals. Furthermore, the majority of birds overlapped at or below chance levels. Overall, song overlapping may not signal aggressive intent but rather ‘nonengagement’, or result from interference avoidance, allowing aggressive residents to better hear an intruder's acoustic output during territorial intrusions.

Individuals of many animal species exchange information through acoustic signals to moderate conflicts over mating partners or territories (Narins, Hödl, & Grabul, 2003; Sales, 1972; Waas, 1991). Physical fighting is not only energetically costly (Briffa & Elwood, 2004), but can also result in exposure to predators or cause injuries (Kelly & Godin, 2001), which may be prevented by signalling competitive abilities or motivation to fight (Galeotti, Saino, Sacchi, & Møller, 1997; Robertson, 1986; Shackleton & Ratcliffe, 2010). Despite the potential benefit of signalling, not all individuals may do so, because individuals vary in how they resolve the trade-off between costs and benefits of a fight depending on individual characteristics and condition (Maynard Smith & Price, 1973). Nevertheless, mutual assessment between competitors through acoustic signals is very common, for example in insects (Greenfield & Minckley, 2010), anurans (Arak, 1983; Reichert & Gerhardt, 2013; Robertson, 1986), mammals (Jennings, Elwood, Carlin, Hayden, & Gammell, 2012; Kitchen, Seyfarth, Fischer, & Cheney, 2003) and birds (Anderson, Searcy, Hughes, & Nowicki, 2012; Anderson, Searcy, Peters, & Nowicki, 2008; Capp & Searcy, 1991; Naguib, Altenkamp, & Griessmann, 2001). However, despite repeated calls for replication in ecological studies (Nakagawa & Parker, 2015), few studies have explicitly tested with sufficient within-study replication (e.g. across years) how aggression levels vary with the occurrence and nature of acoustic signals.

Signals used in agonistic contexts fall into two distinct categories: they convey information on either sender quality or motivation. First, vocalizations can serve as quality signals when morphological or physiological features of the sender restrict their

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production (Gil & Gahr, 2002). Quality signals, therefore, logically vary primarily among individuals, as individuals with insufficient capacity will be unable to, or have difficulty to, produce such signals. Examples of quality signals include vocalization rate, indicative of individual condition (Beani & Dessì-Fulgheri, 1995; Houtmann, 1992; Moller, Saino, Taramino, Galeotti, & Ferrario, 1998), or minimum frequency, indicative of size (ten Cate, Slabbekoorn, & Ballintijn, 2002). Second, vocalizations can convey a motivational message, which is not restricted by any physical features, but related to fluctuations in aggression or excitation (Guilford & Dawkins, 1995). Motivational signals vary, therefore, both among and within individuals, as any individual is able to produce them and may vary in motivational state. Some examples of motivational signals are increased twitter frequencies in European blackbirds, *Turdus merula* (Ripmeester, de Vries, & Slabbekoorn, 2007) and song type matching in song sparrows, *Melospiza melodia morphna* (Burt, Campbell, & Beecher, 2001), both associated with levels of arousal and probability of aggressive interaction.

Because patterns of variation in vocalizations can result from variation in quality (among individuals) and motivation (among and within individuals), study designs are required with repeated measures data, such that among- and within-individual effects can be teased apart (Allegue et al., 2017; Dingemanse & Dochtermann, 2013; Niemelä & Dingemanse, 2018; Westneat et al., 2020). Among-individual variation in behaviour has come to the foreground in adaptive animal personality research, which investigates the repeatable part of repeatedly expressed behaviours (Dingemanse, Kazem, Réale, & Wright, 2010; Réale, Reader, Sol, McDougall, & Dingemanse, 2007; Sih, Bell, & Johnson, 2004). Within-individual variation results instead from short-term internal fluctuations in motivation due to microenvironmental variation in context, and represents a form of reversible plasticity (Bradshaw, 1965; Dingemanse & Wolf, 2013; Piersma & Drent, 2003; Westneat, Potts, Sasser, & Shaffer, 2019). Previous research has already shown that aggressiveness is repeatable and heritable, but simultaneously exhibits reversible plasticity (Araya-Ajoy & Dingemanse, 2017; Betini & Norris, 2012; Thys, Pinxten, & Eens, 2021; Tuni, Han, & Dingemanse, 2018). Along the same lines, acoustic signals also vary among and within individuals (Amy, Sprau, De Goede, & Naguib, 2010; Jacobs et al., 2014; Naguib & Mennill, 2010). Studies with repeated measures of aggression level and signal characteristics are required to disentangle effects of individual quality versus motivation, but are, to our knowledge, rare (Akçay, Campbell, & Beecher, 2014; Nowicki, Searcy, Krueger, & Hughes, 2002).

Birds are one of the most studied taxonomic groups in acoustic signalling research, particularly in the context of aggression (Collins, 2004; Searcy & Beecher, 2009). Birdsong serves a communication function in the context of both mate attraction (Eriksson & Wallin, 1986) and territory defence (Krebs, Ashcroft, & Webber, 1978). In territorial conflicts, acoustic signals convey information about aggressive intent, i.e. motivation to escalate into a physical fight (Cooney & Cockburn, 1995; Krebs et al., 1978). Indeed, various song traits have been hypothesized to signal aggressive intent in birds, including song type matching and frequency matching (Krebs, Ashcroft, & Orsdol, 1981; Shackleton & Ratcliffe, 2010; Vehrencamp, 2001), low-amplitude singing (Dabelsteen & Pedersen, 1990) or wide-frequency bandwidth singing with high trill rates (DuBois, Nowicki, & Searcy, 2009; Szymkowiak & Kuczyński, 2017). One of the most intriguing and controversial traits proposed to signal aggression is song overlapping (Helfer & Osiejuk, 2015; Naguib & Mennill, 2010; Searcy & Beecher, 2011).

Song overlapping occurs when an individual starts singing while its opponent is already singing (Masco, Allesina, Mennill, & Pruett-Jones, 2016), and has been reported for a range of animal taxa (Gerhardt & Huber, 2002; Naguib & Mennill, 2010; Schulz, Whitehead, Gero, & Rendell, 2008). In birds, various studies have proposed that overlapping represents a signal of aggressive intent (Brindley, 1991; Langemann, Tavares, Peake, & McGregor, 2000; Naguib & Kipper, 2006; Naguib & Mennill, 2010), positing that it varies plastically within individuals as a function of (social) context. Importantly, overlapping can also signal individual quality (Bischoff, Tschirren, & Richner, 2009; Kunc, Amrhein, & Naguib, 2006). If so, we expect repeatable among-individual variation in overlapping among repeatedly assayed individuals. The proposed functional value has been supported by studies showing that overlapping and aggression can covary positively (Brindley, 1991; Naguib & Kipper, 2006; van Dongen, 2006). However, various other studies have also shown them to covary negatively (Akçay, Kağan, Avşar, Çabuk, & Bilgin, 2020; Osiejuk, Ratyńska, & Cygan, 2007; Vehrencamp, Hall, Bohman, Depeine, & Dalziell, 2007). These contrasting findings cast doubt on whether overlapping tendency signals aggressive intent or whether deviations from random overlap just aim at reducing signal interference and improving auditory perception (Planqué & Slabbekoorn, 2008; Wilson, Ratcliffe, & Mennill, 2016; Yang, Ma, & Slabbekoorn, 2014). Altogether, the diversity of patterns and apparently contradictory findings make the generality of song overlapping as an aggressive signal an unresolved issue that requires further study (Searcy and Beecher 2009, 2011; Helfer & Osiejuk, 2015).

The great tit, *Parus major*, is a suitable model for studying the relationship between song overlapping and aggression. This territorial passerine readily breeds in nestboxes and frequently engages in territorial conflicts around the nestbox (e.g. Drent, 1987). It is a model organism in behavioural ecology (Davies, Krebs, & West, 2012), commonly used to study the role of song in territory acquisition and maintenance (Akçay et al., 2020; Dabelsteen, McGregor, Shepherd, Whittaker, & Pedersen, 1996; Langemann et al., 2000) as well as within- and among-individual variation in behaviour, including territorial aggression (Araya-Ajoy & Dingemanse, 2014, 2017). Moreover, several studies have addressed the production and perception of song variation in relation to animal personality in this species (Amy et al., 2010; Jacobs et al., 2014; Strauß, Hutfluss, & Dingemanse, 2020). In previous studies, we have already shown that song output during simulated intrusions was negatively correlated with aggression in great tits, while seasonal plasticity in aggressiveness was repeatable, heritable and age dependent (Araya-Ajoy & Dingemanse, 2014, 2017). The contradictory results from studies linking overlapping and aggression (Akçay et al., 2020; Langemann et al., 2000) beg the question of whether overlapping signals aggressiveness in great tits and, if so, whether it signals individual quality, motivation or both simultaneously.

In the current study, we used great tits to quantify song overlapping and aggression and tested whether these behavioural parameters covaried among and within individuals. Importantly, whether overlapping signals aggression can be studied from the sender's or the receiver's perspective, requiring different set-ups, data and analysis. We used an experimental approach with a standardized set-up of a multimodal, simulated, territorial intrusion into the subject's territory, with a taxidermic male model and a song playback (Araya-Ajoy & Dingemanse, 2014, 2017). In 3 consecutive years, we repeatedly tested the same individuals for their relative aggression levels in terms of approach distance and their vocal response behaviour and did so in two ecological contexts (during egg laying and incubation). Consequently, our set-up enabled us to study overlapping and aggression from the sender's perspective rather than addressing how birds respond to being overlapped. We had two main research questions: (1) for birds singing, and independent of song output, is song overlapping associated with aggression; (2) is song overlapping a personality-related (among-individual) signal of aggression or rather a context-dependent (within-individual) signal of motivation?

METHODS

We monitored 12 nestbox plots (established in 2009) in southern Bavaria over a 3-year period (2017–2019); each plot was fitted with 50 nestboxes (Nicolaus et al., 2015). In each year, all boxes were checked twice a week from April onwards to determine life history traits, such as laying date (back calculated, assuming one egg per day was laid), onset of incubation (presence of an incubating female or warm eggs), clutch size and number of fledglings. Ten days after the offspring hatched, the adults were caught with a trapdoor in the box and then individually colour ringed for identification (detailed in Stuber et al., 2013).

Simulated Territorial Intrusions

As part of a long-term study, each male was subjected to four simulated territorial intrusions per year: two during the egg-laying stage (the first and the third day after the first egg was found) and two during the incubation stage (the first and the third day after warm eggs or an incubating female were observed; Fig. 1). Tests were postponed if weather conditions made natural intrusions unlikely and/or might damage our equipment (e.g. snowfall or rain). All trials were performed between 0700 and 1230 hours; the specific starting time was determined by the start of fieldwork and the duration and location of preceding tests, as subsequent tests were not performed in adjacent nestboxes.

During the test, we presented two stimuli to the focal birds: a visual stimulus (taxidermic mount of a male great tit) and an acoustic stimulus (playback of a great tit song). Both stimuli were chosen randomly from our large stock of 23 bird models and 175 song playbacks. All taxidermic mounts were placed within a green-wire mesh for protection and showed a neutral but variable posture. The model and speaker (Shockwave, Foxpro, Lewistown, PA, U.S.A.) were placed 1 m in front of the subject's nestbox. The speaker was placed on the ground (i.e. close to the model), while the model was placed on a 1.2 m wooden pole for consistent visibility across habitats. In 2017, the song stimuli consisted of just 13 recordings of spontaneous singing by local great tits recorded outside our study areas before the breeding season. In 2018 and 2019, we added another 161 playback stimuli made from songs recorded during the simulated territorial intrusions in 2017. Playbacks and speaker settings were adjusted to broadcast all playbacks at the same amplitude. Finally, playbacks were not standardized for equal song duration or overall length, as we preferred to retain variation in natural singing behaviour and hence variation in song duration and singing rate.

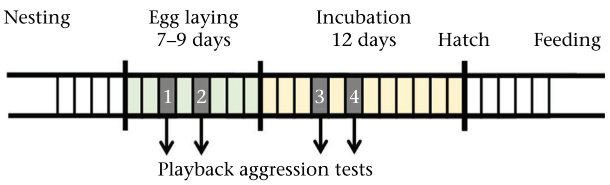

Figure 1. Typical sampling scheme. Durations of each breeding phase, for example egg laying and incubation, are approximations that differ depending on environmental conditions and individual behaviour. While the general pattern was fixed (1 day between tests within a breeding phase), tests were planned but not always executed on the marked days (day 3 and 5 of egg laying, and 4 and 6 of incubation).

Behavioural observations started once the focal male entered a 15 m radius around the nestbox. The observer, 15 m from the nestbox, subsequently counted the songs ('song output') and estimated the minimum distance to the model ('approach distance') for 3 min. We have previously shown that the latter behaviour represents an appropriate proxy for willingness to engage in physical attacks (Araya-Ajoy & Dingemanse, 2014). Subjects not entering the radius or not arriving within 15 min were scored as nonresponsive. All observers were thoroughly trained to reliably spot and identify the sex of great tits, and to categorize and count male vocalizations. For further details on the test procedure, see Araya-Ajoy and Dingemanse (2014; 2017). During the tests, the acoustic response of the focal male was recorded using a directional microphone (Sennheiser ME66/K6) covered with a windscreen and connected to a recorder (TASCAM DR-05, 44.1 kHz sampling rate, 16 bits sample size, WAV format).

Measuring Song Overlapping

We measured song output and determined the extent of overlapping (for those birds that sang) while controlling for song output. We measured overlapping by including all periods with co-occurrence of playback and responder songs, in which the responder had started to sing during the playback song and excluding those cases where overlap was caused by the playback song starting during a song of the responding bird. We compared the observed with the expected overlap duration, which we determined by randomly simulated song arrangements using the SONG package (song overlap null model generator, Masco et al., 2016). We analysed song recordings with Avisoft SASLab Pro v5.2 (Avisoft Bioacoustics, Berlin, Germany). We removed background noise below 2.0 kHz and above 8.0 kHz, by bandpass filtering our recordings between these frequencies. We normalized each file to 75% and created spectrograms for each focal song separately (FFT length = 1024 and window = FlatTop, giving a frequency resolution of 23 Hz, and overlap = 87.5%, giving a temporal resolution of 5.3 ms).

We calculated overlapping in three steps. First, we measured song duration based on start and end times, by using automatic measurements with three threshold levels (−24 dB, start −16 dB, end −12 dB). Second, we also determined the start and end times of one playback song in the recording, in the same way as with songs of the focal male and used these to calculate the temporal position of all other playback songs of the known stimulus. Third, observed and expected durations of the overlapping songs were calculated in R v3.5.1 (R Core Team, 2018) using the SONG package (Masco et al., 2016; Fig. 2). The expected level of overlapping was calculated as the mean length of overlapping of 1000 randomizations, during which the focal bird's songs were randomly rearranged with varying interval lengths between them (SampleGaps method). We then determined a chance-corrected overlapping score, calculated as the observed minus the expected total overlapping time. This score reflects whether an individual overlapped the playback more (positive values), the same (zero) or less (negative values) than expected by chance. Recordings for which any of the three steps could not be taken were excluded from the analysis. This occurred because of elevated noise levels, multiple birds singing or when it was not possible to assign songs to the playback versus the focal bird. For each recording, we also calculated whether the overlap differed significantly from chance. Here, a significant deviation from chance occurred when the observed overlapping value was in the top or bottom 2.5% of the null distribution of 1000 simulated values expected by chance.

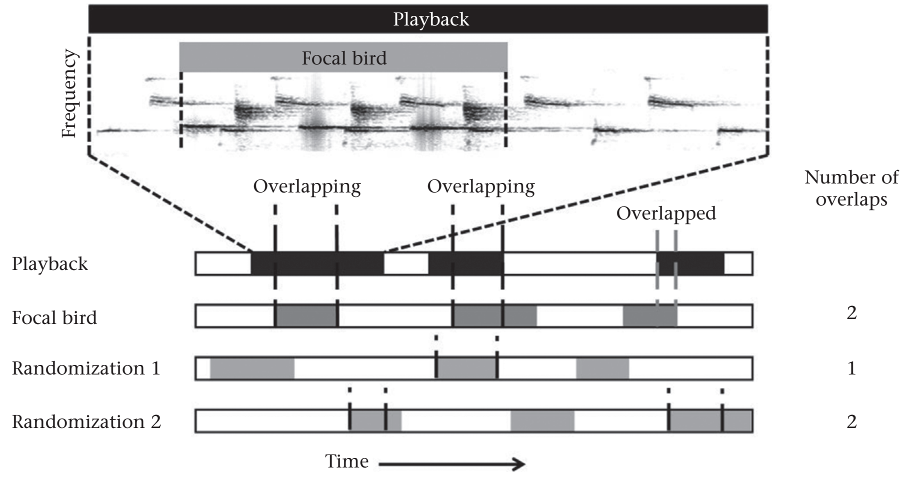

Figure 2. Comparison of playback and focal bird songs to obtain the duration of overlapping songs. Overlapped (i.e. playback song starts while focal male already singing) songs were not considered. Two example randomizations (out of 1000) are shown following an actual example of an overlapping focal bird song. Chance-corrected overlapping was then calculated as the observed length of overlapping (s) minus the mean length of overlapping of 1000 randomizations.

Statistical Analyses

We used univariate linear mixed-effect models with a Gaussian error distribution to test the links between our response variables, (1) song output and (2) chance-corrected overlapping score, and aggression. We multiplied the distance of closest approach by −1 and added the furthest distance from the model a bird showed in our data set to each score, so that the strongest response had the highest score. To account for differences between breeding seasons in overall singing behaviour, we added year (2017, 2018 or 2019) as a fixed-effect factor. Our previous work has shown that aggressiveness, singing and alarm calling can change with breeding context and with test sequence (Araya-Ajoy & Dingemanse, 2014). We thus included nest stage (0 = egg laying and 1 = incubation) and test sequence (0 = first test within a stage and 1 = second test within a stage) as fixed-effect factors in all models. Additionally, because focal birds may overlap more when they sing more, we added the song output of the focal individual as a covariate in an expanded model. As song length may also represent a signal of aggression (Nelson & Poesel, 2011; Osiejuk & Jakubowska, 2017), we added song length of the focal individual as a covariate in a separate run of the expanded model.

Each model also fitted random intercepts for individual identity (song output = 369 and chance-corrected overlapping score = 251 individuals), to estimate among-individual variation. Furthermore, random intercepts were added for playback song recording (song output = 175 and chance-corrected overlapping score = 144 playbacks) and taxidermic model (song output = 18 and chance-corrected overlapping score = 17 models), to control for variation caused by features of the two stimuli. We also added plot-year (i.e. the unique combination of plot and year, 34 levels) to account for spatiotemporal effects that may vary among plots or years, or within years among plots, such as breeding density (see Appendix). These analyses showed that plot-year explained extremely little variation in song output and chance-corrected overlapping (Appendix Table A1), implying that any factor varying at this level is unlikely to have much effect. Finally, we controlled for variation caused by the field observer ($N = 19$) by fitting it as a random effect. In a subsequent analysis, we analysed each year separately, while keeping the model structure the same, to investigate whether effects of aggression on chance-corrected overlapping were consistent across years. The repeatabilities of chance-corrected overlapping and song output were estimated from models where aggressiveness was not fitted as a fixed-effect covariate. This was because our aim was to estimate the trait's overall repeatability rather than that while controlling for (repeatable) variation in aggression (for further discussion, see Dingemanse & Dochtermann, 2013).

In a second step, we aimed to disentangle among- and within-individual effects of aggression on singing behaviour by partitioning the overall effect into these two distinct components (Dingemanse & Dochtermann, 2013; Van de Pol & Wright, 2009). First, effects might exist among individuals if males characterized by specific aggression levels can also be characterized by specific singing characteristics, i.e. specific levels of song output or chance-corrected overlapping. This would indicate that song output and chance-corrected overlapping are personality-related signals rather than signals of short-term motivation to escalate a conflict. Second, effects might exist within individuals if males plastically adjust singing to changes in aggression, indicative of context-dependent motivation. We, thus, reran the initial models on song output and chance-corrected overlapping score after splitting the predictor aggression into two derived variables: the individual's mean aggression ($\bar{x}$) assessing the among-individual effect (β_A) and the observation's deviation from the individual's mean ($x_i - \bar{x}$) estimating the within-individual effect (β_W). The inclusion of individuals with one data point is recommended as it improves the precision of both fixed- and random-effect estimates (Martin, Nussey, Wilson, & Réale, 2011).

All statistical analyses were conducted in R version 3.5.1 (R Core Team, 2018). Linear mixed-effects models were performed using the ‘lmer’ function of the package lme4. We present the mean (β) and 95% credible intervals (CIs) for each fixed and random effect parameter, derived from 2000 simulations implemented using the ‘sim’ function of the package arm. An effect was regarded as statistically significant when its associated 95% CI did not overlap zero (Nakagawa & Cuthill, 2007). All random effect estimates are represented as adjusted repeatabilities, defined as the proportion of the total phenotypic variation not explained by fixed effects.

Ethical Note

This study was approved by the Regierung Oberbayern (permit number ROB-55.2-2532.Vet_02-17-215) in accordance with the ASAB/ABS Guidelines for the use of animals in research. Our experiments were designed to minimize subject discomfort.

RESULTS

General Data

Overall, we performed 2740 simulated territory intrusions of which 1338 had a male of known identity responding. We collected 481 (of 1558 recordings in total) song recordings of 251 birds of a quality sufficient for further analyses (2017: 200; 2018: 140; 2019: 141 recordings). In total, 210 birds were recorded in 1 year only, 38 birds in 2 years and three birds in 3 years. Not all birds responded (i.e. arrived within 15 min) or produced an analysable recording across all four tests in a given year: 107 males yielded one recording, 82 had two, 44 had three, 14 had four, three had five and one had seven recordings.

Of the 481 recordings, 99 (20.6%) overlapped significantly less than expected by chance, while 369 recordings (76.7%) overlapped according to chance. Overlap above chance levels thus occurred rarely (13 recordings; 2.7%). Following chance correction, overlapping scores correlated negatively with song output but not with other traits (see Table 1). A plot of the data showed that birds producing few songs overlapped the recording according to chance levels, while those producing many songs overlapped less than expected by chance (Fig. 3). Aggressiveness, song output, number of alarm calls and the number of attacks were correlated as previously recorded for this population (based on another data set; Araya-Ajoy & Dingemanse, 2014). These insights support the validity of our decision for minimum distance as a suitable measure of aggressiveness.

Covariance Between Aggression, Song Output and Song Overlapping

We found strong support in the raw (unpartitioned) data for a negative overall link between aggression and song output for all years combined (effect of aggressiveness, Appendix Table A1). Song output decreased with increasing aggression. This pattern was observed in all years (Fig. 4, Appendix Table A2), implying that it was not a year-specific effect but rather a general phenomenon. As observed in data collected from the same population in previous years (Araya-Ajoy & Dingemanse, 2014, 2017), we found that birds produced more songs during the incubation than the egg-laying stage (effect of nest stage, Fig. 5). Song output did not change with test sequence (within-stage) and song output of the average bird did not differ between years (Appendix Table A1). Finally, song output showed significant repeatability, regardless of whether the effect of aggression was controlled for or not (see Appendix Table A1).

There was also strong support for a link between chance-corrected overlapping and aggression in the raw (unpartitioned) data (Appendix Table A1). Importantly, year-specific analyses demonstrated a lack of support for this link in 2 of the 3 years of study, indicating that the overall effect was mainly caused by one specific year (Fig. 4, Appendix Table A3). Chance-corrected overlapping did not vary with year, nest stage or test sequence (Fig. 5) but it did covary negatively with the song length of the focal bird during the recording, indicating that birds that sang for longer overlapped the playback for shorter periods (Appendix Table A1). It also covaried negatively with the number of songs the focal bird produced, indicating that birds that sang more showed less overlapping than expected (Appendix Table A1). In contrast to song output, the repeatability of overlapping was low, implying that variation in overlapping was caused by within- rather than among-individual processes (see Appendix Table A1).

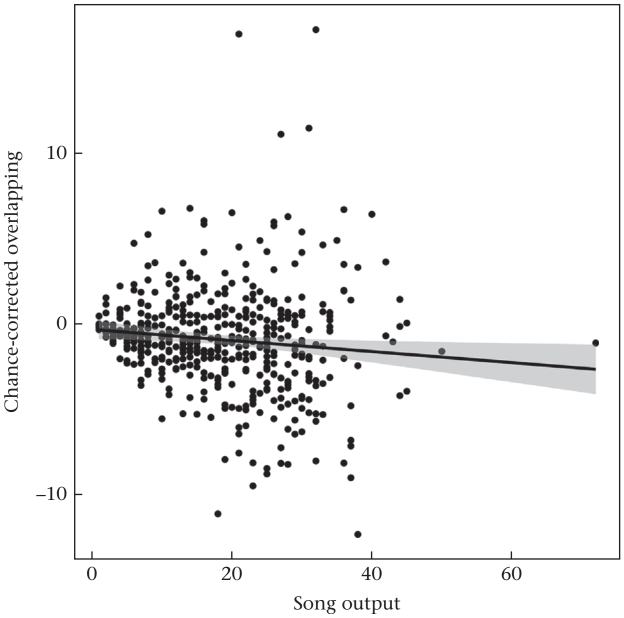

Figure 3. Song output (number of songs sung per 3 min) and chance-corrected overlapping (duration of overlapping compared with expected value) plotted against each other for all 3 years combined ($N = 481$). Black lines represent the regression line, while the grey areas indicate the 95% confidence interval. Graph based on raw data.

Among- versus Within-individual Effects of Aggression

As a next step, we partitioned the patterns in the raw data (Fig. 6) into among- and within-individual effects. This demonstrated that individuals that were on average (over all their observations) more aggressive also, on average, sang less (Fig. 6, Table 2). The same negative effect was observed within individuals: reductions in aggressiveness were associated with increased song output across observations (days) of the same individual (Fig. 6, Table 2).

Applying the same partitioning of the raw data (Fig. 6) to chance-corrected overlapping demonstrated that the average aggression level of an individual did not predict its overlapping behaviour. Specifically, there was no among-individual effect of aggression on chance-corrected overlap (Fig. 6, Table 2). By contrast, changes in aggressiveness and chance-corrected overlapping correlated among observations (recordings) of the same individual, indicating that when birds upregulated their aggression from one observation to the next, they also downregulated their

Table 1
Correlation matrix between chance-corrected overlapping and the four response behaviours measured during the tests

	Chance-corrected overlapping	Aggressiveness	Song output	Alarm calling	Attacks
Aggressiveness	−0.05 (−0.15, 0.05)	–			
Song output	**−0.12 (−0.21, −0.02)**	**−0.25 (−0.30, −0.20)**	–		
Alarm calling	0.04 (005, 0.14)	**0.28 (0.23, 0.33)**	**−0.48 (−0.52, −0.44)**	–	
Attacks	0.02 (−0.12, 0.07)	**0.31 (0.26, 0.36)**	**−0.23 (−0.28, −0.18)**	**0.09 (0.04, 0.14)**	–

Overlapping: $N = 481$; other traits: $N = 1338$. Significant correlations ($P < 0.05$) are shown in bold.

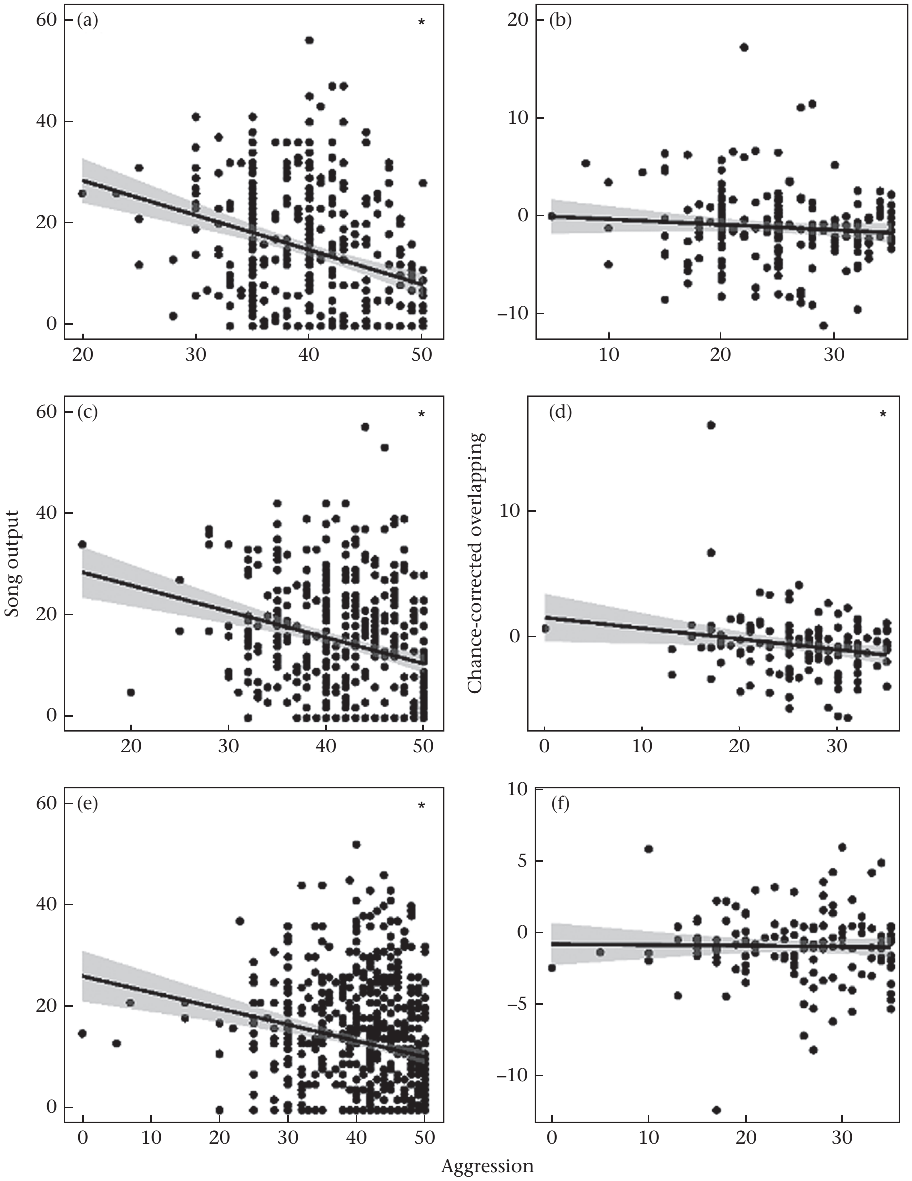

Figure 4. (a, c, e) Song output (number of songs sung per 3 min; $N = 1338$ simulated territory intrusions) and (b, d, f) chance-corrected overlapping (duration of overlapping compared with expected value; $N = 481$ recordings) plotted against the aggression score (approach distance multiplied by $-1 +$ maximum approach distance) for each year separately. (a, b) 2017; (c, d) 2018; (e, f) 2019. Black lines represent the regression line, while the grey areas indicate the 95% credible interval. The '*' symbol in the top right corner indicates cases where patterns of nonzero covariance were supported by the data (i.e. credible intervals not overlapping zero). All graphs based on raw data.

overlapping of the intruder's song (Fig. 6, Table 2). Models where all random effects were excluded except the individual gave the same results (not shown).

DISCUSSION

We used an experimental approach with repeated measures across seasons and multiple years to test whether great tits use song overlapping tendency as a signal of aggressive intent, and whether overlapping tendency correlates with variation in aggression among- and within-individuals. We found that (1) chance-corrected overlap correlated negatively rather than positively with aggressiveness in the data set overall and (2) this relationship existed within but not among individuals. Specifically, within-individual increases in aggression across separate intrusions were associated with within-individual decreases in chance-corrected overlapping. Altogether, our findings imply that overlapping can indeed act as a motivational signal of aggressive intent. However, the correlation between aggression and overlapping was opposite to expectations, while the majority of birds overlapped equal to or less than chance levels. Altogether, these findings imply that the role of overlapping in communicating aggression is not straightforward or prominent and that alternative adaptive mechanisms, such as interference avoidance, might explain our data.

Overlapping as a Signal of Aggression?

Our multiyear study contributes a well-replicated example to the literature (Helfer & Osiejuk, 2015; Naguib & Mennill, 2010; Searcy & Beecher, 2009). Searcy and Beecher (2009) argued that a signal of aggressive intent should (1) occur more often in agonistic

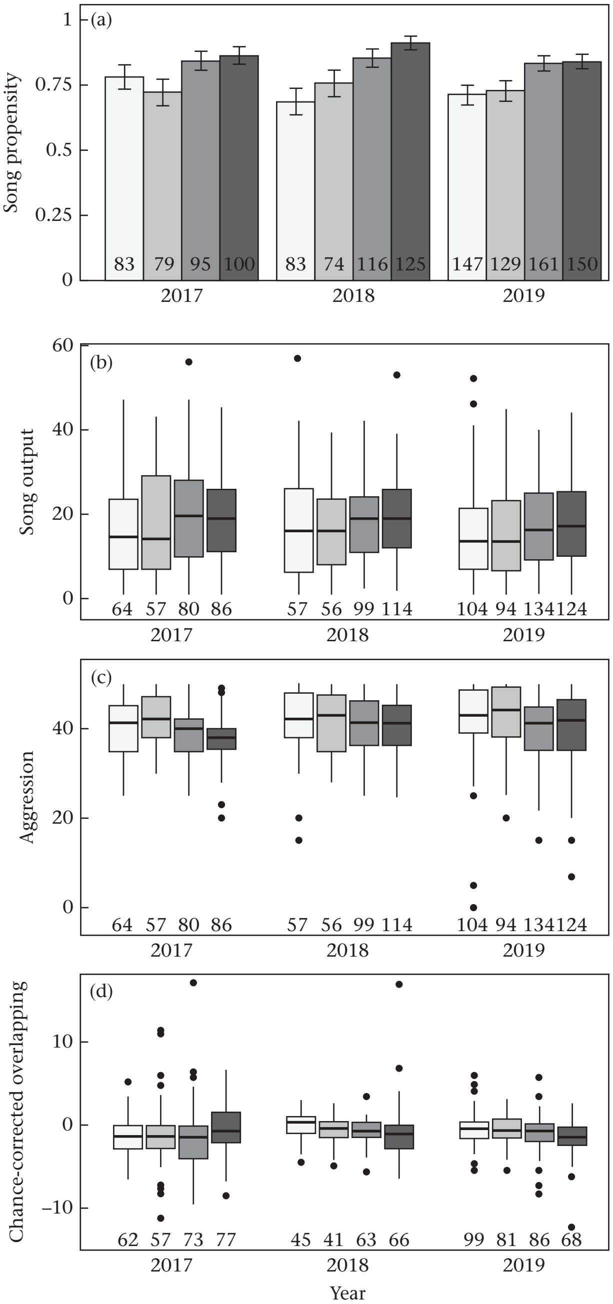

Figure 5. (a) Song propensity (singing no songs or at least one), (b) song ouput (number of songs sung per 3 min), (c) aggression score (approach distance multiplied by −1 + maximum approach distance) and (d) chance-corrected overlapping (duration of overlapping compared with expected value; negative values indicate less overlapping than expected by chance) during the egg-laying and incubation stages within each year. In (a) as song propensity is a binary trait, the average probability of singing per test sequence is shown with standard error. In (b, c, d) the box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers. Within years, the first two bars/box plots represent the egg-laying phase and the last two represent the incubation phase. Numbers at the bottom show sample sizes. Graphs on song output, aggression and overlapping exclude birds without songs.

than in nonagonistic interactions (context criterion), (2) correlate with other aggressive behaviours or predict a subsequent escalation (predictive criterion) and (3) elicit a differential reaction (either stronger or weaker) in receivers (response criterion). We address the literature and our own results here based on these criteria.

Context criterion

Evidence for the occurrence of overlapping varying with context can come from observational, descriptive studies, but is most compelling when derived from experimental studies. Convincing evidence could, for example, come from comparing singing interactions between low-level and high-level arousal conditions, such as before and after playback designed to cause distinct shifts in arousal. We are not aware of any great tit study that has explicitly tested for this context-dependent occurrence of overlapping, but Brindley (1991) reported an example of this in European robins, Erithacus rubecula. Robins overlapped more in response to a nonneighbour song playback, causing relatively high arousal, than to a neighbour song playback, causing only moderate arousal. The overlapping tendency also decreased with the distance between the responding robin and the playback speaker. Similar types of analyses would be possible in great tits but have not been performed.

Predictive criterion

Akçay et al. (2020) recently reported a series of playback experiments in which they tested the correlation between aggression and song overlapping in great tits. They found that a number of behaviours indicative of aggression, such as number of flights, closest approach distance and proportion of time spent within 1 m of the speaker (combined in a principal component) were negatively correlated with overlapping rate. Their study design was similar to ours, in being from within territory boundaries, close to the nest. However, they did not repeat their tests as often as we did, nor did they use a taxidermic mount as a visual stimulus. Despite these differences their findings are corroborated by our own results, as we did find similar patterns. Importantly, neither of the studies provides any evidence for overlapping being a signal of increased aggressive intent.

Response criterion

In a relatively early study on conflict escalation in great tits, Langemann et al. (2000) showed that male great tits exhibited response patterns in three subsequent stages of a playback procedure that differed depending on whether the intruder playback overlapped. The great tits responded strongest to the overlapping playback stimulus in terms of closest approach and number of flights longer than 5 m, although the birds were always exposed to overlapping last, which made playback sequence a confounding factor. In a later study, Amy et al. (2010) showed that overlapping songs induced higher song output and higher switching rates than alternating songs in their interactive playback setting. Using an exceptional set-up, Peake, Terry, McGregor, and Dabelsteen (2001) reported a more indirect test for the response criterion. Focal individuals were eavesdropping on an interaction between two male great tits and were presented one of the two competitors afterwards. Although not all assayed behaviours were affected, when presented with the individual that had been overlapped, birds had a lower song output and made fewer song type switches. Notably, two of the three studies for this criterion found links between overlapping and acoustic traits but not with approach measures.

Specific findings on the response criterion indicated that being overlapped can also influence behavioural responses (Amy et al., 2010; Langemann et al., 2000). To account for this, we tested whether birds that were initially overlapped by the playback differed from birds that were not. We found no evidence for such behavioural differences in song overlapping, song output or aggression, indicating that our results were not biased by birds adjusting their responses to the 'behaviour' of our simulated intruder (see Appendix and Table A4).

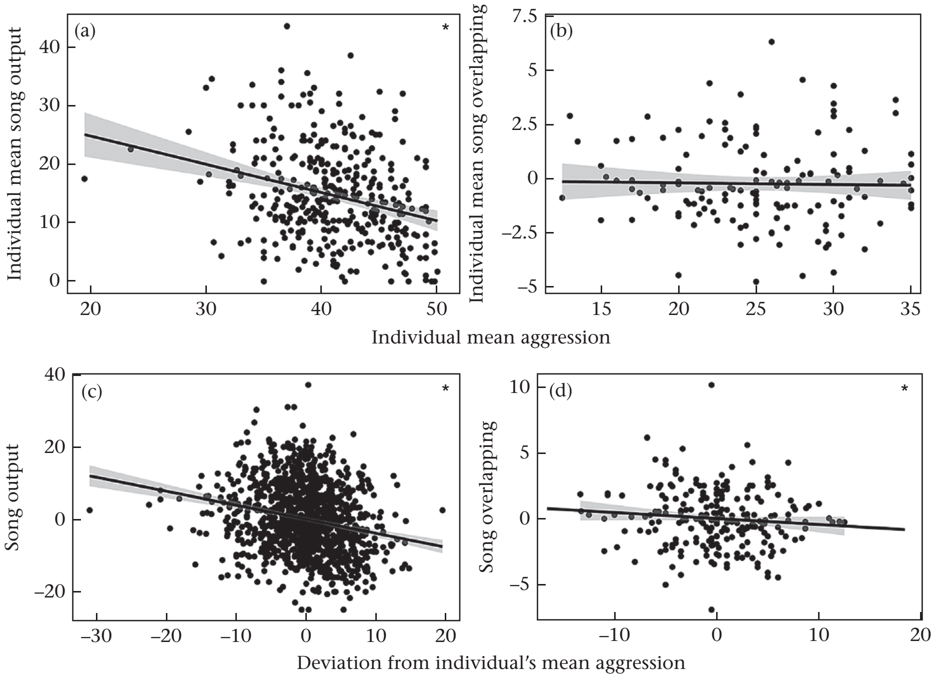

Figure 6. Visualization of (a, b) among-individual and (c, d) within-individual relationships for (a, c) song output (number of songs sung per 3 min) and aggression (approach distance multiplied by −1 + maximum approach distance) and (b, d) chance-corrected overlapping (duration of overlapping compared with expected value; negative values indicate less overlapping than expected by chance) and aggression. Among-individual relationships are visualized by plotting mean values per individual. Within-individual relationships are visualized by plotting each observation's deviation from the individual's mean value for the two focal traits. Black lines represent the regression line, while the grey areas indicate the confidence interval. The '*' symbol in the top right corner indicates cases where patterns of nonzero covariance were supported by the data (i.e. credible intervals not overlapping zero). All graphs based on raw data.

Table 2
The 95% credible interval around the estimated mean parameter estimate for fixed effects on chance-corrected overlapping and song output

	Song output $N = 1338$	Chance-corrected overlapping $N = 477$
	β/σ^2 (95% CI)	β/σ^2 (95% CI)
Fixed effects		
Intercept[a]	9.03 (6.14, 11.94)	−0.71 (−2.01, 0.65)
Aggressiveness		
Among individuals[b]	−1.70 (−2.37, −0.99)	0.12 (−0.29, 0.49)
Within individuals[c]	−1.90 (−2.48, -1.27)	−0.45 (−0.85, −0.06)
Year		
2018	0.92 (−1.40, 3.20)	0.36 (−0.37, 1.06)
2019	−0.77 (−3.02, 1.53)	0.07 (−0.61, 0.76)
Test sequence	0.69 (−0.42, 1.81)	0.20 (−0.39, 0.75)
Nest stage	2.74 (1.59, 3.91)	−0.45 (−1.03, 0.11)
Song output focal bird	–	−0.38 (−0.65, −0.09)
Song length focal bird[d]	–	−0.29 (−0.57, 0.02)
Random effects[e]		
Individual	0.153 (0.147, 0.157)	0.024 (0.021, 0.026)
Playback	0.0048 (0.0042, 0.0053)	0.002 (0.009, 0.001)
Plot-year	0.010 (0.006, 0.013)	0 (0, 0)
Observer	0.03 (0.02, 0.05)	0 (0, 0)
Taxidermic mount	0 (0, 0)	0 (0, 0)
Residual	0.80 (0.78, 0.82)	0.975 (0.973, 0.978)

β is given for fixed effects and σ^2 for random effects. CI: credible interval.

[a] Population mean for the reference of all other fixed effects, i.e. test sequence 0, nest stage 0, year 2017, for birds of average aggression (among-individual aggressiveness) and their average aggressiveness (within-individual aggressiveness).

[b] Mean aggressiveness over all records of the same individual.

[c] Deviation of each observation from an individual's mean aggressiveness.

[d] Estimate from a separate model with the same model structure, except for the exclusion of song output.

[e] Random-effect estimates are presented as adjusted repeatabilities, defined as the proportion of variation not explained by fixed effects.

Interference Avoidance

Based on this overview of the recent literature, we believe that overlapping could serve a signalling function, but there is no support for overlap as a signal of strong aggressive intent, at least not in great tits. Given the general lack of a positive correlation between aggression and overlapping in previous work and our own study, a more likely explanation for a deviation from random overlapping tendency may be related to withdrawal or nonescalation, or with interference avoidance (Ficken, Ficken, & Hailman, 1974; Searcy & Beecher, 2009; Vehrencamp et al., 2007). Indeed, most of our birds either did not show overlap that differed from chance levels or primarily overlapped less (rather than more) than expected by chance. This avoidance seemed to be especially pronounced for individuals that produced more or longer songs. Overall, our evidence for overlap avoidance may suggest great tits seek to increase the audibility of singing competitors, as previously suggested for other species (Wilson et al., 2016; Yang et al., 2014).

Personality or Context-dependent Motivation?

Our study adds to the picture of the signalling role of overlapping emerging from the literature over the past few decades (Akçay et al., 2020; Brindley, 1991; Langemann et al., 2000) and further represents a showcase for the benefits of repeated measurements. The first benefit concerns statistical robustness. Testing the same individuals repeatedly across and within years allowed us to assess the temporal consistency between song output, overlapping and aggression and, indirectly, whether year-specific factors affected these associations. While song output covaried negatively with aggression in all 3 years, song overlapping covaried with aggression in only 1 of these years.

For song output, patterns observed in 1 year are thus representative of within-year patterns generally, whereas for overlapping, this is not the case. Consequently, variation among years in, for example, body condition, which can affect acoustic behaviours (Gil & Gahr, 2002; Gottlander, 1987), is unlikely to affect song output. Within years, we confirmed the findings of Araya-Ajoy and Dingemanse (2014) on song output, showing an increase over the breeding season, potentially indicating a shift in intruder threat, for example via changed risks of paternity loss (Araya-Ajoy et al., 2016), leading to a supposedly weaker response. Song overlapping, even though exhibiting a suggestive pattern in 2019, did not consistently change with breeding context.

Besides the statistical robustness, the second benefit of our repeated measures design is more important as it provides completely novel insights. The repeated sampling of the same individual, especially across years, allows partitioning of long-term repeatable among- (‘animal personality’) and within-individual (‘reversible plasticity’) variation in behaviour (Dingemanse & Dochtermann, 2013). Besides showing misestimated or undetected effects, caused by independent and opposing associations of the two components with a trait of interest (Moiron, Laskowski, & Niemelä, 2020; Van de Pol & Wright, 2009), this partitioning also led to valuable insights into the underlying mechanisms of aggressive signalling. While a within-individual effect indicates motivation or behavioural adjustments to environmental factors (Strauß et al., 2020), among-individual effects can result from genetic differences or early life conditions resulting in differing abilities to express certain behaviours (Bischoff et al., 2009).

We showed a significant within-individual effect of aggression on overlapping and simultaneously no evidence for an among-individual effect. This indicates that changes in song overlapping avoidance resulted from changes in aggression levels between days. This is in line with the idea that overlapping signals context-dependent motivation (DuBois et al., 2009; Ripmeester et al., 2007), rather than a personality-related tendency, signalling an individual's average aggression or typical style of vocal interaction (Amy et al., 2010; Jacobs et al., 2014). In theory, song overlapping can, therefore, provide interacting males with information about the sender's current motivation, while it cannot be used to predict its average aggressiveness. Importantly, the negative within-individual effect implies that the signalled motivation relates to the unwillingness to engage rather than to escalate and show physical aggression.

Conclusion

In conclusion, we have shown that great tits do not generally overlap intruder songs over and above chance levels, and that relationships between aggression and chance-corrected overlap do not imply that overlapping serves as a signal of aggressive intent but rather as a potential signal of nonengagement. Song overlapping tendency deviated from chance levels with birds decreasing rather than increasing song overlap. This suggests that interference avoidance may also be a prominent driver for overlapping tendency. Our multiyear study, with repeated testing across breeding seasons and years, clearly illustrates how long-term data collection with repeated measures can provide statistical robustness and fundamentally new insights into the ecology and evolution of birdsong. We highly recommend repeated measurements as in our study, to allow partitioning of among- and within-individual variation, which will potentially reveal more undetected patterns of biological relevance.

Author Contributions

Alexander Hutfluss: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. Veronika A. Rohr: Methodology, Software, Formal analysis, Investigation, Writing – original draft. Saray Scheidt: Formal analysis, Investigation, Writing – original draft. Linda Steinbichl: Formal analysis, Investigation, Writing – original draft. Eira Bermúdez-Cuamatzin: Conceptualization, Methodology, Writing – review & editing. Hans Slabbekoorn: Conceptualization, Methodology, Writing – review & editing. Niels J. Dingemanse: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Appendix

Effect of breeding density

As discussed by Wesołowski (2011), nestbox populations differ a lot from natural populations in a variety of environmental aspects, such as predation risk and breeding density. These differences can then result in changes in behavioural expression and thus potentially bias comparisons and conclusions drawn from nestbox studies. In our populations, breeding densities, given as breeding pairs/ha, vary considerably between plots (range 1.37–3.46), between years (range 1.69–2.87) and within plots between years (range 0.48–5.02). Even though these differences could affect the behaviours measured in this study (for example, breeding density correlates positively with aggression), we are certain that they do not bias our findings. This is mainly because we accounted for spatiotemporal patterns by adding ‘plot-year’ to all our analyses. By doing so, we corrected for potential variation induced by differences within plots between years. As breeding density mostly varies at this level and because plot-year explained little variation, we can conclude that breeding density is extremely unlikely to affect our response behaviours in any way.

Table A1
The 95% credible interval (CI) around the mean for effects influencing song output and song overlapping

	Song output $N = 1338$	Chance-corrected overlapping $N = 483$
	β/σ^2 (95% CI)	β/σ^2 (95% CI)
Fixed effects		
Intercept[a]	9.15 (6.05, 12.20)	−0.75 (−2.03, 0.56)
Aggressiveness[b]	−2.51 (−3.15, −1.83)	−0.38 (−0.67, −0.08)
Year 2018	0.86 (−1.50, 3.31)	0.41 (−0.24, 1.05)
Year 2019	−0.91 (−3.15, 1.40)	0.09 (−0.60, 0.77)
Test sequence	0.68 (−0.42, 1.80)	0.21 (−0.35, 0.74)
Nest stage	2.72 (1.54, 3.90)	−0.44 (−1.02, 0.15)
Song output focal bird	–	−0.38 (−0.65, −0.09)
Song length focal bird[c]	–	−0.28 (−0.57, 0.001)
Random effects[d]		
Individual	0.153 (0.147, 0.159)	0.023 (0.021, 0.024)
Playback	0.0048 (0.0042, 0.0054)	0.001 (0.001, 0.001)
Plot-year	0.010 (0.006, 0.013)	0 (0, 0)
Observer	0.03 (0.02, 0.05)	0 (0, 0)
Model	0.0005 (0.0002, 0.0008)	0 (0, 0)
Residual	0.80 (0.82, 0.77)	0.976 (0.975, 0.979)
Adjusted repeatability[e]	0.16 (0.15, 0.17)	0.01 (0.01, 0.01)

β is given for fixed effects and σ^2 for random effects.

[a] Reference category; estimate of overlapping length index during test sequence 0, nest stage 0, year 2017 and for attacking birds (aggressiveness = 0).

[b] Aggressiveness measured as approach distance multiplied by −1 + maximum approach distance.

[c] Estimate from a separate model with the same model structure, except for the exclusion of song output.

[d] Random-effect estimates are presented as adjusted repeatabilities, defined as the proportion of variation not explained by fixed effects.

[e] Based on the same model structure but excluding the effects of aggression and focal song number (for overlapping).

Effect of being overlapped

We used a noninteractive playback as a stimulus, which inadvertently resulted in some birds being overlapped by the playback while others were not. We tested whether being overlapped affected their behaviour with a post hoc analysis. Each recording was assigned to one of three categories based on the overlapping of the first song: (1) the focal bird's first song neither overlapped nor was it overlapped (‘no overlap’); (2) the focal bird's first song actively overlapped the playback (‘focal overlapped stimulus’); (3) the focal bird's first song was overlapped by the playback (‘stimulus overlapped focal’). We pragmatically limited this overlap categorization to the very first song, to reduce the number of categories and assumptions. The model was our base model expanded with overlapping category added as a categorical fixed effect. Compared to ‘no overlap’ birds, birds that actively overlapped the intruder showed higher chance-corrected overlapping (see Table A4). This is what one would expect if overlapping is consistent within an observation. Importantly, chance-corrected overlapping did not differ between ‘no overlap’ birds and those that were overlapped by the playback. This latter finding suggested that being overlapped

Table A2

The 95% credible interval (CI) around the mean for effects influencing the song output for the 3 study years separately

	Song output 2017 $N = 356$	2018 $N = 398$	2019 $N = 584$
	β/σ^2 (95% CI)	β/σ^2 (95% CI)	β/σ^2 (95% CI)
Fixed effects			
Intercept[a]	10.72 (5.57, 15.77)	8.43 (3.40, 13.57)	8.23 (4.20, 12.30)
Aggressiveness[b]	−4.72 (−6.20, −3.25)	−3.30 (−4.56, -2.14)	−1.35 (−2.18, −0.53)
Test sequence	0.08 (−2.17, 2.37)	1.24 (−0.83, 3.38)	0.67 (−1.02, 2.27)
Nest stage	1.91 (−0.41, 4.09)	2.98 (0.74, 5.15)	2.74 (1.10, 4.42)
Random effects[c]			
Individual	0.216 (0.204, 0.223)	0.107 (0.098, 0.112)	0.210 (0.202, 0.216)
Playback	0.02 (0.01, 0.04)	0.041 (0.037, 0.043)	0 (0, 0)
Plot-year	0 (0, 0)	0.02 (0.01, 0.03)	0.005 (0.003, 0.008)
Taxidermic mount	0.009 (0.004, 0.01)	0 (0, 0)	0.009 (0.004, 0.02)
Observer	0 (0, 0)	0.03 (0.007, 0.05)	0.06 (0.03, 0.09)
Residual	0.75 (0.72, 0.78)	0.81 (0.77, 0.85)	0.72 (0.67, 0.76)

β is given for fixed effects and σ^2 for random effects.

[a] Reference category; estimate of overlapping length index during test sequence 0, nest stage 0 and for attacking birds (aggressiveness = 0).

[b] Aggressiveness measured as approach distance multiplied by −1 + maximum approach distance.

[c] Random-effect estimates are presented as adjusted repeatabilities, defined as the proportion of variation not explained by fixed effects.

Table A3

The 95% credible interval (CI) around the mean for effects influencing the song overlapping for the 3 study years separately

	Chance-corrected overlapping 2017 $N = 200$	2018 $N = 142$	2019 $N = 141$
	β/σ^2 (95% CI)	β/σ^2 (95% CI)	β/σ^2 (95% CI)
Fixed effects			
Intercept[a]	−2.41 (−4.65, −0.20)	0.78 (−1.19, 2.79)	1.03 (−0.86, 2.92)
Aggressiveness[b]	−0.46 (−1.04, 0.14)	−0.62 (−1.09, −0.18)	−0.15 (−0.52, 0.21)
Test sequence	1.03 (0.02, 2.08)	−0.27 (−1.08, 0.51)	−0.41 (−1.22, 0.34)
Nest stage	−0.12 (−1.15, 1,00)	−0.63 (−1.46, 0.18)	−0.89 (−1.69, −0.10)
Bird song length	−0.25 (−0.74, 0.28)	−0.11 (−0.54, 0.33)	−0.58 (−0.99, −0.17)
Random effects[c]			
Individual	0 (0, 0)	0.09 (0.08, 0.10)	0.154 (0.146, 0.154)
Playback	0 (0, 0)	0.42 (0.41, 0.42)	0 (0, 0)
Plot-year	0 (0, 0)	0 (0, 0)	0 (0, 0)
Taxidermic mount	0 (0, 0)	0.010 (0.006, 0.015)	0 (0, 0)
Observer	0 (0, 0)	0 (0, 0)	0.08 (0.02, 0.14)
Residual	~1.00 (~1.00, ~1.00)	0.48 (0.47, 0.51)	0.77 (0.83, 0.70)

β is given for fixed effects and σ^2 for random effects.

[a] Reference category; estimate of overlapping length index during test sequence 0, nest stage 0 and for attacking birds (aggressiveness = 0).

[b] Aggressiveness measured as approach distance multiplied by −1 + maximum approach distance.

[c] Random-effect estimates are presented as adjusted repeatabilities, defined as the proportion of variation not explained by fixed effects.

Table A4
The 95% credible interval (CI) around the mean for the effects of the focal individual's first song being overlapped, actively overlapping or not showing any overlap with the playback

	Chance-corrected overlapping	Song output	Aggression
	N= 337	*N*= 337	*N* = 337
	β/σ^2 (95% CI)	β/σ^2 (95% CI)	β/σ^2 (95% CI)
Fixed effects			
Intercept[a]	−1.19 (−2.51, 0.11)	15.67 (11.33, 20.04)	29.45 (26.73, 32.28)
First song active overlap	1.36 (0.72, 2.03)	0.04 (−2.19, 2.30)	−0.54 (−1.91, 0.76)
First song overlapped by playback	0.29 (−0.40, 0.97)	−1.00 (−3.46, 1.25)	−1.37 (−2.73, 0.03)
Aggressiveness[b]	−0.36 (−0.66, −0.08)	−1.72 (−2.72, −0.74)	–
Test sequence	0.16 (−0.37, 0.71)	0.37 (−1.42, 2.12)	0.50 (−0.58, 1.58)
Nest stage	−0.29 (−0.87, 0.30)	1.32 (−0.67, 3.22)	−2.54 (−3.69, −1.32)
Bird song length	−0.32 (−0.60, −0.04)	–	–
Random effects[c]			
Individual	0.036 (0.033, 0.039)	0.016 (0.014, 0.017)	0.13 (0.13, 0.14)
Playback	0.007 (0.006, 0.008)	0.010 (0.009, 0.0011)	0.03 (0.03, 0.04)
Plot-year	0 (0, 0)	0 (0, 0)	0.03 (0.02, 0.04)
Taxidermic mount	0 (0, 0)	0 (0, 0)	0.003 (0.002, 0.004)
Observer	0 (0, 0)	0.01 (0.006, 0.02)	0.08 (0.05, 0.10)
Residual	0.96 (0.95, 0.96)	0.96 (0.95, 0.97)	0.72 (0.68, 0.76)

β is given for fixed effects and σ^2 for random effects.

[a] Reference category; estimate of the response variable during test sequence 0, nest stage 0, for attacking birds (aggressiveness $= 0$) and for birds whose first song was not overlapped nor actively overlapped the playback.

[b] Aggressiveness measured as approach distance multiplied by $-1 +$ maximum approach distance.

[c] Random-effect estimates are presented as adjusted repeatabilities, defined as the proportion of variation not explained by fixed effects.

did not affect the focal bird's behaviour. This conclusion was also warranted because we did not find differences between the three categories in song output or aggression. Furthermore, accounting for overlap of the first song did not alter the reported links between aggression and chance-corrected overlapping, or between aggression and song output.