



Sing and do not stray: male rufous-and-white wrens use duets and physical behaviours to guard their mates

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In diverse animal taxa, breeding partners coordinate their vocalizations to produce vocal duets. One seldom-studied hypothesis for duets is the paternity guarding hypothesis, which states that male animals create duets to advertise their partner's mated status and minimize extrapair mating attempts between their partner and other males. We experimentally tested the paternity guarding hypothesis in rufous-and-white wrens, *Thryophilus rufalbus*, a neotropical duetting songbird. We designed a two-part playback experiment: males first experienced a simulated territorial intrusion by a rival male, and were then given opportunities to answer their female breeding partner's songs to create duets. We repeated this experiment during the female's fertile and nonfertile breeding stages. In support of predictions of the paternity guarding hypothesis, male wrens created more duets with their partner's songs during the fertile period compared to the nonfertile period. Additionally, male wrens appeared to physically guard their mates with greater intensity during the fertile period but did not increase their overall song rates, demonstrating that increased duetting rates during the fertile period were a result of a change in male duetting behaviour, rather than a change in song rate. Our study is among the first to experimentally test the paternity guarding hypothesis for duet function, and suggests that male rufous-and-white wrens use both vocal and physical behaviours to guard their paternity.

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In many animals, including diverse species in the tropics, elaborate vocalizations are often produced by both males and females, and in some species breeding partners combine their vocalizations into vocal duets (Geissmann, 2002; Hall, 2004; Slater & Mann, 2004; Stutchbury & Morton, 2001). Vocal duets are highly coordinated acoustic signals where one individual vocalizes and the partner creates a duet by vocalizing in response, either by overlapping the partner's vocalization or by producing a vocalization in quick succession (Hall, 2004). This behaviour occurs in primates (e.g. Caselli, Mennill, Gestich, Setz, & Bicca-Marques, 2015; Geissmann, 2002), frogs (e.g. Tobias, Viswanathan, & Kelley, 1998), insects (e.g. Bailey, 2003), and has been studied most extensively in birds (reviewed in Farabaugh, 1982; Hall, 2004; Tobias et al., 2016).

Vocal duets are multipurpose signals that serve functions in both cooperative and conflict-based contexts (e.g. Benedict, 2010; Mennill & Vehrencamp, 2008). The most widespread and well-

supported function for vocal duets is that they are used to cooperatively defend ecological resources (reviewed in Dahlin & Benedict, 2014; Douglas & Mennill, 2010). An alternative viewpoint is that duets can arise from intersexual conflict between breeding partners; an animal may perform a duet with its partner to prevent their partner from pairing or mating with other animals (Levin, 1996a, 1996b; Rogers, Langmore, & Mulder, 2007; Seddon & Tobias, 2006; Sonnenschein & Reyer, 1983; Tobias & Seddon, 2009). The importance of duets in intersexual conflict, however, remains poorly understood because few studies have used an experimental approach to study this idea (Baldassare et al., 2016; Hall, 2004).

One hypothesis for the function of vocal duets related to intersexual conflict is the paternity guarding hypothesis, which states that male animals answer their breeding partner's vocalizations to create duets in order to advertise their paired status and prevent them from mating with rival males (Hall, 2004; Sonnenschein & Reyer, 1983). Two important predictions arise from this hypothesis: (1) males should answer a higher proportion of female vocalizations to create duets during the fertile period in comparison to other breeding stages; and (2) duets should effectively reduce rates of extrapair offspring (i.e. offspring from males other than the social partner; Hall, 2004). Males in many species have been shown to use

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diverse paternity guarding strategies to reduce alternative breeding attempts during the fertile period, including increased vocalization rates (e.g. Holzman, 2012; Mace, 1987; McElligot & Hayden, 2001; Welling, Koivula, & Lahti, 1995), frequent mating attempts (reviewed in Møller & Birkhead, 1991) and physical mate guarding (reviewed in birds in Birkhead & Møller, 1992; Jormalainen, 1998; Schubert, 2009; Setchell, Charpentier, & Wickings, 2005). However, very few studies have investigated paternity guarding strategies in duetting species, particularly with respect to how duets may function as acoustic paternity guards.

To date, most studies of paternity guarding hypotheses in duetting animals have involved observational analysis of variation in duet rates across different breeding stages. For example, in four species of duetting birds (magpie-larks, *Grallina cyanoleuca*; buff-breasted wrens, *Cantorchilus leucotis*; purple-crowned fairy-wrens, *Malurus coronatus*; and red-backed fairy-wrens, *Malurus melanocephalus*), males do not create more duets with their females during the fertile versus nonfertile periods (Dowling & Webster, 2013; Gill, Vonhof, Stutchbury, Morton, & Quinn, 2005; Hall & Magrath, 2000; Hall & Peters, 2009), suggesting that duets do not function as acoustic paternity guards in these species. However, the paternity guarding hypothesis has only been tested experimentally in two duetting animals. In response to playback simulating the songs of a rival male during the female fertile period, male canebrake wrens, *Cantorchilus zeledoni*, produced more duet-initiation songs (i.e. male songs used to elicit polyphonal duet sequences with females), suggesting that males are more motivated to perform duets with females when they are fertile (Marshall-Ball, Mann, & Slater, 2006). In contrast, in response to playback simulating solo and paired intruders, male red-backed fairy-wrens did not sing more duets with their females in the fertile period compared to prefertile and postfertile breeding stages (Dowling & Webster, 2016), although, in another playback experiment with this species, males that sang more duets with their females had lower reproductive losses to extrapair paternity in their nests (Baldassere et al., 2016). In light of these conflicting results, further experimental investigation of the paternity guarding hypothesis in duetting species is warranted.

In this study, we test the paternity guarding hypothesis by conducting a playback experiment in a neotropical duetting songbird: the rufous-and-white wren, *Thryophilus rufalbus*. Rufous-and-white wrens provide an ideal species for testing the paternity guarding hypothesis for two reasons. First, unlike the aforementioned duetting species, males in this species answer a higher proportion of female songs to form duets during the fertile period, suggesting that duets may serve a paternity guarding function (Topp & Mennill, 2008). Second, rufous-and-white wrens exhibit low levels of extrapair paternity (2% of offspring in 6% of broods), indicating, given the previously stated observational evidence, that duets may play a role in acoustic mate guarding during the fertile period (Douglas, Heath, & Mennill, 2012). We used playback to simulate a rival male near the edge of a subject's territory, creating the impression that a male competitor had entered the subject male's territory. We then used playback to simulate the subject's breeding partner near the centre of the subject's territory, thereby giving males an opportunity to create duets with their breeding partner's songs.

We made a priori predictions about how male rufous-and-white wrens would respond to female songs in the second phase of this experiment. In accordance with the paternity guarding hypothesis, we predicted that males would answer a higher proportion of their female's songs to form duets (i.e. they would create more duets) during the fertile period compared to the nonfertile period. We also predicted that males would attempt to guard their paternity in two additional ways unrelated to duetting. We predicted that males

would increase their independent song rates (i.e. songs that were not part of a duet) during the fertile period compared to the nonfertile period, as has been observed in other studies of temperate songbirds (reviewed in Møller, 1991). We also predicted that males would exhibit a more intense physical response towards the loudspeaker simulating their breeding partner (i.e. they would physically guard the loudspeaker simulating their breeding partner by approaching more closely, more quickly, and remaining near to the loudspeaker for longer) during the fertile period compared to the nonfertile period.

METHODS

Study Species and General Field Methods

We conducted our experiment in 2016 and 2017 with a colour-banded population of rufous-and-white wrens in Sector Santa Rosa of the Guanacaste Conservation Area in northwestern Costa Rica (10°51'N, 85°36'W). Rufous-and-white wrens are neotropical, socially monogamous duetting songbirds found throughout many parts of Central America and northwestern South America in which breeding partners sing loosely coordinated polyphonal duets (details in Mennill & Vehrencamp, 2005). Members of our laboratory have studied this population since 2003; on an annual basis we band birds, map territories, find nests and record songs from most animals in the population (20–55 pairs per year). We banded all of the male playback subjects in this experiment with unique combinations of coloured leg bands to facilitate individual identification. We also banded 11 out of 21 of the females involved in this experiment (52%), and we were confident in our ability to differentiate between the remaining unbanded females based on their territory position and their unique vocal repertoires (see Mennill & Vehrencamp, 2005).

Playback Experiment

We conducted playback experiments to 21 different male rufous-and-white wrens between early May and mid-June across the 2 years of our study (7 pairs in 2016 and 14 pairs in 2017). This time of year coincides with the end of our subjects' nonbreeding season and the beginning of the breeding season (i.e. nest building and egg laying), coincident with the first large rainfall of the year (Topp & Mennill, 2008). Each male received playback on two occasions at different breeding stages. First, we delivered playback during the fertile period of the subject's partner, which we defined as the window from 5 days before to 2 days after females laid their first egg of a clutch (on average, we conducted fertile playbacks 3.7 ± 1.3 days before first egg date; Birkhead, 1998). Second, we delivered playback during the incubation period, which we defined as the period when females were incubating their completed clutch (females usually lay two to five eggs and incubate for 12–15 days; on average, we conducted playbacks on day 6.2 ± 4.4 of incubation). In 19 of 21 cases, we administered playback to focal males first during the fertile period and second during the incubation period. There were two pairs for which we first administered playback during the incubation stage and, due to nest predation, secondly during the fertile period while pairs were reneesting. We determined the breeding stage of each pair by finding and monitoring their nests, and by carefully observing the behaviour of birds in the weeks preceding playback.

Each playback experiment, both during the fertile and nonfertile period, included two stages of playback: the 'intrusion stage' and the 'duetting stage' (Fig. 1). During the intrusion stage, we used a loudspeaker placed within the territory to simulate a neighbouring male encroaching onto the subject's territory, potentially seeking

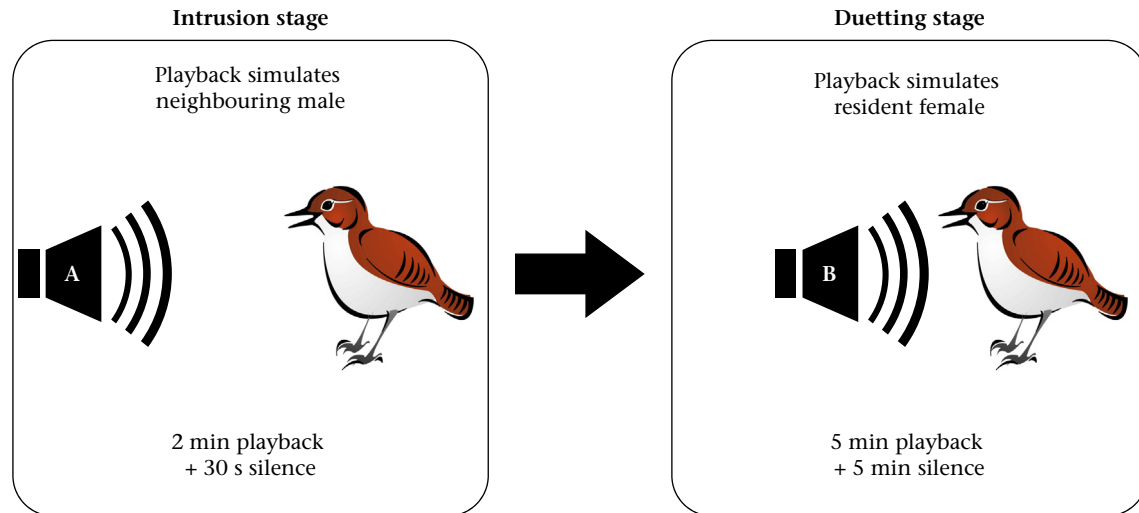


Figure 1. A visual timeline of the two-stage playback experiment used to study the paternity guarding hypothesis for vocal duets in rufous-and-white wrens. During the intrusion stage (left) a loudspeaker near the territory edge simulated the vocalizations of a rival male from the neighbouring territory. This was followed immediately by the duetting stage (right), when a loudspeaker close to the territory centre simulated the vocalizations of the resident female; the subject's response to this speaker provided a test of the paternity guarding hypothesis. Both stages were repeated to each subject when their partner was fertile, and when she was not fertile.

extrapair copulations. We chose to simulate a neighbouring male, rather than a more distant male, because a previous study revealed that extrapair sires were territorial neighbours, and therefore neighbours appear to be the most potent threat to a male's paternity in this population (Douglas et al., 2012). The intrusion stage was a 'priming stage' that established a context of reproductive competition for the territorial male subject.

During the second stage of playback, the duetting stage, we simulated the songs of the resident female using a loudspeaker placed near the centre of the subject's territory, providing the subject male with an opportunity to perform duets with his breeding partner. A similar protocol has proven successful in other species of duetting wrens (Logue, Chalmers, & Gowland, 2008; Rivera-Cáceras et al., 2016; Templeton et al., 2013). Female song output is typically quite low in our study species, and highly variable (Mennill & Vehrencamp, 2008), and therefore we could not rely on the resident female to produce enough songs to test male responsiveness during the duetting stage. During the duetting stage, the subject had the opportunity to respond to his breeding partner's songs broadcast through the loudspeaker (30 songs in total) as well as any songs sung by his partner (these were rare; females produced an average of 5.0 ± 1.2 songs during the duetting stage; $N = 21$). We considered removing females from the subjects' territories during playback, so that the sole female songs available for contribution to duets came from our loudspeakers, but logistical difficulties made this impossible; pilot experiments revealed we could not capture females without simultaneously capturing males. Instead, we controlled for variation in song output from each subject's partner during the duetting stage, as explained below.

Each playback trial consisted of 5 min of preplayback silence, 2 min of neighbour male playback followed by 30 s of silence (i.e. the intrusion stage; 2.5 min in total), followed by 5 min of focal female playback (i.e. the subject's breeding partner) followed by 5 min of silence (i.e. the duetting stage; 10 min in total; Fig. 1). We observed the behaviour of both the male subject and his breeding partner during the intrusion and duetting stages. We did not conduct playback to neighbouring pairs on the same day. On a few rare occasions, neighbouring males approached the territory boundary and sang during our playback trials. We excluded these trials and conducted the playback again during the following day.

The playback apparatus consisted of two camouflaged wireless loudspeakers (Scorpion TX200, FOXPRO Inc., Lewistown, PA, U.S.A.) placed in vegetation 1 m off the ground. For the intrusion stage, we placed the loudspeaker within the subject male's territory, 20–30 m from the edge of the shared territory boundary with the closest neighbouring male. In a few cases when a male had no immediate neighbours, we placed the loudspeaker at the territory edge with the closest possible neighbouring male that the subject could conceivably hear. For the duetting stage, we placed the loudspeaker towards the centre of the subject's territory, and we always placed this second loudspeaker 50 m away from the intrusion stage loudspeaker to ensure that subject males could hear the output from both speakers. Rufous-and-white wrens at our study site exhibit considerable variation in territory size (range 1000–13 000 m²; mean = 5300 m²; Osmun & Mennill, 2011). We played all stimuli at 80 dB SPL, which we measured beforehand with a Casella CEL-24X sound level metre (C-weighting; fast response; measured at 1 m from the loudspeaker). We chose to play our stimuli at 80 dB because it reflects a typical amplitude of rufous-and-white wren songs at our study site, and has been used effectively in previous playback studies with this species (e.g. Kovach, Hall, Vehrencamp, & Mennill, 2014; Mennill, 2006).

During playback, an observer (always Z.A.K.) sat in a position concealed by vegetation, 15–20 m away from the duetting stage loudspeaker, and recorded the experiment using a solid-state digital recorder (Marantz PMD661) and a shotgun microphone (Sennheiser MKH70) mounted on a small tripod. The observer was always positioned closer to the duetting stage loudspeaker to best observe all vocal and physical behaviours of the subject male and his breeding partner. We placed flagging tape at 1 m and 5 m intervals in all four directions around the duetting stage speaker to help estimate the distance of the subject to the loudspeaker. The observer quietly dictated the identity and behaviours of focal birds into the microphone during playback, providing a time-synchronized record of the subject's vocalizations and physical activities.

Playback Stimuli

We generated playback stimuli by isolating songs from recordings of birds in our study population. For neighbour male stimuli, we used recordings of male solo songs collected from the

closest neighbouring individual for each focal pair. No two males received playback from the same neighbouring bird. For focal female stimuli, we used recordings of female solo songs collected from the subject's breeding partner. We created stimuli using Audition software (v. 3.0; Adobe, San Jose, CA, U.S.A.). We selected one song with a high signal-to-noise ratio (assessed visually from spectrograms), filtered out background noise from recordings with a 800 Hz high-pass filter, and standardized the song amplitude to -1 dB. We played songs in both treatments at a rate of one song every 10 s, consistent with natural song rates of this species (Mennill & Vehrencamp, 2005).

Playback Response Measurements

We used Syrinx PC (J. Burt, Seattle, WA, U.S.A.) to visualize audio recordings created during playback trials. We annotated these recordings to produce a time-stamped record of all duets, independent songs and physical behaviours of subject birds. As in previous studies of rufous-and-white wrens, we defined a male-created duet as an instance where a male sang ≤ 1.0 s after the end of his female's song (either a playback song or a song from his actual partner; Mennill & Vehrencamp, 2005). We defined an independent song as either a solo song (i.e. a song that was not preceded or followed by a partner's song by at least 1.0 s) or a song that was sung as the first component of a duet (i.e. a song where the female breeding partner responded, creating a duet). From our annotations, we extracted three measurements of the subject's vocal behaviour, and four measurements of the subject's physical behaviour, during the duetting stage: (1) number of male-created duets in response to partner songs broadcast from the loudspeaker, (2) number of male-created duets in response to actual partner songs, (3) number of independent songs (i.e. solos plus female-created duets), (4) distance of closest approach to the female loudspeaker (m), (5) latency to approach within 10 m of the female loudspeaker (s), (6) time spent within 10 m of the female loudspeaker (s) and (7) number of flights (i.e. the number of times that a bird flew from one perch to another). From duetting variables (1) and (2), we created a single duet responsiveness variable, calculated as the total number of duets created with playback, divided by the total number of female songs the subject had an opportunity to perform a duet with (i.e. 30 songs from playback plus any additional independent songs uttered by the subject's partner). For birds that did not respond to playback, we assigned a distance of closest approach of 50 m (we expect we would have detected any bird within this range) and a latency to approach within 10 m of 600 s (i.e. the total length of the duetting stage). We generated interobserver reliability estimates for a subset of these variables by having a second independent observer analyse a subset of 10 trials and quantify male duet responsiveness and number of male songs. We found very high repeatability both for duet responsiveness ($r^2 = 0.9998$, $P < 0.0001$; a single song across 400 that was classified as a duet by one observer and as a solo by the other observer) and number of male songs ($r^2 = 1.0$, $P < 0.00001$; i.e. identical scores between the two observers).

Data Analysis

We used duet responsiveness and number of male independent songs as our primary acoustic response measurements. To summarize variation in the four physical response measurements (i.e. measurements 4–7, above) we conducted a principal component analysis in JMP (v.12.0; SAS Institute, Cary, NC, U.S.A.; McGregor, 1992). This analysis yielded one principal component (PC1) with an eigenvalue ≥ 1 (2.61), and this component explained 65.2% of the variation in the four original measurements. PC1 scores were positively associated with time spent within 10 m of the female

loudspeaker and number of flights, and negatively associated with distance of closest approach and latency to approach within 10 m of the female loudspeaker. Thus, PC1 provided an indication of the physical response of the male, where higher scores indicated a higher intensity of physical response towards the female loudspeaker, consistent with physical mate guarding.

To test for differences in our key male vocal and physical behaviour variables across female fertility status, we used linear mixed-effects models (LMM) in the lme4 package (Bates, Mächler, Bolker, & Walker, 2015) in R (v.3.2.3, R Core Team, 2016). We created three separate models, with duet responsiveness, number of independent songs and physical response score (PC1) as the three response variables. In each model, we included breeding stage as a categorical predictor (two levels: fertile and incubation) and subject male identity as a random effect. To account for the influence of any habituation effects, we included in the model the time (in days) between playback trials (hereafter referred to as 'playback interval') as a continuous predictor; if habituation influenced playback responses, we expected to see decreased responses at shorter playback intervals.

The behaviour of females during the playback sessions might influence the behaviour of the males (i.e. our playback subjects). To account for the influence of the subject male's breeding partner on his behaviour, we included a 'female response' score in our models as a continuous predictor. To create this variable, we conducted a principal component analysis with female distance of closest approach, latency to approach within 10 m of the speaker, number of independent songs and duet responsiveness as the response variables (see Table A1 for a full summary of female responses during playback). This analysis yielded one principal component (PC1) with an eigenvalue ≥ 1 (3.01), and this component explained 75.4% of the variation in the four original measurements. PC1 scores were positively associated with the female's distance of closest approach and latency to approach within 10 m of the female loudspeaker and negatively associated with number of independent songs and duet responsiveness. Thus, PC1 provided an indication of the overall response of the females, where higher scores indicated a higher intensity response towards the female loudspeaker. We included the interaction between breeding stage and female response score in our initial models, and removed it from our final models when it was not significant. All values are presented as means \pm SD.

Ethical Note

All birds in this study were handled by an experienced bander with the required permits from the Animal Care Committee at the University of Windsor (Permit number: AUPP-13-15) and the Ministerio de Ambiente y Energía (MINAE) in Costa Rica (Permit number: ACG-PI-PC-027).

RESULTS

We conducted a total of 42 playback experiments to the 21 male rufous-and-white wren playback subjects, each receiving playback at two breeding stages (i.e. fertile and nonfertile). Males responded to female playback during the duetting stage in all trials by singing at least one song and approaching to within at least 20 m of the female loudspeaker. Females responded to playback in 29 out of 42 trials (69%); of the 13 trials in which females did not respond, two were in the fertile period and 11 were in the nonfertile period.

Male Vocal Responses to Playback

The duetting behaviour of male rufous-and-white wrens during the duetting stage of playback was influenced by breeding stage.

Males answered a significantly higher proportion of female songs to create duets during the fertile period compared to the nonfertile period (fertile period: $44.0 \pm 22.9\%$ of female songs answered; nonfertile period: $33.0 \pm 22.7\%$; LMM: $t = 2.35$, $N = 21$, $P = 0.025$; Fig. 2). These duets included both responses to female songs simulated by playback (average number of male-created duets in response to playback female songs during the fertile stage: 11.4 ± 7.7 ; nonfertile stage: 8.9 ± 6.8) and responses to actual songs from the female partner (average number of male-created duets in response to female songs during the fertile stage: 4.7 ± 7.8 ; nonfertile stage: 2.4 ± 4.3). Male duet responsiveness was not influenced by playback interval (LMM: $t = 0.30$, $N = 21$, $P = 0.77$), or strength of female response (LMM: $t = 0.60$, $N = 21$, $P = 0.56$). Similarly, male singing behaviour was not influenced by breeding stage: males sang independent songs at similar rates during the duetting stage in the fertile and nonfertile periods (fertile period: 30.6 ± 2.4 songs; nonfertile period: 27.5 ± 2.7 songs; LMM: $t = 0.63$, $N = 21$, $P = 0.54$; Fig. 3). Male independent song rates were not influenced by playback interval (LMM: $t = 0.66$, $N = 21$, $P = 0.52$) or strength of female response (LMM: $t = 0.67$, $N = 21$, $P = 0.51$). Refer to Table A2 for a full summary of linear mixed model outputs.

Male Physical Responses to Playback

The physical behaviour of male rufous-and-white wrens during the duetting stage of playback was influenced by breeding stage. Male physical response intensity scores (principal component scores summarizing four physical response measurements) were significantly higher during the fertile period compared to the nonfertile period, indicating that males responded with greater physical intensity towards the loudspeaker when their females were fertile (LMM: $t = 4.17$, $N = 21$, $P = 0.0003$; Fig. 4). There was a

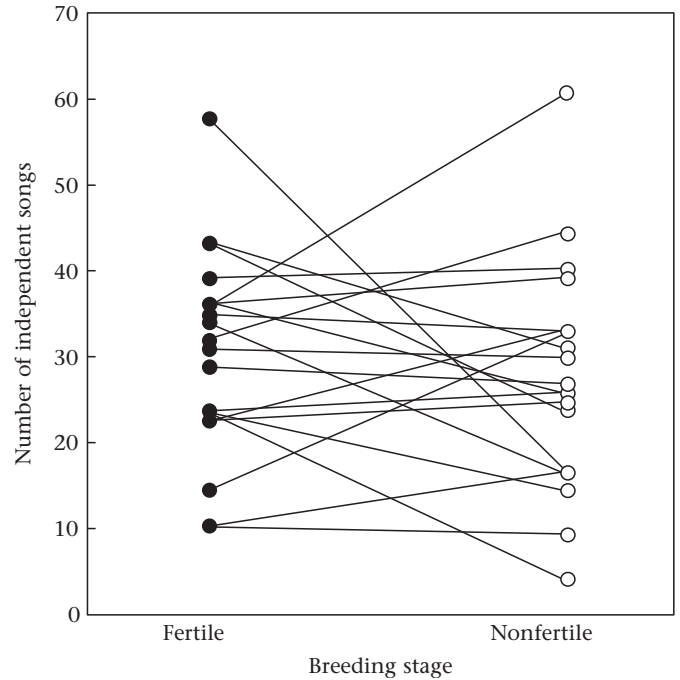


Figure 3. Number of independent songs sung by male rufous-and-white wrens (i.e. solos plus duets where the male sang the first duet component) during the fertile period (black circles) and the nonfertile incubation period (white circles). Graph shows paired comparisons of the number of independent songs by individual males between the fertile and nonfertile breeding stages.

significant interaction between breeding stage and female response score (LMM: $t = 2.51$, $N = 21$, $P = 0.02$): female response scores were more positively correlated with male physical response scores during the fertile period compared to incubation. Overall, with this interaction controlled for, there was no relationship between male

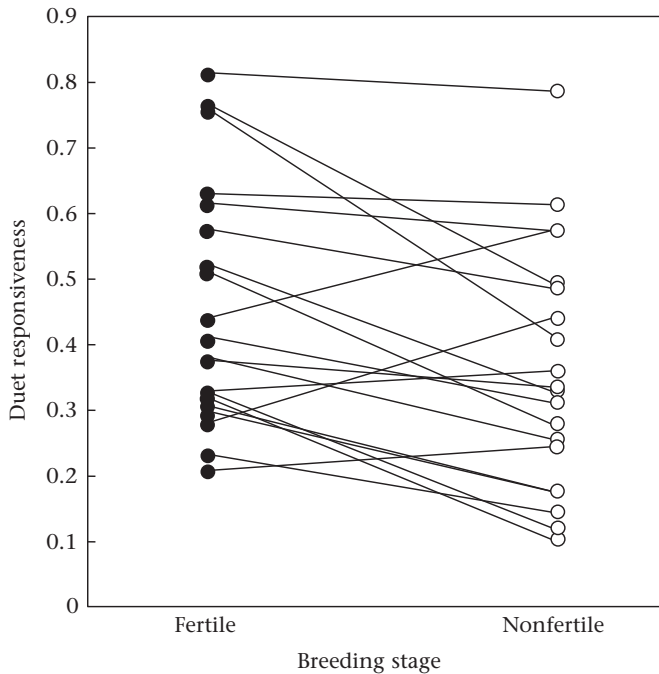


Figure 2. Proportion of female songs, including playback songs and actual songs, to which male rufous-and-white wrens responded by creating a duet during their mate's fertile period (black circles) and the nonfertile incubation period (white circles). Graph shows paired comparisons of the duet responsiveness of individual males between the fertile and nonfertile breeding stages.

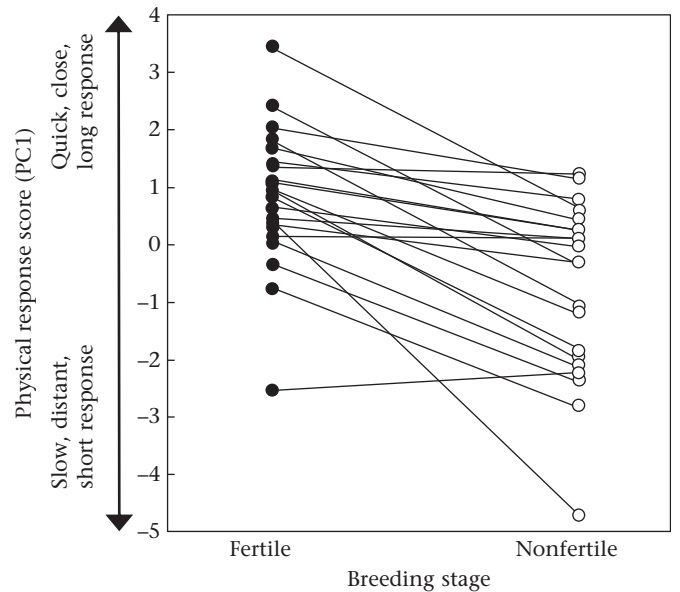


Figure 4. Physical response scores of male rufous-and-white wrens in response to a loudspeaker simulating female songs during the fertile period (black circles) and the nonfertile incubation period (white circles). Graph shows paired comparisons of the physical intensity response (a principal component score summarizing distance of closest approach, latency to respond and duration of response) of individual males between the fertile and nonfertile breeding stages.

physical response score and playback interval (LMM: $t = 0.41$, $N = 21$, $P = 0.68$) or female response score (LMM: $t = 0.87$, $N = 21$, $P = 0.39$). Refer to [Table A2](#) for a full summary of linear mixed model outputs.

DISCUSSION

The playback responses of male rufous-and-white wrens support the paternity guarding hypothesis. Following playback that simulated an intrusion from a neighbouring male, males answered more female songs to create duets when their females were fertile, suggesting that duets play a role in paternity guarding in this species. Males exhibited more intense physical responses towards the loudspeaker simulating their breeding partners during the fertile period compared to the nonfertile period, suggesting that males also use physical mate-guarding behaviours to protect their paternity. Males did not sing independent songs at a higher rate during the fertile period compared to the nonfertile period, revealing that the increased duet responsiveness of males was not simply a by-product of increased song output. Our study provides experimental support for the idea that duets function as acoustic paternity guards, and our results suggest that male rufous-and-white wrens use both duets and physical behaviours to guard their breeding partners during the fertile period.

Acoustic Paternity Guarding

Although cooperative territory defence is widely considered to be a primary function of acoustic duets across many animals, including rufous-and-white wrens ([Mennill, 2006](#); [Mennill & Vehrencamp, 2008](#)), recent evidence suggests that duets are multipurpose signals that can also serve conflict-based functions between breeding partners ([Baldassarre, Greig, & Webster, 2016](#); [Dahlin & Benedict, 2014](#); [Levin, 1996a, 1996b](#); [Rogers et al., 2007](#); [Seddon & Tobias, 2006](#); [Tobias & Seddon, 2009](#)). Few studies have tested the hypothesis that duets are used to acoustically guard male paternity (e.g. [Baldassarre et al., 2016](#); [Marshall-Ball et al., 2006](#)), and consequently there is little evidence that duets function in this way. We found that male rufous-and-white wrens increased their duet responsiveness during the fertile period, suggesting that males used duets to acoustically guard their mates. The increase we observed in duet responsiveness during the fertile stage was moderate (i.e. 11% higher duet responsiveness between fertile and incubation stages), and similar to differences in duet rates observed during natural contexts in this species (during the fertile stage: 52% male duet responsiveness; during the incubation stage: 35% male duet responsiveness; [Topp & Mennill, 2008](#)). Nevertheless, given the higher duet responsiveness during the fertile period, our results provide experimental evidence that duets may function in acoustic paternity guarding, and add to a growing body of literature showing that duets can serve both cooperative and conflict-based functions depending on ecological context ([Dahlin & Benedict, 2014](#); [Mennill & Vehrencamp, 2008](#)).

In four other observational studies of other duetting birds, males did not increase their duet responsiveness during the female fertile period ([Dowling & Webster, 2013](#); [Gill et al., 2005](#); [Hall & Magrath, 2000](#); [Hall & Peters, 2009](#)). There are several possible explanations for why rufous-and-white wrens exhibit different acoustic paternity guarding behaviours from other duetting species. First, birds in our study population live in dense forests with substantial visual obstruction from vegetation ([Mennill & Vehrencamp, 2005, 2008](#)). In a dense environment, males may rely more heavily on acoustic signals; duet responses may be especially useful for advertising the paired status of a bird's partner to intruders during the fertile period if it is difficult to visually track mates ([Logue, 2007](#); [Mennill](#)

& [Vehrencamp, 2008](#)). Second, rufous-and-white wrens exhibit few of the characteristics associated with low levels of extrapair offspring seen in other duetting species studied to date, such as low breeding synchrony (e.g. [Morton, Stutchbury, Howlett, & Piper, 1998](#); [Stutchbury & Morton, 1995](#)), low partnership divorce rates (e.g. [Chu, Koenig, Godinez, McIntosh, & Fleischer, 2002](#)) and high degrees of paternal care ([Albrecht, Kreisinger, & Piálek, 2006](#); [Gowaty, 1996](#); [Morton et al., 1998](#)). Rufous-and-white wrens have highly synchronous breeding, at least at the beginning of the rainy season when we conducted our experiments ([Topp & Mennill, 2008](#)), they sometimes engage in divorce ([D. Mennill, personal observation](#)), and they exhibit lower male parental investment compared to other duetting species ([Gill & Stutchbury, 2005](#); [Hall, 1999](#); [Kahn, 2017](#); [Rogers & Mulder, 2004](#)). Despite this, it is important to recognize the limitations of our experiment to fully explain the influence of duetting behaviour on the low levels of extrapair paternity in rufous-and-white wrens, because it can be difficult to discern the effectiveness of paternity guarding strategies in species with very low levels of extrapair paternity ([Kokko & Morrell, 2005](#)).

Only two studies to date have tested the paternity guarding hypothesis by investigating how duetting behaviour affects male paternity. In crimson-breasted shrikes, *Laniarius atrococcineus*, males that sang more duets with their females did not experience lower rates of extrapair offspring in their nests, suggesting that duets were not effective paternity guards in this species ([Van den Heuvel, Cherry, & Klump, 2014](#)). In contrast, male red-backed fairy-wrens that exhibited stronger duetting responses during simulated intrusions had fewer extrapair offspring in their nests, indicating that duets may have been effective at limiting extrapair mating attempts from rival males ([Baldassarre et al., 2016](#)). Rufous-and-white wrens present an intriguing comparison to these species because they exhibit much lower levels of extrapair paternity, and future studies directly assessing how duetting behaviour affects rates of extrapair paternity in rufous-and-white wrens and across a wide range of duetting species are needed to elucidate the broad evolutionary pressures acting on these behaviours ([Macedo, Karubian, & Webster, 2008](#)).

We presented males rufous-and-white wrens with playback first during the fertile stage, and second during the nonfertile stage, in all but two cases in our experiment (19 of 21 playback subjects). Therefore, we must consider order effects and the possibility that lower levels of duet responsiveness during the nonfertile stage arose due to habituation during the second instance of playback. We cannot discount this explanation but we consider it unlikely. First, the stimuli we broadcast during playback (the subject's partner's song) would be familiar to the male and be heard on a daily basis, making habituation unlikely. Second, the time delay between playbacks was not a significant predictor of male response behaviour in any of our models, as would be expected if habituation was occurring. Third, anecdotally, the two cases where the playback was given first during the nonfertile stage showed the same pattern as the other 19 cases, with higher duet responsiveness during the fertile stage. Finally, we did not find habituation effects to multiple episodes of playback in two other playback studies in this species, suggesting that it would be unlikely to occur in this study with only two instances of playback per subject male ([Hick, Doucet, & Mennill, 2015](#); [Mennill & Vehrencamp, 2008](#)).

Physical Paternity Guarding

Male rufous-and-white wrens exhibited more intense physical responses towards the female loudspeaker during the fertile period compared to the nonfertile period. This suggests that males may have attempted to physically guard their females during the fertile

period. Many other animals have been shown to physically guard their partners during the female receptive period (e.g. Chuang-Dobbs, Webster, & Holmes, 2001; Currie, Krupa, Burke, & Thompson, 1999; Foote, Fitzsimmons, Mennill, & Ratcliffe, 2008; Komdeur, Kraaijeveld-Smit, Kraaijeveld, & Edelaar, 1999; Schubert, 2009; Setchell et al., 2005; Westneat, 1994), including several other duetting species (e.g. Dowling & Webster, 2017; Hall & Magrath, 2000; Hall & Peters, 2009). For example, duetting magpie-larks and buff-breasted wrens exhibit pronounced physical mate guarding strategies during the fertile period and, in both cases, exhibit very low levels of extrapair paternity (Gill et al., 2005; Hall & Magrath, 2000). This indicates that physical mate guarding may be a widespread strategy across duetting species, reflecting the fact that many of these species form stable long-term pair bonds and close physical associations throughout the year (Logue, 2007; Mennill & Vehrencamp, 2008; Tobias et al., 2016). The strong physical mate-guarding behaviour displayed by males in our experiment suggests that low rates of extrapair paternity in our population could be influenced by these physical behaviours in addition to acoustic duets.

Due to the logistical difficulties in removing females from territories, we were unable to control for effects that might have been induced by the presence of the subjects' breeding partners. We do not believe that our experiment was confounded by this for three reasons. First, female response score was not a significant predictor of any of our male response variables, including the important duet responsiveness variable. This suggests that males continued to treat the female speaker as their breeding partner even when their breeding partners responded to playback. It is worth noting that, during our previous studies of this species, we have found no evidence that males create duets with birds other than their social partner (Hick et al., 2015; Kovach et al., 2014; Mennill, 2006; Mennill & Vehrencamp, 2005, 2008), further supporting the idea that they considered the playback to be their breeding partner's voice. Second, similar playback protocols have been used successfully in three other species of duetting wrens, including two studies of black-bellied wrens, *Pheugopedius fasciatoventris*, in which females were not removed from territories (Logue, 2007, 2008; Rivera-Cáceras et al., 2016; Templeton et al., 2013), suggesting that the playback technique of simulating females works effectively in neotropical duetting wrens, even when females are present on the territory. Third, under the alternative territory defense hypothesis (i.e. that males perceived the female loudspeaker as an intruder female, rather than their breeding partner), we would not expect a difference in male aggression between fertile and incubation breeding stages, due to the fact that both stages occur during the wet season when motivation for defending ecological resources should be similar (Ahumada, 2001; Fedy & Stutchbury, 2005; Hall, 2004). Therefore, although previous studies in this species have shown that territory defence is an important function of duets (e.g. Mennill, 2006; Mennill & Vehrencamp, 2008), our results appear to be driven by intersexual conflict during the fertile period.

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Appendix

Table A1

Summary of female rufous-and-white wren responses during the duetting stage of playback

Response variable	Fertile period	Nonfertile period
Distance of closest approach (m)	15.8±15.4	32.5±19.5
Latency to approach within 10 m of speaker (s)	312.3±245.4	506.1±201.9
Time spent within 10 m of speaker (s)	191.3±170.7	31.4±71.7
Number of independent songs	6.8±9.8	3.3±5.4
Number of duets created with male	7.3±9.5	3.5±5.0
Duet responsiveness (%)	22.6±27.0	11.5±16.5

Values are means ± SD.

Table A2

Results of linear mixed models summarizing male rufous-and-white wren responses ($N = 21$) during the duetting stage of playback

	Estimate±SE	<i>t</i>	<i>P</i>
Duet responsiveness			
Breeding stage	0.10±0.04	2.35	0.025
Playback interval	0.01±0.01	0.30	0.77
Female response	0.01±0.02	0.60	0.56
Independent songs			
Breeding stage	2.16±3.40	0.63	0.54
Playback interval	0.30±0.19	0.66	0.52
Female response	0.54±1.12	0.67	0.51
Physical response			
Breeding stage	1.20±0.29	4.17	0.0003
Playback interval	0.02±0.05	0.41	0.68
Female response	0.11±0.13	0.87	0.39
Female response * breeding stage	0.44±0.17	2.51	0.02