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Experimental manipulation of mixed-species flocks reveals heterospecific audience effects on calling

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Animal signallers are subject to audience effects when they alter communication due to changes in the presence or characteristics of receivers. Studies aimed at understanding audience effects have typically examined effects of conspecific audiences on signaller communication. Less work has focused on heterospecific audiences, which present an important avenue of research for species that participate in mixed-species groups. Here we experimentally tested mixed-species flocks of Carolina chickadees, *Poecile carolinensis*, and tufted titmice, *Baeolophus bicolor*, for conspecific and heterospecific audience effects. Birds were trapped from naturally occurring flocks and held in seminatural outdoor aviaries, where we recorded calling. We found that chickadees and titmice were sensitive to the number of conspecifics in flocks when communicating via ‘chick-a-dee’ calls, which are social cohesion calls produced by both species. Chickadees also were sensitive to the number of titmice in flocks, but chick-a-dee calling behaviour in titmice did not differ with regard to the number of chickadees in flocks. Furthermore, when subject to playbacks of simulated risk, chickadees and titmice produced more chick-a-dee calls when more titmice were in a flock. After these playbacks, chickadees produced fewer chick-a-dee calls with increasing numbers of conspecifics in flocks, whereas titmice produced more chick-a-dee calls with increasing numbers of conspecifics in flocks. These results suggest that chickadees and titmice are sensitive to social factors within their mixed-species flocks when communicating, and that chickadees appear more sensitive to heterospecific presence than do titmice. We suggest this is due to the dominance status of the species in these flocks, where titmice are typically dominant over chickadees.

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An ‘audience effect’ occurs when animal signallers alter properties of their communication in response to differences in the presence, characteristics or composition of receivers (Coppinger et al., 2017; Karakashian, Gyger, & Marler, 1988; Zuberbühler, 2008). For example, male house mice, *Mus musculus*, call more in response to female odour if other males are in the area, and males also change the acoustic structure and syllable complexity of their vocalizations (Seagraves, Arthur, & Egnor, 2016). The size of the surrounding audience can also affect communication. For example, green woodhoopoe, *Phoeniculus purpureus*, give longer rally displays when in larger groups (Radford, 2003). Understanding audience effects is important for two reasons. First, audience effects suggest that signallers have volitional control over signal production (Townsend et al., 2017). Second, in some instances, audience effects suggest that some signallers are aware of, and take into account, the perceptual states of receivers when communicating (Coppinger et al., 2017). However, fewer studies focus on the effect of audience composition per se on signaller behaviour.

Audience composition is particularly important in social systems such as mixed-species groups where audiences can vary greatly not only in size, sex and age structure, but also in the distribution of species in the group (Goodale, Beauchamp, & Ruxton, 2017). Indeed, heterospecific communication can be a critical factor in the formation and maintenance of mixed-species groups (Goodale, Beauchamp, Magrath, Nieh, & Ruxton, 2010). Correlational evidence exists for mixed-species audience effects in flocks

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of birds. For example, willow tits, *Poecile montanus*, produce fewer long calls (a call used in flock recruitment) when heterospecific flockmates are in the area (Suzuki, 2012). Forked-tailed drongos, *Dicrurus adsimilis*, also vary calls relative to the heterospecifics present in their audience and produce more terrestrial alarm calls when foraging with pied babblers, *Turdoides bicolor*, compared to when they are foraging alone (Ridley, Child, & Bell, 2007). By investigating how the presence of one species influences the vocal production of another in mixed-species groups, we can better understand the structure and function of those groups and whether heterospecifics may sometimes be the target of signalling or are just unintended eavesdroppers.

The above examples focus on the presence or absence of heterospecifics in the audience, but researchers did not manipulate heterospecific presence or composition of mixed-species groups. Experimental approaches are needed to assess the causal role of audience composition of mixed-species groups on signaller behaviour. Mixed-species flocks of Carolina chickadees, *Poecile carolinensis*, and tufted titmice, *Baeolophus bicolor*, represent a powerful opportunity for experimental studies of mixed-species group audience effects for several reasons. First, for several months over winter, these birds naturally reside in mixed-species groups in which they are the core members (Morse, 1970). These mixed-species flocks in the northern ranges often include satellite species, such as nuthatches and woodpeckers. Diversity of flock participants increases to the south, where up to 10 or more species may participate, including kinglets, creepers, warblers and vireos (Contreras & Sieving, 2011; Dolby & Grubb, 1998; Farley, Sieving, & Contreras, 2008; Morse, 1970). In these flocks, titmice are dominant over chickadees (Waite & Grubb, 1988), and the presence of titmice has negative foraging consequences for chickadees (Cimpich & Grubb, 1994). However, both chickadees and titmice were more likely to solve a novel feeder problem to gain food resources when they were in flocks that contained a greater diversity of heterospecifics (titmice and white-breasted nuthatches, *Sitta carolinensis*, for chickadees; chickadees and nuthatches for titmice), when compared to flocks with a lower diversity of species, like monospecific flocks (Freeberg, Eppert, Sieving, & Lucas, 2017). Additionally, as is the case with many mixed-species parid flocks, there is considerable variation in composition of chickadee and titmouse flocks across the geographical ranges of the species (B. A. Coppinger & T. M. Freeberg, personal observations; see also Ekman, 1989).

Second, both species use a complex, open-ending calling system (the chick-a-dee call) that functions in social recruitment and cohesion (Mahurin & Freeberg, 2008; Randler, 2012). The chick-a-dee call is made up of several distinct note types, with note composition of calls affected by different contexts (Krams, Krama, Freeberg, Kullberg, & Lucas, 2012). Chick-a-dee calls of Carolina chickadees can reflect risk of a predator (Nolen & Lucas, 2009; Soard & Ritchison, 2009), food availability (Mahurin & Freeberg, 2008) and whether a caller is flying (Freeberg & Mahurin, 2013), by changing the ratio of ‘D’ and ‘C’ notes in their calls. Chickadees, tits, titmice and associated species that occur in mixed-species flocks with one another attend to variation in one another's calls (Randler & Vollmer, 2013; Templeton & Greene, 2007). Previous work on this calling system demonstrated that chickadee signalling behaviour is influenced by the surrounding audience. For example, Carolina chickadees produce more complex calls when in larger conspecific groups (Freeberg, 2006). Chickadees also call sooner in response to threats and produce different note compositions of calls when in flocks of familiar conspecifics as compared to flocks of unfamiliar conspecifics (Coppinger, Davis, & Freeberg, 2019; Coppinger, Sanchez de Launay, & Freeberg, 2018). Even though there is evidence that chickadees are sensitive to group size and composition of individuals in chickadee-only flocks, we still do not know what types of audience effects are present in mixed-species flocks containing these birds. However, there is correlational evidence that chickadees and titmice vary their calling behaviour in the presence of heterospecifics when engaged in mobbing a predator (Nolen & Lucas, 2009).

In this study, we examined two main research questions. First, we sought to determine what types of mixed-species flock structure influence calling behaviour in chickadees and titmice. Specifically, we tested for conspecific and heterospecific audience effects in mixed-species flocks. Second, we sought to determine whether the audience effects present in flocks would change after flocks were exposed to simulated risk. We simulated risk by presenting flocks with various auditory stimuli that varied in potential risk. We tested a number of hypotheses to explain signalling in relation to variation in size and composition of groups (summarized in Table 1 and outlined below).

Hypothesis 1: Social Facilitation

Calling behaviour may be enhanced by the presence of both conspecifics and heterospecifics in mixed-species flocks. Research in human social psychology demonstrated that communicative behaviour of individuals is enhanced when in the presence of familiar individuals (Buck, Losow, Murphy, & Costanzo, 1992). Similarly, male domestic chickens, *Gallus gallus domesticus*, call at higher rates in a food context when females are in the immediate environment compared to when alone (Evans & Marler, 1994). In the current experiment, if birds are subject to social facilitation, we would expect birds to call more in the presence of greater numbers of conspecifics and heterospecifics. Increased calling in the context of more conspecifics or more heterospecifics, or both, might be expected if signallers benefit by providing more information or by better managing the behaviour of others through signalling in those larger groups (e.g. pant-hoots and gestures in chimpanzees, *Pan troglodytes*: Roberts & Roberts, 2016; whinny vocalizations in female spider monkeys, *Ateles geoffroyi*: Dubreuil, Notman, & Pavelka, 2015).

Hypothesis 2: Social Inhibition

Counter to social facilitation is social inhibition or social loafing where the presence of others suppresses behaviour. Work in social psychology demonstrated that individuals decreased the number of body movements and paralinguistic vocalizations in the presence of another individual, and that this effect disappeared when observers could not watch the subject (Guerin, 1989). Male chickens display social inhibition effects by reduced calling when rival males are in the immediate environment (Marler, Dufty, & Pickert, 1986). Here, we would expect that, if individuals are subject to social inhibition, they should call less with increasing numbers of conspecifics and heterospecifics present. Decreased calling in the context of more conspecifics or more heterospecifics, or both, might be expected if signallers suffer increased rates of aggression in response to signalling in those larger groups (e.g. pant-grunts by female chimpanzees with larger numbers of males nearby; Laporte & Zuberbühler, 2010).

Hypothesis 3: Oddity Effect

The oddity effect predicts that individuals that are conspicuous or unusual looking in a group may be preferentially attacked by predators (Krause & Ruxton, 2002; Landeau & Terborgh, 1986). Conspicuous individuals in heterospecific groups could be those of the minority species, since they may stand out more compared to individuals of the majority species. If individuals in mixed-species

Table 1
Hypotheses tested with experimental flocks varying in size and species composition

Hypothesis	Prestimulus predictions	Poststimulus predictions
Social facilitation	*Conspecific:* Birds call more with more conspecifics present in flocks *Heterospecific*: Birds call more with more heterospecifics present in flocks	*Conspecific*: Birds call more with more conspecifics present in flocks *Heterospecific*: Birds call more with more heterospecifics present in flocks
Social inhibition	*Conspecific*: Birds call less with more conspecifics present in flocks *Heterospecific*: Birds call less with more heterospecifics present in flocks	*Conspecific*: Birds call less with more conspecifics present in flocks *Heterospecific*: Birds call less with more heterospecifics present in flocks
Oddity effect	If species X is proportionally rare in a mixed-species flock, birds of that species will call less than if species X is proportionally common in mixed-species flocks	The effect of calling less when proportionally rare is stronger in contexts of potential risk
Dilution effect	Minimal effect of conspecific or heterospecific presence on ambient calling rates	Birds in larger flocks call more than birds in smaller flocks; birds in heterospecific flocks call more than birds in same-sized monospecific flocks

For more detailed discussion of each hypothesis, see text.

flocks of chickadees and titmice behave according to the oddity effect, conspicuous individuals should behave in a way that draws less attention to themselves in the flock. Therefore, we predicted that chickadees would call less as they became proportionally rarer in flocks containing titmice, that titmice would also call less as they became proportionally rarer in flocks containing chickadees, and that this effect would be most evident in calling after simulated risk.

Hypothesis 4: Dilution Effect

Mixed-species flocks of birds may be subject to a dilution effect. In the dilution effect, the probability of an individual being attacked by a predator declines as the size of the group increases (Krause & Ruxton, 2002; Foster & Treherne, 1981). The dilution effect, in addition to the many-eyes effect, explains why individual vigilance behaviour decreases as group size increases (Roberts, 1996). Under this hypothesis, individuals should call more with increasing numbers of chickadees and titmice, specifically in conditions of

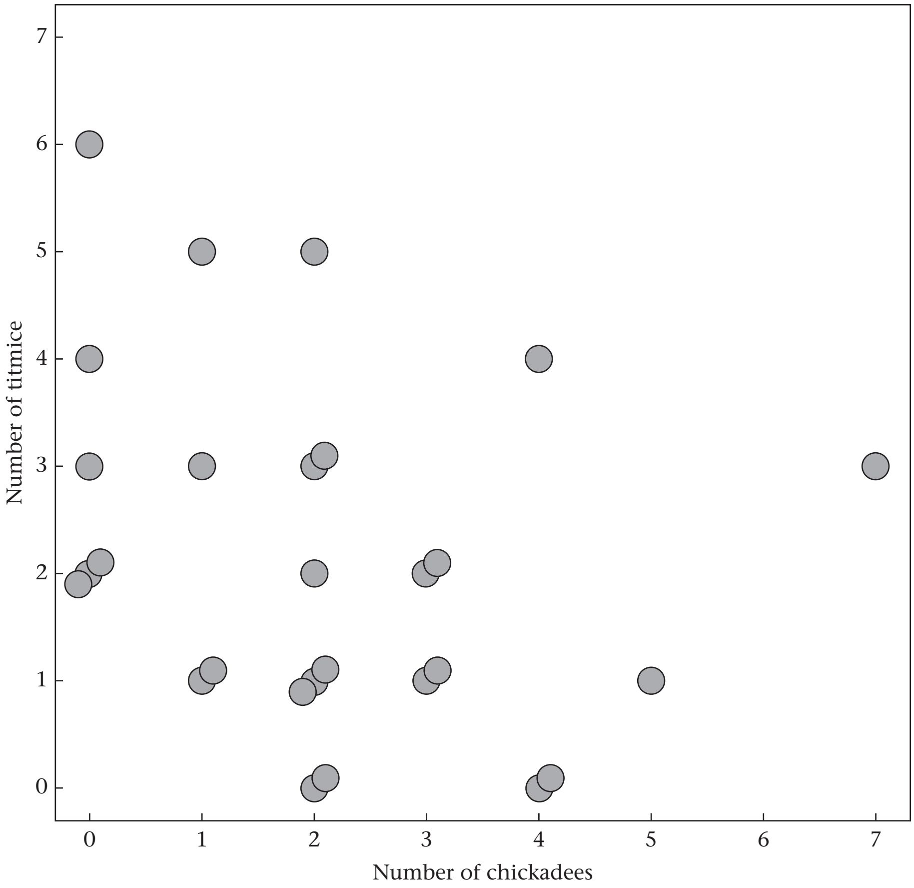

Figure 1. Variation in species composition of experimental flocks.

increased risk, under the assumption that calling in risky contexts increases the caller's likelihood of predation (e.g. increased predation in crested tits, *Parus cristatus*: Krams, 2001; increased nest predation in pied flycatchers, *Ficedula hypoleuca*: Krams, Krama, Igaune, & Mand, 2007). Additionally, mobbing intensity of both chickadees and titmice increases with presence of heterospecifics in flocks (Nolen & Lucas, 2009). For this reason, we predicted to see strong heterospecific audience effects.

METHODS

Subject Collection

Subjects were trapped and individually colour-banded at various sites around the University of Tennessee Forest Resources, Research, and Education Center (UTFRREC), Oak Ridge, Tennessee, U.S.A. during October 2016 – March 2017 and September 2017 – February 2018. Sites contained treadle (Potter) traps mounted on feeding stations baited with black oil sunflower and safflower seeds. All sites were at least 400 m apart to ensure each site sampled from a unique flock of chickadees and titmice (Bartmess-LeVasseur, Branch, Browning, Owens, & Freeberg, 2010). Birds were captured from each site within a 2 h period to help further ensure that we captured birds from the same natural flocks. We trapped and banded 58 chickadees (31 in year 1, 27 in year 2) and 59 titmice (44 in year 1, 15 in year 2) over our 2-year study. Together, these formed 28 different flocks (17 in year 1, 11 in year 2) that spanned a wide range of sizes and species compositions (Fig. 1), likely representing a random subset of wild flocks.

We took blood samples from each bird for sex identification. Blood samples were refrigerated and transported to the University of Tennessee College of Veterinary Medicine. Molecular sex was determined using PCR amplification of chromo-helicase DNA-binding (CHD) genes (Griffiths, Dann, & Dijkstra, 1996) with modifications (Boutette, Ramsay, Potgieter, & Kania, 2002; see Appendix).

Housing

Birds were transported from their site of capture to seminaturalistic outdoor aviaries at the UTFRREC. Experimental flocks contained birds trapped from the same natural flock, as previous research has demonstrated flocks of unfamiliar birds communicate differently than flocks of familiar birds (Coppinger et al., 2018, 2019). There were six aviaries that each measured 6 × 9 m and 3.5 m high. Each aviary had a covered portion and an indoor section so that birds could escape inclement weather. Birds had access to fresh water and ad libitum black oil sunflower and safflower seed and were provided with mealworms and waxworms roughly every other day.

Birds were given at least 7 days to acclimate to the aviaries and to the observer present in the aviaries (see below). During the acclimation period, the observer sat in a designated observation corner and spoke aloud for 5–10 min, allowing birds to acclimate to the experimental procedure. Birds were generally housed in the aviaries for 4 weeks, although this housing period was slightly longer if weather conditions delayed data collection. At the end of the experiment, birds were captured in treadle traps in their aviaries and released at their original site of capture.

Experimental Protocol

We conducted experimental trials between 0830 and 1500 hours (Eastern Standard Time, EST). On trial days, a single observer entered the aviary, set up the audio recording equipment and sat in a designated corner to observe the birds. We connected a Sennheiser ME-64 electret microphone, located on a microphone stand in the middle of each aviary for all recordings, to a Marantz PMD660 Professional Solid State Recorder. Sample rate of the recordings was 44.1 kHz with 16-bit resolution.

Each flock experienced six separate trials, with no more than one trial per day. Each trial included a playback of one of six different auditory stimuli: (1) screech owl, *Megascops asio*, calls; (2) screech owl calls with overlapping Carolina chickadee and tufted titmice mobbing calls; (3) Tennessee Carolina chickadee and tufted titmouse D-note calls; (4) Florida Carolina chickadee and tufted titmouse D-note calls; (5) Tennessee Carolina chickadee gargle calls; (6) Tennessee Carolina chickadee alarm (Z-note) calls. Screech owls are natural predators of chickadees and titmice, who react strongly to auditory and visual stimuli of the owls (Nolen & Lucas, 2009), so stimulus 1 represented high risk. Chickadees and titmice produce mobbing calls when a perched but dangerous predator is detected (Hetrick & Sieving, 2011; Krams et al., 2012), so stimulus 2 also represented high risk. Mobbing calls in these species comprise mostly D notes (Bartmess-LeVasseur et al., 2010; Courter & Ritchison, 2010; Nolen & Lucas, 2009; Soard & Ritchison, 2009), so playback stimuli 3 and 4 likely indicated elevated risk: mobbing but with no indication of a predator. Chickadee gargle calls are often used in conspecific contexts of aggression (Ficken, Ficken, & Witkin, 1978) so stimulus 5 likely represented a moderate level of nonpredatory risk. Chickadee Z-note calls are used in contexts of high alarm, as when an avian predator flies through the area where a flock is foraging (Zachau & Freeberg, 2012), so stimulus 6 likely represented highest risk (Hetrick & Sieving, 2011; Jones & Sieving, 2019). We presented one stimulus playback per trial; flocks received all six stimuli (drawn in random order) but only one trial per 24 h. For each stimulus/trial, we drew one of two examples of each stimulus recording randomly (to minimize pseudoreplication).

We defined three trial phases: prestimulus; stimulus; poststimulus. The prestimulus phase lasted 5 min. The stimulus phase lasted 1 min during playback broadcast. Finally, the poststimulus phase lasted 5 min. We analysed the production of chick-a-dee calls including two or more notes. We were primarily interested in rates of chick-a-dee call production as call rates are sensitive to predator- and threat-related contexts across a wide range of studies (Bartmess-LeVasseur et al., 2010; Courter & Ritchison, 2010; Nolen & Lucas, 2009; Soard & Ritchison, 2009; Templeton, Greene, & Davis, 2005). The observer did not count any gargles, high Z-notes (alarm calls), contact notes or songs produced by experimental birds. Calls were observed ad libitum during the three trial phases, meaning anytime a call was produced, the observer noted which colour-banded individual produced the call aloud on the audio file. Calls were marked as ‘unknown’ when they were produced by a bird who could not be individually identified. Calls produced from unknown callers were not included in the present analyses. We broadcast stimuli using an iHome speaker (model iBT33, SDI Technologies Inc., Rahway, NJ, U.S.A.) mounted 2.5 m high in the aviary, and set speaker playback output to ~75 dB SPL at 1 m from the speaker (based on Mahurin & Freeberg, 2008).

Analysis

B.A.C. coded all the data. T.M.F. independently coded a subset of the total set of sound files (11%) to assess inter-rater reliability. We used Spearman rank correlation to determine the agreement between two coders. The number of titmouse calls that each coder transcribed had high inter-rater reliability ($r_S = 0.998$, $N = 16$ titmice). The number of chickadee calls that each coder transcribed also had high inter-rater reliability ($r_S = 0.993$, $N = 21$ chickadees).

We performed two analyses on the data set to test for (1) general (prestimulus) audience effects and (2) audience effects as a response to sound-induced risk.

General audience effects

We ran two separate generalized linear mixed models (one for chickadees and one for titmice; Proc GLIMMIX in SAS 9.4, SAS Institute Inc., Cary, NC, U.S.A.) to identify any audience effects present in the prestimulus interval. We used a Poisson response distribution with a log link function since the number of calls represents relatively small count data. Bird identification (identified by individual bird bands) was used as a random factor to account for repeated measurements. Number of conspecifics, number of heterospecifics and sex of caller were treated as main effects. Number of conspecifics and number of heterospecifics were calculated relative to an individual bird in a flock, and this was done for all flock members. Sex of caller was based on results from the molecular sexing. We initially included all possible two-way and three-way interactions and squared terms for number of conspecifics and number of heterospecifics to test for nonlinear effects of these variables in the models. We removed nonsignificant higher-order interactions and squared terms from models from lowest to highest *F* statistic. After the removal process, the final chickadee model for calls made in the prestimulus phase included all main effects and no interactions or squared terms, since none were significant (all $P > 0.05$). The final titmouse model for calls made in the prestimulus phase also only included main effects; there were no significant interactions or squared terms (all $P > 0.05$). All analyses were run in SAS (v.9.4), and figures were made in SPSS (v.25.0, IBM Corp., Armonk, NY, U.S.A.), using means of log-transformed counts and 95% confidence intervals (CIs).

Audience effects on response to risk-related playback stimuli

Similar to the analyses done for general audience effects, we ran two separate generalized linear mixed models (Proc GLIMMIX in SAS v.9.4) to assess factors influencing the number of vocalizations given during the poststimulus phase (addressing our second research question). Again, we used a Poisson response distribution with a log link function and individual bird was used as a random factor to account for repeated measurements. Here, main effects included number of conspecifics, number of heterospecifics, sex of caller, stimulus type and the number of calls produced by the focal bird in the prestimulus phase (i.e. a metric for baseline calling rate). All possible two-way interactions and squared terms for number of conspecifics and number of heterospecifics were initially included in both chickadee and titmouse models. The number of prestimulus phase calls was log-transformed to normalize model residuals. We removed nonsignificant higher-order interactions and squared terms from models from lowest to highest *F* statistic. The final chickadee model included all main effects and the squared number of heterospecifics term; there were no significant interactions. The final titmouse model only included all main effects and the squared number of heterospecifics term, and an interaction between number of conspecifics and prestimulus call counts. All analyses were run in SAS (v.9.4), and figures were made in SPSS (v.25.0), using means of log-transformed counts and 95% CIs.

Ethical Note

We have adhered to animal care guidelines (as published by the Association for the Study of Animal Behaviour and the Animal Behavior Society) as well as the Institutional Animal Care and Use Committee of the University of Tennessee (Protocol No. 1326). Birds were humanely captured from wild populations with seed-bated treadle traps. To avoid undue stress, birds were released inside seminatural outdoor aviaries within 1 h after their capture and were held there for the duration of the experiment (roughly 4 weeks). In captivity, birds were provided with ad libitum food and fresh water daily. In addition, birds had access to an indoor enclosure to escape inclement weather, if desired. Birds were monitored daily even if experimental trials did not occur that day. Beyond noninvasive, observational experimental trials and daily maintenance of food reserves, birds were left alone to avoid undue stress. All birds were released at their original site of capture after the experiment ended.

RESULTS

General Audience Effects: Calls Made in the Prestimulus Phase

Chickadees

Chickadees produced fewer calls when more chickadees were in the flock, as compared to when fewer chickadees were in the flock ($F_{1,54} = 15.91$, $P = 0.0002$, model estimate ± SE = -0.471 ± 0.118; Fig. 2a). Chickadees also called less with increased number of titmice in the flock ($F_{1,54} = 5.89$, $P = 0.0186$; model estimate = -0.255 ± 0.105; Fig. 2b). The lack of a significant interaction between the number of conspecifics and the number of heterospecifics in a flock indicates that the influence of additional chickadees and titmice was additive, not relative to proportion of each species in a flock. Additionally, there was no effect of sex of caller on the number of calls that individual chickadees produced ($F_{1,54} = 0.01$, $P = 0.914$).

Titmice

Titmice produced fewer calls with increased numbers of titmice in a flock ($F_{1,55} = 15.64$, $P = 0.0002$; model estimate= -0.705 ± 0.178; Fig. 3a). In contrast to chickadees, which demonstrated a heterospecific audience effect, there was no significant effect of the number of chickadees in a flock on titmouse calling rates ($F_{1,55} = 1.50$, $P = 0.225$; model estimate = -0.160 ± 0.130; Fig. 3b). Like chickadees, the effects of conspecifics and heterospecifics on calling rates were additive; calling rates were not affected by the relative number of each species in a flock. Finally, there was no effect of sex of caller on the number of calls produced in titmice ($F_{1,55} = 1.75$, $P = 0.192$).

Audience Effects in Response to Risk-related Playback Stimuli

Chickadees

There was no effect of stimulus type on poststimulus calling rates in chickadees ($F_{1,333} = 0.46$, $P = 0.496$). However, chickadees that called more before stimulus presentations also called more after the stimulus presentations ($F_{1,333} = 67.03$, $P < 0.0001$; model estimate = 2.020 ± 0.247; Fig. 4). Similar to patterns present in the general call analysis, chickadees produced fewer calls after stimulus playbacks when flocks contained more (as opposed to fewer) chickadees ($F_{1,333} = 3.89$, $P = 0.0495$; model estimate = -0.144±0.073; Fig. 5a). However, chickadees produced more calls after the stimulus with a small number of titmice in a flock compared to when titmice were absent ($F_{1,333} = 5.83$, $P = 0.0163$; model estimate = 0.522 ± 0.216; Fig. 5b); this effect decreased as titmouse flock size increased, as indicated by a trending negative effect of the number of titmice squared ($F_{1,333} = 3.73$, $P = 0.0541$; model estimate = -0.0854 ± 0.0442; Fig. 5b). There was no effect of sex of caller ($F_{1,333} = 0.46$, $P = 0.804$) on calls produced by chickadees after stimulus presentations.

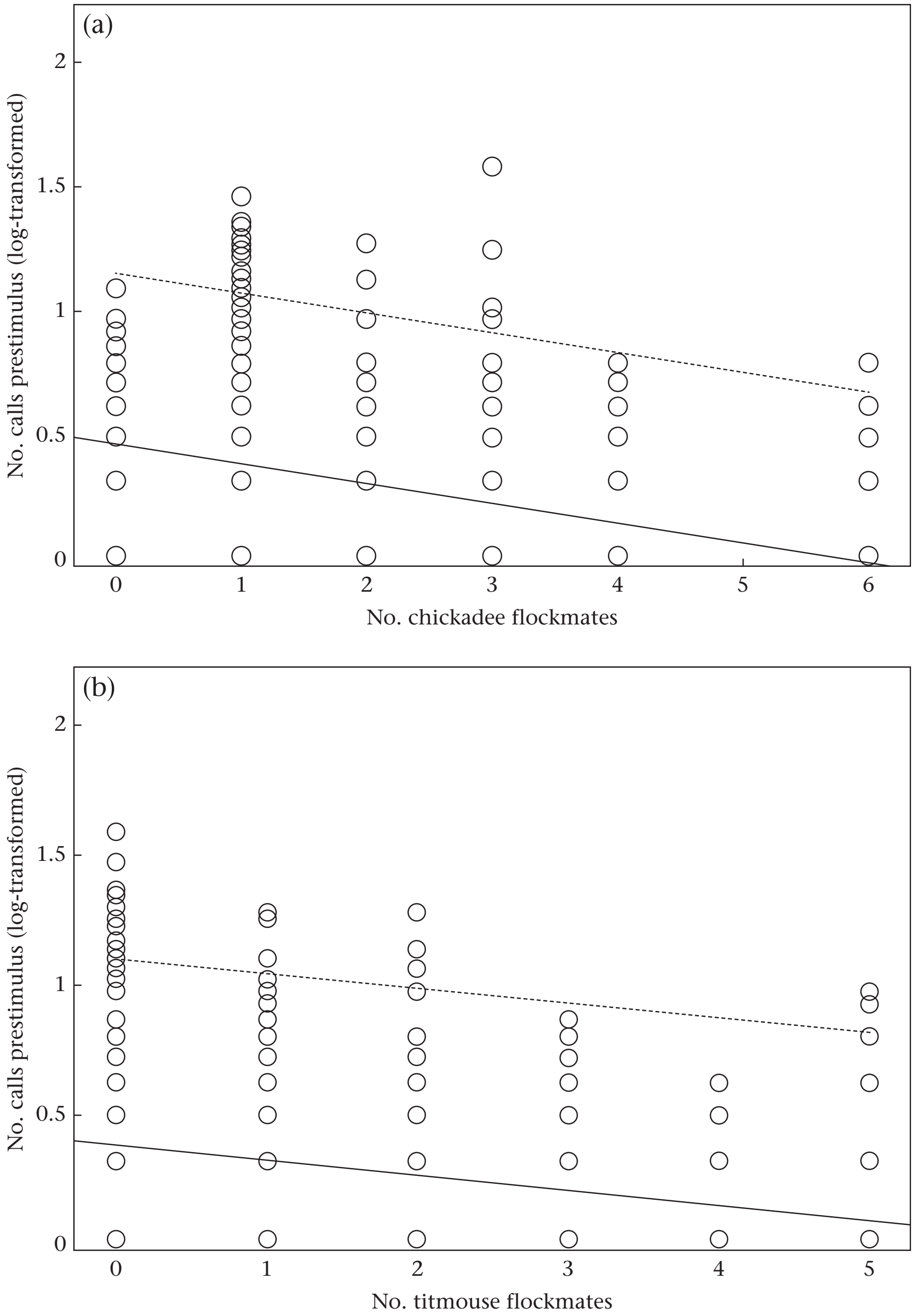

Figure 2. Number of chick-a-dee calls produced by chickadees during the prestimulus period as a function of the number of (a) conspecific chickadee and (b) heterospecific titmouse flockmates. Data points represent a single observational trial for each bird (with six trials per bird maximum), solid lines are lines of best fit, and dashed lines represent upper and lower bounds of 95% confidence intervals (CIs). Darker borders around circles indicate more data points for that number of calls.

Titmice

There was no significant effect of stimulus type on calling rates of titmice after the stimulus presentation ($F_{1.336} = 0.67$, $P = 0.645$). Titmice that called at a higher rate before the stimulus presentations generally tended to call at a higher rate after the stimulus presentations ($F_{1.336} = 15.10$, $P < 0.0001$; model estimate = 1.180±0.304; Fig. 6). However, this effect was complicated by a significant interaction between the effect of calls made before stimulus presentations and the number of titmice in the flock ($F_{1.336} = 8.65$, $P = 0.0035$; model estimate = 0.464 ± 0.158): thus the effect of prestimulus call rates on poststimulus call rates was greater when more titmice were in the flock. The effect of conspecific flock size on call rates during the stimulus presentation (titmice call less with increasing number of titmice in the flock) was also complicated by this interaction term (main effect of conspecific flock size on call rate during stimulus presentation: $F_{1.336} = 13.14$, $P = 0.0003$; model estimate = -0.528 ± 0.146; Fig. 7a). In this case, titmice that called more before stimulus presentation called even more if they were in larger titmouse flocks, but birds that called less before stimulus presentation called even less if they were in larger titmouse flocks. Additionally, titmice called more with increased numbers of chickadees in the flock ($F_{1.336} = 4.63$, $P = 0.0322$; model estimate = 0.495 ± 0.230; Fig. 7b), although this relationship peaked at a flock size of four chickadees, as indicated by a

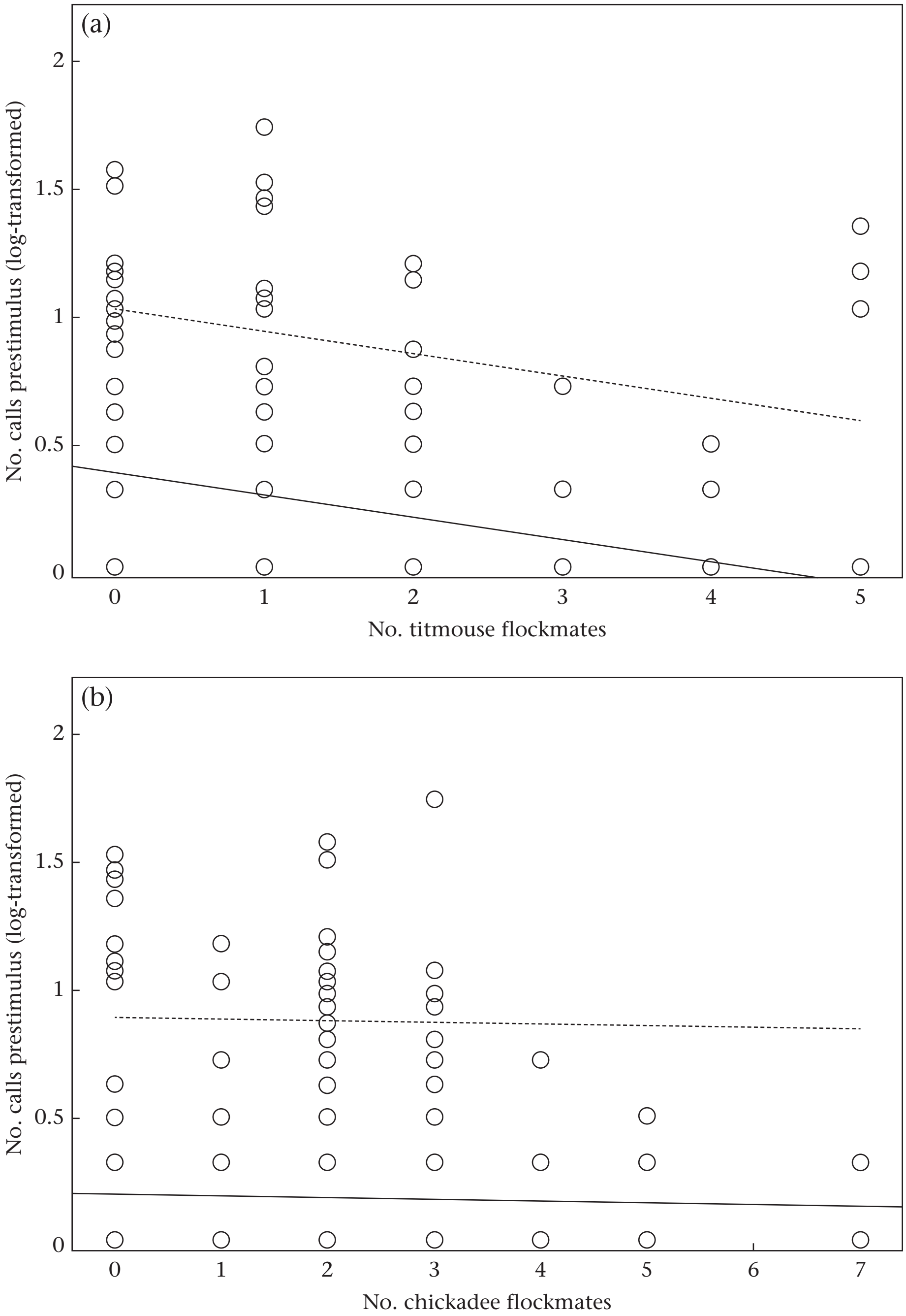

Figure 3. Number of chick-a-dee calls produced by titmice during the prestimulus period as a function of the number of (a) conspecific titmouse and (b) heterospecific chickadee flockmates. Data plotted as in Fig. 2.

significant squared term ($F_{1,336} = 4.38$, $P = 0.0372$; model estimate = -0.116 ± 0.056; Fig. 7b). There was no effect of caller sex on calling rates in titmice after stimulus presentations ($F_{1,336} = 3.21$, $P = 0.0741$).

DISCUSSION

We tested for mixed-species group audience effects on vocal signalling by experimentally manipulating the number of chickadees and titmice present in flocks. We sought to determine what types of audience effects influenced calling behaviour in chickadees and titmice in mixed-species flocks. We found that both chickadees and titmice displayed conspecific audience effects. Whereas chickadees demonstrated heterospecific audience effects in general calling conditions, titmice did not seem affected by the presence of chickadees. In addition, chickadees and titmice both demonstrated conspecific and heterospecific audience effects after simulated risk, although the nuances of conspecific audience effects in titmice were complicated by the effect of prestimulus calling behaviour. In both species, heterospecific audience effects after the stimulus presentations were nonlinear. Finally, the lack of any significant interactions between the number of conspecific and the number of heterospecifics in any of our analyses indicated that the influences of the demonstrated audience effects are additive. In other words, the relative proportion of each species in a flock does not seem to influence calling behaviour of either chickadees or titmice. Instead,

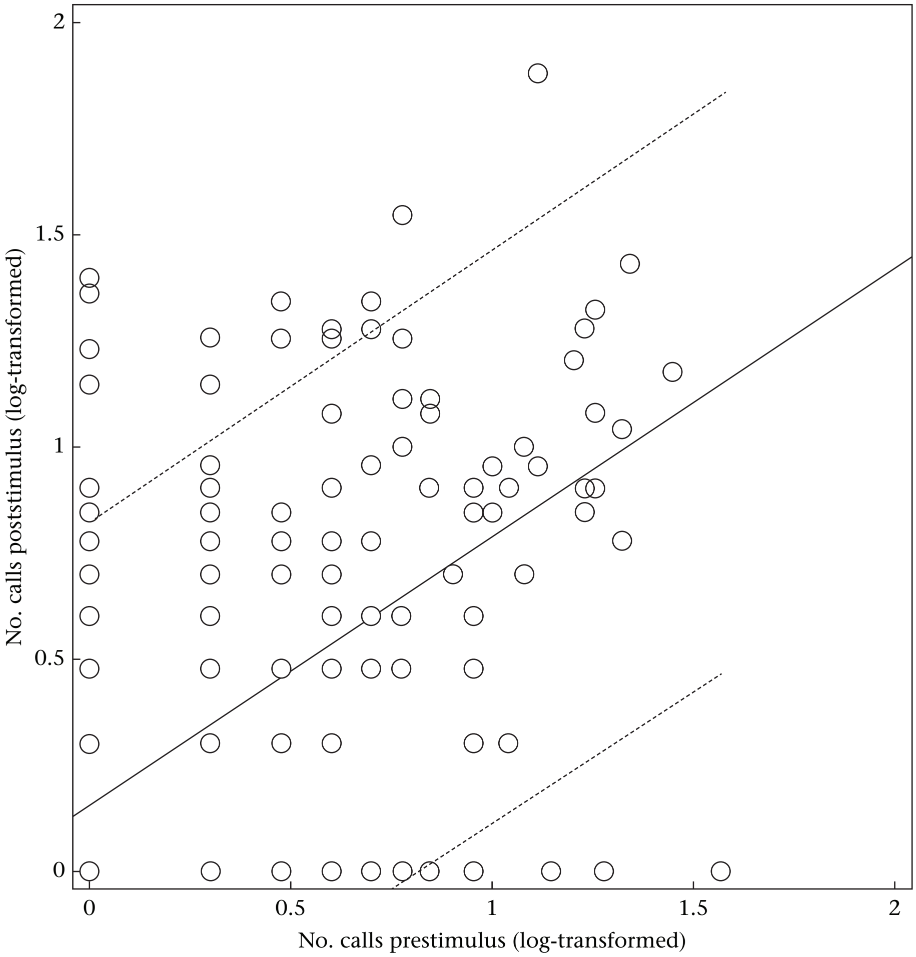

Figure 4. Calling rates after stimulus presentations as a function of calling rates before stimulus presentations for chickadees. Data points represent a single observational trial for each bird (with six trials per bird maximum). Solid line is line of best fit, and dashed lines represent bounds of 95% confidence intervals (CIs).

the effect of the number of heterospecifics on calling rates acts independently of the effect of conspecifics on calling rates.

Audience Effects on General Calling Behaviour

We found evidence of conspecific audience effects on general calling behaviour in chickadees and titmice. Both chickadees and titmice responded similarly to the presence of conspecifics: individuals called less with greater numbers of conspecifics present in the flock. One explanation for these findings may relate to the functional significance of calls produced in ambient conditions. The chick-a-dee call, produced by both chickadees and titmice, functions as a social recruitment call (Mahurin & Freeberg, 2008; Randler, 2012). Birds in small conspecific flocks may have called more to recruit more conspecifics to the flock, whereas this type of social recruitment calling would not be necessary for birds in larger conspecific flocks. This behavioural pattern also occurs in meerkats, *Suricata suricatta*, a social mammal with similarly situational alarm calls used for predator detection; young individuals in small groups give more alarm calls than young individuals in large groups, and the high call number in small groups was associated with increased vigilance (Hollen, Clutton-Brock, & Manser, 2008). Alternatively, larger conspecific flocks present a greater frequency and diversity of social distractions (activity, conflict, social suppression, etc.), and the affective response on individuals may simply inhibit call production.

We found evidence for a heterospecific audience effect in chickadees, but not in titmice. Individual chickadees called less with increased numbers of titmice present in flocks in the prestimulus period. Given the well-documented aggressive dominance of titmice over chickadees (Cimprich & Grubb, 1994), it is most parsimonious to attribute this pattern to social inhibition of the subordinate species. Pravosudov and Grubb (1999) previously found that individual chickadees were more vigilant when housed with a dominant titmouse than when housed with a subordinate conspecific (although individuals reduced vigilance in both dyads as compared to when alone), most likely due to the increased need to attend cautiously to its dominant flockmate. The presence of titmice may inhibit chickadees' calling behaviour because chickadees in the presence of titmice must spend time on heterospecific vigilance. Under this scenario, decreases in chickadee calling in flocks with a large number of titmice may act as a way to avoid conflict with dominant heterospecifics. This hypothesis is also supported by the lack of a heterospecific audience effect in titmice. Other studies have shown that individual titmice reduce vigilance behaviour when housed with a subordinate conspecific (Waite, 1987) or with a chickadee, compared to when housed alone (Pravosudov & Grubb, 1999). In conspecific dyads, subordinate titmice also reduced foraging rates compared to their dominant counterparts, and one explanation for this difference was a trade-off between foraging and conspecific vigilance (Waite, 1987). In our experiment, titmice in a flock containing a majority of

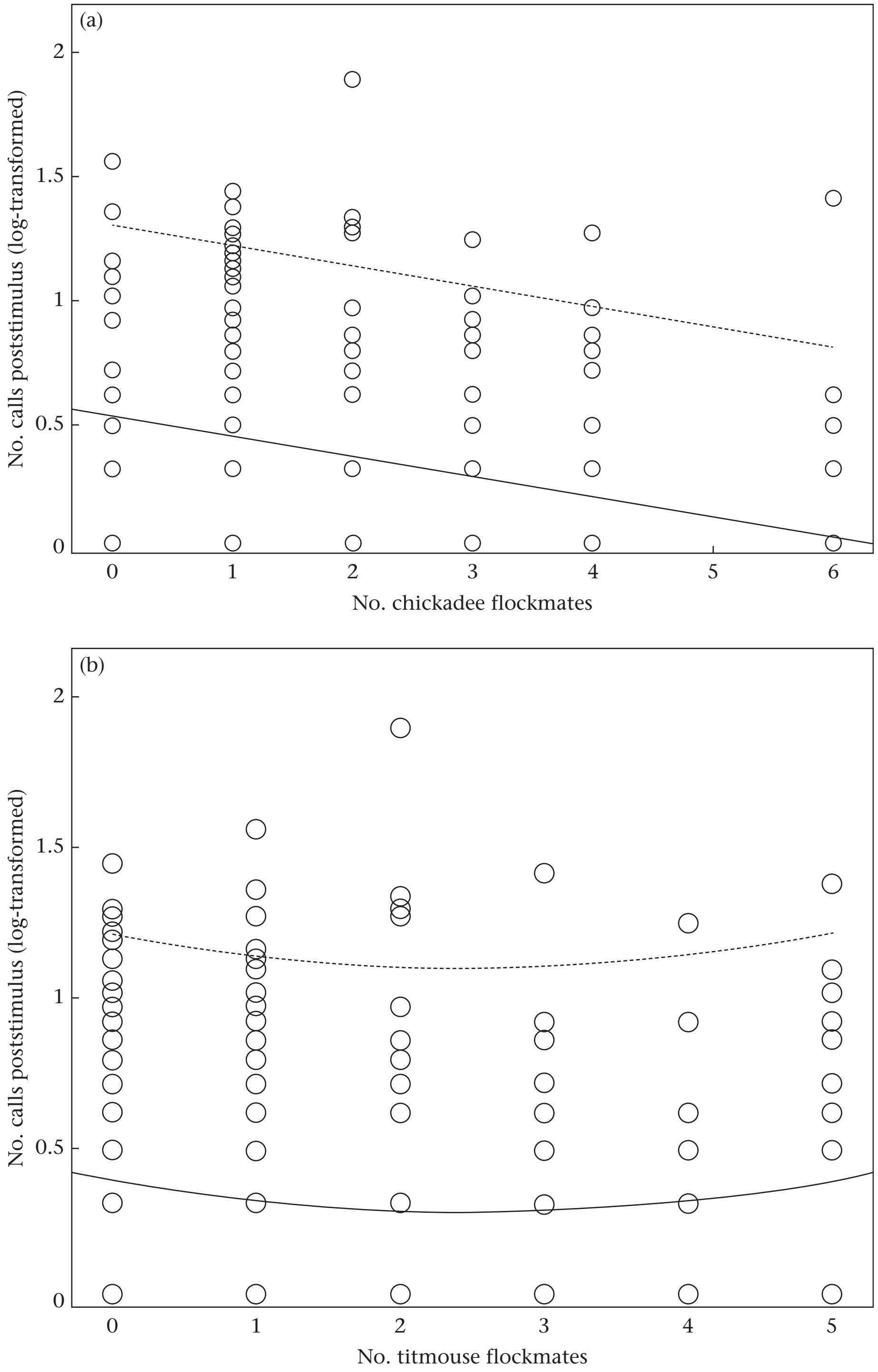

Figure 5. Number of chick-a-dee calls produced by chickadees during the poststimulus period as a function of the number of (a) conspecific chickadee and (b) heterospecific titmouse flockmates. Data plotted as in Fig. 2. Note that the displayed trend contradicts the model effect. The plot uses raw data and does not account for strong influences of other factors in the model.

chickadees would be the most dominant birds in that flock and may benefit from reduced vigilance rates in that flock.

Audience Effects on Calling Responses to Risk-related Playback Stimuli

We also tested for conspecific and heterospecific influences on calling rates in the context of simulated risk. The strongest influence on calling behaviour after simulated risk for individuals of both species was how much those individuals called before simulated risk (which itself was subject to conspecific and heterospecific audience effects). Individuals of both species that called more before simulated risk also called more after.

Calling rates of chickadees in response to the stimuli were affected by additional conspecific and heterospecific audience effects. Similar to prestimulus behaviour, chickadees called less with

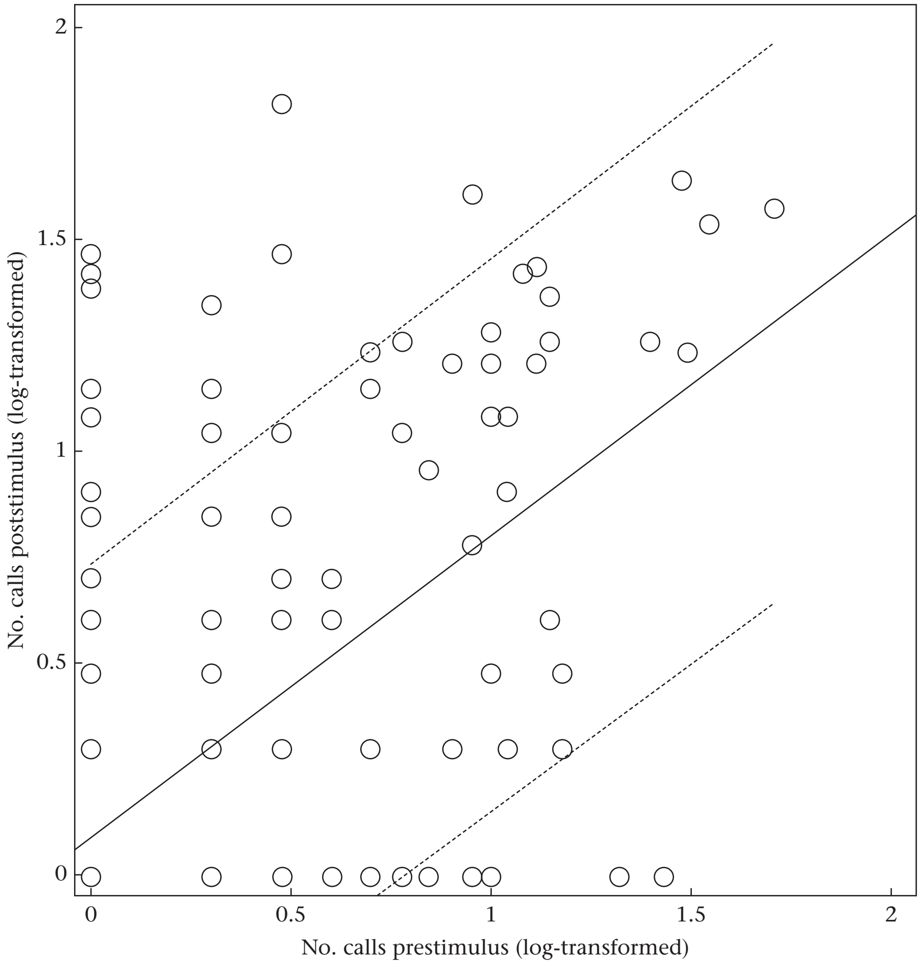

Figure 6. Calling rates after stimulus presentations as a function of calling rates before stimulus presentations for titmice. Data plotted as in Fig. 4.

increased conspecific presence in flocks. Interestingly, while chickadees called less in response to increased presence of titmice in flocks before simulated risk, increases in the number of titmice in a flock led to an increase in calling rate for chickadees after stimulus playbacks. The trending effect of the number of titmice squared term indicates that this heterospecific audience effect weakened as titmice flock size increased. Perhaps the presence of heterospecific titmice offer a safer dilution effect for chickadees than the presence of conspecific flockmates would, since these likely consist of mates in chickadees flocks (Smith, 1984). At low levels of heterospecific presence, chickadees call to recruit more titmice to the flock, but the potential heterospecific dilution benefit decreases in large heterospecific flocks, and either chickadees stand out more, or are subject to more aggression from dominant titmice. Future research should examine this potential function of this heterospecific audience effect. As these social variables also influenced how much an individual chickadee called before the stimulus phase, these findings suggest that chickadees are extremely sensitive to numbers of conspecifics and heterospecifics in their immediate social context when communicating. Previous work with Carolina chickadees posited that interactions among chickadees in complex social groups may influence calling behaviour of individuals (Freeberg, 2006; Freeberg & Harvey, 2008), and our findings in this study further support that claim. In fact, the hypothesis that complex groups influence communication of individuals in those groups (Freeberg, Dunbar, & Ord, 2012) has been suggested for a variety of species including spotted paca, *Cuniculus paca* (Lima, Sousa-Lima, Tokumaru, Nogueira-Fiho, & Nogueira, 2018), ground-dwelling sciurids (Blumstein & Armitage, 1997), species of social mongoose (Manser et al., 2014), whales (May-Collado, Agnarsson, & Wartzok, 2007) and giant otters, *Pterpnira brasiliensis* (Leuchtenbeger, Sousa-Lima, Duplaix, Magnusson, & Mourap, 2014).

Calling rates of titmice in response to simulated risk were also influenced by conspecific and heterospecific audience effects. In groups with more titmice, the effects of prestimulus calling were more pronounced: in flocks that contained greater (as opposed to fewer) numbers of conspecifics, titmice who called more before the stimulus presentation also called more after and, conversely, titmice who called less before the stimulus presentation also called less after. Additionally, while the presence of chickadees did not influence titmice calling rates in the prestimulus conditions, titmice increased calling rates after stimulus presentations as a function of increased number of chickadees in the flock. The significant chickadee squared term suggests that this effect weakened for heterospecific group sizes larger than four. Therefore, we found that increased presence of both conspecifics and heterospecifics

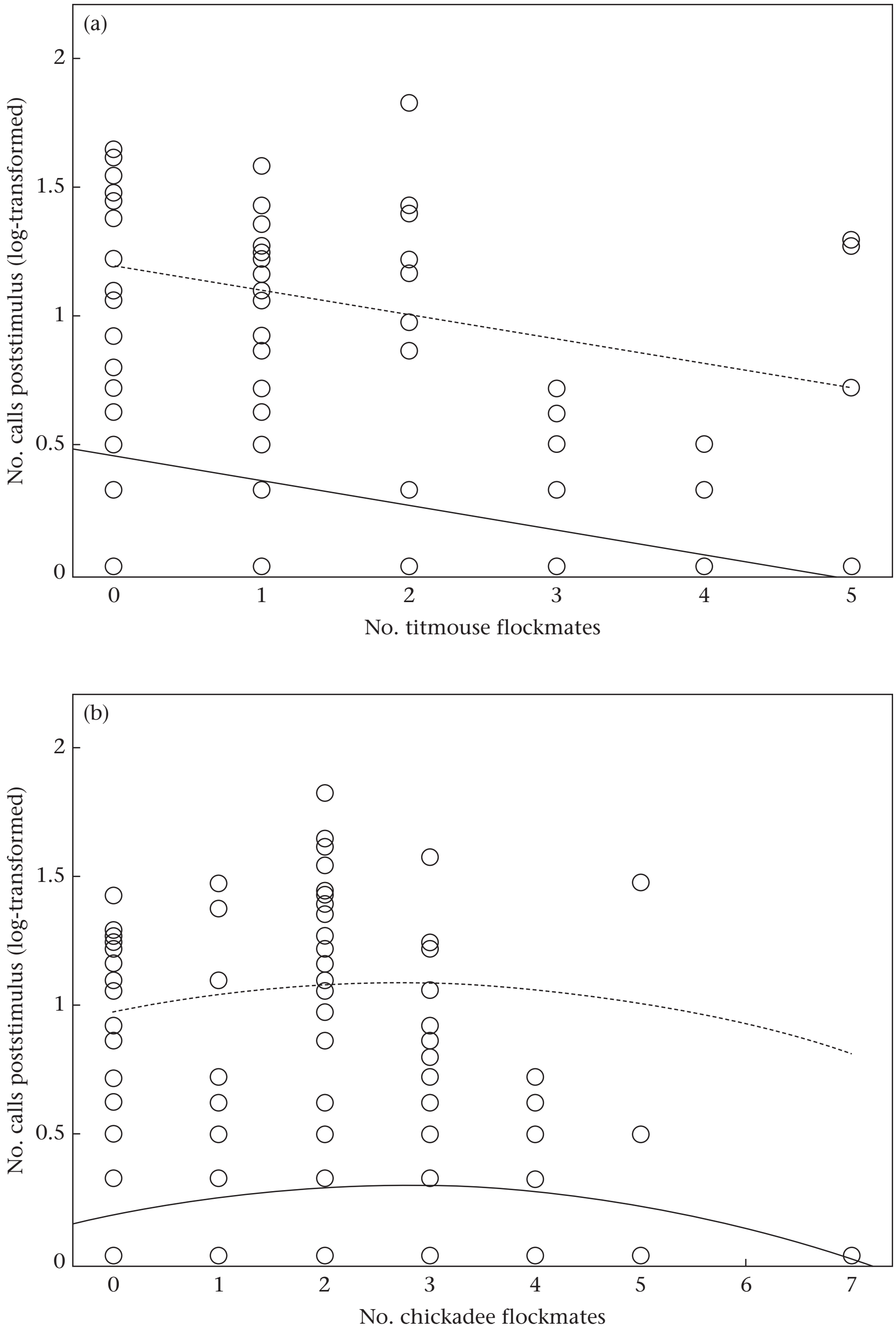

Figure 7. Number of chick-a-dee calls produced by titmice during the poststimulus period as a function of the number of (a) conspecific titmouse and (b) heterospecific chickadee flockmates. Data plotted as in Fig. 2.

increased calling behaviour after simulated risk in titmice. Titmice have been hypothesized to be poor contributors to the information-flow network in these mixed species flocks because of their weak and variable mobbing behaviour, while chickadees are thought be more active and aggressive mobbers (Nolen & Lucas, 2009). Perhaps titmice increase rates of chick-a-dee calls to recruit more aggressive heterospecific mobbers to the scene. While the conspecific audience effect suggests that titmice seek to increase their dilution benefit, titmice may prefer heterospecific mobbers when given the choice, especially considering that overwintering flocks of titmice may include kin. Future studies should examine this idea more closely. Regardless, these results suggest that titmice may benefit from residence in mixed-species flocks, and the audience effects found in our study suggest that the presence of even a few chickadees can increase information flow regarding risk among titmice. Since the Nolen and Lucas (2009) study also included nuthatch mobbing behaviour, future experiments should test for heterospecific audience effects of nuthatches on titmice calling behaviour.

Experimental Evidence for Inhibitive and Facultative Heterospecific Audience Effects Depending on Context

Our study revealed that calling behaviour of both chickadees and titmice is sensitive not just to conspecific flock size, but also to the number of heterospecifics in mixed-species flocks. To our knowledge, this is the first experimental evidence (in terms of manipulations of mixed-species group characteristics) to support heterospecific audience effects in communication. Taken together, our results clearly refute the oddity effect and dilution effect hypotheses (Table 1). Birds did not call more when they were proportionally rarer in these mixed-species flocks, and they did not call more purely as flock sizes increased. Our results for general (prestimulus) calling also do not support the social facilitation hypothesis (Table 1), as birds did not call more with increased number of conspecifics or heterospecifics in flocks. However, both chickadees and titmice increased their calling rates in the presence of heterospecifics under conditions of heightened risk, lending support to the social facilitation hypothesis for poststimulus calling. Similarly, although the social inhibition hypothesis (Table 1) did not seem to be supported by poststimulus calling responses in either chickadees or titmice, the prestimulus calling rates of both species were clearly inhibited by greater numbers of conspecifics and heterospecifics in flocks. Thus, larger conspecific and heterospecific flocks inhibit calling in ambient, low-risk contexts, but the increased numbers of heterospecifics seems to facilitate calling in high-risk contexts. One interpretation of this is the fluid contextualization of a central trade-off faced by mixed-species flock participants: that of minimizing conflict with flockmates versus maximizing antipredator benefits (Goodale et al., 2020). Without incident threats to wellbeing, the costs of aggression in larger, more socially complex groups would reasonably manifest in lower calling rates to avoid drawing attention from dominants. However, when the group's context suddenly shifts to risk, increased calling rates in mixed flocks could function to increase effectiveness of locating and confronting the threat (Caro, 2005) or discouraging the predator from attacking alerted prey (Crofoot, 2012).

Although we found conspecific and heterospecific audience effects for chickadees and titmice, these effects influenced calling behaviour of each species differently, suggesting species level variation in the importance of these variables. Additionally, we found evidence that chickadee general (prestimulus) calling behaviour was inhibited by the presence of titmice, whereas titmice were not influenced by the presence of chickadees, likely due to the fact that titmice are dominant over chickadees. It is possible that for chickadees, facilitation of calling in the presence of titmice when under threat could have a Machiavellian element (Lucas, Gentry, Sieving, & Freeberg, 2018) in that an attacking raptor will always seek the largest prey packet size (Boal & Mannan, 1999). In this case, chickadees can call more and share key information with each other with less incident risk because more, larger-bodied titmice nearby could, essentially, be providing them safe cover. Future work should examine the effect of interspecies dominance on communication and structure of mixed-species groups (Goodale et al., 2010), since these groups of animals are commonly found in mammals (Stensland, Angerbjorn, & Berggen, 2003), birds (Sridhar, 2009) and fish (Ward, Axford, & Krause, 2002). We also demonstrated that chickadees and titmice were further influenced by the presence of conspecifics and heterospecifics when communicating after detection of high-risk acoustic stimuli. Interestingly, the direction of heterospecific audience effects changed for both species after risk. These findings provide more evidence for the socially complex nature of mixed-species groups and that the effects of heterospecific presence may change depending on external context.

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Appendix

Genetic bird sexing was performed as previously described (Boutette et al., 2002). Briefly, DNA was extracted from blood using chelex resin (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's protocol for whole blood. Chromo-helicase DNA-binding protein genes (CHD) were amplified by polymerase chain reaction (PCR) using 5 pmol each of CHD specific primers P2 5′-TCTGGATCGCTAAATCCTTT-3′ and P8 5′-CTCCCAAGGATGAGRAAYTG-3′ and master mix containing taq DNA polymerase (Ex taq, TaKaRa). Cycling parameters were 95 °C for 90 s, 30 cycles at 49 °C for 45 s, 72 °C for 60 s and 94 °C for 60 s with a final extension at 72 °C for 10 min. A negative control without template was included in each amplification run. A 10 μl portion of each PCR product was digested with *Hae*III and resolved on 2% agarose gels containing SYBR green dye. Bird sex was determined based on CHD restriction length fragment polymorphisms. We also measured bird wing chord length, a measurement commonly used to determine sex, to compare the accuracy of wing chord measured to molecular sexing data (see Figs A1, A2).

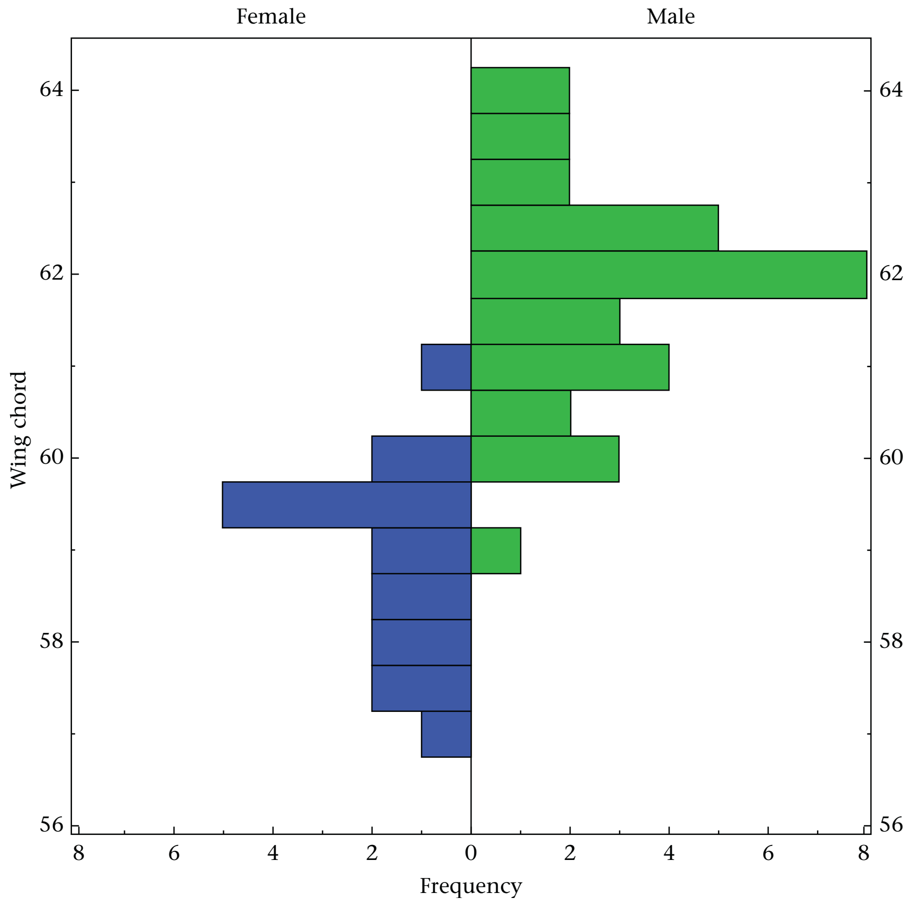

Figure A1. Frequency of male and female Carolina chickadees (determined by molecular sexing) of different wing chord lengths (mm). Blue bars represent frequency of females; green bars represent frequency of males.

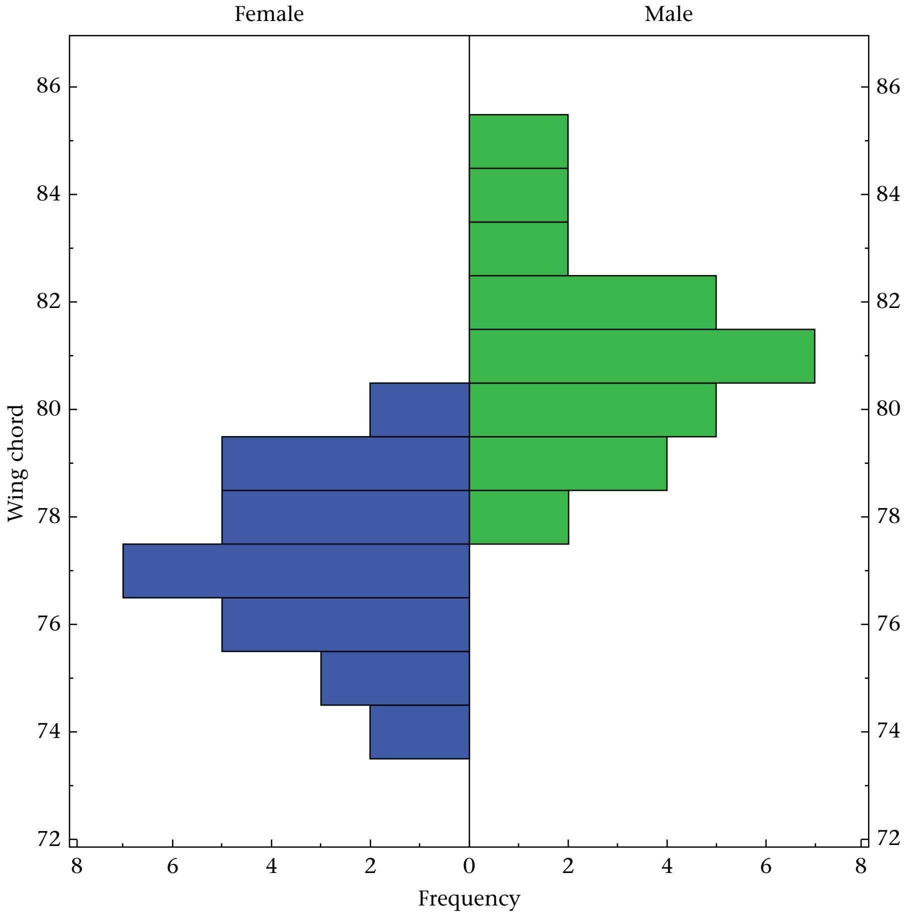

Figure A2. Frequency of male and female tufted titmice (determined by molecular sexing) of different wing chord lengths (mm). Blue bars represent frequency of females; green bars represent frequency of males.