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Original Article

Using playback of territorial calls to investigate mechanisms of kin discrimination in red squirrels

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Kin recognition can facilitate kin selection and may have played a role in the evolution of sociality. Red squirrels (*Tamiasciurus hudsonicus*) defend territories using vocalizations known as rattles. They use rattles to discriminate kin, though the mechanism underlying this ability is unknown. Our objective was to distinguish between the mechanisms of prior association, where animals learn the phenotypes of kin they associate with early in life, and phenotype matching/recognition alleles, where animals use a template to match phenotypes, thereby allowing them to recognize kin without an association early in life. We used audio playbacks to measure the responses of squirrels to rattles from familiar kin, unfamiliar kin, and non-kin. Initial analyses revealed that red squirrels did not discriminate between familiar and unfamiliar kin, but also did not discriminate between kin and non-kin, despite previous evidence indicating this capability. Post hoc analyses showed that a squirrel's propensity to rattle in response to playback depended on an interaction between relatedness and how the playback stimuli had been recorded. Red squirrels discriminated between rattles from close kin ($r = 0.5$) and rattles from non-kin ($r < 0.125$) when the rattles were recorded from provoked squirrels. Squirrels did not exhibit kin discrimination in response to unsolicited rattles. Once we accounted for how the stimuli had been recorded, we found no difference in the responses to familiar and unfamiliar kin. Our study suggests that kin discrimination by red squirrels may be context dependent.

Key words: **context-dependent kin discrimination, kin recognition, local density, playback, *Tamiasciurus hudsonicus*, territorial vocalization.**

INTRODUCTION

Kin recognition is the ability of an individual to recognize its relatedness to other individuals. This involves the expression of a recognizable signal by one individual, and the perception of that signal by another (Hamilton 1964; Beecher 1982). Kin recognition allows individuals to avoid inbreeding (Pusey and Wolf 1996) and to gain inclusive fitness benefits (Hamilton 1964) by mediating social behaviors, such as alarm calling (Sherman 1977). Evidence of kin recognition has been documented in group-living animals, as well as in solitary and territorial animals (Fuller and Blaustein 1990; Sun and Müller-Schwarze 1997; Hare 2004; Flores-Prado and Niemeyer 2010).

Several mechanisms have been proposed to explain how animals recognize kin, including prior association, phenotype matching, and recognition alleles (reviewed by Holmes and Sherman 1982, 1983; Blaustein 1983; Waldman 1987). In prior association, animals learn the phenotypes of specific individuals early in life, when social interactions usually involve kin (e.g., interacting with one's siblings or mother while in the natal nest). In phenotype matching, animals recognize familiar or unfamiliar kin by comparing them to

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a generalized kin template that is based on their own phenotype or on the phenotypes of familiar kin encountered early in life. In recognition alleles, the animal is hypothesized to express and recognize a familial trait, but unlike in phenotype matching, the expression and recognition of that trait is inherited instead of learned (i.e., green-beard effect, Hamilton 1964; Dawkins 1976). Distinguishing between phenotype matching and recognition alleles is often impossible because both allow for the recognition of familiar and unfamiliar kin.

Kin recognition is the process of assessing genetic relatedness, whereas kin discrimination is the differential expression of behavior towards kin. Several studies have found that kin discrimination can be context dependent and can vary between social contexts and with fluctuating environmental conditions. In a few studies on salamanders, kin discrimination varied with predator density (Harris et al. 2003), food abundance, and larval size (Hokit et al. 1996). Another study found that female red-backed salamanders (*Plethodon cinereus*) cannibalized unrelated neonates significantly more often than they cannibalized their own offspring, yet they otherwise did not behave differently towards the 2 groups of young (Gibbons et al. 2003). In eusocial insects, discrimination of nestmates (i.e., kin) has been found to vary with social context (intruder introductions, group interactions, or dyadic interactions, Buczkowski and Silverman 2005), with perceived threat to the colony (amount of nectar in the hive, Downs and Ratnieks 2000; and number of intruders, Couvillon et al. 2008), and with the location of the behavioral assay (either at a natural colony entrance or a test arena, Couvillon et al. 2013). These studies show that multiple factors can influence kin discrimination behavior, and that the absence of kin discrimination does not necessarily mean an absence of kin recognition.

North American red squirrels (*Tamiasciurus hudsonicus*) are solitary, territorial animals capable of discriminating kin in certain contexts. Males and females defend exclusive territories throughout the year (Smith 1968) and use vocalizations known as rattles to establish and signal their presence on a territory (Smith 1978; Lair 1990); physical disputes over territory boundaries are rare (Dantzer et al. 2012). Rattles have individually distinctive acoustic structures (Digweed et al. 2012) and previous research has shown that squirrels respond differently to the playbacks of rattles from kin and non-kin, regardless of whether those rattles were from neighbors or non-neighbors (Wilson et al. 2015). Nepotistic behavior in red squirrels has also been documented in several other contexts. Specifically, females are known to bequeath territories to offspring (Price and Boutin 1993; Berteaux and Boutin 2000; Lane et al. 2015), to nest occasionally with kin during the winter (Williams et al. 2013), and, in rare circumstances, to adopt the orphaned young of close kin (Gorrell et al. 2010). These examples are primarily between pairs of closely related individuals that have close associations early in life (e.g., mother-offspring and littermate pairs): bequeathal occurs only between mother-offspring pairs, nest sharing occurs primarily between mothers and daughters (though there were a few unfamiliar half-siblings nesting together), and adoption occurs only when the orphan's genetic mother and adopting mother were familiar and close kin. In contrast to these examples of kin discrimination, cross-fostering experiments with newborn red squirrels suggest that females do not preferentially allocate parental care to genetic offspring versus foster offspring (Humphries and Boutin 1996; McAdam et al. 2002). Kin discrimination is therefore context dependent in this species.

The objective of this study was to determine whether or not kin discrimination in red squirrels is based on the mechanism of prior association. Previous research showed that red squirrel rattles are individually distinctive (Digweed et al. 2012; Wilson et al. 2015) and used for kin discrimination (Wilson et al. 2015). However, the work on kin discrimination did not address whether red squirrels discriminate between kin with which they had prior associations early in life (familiar kin) and kin with which they had no prior associations early in life (unfamiliar kin). This was because most playback stimuli in the kin treatment of that study involved mother–offspring pairs, who would have interacted early in life while in the natal nest (Wilson et al. 2015). Therefore, we measured the responses of squirrels exposed to the playback of rattles from familiar kin, unfamiliar kin, and non-kin. If red squirrels recognize kin using the mechanism of prior association, we predicted that they would behave less aggressively and, thus, be less likely to rattle in response to rattles from familiar kin than in response to rattles from unfamiliar kin or non-kin. Alternatively, if red squirrels recognize kin by phenotype matching or recognition alleles, then we predicted that they would be less likely to rattle in response to rattles from familiar and unfamiliar kin than in response to rattles from non-kin. Upon finding no evidence of kin discrimination (see Results, below), we conducted a series of post hoc analyses to explore possible contextual factors that might have affected kin discrimination in this study.

METHODS

Study site and subjects

We conducted research on a population of red squirrels that has been studied annually in southwestern Yukon (61°N, 138°W) since 1989 (McAdam et al. 2007). The population lives in open boreal forest that is dominated by white spruce (LaMontagne and Boutin 2007). All individuals in the population were marked with numbered metal ear tags (Monel #1 National Tag and Band Co.) for permanent identification, and with colored wires threaded through their ear tags for visual identification from afar (McAdam et al. 2007). We monitored female reproductive status by live-trapping individuals in Tomahawk traps baited with peanut butter, and we ear-tagged the pups when they reached 25 days of age and were still in the natal nest (McAdam et al. 2007).

As part of our ongoing research program, we generated a multigenerational pedigree for this population (e.g., McFarlane et al. 2015). We established maternal linkages by identifying mothers and their pups while they were still within their natal nests. The few cases of adoption documented in this study population mostly occurred when pups had emerged from the natal nest but were not yet weaned, between 43 and 63 days of age (Gorrell et al. 2010). A single adoption occurred when the pup was only 6 days old. Therefore, adoptions should not have influenced our method of establishing maternal linkages. Paternal pedigree linkages (Lane et al. 2007; McFarlane et al. 2014) were established since 2003 using paternity analysis involving 16 microsatellite loci (Gunn et al. 2005). The paternal linkages were made with 99% confidence using CERVUS 3.0 (Kalinowski et al. 2007). Any unobserved adoptions would have been detected by mismatching genotypes between the pup and mother during the paternity analysis.

Playback trials for this study were conducted on 3 sites: 1 was part of an ongoing food supplementation experiment that started in 2004 (45 ha), and the other 2 were control sites for this same large-scale experiment (40 ha each). As part of this experiment, squirrels on the food-supplemented site were supplied with 1 kg of peanut butter every 6 weeks between October and May each year. The density of squirrels in 2009 was low on the 2 control sites (1.13

and 0.76 squirrels/ha), but was higher on the food supplemented site (2.45 squirrels/ha) due to higher food availability (Dantzer et al. 2013).

Experimental design

The playback experiment followed a 2 × 3 factorial design in which each subject was played a single territorial rattle that varied in terms of its kinship status (familiar kin, unfamiliar kin, or non-kin) and neighbor status (neighbor or non-neighbor). "Kin" was defined as having a pedigree relatedness coefficient (*r*) of at least 0.25. We used a categorical kin variable because we were interested primarily in determining whether kin discrimination was limited to familiar kin or whether it extended to unfamiliar kin as well. Our "familiar kin" treatment referred exclusively to pairs of squirrels that shared a natal nest, as this is the only time in a squirrel's life when they are interacting only with kin. The familiar kin treatment included 15 mother–offspring pairs and 22 litter-mate pairs (full siblings and maternal half-siblings). Male red squirrels do not provide parental care and have no interactions with pups in the natal nest. Our "unfamiliar kin" treatment included 12 father–offspring pairs, 14 non-litter-mate pairs (paternal half-siblings, maternal half-siblings, or full siblings from different litters), and 4 grandparent–grand-offspring pairs (Table 1). "Non-kin" were defined as having a relatedness coefficient of less than 0.125. We included neighbor status in our experimental design to account for the possibility that squirrels behave less aggressively towards their neighbors (i.e., the dear-enemy effect: Fisher 1954; Temeles 1994). "Neighbors" were defined as squirrels with middens located within 100 m of each other, whereas "non-neighbors" were defined as squirrels whose middens were more than 200 m apart. The familiar kin treatment included 24 neighbor and 13 non-neighbor trials, the unfamiliar kin treatment included 16 neighbor and 14 non-neighbor trials, and the non-kin treatment included 16 neighbor and 22 non-neighbor trials.

Playback stimuli

Rattles used as playback stimuli were recorded from squirrels as they moved freely around their territories (*n* = 46), as they emerged from a trap (*n* = 17), or as they rattled in response to rattles that we broadcast from a loudspeaker (*n* = 10). All rattles were recorded using a shotgun microphone (Sennheiser model ME66 with K6 power supply; 40–20 000 Hz frequency response [±2.5 dB]; super-cardioid polar pattern) connected to a Marantz Professional Solid State Recorder (model PMD 660; 44.1 kHz sampling frequency; 16-bit amplitude encoding; WAVE format). The final set of recordings included one recording from each of 73 different adult squirrels. The recordings were from 35 males and 38 females that we recorded in 2005 (1 recording), 2006 (9 recordings), and 2009 (63 recordings). There were 30 rattles recorded on the site with the food supplementation experiment and 43 rattles recorded on the 2 control sites.

Recordings of squirrels with living kin were assigned preferentially to the kin treatments (familiar and unfamiliar kin), as there were a limited number of squirrels with close relatives on our study site. The non-kin stimuli were assigned to subjects at random. In trials for which recordings from 2005 and 2006 were used as stimuli, the vocalizing squirrel from the recording was still alive at the time of the trial in 2009. Most recordings were used only once in the playback experiment; 26 were used to test more than 1 squirrel, though these were used in different treatments for each squirrel.

Rattles used as playback stimuli were not filtered and were not edited to standardize their length. Each stimulus consisted of a single rattle that ranged between 1.5 and 12.3 s in duration (mean ± SD = 4.0 ± 2.3 s). The mean duration of the rattle stimuli ranged from 3.5 ± 2.1 s (mean ± SD) in the non-kin non-neighbor treatment to 4.8 ± 3.2 s (mean ± SD) in the unfamiliar kin non-neighbor treatment, and did not differ significantly among treatments (1-way Anova: $F_{5,\,99} = 0.70$, $P = 0.63$). Rattles were transferred to a SanDisk mp3 player (Sansa e280 model) that supported the WAVE format. Our playback speaker was a custom Saul Mineroff SME-AFS field speaker, with a frequency range of 10–22 500 Hz. The speaker's volume setting was held constant throughout the experiment. At this setting, the rattle peak amplitude averaged 68 ± 3.3 dB (mean ± SD), as measured with a digital sound level meter (RadioShack; C weighting; fast response) held 1 m from the speaker. When broadcast at this amplitude, the rattles were audible to the human ear at up to 120 m away. This is comparable to the only published account of rattle amplitude, which states that red squirrel rattles can be heard up to 130 m away (Smith 1968). The peak amplitude ranged between an average of 66.7 ± 4.5 dB (mean ± SD) in the unfamiliar kin non-neighbor treatment and 68.8 ± 2.8 dB (mean ± SD) in the non-kin non-neighbor treatment, and did not differ significantly among treatments (1-way Anova: $F_{5,\,99} = 0.70$, $P = 0.62$).

Table 1

Kin relationships within each kinship status category and the number of trials completed

Kinship	Kin relationship (subject-stimulus)	No. of trials	Relatedness coefficient
Familiar kin	Mother–offspring	6	0.5
	Offspring–mother	9	0.5
	Full siblings (littermates)	10	0.5
	Maternal half-siblings (littermates)	12	0.25
Unfamiliar kin	Father–offspring	3	0.5
	Offspring–father	9	0.5
	Full siblings (non-littermates)	3	0.5
	Maternal half-siblings (non-littermates)	7	0.25
	Paternal half-siblings (non-littermates)	4	0.25
	Grandparent–grand offspring	2	0.25
	Grand offspring–grandparent	2	0.25
Non-kin	None	38	<0.125
Total		105	

"Familiar" kin denotes individuals with early life associations (i.e., shared a natal nest).

Playback Procedure

Subject squirrels were located by sight, sound, or radio telemetry, and trials were commenced only if the subject was within 20 m of its midden. We used the squirrel's unique color markings to confirm their identity before beginning trials. Once a subject was identified, we set up the speaker approximately 10 m from the subject and concealed it behind a tree, fallen log, or dense vegetation. The observer then sat on the ground approximately 10 m from the subject, such that the line between the observer and subject was perpendicular to the line between the subject and speaker. All trials were completed by a single observer.

Trials consisted of a 3-minute pre-playback observation period followed immediately by the playback stimulus and a 3-minute playback observation period. Throughout the pre-playback and playback periods, we counted each time the subject produced a rattle, looked at the speaker, and approached the speaker. We subjectively scored "looking at the speaker" when we saw head movement by the subject that ended with the squirrel facing the speaker. We defined "approach" as 2 m of continuous travel directly toward the speaker. During the playback period, we audio-recorded the subject using the same recorder as described above. Ten rattles recorded from subjects during the playback period were later used as stimuli in other playback trials on different focal subjects.

We attempted to minimize confounding or obscuring factors during the playback trials. For example, we did not commence a trial if the subject alarm-called as the observer approached (known as a bark; Lair 1990; Digweed and Rendall 2009) or interacted with another squirrel (e.g., chasing) while the observer approached. We also discarded trials if the subject chased an intruding adult ($n = 2$) or juvenile ($n = 2$) squirrel from their territory during the trial, if the subject moved more than 20 m away from the speaker before the trial began ($n = 2$), if the observer lost sight of the subject for longer than 1 min ($n = 18$ during the pre-playback period; $n = 7$ during the playback period), or if the squirrel entered a nest during the pre-playback period ($n = 7$). Discarded trials were attempted again after 3 days. In total, we completed 105 successful trials on 85 individual squirrels between 23 May 2009 and 26 July 2009. There were 63 trials conducted on 45 squirrels on the 2 control sites and 42 trials on 40 squirrels on the food-supplemented site. For the 20 subjects that received 2 trials, each received a different treatment during each trial and the trials were separated by at least 3 days.

Statistical analyses

Response variables in our analyses included 1) whether or not the subject produced a rattle, 2) whether or not the subject looked at the speaker, 3) whether or not the subject approached the speaker, and 4) the latency for the subject to rattle. We considered the first 3 variables to be dichotomous because it was uncommon for squirrels to express these behaviors more than once during each observation period. Latency to rattle was only measured in the playback period and was defined as the time from the start of the playback stimulus to the start of the subject's rattle (measured to the nearest 10 ms using Raven Pro Sound Analysis Software version 1.3).

All statistical analyses were performed in R (version 3.2.2; R Core Team 2015) using the packages "lme4" (Bates et al. 2015) and "survival" (Thernau and Lumley 2009). Our first set of analyses tested whether subjects responded to the playback stimuli by comparing response variables between the pre-playback and playback periods. Separate generalized linear mixed effect models (binomial error distribution; logit link function) were fitted to each of the 3 dichotomous response variables measured in both periods. We included subject identity as a subject variable with random effects to account for the repeated measures obtained during the pre-playback and playback periods, as well as for the multiple trials that were conducted on each of 20 subjects. Period (pre-playback or playback) was included as a categorical variable with fixed effects.

Our second set of analyses tested whether subjects' responses were affected by the kinship status of the playback stimuli. We used separate generalized linear mixed effect models (binomial error distribution; logit link function) to test if the kinship status of the playback stimulus affected 1) whether the subject rattled and 2) whether it looked at the speaker. Approaching the speaker was not included as a response variable in this set of analyses because it did not differ between the pre-playback and playback periods (see results below). In each model, we included subject identity as a subject variable with random effects, and the kinship status (familiar kin, unfamiliar kin, or non-kin) and neighbor status (neighbor or non-neighbor) of the playback stimulus as categorical variables with fixed effects. The 2-way interaction between kinship status and neighbor status was not significant in either model and was, therefore, removed from the final model.

We used a survival analysis approach to test the effect of kinship status and neighbor status on latency to rattle in the playback period. A survival analysis approach was used because it is useful for analyzing time-to-event data and can deal with censored values that result when the event does not occur (e.g., subject squirrels that did not rattle during the 3-minute playback period). We used a Cox proportional hazard model with the playback period data of a reduced dataset ($n = 85$ trials), with kinship and neighbor status as independent variables. We eliminated multiple trials from each of 20 individuals by randomly selecting 1 trial per individual. The 2-way interaction between kinship status and neighbor status was not significant and was, therefore, removed from the final model.

Upon finding no overall effects of kinship or neighbor status (see results below) on any of the response variables, we conducted exploratory post hoc analyses in an attempt to understand the negative results and their inconsistency with previous evidence of kin discrimination in red squirrels (Gorrell et al. 2010; Williams et al. 2013; Wilson et al. 2015). For all exploratory analyses, we used whether or not the subject rattled as the response variable, because this variable can be compared directly with previous studies. Neighbor status was removed from the final models because there were no significant interactions and the main effect of neighbor status was not significant.

There was some variation in the degree of average relatedness within the kinship categories (Table 1), so our first exploratory post hoc analysis examined the relationship between the probability of rattling and known relatedness coefficients derived from the pedigree. We conducted a simplified analysis that treated kinship as a continuous variable and excluded familiarity. Therefore, in contrast to our earlier analyses, this exploratory analysis tested for an overall effect of kin discrimination, regardless of whether kin were familiar or unfamiliar.

Local density was quite variable among squirrels tested in this study, and red squirrels emit rattles more frequently when surrounded by a higher density of conspecifics (Dantzer et al. 2012; Shonfield et al. 2012). For the second exploratory analysis, we tested the effect of local density on rattle responses. Local density (squirrels/ha) was calculated for each subject as the number of squirrels that owned a midden within a 130-m radius (5.31 ha) of the subject's midden. We chose a 130-m radius because rattles from neighboring squirrels are audible up to this distance (Smith 1968).

Recent bioacoustics research has revealed structural differences among rattles recorded from 1) squirrels as they move freely around their territories, 2) rattles recorded as squirrels emerge from a trap, and 3) rattles produced in response to rattles that have been broadcast through a speaker (unpublished data). It is not yet clear how these structural differences affect the natural inter-individual variation in rattle structure (Digweed et al. 2012; Wilson et al. 2015) that is presumably used in discrimination. Therefore, for the third exploratory analysis, we tested whether kin discrimination was affected by the method by which rattles were recorded. We pooled rattles into 2 collection method categories: "unsolicited" included those rattles collected from squirrels moving freely around their territories and "provoked" included those rattles collected from squirrels emerging from traps and those produced in response to rattles broadcast from a speaker.

The effects of local density and recording method were tested separately by fitting an interaction between each of these variables and relatedness (1 model with categorical kinship status and 1 model with continuous relatedness from the pedigree) in the generalized linear models that predicted whether or not a squirrel rattled in response to the playback (see above). A significant interaction would indicate that red squirrels discriminate kin under some circumstances (e.g., local density), but not others. We similarly tested for effects of sex of the subject squirrel, and the date of the playback trial on kin discrimination, but the rationale for these post hoc analyses was weaker, so we did not report these nonsignificant results. We mention them briefly here to be transparent about the scope of our post hoc analyses.

RESULTS

Squirrels were significantly more likely to produce a rattle during the playback period (42% of squirrels) than during the pre-playback period (26% of squirrels; Figure 1; Table 2). Similarly, squirrels were significantly more likely to look in the direction of the speaker during the playback period (44% of squirrels) than during the pre-playback period (3% of squirrels; Figure 1; Table 2). Squirrels were not more likely to approach the speaker during the playback period (7% of squirrels) than during the pre-playback period (2% of squirrels; Figure 1; Table 2), so this variable was not included in subsequent analyses. Subject identity did not improve any of the statistical models, including the models for whether the subject produced a rattle (likelihood ratio test: $X^2 < 0.1$, df = 1, $P > 0.9$), looked at the speaker ($X^2 = 0.9$, df = 1, $P = 0.3$), or approached the speaker ($X^2 < 0.1$, df = 1, $P > 0.9$). Therefore, a subject's behavior in the playback period was independent of its behavior in the pre-playback period and in other playback trials.

Kinship status (familiar kin, unfamiliar kin, and non-kin) and neighbor status (neighbor and non-neighbor) did not have statistically significant effects on any of the response variables, including whether subjects produced a rattle, whether subjects looked at the speaker, or how quickly subjects produced a rattle following the onset of the stimulus (Table 3). These results indicate that red squirrels did not discriminate between playbacks of kin and non-kin. Subject identity did not improve the generalized linear mixed models, including the models for whether the subject produced a rattle (likelihood ratio test: $X^2 < 0.1$, df = 1, $P > 0.9$) or looked at the speaker ($X^2 < 0.1$, df = 1, $P > 0.9$).

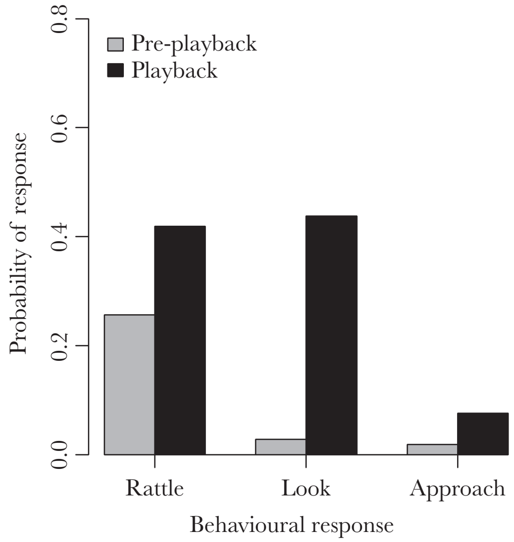

Figure 1
Probability of behavioral responses (producing a territorial "rattle" call, looking at the speaker, and approaching the speaker) of the subject before and during the playback period (*n* = 105 trials).

Table 2
Responses of subject squirrels to the playback rattle

Response variable	Effect	Estimate ± SE	*Z*	*P*
Rattle[a]	Intercept	−1.06 ± 0.22	−4.75	**<0.0001**
	Period (playback)	0.73 ± 0.30	−2.46	**0.014**
Looking at speaker[b]	Intercept	−3.85 ± 0.74	−5.18	**<0.0001**
	Period (playback)	3.54 ± 0.72	4.89	**<0.0001**
Approach the speaker[c]	Intercept	−3.94 ± 0.71	−5.52	**<0.0001**
	Period (playback)	1.44 ± 0.80	1.80	0.072

Responses include whether subjects produced a rattle, looked at the speaker, and approached the speaker. Responses were modeled using three separate linear mixed models (binary response, logit link) with subject identity as a random effect. Estimates are on a log-odds scale and the effects of the factor in the design are reported as the effect of the level in parentheses (e.g., playback) relative to the reference category (pre-playback). Significant *P*-values are in bold (significance level $\alpha = 0.05$). SE = standard error.
[a] Random effect of squirrel identity: variance = 0.
[b] Random effect of squirrel identity: variance = 0.72.
[c] Random effect of squirrel identity: variance = 0.

We conducted exploratory post hoc analyses in an attempt to understand the lack of kin discrimination and the inconsistency of this finding with previous evidence of kin discrimination in red squirrels (Gorrell et al. 2010; Williams et al. 2013; Wilson et al. 2015). Our first exploratory analysis replaced kinship status with known relatedness coefficients (derived from the pedigree as a continuous covariate) as the independent variable in the model with rattling as a response variable to test for an overall effect of kin discrimination regardless of familiarity. The results of the model with relatedness coefficient from the pedigree as a covariate were very similar to the results described above with kinship status. There was no effect of relatedness on any of the response variables (results not shown, see footnote in Table 3).

In our second exploratory analysis, we tested the effect of local density on territorial responses. Local population density (i.e., the number of squirrels with middens within a 130-m radius of the subject's midden) varied from 0.4 to 3.2 squirrels/ha. When local density was included as an independent variable, we found that local density affected the difference in response between unfamiliar kin and familiar kin, but did not affect the difference in response between familiar kin and non-kin (Table 4). However, this effect disappeared when we ran the same model with the relatedness coefficient as a covariate (Table 4).

In our third exploratory analysis, we tested whether kin discrimination was affected by the method by which rattles were recorded. There were 67 trials with unsolicited rattles as the stimulus and 38 trials with provoked rattles. In the models with collection method as an independent variable, we found no significant interaction or main effect of kinship status, when kinship was included as a categorical variable (Table 5). Thus, there was no difference in the responses between familiar kin, unfamiliar kin, and non-kin, and no mediating effect of collection method on responses to these kinship status groups. However, in the model with relatedness included as a continuous variable, we found a significant interaction and a significant main effect of collection method, though the main effect of relatedness was not significant (Table 5). Visual inspection of these results indicated that this interaction between collection method and relatedness was largely driven by the differential response of squirrels to rattles of kin with a relatedness coefficient of 0.5 (Figure 2). Specifically, red squirrels were more likely to rattle in response to provoked rattles if they were from non-kin or less related kin ($r < 0.5$) than from more closely related kin ($r = 0.5$) (Figure 2). Taking the subset of trials that used provoked rattle stimuli ($n = 38$ trials), we found that there was a marginally non-significant effect of relatedness on propensity to rattle (generalized linear mixed model: $Z = -1.89$, $P = 0.058$).

DISCUSSION

We found that across all playback trials red squirrels did not discriminate between familiar kin, unfamiliar kin, and non-kin. Squirrels were just as likely to rattle or look towards the speaker in response to a familiar kin rattle as they were to either an unfamiliar kin or non-kin rattle, and there was no overall effect of the degree of relatedness on their behavioral response. Similarly, squirrels did not discriminate between neighbors and non-neighbors in their behavioral response. We also found no difference in the latency of red squirrels to respond to the playback for either kinship or neighbor status. This lack of effect of kin status on the response of

Table 3

Effects of kinship and neighbor status on the behavioral response from the subject squirrel

Response variable	Effect	Estimate ± SE	Z	P
Rattling[a,b]	Intercept	−0.24 ± 0.36	−0.68	0.498
	Kinship (non-kin)	−0.03 ± 0.48	−0.06	0.952
	Kinship (unfamiliar kin)	−0.12 ± 0.50	−0.25	0.804
	Neighbor status (non-neighbor)	−0.08 ± 0.40	−0.20	0.845
Looking at speaker[a]	Intercept	−0.46 ± 0.37	−1.24	0.216
	Kinship (non-kin)	0.12 ± 0.48	0.26	0.797
	Kinship (unfamiliar kin)	0.23 ± 0.50	0.45	0.653
	Neighbor status (non-neighbor)	0.22 ± 0.40	0.54	0.589
Latency to rattle[c]	Kinship (non-kin)	−0.06 ± 0.41	−0.15	0.879
	Kinship (unfamiliar kin)	−0.20 ± 0.46	−0.43	0.667
	Neighbor status (non-neighbor)	0.17 ± 0.36	0.47	0.637

[a] Responses were modeled using a generalized linear mixed model (binary response, logit link), with subject identity as a random effect. The random effect for both models (rattling and looking at the speaker) had among-individual variance of zero. Estimates are on a log-odds scale and the effects of each factor in the design are reported as the effect of the level in parentheses (e.g., non-kin) relative to the reference category. In this case, the reference category is "familiar kin" for kinship, and "neighbor" for neighbor status.

[b] The model with rattling as a response variable was also run with relatedness coefficient (calculated from the pedigree) in place of kinship status as the kin variable as part of our post hoc exploratory analyses, but the results with respect to statistical significance did not differ from those above and are not shown.

[c] Latency to rattle (range: 4.7–173.9 s, average ± SE = 66.5 ± 8.1 s) was modeled using a Cox proportional hazard model with a reduced dataset ($n = 85$ trials).

Table 4

Effect of local population density and either kinship status or relatedness (coefficient calculated from the pedigree) on the probability of a rattle response from the subject squirrel following the playback

Model	Effect	Estimate ± SE	Z	P
Model with kinship status	Intercept	−1.45 ± 0.89	−1.63	0.103
	Kinship (non-kin)	1.25 ± 1.23	1.01	0.311
	Kinship (unfamiliar kin)	2.51 ± 1.29	1.95	**0.051**
	Local density	0.70 ± 0.49	1.45	0.148
	Kinship (non-kin) × Local density	−0.77 ± 0.68	−1.14	0.256
	Kinship (unfamiliar kin) × Local density	−1.88 ± 0.85	−2.20	**0.028**
Model with relatedness coefficient	Intercept	0.46 ± 0.80	0.58	0.565
	Relatedness	−2.86 ± 2.25	−1.27	0.204
	Local density	−0.44 ± 0.45	−0.98	0.329
	Relatedness × Local density	1.66 ± 1.31	1.27	0.206

Probability of a rattle response was modeled using a generalized linear mixed model (binary response, logit link), with subject identity as a random effect. The random effect for both models had an among-individual variance of zero. Estimates are on a log-odds scale and the effects of each factor in the design are reported as the effect of the level in parentheses (e.g., non-kin) relative to the reference category. In this case, the reference category for kinship is "familiar kin." Significant P-values are in bold (significance level $\alpha = 0.05$).

Table 5

Effect of rattle stimulus collection method (unsolicited or provoked) and either kinship status or relatedness (coefficient calculated from the pedigree) on the probability of a rattle response from the subject squirrel following the playback

Model	Effect	Estimate ± SE	z	P
Model with kinship status	Intercept	−0.34 ± 0.41	−0.81	0.416
	Kinship (non-kin)	−0.36 ± 0.60	−0.60	0.552
	Kinship (unfamiliar kin)	0.23 ± 0.62	0.37	0.709
	Method (provoked)	0.18 ± 0.69	0.26	0.793
	Kinship (non-kin) × Method (provoked)	0.80 ± 0.98	0.82	0.415
	Kinship (unfamiliar kin) × Method (provoked)	−1.06 ± 1.07	−0.99	0.324
Model with relatedness coefficient	Intercept	−0.75 ± 0.40	−1.87	0.062
	Relatedness	1.40 ± 1.20	1.17	0.242
	Method (provoked)	1.31 ± 0.66	1.98	**0.048**
	Relatedness × Method (provoked)	−4.32 ± 1.95	−2.21	**0.027**

Probability of a rattle response was modeled using a generalized linear mixed model (binary response, logit link), with subject identity as a random effect. The random effect for both models had an among-individual variance of zero. Estimates are on a log-odds scale and the effects of each factor in the design are reported as the effect of the level in parentheses (e.g., non-kin) relative to the reference category. In this case, the reference category is "familiar kin" for kinship and "unsolicited" for collection method. Significant *P*-values are in bold (significance level $\alpha = 0.05$).

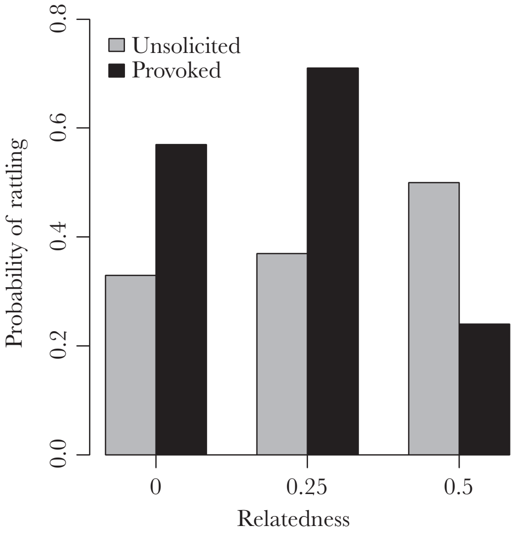

Figure 2

Probability of a rattle response from the subject squirrel during the playback period by relatedness coefficient calculated from the pedigree and the collection method of obtaining the rattle stimulus. Unsolicited rattles were recorded from squirrels moving freely around their territories ($n = 67$ trials), and provoked rattles ($n = 38$ trials) were recorded from squirrels as they emerged from a live-trap or from squirrels responding to a rattle playback.

red squirrels to the playback cannot be explained by the playback stimulus not being detected by the focal squirrels. Subject squirrels were more likely to look in the direction of the speaker following the playback and to rattle in response to the playback, though they were not more likely to approach the speaker. Squirrels, therefore, detected the experimental playbacks but showed no evidence of kin discrimination in how they responded. This absence of kin discrimination precluded us from evaluating mechanisms of kin recognition in red squirrels.

Our findings differ from 2 similar territorial playback experiments previously done on the same population of red squirrels in Kluane (Price et al. 1990; Wilson et al. 2015). The difference in findings between this study and the Wilson et al. (2015) study are surprising, given the similarity in the methods. Indeed, the only differences were the speaker used to broadcast the stimuli (a Saul Mineroff speaker in the present study and a GPX portable stereo in the previous study), the sound level of the stimuli (not measured in dB in the previous study), and the sample size of playback trials (105 trials in this study compared to 53 in the previous study). We found no effect of neighbor status, which is consistent with the results of the Wilson et al. (2015) study, but inconsistent with the Price et al. (1990) study, which found that squirrels were more likely to rattle in response to rattles from non-neighbors compared to rattles from neighbors. In the Price et al. (1990) study, relatedness between subject-stimulus pairs was unknown, and since neighbors tend to be more closely related than non-neighbors (Berteaux and Boutin 2000), it is possible that their results are due to an effect of kin discrimination (as in Wilson et al. 2015) as opposed to discrimination between neighbors and non-neighbors. We found no difference in responses to familiar kin, unfamiliar kin, and non-kin, and no overall effect of the degree of relatedness, which was unexpected given the results of the Wilson et al. (2015) study that found that squirrels were more likely to rattle in response to a non-kin ($r < 0.125$) rattle than to a kin ($r \geq 0.25$) rattle. Despite the lack of discrimination in the responses from red squirrels in this study, the results of the Wilson et al. (2015) study, as well as other documented cases of nepotism in red squirrels, provide strong support that red squirrels are capable of recognizing familiar kin (Price and Boutin 1993; Berteaux and Boutin 2000; Gorrell et al. 2010; Williams et al. 2013), even if they do not always behave differently towards kin and non-kin individuals.

Several studies have found that animals discriminate kin in some contexts, but not in others (Hokit et al. 1996; Gibbons et al. 2003; Harris et al. 2003). The benefits and costs of kin discrimination may fluctuate depending on environmental conditions, even within the same context, so it is possible that under certain conditions red squirrels do not exhibit kin discrimination in their territorial defense behavior. In an attempt to understand our negative results and to reconcile the discrepancy between our results and previous research, we conducted exploratory post hoc analyses to explore possible factors that might have affected kin discrimination. In the red squirrel system, changes in population density and food availability (abundance of spruce cones) are important environmental factors that show large fluctuations from year to year (LaMontagne and Boutin 2007; Fletcher et al. 2010) and affect survival and reproductive success in females (Descamps et al. 2008). We did not have the data to be able to account for food abundance (i.e., amount of food cached by each subject squirrel), but added local population density to our models because of previous evidence indicating that red squirrels emit rattles more frequently when surrounded by a higher density of conspecifics (Dantzer et al. 2012; Shonfield et al. 2012). We found that local density affected the difference in response between unfamiliar kin and familiar kin, but did not affect the difference in response between familiar kin and non-kin. This result did not align with our original predictions. We had expected that, if red squirrels recognize kin by phenotype matching/recognition alleles, they would be less likely to rattle in response to calls from familiar and unfamiliar kin than to calls from non-kin and, alternatively, would be less likely to rattle in response to calls from familiar kin than to calls from unfamiliar kin or non-kin if they recognize kin by prior association. As such, this model suggested that, if anything, there were density-mediated differential responses between familiar and unfamiliar kin rattles, but not differential responses between kin and non-kin. In addition, the effect of density disappeared when we ran the models with relatedness coefficients calculated from the pedigree to test for an overall effect of kin discrimination regardless of familiarity, suggesting that the interaction between density and kinship was most likely spurious resulting from post hoc exploratory data analysis.

We also explored whether the method by which rattle stimuli were recorded affected kin discrimination. Although there was no significant interaction with kinship status in our post hoc analyses, we did find a significant interaction between the degree of relatedness and collection method. We are not able to determine the importance of familiarity in kin discrimination by red squirrels, but the results of the model with relatedness coefficients suggests that when stimuli were recorded by provoking a squirrel to rattle, the subject squirrel in the trial was more likely to discriminate between kin and non-kin (i.e., more likely to rattle at non-kin). This tentatively suggests that the context in which the stimulus was recorded might be important and that kin discrimination in the overall analysis may have been masked by the difference in responses between collection methods. The interaction between collection method and kinship class was not significant (Table 5), but, in this analysis, rattles from kin with relatedness coefficients equal to 0.25 were considered to be kin a priori (see also Wilson et al. 2015). Visual inspection of the results with relatedness coefficients, however, suggests that closely related squirrels ($r = 0.5$) responded differently than more distantly related squirrels ($0 < r < 0.5$; Figure 2), which might have led to heterogeneity in the responses of squirrels to rattles classified as kin ($r \geq 0.25$). Future studies are needed to explicitly test the importance of the degree of relatedness to kin discrimination in red squirrels. These preliminary results suggest that kin discrimination by red squirrels might depend on the circumstances under which the stimulus call is recorded, and suggests that if we had run the experiment using only provoked rattle stimuli we might have detected an effect of kin discrimination. However, we must explicitly acknowledge that this relationship was identified through exploratory post hoc analyses and needs to be tested more rigorously (Simmons et al. 2011; Motulsky 2014). If these results are robust, however, they would suggest that a squirrel's physiological state might influence the structure of its rattles, including those individually distinctive structural features (Digweed et al. 2012; Wilson et al. 2015) that are presumably used in discrimination. This raises the interesting possibility that the receiver obtains information from rattles about the physiological state of the signaler and could be important in assessing the costs and benefits of discriminating kin from non-kin.

Although we found no overall evidence for kin discrimination in red squirrels, our results hint at the possibility that kin discrimination in red squirrels is context dependent. We suspect that the costs and benefits of responding to territorial intrusions by kin and non-kin might be mediated by both environmental and social factors. While we found no evidence that kin discrimination is due to the local density of potential territory intruders, we did find post hoc evidence that kin discrimination might be mediated by the conditions under which the stimulus call is recorded. Our results raise questions about the information contained in the rattles and suggest that they may reflect the current state of stress or aggressiveness of the squirrel. Future studies on kin recognition in red squirrels or other species should explicitly test the importance of environmental or social factors on kin discrimination in order to better understand the costs and benefits of preferential behavior toward kin.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Shonfield et al. (2016).

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