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Recognition of familiarity on the basis of howls: a playback experiment in a captive group of wolves

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Abstract

Playback experiments were conducted with a pack of captive Iberian wolves. We used a habituation–discrimination paradigm to test wolves' ability to discriminate howls based on: (1) artificial manipulation of acoustic parameters of howls and (2) the identity of howling individuals. Manipulations in fundamental frequency and frequency modulation within the natural range of intra-individual howl variation did not elicit dishabituation, while manipulation of modulation pattern did produce dishabituation. With respect to identity, across trials wolves habituated to unfamiliar howls by a familiar wolf (i.e., no direct contact, but previous exposure to howls by this wolf), but not to unfamiliar howls from unfamiliar wolves (i.e., no direct contact and no previous exposure to howls by these wolves). Modulation pattern seems to be an important bioacoustic feature for individual recognition. Overall, our results provide the first experimental evidence that wolves can discriminate individuals based on the acoustic structure of their howls.

Keywords

Canis lupus, howls, individual discrimination, playback, acoustic structure.

1. Introduction

In animals that use vocalizations for social communication, the selective advantage provided by being able to identify and locate distant individuals could promote the evolution of individual identity acoustic signals (Rendall et al., 1996). This would allow individuals to recognize each other by their vocalizations when the transmission of signals in other sensory modalities is

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constrained (Falls, 1982). Penguins, for instance, identify their kin in dense colonies using vocal signatures (Searby et al., 2004), domestic sheep (*Ovis aries*) can recognize their ewes based on their calls (Searby & Jouventin, 2003), Seba's short-tailed fruit bats (*Carollia perspicillata*) recognize their pups based on the individual signature in isolation calls (Knörnschild et al., 2013), and individual recognition has been experimentally shown in dwarf mongooses (*Helogale parvula*) using contact calls (Sharpe et al., 2013).

For group-living animals, the ability to individually recognize group members at a distance is an important adaptation given that, when individuals belonging to the same group are separated, recognizing and maintaining contact with specific individuals can be challenging (Bradbury & Vehrencamp, 2010). Wolves (*Canis lupus*) are group-living canids whose basic social unit is the pack, comprised basically of a mated pair and their offspring (Packard, 2003). Wolf packs occupy wide territories, ranging in Europe from 100 to 500 km^2 (Boitani, 2000). Wolves belonging to the same pack often do not travel together (Demma & Mech, 2009), and it has been suggested that maintaining a loose cohesion with pack mates while travelling separately could increase the chances of finding scattered food sources (Palacios & Mech, 2011). Furthermore, maintaining contact with other pack members is also crucial during territory defence as intra-specific aggression is one of the main causes of natural mortality in wolf populations, and each pack actively defends its own territory from neighbouring packs (Mech & Boitani, 2003). Thus, wolf packs live under conditions that would seem to promote the evolution of individual vocal recognition.

Howls are arguably the most conspicuous wolf vocalizations. Functions attributed to howling often involve some sort of communication among individuals belonging to the same pack. For example, it has been proposed that howls can serve to reunite pack mates in situations where they have been separated (Harrington & Asa, 2003). Howls can also be used as territorial displays, conveying information about pack location and minimizing contact between different packs (Harrington & Mech, 1979). Given these proposed functions, we predict that howls are ideally suited to serve as signals allowing individual recognition. In fact, it has been shown that howls contain information on individual identity (Tooze et al., 1990; Palacios et al., 2007). In Iberian wolves, the acoustic parameters that best discriminate among individuals are howl fundamental frequency and frequency modulation (Palacios et al., 2007). However, the fact that the acoustic structure of howls contains information regarding identity does not imply that wolves use these features to discriminate individuals. Although the ability for discriminating vocalizations from different individuals seems widespread, studies that investigate the acoustic parameters involved in discrimination are rare (Bee & Gerhardt, 2001; Charrier et al., 2003; Searby & Jouventin, 2003).

The purpose of this study was to investigate the acoustic basis of individual discrimination in wolves. We used playback experiments to assess the ability of wolves to discriminate between: (1) familiar howls before and after manipulation of acoustic parameters previously shown to encode individual information (fundamental frequency and frequency modulation; Palacios et al., 2007) and (2) familiar howls to which they had been habituated, unfamiliar howls from a familiar wolf (i.e., the sender of the howl they had been habituated to), and unfamiliar howls from unfamiliar wolves (i.e., no previous exposure to any of its howls). Our experimental design was based on the habituation–discrimination paradigm (Friedman, 1972), which is based on the premise that when subjects habituated to repeatedly presented stimuli respond more intensely to a new stimulus, they perceive it as different from the ones used for habituation (Shettleworth, 2010). This is one of the methods commonly used to evaluate the ability of animals to discriminate among different stimuli and, in particular, it has been used to examine individual recognition in various species such as leopard geckos, *Eublepharis macularius* (LaDage & Ferkin, 2006), bullfrog, *Rana catesbeiana* (Bee & Gerhardt, 2001), red deer, *Cervus elaphus* (Reby et al., 2001), yellow-bellied marmots, *Marmota flaviventris* (Blumstein & Daniel, 2004), giant pandas, *Ailuropoda melanoleuca* (Charlton et al., 2009), rhesus monkeys, *Macaca mulatta* (Rendall et al., 1996) and little brown bats, *Myotis lucifugus* (Kazial et al., 2008).

2. Methods

Playback experiments were conducted in 2010 (from November–December) in Senda Viva Park (Navarra, Spain), a privately-owned nature preserve that houses several species of birds and mammals. We studied a pack of seven Iberian wolves (four adult males and three adult females) held in captivity in a 3320 m^2 enclosure. Three wolves (a male and female siblings and an unrelated female) had been hand reared by the park staff, while the remaining four wolves were from a different litter and had not been tamed. All the wolves were habituated to human presence.

The experimental design was based on the habituation–discrimination paradigm (Friedman, 1972): the subject is initially habituated by repeated exposure to stimulus A before a dishabituation stimulus of type B is presented. During habituation, the response to stimulus A decreases. A restoration in the level of response to stimulus B implies an ability to discriminate between stimuli of type A and B. Finally, a re-habituation stimulus is provided by a re-exposure to a stimulus of type A. A level of response similar to that obtained before the dishabituation stimulus can again be considered as evidence of discrimination between the habituation (A) and the dishabituation (B) stimuli.

Our playback experiments comprised two phases: habituation and habituation–dishabituation (Figure 1). The stimulus played during the habituation phase (familiar stimulus (FS)) was always the same. The response of the

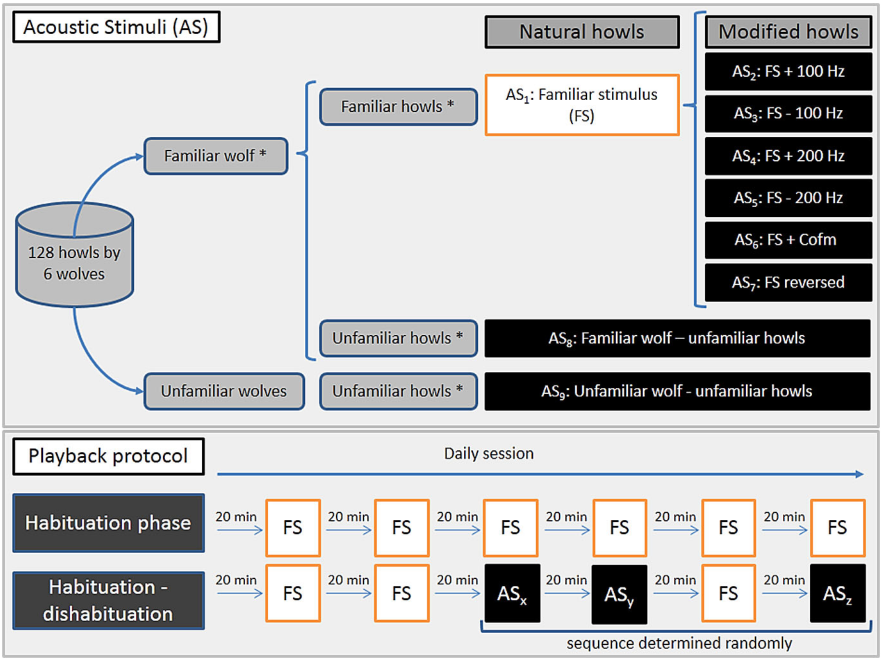


Figure 1. Acoustic stimuli created (upper panel) and playback protocol conducted (lower panel). *From our sample of recorded howls (emitted by six wolves unrelated to Senda Viva's wolves) we randomly selected a wolf (Familiar Wolf) to create the habituation stimulus (Familiar Stimulus) to habituate the group of captive wolves to its howls. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

wolves determined the duration of the habituation phase. In order to maintain the habituation, during the habituation–dishabituation phase the first two stimuli broadcast in a session were always FS. After the first two FS, four additional stimuli were presented in random order. One of these stimuli was again the FS, while the remaining three stimuli were either modified FS howls, unfamiliar howls from the same FS wolf, or unfamiliar howls from an unfamiliar (i.e., no previous exposure to any of its howls) individual (Figure 1).

2.1. Playback stimuli

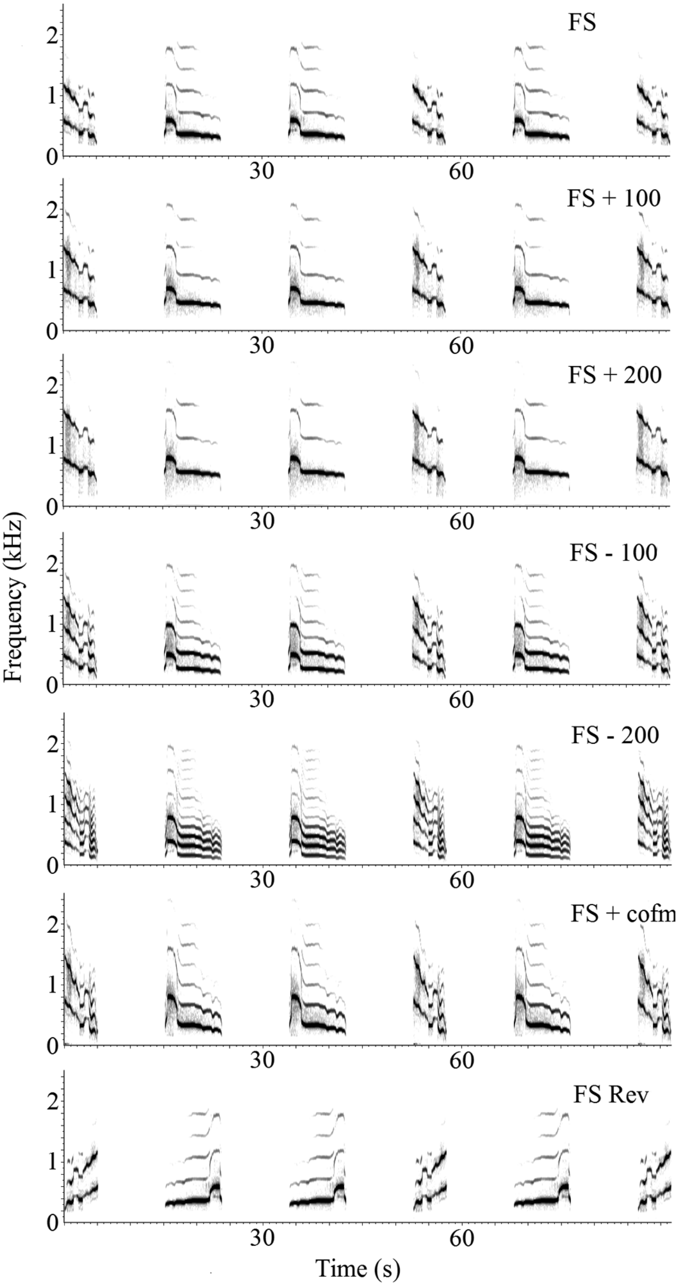
Each acoustic stimulus used for playback consisted of a series of six howls (two howls emitted by the same wolf repeated three times in a randomized order) separated by intervals of 10 s of silence. This interval between consecutive howls is within the range of howl series emitted by wild Iberian wolves (V. Palacios, unpublished data). All the stimuli were created from recordings of 128 howls emitted by six captive wolves unrelated and unknown to the Senda Viva wolves. The amplitude of all the howls was peak normalized using Adobe Audition, version 3.0. We generated nine different acoustic stimuli; three of them included natural howls and the other six were comprised of modified howls.

2.1.1. Natural howls

We created the habituation stimulus (FS) from two natural howls emitted by the same wolf (hereafter ‘familiar wolf’), where both individual and howls were randomly selected from our sample of recorded howls (Figures 1 and 2). The sequence of the howls in FS was randomly selected as well. To investigate whether wolves can distinguish howls emitted by different individuals we also created two different stimulus categories with natural howls: (1) different howls emitted by the familiar wolf (for each stimulus we randomly selected two howls from the familiar wolf different from those included in FS and repeated the howls three times in a random order); and (2) howls emitted by wolves different from the familiar wolf (i.e., each new stimulus created from two howls emitted by a randomly selected wolf different from the familiar wolf; hereafter ‘unfamiliar wolves’) (Figure 1).

2.1.2. Modified howls

To investigate the role of fundamental frequency and frequency modulation in individual discrimination we used stimuli created modifying the howls


FS
FS + 100
FS + 200
FS - 100
FS - 200
FS + cofm
FS Rev
Frequency (kHz)
0
1
2
30
60
Time (s)

from FS (Figures 1 and 2). We used Praat software (version 5.2.08, available online at http://www.fon.hum.uva.nl/praat/) to modify acoustic parameters of howls included in FS. To evaluate the wolves' ability for discriminating changes in the fundamental frequency we created four stimuli, adding or subtracting 100 or 200 Hz to FS (FS+100, FS+200, FS−100 and FS−200) (Praat's procedure: Sound manipulate — To manipulation — Shift pitch frequencies). To determine the magnitude of frequency variation in the modified howls we considered the range of intraindividual variation found in captive Iberian wolves (Palacios et al., 2007). The average range of mean fundamental frequency for howls emitted by the same wolf was 153 Hz. Thus, a 100 Hz variation lies within the intraindividual variation observed, while 200 Hz exceeds the intraindividual variation observed for Iberian wolf howls. We also generated two artificial stimuli that differed from FS in the frequency modulation (Figures 1 and 2). As with the frequency, we considered the intraindividual variation observed for Iberian wolves (Palacios et al., 2007). The average range for the coefficient of frequency modulation of howls emitted by one wolf found in the same sample was 1.92. To produce stimuli with altered frequency modulation, first we generated a stimulus increasing the coefficient of frequency modulation of howls but maintaining the same fundamental frequency as the familiar stimulus (FS+cofm). We increased the fundamental frequency (×2) and subtracted the frequency necessary to obtain the same mean fundamental frequency as FS (Praat's procedure: Sound manipulate — To manipulation — Multiply pitch frequencies — Shift pitch frequencies). This yielded stimuli with coefficients of frequency modulation 0.87 and 0.96 larger than the original howls and with the same mean value of the fundamental frequency (Figure 2). The second stimulus was generated reversing the original howls (FS Rev) (Praat's procedure: Sound modify — Reverse). Thus, we obtained stimuli with the same fundamental frequency and the same coefficient of frequency modulation, but differing from the original howls in the way the frequency was modulated along the howl (Figure 2). These modifications altered only the fundamental frequency and the coefficient of frequency modulation, but duration of howls remained the same.

Figure 2. Familiar stimulus and playback stimuli created modifying the acoustic structure of howls. FS, Familiar Stimulus; FS+100, +200, −100 and −200, familiar stimulus increasing and decreasing the fundamental frequency by 100 or 200 Hz; FS+cofm, FS increasing the coefficient of frequency modulation; FS rev, FS reversing the howls.

2.2. Playback procedure

We conducted a daily session consisting of six trials interspersed by 20-min intervals, beginning at 08:00 (Figure 1). Acoustic stimuli were played back using a digital sound player (Maxtronics mp5 player, 4 GB) attached to an Anchor Explorer Pro amplified speaker (frequency range 0.08–16 kHz; output power 60 W RMS). The stimuli scheduled to be played during each playback session were recorded in a .WAV file using Adobe Audition version 3.0. Each session started with 20 min of silence followed by the six stimuli with 20 min of silence intervals. The first 20 min allowed the observer to enter the hide, minimizing the effect of the observer's presence on the wolves' behaviour. The speaker was located 30 m away from the wolves' enclosure (north side), inside a plastic container that protected it from adverse weather conditions (Figure 3). The container was installed three days before the beginning of the experiments to habituate the wolves to its presence.

To adjust the equalization of the speaker, FS and an audio file of white noise were played six times with six different equalization settings and

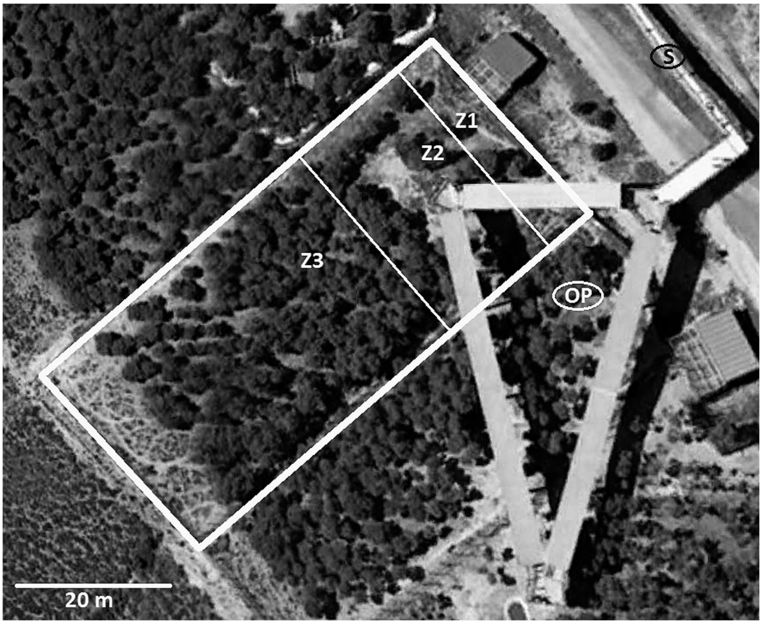


Figure 3. Wolf enclosure sketch. Z1 and Z2, zones where the wolves were visible from the observation point; Z3, area where wolves could not be observed; OP, observation point; S, speaker.

recorded 5 m away from the speaker using a unidirectional Sennheiser MK66 microphone with a K6 power unit (Sennheiser Electric, Wedemark, Germany) attached to a portable solid state recorder Marantz PMD 660 (Marantz, Mahwah, NJ, USA). We generated spectrograms and conducted spectrogram correlations with the original stimuli using Raven Pro 1.4. The largest correlations were obtained with the bass control set in the middle of the range (set to 5) and treble control at 0, and we used this equalization during the playback experiments. Volume level was adjusted to ensure the stimuli were audible by the wolves anywhere inside the enclosure.

2.3. Behavioural observations

Behavioural observations were conducted from a hide installed in a vantage observation point that provided visual access to part of the wolves' enclosure (Figure 3). We divided the enclosure into three zones: zone 1 (250 m^2, the zone closest to the speaker used to broadcast the experimental stimuli); zone 2 (750 m^2, adjacent to zone 1); and zone 3 (2320 m^2, the rest of the enclosure). When the wolves were in zone 3, they could not be observed from the observation point. The visible area was divided into zones 1 and 2 to assess whether the wolves' position changed with respect to the sound source following exposure to the different acoustic stimuli. The subject in each trial was the group of wolves within sight during the tests, and we included in the analyses only trials in which at least four wolves were in zones 1 or 2. To quantify the wolves' response we scored the behaviour of all observable wolves 5 min before and 10 min after the beginning of the stimulus (ad libitum sampling), using a 1-0 recording rule (Lehner, 1996). In addition, the location of the wolves was recorded at the end of every 30-s interval. During the three days preceding the playback experiments, the observer (VP) learned to recognize the wolves individually based on morphological characteristics such as fur design and the presence of distinctive marks (e.g., ear clips, scars).

We selected 11 behaviours to be recorded based on our prior experience with wolves responding to human imitations of howls or howl recordings (Table 1). During the habituation phase we examined the behaviours displayed to determine which followed a habituation pattern and could be considered estimators of the wolves' response to the stimulus. The only behaviour that fulfilled this requirement was Attention (a wolf turns its head towards the speaker and remains with its ears raised and eyes and outer ears

Table 1.
Empirical descriptions of the behaviour categories recorded during the playback experiments.

Behaviour	Description
Attention	Wolf turns head towards the speaker and remains with raised ears, eyes and outer ears facing the speaker during at least 1 s.
Lie down	Wolf lies down without interacting with other pack members.
Approach	Wolf approaches the sound source (i.e., speaker).
Move away	Wolf moves away from the speaker. We define 'move away' as to avoid or escape as reported in the wolf ethogram in Goodmann et al. (2002).
Other directions	Wolf moves in directions other than towards or away from the speaker.
Agonistic behaviours	Threat, aggression and attack behaviours as reported in the wolf ethogram (Mech, 1970; Goodmann et al., 2002), such as beat, show teeth, chase other wolf with tail above the back level, etc.
Submissive behaviours	Defence and submissive behaviours as reported in the wolf ethogram (Mech, 1970; Goodmann et al., 2002), such as escape, inguinal offer, submission (tail between hind legs), active and passive submission, etc.
Greeting	Form of active submission involving two or more pack mates, in which subordinates nip, lick and smell the mouth of the dominants (Mech, 1970). Characterized by ears oriented backward, muzzle–muzzle contacts, tail wagging and, sometimes, whining (Goodmann et al., 2002).
Scratching	Wolf scratches ground with the paws, moving rapidly the front legs, the hind legs or both backwards, displacing vegetation, soil or other material.
Raised leg displays	Raised leg urination (RLU) and raised leg display (RLD) (Goodmann et al., 2002; Harrington & Asa, 2003).
Vocalization	Wolf emits a vocalization. We considered the four vocal types reported by Harrington & Mech (1978): growl, whine, bark and howl.

facing the speaker during at least 1 s). Therefore, following an exploratory analysis of the behaviours displayed by the wolves during the habituation phase, we defined a 'positive response' to the stimulus as at least one wolf showing the behaviour pattern Attention and the 'level of response' in a trial as the number of Attention displayed by all the wolves within sight during the 2 min following the onset of the stimulus. For each trial we considered only one value of level of response taking into account the number of Attention displayed by all the wolves within sight (instead of a level of response for each wolf) to avoid errors due to a wolf exhibiting Attention as a consequence of imitating other pack mates rather than a real interest in the acoustic stimulus.

2.4. Statistical analyses

We conducted binomial tests to analyse the response of wolves to the stimuli. Binomial tests have been used extensively to study discrimination of acoustic stimuli in many taxa such as whooping cranes, *Grus americana* (Fitch & Kelley, 2000), cotton top tamarins, *Saguinus oedipus* (Weiss & Hauser, 2002) and gibbons (Raemaekers & Raemaekers, 1985). Our null hypothesis was that the probability of response (at least one Attention recorded) to a stimulus and lack of response (no Attention recorded) are the same ($p = 0.5$). Thus, if the null hypothesis is rejected, we conclude that the wolves are habituated to the stimulus (absence of response) or, conversely, that the new stimulus leads to a dishabituation (positive response). Response dependence of differences in acoustic parameters was tested by linear regression. To test whether wolves were able to distinguish unfamiliar howls from familiar vs. unfamiliar wolves, we fitted a GLM with a Poisson error distribution and ‘group size’ (number of wolves within sight during the playback trial) as a covariate to specifically compare how the ‘level of response’ (i.e., summation of Attention displayed by all the wolves within sight) to these two types of unfamiliar howls varied across playback trials.

We conducted complementary analyses in order to control for the potentially important variation in individual responses. We pooled data on individual responses within each trial to calculate, for each trial, the number of times the wolves exhibited a response to playbacks (number of Attention). We then fitted a GLMM with number of Attention as the response variable (Poisson error distribution), ‘group size’ and ‘treatment’ (stimulus) as fixed factors, and trial as a random factor. Finally, we fitted a second GLMM using a binomial error structure on non-pooled data to analyse responses at the individual level by using Attention as a binary response variable (i.e., whether a given individual responded or not to a playback in a given trial), ‘group size’ and ‘treatment’ as fixed factors, and trial and ‘individual’ as a random factors. Analyses controlling for the group size effect included all the trials (group size 1–7). Statistical analyses were made with SPSS (12.0) for Windows (SPSS, Chicago, IL, USA) and the R statistical package (R Development Core Team, 2013).

3. Results

We conducted 178 playback trials, 16 during the habituation phase and 162 during the habituation–dishabituation phase (eight trials were excluded due to adverse weather conditions that we judged could affect the results). Of these, 101 were considered valid trials, i.e., at least four wolves were in zones 1 or 2 (10 trials during the habituation phase and 91 trials during the habituation–discrimination phase).

At the beginning of the habituation phase, during the 2 min following the beginning of the stimulus, the wolves displayed only three behaviours apparently as a response to the stimulus: Attention, Approach and Greeting (see Table 1 for descriptions of behaviours). Of these, Attention was the only behaviour that followed a habituation pattern (Figure 4). The frequency of this behaviour decreased during the habituation phase until eventually no wolf showed Attention as a response to the stimulus. The habituation phase concluded after three sessions, because at the end of the third session no wolf exhibited any of these three behaviours as a response to the stimulus.

3.1. Response to modified howls

During the habituation–dishabituation phase, the wolves showed different responses to the different stimuli (Table 2, Figure 4). There was essentially no response to FS during the last four daily trials (Table 2, Figure 4). When stimuli with modified fundamental frequency were played, the number of trials in which the wolves responded (at least one Attention) was no different from those in which they did not respond ($p = 0.5$) (Table 2). However, the first time FS+200 and FS−200 were presented, the level of response was greater than for the rest of trials involving stimuli with modified frequency (the behaviour pattern Attention was recorded 5 and 9 times, respectively). In fact, the first time FS−200 was presented the level of response was similar to that elicited by the first FS trial during the habituation phase. Thereafter, the level of response decreased, probably due to a fast habituation. When stimuli with modified frequency modulation were played, the response was different depending on the type of stimulus (Table 2, Figure 4). While the response to FS+cofm was largely absent, every time FS Rev was played the wolves showed Attention, with the level of response decreasing in successive experiments probably as a result of habituation.

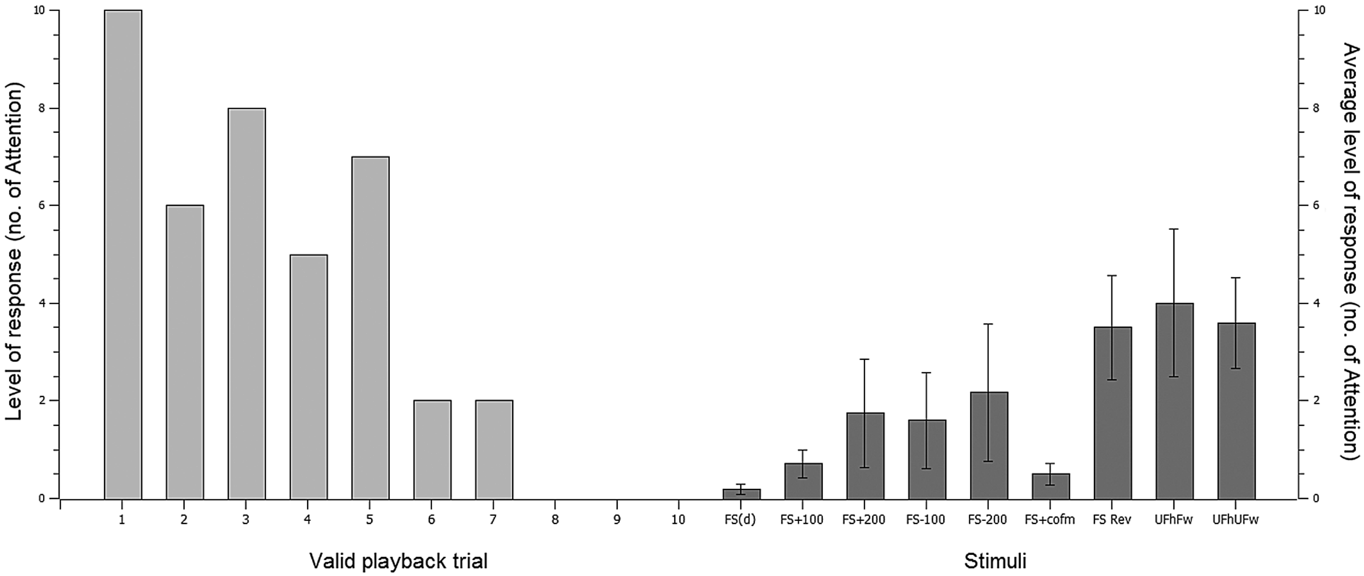


Figure 4. Level of response (number of Attention displayed by all the wolves within sight) during the two min following the beginning of the stimulus. (Left) Level of response obtained to the Familiar Stimulus (FS) in the valid trials ($N = 10$) during the habituation phase. (Right) Average level of response (mean ± SE number of Attention) for each stimulus taking into account all the valid trials. FS (d), FS during the habituation–dishabituation phase; FS+100, +200, −100, −200: FS adding or subtracting 100 or 200 Hz; FS+cofm, FS manipulating only the coefficient of frequency modulation; FS Rev, FS with the howls reversed; UFhFw, unfamiliar howls by the familiar wolf; UFhUFw, unfamiliar howls by unfamiliar wolves.

Table 2.
Response of wolves to different stimuli.

Stimulus	No. of valid trials	Positive response	Binomial test p	Average level of response (mean ± SD)
FS (h)	7^{a}	7	0.008*	5.71 ± 2.98
FS (d)	16^{b}	3	0.009*	0.19 ± 0.40
FS+100	7	4	0.3	0.71 ± 0.76
FS+200	4	3	0.3	1.75 ± 2.22
FS−100	5	2	0.3	1.60 ± 2.19
FS−200	6	4	0.2	2.17 ± 3.43
FS+cofm	6	3	0.3	0.50 ± 0.55
FS Rev	6	6	0.02*	3.50 ± 2.59
UF howls by F wolf	6	6	0.02*	4.00 ± 3.69
UF howls by UF wolves	5	5	0.03*	3.60 ± 2.07

FS (h), familiar stimulus during the habituation phase; FS (d), familiar stimulus during the habituation–dishabituation phase; FS+100, +200, −100, −200, FS adding or subtracting 100 or 200 Hz; FS+cofm, FS manipulating only the coefficient of frequency modulation; FS Rev, FS with the howls reversed; positive response, number of valid trials where the stimulus elicited a positive response (at least one wolf displayed Attention); average level of response, average number of Attention displayed by all the wolves within sight, taking into account the valid trials; UF, unfamiliar; F, familiar.

[a] Habituation phase, only valid trials with positive response considered (total no. of valid trials = 10).

[b] Only FS included in the last four daily trials, because first and second daily stimuli ($N = 30$) were played to reinforce habituation.

* $p < 0.05$.

3.2. Individual discrimination

The wolves discriminated between FS and unfamiliar howls from both the same familiar individual or from unfamiliar wolves (Table 2, Figure 4). We found a strong treatment $*$ playback trial interaction (likelihood Chi test between model with and without the interaction term: differential residual deviance $= -7.522$, $p = 0.0061$). This shows that the response to unfamiliar howls from the familiar wolf, but not to unfamiliar howls from unfamiliar wolves, decreased across playback trials following a habituation pattern (Figure 5). To investigate which acoustic parameter elicited a greater response, we calculated the mean fundamental frequency and the coefficient of frequency modulation of the howls included in the different stimuli. The response to howls from unfamiliar wolves was most intense when the difference between the coefficient of frequency modulation of the first howl of the

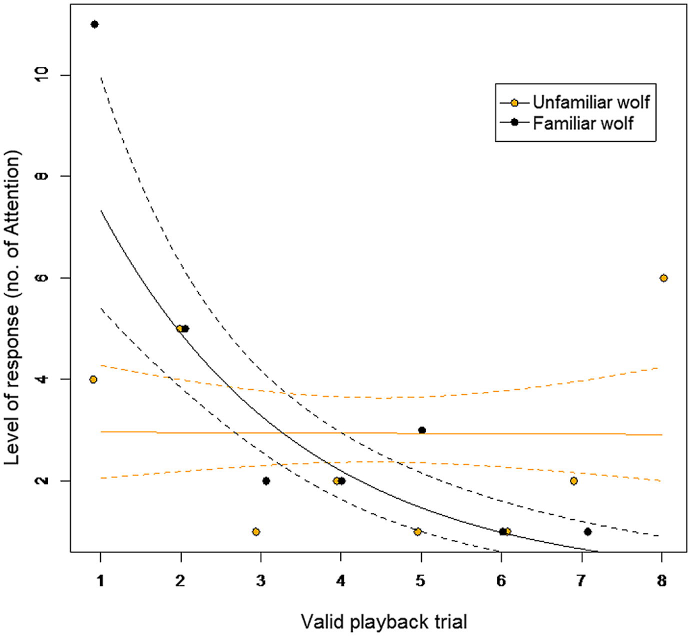


Figure 5. Level of response (i.e., number of Attention; see Methods) of wolves to unfamiliar howls emitted by a familiar wolf or by unfamiliar wolves. Solid lines reflect predicted values for each treatment, while dashed lines show the standard error interval (GLM model: number of attention – treatment $*$ playback trial $+$ group size; Poisson error distribution). Note that random jitter has been introduced in the x axis to distinguish data points from both treatments. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

stimulus and the coefficient of frequency modulation of the first howl of the familiar stimulus was greatest (linear regression: $R^2 = 0.84$, $F_{1,3} = 15.71$, $N = 5$, $p = 0.03$). Regressions of the level of response and differences in the fundamental frequency of howls and the coefficient of frequency modulation for the familiar wolf howls had slopes not significantly different from zero.

3.3. Group size effect

GLMMs controlling for the effect of the different number of wolves within sight across trials (group size effect) confirmed the results above. As expected, we found a significant effect of group size (i.e., number of wolves in sight) on the level of response (number of times Attention was registered responding to a playback), the level of response increased with the number of wolves within sight ($\chi^2 = 8.01$, $p = 0.005$). We also found a highly significant treatment effect ($\chi^2 = 70.28$, $p < 0.001$) on the level of response. Post-hoc Tukey comparisons showed significant differences in the level of response between the following treatment levels: a lower response to FS playbacks than to unfamiliar wolf howls (estimate $= -2.74$, $z = -4.65$, $p < 0.001$), unfamiliar howls from the familiar wolf (estimate $= -2.80$, $z = -4.77$, $p < 0.001$), FS-200 playbacks (estimate $= -2.25$, $z = -3.74$, $p < 0.01$) and FS rev playbacks (estimate $= -2.71$, $z = -4.61$, $p < 0.001$). Tukey comparisons did not detect any significant differences between the latter four treatments. Our second GLMM examining the probability that a given wolf responded or not to a playback yielded equivalent results. As expected, in this case we did not find a significant group size effect on the probability that a given wolf responded to a playback ($\chi^2 = 1.48$, $p = 0.223$), but we found a highly significant treatment effect ($\chi^2 = 58.48$, $p < 0.001$) that was again driven mainly by a significantly lower probability of responding to a FS playback than to unfamiliar wolf howls (estimate $= -3.27$, $z = -4.98$, $p < 0.001$), unfamiliar howls from the familiar wolf (estimate $= -3.10$, $z = -4.81$, $p < 0.001$) or FS rev playbacks (estimate $= -2.77$, $z = -4.25$, $p < 0.001$). Again, Tukey comparisons did not detect any significant differences between the latter three treatments. In this case, no differences between FS and FS$-$200 were found (estimate $= -1.99$, $z = -2.96$, $p = 0.07$).

4. Discussion

Our results show that wolves detected changes in the fundamental frequency of howls outside their natural range of variability, and changes in the frequency modulation pattern of howls. In contrast, manipulations of fundamental frequency and coefficient of frequency modulation within the natural range of intra-individual variation did not elicit a response. Furthermore, wolves detected differences between different howls, even between unfamiliar and familiar howls emitted by the same individual that they were previously familiarized with. Finally, wolves showed habituation to all new stimuli when presented repeatedly, with the exception of responses to unfamiliar calls of unfamiliar wolves.

Playback experiments are a useful tool to investigate whether animals can distinguish the identity of conspecifics by means of vocalizations, as has been reported in the African elephant, *Loxodonta africana* (McComb et al., 2000), banded wren, *Thryothorus pleurostictus* (Molles & Vehrencamp, 2001), barking foxes, *Alopex lagopus* (Frommolt et al., 2003) and black-capped chickadees, *Poecile atricapillus* (Wilson & Mennill, 2010). Using this methodology, it has been shown that domestic dogs distinguish between barks by the same individual emitted in two different contexts and between different individuals which barked in the same context (Molnár et al., 2009). In wolves, playback experiments have been used to investigate the ability of pups and juveniles to discriminate between different sounds (Shalter et al., 1977), to determine whether adult wolves distinguish between pup and adult howls (Harrington, 1986), and to study variation in the responsiveness of wild wolves to unfamiliar individuals (Gazzola et al., 2002).

We are aware of the methodological problems inherent to playback experiments, including pseudoreplication and reduced external validity (Searcy, 1989; McGregor, 2000; Kroodsma et al., 2001). Wolf social organization and spatial ecology make it difficult to conduct playback studies in the wild, and there are few captive packs of wolves with appropriate installations and adequate handling and management conditions. Frommolt et al. (2003) carried out playback experiments with eight barking foxes to investigate individual recognition, McComb et al. (2000) conducted playback on 29 independent groups of elephants, and Molnár et al. (2009) conducted his study on 30 dogs. In contrast, the effective sample size in our study is much lower and, to complicate things even further, the levels of response recorded in different trials are not statistically independent. However, we believe the results presented here are suggestive and could be considered a first step in the demonstration of individual discrimination based on howl acoustic structure in wolves.

Our results also show that wolves discriminate manipulations in the acoustic structure of howls. Experimental signal alteration has been previously applied to assess the cues used for recognition (Tibbetts & Dale, 2007). Changes in the fundamental frequency within the observed range of intraindividual variation induced low levels of response, while the response to changes of 200 Hz (exceeding the observed intraindividual variation) was suggestive of dishabituation the first time the stimulus was played. Although this ability for discriminating differences in frequency exceeding natural signal variation has been reported for other species such as bullfrogs, *Rana catesbesianas* (Bee & Gerhardt, 2001), this is the first time it has been tested with wolves. The fundamental frequency is one of the acoustic parameters that best discriminates individuals in some species such as giant pandas (Charlton et al., 2009), manatees (Sousa-Lima et al., 2002) and wolves (Tooze et al., 1990; Palacios et al., 2007). However, in some species it seems that this variable is less important for individual recognition, possibly because it changes according to the motivational state of the sender (Charlton et al., 2009). In the case of wolves, there is substantial intraindividual variation in the fundamental frequency of howls and it has also been reported that the motivational state of the sender can influence the fundamental frequency of howls (Harrington, 1987). This could explain that changes in the fundamental frequency within the intraindividual range elicited low levels of response.

Increases in the coefficient of frequency modulation within the observed intraindividual range of variation for adult wolf howls did not elicit a response. However, the reversed stimulus induced dishabituation to FS. Frequency modulation does not change during sound propagation, and therefore seems particularly well suited for individual recognition over long distances, or in noisy environments (Slabbekoorn et al., 1998; Charrier et al., 2003). In fact, it has been reported that frequency modulation is important for individual recognition in some species. For example, frequency modulation is crucial for parent-offspring recognition in seals (Charrier et al., 2003), and for individual recognition in macaroni penguins (Searby et al., 2004). In our study, wolves discriminated changes in modulation pattern, suggesting that the frequency modulation pattern may play an important role in wolf acoustic communication and probably in individual recognition.

The results of the playback experiments reported here provide the first evidence that wolves can discriminate among howls according to their acoustic structure. Furthermore, the response to unfamiliar howls emitted by the familiar wolf decreased across trials following a habituation pattern. However, repeated exposure to equally unfamiliar howls emitted by unfamiliar wolves did not result in habituation, and was more intense for wolves emitting howls that differed in the coefficient of frequency modulation with respect to the familiar howls. That wolves discriminate between a familiar wolf and unfamiliar individuals does not necessarily imply that true individual recognition actually exists. Although there is a strong basis for predicting that many species may be able to discriminate between individuals, to determine empirically whether animals are able to identify individuals and which cues they use to do so is difficult (Thom & Hurst, 2004). Wolves produce individually distinct howls (Tooze et al., 1990; Palacios et al., 2007), and the habituation pattern occurred only when unfamiliar howls by the familiar wolf were broadcast, suggesting that wolves treated the familiar wolf uniquely. However, individual recognition is a more complex cognitive capacity than just discrimination, requiring the matching of a specific stimuli with an internally-held template or 'representation' of that individual (Tibbetts et al., 2008). Additional experiments may provide crucial insights to understand the role of acoustic communication in wolf's recognition.

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References

Bee, M.A. & Gerhardt, H.C.C. (2001). Neighbour-stranger discrimination by territorial male bullfrogs (*Rana catesbeiana*): II. Perceptual basis. — Anim. Behav. 62: 1129-1140.

Blumstein, D.T. & Daniel, J.C. (2004). Yellow-bellied marmots discriminate between the alarm calls of individuals and are more responsive to calls from juveniles. — Anim. Behav. 68: 1257-1265.

Boitani, L. (2000). Action plan for the conservation of the wolves (*Canis lupus*) in Europe, Nature and environment, No. 113. — Council of Europe, Strasbourg.

Bradbury, J.W. & Vehrencamp, S.L. (2010). Principles of animal communication, 2nd edn. — Sinnauer Associates, Sutherland, MA.

Charlton, B.D., Huang, Y. & Swaisgood, R.R. (2009). Vocal discrimination of potential mates by female giant pandas (*Ailuropoda melanoleuca*). — Biol. Lett. 5: 597-599.

Charrier, I., Mathevon, N. & Jouventin, P. (2003). Vocal signature recognition of mothers by fur seal pups. — Anim. Behav. 65: 543-550.

Demma, D.J. & Mech, L.D. (2009). Wolf use of summer territory in Northeastern Minnesota. — J. Wildl. Managem. 73: 380-384.

Falls, J.B. (1982). Individual recognition by sounds in birds. — In: Acoustic communication in birds, Vol. 2 (Kroodsma, D.H. & Miller, E.H., eds). Academic Press, New York, NY, p. 237-278.

Fitch, W.T. & Kelley, P. (2000). Perception of vocal tract resonances by whooping cranes *Grus americana*. — Ethology 106: 559-574.

Friedman, S. (1972). Habituation and recovery of visual response in the alert human newborn. — J. Exp. Child Psychol. 13: 339-349.

Frommolt, K.-H.H., Goltsman, M.E. & Macdonald, D.W. (2003). Barking foxes, *Alopex lagopus*: field experiments in individual recognition in a territorial mammal. — Anim. Behav. 65: 509-518.

Gazzola, A., Avanzinelli, E., Mauri, L., Scandura, M. & Apollonio, M. (2002). Temporal changes of howling in south European wolf packs. — Ital. J. Zool. 69: 157-161.

Goodmann, P.A., Klinghammer, E. & Willard, J. (2002). Wolf ethogram (Revised 2002). — Eckhard H. Hess Institute of Ethology, Battle Ground, IN.

Harrington, F.H. (1986). Timber wolf howling playback studies: discrimination of pup from adult howls. — Anim. Behav. 34: 1575-1577.

Harrington, F.H. (1987). Agressive howling in wolves. — Anim. Behav. 35: 1575-1577.

Harrington, F.H. & Asa, C.S. (2003). Wolf communication. — In: Wolves: behavior, ecology, and conservation (Mech, L.D. & Boitani, L., eds). University of Chicago Press, Chicago, IL, p. 66-103.

Harrington, F.H. & Mech, L.D. (1978). Wolf vocalization. — In: Wolf and man: evolution in parallel (Hall, R.L. & Sharp, H.S., eds). Academic Press, New York, NY, p. 109-132.

Harrington, F.H. & Mech, L.D. (1979). Wolf howling and its role in territory maintenance. — Behaviour 68: 207-249.

Kazial, K.A., Kenny, T.L. & Burnett, S.C. (2008). Little brown bats (*Myotis lucifugus*) recognize individual identity of conspecifics using sonar calls. — Ethology 114: 469-478.

Knörnschild, M., Feifel, M. & Kalko, E.K.V. (2013). Mother-offspring recognition in the bat *Carollia perspicillata*. — Anim. Behav. 86: 941-948.

Kroodsma, D.E., Byers, B.E., Goodale, E., Johnson, S. & Liu, W.-C.C. (2001). Pseudoreplication in playback experiments, revisited a decade later. — Anim. Behav. 61: 1029-1033.

LaDage, L. & Ferkin, M. (2006). Male leopard geckos (*Eublepharis macularius*) can discriminate between two familiar females. — Behaviour 143: 1033-1049.

Lehner, P.N. (1996). Handbook of ethological methods. — Cambridge University Press, Cambridge.

McComb, K., Moss, C., Sayialel, S. & Baker, L. (2000). Unusually extensive networks of vocal recognition in African elephants. — Anim. Behav. 59: 1103-1109.

McGregor, P.K. (2000). Playback experiments: design and analysis. — Acta Ethol. 3: 3-8.

Mech, L.D. (1970). The wolf: the ecology and behavior of an endangered species. — The Natural History Press, Garden City, NY.

Mech, L.D. & Boitani, L. (2003). Wolf social ecology. — In: Wolves: behavior, ecology, and conservation (Mech, L.D. & Boitani, L., eds). University of Chicago Press, Chicago, IL, p. 1-34.

Molles, L.E. & Vehrencamp, S.L. (2001). Neighbour recognition by resident males in the banded wren, *Thryothorus pleurostictus*, a tropical songbird with high song type sharing. — Anim. Behav. 61: 119-127.

Molnár, C., Pongrácz, P., Faragó, T., Dóka, A. & Miklosi, A. (2009). Dogs discriminate between barks: the effect of context and identity of the caller. — Behav. Proc. 82: 198-201.

Packard, J.M. (2003). Wolf behavior: reproductive, social, and intelligent. — In: Wolves: behavior, ecology, and conservation (Mech, L.D. & Boitani, L., eds). University of Chicago Press, Chicago, IL, p. 35-65.

Palacios, V. & Mech, L.D. (2011). Problems with studying wolf predation on small prey in summer via global positioning system collars. — Eur. J. Wildlife Res. 57: 149-156.

Palacios, V., Font, E. & Márquez, R. (2007). Iberian wolf howls: acoustic structure, individual variation, and a comparison with North American populations. — J. Mammal. 88: 606-613.

R Development Core Team (2010). R: a language and environment for statistical computing. — R Development Core Team, Vienna.

Raemaekers, J.J. & Raemaekers, P.M. (1985). Field playback of loud calls to gibbons (*Hylobates lar*): territorial, sex-specific and species-specific responses. — Anim. Behav. 33: 481-493.

Reby, D., Hewison, M., Izquierdo, M. & Pepin, D. (2001). Red deer (*Cervus elaphus*) hinds discriminate between the roars of their current harem-holder stag and those of neighbouring stags. — Ethology 107: 951-959.

Rendall, D., Rodman, P.S. & Emond, R.E. (1996). Vocal recognition of individuals and kin in free-ranging rhesus monkeys. — Anim. Behav. 51: 1007-1015.

Searby, A. & Jouventin, P. (2003). Mother-lamb acoustic recognition in sheep: a frequency coding. — Proc. Roy. Soc. Lond. B: Biol. Sci. 270: 1765-1771.

Searby, A., Jouventin, P. & Aubin, T. (2004). Acoustic recognition in macaroni penguins: an original signature system. — Anim. Behav. 67: 615-625.

Searcy, W.A. (1989). Pseudoreplication, external validity and the design of playback experiments. — Anim. Behav. 38: 715-717.

Shalter, M.D., Fentress, J.C. & Young, G.W. (1977). Determinants of response of wolf pups to auditory signals. — Behaviour 60: 98-114.

Sharpe, L.L., Hill, A. & Cherry, M.I. (2013). Individual recognition in a wild cooperative mammal using contact calls. — Anim. Behav. 86: 893-900.

Shettleworth, S.J. (2010). Cognition, evolution, and behavior, 2nd edn. — Oxford University Press, New York, NY.

Slabbekoorn, H. & ten Cate, C. (1998). Perceptual tuning to frequency characteristics of territorial signals in collared doves. — Anim. Behav. 56: 847-857.

Sousa-Lima, R.S., Paglia, A.P. & Da Fonseca, G.A.B. (2002). Signature information and individual recognition in the isolation calls of Amazonian manatees, *Trichechus inunguis* (Mammalia: Sirenia). — Anim. Behav. 63: 301-310.

Thom, M.D. & Hurst, J.L. (2004). Individual recognition by scent. — Ann. Zool. Fenn. 41: 765-787.

Tibbetts, E.A. & Dale, J. (2007). Individual recognition: it is good to be different. — Trends Ecol. Evol. 22: 529-537.

Tibbetts, E.A., Sheehan, M.J. & Dale, J. (2008). A testable definition of individual recognition. — Trends Ecol. Evol. 23: 356.

Tooze, Z.J., Harrington, F.H. & Fentress, J.C. (1990). Individually distinct vocalizations in timber wolves, *Canis lupus*. — Anim. Behav. 40: 723-730.

Weiss, D.J. & Hauser, M.D. (2002). Perception of harmonics in the combination long call of cottontop tamarins, *Saguinus oedipus*. — Anim. Behav. 64: 415-426.

Wilson, D.R. & Mennill, D.J. (2010). Black-capped chickadees, *Poecile atricapillus*, can use individually distinctive songs to discriminate among conspecifics. — Anim. Behav. 79: 1267-1275.