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**ACOUSTIC BEHAVIOUR OF TWO LARGE TERRESTRIAL
MAMMALS IN RELATION TO RESOURCES MAINTENANCE AND
MATING SYSTEMS: WOLF (*Canis lupus*) AND RED DEER (*Cervus
elaphus*) AS MODEL SPECIES**

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To my parents

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SUMMARY

The high variability of vocalization is due to their function and meaning, the habitat where they evolved and the physical constraints of the emitters. The aim of this study were to analyse the acoustic behaviour of two large terrestrial mammals, wolf (*Canis lupus*) and red deer (*Cervus elaphus*), in relation to resources maintenance and mating system. In the first part, I analysed the Italian wolf howl. I found two forms of howl, as reported for American populations. Both types of howl are uttered within the lowest frequencies of the wolf's vocal range, confirming the acoustic adaptation hypothesis, and thus, that low frequencies are more useful for long distance communication. Moreover, I found a group vocal signature into the free ranging wolf packs. This characteristic, shared with other mammals with a complex social structure, is constant over the time, both within biological season and between two consecutive years. In the second part of the thesis, I analysed the acoustic structure of male mating calls in red deer. I provided a detailed description of the vocal repertoire of free-ranging Iberian red deer males, and then I quantified variation of the most commonly vocalized call type -the roar- of three free ranging subspecies, defining the acoustic parameters that differentiate these subspecies. Iberian red deer (*C. e. hispanicus*) repertoire consists of four call types and shows peculiar aspects, as the presence of the "short common roar", a call type completely absent in the repertoire of the others red deer subspecies. Among red deer subspecies exists a strong vocal divergence. Bioacoustic distances showed different degrees of diversification in the roar: *C.e. hippelaphus* present the highest *intra* population variability, followed by *C. e. corsicanus* and *C. e. hispanicus*. Between subspecies, the highest bioacoustic differences were found between *C. e. hispanicus* and *C. e. corsicanus* while this last population and *C.e. hippelaphus* are the most related subspecies. These differences suggest the presence of a strong selective pressure promoting the divergence of the same signals in closely related taxa.

Keywords: acoustic behaviour, wolf, red deer, howl, roar, group signature, acoustic variation, bioacoustic.

RIASSUNTO

I segnali acustici sono altamente variabili. Tale variabilità è data dalla loro funzione e significato, dall'habitat nel quale evolvono e dalle condizioni fisiche dell'individuo che emette il segnale. Scopo del presente studio è stato analizzare il comportamento acustico di due grandi mammiferi terrestri, il lupo (*Canis lupus*) e il cervo (*Cervus elaphus*), in relazione alla difesa delle risorse e ai sistemi riproduttivi.

Nella prima parte di questo studio, ho analizzato l'ululato del lupo appenninico. Come nelle popolazioni nordamericane sono presenti due tipi di ululato. Entrambi i tipi sono emessi entro le frequenze più basse della gamma vocale del lupo, confermando "l'ipotesi dell'adattamento acustico" e cioè, che le basse frequenze risultano maggiormente funzionali per la comunicazione su lunga distanza. Inoltre, nei branchi di lupo è presente un'impronta vocale di gruppo. Questa caratteristica, condivisa con altri mammiferi con una complessa struttura sociale, è costante sia all'interno della stessa stagione riproduttiva sia tra stagioni riproduttive di anni diversi.

Nella seconda parte della tesi, ho analizzato la struttura acustica delle vocalizzazioni di cervo durante il periodo riproduttivo. Qui, ho presentato una dettagliata descrizione del repertorio acustico del cervo iberico ed ho quantificato la variabilità nella vocalizzazione più comune di tre differenti sottospecie, definendo i parametri acustici che differenziano queste popolazioni. Il repertorio del cervo iberico (*C. e. hispanicus*) consiste di quattro principali vocalizzazioni e mostra aspetti peculiari, come la presenza del "short common roar", un tipo di segnale assente nel repertorio delle altre sottospecie. Vi è una forte divergenza vocale tra le sottospecie prese in considerazione. Le distanze bioacustiche mostrano diversi livelli di variabilità nel bramito: *C. e. hippelaphus* mostra la più alta variabilità tra individui, seguito da *C. e. corsicanus* e da *C. e. hispanicus*. Tra sottospecie invece, la maggior distanza bioacustica si ha tra *C. e. hispanicus* and *C. e. corsicanus* mentre quest'ultimo e *C. e. hippelaphus* sono acusticamente i più affini. Queste differenze suggeriscono la presenza di pressioni selettive che promuovono la divergenza dello stesso segnale tra taxa vicini.

INTRODUCTION

Acoustic signals play an important role in animal communication (Hopp et al. 1998). In cooperative or competitive contexts (i.e. territoriality, resources defenses, mating behavior) acoustic communication conveys messages instantaneously and across relative long distances. As other behavioral characteristics, they evolved as result of physical and habitat constraints and in dependence of their function. One of the benefits of acoustic signals is the high degree of variation within each sound type, which enables animals to express variation in meaning (Harrington and Asa 2003).

One of the main constraints affecting acoustic variation in vocal behaviour is the environment (Wiley and Richards 1978). As predicted by the “**the acoustic adaptation hypothesis**” (Morton 1975) habitat differences might cause selection for signal divergence, because different frequencies of sound travel best in different environments or because different habitats have different types of ambient noise (Morton 1975; Wiley and Richards 1982; Baker 2006; Nicholls and Goldizen 2006). Sound pressure and intensity of acoustic signals usually attenuate at rates in excess of the 6 dB per doubling of distance predicted by the inverse square law of attenuation (Wiley and Richards 1978). This attenuation is due to absorption by the atmosphere (scattering by vegetation or atmospheric turbulences), and structure of the habitat (obstacles between the source and the receivers). Moreover, sound attenuation is frequency dependent having these factors their major effects on high-frequency sounds (Konishi 1970; Morton 1975; Wiley and Richards 1978). Over longer distances, signals showed stronger attenuation for both habitat types and transmission heights (Maciej et al. 2011): specifically, at the same amplitude, the higher frequencies of a signal are subject to greater attenuation (travel for much shorter distance) than the lower frequencies in a variety of environments (Konishi 1970; Morton 1975). Acoustic signals evolved for long distance communication are therefore constrained and affected by conveying information with the minimum levels of attenuation, thus is not surprising that, across the repertoire of different mammals species,

the lowest frequencies of their vocal range are involved in long distance communication (elephant: McComb et al. 2000; coyote: Mitchell et al. 2006).

Vocalizations also have the potential to vary between individuals and groups: individually distinct vocalizations, or vocal signatures, have been identified in many social mammals (Symmes et al. 1979; Gould 1983; Conner 1985; Tooze et al. 1990). Group signatures have been generally found in long-distance call variants that work to maintain contact between group members (Townsend et al. 2010). Many group-living mammals are known to produce stereotyped group-specific calls and such group signatures are thought to play a role in territory defence or mate choice (bats: Boughman; cetaceans: Tyack et al. 2000; meerkats: Townsend et al. 2010).

The wolf (*Canis lupus*) is a markedly socially gregarious species, the pack is its basic social unit (Mech 1970) and much of their social behaviour is accompanied by vocalizations (Harrington and Asa 2003). Wolf pups vocalize within hours of birth (Coscia et al. 1991) and adult wolves' vocal repertoire is wide. Wolf vocal signals have been classified either into harmonic and noise sounds according to the context, thus ranging from submissive and friendly to aggressive signals (Shassburger 1987; 1993) or, in relation to the active space, as short-range and long-range calls (Harrington and Mech 1978a).

The howl is the main long-range vocalization (Harrington and Asa 2003). It is important in both intra and inter-pack communication and has several functions. Within a wolf pack, howling may be useful to promote the joining of members (Mech 1966; Theberge and Falls 1967) and to communicate information on individual identity and location (Theberge and Falls 1967; Tooze et al. 1990). Among packs, howling serves to advertise territory ownership and occupation, thus minimizing contact among them (Joslin 1967; Harrington and Mech 1979; Harrington and Asa 2003). This characteristic has been used to develop one of the main monitoring techniques for this elusive species: the "wolf-howling technique" (Pimlot 1960), consisting in the stimulation of resident wolves by playback of howls. It is based on the tendency of wolves to answer to foreign howls (Joslin 1967) to avoid the encounters with the intruders in their own territory (Harrington and

Mech 1979; Harrington and Asa 2003). This method was employed in several studies involving either wolf pack censuses (Harrington and Mech 1982; Fuller and Sampson 1988) or the acquisition of howling data from captive (Frommolt 1999; Palacios et al. 2007) and wild wolves (Harrington and Mech 1978b; 1979; Harrington 1987).

Studies on captive wolves (Tooze et al. 1990; Palacios et al. 2007) showed individual variation of their howling and the fundamental frequency and its modulation were found to be the most effective variable to distinguish individuals (Tooze et al. 1990). However, until now, the hypothesis of group vocal signature in wolves has not been tested.

Despite to solve the same function, call types can differ considerably in closely related species (Mendelson and Shaw 2005) and even in populations within the same species (Proëhl et al. 2006), according to biological constraints operating on their production and perception (Taylor and Reby 2010). Howls of the American wolf populations have been described in two forms: flat, i.e., scarcely modulated, and breaking, i.e., highly modulated and often discontinuous (Harrington and Mech 1978a; Harrington and Mech 1982). However, two other forms have been recently described in captive Iberian wolf: “continuous wavy” and “breaking wavy” howls (Palacios et al. 2007). The Italian Wolf (*Canis lupus italicus* Altobello 1921) is one among six European subspecies, as confirmed by means of molecular analysis (Nowak and Federoff 2002). Two of the main physical characteristics of Italian wolves are their lower weight (25-35 kg for adult male) and smaller size (110-148 cm, tail excluded) than North American and Central European populations (Ciucci and Boitani 1998). These two features are very important parameters as regards the vocalizations, especially when low frequencies are involved (Morton 1977).

Therefore, the aims of this **first part** of this study were: to describe for the first time the structure of howls in a free ranging Italian wolf population, to determine how many howl types characterize the vocal repertoire of the Italian wolf (**Chapter 1**) and to test the hypothesis that free-ranging wolf packs may have a group-specific vocal signature (**Chapter 2**).

As territorial, long distance calls, mating signals also have evolved to maximize the transmission distance in their specific habitat (Hunter and Krebs 1979) and to maximize the signal's localization accuracy (Campbell et al. 2010). However, the main function of sexual signals is to attract potential mates, encoding (or cheating) information on the individual's physical quality and its social status (Morton 1975; McElligott and Hayden 1999; Brumm and Naguib 2009).

Mating signals are obviously subject and forged by sexual selection (Semple and McComb 2000): female mate choice is often based on male courtship signals and local preferences for certain mate traits (Endler and Houde 1995; Griffith et al. 1999) may drive the evolution of different characteristics in sexual signals, both within than between species (Claridge and Morgan 1993; Proëhl et al. 2006, Yu et al. 2011).

The acoustic structure of male mating calls in the red deer (*Cervus elaphus*) is a clear illustration of this variability. This species is divided in several geographic subspecies (for a list see Ludt et al. 2004), and it is characterised by strong vocal activity of the stags during the mating season with a high degree of diversity among geographic subspecies.

During the mating season (from August to October, in the northern hemisphere) stags (adult red deer males) actively defend groups of females, called harem, or compete for and defend territories that contain females (Clutton-Brock and Albon 1979). Only in this season, stags are highly vocal and give several different types of calls, both towards females and male competitors (Clutton-Brock and Albon 1979).

Complexity of vocal behaviour of red deer is increased by the double components of this calls, that it is composed by two main features. The fundamental frequency and the formant frequencies are produced and perceived independently (Fitch and Hauser 2002) and described in the **source-filter theory** for the first time (Fant 1960; Titze 1994, Taylor and Reby 2010). The fundamental frequency is determined by the rate of the vibration of the vocal folds originated by the passage of air through the closed glottis into the larynx (the source); the formant frequencies are resonance peaks produced as the glottal wave travels through the supra-laryngeal vocal tract (filter). While first investigations

considered formants as a human prerogative, further studies found formants as widespread features of the animal calls (Hauser 1993; Fitch 1997; Rendall et al. 1998; Riede and Fitch 1999). Formant frequencies and overall formant dispersion (the frequency spacing between the formants) decrease as the length of the vocal tract (the distance between larynx and lips or nostril) increases (Fitch 1997). The length of the vocal tract is likely to be constrained by body size, hence formants characteristics typically transmit a more reliable indication of caller's physical stature than fundamental frequency (Fitch 1997, Riede and Fitch 1999) and are responsible for individual signature in many mammals species (monkey: Fitch 1997; fallow deer: Reby et al. 1998, Vannoni and McElligot 2007; red deer: Kidjo et al. 2008).

Red deer males have a descended larynx (Fitch and Reby 2001) and are able to drop their highly mobile larynx further down towards the sternum, increasing their vocal tract length (and thus decreasing formant frequencies and spacing) while they vocalize (Fitch and Reby 2001, Reby and McComb 2003a,b). This anatomical adaptation gives to receivers an exaggerated impression of their actual body size, increasing the chances to attract mates and deter competitors (Reby and McComb 2003a). Iberian stags not only have a descended and mobile larynx enabling them to extend their vocal tract during vocalising (Frey et al. 2012) but they also protrude their tongue during the majority of their rutting roars (Frey et al. 2012), increasing the vocal tract length.

Contrary to formants frequencies, several studies suggest that the fundamental frequency is not a reliable index of body size in red deer (Reby and McComb 2003a) as well as in other mammals (Lass and Brown 1978; Rendall et al. 2005), suggesting that very different selection pressures (i.e. habitat constraints, local female preferences) have affected the evolution of this call feature.

The vocal repertoire (different types of calls) of red deer changes among the investigated subspecies: five distinct vocalizations (the common roar, the harsh roar, the grunt roar, the chase bark series and the longer loud bark) were identified in Scottish red deer (Reby and McComb 2003b) while, only common roars and chase bark were found in Corsican deer stags (Kidjo et al.

2008). Finally, only the long common roar has been described in Iberian red deer repertoire (Frey et al. 2012) and thus, the complete Iberian stag repertoire still remain mainly unknown.

All these studies describe the common roar, a tonal sound with well-defined harmonics, as the most common vocalization in the stag's repertoire. However, none of these works attempted to quantify variation in this call, geographic or otherwise.

Therefore, the aims of this **second part** of the study were to use acoustic analyses and automated classification techniques to describe in detail the vocal behaviour of male Iberian red deer in a free-ranging population (**Chapter 3**) and to quantify geographic variation in the most commonly vocalized call type -the common roar- of three free ranging populations: *C. e. hispanicus*, *C. e. corsicanus* and *C.e. hippelaphus* defining the acoustic parameters that differentiate these populations (**Chapter 4**).

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FIRST PART

ACOUSTIC BEHAVIOUR OF WOLF IN RELATION TO RESOURCES MAINTENANCE



Chapter 1

The acoustic structure of wolf howls in some eastern Tuscany (central Italy) free ranging packs.

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THE ACOUSTIC STRUCTURE OF WOLF HOWLS IN SOME EASTERN TUSCANY (CENTRAL ITALY) FREE RANGING PACKS

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ABSTRACT

Italian wolf howls are described for the first time from observations between 2003–2008 of a population living in eastern Tuscany, central Italy. A sample of 37 howls selected among single responses and 128 howls included in the choruses of 7 free ranging packs was recorded and analysed. The mean fundamental frequency of the howls ranged between 274–908 Hz. Two main structures recognised by means of multivariate explorative analysis, in particular Principal Component and Cluster Analysis, were ascribed to breaking and flat howls. Discriminant Function Analysis was applied to the recognised groups with the aim to find a general rule for classification. Howls with different features were correctly assigned to the groups obtained by explorative analysis in 95.8% of cases. The analysis of the variables characterising the structure of the howls suggests that maximum frequency and range of fundamental frequency are the most important parameters for classification, while duration does not appear to play any significant role.

Keywords: *Canis lupus*, acoustic structure, mammal communication, sonogram, fundamental frequency.

INTRODUCTION

Acoustic signals play an important role in animal communication (Hopp *et al.* 1998). In cooperative as well as competitive contexts, acoustic communication conveys messages rapidly, across long distances and without any physical or visual contact. One of the benefits of acoustic signals is the high degree of variation within each sound type, which enables animals to express variation in meaning (Harrington & Asa 2003). Alarm calls can provide important information about the presence and nature of predators (Melchior 1971) and enable the

receivers to choose the appropriate defence reaction (Sherman 1977; Seyfarth *et al.* 1980; Hoogland 1983). Acoustic communication can also be used to estimate the rivals' size and decide whether or not to fight (Davies & Halliday 1978; Clutton-Brock & Albon 1979; Reby & McComb 2003).

In the wolf *Canis lupus*, a markedly socially gregarious species, the pack is the basic social unit (Mech 1970) and much of their social behaviour is accompanied by vocalizations (Harrington & Asa 2003). Wolf pups vocalize within hours of birth (Coscia *et al.* 1991) and adult wolves' vocal repertoire is wide. Wolf vocal signals have been classified either into short-range and long-range calls (Harrington & Mech 1978a) or into harmonic and noise sounds according to the context, thus ranging from submissive and friendly to aggressive signals (Schassburger 1987; 1993). This last is a graded classification, with whine and growl being listed at its extremes. Other vocalizations are listed as whimper and yelp (in submissive and friendly contexts); snarl, woof and bark (in aggressive contexts); moan (not reported in nature). All of them are used in short-range communication (Schassburger 1993).

The howl is the main long-range vocalization (Harrington & Asa 2003). It is important in both intra and inter-pack communication and has several functions. Within a wolf pack, howling may be useful to promote the joining of members (Mech 1966; Theberge & Falls 1967) and to communicate information on individual identity and location (Theberge & Falls 1967; Tooze *et al.* 1990). Among packs, howling serves to advertise territory ownership and occupation, thus minimizing contact among them (Joslin 1967; Harrington & Mech 1979; Harrington & Asa 2003). Packs are more likely to respond and stand their ground when they are at a fresh kill or accompanied by relatively small pups; in fact, carcasses and pups are resources to be guarded on the spot (Harrington & Mech 1979; Harrington & Asa 2003).

Previous papers have considered the behavioural and ecological issues of wolf howling in relation to the defence of resources and aggressive behaviour (Harrington 1987; Harrington & Mech 1979; 1983), to timing of wolf activity (Harrington & Mech 1978b; Gazzola *et al.* 2002; Nowak *et al.* 2007), or to wolf pack census technique (Harrington & Mech 1982; Fuller & Sampson 1988). Some studies considered the acoustic structure of North American wolf howls (Theberge & Falls 1967; Harrington & Mech 1978a; Harrington 1989; Tooze *et al.* 1990); while others, carried out in Eurasia, with the exception of Nikolskii *et al.* (1986) and Nikolskii & Frommolt (1989) that were performed in nature, were limited to captive wolves (Nikolskii & Frommolt 1985; Schassburger 1987; 1993; Frommolt 1999; Palacios *et al.* 2007) and failed to account for the actual influence of captivity on vocalizations (McCarley 1978).

The fundamental frequency (F0) of adults' howls ranges between 150 Hz and more than 1,000 Hz (Theberge & Falls 1967; Harrington & Mech 1978a; Harrington 1989; Tooze *et al.* 1990), which is usually the dominant frequency (Theberge & Falls 1967; Shassburger 1993). The low frequencies and the structure of howls are useful features for long-distance acoustic communication (Harrington & Asa 2003).

The wolf is one of the most widely distributed land mammals. It inhabits all the vegetation types of the Northern hemisphere and environments as different as forests and prairies, tundra, barren ground, mountains, deserts and swamps. Subsequently, the wolf shows high morphological and genetic variability, which accounts for its classification into numerous sub-species (Wayne & Vilà 2003). The Italian Wolf *Canis lupus italicus* is one among six European subspecies, as recently confirmed by means of molecular analysis (Nowak & Federoff 2002). Two of the main physical characteristics of Italian wolves are their lower weight (25–35 kg for adult male) and smaller size (110–148 cm, tail excluded) when compared with North American and Central European populations (Ciucci & Boitani 1998). These two features are also very important parameters as regards the vocalisation process, especially when low frequencies are involved (Morton 1977). Tonal and shape variables, like frequency attributes and modulation, determine – and account for the description of – the structure of howls.

Howls have been described in two forms: flat, i.e., scarcely modulated, and breaking, i.e., highly modulated and often discontinuous (Harrington & Mech 1978a; Harrington & Mech 1982). However, two other forms have been recently described in captive Iberian wolf: continuous wavy and breaking wavy howls (Palacios *et al.* 2007).

This study investigates for the first time the structure of howls in a free ranging Italian wolf population exhibiting inter-pack communication. This is the first study to be conducted on the howls of this subspecies; indeed, it is one of the few studies made on free-ranging European wolves (see also Nikolskii *et al.* 1986; Nikolskii & Frommolt 1989). Therefore, the aims of this paper are: a) to describe the howls of the Italian wolf population and b) to determine how many howl types characterise the Italian wolf.

MATERIALS AND METHODS

Study area

The study area was the province of Arezzo (3,230 km²) in eastern Tuscany, Italy. Altitude ranges between 300 and 1654 m a.s.l. Forests are dominated by deciduous trees and cover about 54% of the area.

Cultivated land and pasture represent 42% of the area and the urban settlement accounts for only 4% of it.

Along this portion of the Apennines, wolves have progressively declined throughout the first half of the last century. In the years of the lowest recorded levels of the Italian wolf population (1950–1970), only a few individuals were reported in these areas (Cagnolaro *et al.* 1974) and only since the early 1990s has the wolf population recovered (Mattioli *et al.* 1995; Scandura *et al.* 2001; Apollonio *et al.* 2004a; Mattioli *et al.* 2004), as a direct consequence of specific conservation laws.

The spatial distribution and reproductive success of wolf packs were monitored from 1998 by means of wolf howling, snow tracking, and molecular analysis in the whole province of Arezzo (Scandura *et al.* 2001; Gazzola *et al.* 2002; Apollonio *et al.* 2004b; Scandura 2005; Capitani *et al.* 2006; Scandura *et al.* 2006). During the field study period (2003–2008), the number of wolf packs ranged from 7 to 11, while the pack size ranged from 2 to 8 individuals.

Data collection and sound analysis

To study wolf vocalizations, we followed the “wolf howling” technique, used for the first time by Pimlott (1960) and consisting in the stimulation of resident wolves by tape-recorded playback of wolf howls. This method was employed in several studies involving either wolf pack censuses (Harrington & Mech 1982; Fuller & Sampson 1988) or the acquisition of howling data from captive (Frommolt 1999; Palacios *et al.* 2007) and wild wolves (Harrington & Mech 1978b; 1979; Harrington 1987).

Wolf howling was performed in summer (from June to October), when the pack activity is focused in a restricted area (home-sites) and the rate of response is consequently higher (Harrington & Mech 1978b; 1979; 1983; Gazzola *et al.* 2002; Nowak *et al.* 2007).

Sampling sites were chosen to cover the whole study area: the approach described as “saturation census” by Harrington and Mech (1982) was adapted to local requirements, mainly dictated by the mountainous topography. Sampling sites were chosen to maximize the range of audibility and minimize sound dispersion and their location and number was such as to completely cover the study area. Each session for eliciting howling was a continuous 15 minute-period. If after the first playback stimulus no answer followed, a second trial was attempted 3 minutes later, after which the operators left the site. However, if there was a response we repeated one or more trials from a place closer to the presumed site of response to obtain higher quality recordings.

Concurrent sessions were performed by two groups of operators so as to verify the effective presence of two adjacent packs. We followed the standard procedure suggested by Harrington & Mech (1982). In particular: i) no session was conducted during rain or strong wind; ii) wolf howling was performed overnight, to minimize the noise related to human activities; iii) two trials, the first one lower in volume, were conducted per site. To standardize the stimulus to the wolves, howls were elicited by playback of recorded chorus howls of a captive wolf pair (duration: 1min, 29 sec).

Audio recording were made with a Marantz CP 430 cassette tape recorder from 2003 to 2006. The recordings were digitised using Raven Pro 1.3 (Cornell Laboratory of Ornithology) with 44,100 Hz sampling rate and 16 bits resolution and saved in “.wav” format. From 2007 we used a M-Audio Microtrack 24/96 handheld digital recorder, keeping the same audio file parameters and format as above. All vocalizations were recorded with a Sennheiser ME67 directional microphone with windshield. Only good quality recordings were used for spectrographic visualizations.

Each answer was classified on the basis of the number of vocalising individuals, as either “choral response” (two or more responding individuals) or “single response” (one responding individual). Since the aim of this study was to characterize howls only, the whimpers, barks and growls that often occurred in the choral responses (Mech 1966; Joslin 1967; Harrington & Mech 1978b; McCarley 1978) were excluded from the analysis. From 2003 to 2008 we analysed 37 howls extracted among the single responses by 3 subjects, and 128 howls belonging to the choruses of 7 packs.

We analysed the vocalizations with Raven Pro 1.3 using Discrete Fourier Transformation (2,048 DFT samples, Hanning window, 21.5 Hz frequency grid, 10 ms time step, 37.5 Hz bandwidth). For each howl the fundamental frequency (F0) was sampled every 0.05 seconds with the cursor, using both spectrogram and spectral views (following Tooze *et al.* 1990; Palacios *et al.* 2007). From the analysis of the collected data, 11 variables were obtained and considered useful for a complete investigation of the howls’ structure (Table 1). Resolutions and variables were consistent with those used in previous works on wolf vocalizations (Tooze *et al.* 1990; Palacios *et al.* 2007). Amplitude parameters and number of harmonic overtones were not considered since they generally depend on the distance between the animals and the recording site (Harrington & Mech 1978a; McCarley 1978).

Statistical analysis

Normal probability plots were used to explore the variable set, with the aim of characterising the shape of their frequency distribution and

TABLE 1
Variables used in the howl characterisation

Pitch variables	Meanf	Mean of the fundamental frequency calculated every 0.05 seconds (Hz)
	Rangef	Difference between maximum and minimum frequencies (Hz)
	Minf	Minimum frequency of the fundamental one (Hz)
	Maxf	Maximum frequency of the fundamental one (Hz)
	Endf	Frequency at the end of the fundamental one (Hz)
Shape variables	Duration	Duration of the howl (s)
	Posmin	Position at which the minimum frequency occurs (time of Minf/Duration) in the howl
	Posmax	Position at which the maximum frequency occurs (time of Maxf/Duration) in the howl
	Cofv	Coefficient of frequency variation (SD/Meanf) \times 100
	Cofm	Coefficient of frequency modulation $\sum f(t) - f(t+1) (n-1) / \text{Meanf} \times 100$
	Abrupt	Number of sudden abrupt changes in frequency (>25 Hz)

correctly choosing central tendency and variability statistics. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis were both used to plot the howl's variance-covariance structures and grouping tendency in a multivariate context.

PCA is a technique able to reduce the original number of variables, forming new uncorrelated variables which are linear composites of the original ones. Only pitch variables and duration were used in order to avoid distortion and to reduce the weight of potential outliers. Hierarchical cluster analysis was carried out to identify relatively homogeneous groups of cases by using the same variables of PCA. Ward's method was used to link groups to each other, while the Euclidean square distance was chosen as a similarity measure. A range of solutions from 2 to 4 clusters was saved in order to investigate the meaning of the multilevel structure of groupings.

Due to the high covariance, plots of the scores of the two principal components represented a fundamental tool to explore the data matrix, particularly when the cases could be discriminated by considering the attribution to the previous cluster solution. In this construction, a cross tabulation procedure clearly showed the nested structure of the clustering process, particularly when different cluster solutions were compared in relation to their relative information. Discriminant Function Analysis (DFA) was used to build a predictive model of group membership based on the 2-cluster solution by using scores of the two principal components as independent variables. Bivariate normality of the groups was checked through Mardia's multivariate test, while equivalence of the covariance matrices was checked through Box's M test. Values of *t* and *Chi* square tests were

computed to explore differences between the two groups, in relation to all pitch and shape variables, and to determine their univariate role in discrimination. Finally, because of the non-homogeneous character of the samples, a Mann-Whitney *U* test was used to compare single and choral howls, pooling data before testing to avoid pseudoreplication (McGregor *et al.* 1992). Statistical analysis was computed with SPSS version 13 (Chicago, Illinois, USA) for Windows and Past version 1.85 (Hammer *et al.* 2001).

RESULTS

Analysed howls show a mean fundamental frequency (F0) value range between 274–908 Hz. F0 of the howls ranges from 21–1,033 Hz, while duration ranges from 0.75–9.55 seconds. The coefficients of frequency variation and modulation spans in the intervals 0.84–45.44 and 0.16–9.09, respectively. Inflexion points of the fundamental (abrupt) range are included in the interval 0–18. The position of the maximum frequency shows a lognormal distribution, so that the maximum F0 occurs during the first quarter in most cases (73% of the howls), while the minimum F0 occurs in the last quarter of the howls in 59% of the sample.

The structure of the howls was determined using PCA and Hierarchical Cluster Analysis as explorative tools. In our sample of 165 howls, PCA indicated that when the two eigenvectors with the highest associated eigenvalues were used, it explained 80% of the total variance.

The first component accounted for about 42% of the variability and was found to correlate with range, maximum of F0 and mean frequency. The second component accounted for about 38% of the variability and was found to correlate with duration (inversely) and minimum and end of F0 (Table 2). A plot of the scores of the two principal components with data discriminated by using a two-cluster solution showed a clear separation which was primarily ascribed to the first component (Figure 1a); on the other hand, plots marked by a three (graph not reported) or four-cluster solution (Figure 1b) displayed separations predominantly due to the second component, as well as a partial overlap between said groups.

Cross tabulation clearly showed the nested structure of the clustering process and, in particular, that cluster 1 tended to include cluster 4, while cluster 2 included cluster 3 completely (Table 3). By taking into account this result and considering that i) most variability was due to the first component and ii) a strict relationship between second component and duration was observed, a two-cluster solution level was preferred to determine the general acoustic structure of recorded howls.

TABLE 2

Values of the variables loads along the two axes generated by Principal Component Analysis with their explained variance. Rangef, Maxf and Meanf are the most important variables to generate the first component (Factor 1) that explain 42% of variability, while Minf, Endf and Duration primarily influenced the second component (Factor 2), explaining 38% of variability. All variables were log-transformed and Varimax rotation procedure applied (for abbreviations see Table 1).

Variables	Factor 1 (42%)	Factor 2 (38%)
Rangef	0.982	-0.136
Maxf	0.933	0.253
Meanf	0.738	0.491
Minf	0.270	0.740
Endf	0.232	0.728
Duration	0.172	-0.942

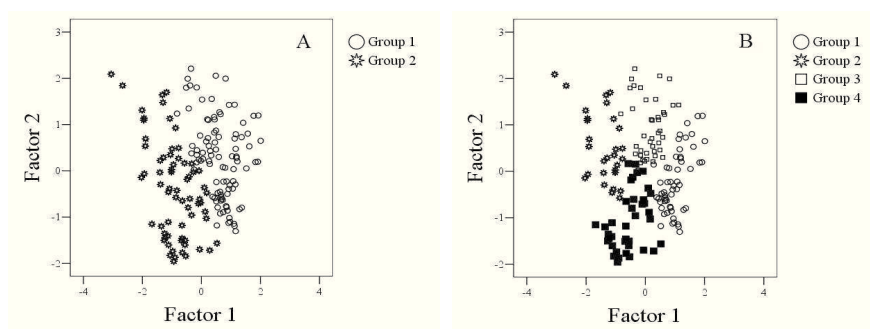


Figure 1. A) Plot of howls grouped by using a 2-clusters solution. Factor 1 and 2 derived by PCA results. Differences between groups of howls are mainly due to the first component -factor 1- (characterized by maximum and range of fundamental frequency). B) Plot of howls grouped by using a 4-clusters solution. Howls in groups 3 and 4 are separated along the second component-factor 2-(characterized primary by duration).

In order to generate a probabilistic rule whereby individual cases could be assigned to the natural groups that had been identified *a priori*, a Discriminant Function Analysis (DFA) was performed. The scores of the first and second components were used as independent variables and the bivariate normality (Table 4) as well as the equivalence of the variance-covariance matrices verified ($p = 0.19$). The function used to discriminate the *a priori* two groups (Table 5) was able to classify correctly about 95.8% of the howls; about 94.1% of the observations pertaining to cluster 1 were assigned to group 1

TABLE 3

Comparison of data pertaining to the 2 and 4-clusters solution by cross-tabulation, with the emerging of the nested structure. Group 1 and 3 of the 4-cluster solution are fully enclosed in group 1 of 2-cluster solution and groups 2 and 4 of the 4-cluster solution in group 2 of 2-cluster solution.

	Group	– 4 – Cluster solution				Total
		1	2	3	4	
2 –	1	55	0	42	0	97
Cluster solution	2	0	29	0	39	68
Total		55	29	42	39	165

with 5.8% error, while about 96.9% of the observations pertaining to cluster 2 were assigned to group 2 with 3.1% misclassification.

Shape variables that were not used in multivariate analyses to elude possible biases due to numerical constraint (not independent variables) showed higher values in group 1 (from now breaking howls) than 2 (from now flat howls) (Coefficient of variation: $t = 11.03$, $d.f. = 163$, $p < 0.001$; Coefficient of modulation: $t = 8.09$, $d.f. = 163$, $p < 0.001$). Regarding flat howls, however, our sample shows higher values of Cofv (mean = 9.97%) with respect to the European and North American populations, often having a rise in pitch at the beginning of the howl (Posmax = 0.22) (Figure 2b). The minimum ($t = 5.827$, $d.f. = 163$, $p < 0.001$) and the end ($t = 5.293$, $d.f. = 163$, $p < 0.001$) frequencies of the fundamental one were higher in the breaking howls, while there were no differences as regards the duration (all variable values split by using DFA solution are reported on Table 6). The same difference between breaking and flat howls was also evident for the number of abrupt changes in pitch ($\chi^2 = 112.726$, $d.f. = 14$, $p < 0.001$).

DFA correctly classified most howls (95.8%). Flat and breaking howls, however, could not be fully distinguished and represented the two halves of the same continuum (Figure 1a). Duration did not affect

TABLE 4

Mardia's multivariate normality test applied to PCA scores to check bivariate normality assumed by discriminant function analysis; normality has tested on the groups of howls. They are discriminated by considering the 2-cluster solution.

	Group 1 (N=98)	Group 2 (N=67)
Ep	5.582	3.145
P	0.2326	0.5339

TABLE 5

Eigenvalue of the discriminant function and canonical correlation value. Wilks' lambda and χ^2 statistics are reported.

Function	Eigenvalue	% of Variance	Canonical Correlation	Test of Function	Wilks' Lambda	χ^2	g.l.	p
1	1.032	100	0.835	1	0.303	193.696	2	<0.001

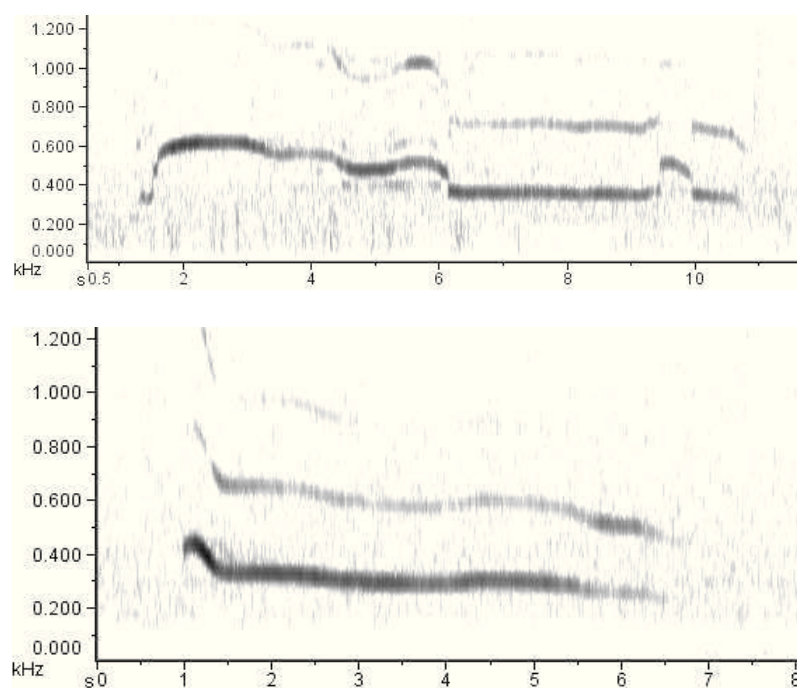


Figure 2. Sonograms representing structures of howls belonging to different groups identified by a 2-cluster solution. Top: example of a breaking howl. Bottom: example of a flat howl. X axis: time in seconds; Y axis: frequency in kiloHertz.

the howls' structures, since breaking and flat howls could both show long as well as short duration (Figure 1b).

Although we could not quantitatively estimate the occurrence of breaking and flat howls in choral responses because we analysed only a selection of good signals, both types of howls were present in all analysed packs. Moreover, breaking howls were more common than flat ones (98 versus 67) and showed higher values in both pitch and shape variables, while flat howls are lower in frequency and relatively constant in form (Figure 2 and Table 6). As regards PCA analysis,

TABLE 6

Grouping according to DFA. Variables list, mean frequencies, standard deviations and range for each group of howls. Shape variables are shown, too. For abbreviations see Table 1.

Variables	Group 1(n=98) Breaking howls Mean \pm SD (Range)	Group 2 (n=67) Flat howls Mean \pm SD (Range)
Meanf	551 \pm 115 (380 -908)	379 \pm 72 (274 - 560)
Rangef	428 \pm 178 (150 \pm 1033)	146 \pm 70 (21 - 323)
Minf	366 \pm 74 (236 \pm 646)	304 \pm 74 (193 - 516)
Maxf	794 \pm 177 (495 - 1356)	450 \pm 80 (301 - 624)
Endf	408 \pm 112 (258 - 796)	329 \pm 83 (215 - 516)
Duration	3.47 \pm 1.60 (0 - 1.00)	4.15 \pm 2.19 (0.75 - 9.55)
Posmin	0.75 \pm 0.33 (0 - 1.00)	0.57 \pm 0.42 (0 - 1.00)
Posmax	0.19 \pm 0.22 (0 - 1.00)	0.22 \pm 0.25 (0 - 0.98)
Cofv	23.75 \pm 8.60 (7.19 - 45.44)	9.97 \pm 5.37 (0.84 - 23.96)
Cofm	3.07 \pm 1.35 (1.15 - 9.09)	1.79 \pm 0.78 (0.16 - 4.41)
Abrupt	4.94 \pm 3.08 (0 - 18)	1.51 \pm 2.12 (0 - 10)

range and maximum F0 were the most useful immediate variables to classify the structures of howls. In single responses breaking and flat howls were both present (19 breaking and 18 flat), showing that wolves vocalize in modulated as well as unmodulated ways, no matter the type of response (single/chorus). Moreover, when we compared the howls from single responses with those from choral ones, no significant difference was found for all the 11 analysed variables. In fact, single

TABLE 7
Comparison between single and choral howls (for abbreviations see Table 1).

	Meanf	Rangef	Minf	Maxf	Endf	Duration	Posmin	Posmax	Cofv	Cofm	Abrupt
Mann-Whitney U	7	11	11	11	10	9	5	12	12	5	12
P	0.229	0.644	0.644	0.644	0.518	0.405	0.116	0.782	0.782	0.116	0.782

and choral howls did not differ significantly in all analysed variables (Table 7).

DISCUSSION

The higher frequencies of a signal are subject to greater attenuation than the lower frequencies in a variety of environments (Konishi 1970; Morton 1980). Accordingly, it is not surprising that wolf long-distance communication mainly employs low frequencies: the wolf's vocal range is between 70 Hz and more than 9,900 Hz (Schassburger 1993), but only the lower frequencies of this range are actually involved in the production of howls. Despite the great variability of howls according to individuals and social contests, howls are uttered within a narrow range of frequencies, thus experiencing minimum levels of attenuation and proving to be apt for long-distance communication (Harrington & Asa 2003). Our results showed that Italian wolves howl with a mean F0 between 274 and 908 Hz (Table 6), values that are consistent with those reported for other wolf populations (Theberge & Falls 1967; Tooze *et al.* 1990, Shassburger 1993; Palacios *et al.* 2007).

We determined two main structures (Figure 2) corresponding to breaking and flat howls, as already distinguished by Harrington and Mech in their study on North American populations (1978a) where they assumed that lower coefficients of variation (Cofv) correspond to flat howls and higher Cofv to breaking howls (< 6% and > 10%, respectively). Iberian wolf howls showed similar values of Cofv (Palacios *et al.* 2007). The higher values of Cofv found in our flat howls sample is probably due to a characteristic rise in pitch at the very beginning of the howl. At present, we cannot evaluate the importance of this difference concerning flat howls; further investigations that take into account wider areas and samples are necessary to understand whether this high frequency starting can be said to be a systematic feature of Italian wolves.

We were not able to support the division into continuous and breaking howls that was found in the Iberian wolf (Palacios *et al.* 2007), probably because many howls presented both breaking point and modulated fractions (Figure 2). Howls structures were so intrinsically variable that further basic groups could not be identified, with the exception of those based on factor 2, being mainly characterized by duration (Figure 1b).

Duration was an important variable characterizing factor 2 of PCA (Table 2). Duration may be an honest indicator of body size, given that lung capacity limits the airflow necessary to vocalize (Fitch & Hauser 2002), but it does not influence the howls' structure.

As regards the meaning of the two structures, Harrington (1989) suggested that highly modulated howls could serve to disguise inter-

pack information about pack size or to obtain a Beau Geste effect, that is to make the receivers overestimate the number of senders (Krebs 1977). We suggested that those highly modulated howls could correspond to breaking howls. Flat howls, lower in pitch and less modulated, can increase the hostility of the signal (Morton 1977). Birds and mammals use relatively low-frequency sounds in hostile-aggressive contexts and higher frequency sounds in friendly ones. In mammals, the relationship between low frequency sounds and aggressiveness is particularly evident (August & Anderson 1987).

Many factors, however, can affect the type of howls (distance between members of the same pack, health and motivational status, etc), so that further investigations are necessary to understand the meaning of the different acoustic structures in conveying information about wolf behaviour.

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Chapter 2

Group specific vocal signature in free-ranging wolf packs

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Group specific vocal signature in free-ranging wolf packs

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Acoustic communication conveys a variety of information that is a helpful tool for animal conservation. The wolf is an elusive species, which can be detected through the howls that individuals emit. In this study we investigated the acoustic features of wild wolf pack howls from five locations in the province of Arezzo, Italy. We tested the hypothesis that each group had a distinctive vocal signature. Our results showed that these wolf packs emitted howls with significantly distinctive acoustic structures. We hypothesized that group-specific vocal signatures require temporal stability to be functional. Indeed, we did not find any statistical differences in howls collected from the same location during the same season or for 2 consecutive years. We suggest that the acoustic features of howls can be used to distinguish wolf packs in the wild.

KEY WORDS: wolf, howling, acoustic communication, group signature, vocalizations.

INTRODUCTION

Animal communication is not only just a subject for behavioural studies, but acoustic signals also convey diverse information that can be used to census individuals as well as groups of the same species (MCGREGOR 2005). Bioacoustic research is known to provide useful insights for the census and the monitoring of species, a central criterion for the conservation of animal diversity (BAPTISTA & GAUNT 1997).

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The wolf (*Canis lupus*) is one of the most widely distributed land mammals and a protected species under the Bern Convention and the European Council Directive 92/43/EEC ('Habitat Directive'). However, this species is endangered in several European countries, or was severely threatened until recently (PROMBERGER & SCHRÖDER 1993; APOLLONIO et al. 2004). The pack is the social unit of a wolf population and generally consists of a breeding pair and their offspring (MECH 1970). Acoustic signals play a key role in wolf social behaviour (HARRINGTON & ASA 2003). The howl is considered the main long-distance vocalization and its structure has been widely investigated in both wild and captive populations (HARRINGTON 1989; TOOZE et al. 1990; PALACIOS et al. 2007; PASSILONGO et al. 2010). The howl is a long sound, whose fundamental frequency (F0) generally ranges between 150 and 1300 Hz in adults. Its characteristics are stable over distance, as observed in a closely related species, the coyote (*Canis latrans*) (MITCHELL et al. 2006). Howling is a relevant vocalization with several functions, regulating intra and inter-pack interactions, such as social spacing, defence of resources and mate attraction (JOSLIN 1967; HARRINGTON & MECH 1979; HARRINGTON & ASA 2003). Howling is also involved in the coordination of social activities such as the re-joining of separated members to the pack (MECH 1966; THEBERGE & FALLS 1967). Wolf chorus howls are a series of vocalizations emitted by a pack, in which one wolf begins howling, with some or all other members forming the chorus (JOSLIN 1967). Importantly, howling can provide information on individual identity and position (THEBERGE & FALLS 1967; TOOZE et al. 1990).

Since wolves tend to respond to vocal stimuli, tracking wolf-howling is a technique that enables operators to locate packs even in areas with dense vegetation, where direct observation is difficult. Wolf-howling tracking was described by PIMLOTT (1960) and requires an observer either to playback recorded howls, or to produce human imitations of them. When the pack is within hearing distance, the wolves may reply by howling back (JOSLIN 1967). Packs are more likely to respond when pups and/or food resources are present (HARRINGTON & MECH 1979; HARRINGTON & ASA 2003). This method was used in several studies on wolf pack behaviour as well as to census wolf packs (HARRINGTON & MECH 1979; FULLER & SAMPSON 1988). Elicited howls have been used to acquire information on wolf behaviour related to territorial maintenance, resources defence and activity rhythms (HARRINGTON & MECH 1978a; GAZZOLA et al. 2002; NOWAK et al. 2007).

In captivity, individual wolves can be recognized by the characteristics of their howling (TOOZE et al. 1990; PALACIOS et al. 2007) and the fundamental frequency was found to be the most effective variable to distinguish individuals (TOOZE et al. 1990). Individual vocal features have been recognized in a large variety of taxa, from birds (PEAKE et al. 1999) to several mammalian species, including canids (DURBIN 1998; DARDEN et al. 2003; FROMMOLT et al. 2003; HARTWIG 2005). It was recently shown that it was possible to distinguish individuals within a group of conspecifics by virtue of their vocalizations both in birds (BAKER 2004; RADFORD 2005) and in mammals (BOUGHMAN 1997; CROCKFORD et al. 2004; TOWNSEND et al. 2010). No research has yet addressed the potential for group-specific differences, especially in the wild.

Individual recognition by vocal print has been proposed as a possible species conservation tool (DARDEN et al. 2003; HARTWIG 2005), even if it was noted that there are cases in which a species may alter its vocalizations in relation to the territory in which it is located (WALCOTT et al. 2006). However, there are only a few reports of acoustic identification used as a monitoring tool for mammals in the wild (O'FARRELL & GANNON 1999; OSWALD et al. 2007).

In this study we tested the hypothesis that free-ranging wolf packs have a group-specific vocal signature by analysing howls extracted from the choral responses of five wolf packs in central Italy.

MATERIALS AND METHODS

Data were collected during the years 2007, 2008 and 2009 from June to October in the province of Arezzo (3230 km²), eastern Tuscany, Italy. The topography of this area is mountainous ranging from 300 to 1654 m a.s.l. and more than 50% of the area is covered by forests. From 1998 to 2010 spatial distribution and reproductive success of wolf packs in the province of Arezzo were monitored using wolf howling tracking, snow tracking and molecular analyses (SCANDURA et al. 2001, 2006; GAZZOLA et al. 2002; APOLLONIO et al. 2004; SCANDURA 2005; CAPITANI et al. 2006; IACOLINA et al. 2010). During the field study, the number of wolf packs in the province ranged from 7–13, while the pack size ranged from two to eight individuals, with a mean of 4.5 individuals.

Wolf howling tracking was performed in summer, when the pack activity was focused on a restricted area (home-site) due to the presence of pups, and the response rate to vocal stimuli was high (HARRINGTON & MECH 1978b, 1979, 1983; GAZZOLA et al. 2002; NOWAK et al. 2007). Sampling sites were chosen to maximize the audible range and minimize sound dispersion, while their location and number were planned to cover the whole study area. Two groups of operators conducted concurrent sessions to determine the presence of two adjacent packs. To elicit the vocalizations of wolves, we used a playback of recorded chorus howls by a captive wolf pair (duration: 1 min 29 sec). Trials were carried out at night and in good weather conditions, i.e. with low wind and no rain, using a tape player connected to an amplifier with an output of 40 W and an exponential horn with high emission directionality (120° horizontal coverage and 60° vertical), as described in detail in PASSILONGO et al. (2010).

In order to analyse vocalizations of free-ranging wolf packs, we selected the five locations out of those reported by census data to have the highest number of recorded vocalizations: Lignano (LI), Vallesanta (VS), Catenaia (CT), Camaldoli (CM), and Pratomagno (PM) (Fig. 1).

Vocalizations were recorded using a Sennheiser microphone with windshield (ME67 head with K6 power module) and a digital recorder (M-Audio Micro TRACK 24/96) with a sampling rate of 44.1 kHz and 16 bits accuracy. Analysis of recorded howling was performed using Raven Pro 1.3 (Cornell Lab of Ornithology). Spectrogram parameters selected for the analysis were: 2048-point discrete Fourier transform; frequency resolution: 21.5 Hz; filter bandwidth: 37.5 Hz; time overlap: 10 msec; Hanning window. For the purposes of this study, we analysed only howling (flat and breaking) and did not consider other types of vocalizations such as whimpers, barks and growls, that often occur in choral responses (MECH 1966; JOSLIN 1967; HARRINGTON & MECH 1978b; MCCARLEY 1978). Howls by pups, recognizable until 6/7 months of age for their high frequency and instability of the vocal structure due to physical growth (HARRINGTON & MECH 1978b; HARRINGTON & ASA 2003), were not taken into consideration. We measured the entire length of the fundamental frequency (F0) of the howl (Fig. 2) every 0.05 sec to obtain 12 variables for each howl, as in previous studies on wolf vocalization (TOOZE et al. 1990; PALACIOS et al. 2007; PASSILONGO et al. 2010) (Table 1, Fig. 2). Harmonics, which were sometimes visible in the spectrogram, were not considered for the analysis.

Statistical analysis

Univariate and multivariate non-parametric methods were used to test vocal differences (i) between years in the same pack, and (ii) among packs. Each vocal variable difference between years were tested by Mann Whitney U test and among packs by Kruskal-Wallis test (ZAR 1996; HAMMER et al. 2001). A one-way PERMANOVA, based on a similarity matrix created on Gower similarity criteria, was used to test the null hypothesis (H₀) that there were no differences among

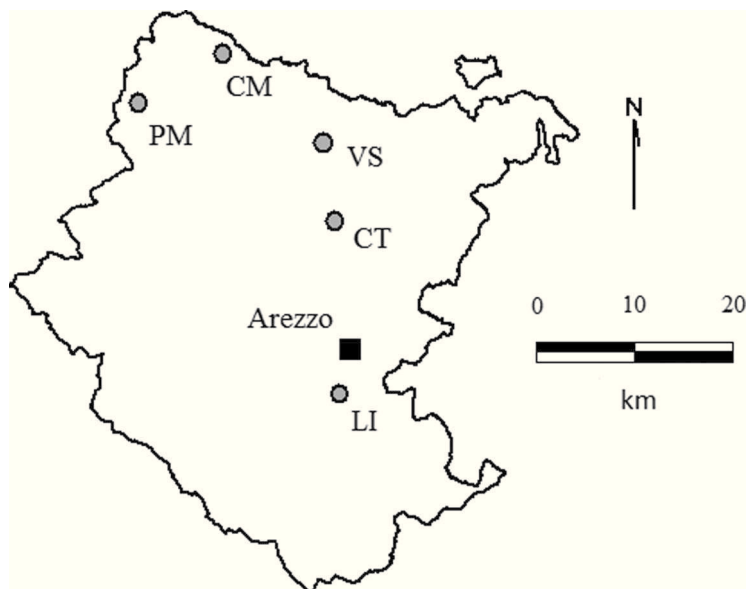


Fig. 1. — Geographical distribution of the analysed packs.

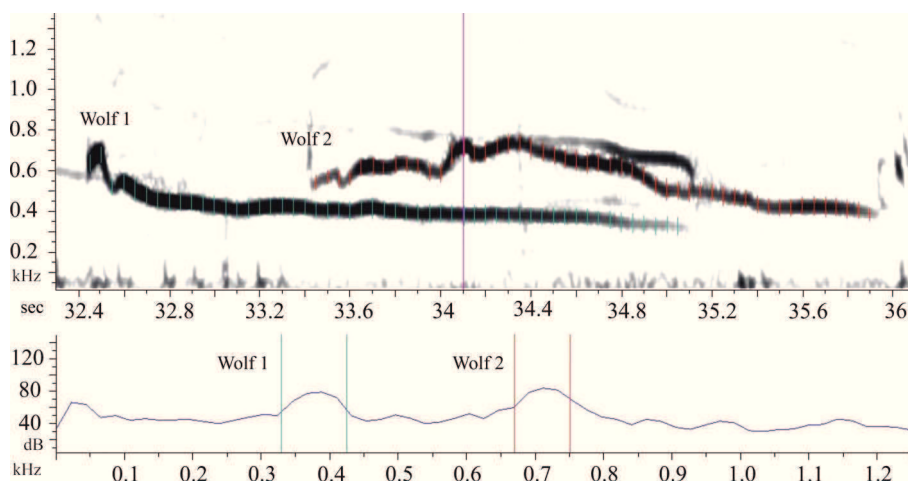


Fig. 2. — Spectrogram with spectrum of an example of howls analyzed. Purple line shows the position of the spectrum at 34.1 sec. Blue and brown bars in the spectrum show the peak frequency of each howl in that time span. Peak frequency was collected every 0.05 sec from the beginning to the end of each howl.

acoustic variables of packs; post hoc pair-wise tests were applied after PERMANOVA (ANDERSON 2001). Principal coordinates analysis was used to visualize their relationships (TORGERSON 1958), by using the Gower similarity matrix among samples. Spearman correlation indexes among the scores of the samples were computed for each PCO axis. The values related to each variable were then considered to facilitate interpretation of the meaning of the axes. Vectors of the variables

Table 1.

Description of variables used for the analysis of wolf howls.		
<i>Pitch variables</i>	Meanf	Mean of the fundamental frequency calculated every 0.05 sec (Hz)
	Modf	Mode of the fundamental frequency calculated every 0.05 sec (Hz)
	Rangef	Difference between maximum and minimum frequencies (Hz)
	Minf	Minimum frequency (Hz)
	Maxf	Maximum frequency (Hz)
	Endf	Frequency at the end of the howl (Hz)
	<i>Shape variable</i>	Duration
Posmin		Position at which the minimum frequency occurs (time of Minf/Duration) in the howl
Posmax		Position at which the maximum frequency occurs (time of Maxf/Duration) in the howl
Cofv		Coefficient of frequency variation (SD/Meanf) \times 100
Cofm		Coefficient of frequency modulation $\Sigma f(t)-f(t+1) (n-1)/\text{Meanf} \times 100$
Abrupt		Number of sudden abrupt changes in frequency (> 25 Hz)

were superimposed on the PCO plot to improve graphical results. To avoid multicollinearity the variables Meanf and Cofv, were excluded from the multivariate analysis. All statistical analyses were performed using SPSS 18 (Chicago, Illinois, USA), PRIMER v. 6.1 (CLARKE & GORLEY 2006) and PERMANOVA+ for PRIMER routines (ANDERSON et al. 2008).

RESULTS

From 2007 to 2009, in the five locations considered, wolves replied to 59 of the 180 trials (33%). A total of 271 howls were found to be suitable for a quantitative analysis (Table 2). The presence of pups was recorded in all packs, which ranged from four to seven individuals (Table 2). Each trial was obtained from a minimum of three wolves with the exception of PM where we used a minimum of two wolves per trial.

No significant difference among most variables in the CM pack between 2008 and 2009 were observed, with the exception of duration ($N = 48, 24$; $U = 269$; $P < 0.001$) and posmin ($N = 48, 24$; $U = 401$; $P < 0.05$), demonstrating some persistence in the structure of CM howls in subsequent years.

Univariate comparisons among packs showed significant differences for 10 out of 12 variables analysed; the only variables that showed no difference among howls by different packs were the posmin and posmax (Table 3). Multivariate comparisons among howls by different packs showed significant difference in their structure as a whole (pseudo-F = 8.6956; $df = 5$; $P < 0.0001$, PERMANOVA test). The pair-wise test among groups was used as a post-hoc test and showed significant differences among all packs, with the exception of VS vs CT, and CM 2008 vs CM 2009 (Table 4). The two-dimensional scatter plot of principle coordinates (Fig. 3) shows that LI pack clusters apart and is characterized by stable and long duration howls. In fact these howls are distributed on the positive side of the PCO_1 (49.9% of total variance explained) which is inversely correlated with Rangef ($R = -0.86$), Abrupt ($R = -0.54$), Cofm ($R = -0.45$),

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Table 2.

Summary of data collected during the study period. Minimum number of wolves was estimated by means of visual inspection of the narrow band spectrograms.

Pack	Study period	Trials	No. of successful trials	Analyzable trials	No. of howls analyzed	Minimum no. of adult wolves (no. of puppies)
LI	07/10/2007	15	5 (33%)	4	39	4(3)
VS	05/6/2008–10/10/2008	64	10 (16%)	6	31	4(3)
CT	30/7/2008–09/9/2008	42	14 (33%)	11	91	3(2)
CM08	19/8/2008–13/10/2008	29	11(38%)	8	24	3(2)
CM09	13/8/2009–13/9/2009	11	9 (82%)	6	48	4(2)
PM	06/8/2009–23/8/2009	29	10 (34%)	5	38	2(2)
		180	59 (33%)	Tot 40	Tot 271	Mean 5.66

Table 3.

Results of univariate comparison among packs of the variables considered for the analysis (Kruskall Wallis with Monte Carlo exact test).

Variables	χ^2	df	<i>P</i>
Meanf	107.966	5	<0.0001
Modf	83.050	5	<0.0001
Rangef	61.605	5	<0.0001
Minf	85.010	5	<0.0001
Maxf	106.943	5	<0.0001
Endf	80.335	5	<0.0001
Duration	61.874	5	<0.0001
Posmin	4.511	5	0.478
Posmax	6.346	5	0.274
Cofv	25.746	5	<0.0001
Cofm	29.753	5	<0.0001
Abrupt	54.024	5	<0.0001

Maxf ($R = -0.56$), and on the positive side of the PCO_2 (17.5% of total variance explained) which is directly correlated with Duration ($R = 0.41$) and inversely correlated with Endf ($R = -0.74$).

DISCUSSION

In this study we tested, for the first time, the hypothesis that group-specific vocal signatures exist for wolves. Our results are from wild populations and a limitation is that it was not possible to exactly estimate the contribution of each wolf in the

Table 4.

Results of PERMANOVA paired test among packs. Packs are labeled as follows. Number of howls in brackets.

Packs	Paired test <i>P</i> values					
	LI(39)	VS(31)	CT(91)	CM 08(24)	CM 09(48)	PM(38)
LI	–					
VS	<0.001	–				
CT	<0.001	0.467	–			
CM 08	<0.001	0.007	0.002	–		
CM 09	<0.001	0.003	<0.001	0.595	–	
PM	<0.001	<0.001	<0.001	<0.001	<0.001	–

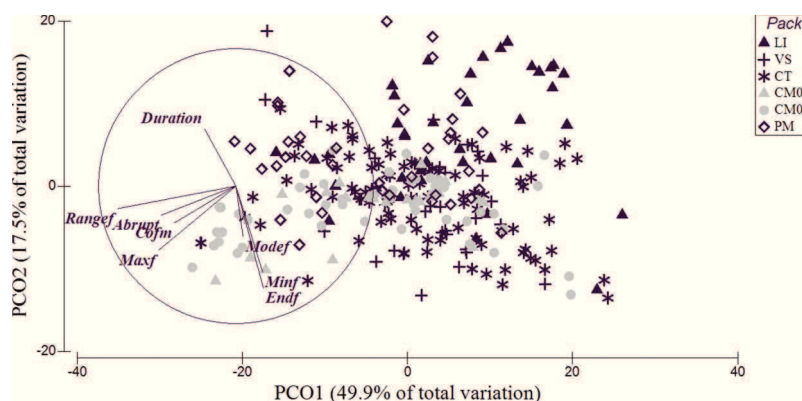


Fig. 3. — Two-dimensional scatter plot of first and second principal coordinates axis of 10 acoustic parameters of howls recorded for 6 wolf packs in the province of Arezzo.

pack. On the other hand, we collected vocalizations from choruses of howling composed by a minimum of two to three wolves per trial so that the possibility that the group vocal signature of a pack is due to the vocal characteristics of a single individual should be minimal. To investigate the presence of a group specific vocal signature, we decided to collect howls from wild populations living in an interconnected territory. HARRINGTON & MECH (1979, 1983; HARRINGTON 1987) suggested that howling serves: (1) to maintain or to increase the distance between packs, (2) to help establish and preserve exclusive territories, (3) to reduce the probability of contact with unfamiliar wolves or packs. Further, they suggested that howls might hold promise as a non-invasive conservation management tool to recognize packs in the wild. Our results revealed significant differences among howls emitted by different packs supporting the hypothesis that packs have a group-specific vocal signature suitable for census and monitoring. Several mammalian species show group-specific vocal signatures (primates: CROCKFORD et al. 2004; CHENEY & SEYFARTH 2007; HERBINGER et al. 2009; bats: BOUGHMAN 1997; cetaceans: FORD 1991; TYACK 2000); all these species, including

wolves, have complex social structures, defend their territory from intruders, and live in habitats where long-range acoustic communication is employed to convey messages. To be functional, a group-specific vocal signature requires stability over time. Therefore, we tested the acoustic stability of the howls in different years. In Camaldoli, howls recorded in two different years (2008–2009), and very likely emitted by the same pack (CM), were compared and no significant differences were observed. Our data seems to support the hypothesis that group howling has a vocal stability because we found that a similar acoustic structure was maintained over 2 years, despite possible changes in pack composition due to high winter mortality, new births and dispersion of young individuals.

In the two packs living in CT and VS no statistical differences were recorded that could help distinguish the two groups' howls. This could be accounted for by the proximity of the two locations and by the migration of at least one female from the CT to the VS pack. The migration was tracked using genetic analysis of droppings and the individual was reported to belong to the CT pack in 2003 and to the VS pack in 2008 (M. SCANDURA pers. comm.).

Howls recorded in CM are characterized by higher frequencies of Maxf and Rangef compared to other packs, and in particular compared to LI, the most geographically distant, which has lower frequencies in maxf, rangef and a low number of abrupts. The acoustic structure of CT and VS have intermediate values compared to CM and LI and they are located between the two packs described above. However, it should be noted that pack PM, geographically closest to CM, has vocal characteristics that are not clearly explainable from a geographical point of view. This suggests that the distance among packs increases the differences in the vocalizations, but the reduced number of packs sampled does not allow us to go into further detail.

Identifying individuals using acoustic cues is a non-invasive method that has been the focus of much work in bioacoustics as well as in behavioural sciences (TERRY et al. 1995) and could be especially useful when species have nocturnal acoustic activity (DARDEN et al. 2003). The wolf is a gregarious and territorial species. Howls therefore should carry a group-specific vocal signature and our results support this hypothesis. We conclude that group-specific vocal signatures could be used as a non-invasive tool to recognize packs and for the management of this species. It is possible that group-specific vocal signatures represent a sort of cultural tradition, but further studies are necessary to determine whether group signatures are due to genetic features, are acquired, or are due to a mixture of both.

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SECOND PART

ACOUSTIC BEHAVIOUR OF RED DEER IN RELATION TO MATING SYSTEMS

Chapter 3

Iberian red deer stags vocalizations: quantifying repertoire size and structure of the calls

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Iberian red deer stags vocalizations: quantifying repertoire size and structure of the calls

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Abstract

Iberian deer (*Cervus elaphus hispanicus*, Hilzheimer 1909) are geographically isolated and characterised by different morphological traits from other *Cervus elaphus* subspecies. The aim of this study was to provide a detailed description of the vocal repertoire of free-ranging males during the rut. Results revealed that: 1) the acoustic repertoire of Iberian deer consists of four main types of calls: a long common and semi-harsh roar, a short common roar and a series of chase barks 2) semi-harsh roars are longer and higher pitched compared to the other types of calls, suggesting a stronger effort during their emission; 3) the arrangement of the call types within bouts is not random: while single and bout-opening vocalizations are almost systematically long or semi-harsh common roars, mid-bout vocalizations are typically short common roar and end of bout vocalisations are either long or short common roars. The divergence of the Iberian red deer vocalizations with those of other documented European red deer subspecies (*Cervus elaphus scoticus*, *Cervus elaphus corsicanus*) suggests that different selective pressure operate on the sexual signals of these closely related taxa.

Introduction

Vocal behaviour can differ considerably in closely related species [1] and even in populations within the same species [2,3,4]. Vocalizations vary according to their function [5] and to biological constraints operating on their production and perception [6]. Moreover, as predicted by “the acoustic adaptation hypothesis” [7] particular associations exist between signals and the environment in which they are transmitted; because frequencies propagate differently through closed vs. open environments [8,9], populations evolving in different habitats can develop vocalizations with different features [7,10] despite having similar functions.

Ecological factors have been found to generate directional selection on mating signals and mating preferences [11,12]. Sexual signals can evolve to maximize the transmission distance in their specific habitat [13], to maximize the signal’s localization accuracy [14], or to encode information on the individual’s physical quality and social status [15-18].

Moreover, female mate choice is often based on male courtship signals [19], thus mating signal variation within the species is also subject and forged by sexual selection [20]. Thus, vertebrate vocal communication is highly variable, both within and between species, and this variability concerns both the acoustics of the calls and the context in which they occur. The acoustic structure of male mating calls in polygynous deer is a clear illustration of this variability, with a high degree of diversity both among species of the Cervinae and also among geographic subspecies of *Cervus elaphus* (Linnaeus, 1758) [21]. This species, divided in several geographic subspecies (for a list see [22]), is characterised by strong vocal activity of the stags during the mating season. During the mating season (from August to October, in the northern hemisphere) stags defend and actively herd groups of females called harem) [23] or compete for and defend territories where females are subsequently attracted [24,25]. Harem-holders feed little compared to the rest of the year and spend much time collecting and herding hinds and chasing off young stags [23]. Throughout the mating

season stags are highly vocal, and give several different types of calls, both towards females and male competitors [23].

The acoustic structure and patterns of calls changes among the investigated subspecies (*C. e. scoticus*, [17,21] Reby and McComb, 2003a, b; *C. e. corsicanus* [26]), along with other behavioural [27,28] and morphological features (body size:[29]; cranial measurements:[30,31]).

The composition of vocal repertoires varies between geographical subspecies of red deer: studies of male vocal behaviour in Scottish red deer have identified five distinct vocalizations, which vary in relation to their acoustic structure and their contexts of emission: the common roar, the harsh roar, the grunt roar; and two kinds of barks: the chase bark series and the longer loud bark [21]. In contrast, only common roars and chase bark have been described in Corsican deer stags [26].

In addition, the overall variation of spectral components (i.e. the range of frequencies used for calls) varies between red deer subspecies. This variation is best explained using the “Source-filter” theory of voice production [32], where the fundamental frequency and the vocal tract resonances are described as two key features that are produced and perceived independently: the fundamental frequency and the formant frequency [33]. The fundamental frequency (F0) is produced at the source (the larynx) and constitutes a highly distinctive and variable component of mammal calls [34]. F0 is determined by the rate of the vibration of the vocal folds caused by the passage of air through the closed glottis and it is perceived as the pitch of the sound. Several studies suggest that F0 is not a reliable index of body size in mammals [35,36] indicating that other physical or physiological factors may influence the variation of this acoustic feature, or that is it relatively unconstrained and dynamic. Corsican deer roars have a mean F0 of around 30 Hz [26], which is considerably lower than the mean F0 of 112 Hz reported in the larger Scottish deer [21], in contradiction with the general prediction that larger animals should have larger and heavier vocal folds and thus produce lower frequencies [37,38].

Formant frequencies are resonance peaks produced as the glottal wave travels through the supra-laryngeal vocal tract [32]. Formant frequencies and overall formant dispersion (the overall frequency spacing between the formants) should decrease as the length of the vocal tract (the distance between larynx and lips or nostril) increases [39] and, because the length of the vocal tract is likely to be constrained by body size, formants characteristics typically provide a more reliable indication of body size to receivers than fundamental frequency [39-41].

Red deer males have a descended larynx [42], and are able to drop their highly mobile larynx further down towards the sternum, which allows them to increase their vocal tract length while they vocalize [25,21,42]. Iberian deer (*Cervus elaphus hispanicus* Hilzheimer1909) not only have a descended and mobile larynx enabling them to extend their vocal tract during vocalising [43], but they also protrude their tongue during of the majority of their rutting roars [43]. Although the acoustic structure of red deer male mating calls has been extensively studied [17,21,26,43], no systematic and quantitative classification of the repertoire of this species has been previously reported.

Iberian red deer inhabit the Iberian Peninsula and are currently geographically isolated from other red deer populations in Eurasia and Maghreb [44,45]. This subspecies is smaller than Central European subspecies (weight: 80-160 Kilograms) [45], but bigger than the Corsican deer. Compared with the Eastern European red deer's, its coat is darker and its skull is lighter [46,44]. The aim of this study is to describe the vocal behaviour of male Iberian red deer in a free-ranging population. More specifically to assess the quantitative and qualitative acoustic variation of vocal signals in order to categorise them into call types, we examine the arrangement of these call types into call sequences. We then compare the vocal behaviour of Iberian deer males with that previously described in other subspecies of red deer.

Materials and Methods

The study area in Doñana National Park (Andalucía, Spain) included a western area with Mediterranean shrub land and an eastern area with a marsh (which was dry during the period of study) separated by an ecotone, a long narrow strip of land with meadows and rushes. The climate is typically Mediterranean with hot, dry summers and mild, wet winters. All field work was carried out with the permits from the authorities of the National Park.

The red deer rut in Doñana usually takes place between the 1st and the 25th of September [48]. During this period, males typically move from their home ranges to the area used by females in the ecotone and use their harem-holding or territorial tactics to monopolize females [47,49,50].

Data collection took place during the 2010 rutting season. To investigate and categorize repertoire size, calls were recorded from adult males individually identified by the size, shape and branching pattern of their antlers. Call recording and observations were carried out during the last 3-4 hours before dusk, which is the period of maximum activity for red deer in South Western Spain [51]. Observation sites were conveniently selected in relation to the places where most deer activity was recorded during previous years and observations were carried out from fixed blinds. Recording distances ranged between 70 to 200 meters. Over 60 hours of vocal displays from 13 adult males were recorded, from which a total of 115 bouts (334 vocalizations) were extracted and analyzed.

A subset of calls was recorded from a car along a field path route close to the males territories in order to minimize the distance (mean 68 m) between the microphone and the rutting deer. All distances were checked by a Leica Range Master CRF 900 7x24 telemeter.

Audio tracks were recorded with a hand-held Sony PCM D-50M digital recorder, with 44,100 Hz sampling rate and 16 bits accuracy, and saved in “.wav” format. Vocalizations were recorded with a Sennheiser directional microphone with windshield (ME67 head with K6 power module).

Acoustic analysis

All analyses were performed on a HP Compaq nx7400 with a SoundMAX integrated Digital HD Audio soundcard using Praat version 5.2.13 DSP package for Windows [52]. Narrow-band spectrograms of the vocalizations were edited (FFT method; window length = 0.03 s; time step = 1000; frequency step = 250; frequency resolution = 20 Hz; Gaussian window shape).

Pitch values for each call were extracted using a forward cross-correlation [to pitch (cc) command] algorithm in Praat. The time step in the analysis was 0.03 s and the specified expected values for limits of pitch ranged between 30 and 300 Hz. Pitch variables included in the analysis were: mean (Mean F0); minimum (Min F0); maximum (Max F0) and range (Range F0). Duration of the calls (Duration) was also calculated (table 1). The presence of Deterministic Chaos (DC), characterized by widespread energy and weak harmonic structure [53] was also investigated using visual inspection of the narrow-band spectrograms.

Formant analyses were computed on the subset of calls recorded during car transects, in order to minimize the effect of sound propagation on the spectral envelope of the vocalization. Because Iberian deer are characterized by a relatively high F0, with harmonic spacing of the same order of magnitude as the formant frequencies predicted from the animal vocal tract, formant frequencies are typically a poorly defined feature of their calls, and therefore difficult to measure. In order to estimate the values, we decided to measure formant centre frequencies on short segments located at the end of the calls, where the relatively low fundamental frequency (average: 126 Hz measured in a random sample of 20 calls from 4 animals) highlights the resonant properties of the vocal tract. This section is also characterized by minimal formant frequencies [17,21], reflecting the maximal vocal tract extension reached when the stag lowers its larynx all the way down to the sternal limit. Cepstral smoothing using a 150 Hz bandwidth filter was applied to the spectrum in order to remove the effect of the source (F0) with the command [cepstral smoothing] in Praat object window, and to highlight the effect of the filter (Figure 1). The values of the first 8 formants were extracted with the command [to formants] in Praat. We calculated formant dispersion (Df) and estimated VTL (eVTL)

according to Reby & McComb [21,24]. When the supra-laryngeal vocal tract is approximated to a straight uniform tube, closed at one end (the glottis) and open at the other end (the mouth), the spacing between any two successive formants can be approximated as a constant, and formant frequencies can be plotted as $F_i = D_f (2i-1)/2$. Since $F_i = D_f (2i-1)/2$, the slope of the linear regression gives the best estimate of D_f for our vocal tract model. Subsequently, formant dispersion (D_f) can be used to estimate vocal tract length by the equation $eVTL = \frac{c}{(2D_f)}$, where c is the speed of sound in air (350 m/s) and D_f is the formant dispersion [6,39].

All formants values were verified by visual inspection of narrow band spectrograms. The parameters were set as follows: maximum formant: 5000 Hz; maximum number of formants: 20; window length: 0.1 s; time step, 0.04 s. Calls with spurious values were excluded from the analysis. The resulting sets of 15 to 18 calls from 6 individuals were used for formant analyses. Because calls were selected on the basis of the quality of the formant frequencies (mostly visible in the long low pitch calls), these data were excluded from the repertoire analyses, to avoid bias in the sample. As calls are generally emitted in sequences (bouts), separated by periods of silence, we calculated the overall duration (TotDur), the number of units in the bouts (Calls/Bout) and the relative duration, i.e., total duration divided by the number of calls (TotDur/Calls). The position of each call within the bout was assigned as: first, middle, last, or single call. A total of 21 variables were analyzed from calls and bouts (Table 1). Vocalizations with high levels of background noise were excluded from the analysis.

Statistical analysis

Explorative cluster analysis was used to detect the presence of relatively homogeneous groups of cases; because one variable (deterministic chaos) was binary, analysis was performed on the dissimilarity matrix instead of the original dataset [54]. A series of agglomerative hierarchical clustering was performed with “AGNES” (AGglomerative NESTing) function in the library

“cluster” of R, changing the number of input variables (from 2 to 6) until reaching the highest Silhouette value. Ward’s method was used to link groups to each other, and the Euclidean squared distance was chosen as a similarity measure.

Silhouette Information was computed as a method of interpretation and validation of clusters of data [55]. Silhouette plots for different cluster solutions (from 2 to 8 clusters) were compared and the cluster with the highest values was chosen as the best solution. Silhouette was defined as follows:

$$s(i) = b(i) - a(i) / \max(a(i), b(i)) \in [-1, 1]$$

where $a(i)$ is the average distance from the i^{th} point to the other points in its cluster, and $b(i, k)$ is the average distance from the i^{th} point to points in another cluster k .

The average $s(i)$ of a cluster is a measure of how tightly all the data in the cluster are grouped. Thus, the $s(i)$ of the entire dataset is a measure of how appropriately the data has been clustered. $s(i)$ close to zero means that the assessment is on the border of two natural clusters; observations with a negative $s(i)$ were probably grouped into the wrong cluster.

Because of the unbalanced group samples, generating heteroscedasticity among groups, non parametric Kruskal-Wallis test were used to compare acoustic parameters among overall groups while to explore differences between pairs of groups, nonparametric simultaneous 0.95 confidence intervals for relative Tukey contrast effects were computed with the “nparcomp” function in R [56].

In order to ascertain the accuracy of our cluster solution with an inferential methodology, Discriminant function analysis (“lda” function in the “MASS” library of R) was used to build a predictive model of group membership based on the cluster solution (grouping variables).

Due to the high degree of correlation among pitch variables, and the presence of one binary variable (DC), Multiple Factor Analysis for mixed data (“MDFA”, in the library “FactoMineR” in R) was used to obtain new uncorrelated variables that are linear composites of the original ones. Subsequently, scores of the coordinates were used as independent variables in the DFA.

In order to find relationships between the type of call and the position in the sequence, groups identified according to DFA results were tabulated with the position of the calls in the bout and the independence of the variables was tested by means of chi-squared test.

Only descriptive statistics (mean and SD) were computed on formants frequencies, formant dispersion and estimated VTL.

Probability was two tailed, and $p < 0.05$ was considered significant. All analyses were computed with R 2.14.0 statistical software (R Development Core Team 2010) [57].

Results

Repertoire classification. Using our sample of 334 vocalizations from 13 individuals, the highest average silhouette classification score (0.62) was achieved by a four-groups solution (Figure 2) based on DC, duration and maximum fundamental frequencies as input variables. Single silhouette values were 0.52 for the first group (N=121); 0.65 for the second group (N=22), 0.68 for the third group (N=177), while few others calls (N=14) were classified in a fourth group (silhouette score=0.58) (Figure 2).

Calls belonging to the 1st group is homologous to that of Corsican and Scottish red deer “long common roars” [17,21,26]; calls belonging to the 2nd group are comparable to the “semi-harsh roars” found into Scottish red deer repertoire [17,21], and are characterized by the occurrence of deterministic chaos, a higher F0 and longer duration than the long common roars. The others identified types of calls are: the short common roar (group 3), and the series of chase barks (group 4), previously reported in the repertoire of the Scottish red deer stags [17,21].

Long common and semi-harsh roar (Table 2) were longer (1.83s and 2.05s respectively) than short common and chase bark (0.43s and 0.37s) and higher in pitch (MaxF0: 208Hz and 223Hz versus 140 and 125 Hz). DC is a strong selection variable, being present in the second and in the fourth group exclusively (Table 2).

Univariate comparison (Kruskal-Wallis tests) among the four groups (Table 3 and Figure 3) showed highly significant differences among all variables analyzed, as well as among those not included in the explorative cluster analysis. Post hoc tests revealed the origins of these differences: with the exception of the Minimum F0, that differed only between long common (127 Hz) and short common roar (115 Hz), long common and semi-harsh differed from short common roar for all analysed variables, while only duration and Max F0 (as well as the presence of DC in group 2) differed statistically between long common and semi-harsh roar. Short common roar and chase bark only differed in the presence of DC: completely absent in the short common roar and widespread in

chase bark, although difference in duration was also marginally significant (p-value of 0.07; Table 3).

Factor analysis was computed to explore relationships among variables and explain variability. The first dimension accounted for 54% of the variance and was found to be associated with the mean, the maximum fundamental frequency and duration; the second dimension accounted for 23% of the variance and was found to associate with the minimum and the range of F0, while the third dimension was mainly due to DC and explain the 16% of the variance (Table 4). A plot of the scores of the first (MaxF0, MeanF0 and Duration) and third (DC) coordinates with calls grouped using a 4-cluster solution showed a clear division of the calls on both axes (Figure 4). The Discriminant function analysis (Table 5) based on the calls identified in the cluster analysis and first three dimensions (factors) as discriminant variables were computed. Although the first discriminant function was mainly influenced by factor 3 (mostly related with DC), an overall highly correct classification emerged from the analysis: 94.9% of the calls being classified into the correct “call type” group; with 12 observations from long common being misclassified into short common roar, 3 calls from short common to long common roar and 1 observation from chase bark to semi-harsh roar. A spectrogram of the identified call types is shown in Figure 5.

Despite the low number of calls belonging to semi-harsh roar and chase bark (22 and 14 respectively) with respect to the total sample (334 calls), out of the 13 individuals recorded, 9 emitted semi-harsh roars and 4 emitted chase barks.

While overall formant frequency values (Table 6) were similar to those reported in [43] Frey et al. 2012, formant dispersion, calculated as minimum values of the eight formants by linear regression was 250 Hz and the average estimated vocal tract lengths during roaring (eVTL) was 70.4 cm (Table 6), showing lower values than those reported in [43].

Bout patterns. Considering the number of calls and their temporal aggregation, calls are emitted in sequences (bouts). The 115 analyzed bouts were composed of a variable number of sound units (1-12), a pattern previously reported in Iberian red deer [43] and other European subspecies [17,26], with a mean of 3.19 calls per bout. More than 40% of the bouts were composed by one call only. Overall duration of the bouts ranged from 0.85 s to 11.56 s with a mean of 4.36 ± 2.57 s, while relative duration of the bouts (overall duration divided by number of calls) ranged between 0.23 and 3.87 with a mean of 1.64 ± 0.83 s.

DC was present in 22% of calls of all the analyzed bouts. Contingency table (Table 7) and Chi-squared test (Chi-squared = 164.04; df = 9; $p < 0.0001$) showed a clear correlation between type of call and position within the bout. Bouts opened with long common and semi-harsh roars in 68 out of 76 cases, and short common roars and chase barks were typically emitted in the middle of the bouts (80% of the cases).

Discussion

We identified four types of calls in the rutting vocal repertoire of the Iberian red deer stag. These calls are distinguished by a combination of their duration, the maximum of their fundamental frequency and the occurrence of deterministic chaos. The acoustic structure of the long common roar is homologous to that of Corsican and Scottish red deer “common roars” [17,21,26], and is likely to play the same role in both male–male competition [23,58] and female mate choice contexts [34,59,60]. However, the fundamental frequency of long common roars in Iberian red deer (mean: 183.60 ± 23.24 Hz), confirming 43 Frey et al. 2012, is much higher than in Scottish red deer (111.7 Hz [17]) and in fact is the highest reported amongst European red deer subspecies [17,21,26]. Investigations of F0 in the calls of Scottish red deer stags have failed to identify an intraspecific correlation between fundamental frequency and body size within species [17]. Moreover, Corsican deer males have the lowest F0 [26] of the European red deer subspecies despite being the smallest, suggesting that the F0 differences observed between geographically distinct subspecies are not simply a direct consequence of size differences between these taxonomic groups.

In Scottish red deer, oestrous females do show a preference for high-pitched roars [34], suggesting that a relatively high pitched voice may be sexually selected for in this species. Recent playback experiments contrasting the response of oestrous Scottish red deer hinds to homo- or hetero-specific sika deer (*Cervus nippon*) male sexual calls have shown that while red deer females typically prefer their own species vocalisations, a small proportion of individuals appear to prefer high-pitched heterospecific sika moans [62]. Our observations suggest that in Iberian red deer, selection may have favoured a relatively high F0, possibly at the expense of the availability of the information on body size typically provided by the formant frequencies.

While source (F0) and filter (formant) components can be assumed to be independently produced, the periodicity of the source signal (the glottal wave) affects the frequency spacing of the harmonics, and consequently the spectral resolution of the formant frequencies [63]. Roars

delivered with a high fundamental frequency are characterised by decreased density of harmonics, and consequently by a poorer sampling of the formant envelope [61,63].

According to the source filter theory of voice production, formant frequencies and overall formant dispersion increase when the length of the vocal tract decreases [64]. In red deer, despite the fact that males have a mobile larynx, the minimum formant frequencies achieved during full vocal tract elongation to provide an honest indication of body size [17,42]. While the high F0 of Iberian deer calls makes measuring formants rather unreliable, and often impossible, formants are clearly visible on the spectrograms (Figure 1). When measuring formant frequencies during to a short terminal section of the calls, where F0 is low enough to highlight the vocal tract resonances, we found that the minimum formant frequencies achieved by Iberian stags were higher than those reported for the larger Scottish red deer [17]. The fact that our mean eVTLs are slightly shorter than the ones reported in [43] could be explained by the fact that while Frey and colleagues [43] measured formant frequencies analysis on harsh roar segments, we mostly measured formants during the low-pitched segments at the end of long common roars. Indeed, taking into account only roars containing chaotic segments, when stags may put more effort in fully extending their vocal tract, Df and eVTL (230 Hz and 76.1 cm respectively) become more similar to those reported in [43] (228.15 Hz and 76.7 cm).

Semi-harsh roars are characterized by the occurrence of deterministic chaos. This is comparable to Scottish red deer, where common roars often contain segments of deterministic chaos, particularly in the section of the roar where the formants reach their minimum values [17,21]. While relatively rare, calls containing deterministic chaos were found in the vocal repertoire of 10 individuals out of 13, suggesting that they are a regular feature of the mating calls of this subspecies. Interestingly, roars containing DC (“semi-harsh roars”) were also characterised by a higher F0 and longer duration than the other long common roars, suggesting that animals may put more effort into these vocalisations, and that deterministic chaos may be a by-product of vocalising at a higher F0 over a

longer period. Deterministic chaos is a non-linear phenomenon (NLP) which has been attributed to the desynchronization of the left and right vocal fold [53,67]. It is part of the normal acoustic output of several species and generally believed to function to increase the auditory impact of calls [68,69,70]. However, in common chimpanzee [71] the absence of NLP in the signal has been interpreted as a cue of good physical condition of the signaler (based on the assumption that higher quality individuals can maintain vocal fold stability and produce periodic calls at higher frequencies and amplitudes) therefore minimizing the occurrence of NLP in their acoustic output.

Interestingly we did not identify a distinct, entirely harsh type of roar, as has been reported in the Scottish subspecies. In Scottish red deer, *harsh roars* are characterized by poorly defined or no harmonics and little or no formant modulation, reflecting the fact that the VT is fully extended throughout the vocalization [17]. Harsh roars are given by red deer stags during intense male contests [17,58], and may also function to attract and retain female attention more [13]. The Iberian red deer repertoire appears to lack such a call type as all our calls contained at least one segment with a clear F0 contour and visible harmonics structure. Moreover, formant frequencies clearly drop during the beginning of all analysed calls (Fig 1) indicating that the vocal tract is extended during, rather than immediately before the vocalisation.

We identified 2 further types of calls in our sample: the short common roar, and the series of chase barks. While chase barks have previously been reported in the repertoire of the Scottish red deer stags [17,21] studies of other red deer subspecies (including Iberian red deer) have not reported quantitatively nor qualitatively distinct “short common roar” [17,21,26,43]. However, mostly of the measured acoustic variables take significantly different values in this variant than in long common roar, suggesting that short common roar can be considered as distinct call types from the long common roar. The prominent difference of these vocalizations is the duration, which is dramatically shorter than in long common roars (short common roar: 0.43 ± 0.21 s). Moreover, we measured a difference of 7 dB in relative intensity between calls of group three and those of the others groups

(as estimated from calls recorded in the same sequence), suggesting that short common roars are produced with a lower intensity. The fact that these calls are shorter and tend to be produced with a lower intensity indicate that they may be associated with a lower vocal effort.

We observed that call types were not randomly arranged within bouts: the type of call and its position in the bout are strongly correlated. Opening vocalizations are usually long common roars and semi-harsh roars, whereas short common roars and chase barks are usually found in the central part of the bout. Single vocalizations are almost always long common roars. Accordingly, we suggest that the combination of the different types of calls and their position within a vocalization may reflect variation in physical effort required for their emission.

In conclusion, in contrast with that of the Scottish red deer, the vocal repertoire of the Iberian red deer male is characterized by the presence of the short common roar and by the lack of an entirely harsh roar. Moreover, the long common roar, a call type shared with the other subspecies, shows the highest F₀ in European deer. Experimental investigations are now needed to understand the selective pressure that affects the evolution of vocal behaviour in Iberian red deer as well as in other subspecies.

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Tables

Table 1. List and description of variables used in the analysis.

Call variables	F0-related	
	Mean F0	Mean of the fundamental frequency (Hz)
	Range F0	Difference between maximum and minimum frequencies (Hz)
	Min F0	Lower frequency of the fundamental one (Hz)
	Max F0	Higher frequency of the fundamental one (Hz)
	Time- related	
	Duration	Duration of the call (s)
	PosBout	Position into the bout: First-Middle-Last-Single
	Non linearity-related	
	DC/Not DC	Occurrence of deterministic chaos
	Formants-related	
	F ₁ -F ₈	Minimum values of the first 8 formant frequencies (Hz)
	Df	Formants dispersion (Hz)
	eVTL	Estimated vocal tract length (cm)
Bout variables	DurTot	Duration of the whole bout (s)
	Calls/Bout	Number of units into the bout
	Dur/Calls	Duration of the bout divided for the number of calls (s)

Table 2. Groupings generated from Cluster analysis.

Variables		Groups by Cluster							
		Long Common Roar		Semi-harsh Roar		Short Common Roar		Chase Bark	
DC	Absence	121		0		177		0	
	Presence	0		22		0		14	
Duration	Mean±SD	1.83	±0.54	2.05	±0.61	0.43	±0.21	0.37	±0.42
	Range	0.8	4.2	1	3.9	0.1	1.2	0.1	1.2
MaxF0	Mean±SD	208.48	±30.54	223.64	±27.45	140.05	±32.75	125.57	±41.9
	Range	135	302	164	282	34	247	56	190
MeanF0	Mean±SD	183.60	±23.24	198.86	±39.47	132.05	±33.43	118.29	±39.48
	Range	133	241	126	302	27	244	56	190
MinF0	Mean±SD	127.89	±29.98	132.50	±41.04	115.78	±35.56	105.71	±46.37
	Range	27	225	48	204	28	242	33	190
RangeF0	Mean±SD	80.60	±38.37	91.14	±40.57	24.27	±23.12	19.86	±37.49
	Range	10	200	21	166	0	112	0	139

Variables list, mean frequencies, standard deviations and range for each group of calls. For abbreviations, see Table 1.

Table 3. Kruskal Wallis and non parametric post hoc Tukey tests. Calls are labelled as follow:
Long common roar-LCR; Semi-harsh roar-SHR; Short common roar- SCR; Chase bark- CB.

Variables	Kruskal-Wallis rank sum tests			Post hoc Tukey tests				
	chi-squared	df	p-value	Comparison	95% CI		t-value	p-value
Dur	247.2561	3	<0.0001	LCR vs SHR	0.47	0.77	2.11	0.034
				LCR vs SCR	0.00	0.01	-16.89	<0.0001
				LCR vs CB	0.00	0.06	-8.41	<0.0001
				SHR vs SCR	0.00	0.02	-8.89	<0.0001
				SHR vs CB	0.00	0.09	-5.74	<0.0001
				SCR vs CB	0.09	0.60	-1.77	0.077
MaxF0	203.7468	3	<0.0001	LCR vs SHR	0.49	0.79	2.46	0.014
				LCR vs SCR	0.03	0.09	-13.82	<0.0001
				LCR vs CB	0.01	0.16	-6.21	<0.0001
				SHR vs SCR	0.01	0.07	-10.36	<0.0001
				SHR vs CB	0.00	0.10	-5.93	<0.0001
				SCR vs CB	0.19	0.64	-1.08	0.28
MeanF0	179.2215	3	<0.0001	LCR vs SHR	0.43	0.78	1.63	0.104
				LCR vs SCR	0.04	0.12	-13.09	<0.0001
				LCR vs CB	0.01	0.28	-4.26	<0.0001
				SHR vs SCR	0.02	0.18	-6.45	<0.0001
				SHR vs CB	0.01	0.23	-4.77	<0.0001
				SCR vs CB	0.19	0.63	-1.15	0.251
MinF0	13.3056	3	0.004	LCR vs SHR	0.34	0.73	0.50	0.618
				LCR vs SCR	0.31	0.48	-3.19	0.001
				LCR vs CB	0.16	0.60	-1.49	0.135
				SHR vs SCR	0.21	0.57	-1.66	0.097
				SHR vs CB	0.13	0.59	-1.63	0.103
				SCR vs CB	0.23	0.69	-0.51	0.611
RangeF0	166.7496	3	<0.0001	LCR vs SHR	0.41	0.75	1.23	0.219
				LCR vs SCR	0.06	0.14	-13.27	<0.0001
				LCR vs CB	0.01	0.38	-3.31	0.001
				SHR vs SCR	0.03	0.17	-7.46	<0.0001
				SHR vs CB	0.01	0.38	-3.30	0.001
				SCR vs CB	0.11	0.59	-1.75	0.08

Univariate comparison (Kruskal-Wallis tests) among the four groups showed highly significant differences among all variables analyzed. Differences in acoustic parameters between group pairs from Tukey Post Hoc tests are shown.

Table 4. Factor Analysis.

	Functions		
	1	2	3
DC	0.05	0.02	0.94
Duration	0.67	0.12	
MaxF0	0.95		
MeanF0	0.86	0.13	
MinF0	0.20	0.78	
RangeF0	0.53	0.40	
Variance explained (%)	54.23	23.90	16.04
Variance cumulative (%)		78.13	94.17

Values of the variables loads along the first three axes generated by Factor Analysis with their explained variance. Values <0.1 are not reported, values > 0.5 are in bold. For abbreviations see Table 1

Table 5. Structure matrix from the Discriminant Function Analysis.

	Functions		
	1	2	3
fac1	0.50	-0.94	-0.12
fac2	-0.69	0.29	-0.79
fac3	4.20	0.48	-0.14
Variance %	0.87	0.12	0.02

The covariance coefficients represent the contribution of each variable (factors generated from Factor Analysis) to the discrimination of the types of calls. Factor 3 (main due to DC) has the strongest associated weight with the first linear discriminant function. Explained variance is reported. Highest coefficients for each discriminant function are in bold.

Table 6. Formant frequencies.

F1	F2	F3	F4	F5	F6	F7	F8	Df	eVTL
204±12	407±39	626±42	795±26	1089±29	1385±24	1646±50	1900±60	250.18±6	70.46±5.7

Frequencies of the first eight formants (F1-F8) - Hz, formant dispersion (Df) - Hz, and estimated vocal-tract length (eVTL) - cm, in 6 Iberian deer male (Mean and SD are reported).

Table 7. Bouts patterns.

			Long Common Roar	Semi-harsh Roar	Short Common Roar	Chase Bark	
PosBouts	First	Count and (%)	58 (77%)	9 (12%)	7 (9%)	2 (2%)	76(100%)
	Last		19 (27%)	4 (6%)	43 (62%)	4 (6%)	70(100%)
	Middle		20 (13%)	2 (1%)	126 (80%)	9 (6%)	157(100%)
	Single		21 (67%)	6 (19%)	4 (13%)	0	31(100%)
			(N= 118)	(N=21)	(N= 180)	(N= 15)	Total

Cross tabulated assignment of calls to the groups in relation to the positions into the bouts. Groups are based on DFA model. Difference in sample between first and last calls is due to the exclusion of some calls because of background noise or overlapping.

Figures

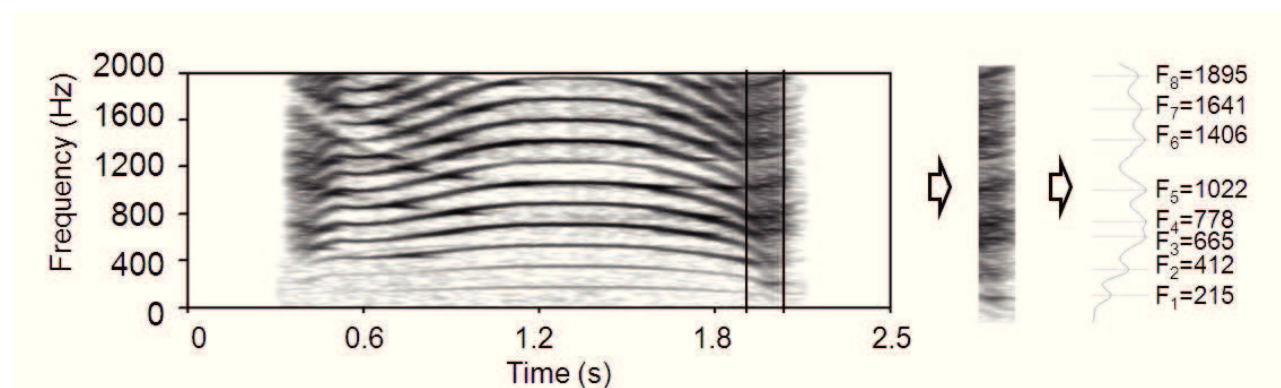


Figure 1. Extraction of the minimum frequency of individual formants in a red deer roar. The section of the spectrogram where individual formants reach their lowest frequencies in the roar was selected. Cepstral smoothing using a 150 Hz bandwidth filter was applied to the spectrum in order to highlight the effect of the filter, removing the effect of the source (F₀) Mean fundamental frequency in the selected segment is 121 Hz. Note formants dropping until the middle of the call.

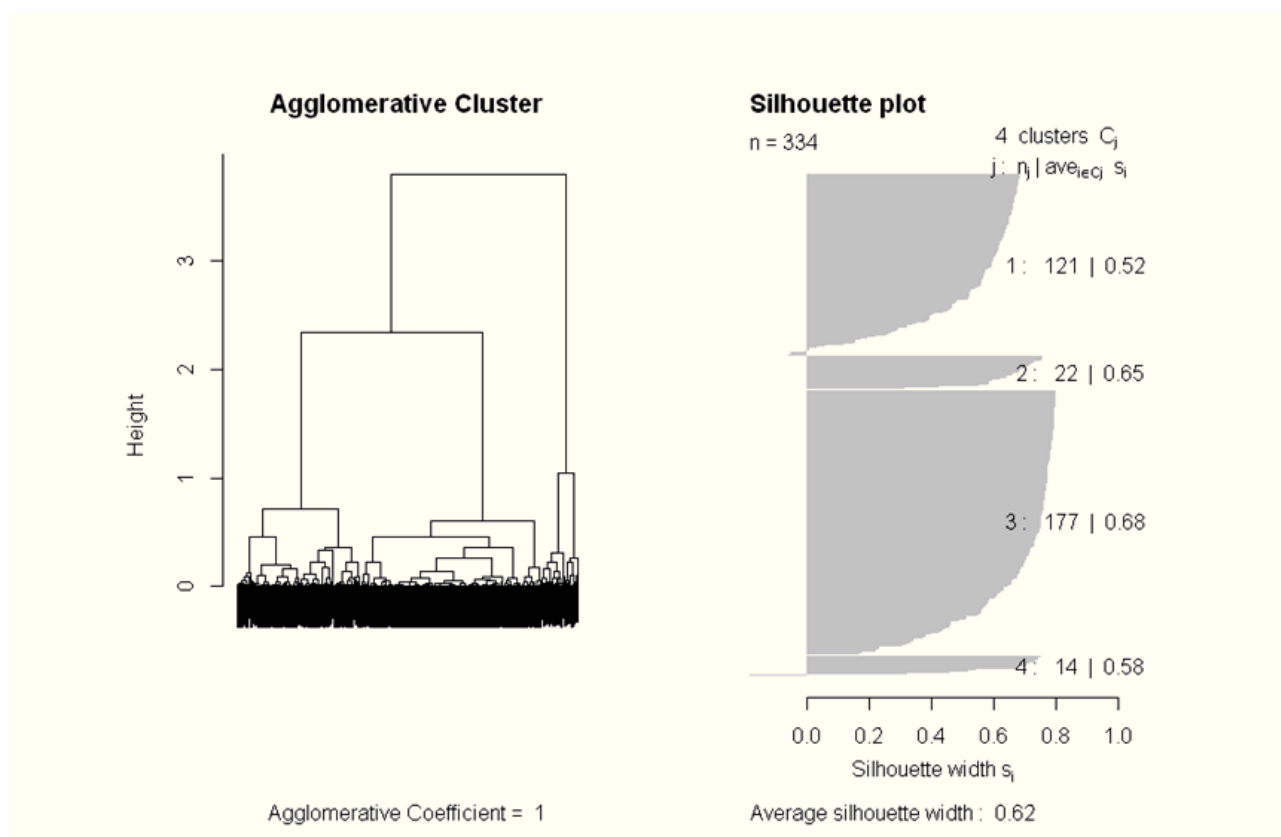


Figure 2. Cluster tree and Silhouette graphic. Cluster analysis was used to detect the presence of relatively homogeneous groups of calls. Silhouette Information was computed as a method of interpretation and validation of clusters of data; the highest average silhouette classification score (0.62) was achieved by a four-groups solution based on DC, duration and maximum fundamental frequencies as input variables

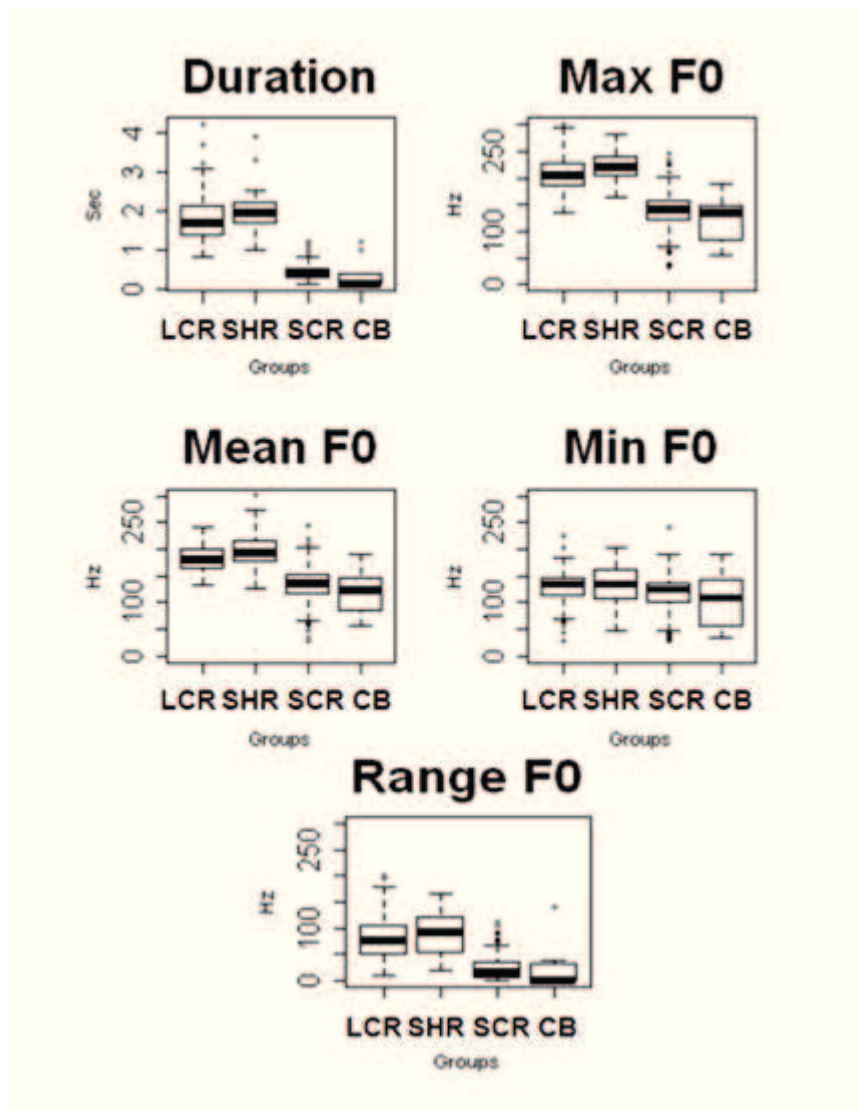


Figure 3. Box plots of variables, split by 4-cluster solution. Calls are labelled as follow: Long common roar-LCR; Semi-harsh roar-SHR; Short common roar- SCR; Chase bark- CB.

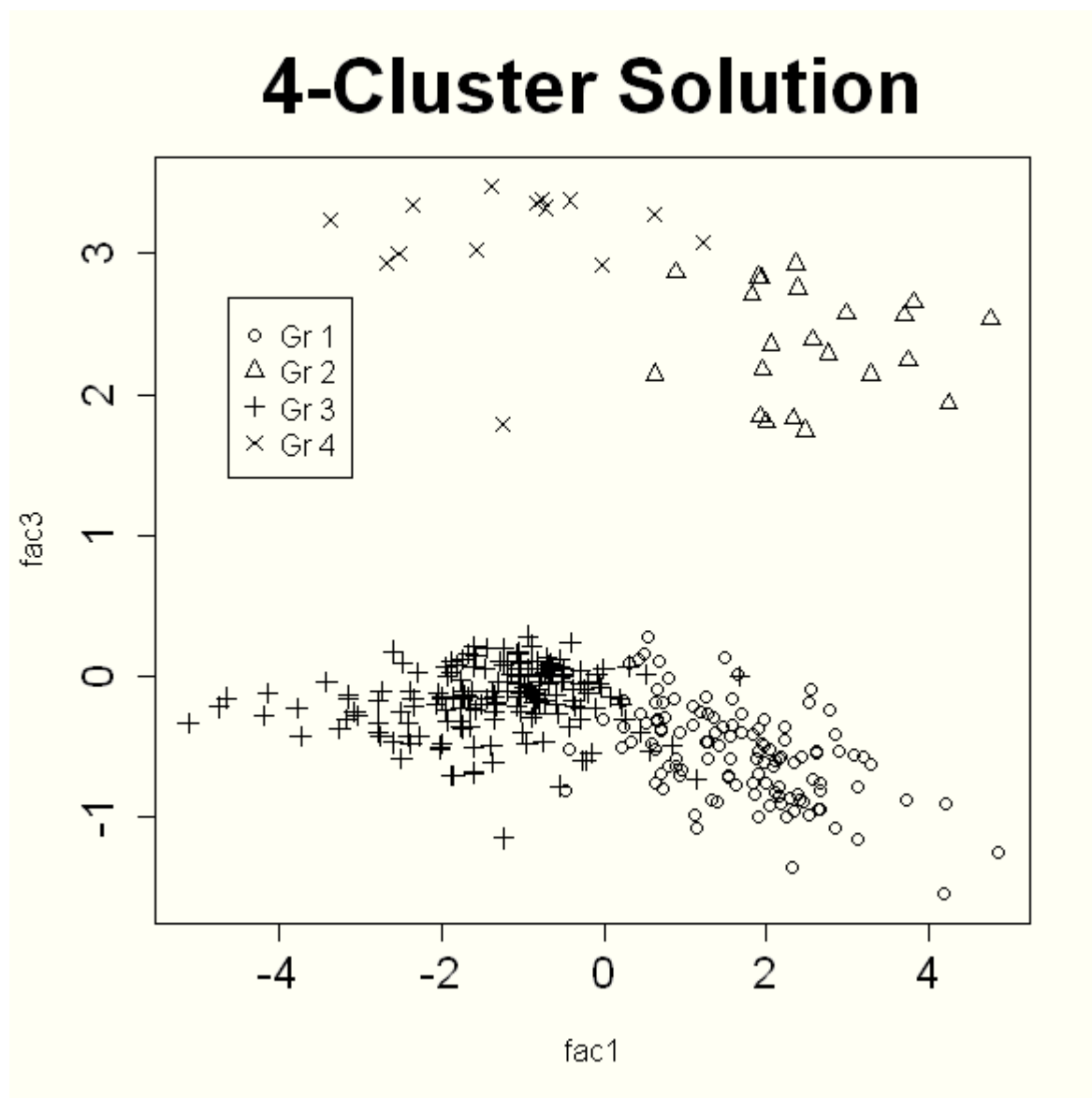


Figure 4. Plot of calls grouped by using a 4-clusters solution. Axes derived by factor analysis. Factor 1 is mainly due to Duration, Mean F0 and Max F0, Factor 3 represents DC. Factor 2 is mainly due to Min F0 and Range F0, thus not relevant in calls classification.

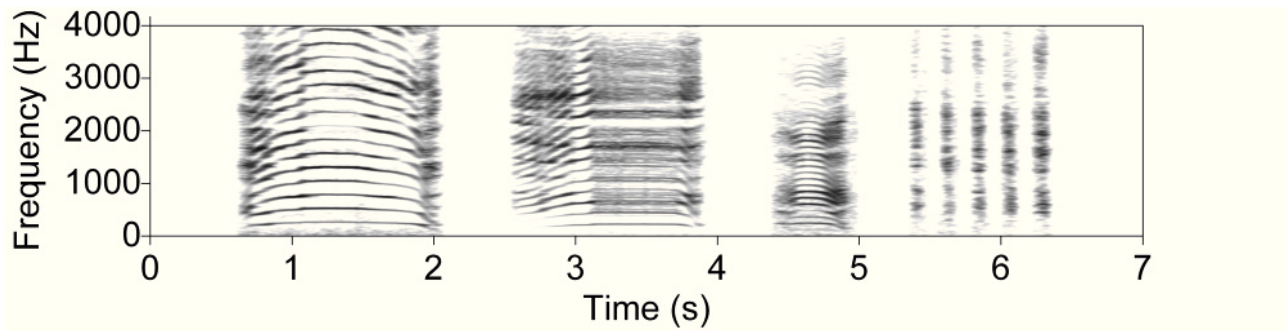


Figure 5. Spectrogram showing iberian deer repertoire. From the left: long common roar; semi-harsh long common roar, short common roar and a series chase barks.

Chapter 4

Geographic variations in the roar of red deer stags

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Geographic variations in the roar of red deer stags

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Abstract

Geographical variations often influence phenotypic aspects as behavioral traits; acoustic behavior can deeply differ between populations within a species. Sound frequencies propagate differently through closed or open environments. These properties of the signals can lead to the evolution of different characteristics in the calls of the same species in different habitats. Acoustic behavior in cervids is highly variable, also among geographic subspecies of *Cervus elaphus*. This species, divided in many subspecies with different morphological and behavioral traits, shows a strong and variable vocal activity. We analyzed the acoustic structures of the main mating call - the roar - in three different free ranging red deer population: *C. e. hispanicus* (Doñana, Spain), *C. e. corsicanus* (Pattada, Sardinia) and *C. e. hippelaphus* (Casentino, Italy).

The aims of this study were to compare quantitatively the stag's roars and explore the vocal patterns of these three red deer populations.

Our results revealed that a strong vocal divergence exist among subspecies, for all pitch related variables, as well as the duration of the signals. Differences in acoustic patterns are not simply explained by body weight, being Corsican red deer the smallest one more acoustically related with European red deer (the bigger one) than with the Iberian.

The different acoustic structure among red deer populations, suggests the presence of a strong selective pressure promoting the divergence of the same signals in the same species.

Further investigations are needed to clarify the role of geographic factors, being able to influence and forge the evolution of behavioral patterns in mammals as well as phylogenetic and physical constraints.

Keywords: red deer, geographic variation, roars, fundamental frequency

Introduction

Variation among different species and their subspecies can be examined at different levels. While early phylogenetic classifications essentially relied on morphological features such as coat colour, body shape and size (Ellerman and Morrison-Scott 1951; Flerov 1952); they have been largely replaced by molecular approaches (e.g. uric acid: Christen et al. 1970; e.g. ribosomal RNA: Mindell and Honeycutt 1990; e.g. mitochondrial DNA: Cantatore et al. 1994, Hartl et al. 1995, Zachos et al. 2003). However, more recently, behavioural characters too such as mating or vocal behaviour have been used for investigating phylogenetic relationships (Cap et al. 2000; Cap et al. 2008; Yu et al. 2011).

Acoustic behaviour can differ significantly in strongly related species (Mendelson and Shaw 2005) and also in different populations within the same species (Claridge and Morgan 1993; Proëhl et al. 2006, Yu et al. 2011). Calls variation is due to their functions (Reby and Charlton 2012) and to the physical constraints operating on their emission and perception (Taylor and Reby 2010). Finally, habitat properties (low frequencies propagate better than high frequencies in closed environments (Wiley and Richards 1978; Maciej et al. 2011)) can lead to the evolution of vocalizations with different features, despite similar functions (Hunter and Krebs 1979).

Geographic variation in sexual signals can be generated by ecological factors (Schluter 2001; Coyne and Orr 2004). For instance, sexual signals might evolve to maximize transmission distance, i.e. the active space, in the habitat (Ryan et al. 1990), or to convey information on the caller and his status (Davies and Halliday 1978; Clutton-Brock and Albon 1979; Reby and McComb 2003a).

Moreover, mating signal variation within the species is also subject and forged by sexual selection (Rayan 1985) and female mate choice often differs in local preferences for mate qualities (Endler and Houde 1995; Griffith et al. 1999). As a consequence, vertebrate vocal communication is highly variable, both within and between species.

Old World deer form a morphologically and ecologically diverse subfamily among the family Cervidae (true deer) that inhabits a variety of terrestrial environments (Pitra et al. 2004). Deer

species show a high degree of diversity in the calls, both within the Cervinae subfamily and among geographic subspecies of *Cervus elaphus*.

The acoustic structure and patterns of calls changes among the investigated subspecies *C. e. Scoticus* (Reby and McComb 2003a, b), *C. e. corsicanus* (Kidjo et al. 2008), *C.e. hispanicus* (Passilongo et al. submitted) along with other behavioural (Cap et al. 2002, 2008) and morphological features (body size: Dolan 1988; cranial measurements: Geist 1991, 1992). Although the vocal repertoires of different subspecies have been investigated, few of these observations were made under natural conditions, and none attempted to quantify variation in these repertoires, geographic or otherwise.

Corsican deer roars have a mean F0 of around 30 Hz (Kidjo et al. 2008), which is considerably lower than the mean F0 of 112 Hz reported in the larger Scottish deer (Reby and McComb 2003a), in contradiction with the general prediction that larger animals should have larger and heavier vocal folds and thus produce lower frequencies (Morton 1977, Ey et al. 2007). Iberian red deer show the highest fundamental frequency among European red deer (180 Hz, Passilongo et al. 2012). To our knowledge, the male acoustic repertoire of *C.e. hippelaphus* (hereafter European red deer), one of the European mainland red deer (Pitra et al. 2004), still remain largely unknown.

Highly variability among acoustic pattern of calls is also explained using the “Source-filter” theory of voice production (Fant 1960), according to which the main components of the calls, the fundamental frequency and the formant frequencies, are produced and perceived independently (Fitch and Hauser 2002). The fundamental frequency (F0) is produced at the source (the larynx) and constitutes a highly distinctive and variable component of mammal calls (Reby et al. 2010). Formant frequencies are resonance peaks produced as the glottal wave travels through the supra-laryngeal vocal tract (Fant 1969). Formant frequencies and overall formant dispersion (the frequency spacing between the formants) should decrease as the length of the vocal tract (the distance between larynx and lips or nostril) increases (Fitch 1997) and, because the length of the vocal tract is likely to be constrained by body size, formants characteristics typically transmit at the

receivers a more reliable indication of body size than fundamental frequency (Fitch 1997, Riede and Fitch 1999).

F0 is determined by the rate of the vibration of the vocal folds caused by the passage of air through the closed glottis and it is perceived as the pitch of the sound. Several studies suggest that F0 is not a reliable index of body size in red deer (Reby and McComb 2003) as in other mammals (Lass and Brown 1978; Rendall et al. 2005), suggesting that very different selective pressures have affected the evolution of this feature in closely related taxa.

Thus, the aims of this study were to quantify variation in the fundamental frequency of the most commonly vocalized call type -the roar- of three free ranging subspecies and define acoustic parameters that differentiate populations. More specifically, to assess the quantitative and qualitative acoustic variation of vocal signals of two Mediterranean subspecies of red deer: the Iberian red deer *C. e. hispanicus* and the Corsican red deer *C. e. corsicanus* and a continental red deer subspecies: *C.e. hippelaphus*.

Materials and methods

Study areas and populations

Roars of *C. elaphus sp* were recorded in 3 geographic areas during the years 2010 and 2011 (Figure 1): the northern Apennines (Tuscany, Italy), the Monte Lerno Forest (Sardinia, Italy) and the Doñana Park (Andalucía, Spain).

Calls from *C. elaphus hippelaphus* were recorded in the northern Apennines, comprise approximately two thirds 223 km² of the Foreste Casentinesi National Park, plus surrounding zones open to human exploitation. Altitude ranges between 400 and 1658 m a.s.l. Forest cover exceeds 80% and the vegetation comprises species typical of temperate-sub-Mediterranean zones.

In this part of the Apennines, a reintroduced population of red deer from the eastern Alps (Mazzarone et al. 1997) is spreading out; the species is protected within the National Park but are hunted outside it in the August-January period since 2000. Calls from *C. e. hispanicus* were recorded in Doñana National Park, included a western area with Mediterranean shrub land and an eastern area with a marsh (dry during the period of study) separated by an ecotone, a long narrow strip of land with meadows and rushes. The climate is typically Mediterranean with hot, dry summers and mild, wet winters.

Calls from *C. e. corsicanus* were recorded in the Forest of Monte Lerno covers an area of 2853 hectares entirely included in the territory of Pattada, in the Province of Sassari, where the species were reintroduced in 2003. Altitude ranges between 400 and 1039 m a.s.l. Vegetation cover is extremely heterogeneous due to a strong human pressure and fires, but is basically constituted by Mediterranean maquis degraded at various stages. The climate of the area is semi-continental type, with wet winters and moderately dry summers and rainy spring and autumn.

Calls were recorded from a four wheel car close to the males' territories in order to minimize the distance between the microphone and the rutting deer. For each male, selected roars had been recorded on the same day. Because vocalisations produced within the same bout (a sequence) are more likely to be acoustically homogeneous than vocalisations emitted in different bouts (Briefer et

al. 2010), each analyzed roar were extracted from different bout. A total of 210 roars from 21 males (10 calls from 7 males for each of the 3 populations) were extracted and analyzed.

Acoustic analysis

Audio tracks were recorded with a hand-held Sony PCM D-50M digital recorder, with 44,100 Hz sampling rate and 16 bits accuracy, connected with a Sennheiser directional microphone with windshield ME67 head with K6 power module) and saved in “.wav” format.

All analyses were performed on a HP Compaq nx7400 with a SoundMAX integrated Digital HD Audio soundcard using Praat version 5.2.13 DSP package for Windows 47 P. (Boersma and D. Weenink, University of Amsterdam, The Netherlands). Narrow-band spectrograms of the vocalizations were edited (Gaussian window shape, window length = 0.03 s; frequency step =250; frequency resolution = 20 Hz;).

Pitch values for each call were extracted using a forward cross-correlation [to pitch (cc) command] algorithm in Praat. The time step in the analysis was 0.03 s and the specified expected values for limits of pitch were setting in dependence of the range of each population (between 20 and 300 Hz). Pitch variables included in the analysis were: mean (MeanF0), lower (MinF0) and the higher (MaxF0) of fundamental frequency (F0). We also measured F0 perturbation as Jitter and Shimmer. Jitter is a measure of period-to-period fluctuations in fundamental frequency (Li et al. 2007) Shimmer is the mean absolute difference between the amplitudes of consecutive F0 periods divided by the mean amplitude of F0 (Li et al. 2007, Briefer et al. 2010). To increase the validity of our jitter measurements, we averaged three measurement values (local, relative average perturbation, and 5-point period perturbation quotient) (Titze and Liang 1993, Charlton et al. 2009). Finally, we calculated the duration of the calls (Dur).

Statistical analysis

Descriptive statistics (Mean and SD) were computed for all extracted variables. To test differences between populations for all call parameters, we used one-way Anova, followed by Bonferroni post hoc tests to ascertain which population pairs differed in which call parameters. Equal variances were verified by Leven tests. We computed between and within population coefficients of variation (CV_b and CV_i, respectively) as follows: $CV = 100 * (1 + 1 / (4 * n)) * (SD / X_{mean})$, where SD is the standard deviation, X_{mean} is the mean of the sample and n is the sample size (Sokal and Rohlf 1995). We then calculated a potential of area coding (PAC) for each parameter with the ratio CV_b/mean CV_i, where mean CV_i is the mean value of the CV_i of all individuals. Coefficients of variation ($CV = SD * 100 / \text{mean}$) were calculated for every call parameter within and among populations to find out which call parameters are most distinctive. For a given parameter, a PAC value greater than 1 indicates that this parameter is likely to be used for population recognition because its intra-area variability is smaller than in inter-area variability (Robisson et al. 1993).

Hierarchical Cluster Analysis and dendrogram (method: Ward; distance: Euclidean) on individual male means for all roar variables were also performed to test bioacoustic distances among populations.

Analyses were performed using R 2.14.0 statistical software (R Development Core Team 2010) with significance levels set at $P < 0.05$.

Results

Mean values of the vocal parameters obtained for *C.e.corsicanus*, *C.e.hispanicus* and *C.e.hippelaphus* are shown in Table 1. All means of pitch related variables were lower for *C.e.corsicanus*, medium for *C.e.hippelaphus* and higher for *C.e.hispanicus*, except Jitter and Shimmer, which showed the opposite trend. Duration was constant between Hispanic and Sardinian populations, while lower for Apennine population (Table 1).

Univariate comparison (ANOVA) among the 3 populations showed on the whole significant differences for all analyzed variables (Table 2). Bonferroni post hoc tests were statistically significant ($p < 0.05$) for all pair-wise comparisons with few exceptions: Dur between Iberian and Corsican red deer (Mean differences = -0.001; SE = 0.16; $p = 1.000$), MinF0 between Red and Iberian red deer (Mean differences = 17.513; SE = 5.51; $p = 0.094$) and Shimmer between Corsican and Red deer (Mean differences = -0.174; SE = 0.10; $p = 1.000$).

Predictive Area Coding showed values > 1 for all parameters (Table 2); however, pitch contour variables (MeanF0, MaxF0 and MinF0) reached the highest PAC. Coefficients of variation were highly different among population. The highest variability in Dur were found in *C.e.hippelaphus*, while the pitch shape parameters were more variables in *C.e. corsicanus*; F0 perturbation were more variable in *C.e. hispanicus*.

Hierarchical Cluster Analysis on individual male means, showed only one individual misclassified from *C.e.hippelaphus* to *C.e.corsicanus* (Figure 2). The first node divided Corsican red deer from the other two populations assigning however, one individual of *C.e. hippelaphus* to this group. The second node divided Iberian and European red deer.

Distance matrix (Table 3) revealed bioacoustic distances intra and inter populations: the lower bioacoustic diversity, were found in Iberian red deer (0.755), while the higher values intra population were found in European red deer (1.493), however also Corsican red deer showed high bioacoustic diversity (1.345). Among populations, the highest differences were found between

Corsican and Iberian red deer (3.768), followed by the distance between Iberian and European red deer (2.928) and finally between Corsican and European red deer (2.629) (Table 3).

Discussion

Our results revealed that a strong vocal divergence exist among subspecies, for all fundamental frequency related variables, as well as the duration of the signals.

The characteristics of the fundamental frequency are determined by the tension and the size of the vocal folds: the tenser the vocal folds, the higher their oscillation rate, and hence the fundamental frequency, and *vice versa* (Ey et al. 2007). Although vocal fold length is proportional to body size (Ey et al. 2007), this assumption is not verified among red deer subspecies. Corsican red deer, the smallest one (88 Kg, Kidjo 2007), displayed the lower F0 (mean F0= 41 Hz) among the three investigated subspecies, Iberian red deer (adult males weight: 160 Kg (Carranza 2004)) present the highest F0 (mean F0= 171 Hz) while the heaviest subspecies *C.e. hippelaphus* (195 Kg, Bonnet & Klein 1991) show a mean F0 of 125 Hz.

Anatomical investigation are needed to clarify the relation of the vocal fold with the body size; however, a strong allometry among body parts of Corsican red deer (short-legged red deer with a disproportionately long head (Vigne 1983) is well documented.

Voice perturbation analysis (Jitter and Shimmer values) is useful to determine the degree to which vocal fold vibration is aperiodic and among populations; our results show that F0 perturbation is inversely related with the F0 values. Perceptually, such acoustic phenomena seem harsh and ‘noisy’ (Fitch et al. 2002) and instead, harshness progressively rise from Iberian (the highest F0), to European and Corsican red deer (the lowest F0) (Figure 3). High jitter is related with rise in testosterone and excitement in humans (Li et al. 2007) as well in other animals (Li et al. 2007, Charlton et al. 2010) and generally believed to function to increase the auditory impact of calls (Owren and Rendall 2001; Fitch et al. 2002). Male red deer utter their roars exclusively during the mating season, when the excitement reach its highest level. However, geographic differences in hormones levels has been observed in mammals (glucocorticoids: Mateo 2006) and birds (testosterone: Moore et al. 2002, Horton et al. 2010). It could be interesting compare testosterone of

these population to verify if the evolution of this non-linearity is an under-product of the pitch frequencies, or if F0 perturbation is related to different emotional levels caused for instance by a differential resource distribution (i.e. oestrus females) or by competition levels, showing therefore an hormonal control.

Pair wise comparison and Predictive Area Coding show very lower overlap among values of the acoustic parameters highlighting the structural differences of roars although it serves at the same function. Coefficients of variation of the three populations, as well as bioacoustic distances *intra* populations, show different degrees of diversification in the acoustic patterns of the subspecies: European deer show the highest *intra* population diversity, followed by Corsican red deer and Iberian red deer. Considering *inter* population acoustic variations, Corsican and European Red deer are the most related subspecies while the highest differences were found between Corsican and Iberian red deer.

Various hypotheses have been proposed to explain geographic variations in sexual signals (reviewed in Gerhardt and Huber 2002): differences in habitat acoustics may lead to differences in signals; another hypothesis is related to arbitrary differences in local preferences for certain mate traits (Endler and Houde 1995; Griffith et al. 1999), moreover, calls variation may arise from genetic drift and finally, acoustic signals can be sensitive to diverse stochastic forces (Campbell et al. 2010). All these factors however are clearly mediated by the expression of the genes, but the mechanisms involved in this expression are far to be understood.

The relatively high deer diversity in modern fauna is often attributed to a burst of Pleistocene speciation (Geist 1987, Pitra et al. 2004). Pleistocene was an exceptionally speciose period for deer. High variability in acoustic behavior of *C.e.hippelaphus* could be evolved in the variety of environment of the Middle Europe, mostly closed forest; while Corsican red deer colonized the Tyrrhenian Islands relatively recently, via mainland Italy during the height of the last glaciation (22–18 000 years before present) (Hmwe et al. 2006), Iberian red deer evolved independently from the other subspecies in the Iberian Peninsula (Zachos and Hartl 2011), and are still geographically

isolated from other red deer populations in Eurasia and Maghreb (Carranza 2004). Iberian Peninsula is characterized by different vegetation cover and density from the Central Europe it thus possible that environmental characteristics, such as open forest, strongly favoured the evolution of calls with high frequency (constrained in variation) and therefore a better propagation in open areas.

As concern phylogeography of European Red Deer, Skog and colleagues (2009) identified the three lineages, based on mitochondrial DNA, that displayed a phylogeographical pattern dividing individuals into western European, eastern European and Mediterranean (Sardinia, Spain and Africa) groups, suggesting contraction into three separate refugia during the last glaciation, a pattern shared with many other European mammals (Hewitt 2004). While studies on genetic variation (Zachos et al. 2003; Hmwe et al 2006) found Sardinia and Spain most closely related in terms of net nucleotide diversity and haplotype distribution, other study (Ludt et al. 2004) found the subspecies *C. hippelaphus* and *C. hispanicus*, together with other Western Europe populations not differentiated by the mtDNA cytochrome b sequence data.

Despite of controversial genetic relationship, the different acoustic structure among red deer populations, suggests the presence of a strong selective pressure promoting the divergence of the same signals in the same species. Differences in local selection pressures on signal transmission efficiency, sexual selection or competition in calling assemblages may be potential causes (Wollermann and Wiley 2002).

Although these differences could be hardly related with certainty to genetic, or ecological factors, being these factors strongly interconnected, this study confirms the evidence that vocal behaviour is a peculiar and distinctive behavioural trait, useful in the description and understanding of closely related but geographically distinct taxa. Further investigations are needed to clarify the role of geographic factors, being able to influence and forge the evolution of acoustic signals in mammals as well as phylogenetic and physical constraints.

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Tables

	<i>C.e. corsicanus</i>	<i>C.e.hispanicus</i>	<i>C.e.hippelaphus</i>
Dur(s)	1.80 ± 0.56	1.80 ± 0.44	1.32 ± 0.48
MeanF0(Hz)	41.34 ± 10.40	172.70 ± 27.64	125.28 ± 27.02
MinF0(Hz)	29.85 ± 6.27	98.95 ± 30.01	81.43 ± 16.85
MaxF0(Hz)	57.92 ± 15.62	201.15 ± 31.91	161.00 ± 40.45
Jitter(%)	4.55 ± 2.45	0.82 ± 0.95	2.75 ± 2.50
Shimmer(%)	15.19 ± 3.27	5.37 ± 2.55	15.02 ± 3.75

Table 1. Descriptive statistics. Pitch related variables and duration. Mean and standard deviations for each variable are shown by populations.

	<i>CVcors</i>	<i>CVhispl</i>	<i>CVhippl</i>	<i>CVb</i>	<i>CVi</i>	PAC	F	p
Dur	31.07	24.79	36.71	33.19	30.86	1.08	5.55	0.013
MeanF0	25.24	16.06	21.65	52.32	20.98	2.49	86.89	<0.0001
MinF0	21.09	30.44	20.76	50.88	24.10	2.11	45.78	<0.0001
MaxF0	27.06	15.92	25.21	48.56	22.73	2.14	69.54	<0.0001
Jitter	54.09	116.73	91.08	95.58	87.30	1.09	24.02	<0.0001
Shimmer	21.59	47.68	25.07	47.36	31.45	1.51	59.07	<0.0001

Table 2. Predictive Area Coding and ANOVA. Coefficient of variation between and within population and Predictive Area Coding (PAC), calculated as the CVb/CVi . Parameters with a PAC <1 indicate are not useful for classified the population (variability intra population higher than variability among populations). ANOVA results testing for differences in call parameters among the 3 populations.

Subspecies	<i>C.e. corsicanus</i>	<i>C.e.hispanicus</i>	<i>C.e.hippelaphus</i>
<i>C.e. corsicanus</i>	1.345	3.768	2.629
<i>C.e.hispanicus</i>		0.755	2.928
<i>C.e.hippelaphus</i>			1.493

Table 3. Bioacoustic Euclidean distance among 21 individuals of the 3 populations on rescaled acoustic parameters. Both intra than inter population distance is reported.

Figures



Figure 1. Study areas. Map of the western Europe, showing the locations of sampling sites.

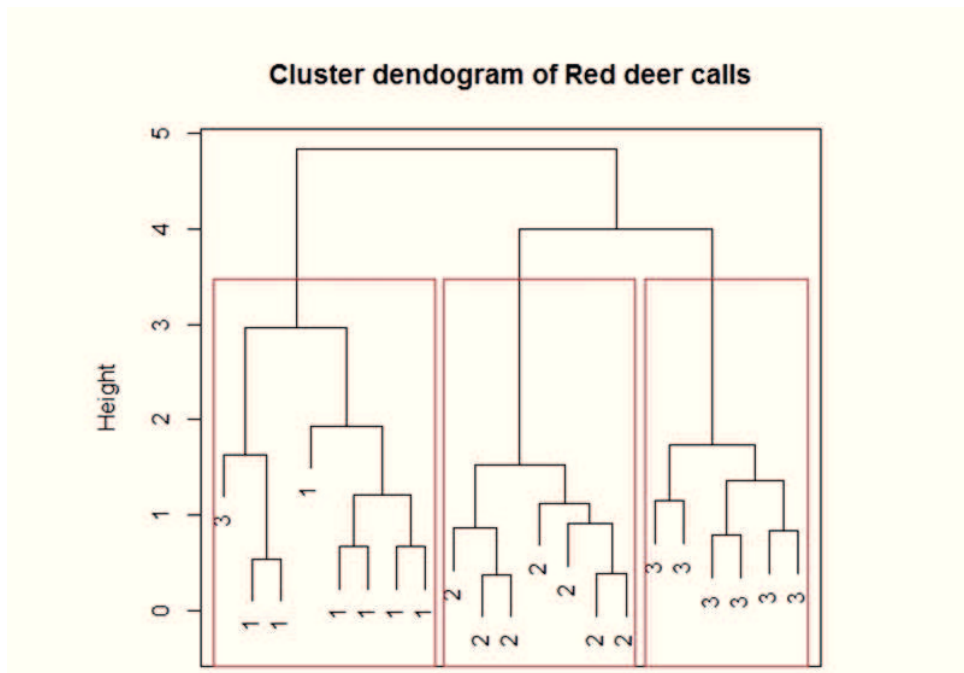


Figure 2. Cluster Analysis. Dendrogram of the variation between male calls from different populations of Red deer. 1: Corsican deer; 2: Iberian red deer, 3: European Red deer.

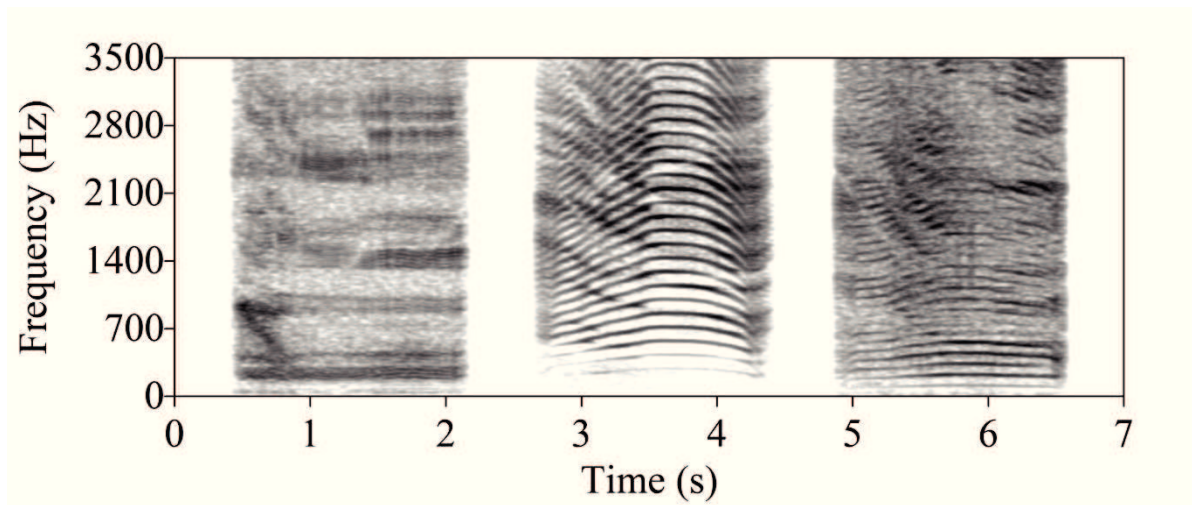


Figure 3. Spectrogram showing roars from the three populations. From the left: *C.e.corsicanus*, *C.e.hispanicus* and *C.e.hippelaphus*.

CONCLUSIONS

As predicted by the “Acoustic Adaptation Hypothesis” (Morton 1975), wolf long-distance communication mainly employs low frequencies. Wolf’s vocal range is between 70 Hz and more than 9900 Hz (Schassburger 1993), but only the lower frequencies of this range are actually involved in the production of howls. My results confirmed this hypothesis, showing Italian wolf howl a fundamental frequency consistent with those reported for other wolf populations (Theberge and Falls 1967; Tooze et al. 1990, Schassburger 1993; Palacios et al. 2007) and never exceeding the value of 1356 Hz (**Chapter 1**). In this study I documented two main howl structures, breaking and flat howls, as already distinguished by Harrington and Mech (1978) in their study on North American populations. On the contrary I can’t support the division into continuous and breaking howls found in the Iberian wolf (Palacios et al. 2007). Italian howls structures were so intrinsically variable that further basic types of howl could not be identified.

We also tested, for the first time, the hypothesis that howls intra pack variability were lower than inter pack variability (**Chapter 2**). My findings indicated that howls emitted by wolf packs were acoustically distinct ones each other except in the case of the two adjacent packs. Several primate, bat and cetacean species show group-specific acoustic differences and are able to discriminate between familiar and unfamiliar individuals (primates: Cheney and Seyfarth 1990, 2007; Crockford et al. 2004; Herbinger et al. 2009; bats: Boughman 1997; cetaceans: Ford 1991; Tyack 2000). Most of these species exhibit a complex social structure and defend their own territory from the intruders (Towensed et al. 2010), as the wolves do. Moreover some of them live in environments with scarce visibility, where visual cues are useless to convey messages.

The ability to recognize individuals and determine their location based on vocalizations allowed wolves to use auditory cues to coordinate social activities. Moreover, the differentiation between familiar and unfamiliar howls is highly advantageous for lone individuals (Tooze et al. 1990). While discrimination requires that individuals or groups differ enough at one point in time to be separated, identification, as vocal signature, needs that vocal signals remain constant enough to be associated

with that individual or groups for periods of time (Terry et al 2005). My results showed that packs were able to maintain the same acoustic structure during the same season as well as in two consecutive years. Further investigations are needed to clarify the role of cultural (vocal learning) and genetic transmission of group vocal signatures. Anyway, wolves that take part into a chorus tend to high frequencies modulation among their howls and this characteristic is stable over the time,

As in the case of wolves among red deer subspecies too exist a strong vocal divergence, both in terms of frequency range of the most common call type -the long common roar -as well as in the whole repertoire. My results revealed that the acoustic variation was related to fundamental frequency and the duration of the signals (**Chapter 4**).

Bioacoustic distances documented different degrees of diversification in the roar of the subspecies. *C.e. hippelaphus* showed the highest *intra* population diversity, followed by *C. e. corsicanus* and *C. e. hispanicus*. The low variation in the long common roar of Iberian red deer was compensated with the presence of the “short common roar” (**Chapter 3**), absent in the repertoire of the others investigated red deer subspecies. In Iberian deer long common and short common roar were structurally and functionally separated, the first selected on the high F0 (the highest among red deer subspecies), the second for carrying formants and their cues.

Considering *inter* population acoustic variations, the highest bioacoustic differences were found between Corsican and Iberian red deer, while Corsican and European red deer were the most related subspecies. These geographic variations in sexual signals may be related to differences in habitat acoustics or to female local preferences (Endler and Houde 1995; Griffith et al. 1999), but it is hardly to determinate the relative importance of these factors (Delgado 2007).

As concern phylogenetic studies, some authors (Zachos et al. 2003; Hmwe et al 2006) found Sardinia and Spain most closely related in terms of net nucleotide diversity and haplotype distribution, while Ludt and colleagues (2004) found the subspecies *C. hippelaphus* and *C. hispanicus* were not differentiated by the mtDNA cytochrome b sequence data. Despite of

controversial genetic relationship, the different acoustic structure among red deer populations suggests that different selective pressure operate on the sexual signals of these subspecies.

Further investigation involving the bioacoustic distances among other red deer subspecies are needed to clarify the origins of these differences and relationships. My study confirms that behavioural patterns may complement morphological and molecular information to understand evolutionary relationships among closely related taxa.

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