Diversity of *Staphylococcus aureus* clones in wild mammals in Aragon, Spain, with detection of MRSA ST130-*mecC* in wild rabbits

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Running title: *S. aureus* in wild mammals

Abstract

Aims: To determine the *S. aureus* carriage rate in wild mammals in Aragon, northern Spain, to analyse their antimicrobial resistance phenotype/genotype and to characterize the recovered isolates.

Methods and results: Nasal and rectal swabs of 103 mammals were collected in Aragón during 2012-2015. Antimicrobial susceptibility, the presence of antimicrobial resistance genes and virulence factors were investigated. Molecular characterization was carried out by *spa*, MLST, *agr* and SCC*mec*. *S. aureus* were recovered from 23 animals (22%). Four out of the 23 *S. aureus* were MRSA. Three MRSA were *mecC*-positive and were isolated from European rabbits and were typed as t843 (ascribed to CC130). The remaining MRSA was a *mecA*-carrying isolate from European hedgehog, typed as ST1-t386-SCC*mec*IVa-*agr*III and it harbored the *blaZ*, *erm*(C), *ant*(6)-Ia and *aph*(3´)-IIIa resistance genes. A high diversity of *spa*-types was detected among the nineteen MSSA isolates, which showed high susceptibility to the antimicrobials tested. The *tst* gene and different combinations of staphylococcal enterotoxins were found.

Conclusions: *S. aureus* were detected in nasal and rectal samples of wild mammals. Wild rabbits could be a reservoir of *mecC*-MRSA.

Significance and Impact of Study: This work provides information on the presence and characteristics of *S. aureus* from mammals in a defined geographic region in Spain.

Keywords: free-living animals, *S. aureus*, *mecC*-MRSA, *mecA*-MRSA, CC130

Introduction

Staphylococcus aureus is a ubiquitous microorganism widely disseminated in the natural environment, such as in the water, air, soil, humans and animals (Hennekinne *et al*. 2012). *S. aureus* is part of the normal microbiota of the skin, the nose and mucous membranes of healthy humans and of a wide range of animal species, particularly mammals and birds. However, it is also recognized as one of the most important human opportunistic pathogens, causing several diseases of diverse severity, including skin and soft tissue infections, endocarditis and necrotizing pneumonia, among others.

The pathogenicity of *S. aureus* is due to the presence of variable virulence factors, such as the staphylococcal enterotoxins (SEs), which are responsible for food poisoning (Argudín *et al*. 2010), and the toxic shock syndrome toxin (TSST). Moreover, *S. aureus* have also a great capacity to acquire multiple resistance mechanisms to several antimicrobial agents (van Belkum *et al*. 2009), which limits their therapeutic options. In this regard, it is especially relevant methicillin-resistant *S. aureus* (MRSA), carrying *mecA* or *mecC* genes, which represents a major public health concern, and it is considered as one of the main pathogens causing nosocomial infections (McCarthy *et al*. 2012).

S. aureus has been recovered from several healthy or sick animal species worldwide, including livestock, companion animals and free-living animals (Monecke *et al*. 2016). Some genetic lineages of MRSA are frequently detected in animals, which is the case of CC130. This clonal complex was initially associated with methicillin-susceptible *S. aureus* (MSSA) from animals, but in recent years, it has gained interest because of its relationship with the methicillin-resistance determinant *mecC* and the SCC*mec* (staphylococcal cassette chromosome *mec*) type XI. Moreover, though CC130 is considered a livestock-associated (LA) MRSA, it has also been found causing human illness (Pantosti 2012).

Free-living animals are not directly in contact with antimicrobial agents, but they can be colonized or infected via human and livestock sources, among others. In this context, wildlife could act as reservoir and vehicle of transmission of antimicrobial resistance determinants (Carroll *et al*. 2015). In recent years, there has been growing interest in the molecular epidemiology of *S.*

aureus in animals due to the increasing number of genetic lineages associated with animal hosts and its evidenced zoonotic potential.

Previous studies performed in Spain, some of them by our group, have determined the prevalence and characteristics of *S. aureus* isolates in different wild mammals and from different geographic regions (Porrero *et al*. 2013; Porrero *et al*. 2014; Gómez *et al*. 2014; Mama *et al*. 2019). In these studies, animals from Aragón, a geographic region located in the North of Spain, were scarcely represented (Porrero *et al*. 2013; Porrero *et al*. 2014). Aragón is an important territory with a great wildlife biodiversity and natural resources, located in the way of travelling of migratory birds. For this reason, the objective of the present study was to analyse the carriage rate of *S. aureus* recovered from wild mammals from Aragón, to determine their antimicrobial resistance profiles, as well as to perform the genotypic characterization of the recovered isolates.

Material and methods

Sampling and bacterial isolation

Nasal and rectal swabs from 103 free-living mammals were collected from 2012 to 2015 in the Aragón region in northern Spain. The number and species of animals studied are included in the Supplementary Table S1. These animals were sampled at the arrival to the Reference Centre of Wildlife Recovering La Alfranca (CRFSA) or after hunting process in the case of game animals. Samples were inoculated into Brain Heart Infusion broth (Conda, Madrid, Spain) with 6.5% NaCl and incubated at 37ºC for 24 hours. An aliquot of 20 µl was subcultured on Mannitol Salt Agar (Becton-Dickinson, Sparks, MD, USA) and Oxacillin Resistance Screening Agar-Base (Oxoid, Basingstoke, Hampshire, UK) containing 2 mg/L of oxacillin and incubated for 24 hours at 37ºC. Identification of *S. aureus* was performed by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) (Bruker Daltonik, Bremen, Germany) system following the standard extraction protocol using formic acid recommended by Bruker. One *S. aureus* isolate per sample was selected and further characterized.

All procedures were carried out under Project Licence PI11/057 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Antimicrobial resistance profile

Antimicrobial susceptibility was performed by disk-diffusion method in accordance with the European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST, 2017), with the exception of streptomycin and fusidic acid for which the recommendations of Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM, 2017), were used. The following antimicrobial agents were tested (µg/disk): penicillin (10 units), cefoxitin (30), erythromycin (15), clindamycin (2), gentamicin (10), tobramycin (10), streptomycin (10), tetracycline (30), ciprofloxacin (5), chloramphenicol (30), linezolid (30) and trimethoprim-sulfamethoxazole (1.25 + 23.75).

On the basis of the resistance phenotype of each isolate, the presence of 10 antimicrobial resistance genes was investigated by PCR: *mecA*, *mecC*, *blaZ*-SCC*mec*XI, *blaZ*, *erm*(A), *erm*(B), *erm*(C), *str*, and *ant*(6)-Ia (Sutcliffe *et al*. 1996; Clark *et al*. 1999; Poulsen *et al*. 2003; Schnellmann *et al*. 2006; Cuny *et al*. 2011; García-Álvarez *et al*. 2011). The *aph*(3´)-IIIa and *vga*(A) genes were tested in CC1 strains (van de Klundert *et al*. 1993; Lozano *et al*. 2012).

Molecular characterization

The *spa*-typing was performed in all isolates as previously described (Shopsin *et al*. 1999) and the obtained sequences were analysed using Ridom® Staph-type software. Multilocus-Sequence-Typing (MLST) was performed in selected isolates (Enright *et al*. 2000), and according to the sequence-type (ST), the isolates were ascribed to the different clonal complexes (CC). The CC was assumed for some isolates, according to its specific *spa*-type. All isolates were

characterized by *agr*-typing (Shopsin *et al*. 2003), and MRSA strains were subjected to SCC*mec*-typing following standard methodology (Zhang *et al*. 2005; Cuny *et al*. 2011).

Virulence gene content

The presence of the virulence genes encoding toxic-shock syndrome toxin (*tst)*, Panton-Valentine leukocidin (*lukF*/*lukS*-PV) and exfoliative toxins (*eta*, *etb* and *etd*) was checked by PCR in all *S. aureus* isolates (Lina *et al*. 1999; Jarraud *et al*. 2002; Yamaguchi *et al*. 2002). The five genes of the immune-evasion-cluster (IEC) system (*scn*, *chp*, *sak*, *sea* and *sep*) were studied by PCR (van Wamel *et al*. 2006); the *scn* gene, encoding the staphylococcal complement inhibitor is considered the marker of the IEC system. Furthermore, the presence of 18 SEs (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser* and *seu*) was carried out by three multiplex PCR as described elsewhere (Hwang *et al*. 2007).

Results

Twenty-three animals out of the 103 analysed (22.3%) were *S. aureus*-carriers. Positive samples included 11 wild boars, four red deers, four European mouflons, three European rabbits and one European hedgehog. Moreover, *S. aureus* isolates were detected in all mouflons tested, as well as in 65% of wild boar, 44% of deers, and 8% of wild rabbits. A higher number of isolates were recovered from nasal samples (n=17) than from rectal swabs (n=6); and none animals were positive for both nasal and rectal samples. Table 1 shows the characteristics of the 23 *S. aureus* isolates recovered in this study.

Among the 23 *S. aureus* recovered, four were MRSA; three of them harbored the *mecC* gene and the remaining one was *mecA*-positive. All three MRSA-*mecC* strains were recovered from wild rabbits, were typed as t843 (ascribed to CC130), and showed resistance only to β-lactam antimicrobials (penicillin and cefoxitin). The *mecC*-positive strains carried the SCC*mec* XI, and harbored the specific *blaZ* allotype included in this SCC*mec*. The *mecA*-positive *S. aureus* strain was recovered from the nasal sample of a European hedgehog, and was typed as: *spa*-type-t386, sequence-type ST1, clonal complex CC1, SCC*mec*-IVa and *agr*-type-III. This CC1 MRSA strain showed resistance to penicillin, cefoxitin, erythromycin, streptomycin and inducible re-

sistance to clindamycin and harbored the *blaZ*, *erm*(C), and *ant*(6)-Ia genes; in addition, this strain was positive for *aph*(3´)-IIIa gene, but lacked *vga*(A) gene.

Among the 19 MSSA, 10 different *spa*-types were detected (t1125, t1534, t1535, t3750, t6056, t6386, t7174, t11225, t11230 and t11233), being *spa*-types t1535 and t3750 the predominant ones in wild boar and t6056 (ST133) in European mouflon (Table 1). *S. aureus* belonging to CC130 (*agr*-III) but lacking methicillin resistance determinants were recovered from samples of red deer and wild boar. All but one MSSA showed susceptibility to all antimicrobials tested. The *blaZ* gene was present in the unique penicillin-resistant strain.

Regarding the presence of virulence factors, all *S. aureus* lacked the Panton-Valentine leukocidin (*lukF*/*lukS*-PV), as well as the exfoliative toxins *eta*, *etb* and *etd*. The presence of the *tst* gene encoding the TSST-1 was detected in one MSSA isolate ascribed to CC522 recovered from a nasal sample of a wild boar. Further, *S. aureus* harbored different staphylococcal enterotoxins (number of isolates): *sec* (1), *seg* (1), *seh* (2), *sel* (1), *ser* (1) and operon *egc* (enterotoxin gene cluster)-like (1). All MRSA and MSSA isolates lacked the IEC system.

Discussion

In this work, we describe and characterize the *S. aureus* isolates detected in healthy free-living mammals from a defined and representative Spanish geographic region. A high rate of *S. aureus* carriage (22%) was detected among the analyzed animals. European rabbit was the most representative species, and although the *S. aureus* carriage rate was relatively low (8%), isolates were relevant because all of them were MRSA and carried the emergent *mec*C gene.

A high *S. aureus* carriage rate was detected in wild boars (65%), which is in accordance with previous data obtained in Portugal and Germany (Sousa *et al*. 2017; Seinige *et al*. 2017), although lower colonization rates were also reported in other studies (Porrero *et al*. 2014; Mama *et al*. 2019). Although the lineage MRSA CC398 has been reported in wild boar (Porrero *et al*. 2013; Sousa *et al*. 2017; Mama *et al*. 2019), the presence of MRSA isolates in this animal species is scarce, as has been evidenced in our study. It is important to highlight that the four European mouflons tested were *S. aureus*-carriers and three out of the four isolates recovered were

194 typed as t6056-ST133. Neither scavengers nor carnivorous mammal's species were *S. aureus*-
195 carriers, so the diet does not seem to be a key risk factor for *S. aureus* colonization.
196 Nares and rectum samples were analysed in the studied animals, but none was *S. aureus*-
197 positive for both type of samples. In this sense, 17 of the 23 *S. aureus* recovered in this study
198 were obtained from nasal samples. This fact was expected considering that one of the major
199 colonization sites of *S. aureus* are the nares, in both humans and animals. However, it is also
200 important to note that some animals would have been considered as negative *S. aureus*-carriers
201 if samples from rectum had not been taken.
202 The *mecA*-positive strain was isolated from the nasal sample of a European hedgehog. MRSA
203 have already been detected in this animal species (Monecke *et al*. 2013; Monecke *et al*. 2016),
204 but in those cases, the methicillin-resistance determinant was *mecC*. Our MRSA-*mecA* strain
205 was typed as t386-ST1/CC1-SCC*mec*IVa-*agr*III. The lineage CC1 is primarily associated with
206 MSSA from humans and with community-acquired (CA) MRSA, although it is also frequently
207 found in farm-animals (Alba *et al*. 2015; Monecke *et al*. 2016). According with Alba *et al*.,
208 (2015), the *aph*(3´)-IIIa and *vga*(A) genes could be genetic markers of MRSA CC1 of human
209 and animal origin (mostly pigs), respectively. The presence of *aph*(3´)-IIIa and the absence of
210 both *vga*(A) and IEC system in our MRSA CC1 strain, open questions about its potential origin.
211 In our study, all *mecC*-MRSA strains were isolated from nasal samples of European rabbit and
212 were typed as t843-ST(CC)130. The European rabbits sampled were hunted animals destined to
213 food consumption and therefore, the potential risk for human health should be assessed. The
214 *blaZ* allotype within the SCC*mec*-XI that harbor the *mecC* isolates, presents a 67% amino acid
215 identity with the previously known *blaZ* gene of *S. aureus* (Shore *et al*. 2011), and have been
216 demonstrated its penicillinase activity (Gomez *et al*. 2014). The *mecC* gene has been previously
217 detected in farmed rabbits from Belgium from a case of highly virulent cutaneous infection in
218 1995 (Devriese *et al*. 1996; Paterson *et al*. 2012), and in brown hares in Germany (Monecke *et*
219 *al*. 2016). Originally, the CC130 was associated to MSSA, but in recent years it is being of par-
220 ticular concern due to its relationship with the *mecC* gene. This clonal complex is known to be a
221 livestock-lineage mostly related with ruminants but also found in wild animals, and the envi-

ronment (García-Álvarez *et al*. 2011; Gómez *et al.* 2014; Gómez *et al.* 2016). The presence of the *mecC* gene in clinical isolates in Spain is still unusual, as evidenced by a recent multicenter study conducted in twelve Spanish hospitals among non-β-lactam-susceptible MRSA isolates (common phenotype of *mec*C strains) (Ceballos *et al*. 2019). Nevertheless, sporadic detections of clinical *mec*C MRSA strains have been reported in Spain, corresponding to the lineage t843 and ST130/ST1945 (included in CC130) (Romero-Gómez *et al*. 2013; García-Garrote *et al.* 2014; Cano-García *et al*. 2015; Benito *et al*. 2016), similar to the *mecC*-MRSA isolates detected in this study.

The molecular typing revealed a high heterogeneity of genetic lineages among MSSA isolates, with the detection of 10 different *spa*-types. In this study, the CC130 has been detected in one/third of MSSA isolates, linked in most cases to *spa*-type t1535 (although a non-typeable *spa* strain was also detected, ascribed to ST130). The *spa*-type t1535 has been found in animals in Spain but harboring the *mecC* gene (Gómez *et al*. 2014; Gómez *et al*. 2015). Isolates belonging to CC5 (*spa*-types t7174 and t1125) are also found in our work. This finding is worth nothing since it is a human-associated lineage related to MRSA strains, but in recent years it has been disseminated in livestock (Hasman *et al*. 2010) and pets (Gharsa *et al*. 2015). There are several *spa*-types that seem to be widely spread in wild mammals, like in the case of t7174, t6386, t3750, t11230, and t11233, previously reported in Spain (Porrero *et al*. 2014). The *spa* t3750 (ST2328), detected in three out of eleven MSSA isolates of wild boar, was the most prevalent *spa*-type among MSSA in this animal species in previous studies conducted in Spain and Portugal (Porrero *et al*. 2014; Sousa *et al*. 2017; Mama *et al*. 2019). The clonal complex CC133 has been detected in the isolates recovered from European mouflon. This genetic lineage was reported in MSSA isolates in wildlife in Spain (Porrero *et al*. 2014), and is commonly observed among ruminant animals (Eriksson *et al*. 2013), as is the case of the European mouflon. Regarding antimicrobial resistance, all our MSSA isolates showed susceptibility to all antimicrobials tested, with one exception (with *blaZ* gene). Wild animals are not supposed to be under the pressure of antimicrobials agents, and the low rate of resistance might be due to the low relationship with human or veterinary environments.

The *tst* gene, encoding the toxic-shock syndrome 1 toxin, was detected in one MSSA isolate ascribed to CC522. The presence of this gene in free-living animals has been previously reported in *S. aureus* belonging to CC30 of white storks in Spain (Gómez *et al*. 2016). Moreover, CC522 strains carrying *tst* gene were frequently detected in sheep in Tunisia (Ben Said *et al*. 2017). The presence of the enterotoxins *sec* and *sel* in the MSSA-CC522 is noteworthy since it is known the existence of a bovine staphylococcal pathogenicity island (SaPlbov) which encodes either *sec, sel* and *tst* (Fitzgerald *et al*. 2001).

Staphylococcus aureus is also the causal agent of food poisoning due to the production of different enterotoxins. Although the main source of food contamination is food-handler carriers of enterotoxin-producing *S. aureus*, this bacterium could also be present in food of animal origin (Argudín *et al*. 2010). Since certain *S. aureus* isolates were recovered from game animals destined to food consumption (European rabbit, wild boar, red deer, and European mouflon), it was considered important to determine the enterotoxin gene content. The operon *egc*-like, which comprises up to six enterotoxin genes (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*) was detected in one isolate recovered from a wild boar. As expected, the MRSA-*mecA* strain harbored the enterotoxin gene *seh*, which seems to be ubiquitous in CC1/ST1 strains independently of their origin (Alba *et al*. 2015), and it is supposed to be on a transposon (Lindsay 2011).

Summing up, *S. aureus* is found in nasal and rectal samples of free-ranging mammals in Aragon (Spain). Remarkably, wild rabbits could be a reservoir of MRSA carrying the *mecC* gene, which could be a risk for human health as well as be a vehicle to enter in the human food chain. Some mammal species are frequently colonized by MSSA such as wild boars, mouflons and deers. The MSSA population was very heterogeneous with the detection of different genetic lineages related with both humans and animals. A high susceptibility to the tested antimicrobials was detected what might be explained by the absence of selective pressure in this environment.

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Conflict of interest

No conflict of interest declared.

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Table 1. Phenotypic and genotypic characterization of the 23 *S. aureus* isolates recovered from free-living mammals.

Strain	Animal species	Origin[a]	Molecular characterization				Antimicrobial resistance		Virulence factors	
			spa-type	ST/ CC[b]	SCC*mec*	*agr*	Phenotype[c]	Genotype	Enterotoxins	Other genes[e]
C6483	European hedgehog	N	t386	ST1/CC1	IVa	III	PEN-FOX-ERI-CLI[I]-STR	*mecA*, *blaZ*, *erm*(C), *aph*(3')-IIIa, *ant*(6)-Ia	*seh*	-
C8483	European rabbit	N	t843	ST130/CC130	XI	III	PEN-FOX	*mecC*, *blaZ*-SCC*mec*-XI	*seg*, *seh*	-
C8488	European rabbit	N	t843	(ST130/CC130)	XI	III	PEN-FOX	*mecC*, *blaZ*-SCC*mec*-XI	-	-
C8500	European rabbit	N	t843	(ST130/CC130)	XI	III	PEN-FOX	*mecC*, *blaZ*-SCC*mec*-XI	-	-
C6771	Red deer	N	t1535	(CC130)	-	III	PEN	*blaZ*	-	-
C8646	Wild boar	N	t1535	(CC130)	-	III	Susceptible	-	-	-
C8607	Wild boar	R	t1535	(CC130)	-	III	Susceptible	-	-	-
C8608	Wild boar	R	t1535	(CC130)	-	III	Susceptible	-	-	-
X717	Wild boar	R	t1535	(CC130)	-	III	Susceptible	-	-	
C8516	Red deer	N	NT	ST130/CC130	-	III	Susceptible	-	-	-
C8493	Wild boar	N	t7174	(CC5)	-	II	Susceptible	-	[*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][d]	-
C6770	Red deer	N	t1125	(CC5)	-	II	Susceptible	-	-	-
C8611	Wild boar	N	t1534	(CC522)	-	I	Susceptible	-	*sec*, *sel*	*tst*
C8609	Red deer	R	t11225	(CC425)	-	II	Susceptible	-	-	-

C8613	Wild boar	N	t6386	(CC425)	-	II	Susceptible	-	-	-
C8508	European mouflon	N	t6056	ST133/CC133	-	I	Susceptible	-	-	-
C8510	European mouflon	R	t6056	(ST133/CC133)	-	I	Susceptible	-	-	-
C8513	European mouflon	N	t6056	(ST133/CC133)	-	I	Susceptible	-	-	-
C8614	Wild boar	N	t3750	ST2328 /Singleton	-	III	Susceptible	-	-	-
C8491	Wild boar	N	t3750	(ST2328) /Singleton	-	III	Susceptible	-	-	-
C8496	Wild boar	N	t3750	(ST2328) /Singleton	-	III	Susceptible	-	-	-
C8610	Wild boar	N	t11230	ST2328 /Singleton	-	III	Susceptible	-	-	-
C8506	European mouflon	R	t11233	ST3237	-	IV	Susceptible	-	*ser*	-

NT, non-typeable.

[a]N: nasal sample; R: rectal sample.

[b]ST: sequence type; CC: clonal complex; In brackets when the ST or CC is assumed according to the *spa*-type.

[c]PEN: penicillin; FOX: cefoxitin; ERI: erythromycin; CLI: clindamycin; STR: streptomycin; I: inducible resistance.

[d]*egc*-like cluster (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*).

[e]Virulence genes studied include *tst*, *lukF*/*lukS*-PV, exfoliative toxins *eta*, *etb* and *etd*, and IEC system genes (*scn*, *chp*, *sak*, *sea* and *sep).*

Supplementary material

Table S1. Bird species and number of animals sampled.

Animal species	Number of animals tested
Griffon vulture (*Gyps fulvus*)	14
White stork (*Ciconia ciconia*)	11
Eurasian eagle-owl (*Bubo bubo*)	11
Western marsh harrier (*Circus aeruginosus*)	10
Eurasian sparrowhawk (*Accipiter nisus*)	9
Common kestrel (*Falco tinnunculus*)	8
Common buzzard (*Buteo buteo*)	5
Long-eared owl (*Asio otus*)	5
Black kite (*Milvus migrans*)	5
Golden eagle (*Aquila chrysaetos*)	4
Northern goshawk (*Accipiter gentilis*)	4
Barn owl (*Tyto alba*)	3
Eurasian scops owl (*Otus scops*)	2
Barn swallow (*Hirundo rustica*)	2
Red kite (*Milvus milvus*)	2
Common wood pigeon (*Columba palumbus*)	2
Bearded vulture (*Gypaetus barbatus*)	2
Eurasian collared dove (*Streptopelia decaocto*)	2
Booted eagle (*Hieraaetus pennatus*)	1
Short-toed snake eagle (*Circaetus gallicus*)	1
Eurasian hobby (*Falco subbuteo*)	1
Mallard (*Anas platyrhynchos*)	1
Common little bittern (*Ixobrychus minutus*)	1
Common house martin (*Delichon urbicum*)	1
Great cormorant (*Phalacrocorax carbo*)	1
Merlin (*Falco columbarius*)	1
Common starling (*Sturnus vulgaris*)	1
Grey heron (*Ardea cinerea*)	1
Yellow-legged gull (*Larus michahellis*)	1
Common crane (*Grus grus*)	1
Common blackbird (*Turdus merula*)	1
European turtle dove (*Streptopelia turtur*)	1
Common swift (*Apus apus*)	1

Table S2: Mammals species and number of animals sampled.

Animal species	Number of samples tested
European rabbit (*Oryctolagus cuniculus*)	38
Wild boar (*Sus scrofa*)	17
European hedgehog (*Erinaceus europaeus*)	11
Red deer (*Cervus elaphus*)	9
American mink (*Neovison vison*)	6
European mouflon (*Ovis orientalis musimon*)	4
Eurasian otter (*Lutra lutra*)	3
European badger (*Meles meles*)	3
Red fox (Vulpes vulpes)	3
Beech marten (*Martes foina*)	2
European hare (*Lepus europaeus*)	2
Iberian ibex (*Capra pyrenaica*)	1
Least weasel (*Mustela nivalis*)	1
European roe deer (*Capreolus capreolus*)	1
Common genet (*Genetta genetta*)	1
European free-tailed bat. (*Tadarida teniotis*)	1