# Patterns and Behavioral Determinants Related to Wolf Predation on Free-Ranging Horses


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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ______/______/_________

MSc

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# Abstract

In northern Iberian Peninsula, free-ranging horses are a relevant prey in wolf diet, representing an ecological context poorly represented worldwide, despite the range overlap between both species. In northwestern Portugal and Galiza, an autochthonous breed of mountain ponies, the *Garrano* horses, is raised under a free-ranging regime since centuries ago, providing a stable food resource for wolves. However, wolf predation on these horses leads to important socio-economic conflicts. In Portugal, wolf damages are compensated by law to mitigate the losses of livestock owners while reducing illegal killing of wolves. However, the compensation system is not as efficient as it would be required and new solutions are needed to minimize wolf predation and adequately compensate owners while still protecting the wolf.

This study aims to characterize patterns and determinants related to wolf predation on horses, by targeting two main approaches: i) characterize wolf-horse conflict in Alto Minho region (northwestern Portugal), by using available data from 2016 to 2018, together with information on mortality causes of *Garrano* horses, to quantify the magnitude of wolf damages, in terms of number of attacks and compensation values, determine the spatial and temporal variation of wolf damages to free-ranging horses and determine the relevance of wolf predation in the mortality causes reported to *Garrano* horses, by considering both sex and age classes; ii) investigate wolf-horse interactions in Serra d'Arga (Viana do Castelo), by taking advantage of available data obtained during 2017 and 2018 in wolf monitoring programs (conducted by CIBIO) and behavioral studies on horse herds (conducted by Kyoto University) in order to determine the relevance of horses in wolf diet and prey selection, estimate wolf predation rates on horses based on GPS telemetry of a collared wolf, and identify interactions between horses and wolves, using innovative procedures based on non-invasive genetics.

Results for wolf-horse conflict in Alto Minho showed that horses comprise 20% of all wolf attacks to livestock declared to ICNF, with summer showing the highest number of attacks and Viana do Castelo being the most affected municipality, receiving 60% of the economic compensations paid for wolf damages on horses. The main cause of *Garrano* mortality was animals reported to be lost (n=592; 59%) followed by wolf predation (n=363; 37%). Foals were the most affected age class by wolf predation (80%), particularly males (83%), with the highest mortality reported during august and september (52%).

Results for wolf-horse interactions in serra d'Arga showed that free-ranging horses comprise the majority of wolf diet both in 2017 (F.O.=81.4%; Biomass=85%) and 2018

(F.O.=84%; Biomass= 92.9%), and have a strong positive selection by wolves. Predation impact was approximately 41% of the annual horse population, with estimated kill rates of 6 horses/month and 73 horses/year. Considering the estimated group size of the local pack, kill rates reached 0.5 horses/wolf/month and 6.1 horses/wolf/year. The genetic individual identification of 46 horses consumed by wolves, identified either in wolf scat remains and/or in predation sites, together with the genetic identification of horse fecal samples, provided by Kyoto University, joined together 164 different *Garrano* genotypes and allowed us to reconstruct 14 horse families. Overall, predation seems to be biased towards males (30% in males and 22% in females) in relation to their availability in the area. It was also analyzed patterns of horse consumption with the genetic identification of which wolves consumed the same horse and estimation of distances between scats and/or predation sites containing the same dead horse.

Based on the obtained results, several measures are proposed to mitigate wolf predation on horses related to herd management, damage prevention measures and alternative compensation systems to safeguard the traditional husbandry system of *Garrano* horses as well as wolf conservation. Also, several areas of research focusing wolf-horse interactions should be further developed in order to support best practices to minimize the risk of wolf predation. In conclusion, this work provided valuable insights on patterns and methodological approaches related to wolf predation on horses, allowing new research opportunities regarding predator-prey interactions.

**Key words:**

*Garrano* breed, Human-wolf conflict, Non-invasive genetics, Predation rates, Prey selection, Socioeconomic analysis, Wolf diet, Wolf-horse interactions;

# Resumo

No norte da Península Ibérica, os cavalos pastoreados em regime de liberdade constituem presas relevantes na dieta do lobo, representando um contexto ecológico pouco expressivo a nível mundial. No Noroeste de Portugal e Galiza, uma raça autóctone de póneis, os Garranos, são criados em regime livre desde há séculos, constituindo um recurso alimentar estável para o lobo. Contudo, a predação do lobo nestes cavalos gera importantes conflitos socioeconómicos. Em Portugal, os ataques de lobo nas espécies pecuárias são compensados de modo a mitigar as perdas dos criadores de gado, reduzindo assim a perseguição ilegal a este carnívoro. No entanto, o sistema de compensação económica é pouco eficiente e são necessárias novas soluções para minimizar a predação de lobo e compensar adequadamente os criadores, de forma a também proteger o lobo.

Este estudo tem como objetivo caracterizar os padrões e determinantes relacionadas com a predação de lobo em cavalos em regime livre, visando duas abordagens principais: i) caracterização do conflito entre lobo e cavalo na região do Alto Minho (Noroeste de Portugal), usando informação disponível de 2016 a 2018, e informação sobre as causas de morte de Garranos, de forma a quantificar a magnitude dos danos de lobo em termos de número de ataques e valores de compensação, determinar as variações temporais e espaciais desses danos e determinar a relevância da predação de lobo nas causas de mortalidade de Garranos, considerando sexo e classe etária; ii) investigar interações lobo-cavalo na Serra d'Arga (Viana do Castelo), utilizando dados obtidos em 2017 e 2018 no âmbito de programas de monitorização do lobo (realizados pelo CIBIO) e estudos comportamentais sobre Garranos (realizados pela Universidade de Kyoto), de forma a determinar a relevância de cavalos na dieta do lobo e seleção de presas, estimar taxas de predação de lobo em cavalos através da telemetria GPS de um lobo marcado, e identificar interações entre cavalos e lobos, usando procedimentos inovadores baseados em genética não-invasiva.

Resultados do conflito entre lobo e cavalo no Alto Minho demonstram que os cavalos representam 20% de todos os ataques de lobo a espécies pecuárias declarados ao ICNF, com a maior incidência de ataques no Verão e sendo Viana do Castelo o município mais afetado, recebendo 60% das compensações pagas por danos de lobo a cavalos. A principal causa de morte foi desaparecimento (n=592; 59%) seguida por predação de lobo (n=363; 37%). Os potros foram a classe etária mais afetada por predação de lobo (80%), particularmente machos (83%), com a mortalidade mais elevada durante agosto e setembro (52%).

Resultados das interações entre lobo e cavalo na Serra d'Arga revelam que os cavalos pastoreados em regime livre constituem a maioria da dieta do lobo em 2017 (F.O.= 81.4%; Biomassa = 85%) e 2018 (F.O.= 84%; Biomassa = 92.9%), sendo alvo de uma forte seleção positiva por parte do lobo. O impacto da predação foi, aproximadamente, 41% do efetivo anual de cavalos, com taxas de morte de 6 cavalos/mês e 73 cavalos/ano. Considerando a estimativa de lobos presentes na alcateia local, foram obtidas taxas de morte de 0.5 cavalos/lobo/mês e 6.1 cavalos/lobo/ano. A identificação genética individual de 46 cavalos consumidos pelo lobo através da sua identificação em excrementos de lobo e/ou locais de predação, juntamente com a identificação genética de cavalos através de amostras fecais fornecidas pela Universidade de Kyoto, permitiu a obtenção de 164 genótipos e reconstruir 14 famílias de cavalos. Em geral, a predação parece incidir maioritariamente nos machos (30% em machos e 22% em fêmeas) em relação à sua disponibilidade na área. Foram também analisados os padrões de consumo de cavalos através da identificação genética de quais os lobos que consumiram o mesmo cavalo e a estimativa das distâncias entre os dejetos e/ou locais de predação com o mesmo cavalo morto.

Com base nos resultados obtidos, são propostas medidas para mitigar a predação de lobo em cavalos focadas na gestão das manadas, medidas de prevenção de ataques e sistemas alternativos para compensação de danos, de forma a salvaguardar o sistema de criação tradicional dos Garranos, assim como a conservação do lobo. Além disso, várias áreas de investigação devem ser desenvolvidas com o objetivo de apoiar as melhores práticas para minimizar o risco de predação de lobo. Em conclusão, este trabalho aporta informação importante sobre os padrões e abordagens metodológicas relacionadas com a predação do lobo sobre cavalos, criando novas oportunidades de investigação sobre as relações predador-presa.

**Palavras-chave:**

Análise socioeconómica, Conflito homem-lobo, Dieta de lobo, Genética não-invasiva, Interações lobo-cavalo, Raça Garrana, Seleção de presa, Taxas de predação;

# Table of Contents

# List of Figures

# List of Tables

# List of Abbreviations

**ACERG** – Associação de Criadores de Equinos de Raça Garrana

**Biomass –** Consumed Biomass

**CIBIO –** Research Centre of Biodiversity and Genetic Resources, University of Porto

**F.O. –** Frequency of Occurrence

**GPS –** Global Positioning Systems

**ICNF –** Institute for Nature Conservation and Forest

**LGD –** Livestock Guarding Dog

**NW –** Northwest

**QGIS -** Quantum Geographic Information System

**SPREGA** – Sociedade Portuguesa de Recursos Genéticos Animais

# 1. Introduction

## 1.1. The role of livestock depredation on Human-carnivore conflict

Large carnivores are among the most iconic and charismatic species in the world and are responsible for important ecosystem functions, such as limiting populations of both herbivores through predation and mesocarnivores through intraguild competition (Treves and Karanth 2003; Ripple *et al.* 2014). Many ecosystem services are also under the indirect influence of carnivores, as these predators are capable of buffering the effects climate change, reestablish native plant diversity and enhance biodiversity (Estes 1996; Ripple and Beschta 2005; Ripple *et al.* 2014). In fact, large carnivores are often used as flagships, umbrellas, biological indicators and even keystone species due to their essential ecosystem function, promoting the conservation of biodiversity (Linnell *et al.* 2000; Ordiz *et al.* 2013). However, their strong regulatory effects on prey species become one of the main causes of conflicts with humans when attacks to livestock are involved, raising important sociopolitical challenges that hinder conservation efforts (Treves and Karanth 2003; Graham *et al.* 2005; Dickman 2010; Ripple *et al.* 2014).

Large carnivores have wide ranges, occur at low densities and are positioned at the top of trophic webs, traits that make them vulnerable to extinction, particularly whenever feeding on animals with interest for humans, such as livestock (Treves and Karanth 2003; Graham *et al.* 2005; Ripple *et al.* 2014). In fact, this behavioral trait has led, for centuries, to an intense human persecution towards large carnivores, which in combination with habitat loss and fragmentation, has contributed to their sharp decline worldwide (Chapron *et al.* 2014; van Eeden *et al.* 2018). Human-carnivore conflicts related to livestock depredation are particularly relevant for grey wolves (*Canis lupus*) and are becoming an increasing concern, as human population increases and wolf populations recover and expand towards human-dominated landscapes (Graham *et al.* 2005; Dickman 2010). As a consequence, people who depend on livestock as livelihood suffer important economic losses, becoming antagonistic towards wolves and motivating an intense direct persecution (Treves and Karanth 2003; Graham *et al.* 2005; Dickman 2010).

## 1.2. Wolf predation on livestock

Wolves have an opportunistic behavior allowing them to hunt primarily wild prey and, when easily available, also livestock (Mech 1970; Meriggi and Lovari 1996; Newsome *et* *al.* 2016). Wherever domestic animals and wolves occur together, there will be depredation (Chavez and Gese 2006; Linnell and Cretois 2018), and this is a common scenario particularly in Asia (Suryawanshi *et al.* 2013) and southern Europe (Meriggi and Lovari 1996; Newsome *et al.* 2016). In fact, Portugal, Greece, Croatia, France and Italy are the European countries with the highest livestock depredation rates due to wolves (Figure 1), apparently as a consequence of the husbandry systems based on free-ranging livestock together with low densities of wild prey (Linnell and Cretois 2018). Most studies on wolf predation focus on the ecological perspective, describing the interactions between wolves and prey and how they are influenced by the landscape and human presence, while knowledge regarding the socio-economic perspective, including human-wolf conflict and quantification of livestock losses and economic costs associated, is still limited and needs further study. Of all domestic species consumed by wolves, sheep are by far the most predated, followed by cattle, goats and, with lower expression, horses (Meriggi and Lovari 1996; Newsome *et al.* 2016; Linnell and Cretois 2018), as can be seen in Figure 1.

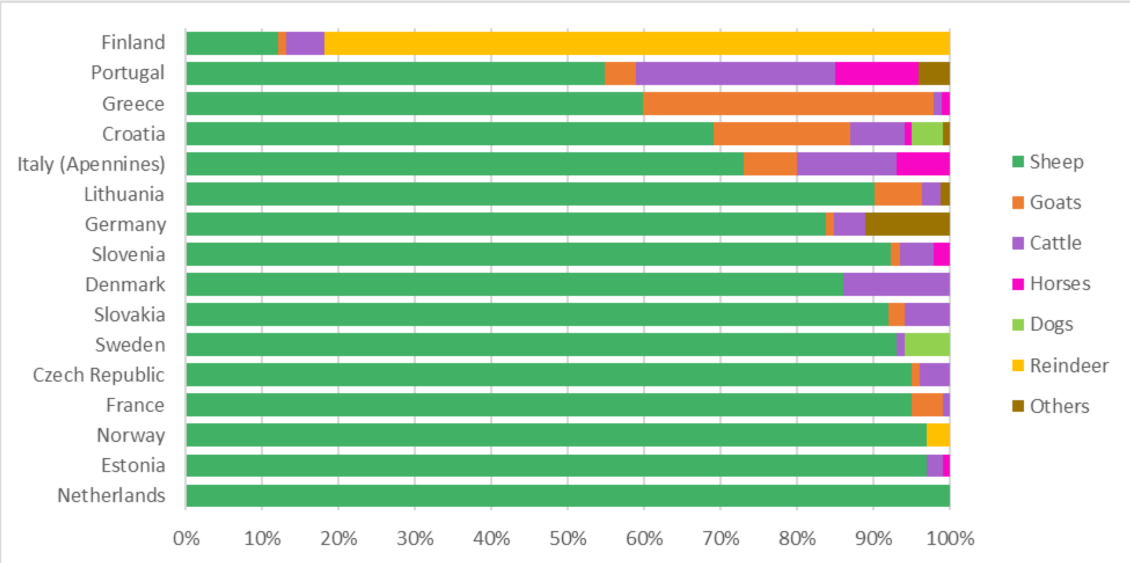


**Figure 1**. Relative representation (%) of different livestock species in compensation payments attributed to wolf predation in different European countries. Table adapted from Linnell and Cretois (2018).

Abundance of unprotected livestock leads to a shift in wolf diet with high economic costs. From 1991 to 1995, the cost of economic compensations in Italy was one of the highest in Europe, ranging between 1,445,000€ and 1,688,000€ (Boitani *et al.* 2010). In particular, the region of Lazio had the highest depredation costs, involving 727,000€ paid as compensation to the livestock owners, having a maximum period of 30 days to verify the claim, while the region of Liguria only paid 2,500€ and the verification of the claim was done as soon as possible (Boitani *et al.* 2010). During the same period, France was paying 5,000€ per wolf per year, which was lower than Sweden and Norway that were paying, respectively, 14,000€ and 10,000€ per wolf per year (Boitani *et al.* 2010). However, unlike France, these northern countries also included subsidies for prevention measures (Boitani *et al.* 2010). Furthermore, in 1999, Spain reported livestock depredation damages that surpassed 340,000€ (Blanco 2003). Regarding the United States, compensation costs are generally lower, as in the states of Idaho, Wyoming and Montana the government paid in average 11,076.79$ per year (~ 9,980.96€), between 1987 and 2003, reaching the maximum of 41,230.32$ (~ 37,153.88€) in 2003 (Muhly and Musiani 2009).

Similarly, in Portugal, compensation costs stabilized around 700,000€ per year since 2001, with 43% of this amount (approximately 300,000€) corresponding to the region of Peneda-Gerês National Park (NW Portugal) due to high predation rates on cattle and free-ranging horses (Milheiras and Hodge 2011; Pimenta *et al.* 2018). Such high costs can only be minimized by changing husbandry and guarding practices, without disregarding the socioeconomic constraints of the rural communities (Treves and Karanth 2003). Traditionally, several mitigation measures have been proposed to reduce wolf predation, such as enclosures to confine the animals at night and during winter, when they are most vulnerable to predators (Kaczensky 1998), as well as the presence of shepherds and livestock guarding dogs to defend the herds (Dickman 2010; Rigg *et al.* 2011). Overall, topics focusing on the intensity, patterns and determinants of wolf predation on livestock related to the most consumed livestock prey species are already well studied (see e.g. Llaneza *et al.* 1996; Vos 2000; Hovens and Tungalaktuja 2005; Newsome *et al.* 2016), especially considering domestic species like sheep or cattle that are often main prey for wolves across the world (Linnell and Cretois 2018). However, determinants of wolf predation on other domestic prey species that are occasionally consumed worldwide is still largely unknown, as is the case of horses (*Equus caballus*). Wolf predation on horses is usually considered occasional worldwide, although, in some areas, predation can become high and raise important conservation and economic implications.

## 1.3. Wolf predation on horses

Horses are considered an occasional prey for wolves (Newsome *et al.* 2016), despite the range overlap between both species. Feeding ecology is probably the most studied ecological trait in wolves, however most studies, including reviews on wolf diet (e.g. Meriggi and Lovari 1996; Newsome *et al.* 2016), often disregard occasional prey items, only including them on the category “Other prey”, which are poorly described or quantified. Based on a review of 107 available studies focusing wolf diet and predation, conducted in the scope of this thesis, horses were reported in 50 studies (47%) from 10 regions worldwide (Figure 2). This review demonstrated that despite the several studies reporting wolf predation on horses in some areas of the world, very few actually described horse depredation and fully characterized predation or consumption rates. Equids are usually only mentioned as an occasional portion of wolf diet and are never considered an important prey, therefore, this subject is poorly studied worldwide. Wolf predation on horses is located mainly in Central Asia and Iberian Peninsula, however, it can also occasionally occur in other regions of southern Eurasia, with much less importance in wolf diet. On the other hand, North America reports almost no predation on horses, with very few studies mentioning horses in wolf diet (Musiani *et al.* 2003; Haney *et al.* 2007), possibly attributed to higher availability of wild prey, adequate horse husbandry practices and limited range overlap between feral horses and wolves (Chavez and Gese 2006). In the United States, the feral horse populations are maintained in public areas administered by the Bureau of Land Management (BLM) (Beever 2003; Garrott *et al.* 2007), where the animals are frequently managed and contact with predators is minimal, which may explain the residual wolf predation despite some overlap between the distributions of both species in western United States (Chavez and Gese 2006; Abella 2008; Treves and Bruskotter 2011; Beschta *et al.* 2013). Besides, domestic horses are also well guarded by the owners and confined at night, thus reducing the risk of wolf attacks.

In Eurasia, some countries, such as Bulgaria and Italy, report wolf attacks on free ranging horses, yet sheep and goats are still the most preyed species (Cozza *et al.* 1996; Kaczensky 1998; Mertens and Promberger 2001; Iliopoulos *et al.* 2009; Rigg *et al.* 2011). However, in some areas of southern Europe and central Asia, particularly NW Iberian Peninsula and Mongolia, respectively, horses can become the most relevant prey for wolves (Figure 2) ( Llaneza *et al.* 1996; Meriggi and Lovari 1996; Hovens and Tungalaktuja 2005; López-Bao *et al.* 2013). In these areas, equids seem to be positively selected (Hovens and Tungalaktuja 2005; Álvares 2011; Lagos and Bárcena 2018), meaning that wolves consume horses in higher proportion than their availability in the area. The reasons for this preference can be related to the fact that modern grey wolves preferably prey on large ungulates weighting up to 300kg and with an escaping tactic towards wolf attacks (Mech 1970). Accordingly, wild ungulates evolved to minimize wolf predation, developing effective anti-predator behaviors (Mech and Peterson 2003). Such characteristics, in Europe, are represented by red deer (Jędrzejewski *et al.* 2000) together with mountain ponies, the few prey species frequently reported as being positively selected by wolves (Vos 2000; Llaneza and López-Bao 2015).

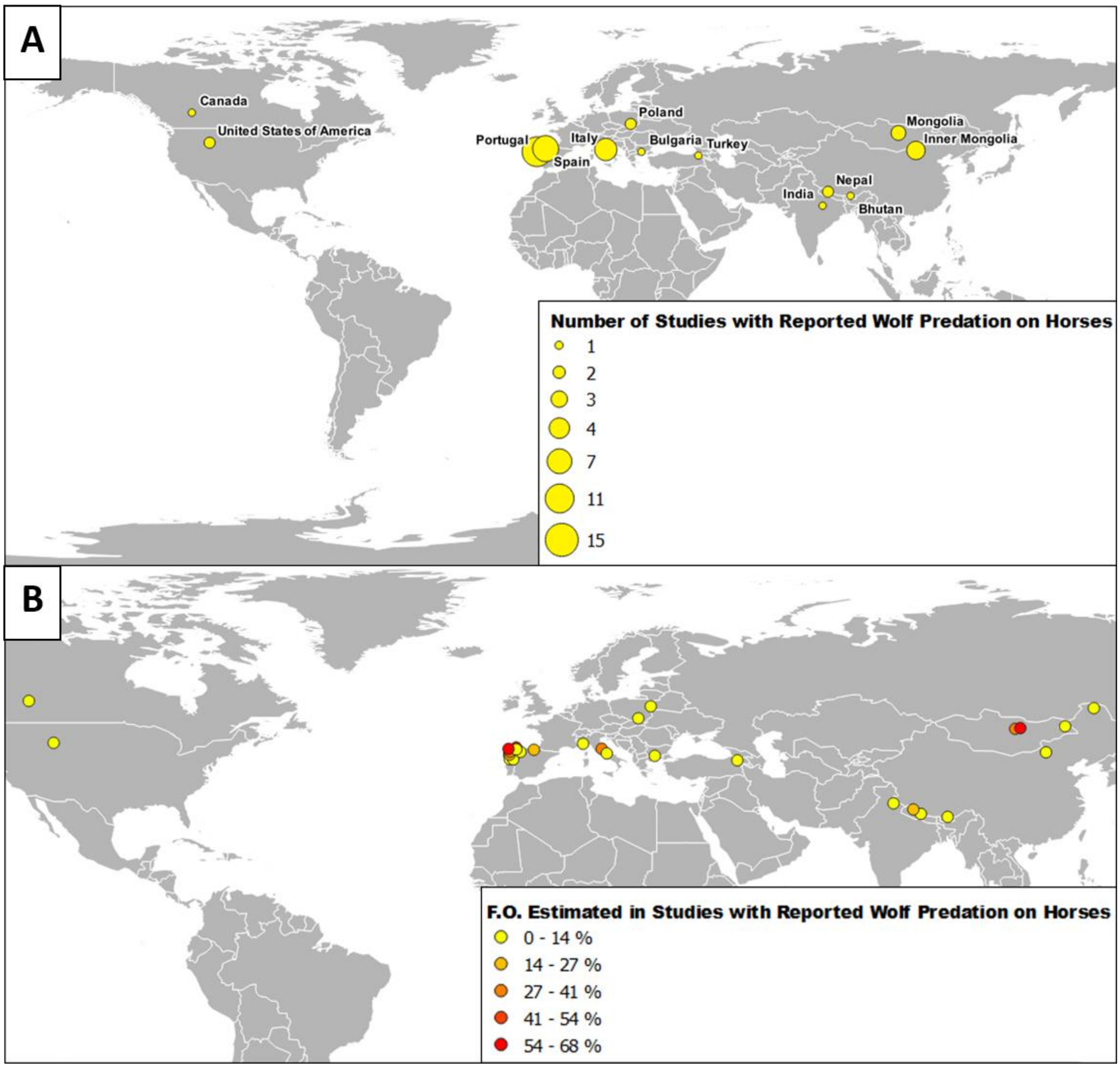


**Figure 2**. Location of studies reporting wolf predation on horse worldwide, with reference to the number of compiled studies per country (A) and reported frequency of occurrence (F.O.) of horses in wolf diet (B). Circles indicate each study reporting horse depredation in the following countries: Portugal (n=15 studies), Spain (n=11), Italy (n= 7), Inner Mongolia-China (n=4), Mongolia (n=3), India, Nepal and United States of America (n=2 each), Poland, Bulgaria, Turkey and Canada (n=1 each) (see Table S1 for bibliographic references).

Predation on domestic horses always involves economic losses for the owners, who not only lose the animal but also have to replace it in order to keep its livelihood. Besides, endangered wild equids are also predated by wolves, raising important conservation implications as wolves may keep the population from growing or recovering due to intensive predation on foals (van Duyne *et al.* 2009). Regarding wolf predation on wild equids, in Mongolia (Central Asia), Przewalski horses (*Equus ferus przewalskii*) and Mongolian Kulans (*Equus hemionus kulan*) are frequently attacked by wolves (Figure 3), not only limiting the population size of these endangered equids but also seemingly changing group behavior as a behavioral response to wolf predation (Feh *et al.* 1994; Kaczensky and Walzer 2003).

Kulan *(Equus hemionus kulan)* Przewalski Horse *(Equus ferus przewalskii)*

**Figure 3**. Mongolian wild equids reported as being regular prey for wolves: Kulan (*Equus hemionus kulan*) and Przewalski Horse (*Equus ferus przewalskii*).

Przewalski horses were extinct in the wild during the 1960s and the survival of this last species of wild horse was only ensured by keeping individuals in captivity (Kaczensky and Walzer 2003). A reintroduction program in their original range started in 1992, by releasing 84 horses into the wild in Hustai National Park, located in central Mongolia (van Duyne *et al.* 2009). After several reintroductions, the population started to increase slowly, however, remained highly inbred (Bouman 1977). The last population size assessment by IUCN Red List in 2014 revealed an increasing population trend, with 387 wild Przewalski horses in Mongolia, at three reintroduction sites, and a total of 1,988 captive and semi-captive individuals (King *et al.*, 2015). The reintroduction efforts of Przewalski horses is greatly impacted by the presence of wolves, their main natural predator, which resulted in frequent losses and seems to be restricting population growth in some areas (Kaczensky and Walzer, 2002). In fact, in a reintroduced population of Przewalski horses in Hustai National Park, Mongolia, van Duyne *et al.* (2009) reported 5 to 15 foals killed each year, of approximately 40 foals born, which despite horses comprising a small portion of the wolves local diet, had a population impact of almost 40% of all foals born each year. Regarding Kulans, currently considered as Near Threatened (NT) with an estimated population size of, approximately, 42,000 individuals with a stable population trend (Kaczensky *et al.*

2015), Kaczensky and Walzer (2002) reported behavioral changes in this wild equid which were attributed to wolf predation, namely increase of group size and establishment of bonds between males and females. Similarly to horses, Kulans live in family groups and do not maintain territories, increasing their group size in response to the presence of cooperatively hunting predators (Feh *et al.* 1994). As wolves are Kulans' main predator in Mongolia, the bonds between males and females are thought to be created in the presence of high levels of wolf predation in order to better defend their offspring (Feh *et al.* 1994).

Furthermore, domestic horses are also a major part of wolf diet in Mongolia, especially at the end of winter, when mortality by starvation has its peak and wolves scavenge on the carcasses (Hovens and Tungalaktuja 2005). For domestic horses, Mongolians maintain the mares and foals close to the settlements from june to october in order to get the milk from the females, therefore consumption of domestic horses by wolves decreases greatly in these months (Hovens and Tungalaktuja 2005). As it is expected, whenever there is predation on domestic species, there is socioeconomic conflict between the local people and the predators. Mongolians are pastoralists and fully rely on their livestock, with almost every family reporting losses on domestic horses and raising a strong need to reduce predation on livestock (Hovens and Tungalaktuja 2005; van Duyne *et al.* 2009). Likewise, wolf predation on domestic horses has also been widely reported in mountainous areas of northern Iberian Peninsula, where free-ranging mountain ponies belonging to autochthonous breeds are a main prey for Iberian wolves (Álvares *et al.* 2000; Vos 2000; López-Bao *et al.* 2013; Fagundez *et al.* 2017).

## 1.4. Ecological interactions between wolves and horses in Iberian Peninsula

To fully understand wolf predation on free-ranging mountains ponies in Iberian Peninsula, it is important to address how both species evolved and interact since pre-historic times. The first reference of horses morphologically similar to modern autochthonous breeds in Iberian Peninsula dates from the late Paleolithic (aprox. 40,000 years ago), in which rock paintings depict wild equids with similar phenotypic traits to the horses found today (D'Andrade 1938; Morais *et al.* 2005). The earliest evidences of horse domestication are found in the Eurasian steppes, where is presently situated Ukraine and Kazakhstan (Warmuth *et al.* 2012), approximately 4,000 years BC (Levine 2005; Lira *et al.* 2010; Warmuth *et al.* 2011). During the Holocene, Eurasian steppes became dominated by forest, making these closed habitats unsuitable for horses (Warmuth *et al.* 2011), and, therefore, the natural range of the wild horse suffered a sharp decrease as a consequence of these ecological and climatic changes (Levine 2005). Horse domestication is thought to have occurred with a small number of stallions while the restock of the herds was accomplished with wild females, as they were easier to handle (Cieslak *et al.* 2010; Warmuth *et al.* 2011, 2012). In fact, Warmuth *et al.* (2012) suggested that introgression from wild lineages was mainly female mediated, which would explain the high levels of matrilineal diversity.

The evolution of Iberian horses during glacial refugia is considered to have occurred differently from other Eurasian breeds, since Iberian Peninsula was separated from Europe by the mountain range of Pyrenees (Cieslak *et al.* 2010). In fact, several hypotheses have been proposed regarding the domestication process of horses in Iberia, where the glacial refugia may have ensured the survival of these wild equids. One hypothesis states that northern Iberian breeds (e.g. *Garrano*, *Asturcón* and *Losino*) are originated from Celtic migrations into Iberia after 1,500 BC, that settled along the Cantabrian range and evolved into their current form (Royo *et al.* 2005; Lira *et al.* 2010). These northern Iberian breeds share similar phenotypic traits with British ponies from northern Europe, specifically the Exmoor pony, suggesting a common origin (Royo *et al.* 2005; Lira *et al.* 2010). A similar pattern happened for the 3 current southern Iberian breeds (e.g. *Lusitano*, *Sorraia* and *Andalusian*) that were originated in North Africa and then brought to Iberia, where they evolved and still share phenotypic traits with northwestern African horses such as the Barb horse breed (Royo *et al.* 2005; Luís *et al.* 2006; Lira *et al.* 2010). A second hypothesis suggests the existence of a domestication event in Iberian Peninsula during the Chalcolithic-Bronze Age, where relic populations of wild Iberian horses were used in the domestication process together with introgression from domestic horses brought during Celtic immigrations (Lira *et al.* 2010). Regardless of the origin, the northern Iberian ponies, even after domestication, were always maintained in a free-ranging husbandry system, thus, always available to wolves during millennia (ACERG 2000). In fact, the first wolf-like canid appeared in Iberia during Early Pleistocene, approximately 2 Ma ago (Bartolini *et al.* 2017), coexisting since that time with several subspecies of ancient wild horses (*Equus ferus*) (Cardoso 1993; Flower and Schreve 2014). In this ecological context, characterized by a megafaunal community with several species of ungulates and large carnivores coexisting, is expected that wolves could become specialized in horses as a main prey, as these animals present a body mass (approx. 400kg) and anti-predator behavior (frequently escape by running under an attack, avoiding standing their ground and lashing out at predators) which makes them particularly suitable and vulnerable in relation to wolf's ecomorph and hunting tactics, respectively (Zimen 1976; Mech *et al.* 2003; Leonard *et al.* 2007).

Currently, Iberian wolves (*Canis lupus signatus* Cabrera 1907) feed mostly on livestock across the entire range, due to scarcity of wild prey and high availability of domestic animals under extensive grazing (Meriggi and Lovari 1996; Vos 2000; Barja 2009; Lagos 2013; Torres *et al.* 2015; Yilmaz *et al.* 2015; Linnell and Cretois 2018). Among consumed domestic species, Iberian wolves frequently prey on free-ranging domestic horses whenever they are available and which can locally or seasonally comprise the majority of wolf diet (Llaneza *et al.* 1996; Álvares *et al.* 2000; Vos 2000; Álvares 2011; Lagos 2013). Furthermore, during the last few decades, wolf diet in Iberian Peninsula has been shifting from a broad diet based on medium-sized domestic species (e.g. goats and sheep) during the 1970s to a narrowing diet based mostly on large domestic ungulates under extensive grazing, such as cattle and horses, as detected nowadays (Lagos and Bárcena 2015; Llaneza and López-Bao 2015). Based on a compilation of 25 studies focusing wolf diet in Iberian Peninsula in which consumption of horses is mentioned (Figure 4; Table S1), is possible to depict that domestic horses are reported as an important prey for Iberian wolves in Northern Portugal and across the Cantabrian mountains, with higher levels of consumption in Peneda-Gerês region (Portugal) and in the Autonomous communities of Galicia and Asturias (Spain). Some of these studies reporting low levels of horse consumption (e.g. F.O. < 10%) are considered to be result of a scavenging behavior from domestic horses already found dead in dumpsites, such as the case in Arada and Trancoso, located in the Portuguese wolf subpopulation in the south of Douro river (Quaresma 2002). In fact, in some regions of Iberian Peninsula it was frequent, until recently (before the EU Sanitary Regulation on Livestock Disposal), to leave in selected dumpsites dead horses together with other equids, such as donkeys and mules, in order to be consumed by scavengers, resulting in their occurrence in wolf diet by scavenging and not necessarily as an act of predation (Carreira and Petrucci-Fonseca 2000; Casimiro 2017; Lagos and Bárcena 2018; Pimenta *et al.* 2018). However, the majority of the Iberian studies reporting the consumption of domestic horses involve active wolf predation on free-ranging horses, mostly belonging to autochthonous breeds of mountain ponies.

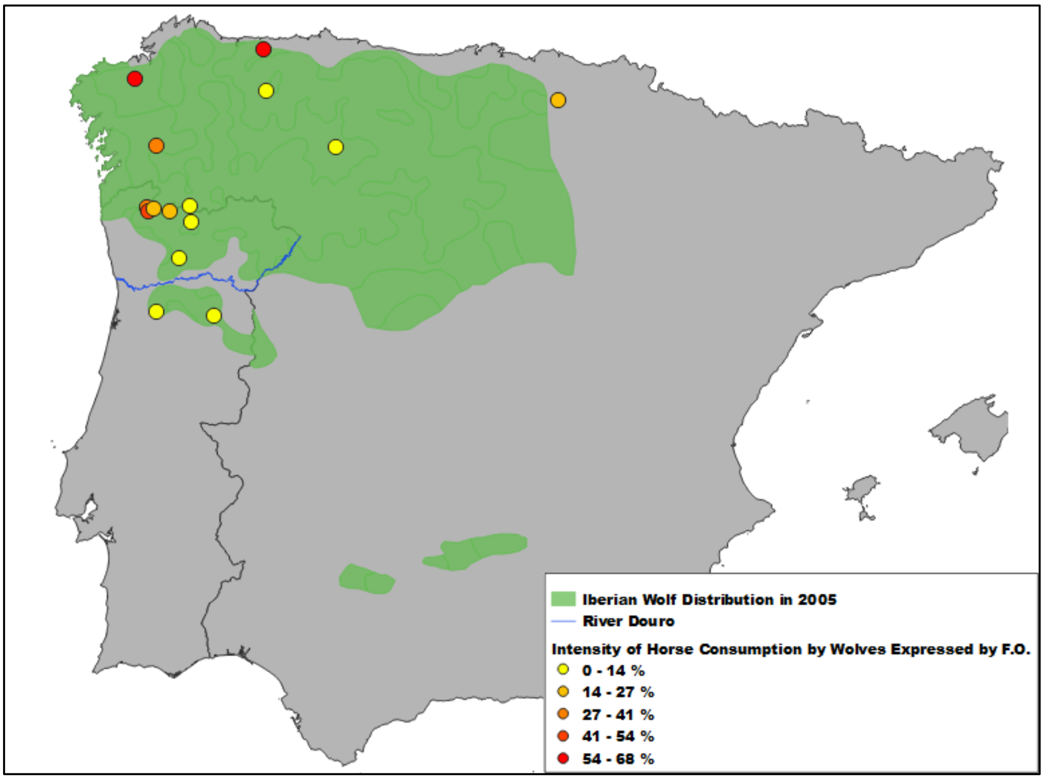


**Figure 4**. Location of studies reporting horse consumption by wolves in Iberia Peninsula in relation to wolf distribution estimated in 2005 (Álvares *et al.* 2005). Color code indicates the magnitude of frequency of occurrence (F.O.) of horses as prey in wolf diet. Circles indicate each study on wolf diet reporting domestic horses as prey item, in the following regions (some studies report the same study area). Portugal (N=15 studies): Vez/Soajo, Pitões das Junias, Larouco, Peneda-Gerês National Park, Alvão Natural Park, Leiranco, Arada, Trancoso, Peneda-Gerês National Park. Spain (N=10 studies): Galicia, Basque Country, northwestern and southwestern Asturias and Coruña (see Table S1 for bibliographic references).

Local breeds of mountain ponies (weighing between 250 and 350 kg), such as the *Asturcón*, *Pottok* and *Losino*, autochthonous from the Cantabrian mountain and Pyrenees in northern Spain, and the *Garrano*, autochthonous from Portugal and Galiza (Figure 5), are free-ranging all year, during day and night (Aramendia 1984; Álvarez-Sevilla *et al.* 1995; Martinez Saiz *et al.* 1996; Linnartz and Meissner 2014; Pereira 2018). Given their free-ranging husbandry system, these horses become a more accessible prey in relation to other domestic animals that are confined at night and therefore, becoming strongly selected by wolves (Álvares, 2011; Lagos 2013; Lagos and Bárcena 2015).

**Figure 5.** Autochthonous breeds of mountain ponies in Iberian Peninsula reported as prey species for Iberian wolves: (top row, from left to right) *Garrano*, *Losino*, (bottom row, from left to right) *Asturcón* and *Pottok. Losino, Asturcón* and *Pottok* are autochthonous from northern Spain while *Garrano* is autochthonous from Portugal and Galiza.

Foals are particularly vulnerable during the first months of age despite the protection of the herd, thereby increasing predation rates during summer (Llaneza *et al.* 1996; Vos 2000; Lagos 2013; Fagundez *et al.* 2017). Adult horses seem less likely to be killed by wolves, possibly due to their ability to inflict potentially fatal injuries during defensive behavior, but in fact they are reported to be regularly consumed especially in autumn and winter (Álvares, 2011; Lagos, 2013). During winter, adult horses are in poorer body condition in consequence of lower food availability and harsh weather, which in turn increases mortality either as a result of the environmental conditions, and in this case wolves will scavenge on the carcasses, or by wolf predation on weak animals (Lagos 2013; Llaneza and López-Bao 2015; Fagundez et al. 2017). Disease or injury also contribute to the natural mortality of free-ranging horses, providing large amounts of biomass for wolves to consume during periods of lower prey availability (Lagos 2013). Furthermore, Lagos (2013) also suggested the existence of differences between sexes on horses killed by wolves, indicating that females are less preyed than males. Males have higher energetic and nutritional costs as a result of high metabolic and growing rates, along with higher exposure to injuries during mating season and herd defense, factors that may lead to an increase in mortality risk by wolves (Berger 1983; Garrott 1991; Lagos 2013). Nevertheless, gestating and lactating females are also found in poorer condition during winter, as a consequence of low quality and availability of food, contributing to an increase in mortality rates (Berger 1983; Garrott 1991; Miranda 2000). Overall, despite both sexes maximizing their reproductive success differently, with male competition to produce more offspring (Asa 1999) and lactating females that have high nutritional demands in order to produce healthy foals while also preserving their own health (Cameron *et al.* 2000), males seem to support the highest energetic costs and, consequently, higher mortality rates by predators (Berger 1983). Iberian free-ranging horses, particularly *Garranos*, are also exposed to high incidence of diseases such as Equine Infectious Anemia Virus (Carvalho 2008; Abreu 2010) and intestinal parasites from the genus *Strongylus* (Madeira de Carvalho 1993), which may become debilitating and even fatal without proper sanitary care from the owners, leading to higher mortality rates.

The current abandonment of rural lands and traditional agricultural practices throughout Iberian Peninsula has contributed to the decline of horse husbandry, as they are not used for transportation any longer (López-Bao *et al.* 2013), and is currently leading to a decrease of the herds occurring in the mountains. However, the declining horse numbers in the mountains have important ecological implications as these free-ranging horses are pivotal for Iberian ecosystems. Horses have a strong impact on the landscape by changing vegetation cover, as horse grazing is essential to control plant biomass by decreasing shrub cover and, consequently, the risk of fires, to maintain open heathlands including some protected habitats, to increase plant diversification and floristic composition, to increase seed dispersal through defecation, as well as to increase the diversity of arthropod communities in heathlands (García *et al.* 2009; López-Bao *et al.* 2013; Fagundez *et al.* 2017). Besides, free-ranging horses provide a stable food resource for wolves, leading to less wolf attacks in more economically valuable livestock species, such as cattle and goats (López-Bao et al. 2013; Llaneza and López-Bao 2015). Under this ecological context, horse-wolf interactions have an important role in trophic webs and ecosystem functioning, controlling, by wolf predation, the abundance of these large herbivores, promoting habitat heterogeneity, and preserving plant and animal diversity (Ripple *et al.* 2014). Additionally, horses are not only essential as an important source of food for wolves by predation but also in the form of carrion for several species of scavengers during winter, when there is high horse mortality due to environmental conditions and low food availability (Lagos 2013; Llaneza and López-Bao 2015; Fagundez et al. 2017).

## 1.5. *Garrano* Horse Breed: characteristics and husbandry practices

*Garrano* horses were first described by Ruy d'Andrade (1938), being characterized as having a straight or concave head profile, short legs with small and cylindrical hooves, straight shoulders, round abdomen and dark brown coat, with abundant mane and tail. These horses are small sized, with average weight of 290 kg and height of 1.30 meters at withers (Morais *et al.* 2005; Pereira 2018). The *Garrano* breed from northern Portugal is considered to be the same existing in most Galiza, an autonomous community from northwestern Spain (Linnartz and Meissner 2014; Pereira 2018). In Portugal, *Garrano* horses are considered to be native from the northwestern region of the country (mostly Viana do Castelo and Braga districts) (Figure 6), although they were later dispersed throughout the country as a valuable mean of transportation (Sampaio 1998; ACERG 2000).


**Figure 6.** Distribution of the *Garrano* horse in Portugal (in green). Figure adapted from ACERG (2000).

Since historical times, *Garranos* are raised under a free-ranging regime in the mountains of north-west Portugal and Galiza. Despite the domestication process, the free-ranging nature of this breed has maintained genetic and morphological characteristics that allowed them to survive in the harsh mountain environments and to display strong antipredator behavior (ACERG 2000; Linnartz and Meissner 2014; Pereira 2018), a trait no longer present in most livestock species (Mignon-Grasteau *et al.* 2005). The range of the *Garrano* overlaps with the area where Iberian wolves have persisted for centuries, being exposed to high predation risk and where wolves act as a strong element of natural selection, preserving only the fitted, well adapted and more resilient individuals (ACERG 2000; DeSilvey and Bartolini 2018).

*Garrano* horses have always been an important livestock species in the traditional way of life of rural communities from northern Portugal, according to detailed descriptions in several historical documents since XVIII^th^ century and ethnographic studies from mid XX^th^ century (Dias 1948; Fontes 1977; Coutinho 1997; Sousa and Alves 1997) (Table S2). Rural populations in northern Portugal have always depended on livestock for food, work labor and transportation (Dias 1948; Fontes 1977; Coutinho 1997). Traditionally, *Garrano* horses were used mostly for transportation but also for breeding and food, particularly in Alto Minho region (NW Portugal), where since XVIII^th^ century there are detailed descriptions stating a traditional free-ranging husbandry system, in which large numbers of horses were left unattended in the mountains during all year (Sousa and Alves, 1997). Sousa and Alves (1997) report the number of horses and donkeys in Viana do Castelo district in 1792, showing how important horses were to farmers, in terms of personal transportation.

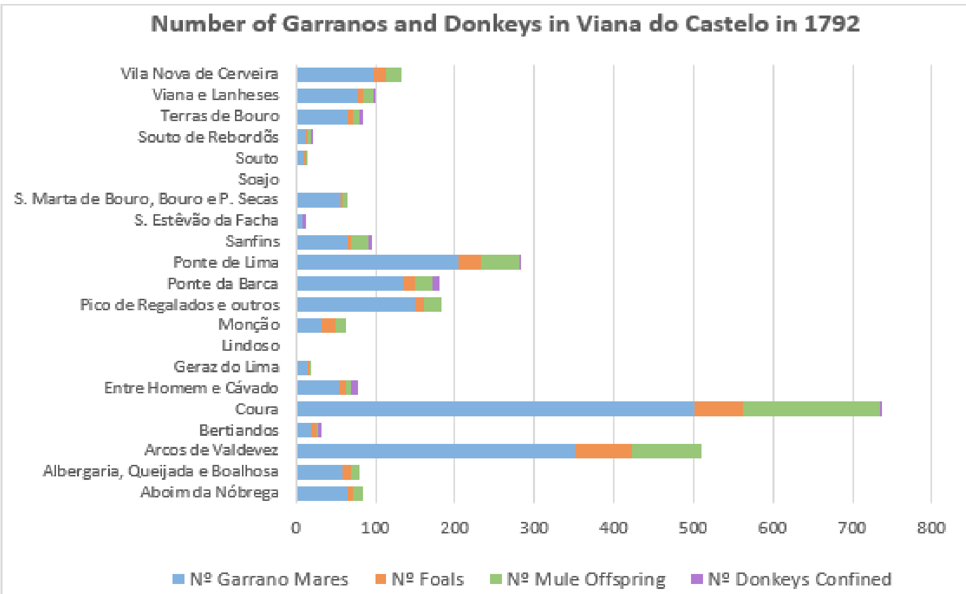


**Figure 7.** Number of *Garrano* horses (mares and foals) as well as mules and donkeys in each municipality belonging to Viana do Castelo district, in 1792. Adapted from Sousa and Alves (1997).

Often called as “bestas”, “burras” or “gado grosso” when in association with cattle (Dias 1948; Silva 1985; Sousa and Alves 1997; Capela 2005), horses, and specifically *Garranos*, weren’t so valued and numerous as other livestock species, such as cattle, sheep and goats. Most households possessed one or a few horses, which were kept in the mountains without direct attendance or protection from the farmers, naturally grouping in herds composed by animals from different owners (Fontes 1977). Historic references describe these herds as feral, where animals didn’t recognize the owner nor allowed approaching the herd (Dias 1948; Fontes 1977: see Table S2). In fact, historic references also recognized that these horses didn’t need any protection and care from the shepherds, as they appeared well-nourished by feeding in natural vegetation, particularly spiny scrublands (e.g. *Ulex sp*), and were wild enough to show strong antipredator behavior for defense from wolf attacks (Dias, 1948; Fontes, 1977: see Table S2). Descriptions report that in order to defend from wolf attacks, adult mares would group in a circle keeping foals inside, with their back towards the outside as kicking was their mean of defense, while males remained outside the group, facing and charging towards the predator  (Fontes 1977; see Table S2). Even so, it was common for wolves to kill horses, although occasionally or with less intensity to what was reported for cattle, goats and sheep.  (Dias 1948; Fontes 1977; Fontes, 1977: see Table S2). It is possible that some decades or centuries ago wolf predation on horses wasn’t as high as nowadays due to higher availability of other wild and domestic prey, particularly as carrion.

In 1948, it was estimated that approximately 40,000 *Garranos* existed in Portugal, however, due to changes in traditional rural living, husbandry practices, transportation and rural abandonment, the number of *Garranos* and number of owners has been sharply decreasing in the last few decades (ACERG 2000; Pereira 2018). Although large numbers of free-ranging horses still occur in mountain pastures due to EU economic subsidies for livestock production, only a small portion are actually pure *Garrano* horses. As a result, the  population size of *Garranos* in northern Portugal at the end of XX$^{th}$ century was estimated in less than 2,000 individuals (Portas and Leite 2000). In recent times, horse owners started to frequently introduce and cross *Garrano* horses with other non-native horse breeds for meat production purposes due to their bigger size and with wider variation in coat color, including white and light-colored individuals easier to detect in the landscape (Morais *et al.* 2005), which are poorly adapted to the harsh local conditions. When analyzing the evolution of the equine population in northern Portugal, based on official statistics from INE (Instituto Nacional de Estatística) between 1972 and 2016, it is evident an increase until 1993 followed by several fluctuations (Figure 8). Nevertheless, the effects of rural abandonment are very clear based on the steady decline in the number of farms with horses (Figure 8). However, the majority of these horses are not pure *Garranos*, thus, it does not reflect the current trends of the population of this autochthonous breed.

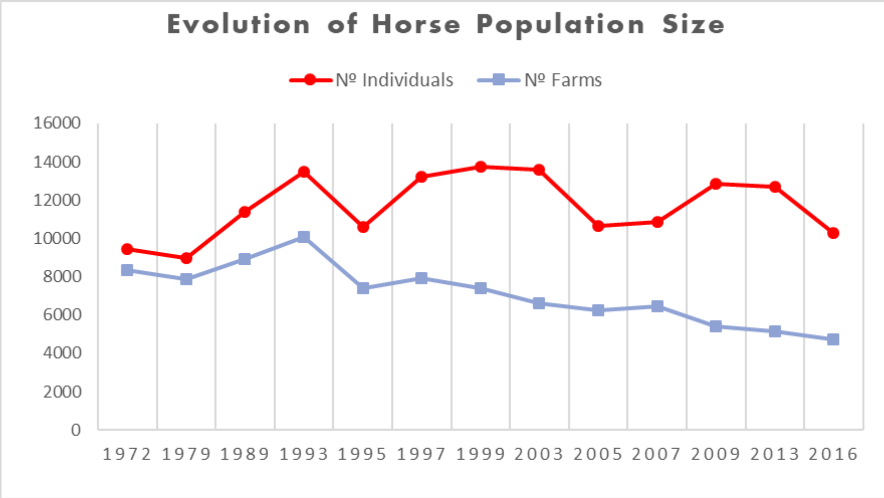


**Figure 8.** Evolution of the number of farms with horses and number of horses, in northern Portugal, between 1972 and 2016, based on agricultural census from Instituto Nacional de Estatística (INE).

Given the sharp decline in the population size of *Garrano* horses since mid XX$^{th}$ century (Figure 9), in 1995 was established an association (ACERG - Associação de Criadores de Equinos de Raça Garrana) in order to protect the *Garrano* and its genetic heritage as an endangered autochthonous horse breed. ACERG is responsible for a registration book of this horse breed – the Stud Book – where pure blooded *Garranos* are registered and owners receive financial support according to total number of animals and mare productivity in order to contribute for the conservation of this endangered horse breed (Bessa Abreu 2010). This initiative led to an increase in *Garrano* population and, in 2018, were registered 1,648 individuals (Figure 9).

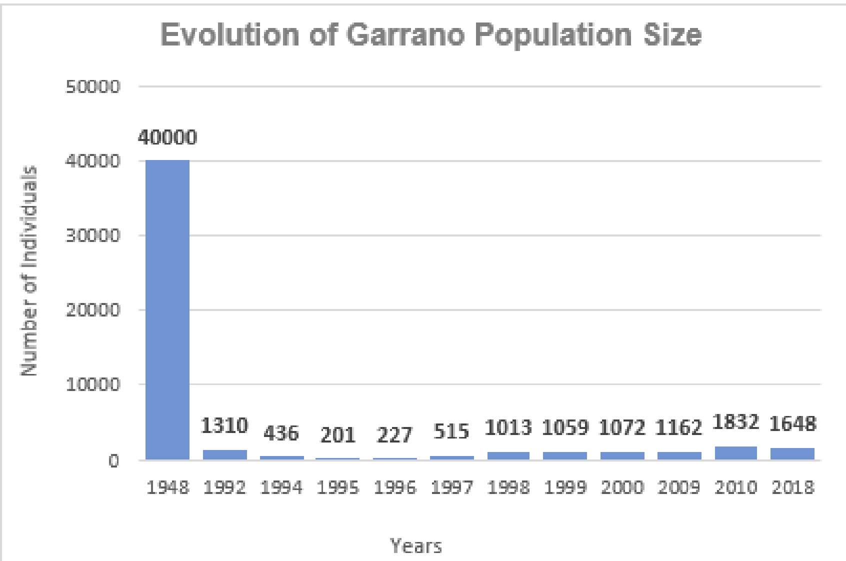


**Figure 9.** Evolution of *Garrano* horses population size between 1948 and 2018 based on data from Serôdio (1992); Gomes (1996); ACERG (2000); Portas and Leite (2000); Bessa Abreu (2010) and Pereira (2018).

However, *Garrano* populations are showing very low productivity, mostly attributed to wolf predation (Gomes 1996; Pereira 2018). The apparent high predation from wolves, raises major conflicts with horse owners and important concerns on the impact of this endangered large carnivore on *Garrano* populations, potentially hindering the efforts for the recovery of this autochthonous breed.

## 1.6. Horse-wolf conflict and damage prevention in Portugal

Wolf population in Portugal is estimated in approximately 300 individuals (± 60 breeding packs), being fully protected by law since 1989 and classified as Endangered in the Portuguese Red Data Book (Pimenta *et al.* 2005). However, Iberian wolves occur in a human-dominated landscape with low availability of wild prey, leading to high levels of livestock depredation (Pimenta *et al.*, 2017). This situation is particularly relevant in northwest Portugal (Figure 10), where extensive husbandry systems of cattle and also free-ranging horses leave the herds unattended and with no protection against predators, increasing conflicts with livestock owners which leads to high levels of illegal persecution towards this carnivore (Álvares 2011; Pimenta *et al.* 2017, 2018).

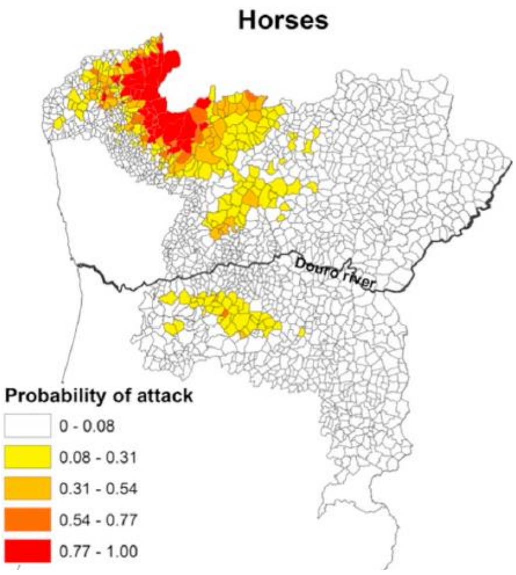


**Figure 10.** Predicted probability of wolf predation occurrence on horses per parish in the Portuguese wolf range. Figure adapted from Pimenta *et al.* (2018).

Wolf predation on domestic horses raises important socio-economic conflicts with owners, despite wolf damages being compensated by law (Milheiras and Hodge 2011). Compensation systems are one of the main tools for mitigating conflicts with large carnivores worldwide (Pimenta *et al.* 2017) and can be quite successful if applied correctly. In Portugal, the damage compensation system was implemented to mitigate the losses of livestock owners while also reducing illegal killing of wolves, however, there are many constraints in this system (Milheiras and Hodge 2011). Whenever a domestic animal is reported to have been killed by a wolf, rangers from the Institute for Nature Conservation and Forests (ICNF) visit the kill site in order to assess cause of death, verifying if wolves were responsible for the damage (Milheiras and Hodge 2011). The appropriate economic compensation for the owner is provided only if there are evidences of a wolf kill and if there are met the requirements related to damage prevention measures (e.g. presence of shepherd and livestock guarding dogs) as well as the sanitary condition of the killed animal (Milheiras and Hodge 2011; Pimenta *et al.* 2017). However, the compensation system is flawed and inefficient, leading to delayed payments often insufficient to cover the losses, exacerbating the animosity between local people and wolves (Milheiras and Hodge 2011). On the other hand, feral dogs are often found roaming in the mountains and can occasionally kill livestock, although in these cases, livestock killings are frequently mistakenly attributed to wolves, resulting in wrongly attributed compensations (Boitani *et al.* 2010; Echegaray and Vilà 2010). Moreover, when carcasses are not found and the assessment of cause of death is not possible, owners do not receive any compensations (Milheiras and Hodge 2011). This is particularly relevant for free-ranging horses, as foals are completely consumed within few days or hours (Fagundez *et al.* 2017), preventing rangers from finding the carcasses and determining if it was a wolf kill (Lagos 2013). Finally, the efficient and legally required measures to prevent wolf attacks, such as fencing or the presence of shepherds with livestock guarding dogs, are nonexistent or difficult to implement for free-ranging horses, preventing owners from receiving any compensations (Milheiras and Hodge 2011).

Prevention of wolf damages in free-ranging horses must be assured by the correct management of horse herds by their owners, such as controlling herd size and composition as well as selecting grazing areas with less risk of predation. However, despite the wide available knowledge regarding the relevance of horses in wolf diet, there is few information concerning patterns and factors influencing wolf predation on horses, particularly in Iberia Peninsula. A recent study focused in Portugal, suggests the free-ranging husbandry system as the main cause for the high levels of horse predation, as the animals are unconfined all year, day and night (Pimenta *et al.* 2018). In fact, Pimenta *et al.* (2018) concludes that the reduction in the number of horses roaming freely is the main factor that could reduce predation on horses, which is difficult to apply given the traditional husbandry system that horses have been raised since centuries ago. However, other studies have suggested several behavioral traits as determinant in reducing wolf predation risk, such as group size, habitat use and avoiding removal of males or dominant adult females, which contributes to the collapse of herd social structure and increase lone dispersing individuals that are more vulnerable to wolves (Gomes 1996; Rio-Maior *et al.* 2006; Álvares 2011). In absence of effective systems and measures for prevention of wolf damages on free-ranging horses, new solutions are needed to minimize horse exposure to wolf predation risk and adequately compensate owners while still protecting both wolves and horses. Such solutions can only be accomplished with further behavioral studies on wolf-horse interactions using several methods such as telemetry of collared wolves and non-invasive genetics. GPS telemetry enables the estimation of predation rates, prey selection and consumption rates, by measuring the clustering of localizations on a single area and how long the individual remains there (Palm 2001; Sand *et al.* 2005). In addition, the use of genetic tools could allow the study of consumed prey by analyzing wolf scats for genetic identification of the individuals consumed, providing valuable information regarding predation on horse herds without resorting to invasive methods. Also, new procedures for damage compensation in free-ranging horses should be evaluated, such as indirect payments for wolf predation risk, as already applied in northern Europe in areas with other species of free-ranging livestock, such as reindeers (*Rangifer tarandus*) (Swenson and Andrén 2003; Persson *et al.* 2015).

## 1.7. Goals and Hypothesis.

The main goals of this study are to characterize wolf-horse conflict and investigate patterns and determinants related to wolf predation on free-ranging horses, allowing a more accurate understanding of factors that influence wolf predation on horses, such as herd size and sex/age of the individuals. In order to address these main goals, the study will focus two different geographical contexts.

To characterize wolf-horse conflicts (Chapter I), this study will focus on Alto Minho region (northwestern Portugal) by using available data on official statistics of wolf damages to livestock from ICNF and information on mortality causes of *Garrano* horses from ACERG. With this approach, this study aims to target the following specific goals:

i) Quantify the relevance of wolf damages on horses in relation to all livestock species, in terms of number of attacks and compensation values;
ii) Determine the spatial and temporal variation of wolf damages on free-ranging horses;

iii) Quantify socio-economic costs due to compensation values related to wolf predation on free-ranging horses

iv) Determine the relevance of wolf predation in the mortality causes reported for *Garrano* horses, considering both sex and age classes;

To investigate patterns and determinants related to wolf predation on free-ranging horses (Chapter II), this study will focus on a smaller area located in Serra d'Arga (Viana do Castelo, northwestern Portugal), by taking advantage of available data obtained in 2017 and 2018 in the scope of on-going wolf monitoring programs (conducted by CIBIO) as well as behavioral studies on horse herds (conducted by Kyoto University). With this approach, this study aims to target the following specific goals:

i) Determine the relevance of horses on wolf trophic ecology by assessing wolf diet and prey selection;

ii) Analyze temporal patterns on spatial overlap between wolves and *Garranos* to assess the risk of predation;

iii) Estimate wolf predation rates on horses considering both sex and age, based on GPS telemetry locations of a collared wolf;

iv) Identify interactions between horses and wolves using innovative procedures based on non-invasive genetics. A database of individual identifications of *Garranos*, which resulted from the combination of individual genetic identifications of horses dung collected by Kyoto University as well as the genetic identification of both horses consumed by wolves and the wolves who consumed them, will allow the reconstruction of the horse herds genealogies and the assessment of which individuals or families may be more susceptible to predation.

Hopefully, the expected results will allow the scientific basis to further understand wolf-horse interactions and support effective measures to both reduce predation on free-ranging horses and ensure an adequate financial support of the owners, while maintaining a stable wolf population. Based on these main goals, we hypothesize that free-ranging horses are the main prey for wolves in Serra d'Arga, particularly during spring and summer (Lagos 2013), being positively selected in relation to other livestock species (Fagundez *et al.* 2017; Lagos and Bárcena 2018). We further hypothesize that horses and herds are not equality predated, with foals being more predated than adults and males more predated than females (Lagos 2013; Fagundez *et al.* 2017; Lagos and Bárcena 2018). Furthermore, the determination of individual genetic profiles of horses consumed by wolves based on remains found in wolf scats has not been done before, which opens new possibilities to the study of prey-predator interactions as well as the trophic ecology of carnivore species.

# 2. Chapter I: General patterns of wolf-horse conflict

## 2.1. Study Area – Alto Minho

To address the socioeconomic conflict related to wolf damages on free-ranging horses, the study area included the wolf range in Alto Minho region. Alto Minho is located between Lima river, as the southern border, and Minho river, as the northern border which separates Portugal from the Spanish region of Galiza. This region comprises 10 different municipalities: Arcos de Valdevez, Caminha, Melgaço, Monção, Paredes de Coura, Ponte da Barca, Ponte de Lima, Valença, Viana do Castelo e Vila Nova de Cerveira (Figure 11) (Alves *et al.* 2014). The Alto Minho region is one of the most humanized areas in Portugal, with a human population density of 107.7 inhabitants/km2 (PORDATA 2014), mostly located along river valleys where the most fertile soils can be found (Roca *et al.* 2002).

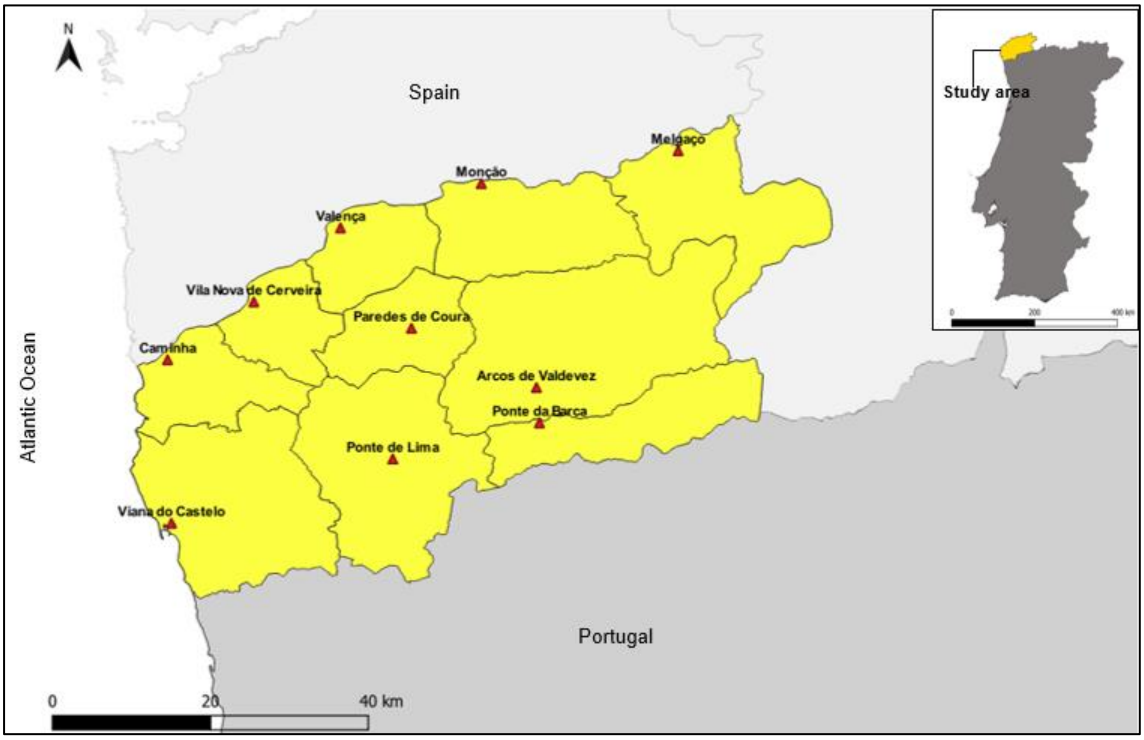


**Figure 11.** Location of the study area comprising Alto Minho region (shaded in yellow), including the 10 municipalities covered by this region.

The study area is included in the EuroSiberian biogeographic region, being characterized by a typical Atlantic climate, with a mean annual temperature of approximately 14ºC and high annual precipitation, that can reach 3,400mm in mountainous areas, presenting mild winters and cooler summers (Rodrigues 2009). Precipitation is irregular during the year, being particularly intense between october and february and scarce between april and september (Rodrigues 2009). Most study area is characterized by mountainous terrain (Roca *et al.* 2002), displaying steep cliffs and pronounced valleys, with the highest altitudes in Soajo-Peneda (1,416m) and Gerês mountains (1,508m) (Álvares 2011). The western border of the study area coincides with a coastal zone, presenting rocky beaches but also large, continuous areas of sand dunes. The landscape is very heterogeneous, with villages and agricultural land predominating along river valleys, whereas scrublands, oak forest patches and forest plantations are the most common land cover types in the mountains. Human activities occur throughout the territory, even in mountainous areas where there are extensive livestock grazing, tourism, hunting and infrastructure development (e.g. wind farms and roads) (Álvares 2011).

Alto Minho region is rich in natural resources and biodiversity, being included in a large part of Peneda-Gerês National Park as well as areas belonging to Natura 2000 Network and Sites of Community Importance (SCI), in which is included Serra d'Arga and Corno do Bico (Alves *et al.* 2014). This region harbors a very diverse flora, with forests of oak (*Quercus robur*), cork oak (*Quercus suber*), chestnut (*Castanea sativa*), strawberry (*Arbutus unedo*) and pine trees (*Pinus pinaster*), among other autochthonous species (Álvares 2011; Alves *et al.* 2014). Fauna is also very rich, comprising species such as the wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and Iberian wolf. The wolf population is stable within the study area, comprising 7 packs (Arga, Cruz Vermelha, Boulhosa, Peneda, Soajo, Vez and Laboreiro), ranging from 4 to 9 individuals per pack and which tend to use the less populated areas at higher elevation as core areas, although pack home ranges still encompass settlements, roads and wind farms (Álvares 2011; Rio-Maior *et al.* 2019). There are significant conflicts with humans due to heavy predation on livestock, resulting in high levels of poaching as an important source of wolf mortality (Vos 2000; Álvares 2011; Pimenta *et al.* 2017). Occupying the same areas as wolf packs, there are also high densities of free-ranging *Garrano* horses, an autochthonous breed from northern Portugal and particularly abundant in Alto Minho, where is considered to be the region of origin for this horse breed (e.g. "solar da raça") (Lima 1872).

## 2.2. Methods and Data source

**Wolf damages on free-ranging horses declared to ICNF**

To assess the socioeconomic conflict related to wolf damages in free-ranging horses, we used data from official statistics provided by ICNF regarding the number of declared wolf attacks on livestock (e.g. sheep, goats, cows, donkeys, horses, dogs and pigs), during 2016 and 2017, and including information on date, location (parish and municipality) and compensation value attributed to each killed animal. These data allowed the quantification of the conflict between horses and wolves, by estimating the proportion of horses killed in relation to other livestock species as well as the spatial (municipality) and temporal (month and season) patterns of wolf damages on horses. Seasons were considered as follows: winter (dec-feb), spring (mar-may), summer (jun-aug) and autumn (sep-nov). Moreover, it was also quantified the economic costs (in Euros) associated to the compensation of wolf damages on horses, in relation to all livestock species, each killed horse and per municipality. The significance of the statistical analysis was tested with $X^2$ test (Zar 1999).

**Mortality causes in *Garrano* horses reported to ACERG**

To assess the relevance of wolves on the mortality causes of free-ranging *Garrano* horses, we used unpublished data provided by ACERG regarding mortality data reported by owners of horses registered in the Stud Book as pure *Garranos*, between 2016 and 2018 and including information on cause of death, sex, age and location (municipality). These data allowed the quantification of wolf attacks as a mortality cause in *Garrano* horses, by analyzing spatial (municipality) and temporal (month) patterns on *Garrano* mortality by wolves as well as the proportion of each age class in male and female horses in relation to each mortality causes, including wolf predation. For this analysis, we considered 3 age classes: a) foals, individuals with less than one year old; b) fillies (females) and colts (males), individuals between one and three years old; and c) adults, individuals with more than three years old. The significance of the statistical analysis was tested with $X^2$ test (Zar 1999).

It is important to note that ACERG information regards only the individuals registered in the Stud Book as pure *Garranos*, which are the focus of conservation efforts to protect this endangered autochthonous breed. In contrary, ICNF data includes all breeds of horses, therefore providing a wider view on the socioeconomic conflict related to wolf damages on free-ranging horses.

## 2.3. Results

### 2.3.1. Wolf Damages on Free-Ranging Horses

**The relevance of horses in damages to livestock**

The number of wolf attacks on livestock declared to ICNF in 2016 and 2017, showed that sheep (n = 192; 55%) were the most predated species, followed by horses (n = 69; 20%) and cows (n = 65; 19%) (Figure 12). There were significant differences between years, with 2016 having the highest number of losses for all species ($X^2$: $p < 0.05$, except for cattle and dogs) except for goats and horses, that registered higher number of declared attacks in 2017. Regarding free-ranging horses, there was a significant increase of declared wolf attacks from 2016 (n=30) to 2017 (n = 39) ($X^2$: $p < 0.05$). In contrast, wolf attacks between 2016 and 2017 had a significant increase for goats (n = 4 and n = 16 respectively), while a clear reduction for sheep (n = 122 and n = 70 respectively) ($X^2$: $p < 0.05$) and cows (n = 41 and n = 24 respectively) ($X^2$: $p > 0.05$). Only in 2017 was declared a wolf attack on domestic dogs (n = 1) ($X^2$: $p > 0.05$).

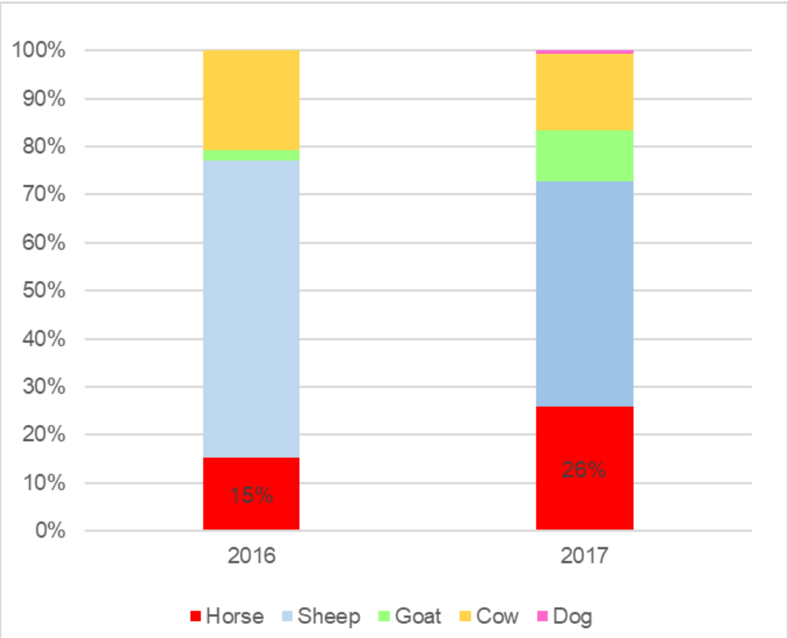


**Figure 12.** Proportion in the number of wolf attacks on free-ranging horses in relation to other domestic animals, declared to ICNF in 2016 and 2017 for Alto Minho region.

**Temporal variation by month and season**

Regarding the monthly variation in wolf attacks on horses in 2016 and 2017, august (n = 5, in 2016) and february (n = 8, in 2017) showed the highest number of reported losses, while january, october (n = 1 each in 2016) and september (n = 1 in 2017) had the lowest (Figures 13 and 14). Considering seasons, summer (n = 10 in 2016; n = 11 in 2017) showed the highest number of reported attacks to horses, followed by spring (n = 9 in both years) and autumn (n = 5 in both years). On the other hand, winter showed the highest variation between years (n = 6 in 2016; n = 14 in 2017).

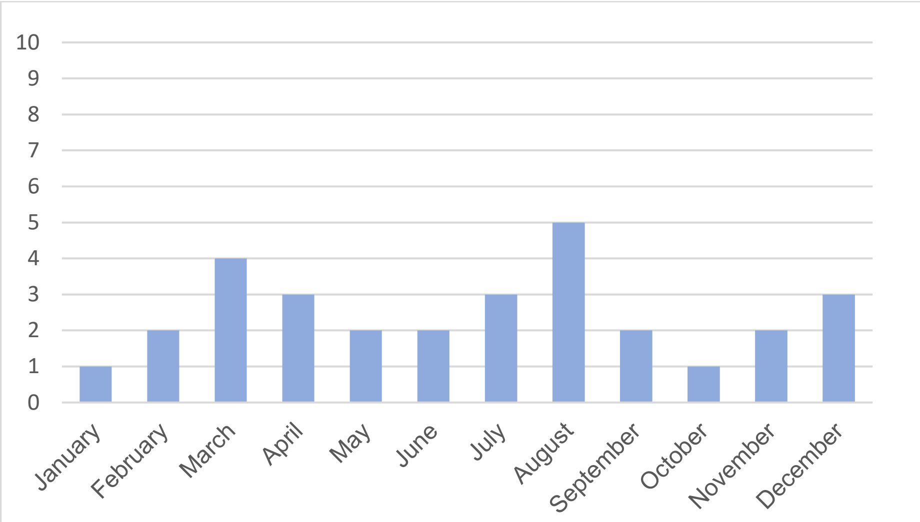


**Figure 13.** Monthly variation in the number of wolf attacks to free-ranging horses, declared to ICNF in 2016

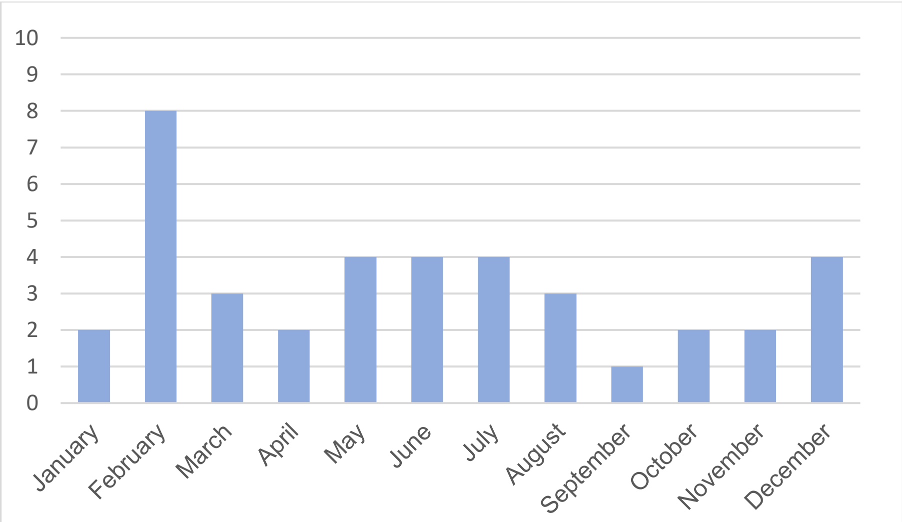


**Figure 14.** Monthly variation in the number of wolf attacks to free-ranging horses, declared to ICNF in 2017.

## Spatial variation by municipality

Considering the geographical variation of wolf damages to horses in Alto Minho, only 4 municipalities in the study area had declared losses. Viana do Castelo showed the highest number of wolf attacks to horses (n=41; 60%), with an increase in the number of losses from 2016 to 2017 (n=17 and n=24, respectively) ($X^2$: $p > 0.05$). In contrary, Paredes de Coura (n = 9 in 2016; n = 10 in 2017) and Ponte de Lima (n = 4 in both years) showed a constant number of wolf attacks during both years, while Vila Nova de Cerveira only reported one horse killed by wolves in 2017 ($X^2$: $p > 0.05$).

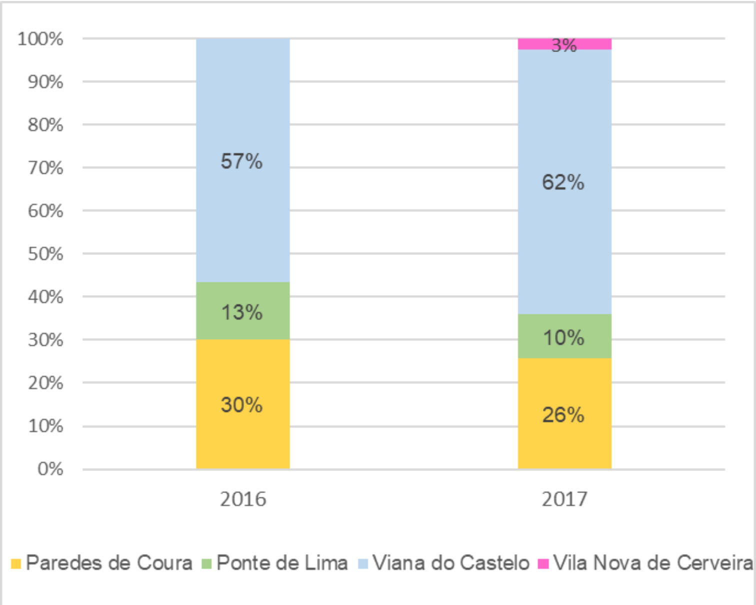


**Figure 15.** Proportion in the number of wolf attacks to free-ranging horses in each municipality, based on declared damages to ICNF in 2016 and 2017.

## Compensation values for wolf damages on horses

The total value of economic compensations for wolf damages on livestock paid by ICNF in 2016 and 2017 for Alto Minho region, was 48,546.36 euros. Compensation for damages on free-ranging horses comprised 12,824 euros, representing almost 26% of the total value compensated for all livestock species, and comprising lower values in 2016 (5,384 euros) than in 2017 (7,440 euros) (Figure 16). In average, ICNF paid 191.40 euros for each horse reported as killed by wolves, with maximum values of 400.00 euros and minimum values of 75.00 euros. Regarding the costs of compensation for wolf damages to horses in each municipality, Viana do Castelo is clearly the most affected by wolf predation, followed by Paredes de Coura and Ponte de Lima (Figure 16). Vila Nova de Cerveira only received economic compensations in 2017.

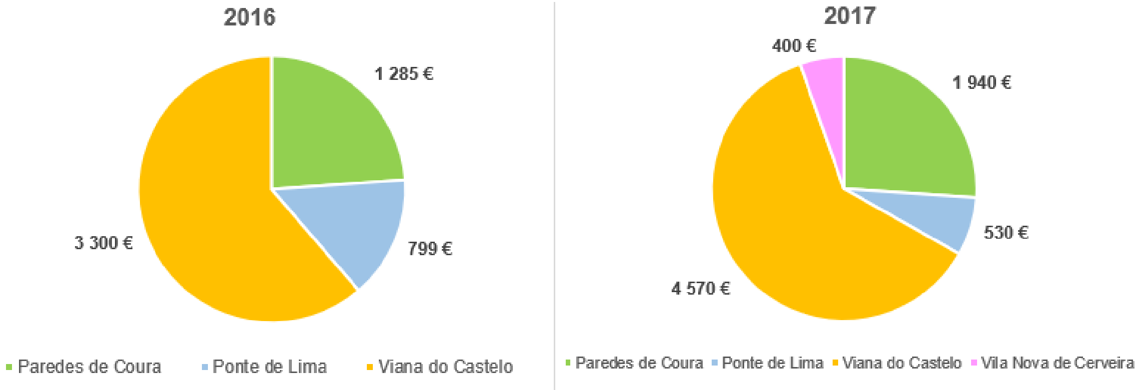


**Figure 16.** Values of economic compensations paid by ICNF for wolf attacks to horses, per municipality of Alto Minho region in 2016 and 2017.

### 2.3.2. Mortality causes reported in *Garrano* horses

**Temporal and spatial variation on *Garrano* mortality by wolves**

The main causes of mortality reported to ACERG by owners of *Garrano* horses in Alto Minho region, between 2016 and 2018, were Disappeared (n=592; 59%), followed by Wolf Predation (n=363; 37%). Other reported causes of mortality (n=41; 4%) were attributed to Natural Death (n=3.5%), Death at Birth (n=0.3%), Sold for Slaughter (n=0.2%) and Accident (n=0.1%) (Figure 17).

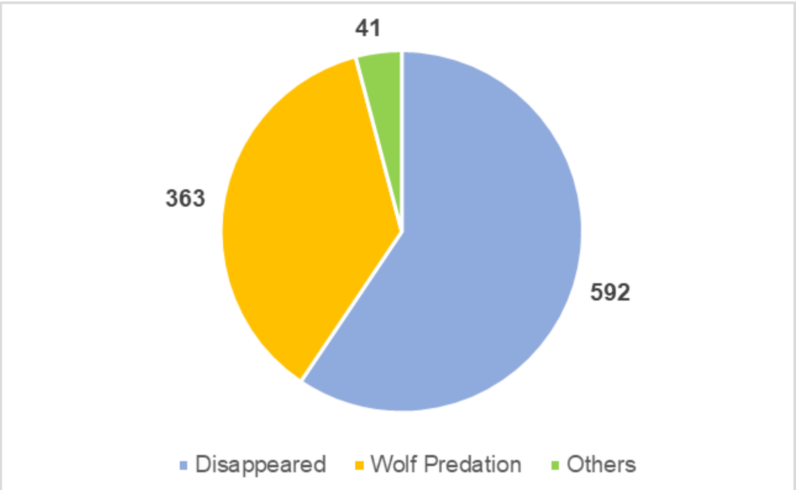


**Figure 17.** Causes of mortality in *Garrano* horses reported to ACERG in Alto Minho, between 2016 and 2018. The class "Others" included Natural Death, Death at Birth, Accident and Sold for Slaughter.

Focusing only on the two main causes of mortality (which may be related directly or indirectly to wolves), the number of *Garranos* reported as being lost (disappeared) and as killed by wolves was fairly constant across all the 3 years analyzed (Figure 18). In 2017 was a slight decrease in mortality due to wolf predation, although with no significant differences ($X^2$: $p > 0.05$). Wolf predation reached a maximum of 202 individuals killed per year, while the number of Disappeared horses reached 302 individuals.

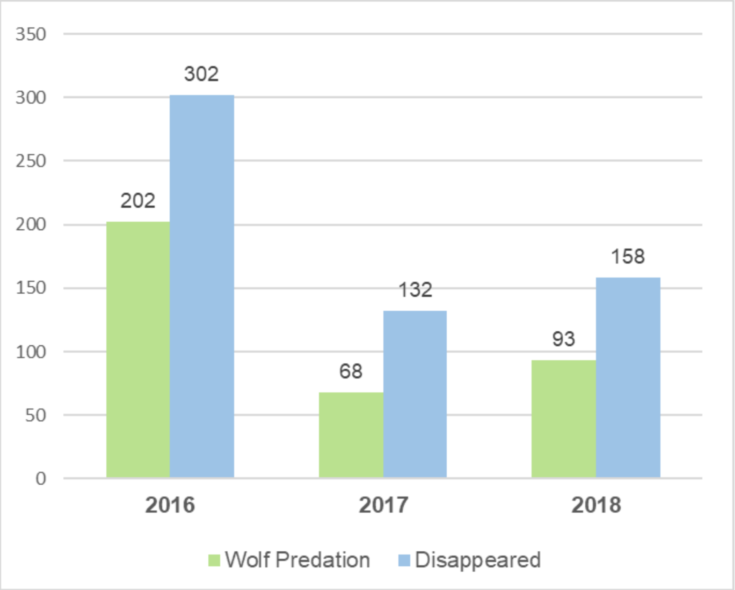


**Figure 18.** Number of individuals lost to the two main causes of mortality in *Garrano* horses reported to ACERG in Alto Minho, per year between 2016 and 2018.

Regarding the spatial variation in the number of *Garranos* reported as lost or as killed by wolves, Valença and Vila Nova de Cerveira are the only municipalities with no reported horses killed by wolves (Figure 19). In contrast, Arcos de Valdevez and Monção show the highest values of wolf predation, reaching 73% and 17% of reported mortality, respectively.

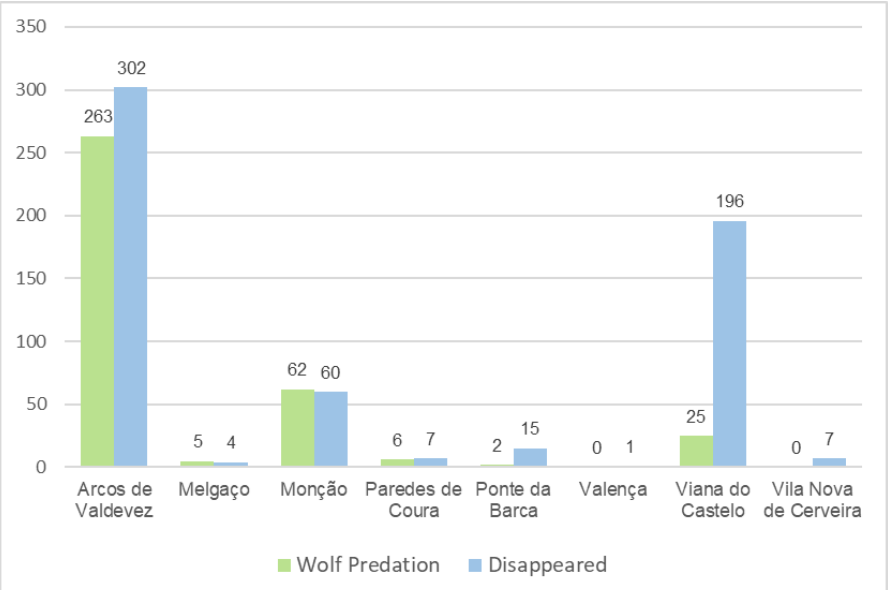


**Figure 19.** Number of individuals lost to the two main causes of mortality in *Garrano* horses reported to ACERG in Alto Minho between 2016 and 2018, per municipality.

**Sex and age patterns on *Garrano* mortality by wolves**

Considering the sex of *Garrano* horses reported to be killed by wolves between 2016 and 2018 in Alto Minho, 157 (43%) were males and 206 (57%) females, while, regarding age classes, there were 277 (76%) foals with < 1 year old, 55 (15%) individuals with age between 1 and 3 years old, that are colts (males) or fillies (females), and 31 (9%) adults with > 3 years old. Foals of both sexes where the most affected age class by wolf predation, representing almost 80% of the individuals reported to have been killed by wolves. Furthermore, 83% of the males predated by wolves were foals, while only 71% of the predated females belonged to this age class, suggesting that males seem to be more vulnerable to predation when they are younger than 1 year old. Besides, females are more killed than males when they are both fillies, with ages between 1 to 3 years old, and adults with more than 3 years old (Figure 20). These results may reflect the population structure of free-ranging horses in the mountains, since there are more females than males older than 1 year old, as a result of the traditional management where male foals are usually removed by owners.

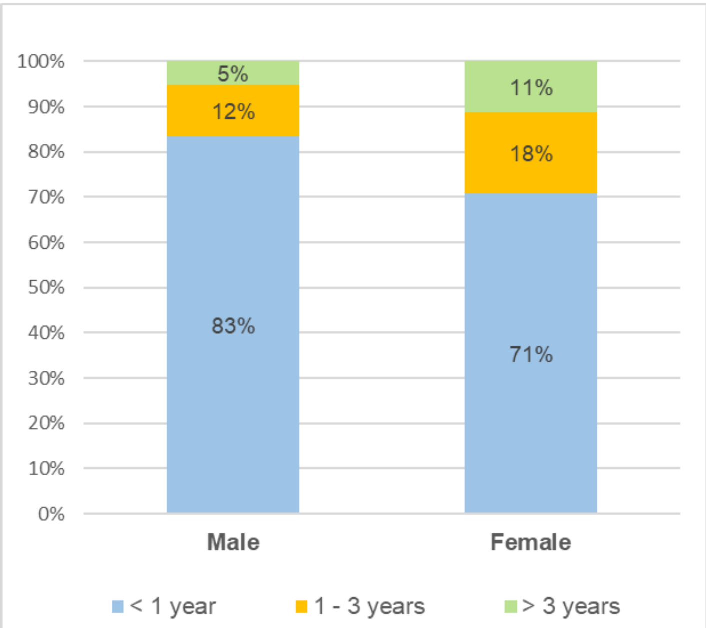


**Figure 20.** Proportion of each age classes in *Garrano* horses reported to ACERG as being killed by wolf predation in Alto Minho between 2016 and 2018, considering males and females. Individuals with less than 1 year old are foals, between 1 and 3 years old are colts (males) and fillies (females), and older than 3 years old are adults.

Regarding *Garranos* reported as lost (Disappeared), 250 (42%) were males and 342 (58%) females, while regarding age classes there were 218 (37%) foals with < 1 year old, 188 (32%) individuals with age between 1 and 3 years old, that are colts (males) or fillies (females) and 186 (31%) adults with > 3 years old. Again, most males are reported as being disappeared when they were less than 1 year old (45%), while only 31% of the females were lost as foals (Figure 21). On the other hand, considering lost individuals with age classes older than 1 year old, 70% were females while only 55% were males. These results regarding the proportion of each age class of females and males that disappeared, although with more subtle differences, are in accordance with the ones obtained for wolf predation, and should also reflect the population structure of these horses in the mountains.

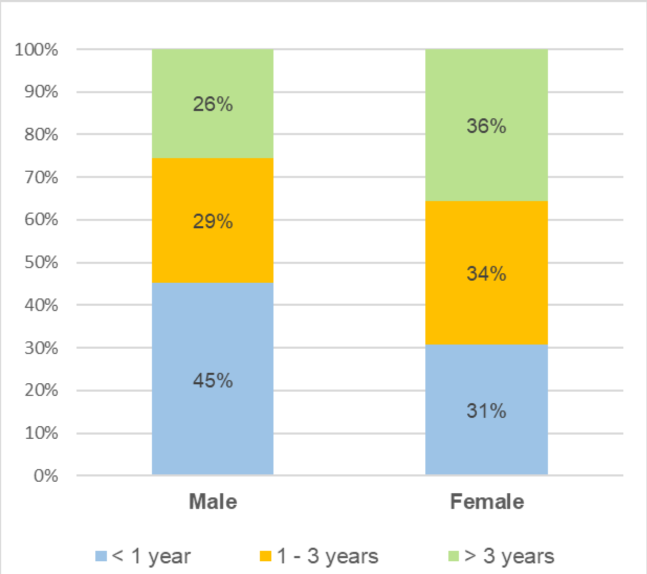


**Figure 21.** Proportion of each age classes in *Garrano* horses reported to ACERG as being lost (disappeared) in Alto Minho between 2016 and 2018, considering males and females. Individuals with less than 1 year old are foals, between 1 and 3 years old are colts (males) and fillies (females), and older than 3 years old are adults.

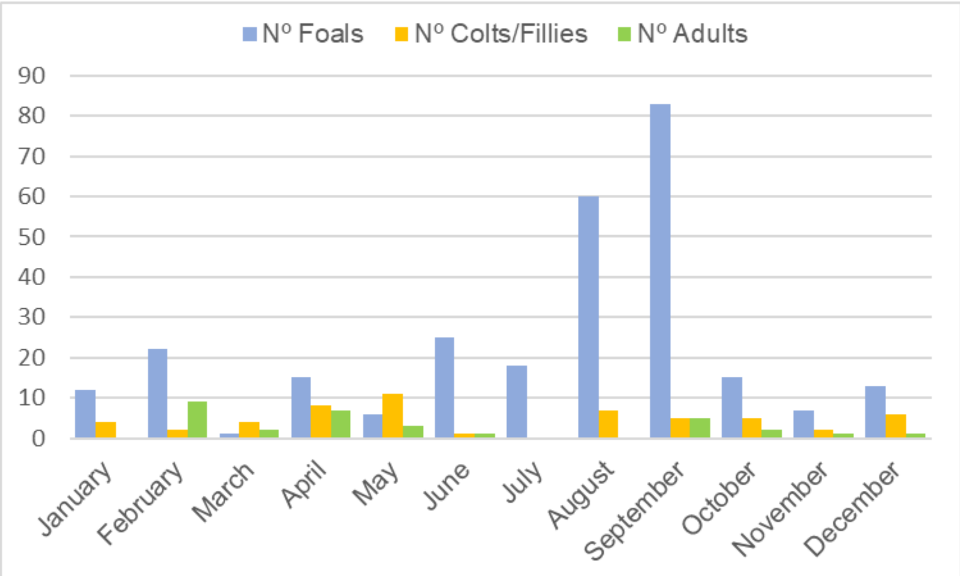


**Figure 22.** Monthly variation in the number of *Garrano* horses reported to ACERG as being killed by wolf predation in Alto Minho between 2016 and 2018, considering foals (< 1 year old), colts and fillies (1 to 3 years old) and adults (> 3 years old).

Focusing on the mortality causes of *Garrano* horses according to each age class, foals (< 1 year old) show the highest mortality due to wolf predation during august and september (52% considering both months) while the lowest is in march (Figure 22). However, when considering losses by disappearance, december is the month with higher values (36%), followed by july (15%), while may has the lowest number of losses (0.5%) (Figure 23). All other months have similar values for wolf predation mortality.

Moreover, mortality of colts/fillies (1 to 3 years old) and adults (> 3 years old) is very similar, registering slightly higher mortality due to wolf predation during spring, approximately 40% for both age classes. Considering lost (disappeared) horses, colts/fillies still suffered 35% (n=66) mortality during spring while adults registered higher mortality during winter (n=69; 37%).

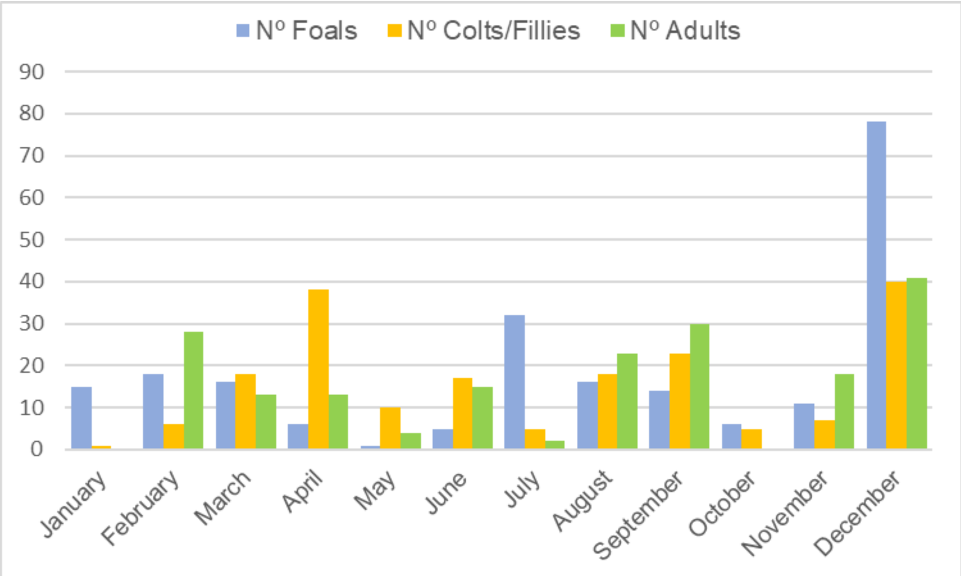


**Figure 23.** Monthly variation in the number of *Garrano* horses reported to ACERG as being lost (disappeared) in Alto Minho between 2016 and 2018, considering foals (< 1 year old), colts and fillies (1 to 3 years old) and adults (> 3 years old).

## 2.4. Discussion

The assessment of the socioeconomic traits associated to wolf damages on livestock is essential to fully understand the management implications related to prey-predator interactions and minimize properly the conflict between wolves and livestock breeders (Dickman 2010). This challenge is particularly relevant when an endangered and autochthonous livestock breed is involved, as the case of *Garrano* horses. The descriptive approach used in this chapter based on available datasets from different institutions (ICNF and ACERG) provides valuable and innovative insights to better contextualize at a regional scale: i) the conflict related to wolf predation on free-ranging horses, in terms of declared attacks and respective compensation value; and ii) the relevance of wolves as a mortality cause for pure *Garrano* horses, in terms of sex and age classes more affected.

Free-ranging horses comprise approximately 20% of all wolf attacks declared to livestock in Alto Minho but represent more than 25% of the compensation value for wolf damages, reflecting the higher economic value of horses in relation to other livestock species, such as goats and sheep, as previously reported at both a regional (Álvares 2011) and national level (Pimenta *et al* 2018). However, the proportion of horses in relation to all livestock species killed by wolves seem to show an increasing trend, as suggested by the considerable increase of horse damages declared to ICNF between 2016 and 2017, in the study area. This increasing trend in wolf damages may be related to the increasing size of wolf population in Alto Minho, particularly in Viana do Castelo municipality which comprises more than half of the reported wolf attacks to ICNF in 2016 and 2017 and where occurs a wolf pack – Arga pack – that had a much smaller pack size in 2016 (estimated in 6 wolves: Nakamura *et al.* 2017) than in 2017 (estimated in 11 wolves: Nakamura *et al.* 2018), which would eventually cause an increase of wolf attacks on livestock and, particularly, on horses. Higher values of declared wolf damages are coincident with two vulnerable periods for horses: breeding season, including late spring and summer, when foals have few months old and are particularly vulnerable to predators, and winter, when the harsh conditions and low availability of food contribute to poor body condition and high mortality risk, as already reported in previous studies (Garrott 1991; Álvares 2011; Lagos 2013).

At a spatial context, Viana do Castelo and Paredes de Coura are the municipalities with the majority of declared damages to horses, reflecting an overlap between high wolf densities with high number of free-ranging horses, as already described in historical references since XVIII$^{th}$ century (Sousa and Alves 1997). Viana do Castelo registered more than twice the number of attacks compared to Paredes de Coura, considering the data from ICNF. On the other hand, ACERG reports higher predation in Arcos de Valdevez, suggesting that the majority of horses killed by wolves in the municipality are pure *Garranos*, however, since data from ICNF does not include that municipality, we cannot compare the values. Yet, by comparing Viana do Castelo, one of the most affected municipalities common to both databases, ICNF declares approximately 60% mortality in both years, while ACERG reports around 23% mortality, which could indicate that a considerable proportion of the horses declared are not pure *Garranos*. When comparing the number of horse losses in Alto Minho declared to both ACERG and ICNF, during the same time period (2016 and 2017) and for the same municipalities with common data (Viana do Castelo, Paredes de Coura and Vila Nova de Cerveira) there are great discrepancies, demonstrating how difficult is to quantify adequately the losses by wolf predation. When considering only wolf predation as the mortality cause reported to ACERG (n = 13 in 2016; n = 8 in 2017), the declared wolf attacks to ICNF (n = 26 in 2016; n = 33 in 2017) are much higher, particularly for 2017. As ICNF data includes data on all horses reported as killed by wolves, it is expected that the numbers are higher than the numbers provided by ACERG for wolf predation, which only represents the associated owners with pure *Garrano* horses. However, by joining the two mortality causes reported to ACERG which can be assumed to result from wolf predation (Disappeared and Wolf Predation), the number of declared losses to ACERG (n = 85 in 2016; n = 80 in 2017) becomes more than double compared with the ICNF values for the number of losses by wolves. In this case, *Garranos* reported to ACERG as being lost may be result of wolf predation or scavenging which may hamper the detection of remains from dead horses (either by their total consumption or their concealment in dense vegetation), therefore, explaining the lack of proof to declare these kills to ICNF. In fact, the lack of remains found in the field to allow the confirmation of a wolf attack, especially when considering foals that are consumed entirely in a few days, is a great limitation for compensations to be paid and an important source of conflict with livestock owners (Milheiras and Hodge 2011).

Mortality causes of pure *Garrano* horses reported by ACERG are mostly attributed to lost animals (disappeared) and wolf predation, with the number of disappearances almost doubling the number of kills by wolves. Although mortality by wolf predation can be the most likely cause despite the absence of evidences of a wolf kill, disappearances can not only be attributed to wolf predation, but also to other causes such as natural death by disease and injury or even by robbery, reflecting a poor herd management and control by the owners. In this context, is important to note the extremely low incidence of reported causes of natural mortality in *Garrano* horses in ACERG dataset, which may suggest that given their free-ranging husbandry system, debilitated animals that would eventually die can become predated or scavenged by wolves. In fact, previous studies that indeed mention other causes of horse mortality, usually report low percentages. Gomes (1996) reported accident as the second main cause of foal mortality (6.7%), followed by disease (3.4%), while Lagos (2013) reported 3.3% mortality on individuals with more than 1 year old, resulting from malnutrition, disease and accident. As a result of wolves and the harsh environment conditions where *Garranos* occur, horse owners seem to have a poor economic profit from their activity, as suggested by the small number of horses (0.2%) reported to be killed for meat (e.g. sold for slaughter).

Regarding sex and age of the individuals, the majority of both males and females reported to have been killed by wolves or lost were younger than 1 year old, which is in accordance with results from previous studies (Gomes 1996; Lagos 2013; Fagundez *et al.* 2017; Lagos and Bárcena 2018). Furthermore, the proportion of killed or lost foals was higher in males than in females, which could be attributed to behavioral traits of males such as wandering farther away from their mothers, being more prone to injuries and/or expose to predators, or their larger body size which could increase their vulnerability to predation (Garrott 1991; Lagos 2013). On the other hand, Monard *et al.* (1997) hypothesized that differential foal mortality is related to environmental conditions. Unfortunately, very few studies address this subject, therefore, more research is needed to understand why mortality seems to be biased towards male foals. During their first year of age, foals have higher mortality by wolf predation during august and september, when they are a few months old and start exploring their environment and straying from their mothers, consequently being more exposed to predators (Lagos 2013). On the other hand, foals have also highest mortality by disappearance during December, which can be attributed to poor body condition due to the harsh climatic conditions and lower availability of food (Lagos 2013). After foals, the highest proportion of both males and females reported to have been killed by wolves comprised individuals between 1 and 3 years of age (colts and fillies), reaching the highest values during spring, which can be related to the fact that after most of the foals are consumed, wolves could start hunting the second most vulnerable class, the juveniles. Contrastingly, adult mortality by wolf predation was higher in winter, likely a result of harsh weather and poor body condition, which could indicate that the evidence of predation found in the carcass was actually a result of scavenging. The higher incidence of mortality due to both disappearances and wolf attacks in females with more than 1 year old, in comparison with males with the same age, can be related to when they start dispersing in search of other groups, becoming more exposed to predators without the protection of the herd. Disease and injury are also factors to be taken into account, since breeding females often become weakened after giving birth and during lactation, consequently increasing the probability of being targeted by wolves (Garrott 1991).

Wolf damages on horses involve a considerable economic cost for their owners. The reduction of compensation values in the study area between 2016 and 2017 may be related to the delay on the payments by ICNF, which encourages further disbelief in the compensation system and may lead to a reduction of the number of declared losses from one year to the next (Milheiras and Hodge 2011). The individual values of economic compensations paid by ICNF are low (average of 200 euros per horse), considering that the owners not only lose the animal but also have a short period of time to replace it in order to benefit from subsidies for livestock production. In fact, considering the dwindling *Garrano* population, it becomes difficult to find another animal to replace the lost horse. Besides, the status of endangered autochthonous breed for *Garrano* horses should be taken into consideration when paying for wolf damages. Many free-ranging horses found in northern Portugal are not pure *Garranos*, having been crossed with other more robust breeds to fulfill the needs of the owners (Morais *et al.* 2005). Nevertheless, ACERG maintains records of most pure *Garranos* still existing in Portuguese mountains, which should be considered when compensating the owners. By providing higher values and faster payments for compensations to pure *Garrano* owners, using ACERG's database to confirm the identity of the owner and the horse, it would become a stronger incentive towards maintaining the traditional husbandry practices and safeguarding this iconic livestock breed. The current economic compensation system does not benefit the horse owners, endangering not only the *Garrano* population but also the wolf population as well, since owners are likely to kill wolves illegally to prevent further damages to livestock (Álvares 2011; Milheiras and Hodge 2011). In the context of a free-ranging livestock species, a more suitable alternative to the compensation system in Portugal is currently applied in Swedish reindeer husbandry area, where the payments are issued according to the detection of reproduction in wolverines (*Gulo gulo*), in order to offset any reindeer losses (Zabel and Uller 2008; Persson *et al.* 2015). In this case, the economic compensation is dependent on conservation outcomes, which implies the number of carnivore reproductions, and not on livestock losses (Zabel and Uller 2008; Persson *et al.* 2015), unlike the ex-post system of compensation enforced in Portugal, which is poorly fitted for free-ranging horses. With the procedure applied in Sweden, reindeer herders do not need to search the dead animal to receive the payment and are encouraged to maintain a healthy carnivore population (Zabel and Uller 2008). In fact, after the implementation of this compensation system, the Swedish wolverine population increased due to the protection of breeding females from illegal killing (Persson *et al.* 2015). A similar compensation scheme in Portugal would ensure the survival of the wolf population while fairly compensating owners of free-ranging horses for their losses.

# 3. Chapter II: Patterns of wolf predation on horses: Insights from Serra d'Arga

## 3.1. Study Area – Serra d'Arga

This study was conducted in the main plateau of Serra d'Arga (41º49'13.890''N, 8º42'39.662''W), occupying a total of 5,331 hectares and comprising 4 parishes – Arga de Cima, Arga de Baixo, Arga de São João and Montaria (Figure 24) – which belong to the municipalities of Caminha and Viana do Castelo, located in Alto Minho region (INE 2012).

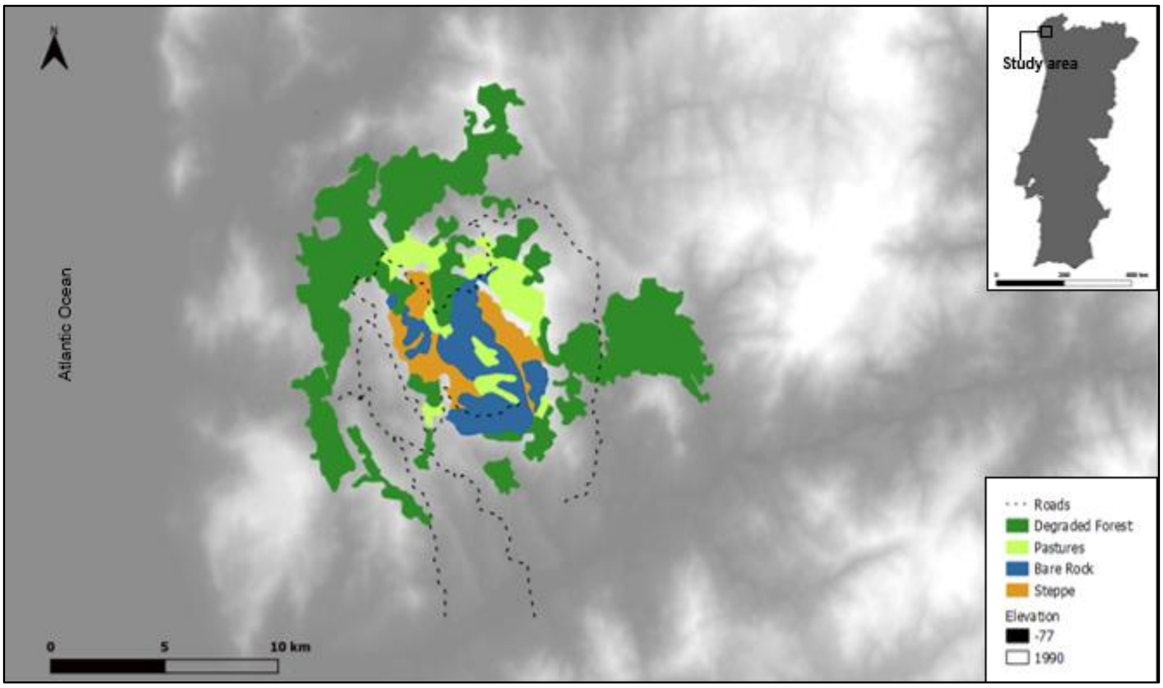


**Figure 24.** Location of Serra d'Arga and characterization of vegetation cover in the study area comprising the main plateau.

Serra d'Arga is integrated in Rede Natura 2000 network and was classified as a Site of Community Importance (SCI) due to the high value of its environment, ecosystems and landscape as well as the presence of several protected species, including the Iberian wolf (ICNB 2005; Pereira *et al.* 2008). This mountain range is located in northwestern Portugal, with a maximum altitude of 825 meters and characterized by a strong Atlantic climate with high values of mean annual precipitation, which can surpass 2,800 millimeters per year (Pereira *et al.* 2008; Álvares 2011). Annual mean temperatures are around 12.2ºC and humidity averages 70% (Gonçalves *et al.* 2016). In the study area, there are two main vegetation types, namely Atlantic wet heathlands and *Nardus* grasslands, both listed in Annex I of the EU Habitats Directive (Gonçalves *et al.* 2016). When comparing vegetation at higher and lower altitudes of the mountain, the plateaus at higher altitudes are characterized by wetlands and shrubland and extensive rock outcrops, while lower altitudes are occupied by agricultural fields and forests, mainly pine (*Pinus pinaster*) and eucalyptus (*Eucaliptus globulus*) (ICNB 2005; Pereira *et al.* 2008; Gonçalves *et al.* 2016).

Serra d'Arga is one of the few mountains in coastal Portugal with low human presence, where local people still survive from traditional practices such as agriculture and livestock husbandry (Primavera *et al.* 2015). The presence of one wolf pack – Arga pack - was confirmed regularly until 2005, when it became absent during a period of 8 years, until its reappearance was again confirmed in 2013 due to the natural dispersal of wolves from neighboring packs (Primavera *et al.* 2015; Nakamura *et al.* 2019). After its return, this pack have been reproducing regularly every year since 2014, with an estimated pack size of 11 (5 adults and 6 pups) and 12 (5 adults and 7 pups) individuals in 2017 and 2018, respectively (Nakamura *et al.* 2019). Human persecution towards wolves is still intense in Serra d'Arga, due to livestock depredation, and along with continuous habitat fragmentation originated by forest fires and highways that cross the mountain range, the viability of this wolf pack might be compromised (Álvares 2011; Primavera *et al.* 2015). Wolf breeding season can be divided in two main periods: parturition and dependency to rendezvous sites. Parturition normally occurs in late may and pups remain in the dens, fully dependent on milk, until june (Rio-Maior *et al.* 2018). From july to september, pups are moved from the dens to rendezvous sites and start to feed on meat brought by the adults (Rio-Maior *et al.* 2017). By the end of this period, pups start traveling with the pack and hunt, thus, being accounted as part of the effective size of the wolf pack.

The *Garrano* population occurring in Serra d'Arga is owned by local people, although the horses are free-ranging all year, with occasional removal by the owners (Ringhofer *et al.* 2017). In 2017 and 2018, the *Garrano* population in Arga was estimated in, respectively, 201 and 177 individuals, distributed by 30 herds (Kyoto University, unpublished data). As consequence of the prolonged absence of a wolf pack in Serra d'Arga, the population of *Garranos* occurring in this mountain may not be familiarized to this predator and become more susceptible to predation.

## 3.2. Methods

### 3.2.1. Scat analysis for detection of consumed horses

Scats attributed to wolves collected in the field in the scope of on-going wolf monitoring projects conducted by CIBIO were already genetically analyzed for species identification and individual identification. Species identification was assessed through the amplification of an approximately 425 bp sized fragment of the mtDNA control region, and then samples with wolf mtDNA were genotyped for a set of 18 microsatellites for individual identification. DNA extractions and amplification reactions were conducted in a dedicated non-invasive genetics laboratory in CIBIO and monitored by negative controls to detect possible contaminations (for further details on the molecular procedures for scat analysis see: Nakamura *et al.* 2017). For each collected scat was registered the precise location and date of collection.

All scats genetically identified as wolf (n=185), collected in Serra d'Arga during 2017 and 2018, were considered for this analysis, with a total of 55 and 130 samples analyzed, respectively. The main goal of this analysis was i) to identify prey remains in wolf scats to investigate horse consumption and prey selection by wolves, as well as ii) to collect horse remains in wolf scats to identify genetically the individuals consumed by wolves.

Diet analysis was performed by washing the scats with disinfection of all material used for each sample with 96% ethanol, in order to prevent contamination for posterior genetic analysis (see chapter 3.2.5). Each scat was submerged in a tray filled with water to desegregate the macro-components, followed by a second wash in a sieve with a mesh size of 1 mm to eliminate microscopic residue. The organic material left was placed in a petri dish, properly identified, and placed in an oven to dry, at a mean temperature of 65ºC during, approximately, 1 day. The point-frame method was used to determine diet composition and collect horse remains for posterior genetic analyses (see chapter 3.2.5). The point-frame method is a standardized procedure to identify macroscopic prey remains, allowing the systematic sampling of the scats with reduced effort and processing time while still providing a rigorous approach (Ciucci *et al.* 2004). The point-frame was constructed with an aluminum box and a plastic bar with 10 pins, placed perpendicular to the box, adapted to slide along the box (Figure 25), following the description in Chamrad and Box (1964) and Ciucci *et al.* (2004). For this analysis were used 3 aluminum square boxes with 10 cm, 8 cm and 6 cm squares depending on the size of the sample. When placing the frame with the pins on top of the box, the number of pins falling inside would depend on the size of the box, specifically, inside the 10 cm square box would fit 100 pins, while inside the 8 cm square box would only fit 64 pins and, finally, inside the 6 cm square box would fit 36 pins.

**Figure 25.** Point-frame method, with 10 cm aluminum box and containing a wolf scat sample.

The faecal sample was evenly spread in the box and the items touching each pin were recorded. The items recorded were categorized as prey hair, wolf hair, bone, cartilage, vegetal material, mineral material and insects. If the item recorded was prey hair, then it was collected and used for identification of consumed prey. Hair identification was done according to Debrot *et al.* (1982) and Teerink (1991). In order to conduct a more accurate analysis, particularly for hairs from domestic ungulates which have many similarities leading to uncertainty in the identification of the *taxon* (De Marinis and Asprea 2006), a reference collection elaborated by Casimiro (2017) was also used as a guide, as recommended by Spaulding *et al.* (1973) and De Marinis and Asprea (2006). With the hairs collected from each scat sample, the consumed prey was identified through microscope observation of cuticular pattern, medulla and cross-section. Only prey classes related to food items were considered in the analysis, which excluded purgative plants or other vegetal material ingested unintentionally, mineral material, wolf hairs and insects, considered as non-food items.

Horse remains (n = 35 and n = 89 from 2017 and 2018, respectively) were brought to the laboratory for genetic individual identification (see chapter 3.2.5). In total were obtained 124 samples, from which one sample only contained bones but was still used for genetic identification of the consumed individual horse, since the probability of being a horse was high. Furthermore, 2 samples were excluded from the analysis, one in each year, as there was not enough material for genetic identification (only a few horse hairs without roots).

### 3.2.2. Wolf diet expressed in Frequency of Occurrence, Consumed Biomass and Prey selection

To quantify each prey species found in a total of 149 wolf scats, it was used the Frequency of Occurrence (F.O.) and Consumed Biomass (Biomass), as they are the most common approaches for this type of analysis (Ciucci *et al.* 1996). Frequency of Occurrence is expressed in absolute percentages of the number of occurrences of each prey item in relation to the total of occurrences of all identified prey. In addition, it was estimated the Consumed Biomass to complement the analysis, since F.O. can be biased due to the overestimation of large or less consumed prey and underestimation of common prey, as a consequence of different surface/volume ratio between species (Ciucci *et al.* 1996). The estimation of the Consumed Biomass of each prey class was performed using the model of Ciucci *et al.* (2001), represented as the linear regression:

$$\boldsymbol{y = 0.274 + 0.011x}$$

where the dependent variable ($y$) represents the biomass ingested per collected scat and the independent variable ($x$) represents the mean live weight (kg) of each prey class identified in the scat. The total amount of biomass for each prey class is obtained by multiplying the value of ($y$) by the number of scats in which the corresponding prey species was found. Information regarding the mean weight (kg) of each prey species was obtained from published data and official statistics (see Table S3 in Supplementary Material).

Regarding prey selection, it was quantified using Ivlev’s Selectivity Index (D), modified by Jacobs (1974):

$$\boldsymbol{D = \frac{(r - p)}{(r + p - 2 \cdot r \cdot p)}}$$

with ($r$) being the proportion of each prey class in wolf diet and ($p$) the proportion of that prey in terms of population in the study area. The index varies between -1, representing the complete avoidance of the species, and 1, representing maximum positive selection. When the index equals 0, then the consumption of that prey class is proportional to its abundance in the study area. To determine availability of domestic and wild ungulate species in the study area in order to assess prey selection in wolf diet, it was used information provided by Kyoto University regarding the *Garrano* population size, official statistics for livestock species and published data for species of wild ungulates (see Table S4 in Supplementary Material). The population size of each livestock species occurring in the study area was obtained from the agricultural statistics “Recenseamentos Gerais da Agricultura” (INE 2011). There are no available information regarding population estimates of wild ungulates in Serra d’Arga, therefore the population size of roe deer was estimated based on population density estimated in Peneda-Gerês National Park (Ferreira 2003) and population size of wild boar was estimated using abundance values estimated for all Portugal (Terras de Sicó 2019).

### 3.2.3. Estimates on wolf predation rates

The estimation of wolf predation rates on horses was based on information collected by GPS telemetry of a collared wolf (Lobo#11537 – “Minho”), an adult male resident in Arga pack tracked from 24.07.2018 (date of capture) to 10.11.2018 (date of collar failure). The GPS collar was programmed for giving wolf positions at 20 minutes intervals, during 10 days at the beginning of every month, starting on the second day of each month, at 00h00, until the eleventh day, at 23h59. After downloading the wolf positions every 2 days, they were plotted using ArcView, a metric grid system. A cluster was defined when more than 2 positions were obtained within 100 meters radius, during consecutive periods of time, following the procedure described by Anderson and Lindzey (2003).

When potential clusters were identified, the sites were located on the field with hand-held GPS and searched for prey remains, being categorized as feeding sites (with prey remains both related to predation events or scavenging), resting sites (nocturnal and diurnal) or other sites with wolf activity. On feeding sites, a kill-site (or predation site) was defined when a carcass was still fresh and displayed signs of predation, such as hematomas and blood, while scavenging sites had old remains without evidence of predation or a recent kill. At feeding sites, whenever a fresh horse carcass was found, the sex and the age of the individual was assessed if possible and tissue samples were collected to be used for genetic identification of the individual horse. During the field assessment of predation clusters 19 tissue samples of horses were collected. Age of killed horses was assessed through observation of dental wear (Evans *et al.* 2007) and classified as foals (< 1 year old) and adults (> 1 year old) with reference with the estimated number of years of age. Regarding the assessment of sex from killed individual horses with few remains, some carcasses allowed checking dentition, by considering the presence of canines as being males, and their absence as being females (Lowder and Mueller 1998).

In some cases, although the head was still intact it wasn't possible to open the mouth without cutting the lips, thus, that procedure was not done since it could have a negative influence of the economic compensation paid by ICNF.

When each site was visited, the findings were recorded based on the evidence found, such as prey remains (carcasses, bones, hairs, blood, etc.) and wolf beds, as well as wolf GPS coordinates and habitat characteristics. Finally, it was possible to estimate monthly, annual and individual wolf predation rates based on the number of dead horses found in kill sites and the period in which the GPS collar sent positions every 20 minutes for the analysis of the predation clusters.

### 3.2.4. Spatial assessment of wolf predation risk

In order to determine wolf predation risk in the study area, it was considered both the locations of the GPS collared wolf (Lobo#11537 – "Minho") between july and november 2018 and the locations of wolf scats detected monthly in transects distributed in the study area between 2016 and 2018, which were spatially analyzed with Kernel Density Estimation in R (R Development Core Team 2011), using the packages 'ks', 'sp', 'rgdal', 'raster', 'spatialEco' and 'rgeos' (Pebesma and Bivand 2005; Hijmans *et al.* 2014; Duong 2015; Evans 2015; Bivand *et al.* 2016, 2018). To estimate wolf core areas, we used 50% of the GPS locations from the collared wolf, while to estimate the wolf range we used 95% of the GPS locations. By estimating those areas, it was possible to determine which regions were more frequently used by Arga pack and, consequently, assumed as where the predation risk was higher. Additionally, it was also estimated the areas with highest abundance of wolf scats collected in the field in 2016, 2017 and 2018, also using Kernel 50% and Kernel 95%, to determine if the areas with higher activity of the collared wolf would match the areas with higher number of collected wolf scats. For this approach, were considered two time periods: may – october as representative of wolf breeding season and november – april as representative of non-breeding season. To assess if the horse population in the study area overlaps with areas with higher wolf predation risk, we compared the location of the horse herds in Serra d'Arga plateau, provided by the Kyoto University team, with the estimated wolf range based on the two datasets (e.g. telemetry and detected scats). The herd locations were photographed by drone and georeferenced, only during the day in 2018. All spatial analyses were performed in QGIS (version 3.6.2. Noosa), an open source of Quantum Geographic Information System.

### 3.2.5. Genetic identification of horse remains from wolf scats and predation clusters

A total of 122 horse remains (bone, cartilage and hair samples) found in wolf scats were used for the individual genetic identification of consumed horses. Additionally, 19 tissue samples collected in the field from dead horses found in predation clusters of the GPS collared wolf were also analyzed for individual identification.

**DNA extraction**

The remains collected from the wolf scat analysis were washed with TE at 55ºC for 24h, followed by DNA extraction and purification using the DNeasy® Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA). The protocol for digestion of the tissues was adapted for 1000µL of buffer and 35µL of Proteinase K to all samples, followed by incubation at 37ºC with agitation for a minimum of 48h. After this step, the original protocol was followed according to the manufacture. The samples collected from predation clusters included 16 tissue samples and 3 bone samples. DNA from these samples was extracted using the EasySpin® Nucleic Acid Extraction Tissue Kit (Citomed, Lisbon, Portugal). For bone samples, an additional incubation at 37ºC with agitation for 48h was performed for a more thorough digestion. The products of extraction were analyzed by electrophoresis on 0.8% (w/v) agarose NA gel in 1x TBE buffer containing 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0. Afterwards, the gel was visualized by UV transilluminator.

**PCR assays**

DNA was amplified by two consecutive PCRs, using a pre-amplification protocol (Smith *et al.* 2011) . Both reactions were performed with a final volume of 10uL, using 5µL of the Multiplex PCR kit (QIAGEN), 1µL of a primer mix and 3µL and 1,5 µL of DNA, for the first and the second PCR, respectively. All samples were amplified for two primer mixes, each containing different markers (Table 1). The first PCR (Pre PCR) was performed with the following conditions: initial denaturation at 95ºC for 15 minutes, followed by an initial phase of 9 cycles of 30 second denaturation at 95ºC, 90 second annealing at 62ºC with a decreasing of 0.5ºC per cycle and extension of 30 seconds at 72ºC. The final phase comprised 11 cycles, with denaturation for 30 seconds at 95ºC, annealing for 90 seconds at 58ºC and extension for 30 seconds at 72ºC, followed by a final extension of 15 minutes at 60ºC. The 2 uL of this first PCR products were used as template for the second PCR (Post PCR) with the following conditions: initial denaturation at 95ºC for 15 minutes, followed by an initial phase of 9 cycles of 30 second denaturation at 95ºC, 1 minute annealing at 62ºC with a decreasing of 0.5ºC per cycle and extension of

30 seconds at 72ºC. The final phase comprised 36 cycles, with denaturation for 30 seconds at 95ºC, annealing for 45 seconds at 58ºC and extension for 30 seconds at 72ºC, followed by a final extension of 30 minutes at 60ºC. Conditions of both PCRs are present in the table below (Table 2). Additionally, we adapted the protocol established by Hasegawa *et al.* (2000) to determine the sex of horses found in wolf scats and in predation clusters by simultaneously amplifying the genes SRY and AMEL.

**Table 1.** Microsatellite markers used for the genetic identification of horses (in remains collected in wolf scats and samples taken in the field from dead horses), which are recommended by the International Society of Animal Genetics for this purpose, and the two markers used for sex determination, following Hasegawa *et al.* (2000).

| *Locus* | | *Primer Sequences* | | *Fragment Length* |
|---|---|---|---|---|
| | | **Primer Forward** | **Primer Reverse** | |
| AHT4 | Primer Mix 1 | AACCGCCTGAGCAAGGAAGT | CCCAGAGAGTTTACCCT | 144 - 164 |
| ASB23 | | GAGGGCAGCAGGTTGGGAAGG | ACATCCTGGTCAAATCACAGTCC | 175 - 211 |
| HMS3 | | CCATCCTCACTTTTTCACTTTGTT | CCAACTCTTTGTCACATAACAAGA | 148 - 170 |
| HMS7 | | TGTTGTTGAAACATACCTTGACTGT | CAGGAAACTCATGTTGATACCATC | 165 - 185 |
| HTG6 | | GTTCACTGAATGTCAAATTCTGCT | CCTGCTTGGAGGCTGTGATAAGAT | 84 - 102 |
| VHL20 | | CAAGTCCTCTTACTTGAAGACTAG | AACTCAGGGAGAATCTTCCTCAG | 87 - 105 |
| ASB17 | Primer Mix 2 | ACCATTCAGGATCTCCACCG | GAGGGCGGTACCTTTGTACC | 87 - 129 |
| ASB2 | | CCACTAAGTGTCGTTTCAGAAGG | CACAACTGAGTTCTCTGATAGG | 216 - 250 |
| HMS1 | | CATCACTCTTCATGTCTGCTTGG | TTGACATAAATGCTTATCCTATGGC | 170 - 186 |
| HMS2 | | CTTGCAGTCGAATGTGTATTAAATG | ACGGTGGCAACTGCCAAGGAAG | 222 - 248 |
| HMS6 | | GAAGCTGCCAGTATTCAACCATTG | CTCCATCTTGTGAAGTGTAACTCA | 151 - 169 |
| SRY | Sex determination | CTTAAGCTTCTGCTATGTCCAGAGTATCC | GCGGTTTGTCACTTTTCTGTGGCATCTT | 429 |
| AMEL | | CCAACCCAACACCACCAGCCAAACCTCCCT | AGCATAGGGGGCAAGGGCTGCAAGGGGAAT | 160 - 200 |

**Table 2.** PCR conditions for the two consecutive amplifications of the equid markers used for individual genetic identification of horse samples.

| Pre PCR | | | Post PCR | | |
|---|---|---|---|---|---|
| Temperature (ºC) | Duration | Nº of Cycles | Temperature (ºC) | Duration | Nº of Cycles |
| 95ºC | 15' | | 95ºC | 15' | |
| 95ºC | 30'' | 9 | 95ºC | 30'' | 9 |
| 62ºC | 90'' (-0.5ºC) | | 62ºC | 1' (-0.5ºC) | |
| 72ºC | 30'' | | 72ºC | 30'' | |
| 95ºC | 30'' | 11 | 95ºC | 30'' | 36 |
| 58ºC | 90'' | | 58ºC | 45'' | |
| 72ºC | 30'' | | 72ºC | 30'' | |
| 60ºC | 15' | | 60ºC | 30' | |
| 12ºC | ∞ | | 12ºC | ∞ | |

The reaction was performed with a volume of 5µL of *Taq* PCR Master Mix, 0.4µL of primer SRY Forward, 0.4µL of primer SRY Reverse, 0.4µL of primer AMEL Forward, 0.4µL of primer AMEL Reverse, 0.4µL of tail FAM and 1.5µL of DNA, and the following conditions: denaturation at 95ºC for 15 minutes, followed by 40 cycles of 30 seconds denaturation at 95ºC, 45 seconds annealing at 60ºC and 45 seconds extension at 72ºC (Table 3). Lastly, there was a final extension at 60ºC for 5 minutes.

**Table 3.** PCR conditions for the amplification of the genes SRY and AMEL, used to determine the sex of the individuals.

| Temperature (ºC) | Duration | Nº of Cycles |
|---|---|---|
| 95ºC | 15' | |
| 95ºC | 30'' | |
| 68ºC | 45'' | 40 |
| 72ºC | 45'' | |
| 60ºC | 5' | |
| 12ºC | ∞ | |

PCR products of the second PCR were analyzed by electrophoresis on 2.0% (w/v) agarose NA gel in 1x TBE buffer containing 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0. The bands in the gel were visualized using a UV transilluminator. A 100bp DNA ladder, NZYDNA Ladder V, was used as a molecular weight marker. The separation of the amplified products and reading of the fragments was performed with the sequencer 3130xl *Genetic Analyzer* (Applied Biosystems).

The resulting sequences were then analyzed with GenAlEx 6.5. (Peakall and Smouse 2012). First, we calculated the consensus for all samples, from the replicate genotypes obtained from the PCRs (2 repetitions per sample). The allele would only be considered if appeared in both replicates. After the construction of the consensus, all genotypes with more than 25% of missing data were eliminated from the dataset (n = 2). Since the scat samples could contain remains from more than one individual, we also used GenAlEx 6.5. to search for matching genotypes. When multiple genotypes matched, only one was used for the analysis. Stochastic errors such as allelic dropout and false alleles are particularly important for parentage analysis, as could lead to the wrong exclusion/inclusion of parents as a result of failed amplification of one allele of a heterozygote or misgenotyping of a true allele (Johnson and Haydon 2007). Therefore, we estimated the error rates and allele frequencies for the 11 loci used for genetic identification, using GIMLET 1.3.2. (Valière 2002) and GENEPOP (Raymond and Rousset 1995).

### 3.2.6. Pedigree analysis of horses genetically identified

The pedigree analysis was performed using a dataset comprising 46 genotypes obtained from the genetic identification of horse remains found in wolf scats, 16 genotypes obtained from 19 tissue samples collected at predation clusters and 136 genotypes owned by Kyoto University from the same horse population. The genotypes of individual horses (with a given name) owned by the Kyoto team and obtained by using non-invasive genetics from horse dungs, were previously genotyped for the same panel of markers in the same laboratory (CIBIO). Only one sample per individual was used in this analysis (n=164). Genealogies were searched using COLONY 2.0.6.5. (Jones and Wang 2010; Wang 2013), which estimates maternity, paternity, full siblings and half siblings, using maximum likelihood methods. According to the software requirements, mothers, fathers and offspring must be specifically identified in different files. However, because this information was not available, we classified all individuals (n=164) as possible offspring, all females as possible mothers (n=101) and all males as possible fathers (n=68). For some individuals (n=5) it was not possible to determine the sex, thus, they were included as both possible mothers and possible fathers.

We first run COLONY using the model FPLS, the combination of full likelihood (FL) and pairwise likelihood methods (PLS), considering the mating system with polygamous males and monogamous females, known allele frequencies, diploid and dioecious species, 1 long run and strong sibship prior. In order to produce a robust analysis, results were only accepted if the confidence in the parent pair and full sibship was above 90%. On a second run of COLONY, we included information for very consistent and highly probable relationships (P > 0.90) from the first run, including full siblings, mother-offspring and father-offspring, using more powerful parameters of the software (long run) to solidify the results before constructing the genealogies. We used the software PROGENY Clinical (Progeny Genetics LLC, Delray Beach, FL, www.progenygenetics.com), an online Pedigree tool, to construct the genealogies.

## 3.3. Results

### Horse consumption and selection by wolves

The analysis of wolf diet in Serra d'Arga, based in scats genetically validated, revealed that horse was the main prey both in 2017 (F.O.=81.4%; Consumed Biomass=85%) and 2018 (F.O.=84.0%; Consumed Biomass=92.9%) (Table 4). Cattle was the second most frequent prey in 2017 (F.O.=9.3%; Consumed Biomass=13.1%), while in 2018 both cattle and wild boar were the second most frequent prey, although with low relevance (n=3; F.O.=2.8%), and with consumed biomass displaying different values (cattle: Biomass=4.2%; wild boar: Biomass=0.9%) attributed to the contrasting mean weights attributed to both species. Goats were also consumed in both years, with more expression during 2018. Besides wild boar, the other wild prey detected was the roe deer, only found in 2017 with very low values (F.O.=2.3%; Biomass=0.4%), which can be expected as its density is presumably low in the study area. Overall, livestock were the main source of food for wolves (2017: Biomass=98.9%; 2018: Biomass=99.1%) while wild prey was poorly represented in wolf diet (2017: Biomass=1.1%; 2018: Biomass=0.9%).

**Table 4.** Wolf diet in Serra d'Arga, in 2017 (N=43 scats) and 2018 (N=106 scats), expressed in identified prey items, number of detections (N), Frequency of Occurrence (F.O.) and Consumed Biomass (Biomass).

| | 2017 | | | 2018 | | |
|---|---|---|---|---|---|---|
| Prey Class | N | F.O. (%) | Biomass (%) | N | F.O. (%) | Biomass (%) |
| *Equus caballus* | 35 | 81.4 | 85.0 | 89 | 84.0 | 92.9 |
| *Bos taurus* | 4 | 9.3 | 13.1 | 3 | 2.8 | 4.2 |
| *Capra hircus* | 2 | 4.7 | 0.8 | 11 | 10.4 | 2.0 |
| *Capreolus capreolus* | 1 | 2.3 | 0.4 | | | |
| *Sus scrofa* | 1 | 2.3 | 0.7 | 3 | 2.8 | 0.9 |

The Ivlev's Selectivity index used to measure prey selection by wolves showed a strong preference towards free-ranging horses in both years (D=0.94 in 2017; D=0.95 in 2018) suggesting that this prey species is consumed in a much higher proportion than expected given their local availability (Figure 26). For all other prey species, either livestock or wild ungulates, wolves displayed negative selection. Moreover, in 2017, cattle showed no selection (D= -0.04), while in 2018 was negatively selected (D= -0.59).

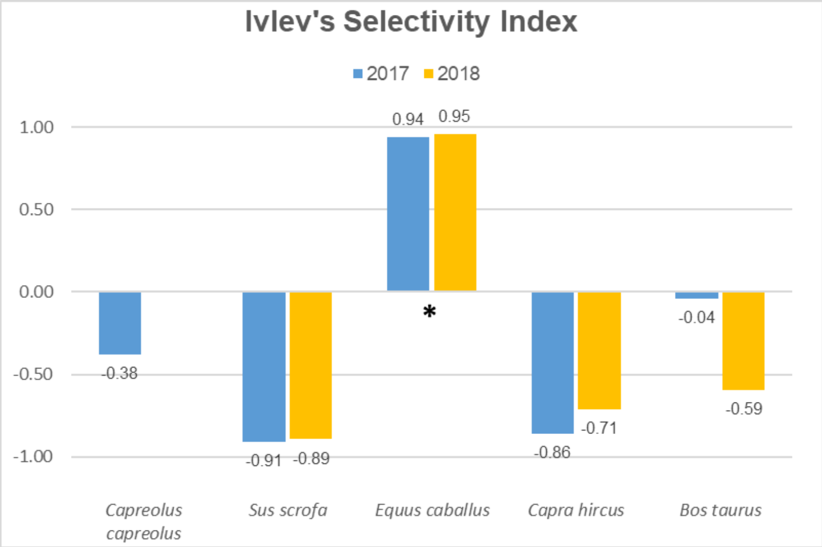


**Figure 26.** Prey selection by wolves measured by Ivlev's Index in Serra d'Arga, during 2017 and 2018, according to the prey availability in the study area and F.O. in wolf diet. The index varies between 1 (maximum positive selection) and -1 (rejection of the species). *Species with significant differences between occurrence in wolf diet and availability in the study area ($\chi^2$: $p<0.05$).

## Wolf predation rates on horses

Based on the GPS positions of a collared wolf in Serra d'Arga between august and november 2018, a total of 28 clusters were located and visited, from which 19 (68%) were considered feeding sites with prey remains, 5 (18%) were considered resting sites and 4 (14%) were considered unknown since there were no evidences of feeding sites (presence of remains) nor evidence of resting sites (absence of beds) (Table 5). Regarding the 19 feeding sites, all (100%) presented horse remains, from which 8 (42%) were considered kill sites, as there were evidences of predation, and 11 (58%) were considered unknown, as there were no signs the horse had been killed by wolves and it could have been a scavenging event (Table 5).

**Table 5.** Type and number of clusters found from the collared wolf in the study area, between 2nd of august and 10th of november 2018.

| *Clusters* | *Nº Clusters* |
|---|---|
| **Feeding site** | **19** |
| Predation site | 8 |
| Unknown | 11 |
| **Resting Site** | **5** |
| **Other** | **4** |
| **Total** | **28** |

Overall, from the 19 horses found in feeding sites from the collared wolf there were 6 males, 6 females and 7 individuals for which was not possible to assess the sex. Regarding the estimated age of the horses found at feeding sites, most of the individuals (n=9; 47%) were adults with more than 4 years old (Table 6). Only 2 horse carcasses (11%) were classified as foals with less than a year old, while for 8 carcasses (42%) were not possible to find enough remains to perform age estimation.

**Table 6.** Estimated age of the horse carcasses found at feeding sites from the collared wolf in the study area between 2nd of august and 10th of november 2018.

| *Age* | *Nº Horses* |
|---|---|
| **Foals** | **2** |
| < 1 month | 1 |
| 7 months | 1 |
| **Adults** | **9** |
| > 4 years | 3 |
| > 7 years | 2 |
| 7/10 years | 1 |
| 15/20 years | 1 |
| 20/25 years | 2 |
| **Unknown** | **8** |
| **Total** | **19** |

Considering the number of dead horses found in predation clusters, it was possible to calculate the predation rates of the wolf pack present in Serra d'Arga. In the field, at kill sites we found 8 dead horses during 40 days of intensive tracking, which suggests a kill rate of 6 horses/month, between august and november. If we extrapolate these same kill rates to a whole year (365 days), then the annual kill rate of the pack would be 73 horses/year. Considering that during the monitoring period for the predation clusters (between august and november 2018), Arga pack was estimated to have a group size of 12 wolves (including pups that at this time were already feeding only on meat and hunting together with the pack) the kill rates per month and per wolf are 0.5 horses/wolf/month and 6.1 horses/wolf/year. Horse population in Serra d'Arga was estimated roughly in 177 individuals during 2018, including adults, colts/fillies and foals (data provided by Kyoto University), which considering 73 horses/year killed by wolves, translates into an annual wolf predation impact in this horse population of approximately 41%.

### Spatial patterns in wolf predation risk

The assessment of areas with higher predation risk, showed that wolf range in Serra d'Arga based on Kernel 95% applied to the collared wolf locations and, presumably, reflecting the space use of the entire wolf pack, occupies a large area located in the plateau and few areas at lower altitude (Figure 27). Furthermore, there are 5 main core areas (based on Kernel 50%), in the center and north of the plateau, which corresponds to main resting and breeding areas. The same approach using Kernel 95% and Kernel 50% applied to the wolf scats collected in the field during 2016, 2017 and 2018, and divided in two periods: may – october (breeding season) and november – april (non-breeding season), showed that the area with highest abundance of wolf scats during breeding season (may – october) in all 3 years, estimated with Kernel 50% is located in the main core area identified by the collared wolf , further confirming the location of the pack's breeding site (see Figure S1 in Supplementary Material). Outside wolf breeding season, wolf range is much larger, with core-areas occupying a wider extension at lower altitudes but still overlapping the same location in accordance with the main core areas estimated by the locations of the collared wolf (see Figure S2 in Supplementary Material). Furthermore, when using Kernel 95%, the exact same pattern is observed in both periods, during 2016, 2017 and 2018, only covering a larger area (see Figure S3 and S4 in Supplementary Material).

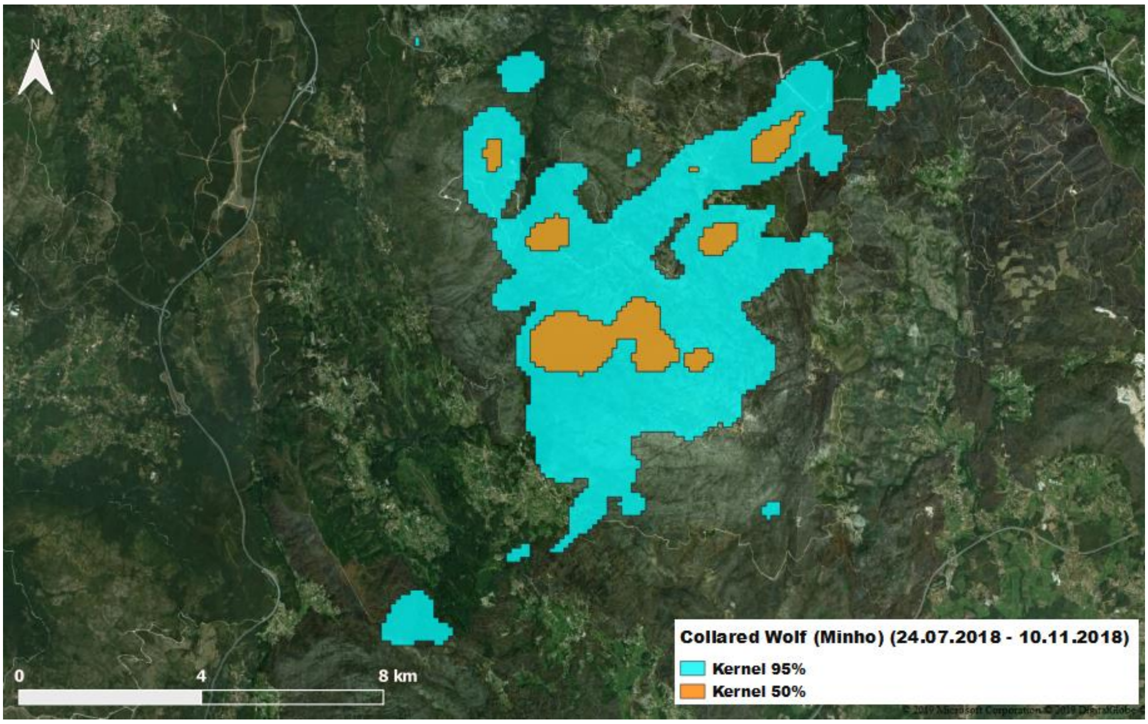


**Figure 27.** Wolf range (blue) and wolf core-areas (orange) in Serra d'Arga, estimated by Kernel Density Estimation with 95% and 50% of the GPS positions of the collared wolf, respectively, obtained between 24th of july and 10th of november of 2018.

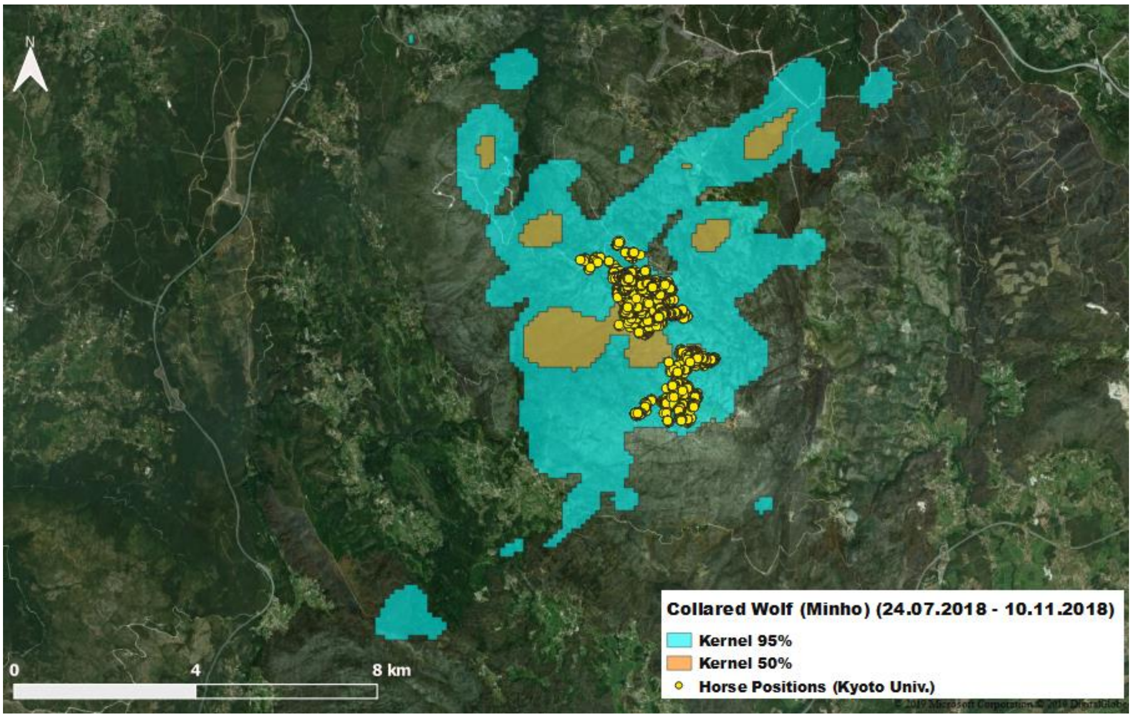


**Figure 28.** Wolf range (blue) and wolf core-areas (orange) in Serra d'Arga, estimated by Kernel Density Estimation with 95% and 50% of the GPS positions of the collared wolf, respectively, obtained between 24$^{th}$ of july and 10$^{th}$ of november of 2018, in relation to the diurnal positions of the horse herds (yellow), provided by Kyoto University.

The overlap between the diurnal locations of horse herds in Serra d'Arga plateau (provided by Kyoto University) and the areas with higher predation risk by wolves, revealed that the area occupied by horse herds is located mostly within wolf range (75%), but slightly overlapping one of the largest core areas of the wolf pack, where 25% of herd locations are included (Figure 28).

However, by overlapping the locations of the predation clusters found in the field between august and november of 2018, with the estimated areas of higher risk of predation, it shows that most (84%) of the clusters fall within the estimated home range of the wolf pack while few clusters (16%) are located inside the wolf core areas scattered throughout the plateau (Figure 29). Moreover, the dead horses found in clusters that indeed overlap with wolf core areas are found in the northern section of the plateau, far from the diurnal locations of sampled horse herds and corresponding to smaller wolf core areas, possibly resting sites far from the main core area, where the pack breeding site is located. These dead horses found in the northeastern section of the plateau may correspond to other individuals or herds than the ones monitored by the Kyoto University team and, therefore, not considered in the study, or may result from wide nocturnal or seasonal movements of the same horse herds that were sampled in the central plateau by Kyoto team.

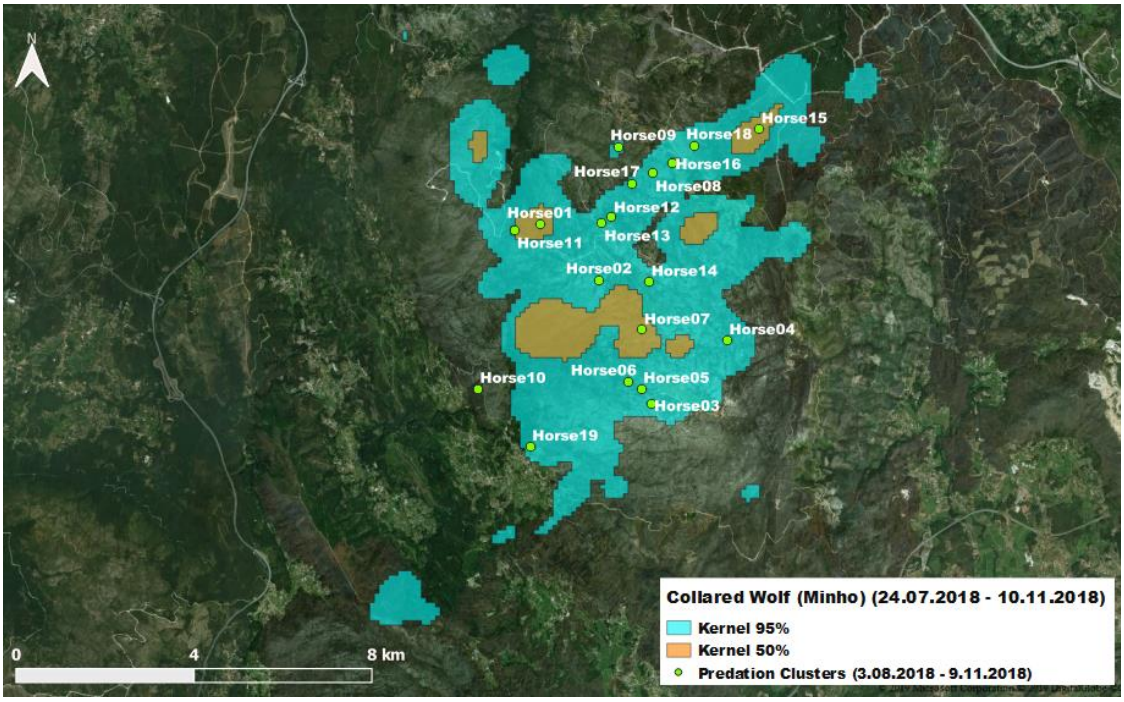


**Figure 29.** Wolf range (blue) and wolf core-areas (orange) in Serra d'Arga, estimated by Kernel Density Estimation with 95% and 50% of the GPS positions of the collared wolf, respectively, obtained between 24th of july and 10th of november of 2018, in relation to the locations of the predation clusters (green) found in the field by GPS telemetry of the collared wolf.

## Genetic Identification of horses consumed by wolves in relation to horses sampled by Kyoto University

The initial dataset comprised 198 genotypes of free-ranging horses, including 46 genotypes obtained from horse remains found in wolf scats, 16 genotypes obtained from horse carcasses found in predation clusters and 136 genotypes obtained from scat samples collected in the field by Kyoto University. The 46 horse genotypes obtained from remains in wolf scats, resulted in 31 different individuals, including 17 females, 10 males and 4 individuals with unknown sex. From the horse samples collected in predation clusters was possible to identify 15 different individuals, including 9 females and 6 males, of which 6 individuals are the same than the ones identified in wolf scats. Finally, after eliminating samples corresponding to the same individuals, we combined our dataset of 28 genotypes (18 genotypes, from scats and clusters, were eliminated because they were also represented in the Kyoto University database) with the dataset with 136 genotypes provided by Kyoto University, obtaining a final dataset 164 individuals. We estimated allelic dropout and false allele rates for all 11 loci, obtaining average values of 0.136 (minimum 0.056 and maximum 0.235) and 0.007 (minimum 0.000 and maximum 0.024), respectively. Furthermore, we also estimated individual heterozygosity in order to assess the genetic diversity of the population, obtaining an average individual heterozygosity of 0.720, which is considerably high for such a small population. The 46 individuals (26 females, 16 males and 4 unknown sex), identified either in wolf scat remains and/or in predation clusters, were individuals consumed by wolves and represent 28% of the total horse database (n = 164). After comparing the number of individuals identified in 2018 (22 females and 14 males) with the total number of females (n = 102) and males (n = 46) present in Arga during that year, estimated by Kyoto University, males seem to be more predated than females (30.4% and 21.5%, respectively). Despite being a small difference, predation is apparently biased towards males, who seem to be positively selected in relation to their availability in the area. Due to the lower sample size of wolf scats and absence of predation clusters in 2017, we did not compare the number of individuals predated (4 females and 2 males, found in wolf scats) with the population size (114 females and 47 males).

As the wolf scats were previously analyzed for individual identification of the wolf, it was also possible to identify how many and which wolves consumed each horse (Figure 30). We observed that, on average, 2 to 3 wolves consumed each horse, reaching a maximum of 4 wolves eating the same horse (male Taiji). Moreover, 2 wolves – LAM211 and LAM214 – were the only consuming 2 different horses each in our sample (wolf LAM 211 consumed horse males Taiji and EqW013 while wolf LAM214 consumed horse females Shima and Oyama_18, a foal born in 2018). There were also 3 wolf scats containing each remains of different horses (horse male Taiji and females Oyama_18 and Shima), however, it was not possible to identify the wolf who consumed those 3 horses and, therefore, it was not possible to assess if it was a single wolf or different wolves who consumed these horses.

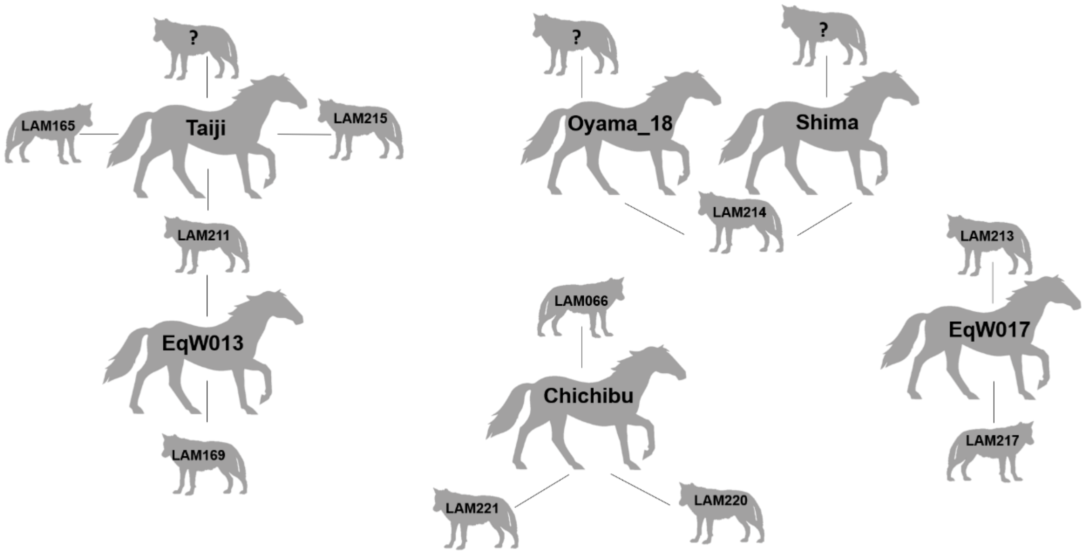


**Figure 30** Individual identification of horses from wolf scats as well as individual identification of wolves who consumed horses. Wolves marked as "?" were not individually identified, therefore, it can be a single individual or multiple individuals.

From the 31 horses that were identified in the 46 wolf scats analyzed, 20 were found in only 1 scat, 8 horses each appeared in 2 different scats, 2 horses each appeared in 3 different scats and 1 horse was found in 5 wolf scats. Furthermore, since all wolf scats were georeferenced when collected, it was possible to estimate the distance between the scats containing remains of the same horse as well as the distance between wolf scats and the corresponding horse found in a predation clusters (when the same horse was found both in predation clusters and wolf scats). The distance between wolf scats containing the same horse ranged between 201 and 2,065 meters, averaging on, 1,122 meters (Figure 31). On the other hand, the distance between wolf scats containing a horse found in predation clusters and the respective predation cluster ranged between 289 and 3,108 meters, averaging a distance of 2,162 meters. There were also 2 predation clusters with remains of the same horse (EqW016), separated by 182 meters, while the wolf scats with remains of that horse were found over 3 km away from those sites. The 2 clusters could be a result of the wolf pack dragging the carcass when feeding, which would explain the proximity between the two kill sites.

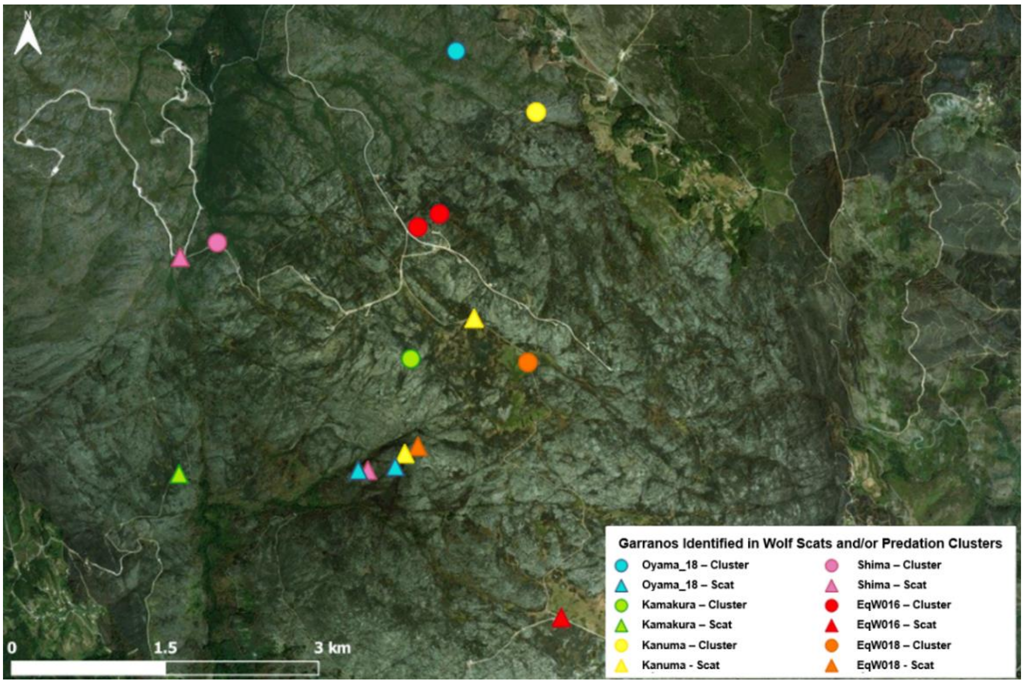


**Figure 31**. Location of horses identified from remains in wolf scats and/or predation clusters. All circles represent individual horses identified from predation clusters and all triangles represent individual horses identified from wolf scats.

The final dataset used to construct the pedigree comprised 164 genotypes, attributed to 96 females, 63 males and 5 individuals with undetermined sex. COLONY established relationships between 77 individuals, comprising 44 females, 32 males and 1 individual with unknown sex, which corresponds to 47% of the total number of individuals in the database. These relationships are grouped in 14 genealogies representing 14 families of free-ranging horses in Serra d'Arga (Figure 32).

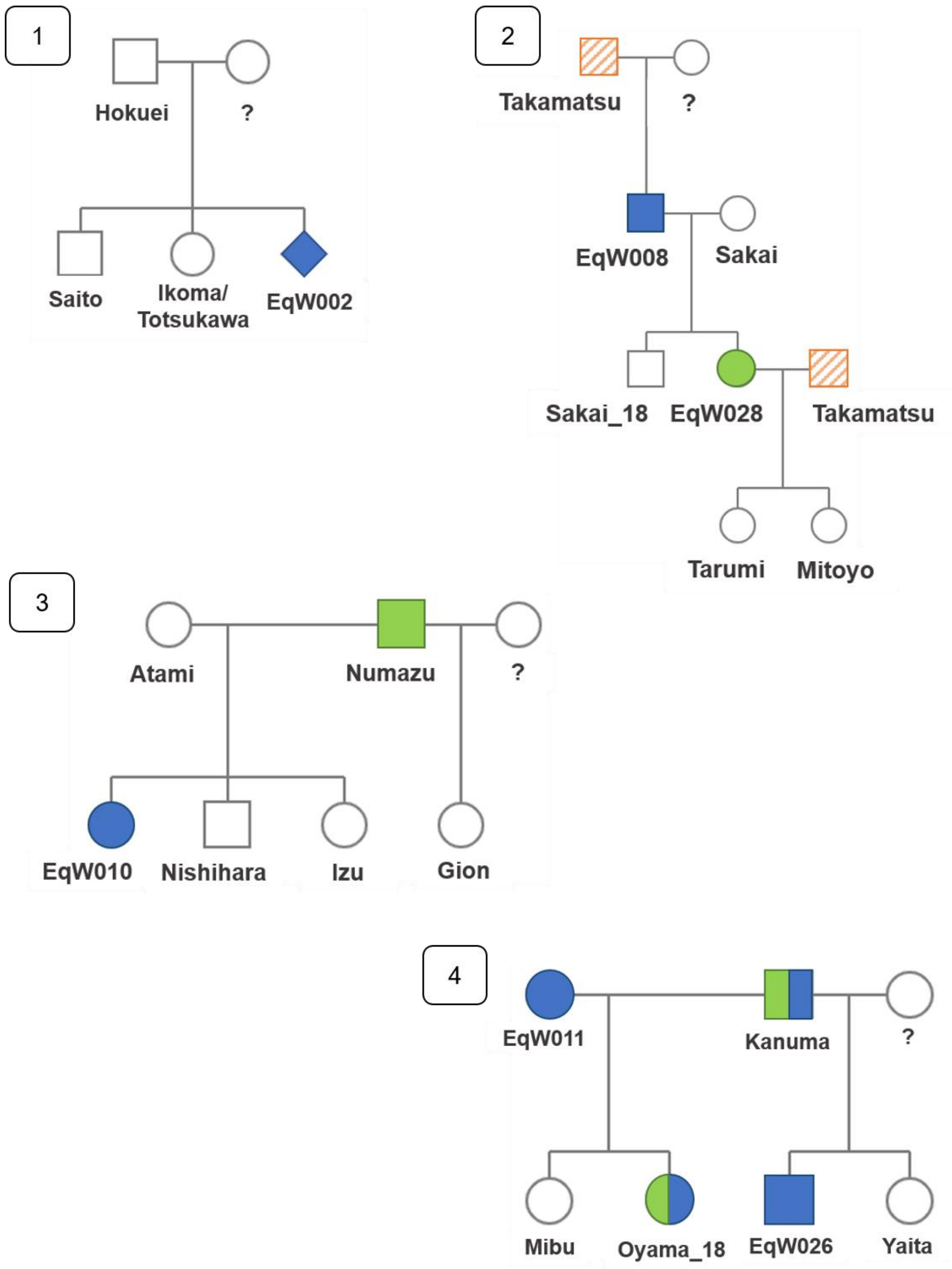


**Figure 32.** Pedigree of 14 horse families constructed with the genotypes obtained from horse remains found in wolf scats and samples collected from predation clusters (EqWXXX) together with faecal samples collected by Kyoto University (Name or EqNIXXX). Blue individuals were obtained from wolf scats, green individuals were obtained from predation clusters and blue/ green individuals were found in both wolf scats and predation clusters. The horse male Takamatsu (in orange) was found to be the breeding male twice in the same genealogy.

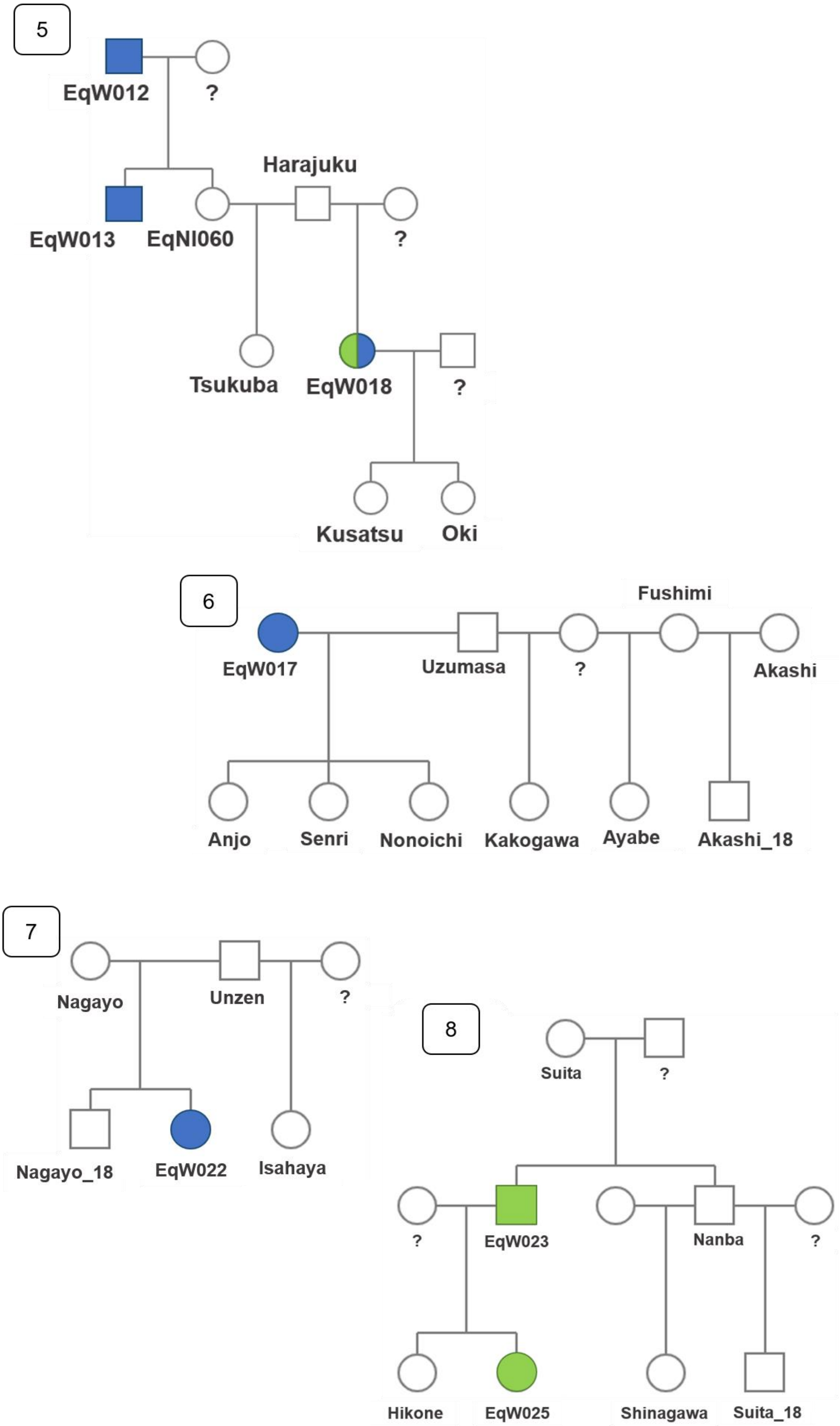


**Figure 32.** (Cont.)

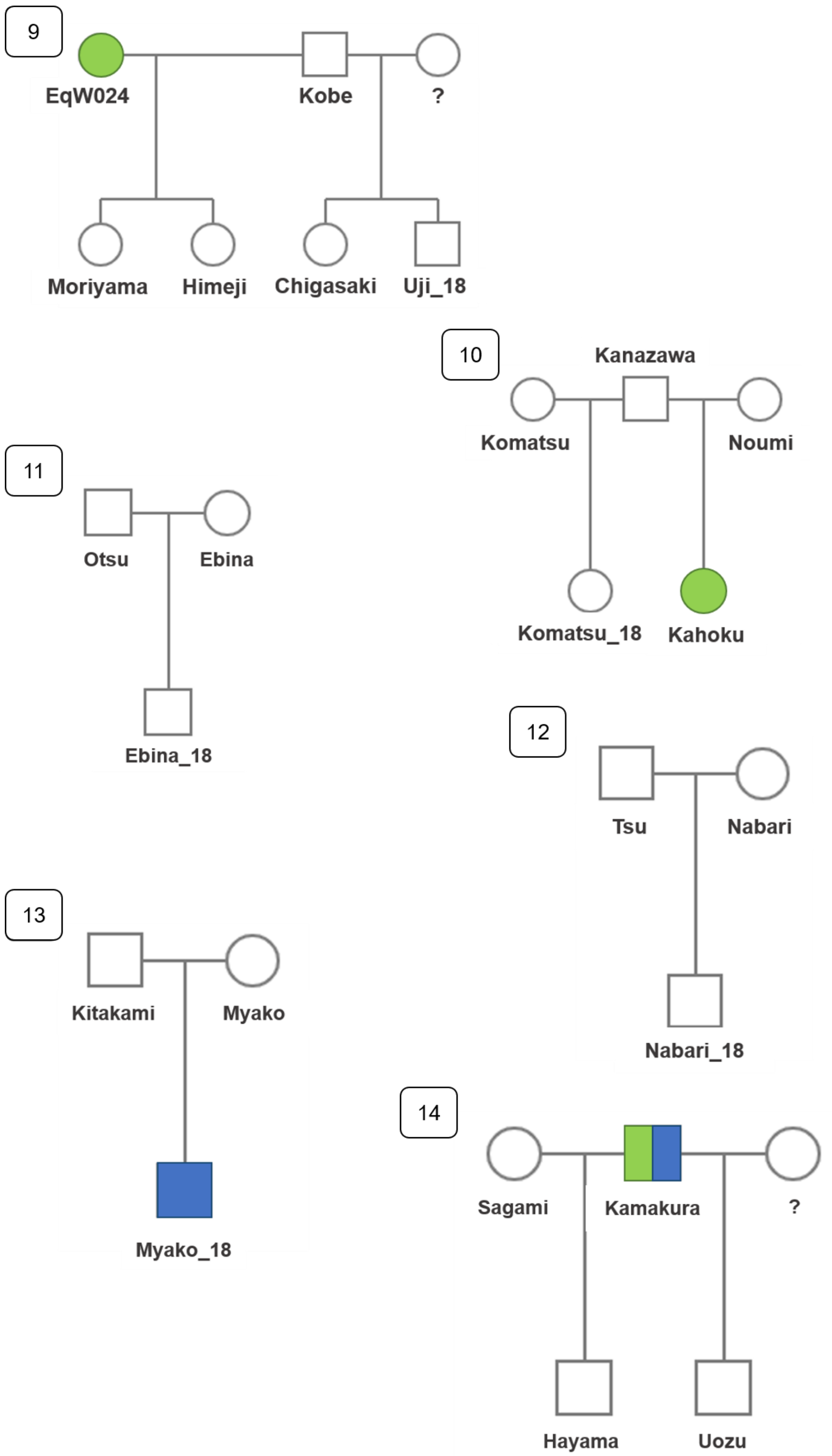


**Figure 32.** (Cont.)

We observed 9 horse families out of 14 with a single male and multiple females, which can be used as a proxy for a herd. For the remnant 5 families, each male bred with a single female. Breeding pairs have in average 1.6 offspring. One male (Takamatsu) belonging to family 2 (Figure 32) has reproduced twice, the first with the progenitor female of the family (not present in the dataset) producing a male offspring (EqW008), and later with his granddaughter EqW028, producing 2 daughters (Tarumi and Mitoyo). This represents the only inbreeding case that we could detect among the analyzed samples.

Twenty individual horses included in the family pedigrees were consumed by wolves, corresponding to 10 females (~22%), 9 males (~ 29%) and one individual with undetermined sex, composing approximately 26% of the individuals in the pedigree. Considering the putative age class of the 20 individuals consumed by wolves, 11 (55%) were breeding adults (parents with at least one detected offspring) while 9 (45%) were non-breeding juveniles (offspring with no detected reproduction), suggesting a proportion slightly biased towards adults. Among all families with individuals consumed by wolves, only one male (Kanuma) lost 2 foals (Family 4 in Figure 32), while 10 males (Hokuei; Takamatsu; EqW008; Numazu; EqW012; Harajuku; Unzen; EqW023; Kanazawa; Kitakami) and 7 females (Sakai; Atami; EqW011; Nagayo; Suita; Noumi; Myako ) lost only one foal each (Figure 32). From these 18 individuals who lost foals, the males EqW008, Numazu, Kanuma, EqW012 and EqW023 and the female EqW011 were killed by wolves. On average, wolf kills represent 25% of the individuals in a horse family and only two horse families were apparently not affected by wolf predation during the studied period (Family 11 and 12 in Figure 32).

When comparing the obtained horse family pedigrees with the observational data provided by Kyoto University during 2017 and 2018, it was possible to obtain by non-invasive genetics a total of 8 mother/foal relationships: Family 2 – Sakai/Sakai_18; Family 3 – Atami/Izu; Family 6 – Akashi/Akashi_18; Family 7 – Nagayo/Nagayo_18; Family 10 – Komatsu/Komatsu_18; Family 11 – Ebina/Ebina_18; Family 12 – Nabari/Nabari_18 and Family 13 – Myako/Myako_18 (Figure 32). Furthermore, horse families 7 and 10 seem to match 2 herds observed by Kyoto team, despite not including all the observed individuals in the field, as Family 7 has 4 of the 5 individuals identified in the field, while Family 10 has all individuals observed in the field by Kyoto team as part of this herd. We also compared the individuals consumed by wolves with the genotype data provided by Kyoto University and it was possible to identify 6 of the 20 individuals predated (Numazu; Kanuma; Oyama_18; Kahoku; Myako_18; Kamakura). We could not find a pattern within the pedigree in terms of which families suffered higher predation, however, it needs to be taken into consideration that this was an experimental approach not yet optimized.

## 3.4. Discussion

In this chapter, the multi-level approach resorting to different methodological approaches provided valuable and innovative insights on horse-wolf interactions at an ecological and behavioral point of view. The analysis of wolf diet revealed that horses compose more than 80% of the consumed biomass in a local wolf pack in consecutive years, showing a strong positive selection by wolves in contrary to all other prey species, including both livestock and wild ungulates. Although the positive selection towards free-ranging horses was already described in Portuguese wolf packs, the relevance of this prey species in wolf diet from Serra d'Arga is much higher than the one reported in previous diet studies from Portugal (Vos 200; Álvares 2011; Casimiro 2017). Despite roe deer being a preferred prey in most studies focusing wolf diet in Europe (see e.g. Mattioli *et al.* 2011; Stoynov *et al.* 2014; Meriggi *et al.* 2015; Lagos and Bárcena 2018), the negative selection towards roe deer reported in Serra d'Arga can be a result of the low density of this wild ungulate (which only recently has been recolonizing the study area) together with the high availability of free-ranging horses, as it has been considered a determining factor in prey selection (Lagos and Bárcena 2018). However, considering that the density of roe deer in our study was estimated from values obtained almost 20 years ago in a neighboring region (Peneda-Gerês National Park), obtained values for Ivlev's Selectivity Index most likely do not correspond to the true abundance of this species in Arga, even if its abundance in the area is low. On the other hand, wild boar densities in Serra d'Arga seem to be considerably high (10 individuals/$km^2$) (Terras de Sicó 2019) and possess the optimal body size for a wolf prey (Mori *et al.* 2017), however, the presence of boar in wolf diet is very low. Wild boar consumption doesn't seem to be related to its abundance in the area (Nores *et al.* 2008), which could be attributed to the effective anti-predator strategies of this species or the higher abundance of easier prey such as livestock, particularly free-ranging horses. In fact, the high relevance of livestock species as prey for wolves in our study area may be related to the fact that livestock has lost anti-predator defenses during the domestication process and constitute easier and more available prey, likely being preferred in relation to wild prey (Mattioli *et al.* 2011). Considering the high density of free-ranging horses in Serra d'Arga, it is probable that the surplus killing that was observed is related to its abundance (Graham *et al.* 2005) and the lack of sharp anti predatory behaviors against wolves due to the recent recolonization of this predator in the study area (Primavera *et al.*, 2015), factors that may expose horses more vulnerable to wolf predation than the remaining domestic species. Finally, it is important to note that our results on wolf trophic ecology may be affected by the difference between the number of wolf scats collected in both years, as the scats from 2017 were collected during the yearly wolf population monitoring, while the scats from 2018 were also collected during the monitoring program but more intensively for this study. This different sample size of wolf scats used for diet analyses between both years might have influenced the detection of occasional prey or their observed values in Frequency of Occurrence and Consumed Biomass, however, it will not influence the high relevance of free-ranging horses on wolf diet that was detected in Serra d'Arga. Moreover, the accurate assessment of prey selection in our study area may be influenced by the lack of updated data regarding local abundance of livestock and wild ungulate species. The used data is outdated or referring to other spatial contexts, especially regarding wild ungulates such as roe deer and wild boar, which may influence our results. In order to produce robust results regarding prey selection, reliable data sources are absolutely needed, which emphasizes the strong need for regular and updated census of both wild ungulates and livestock species.

Cluster analysis based on the GPS positions obtained from a single collared wolf were essential for the quantification of wolf predation rates in the study area, providing the first available data on this topic for Portugal despite the short time period of monitoring. All feeding sites involved horses, in which the majority (n=9) were adult individuals while only 2 had remains of foals. These results may be related to the fact that foals are consumed too quickly and thoroughly to leave remains, which decreases the probability of finding remains of small prey in the field as already reported in other studies based on GPS telemetry (Sand *et al.* 2005; Palacios and Mech 2011). Accordingly, despite not being possible to assess the age of 8 carcasses, we can assume that horses were more than 1 year old. Furthermore, the majority of foals in the study area had already disappeared before the monitoring period of the collared wolf (august – november), most likely as a result of wolf predation, which probably contributed to the high number of juveniles and adults found at feeding sites. There is also the possibility that some sites categorized as unknown are actually feeding or kill sites, in which small-sized prey (possibly foals) was consumed thoroughly and no remains were found. The same could have happened with resting sites where no wolf beds were found. Predation by wolves and other carnivores, such as mountain lions (*Puma concolor*) on adult horses is not commonly reported, since predators usually only target foals and juveniles (Turner *et al.* 1992; Turner and Morrison 2001; van Duyne *et al.* 2009; Lagos 2013). However, the high mortality on adult horses found in Serra d'Arga could be related to lower availability of alternative prey species such as livestock, which are confined at night and well-guarded by dogs during daytime, together with wild prey given its low diversity and abundance existing in the study area. Thus, since horses are the most available prey in Arga, after all the foals were consumed, wolves seemed to target juveniles and adults, representing a unique context worldwide.

The predation rate estimated from the clusters analyses demonstrate the strong pressure from wolves that the *Garrano* population in Serra d'Arga are facing, losing approximately 41% of the individuals each year. The obtained kill rates of 6 horses/wolf/year in Serra d'Arga are much higher than the ones obtained in other areas with high horse depredation, such as Vez/Soajo and Pitões, with respectively 3.7 horses/wolf/year and 1.6 horses/wolf/year based on declared wolf attacks (Álvares 2011). Furthermore, the kill rates and predation rates in this study were estimated from the locations obtained only during the first 10 days of every month between august and november, possibly underestimating the obtained values. Wolf predation in Serra d'Arga is expected to be high as horse herds occur within areas of high predation risk, largely overlapping with the home range of Arga pack (estimated with Kernel 95%) and with one of the largest wolf core areas (estimated with Kernel 50%).

Using the 19 dead horses found in feeding clusters and the wolf scats collected during the same period of the clusters, it was possible to infer the trophic strategy of the wolf pack and determine if the horses consumed by wolves were a result of predation or scavenging. Between 24 of july and 10 of november of 2018 were collected 35 wolf scats from which were genetically identified 19 different horses. As the number of horses found in feeding clusters is the same as the number of horses identified in the wolf scats collected during the same period, it suggests that the wolves are indeed preying on horses and not scavenging. Despite the number of individuals identified from the wolf scats and the number of individuals found in feeding clusters being the same, only 6 horses were simultaneously identified in both scats and predation clusters. Such result could be attributed to horses consumed outside the time-frame of the clusters and, therefore, were not sampled. Moreover, it was only possible to obtain 16 genotypes from the 19 clusters, which also influences the mismatch between the number of horses found and number of individuals identified. Genotyping errors due to low DNA quality can also be possible, although a rigorous procedure of replicates was implemented to decrease the possibility of consensus errors. Lastly, this mismatch could be related to the fact that some of the horses found in these cluster probably belonged to other herds that weren´t sampled as they were detected in the northeastern part of Serra d'Arga, relatively far from the central plateau where horse herds were monitored by Kyoto University. Nevertheless, it is safe to assume that, despite this mismatch, wolves are in fact predating the horses and not scavenging. Additionally, considering such high predation, the fact that owners are obliged by law to dispose the carcasses of dead animals only contributes to a continuous decline of the population by not allowing wolves to scavenge on individuals that have died from disease, accident or natural causes and, consequently, increasing depredation (López-Bao *et al.* 2013; Lagos and Bárcena 2015).

To our knowledge, this is the first study that used consumed remains collected from wolf scats to individually identify the prey by molecular analyses. We could achieve the genetic identification of 46 horse remains from the initial 122 remains that were selected for this analysis, meaning a success of approximately 40%. Several factors might have contributed to this result, such as the low-quality DNA present in the horse remains and the presence of PCR inhibitors and/or contaminations in the wolf scats. It has to be taken into account that the prey was consumed and, therefore, all tissues went through the digestive system of the predator, likely compromising the quality and quantity of the DNA. Nonetheless, error rate estimates were low (mean allele dropout rate 13.6% and mean false allele rate of 0.7%), and comparable to error rates observed for other sources of non-invasive DNA such as scat samples (Godinho *et al.* 2015; Nakamura *et al.* 2017). The fact that genetic individual identification of the prey consumed by carnivores has not been attempted before, naturally creates difficulties in terms of optimization of the protocols and correct processing of the samples. Nonetheless, this is a remarkable result of this work that can be applied further on horses and on other prey species that leave indigestible remains in predator scats.

In terms of the genetic diversity of the 164 horses identified in Serra d'Arga, individual heterozygosity was high, similarly to what was already reported by Morais *et al.* (2005) for other *Garrano* populations. Despite our study area harboring a small, isolated population of horses, it is known that owners frequently introduce horses from other populations or even breed the *Garranos* with other horse breeds to produce horses with more suitable characteristics (Morais *et al.* 2005), becoming expected an increased genetic diversity (Gomes 1996). In fact, as small and isolated populations are often inbred and with high levels of homozygosity, the introduction of new genes from the new breeding individuals is most likely the cause of such high heterozygosity (Gomes 1996; Morais *et al.* 2005). However, domestic horses used in these introduction actions are often not adapted to the mountain and may have never contacted with predators, being more susceptible to harsh environmental conditions and lower availability of food, as well as extremely vulnerable to predation. Accordingly, the introduction of poorly adapted individuals in the *Garrano* population may lead to a reduction of fitness (Gomes 1996). Furthermore, the genetic analysis showed that males were more susceptible to predation than females as, from the 77 individuals included in the genealogies, 10 females and 9 males were killed by wolves, which represents approximately 22% predation in females and 30% predation in males. These results can be expected since males protect the herds against predators and competitor males, being more prone to injury and, consequently, higher mortality risk by wolves (Garrott and Taylor 1990; Garrott 1991). Furthermore, males are also more prone to disperse from herds, becoming solitary or grouped in small herds of bachelors (Garrott 1991), which can also become more vulnerable to wolf predation.

Predation is usually studied with GPS telemetry by analyzing feeding clusters, in order to assess kill rates and predation rates (e.g. Anderson and Lindzey 2003; Sand *et al.* 2005; Palacios and Mech 2011). However, capturing and collaring predators not only is an extremely invasive process but also requires an enormous effort due to their elusive behavior (Bangs *et al.* 2006). In this context, the innovative approach developed in our study comprising the genetic identification of consumed individuals in the scats of a predator, provides a high potential for predation to be studied in a less invasive, time consuming and expensive way. Furthermore, collared animals usually have short life spans as data is collected only during few months due to mortality events or battery failure, as demonstrated by the short monitoring period of the wolf collared in our study which encompassed only 4 months, approximately. In contrary, a genetic approach based on scat analysis can be performed during longer periods and resorting to much larger sample sizes. Furthermore, and as demonstrated in this study, the genetic analyses of wolf scats allow the individual identification of both prey and predator, providing a huge potential to not only assess predation, but also study the carnivore population dynamics and behavioral traits. In fact, this study was successful in identifying genetically which and how many wolves consumed a single prey carcass, as well as determining the distances between the kill site and the scats containing remains of the consumed prey. Such analysis creates new opportunities for the study of predator-prey relationships as well as provide important insights on wolf social behavior, such as estimation of pack size and pack cohesion.

Our genetic approach also allowed valuable insights on the social structure of free-ranging horses. Despite the genealogies only allowed the identification of 47% of the individuals detected by field surveys, it was possible to reconstruct 14 horse families. Among the detected genealogies, family 4 (n =7 individuals) reported the highest mortality due to wolves with approximately 67%, while the families 11 and 12 (n = 4; n= 3, respectively) reported no mortality events. However, in comparison with other families with the same number of individuals, mortality values were quite different. Two other families also with 7 individuals showed a mortality of 33% (Family 3) and 16.7% (Family 9). Likewise, for family 13 (n= 3 individuals), mortality reached 33%. Such disparity could be attributed to the constitution of the groups (e.g. number of females and foals), weakness and/or inexperience of the male. In fact, in the families 3 and 4, the male was killed by wolves, while family 9 only lost one foal, which could support the hypothesis of a weak and/or inexperienced male. Regarding the age of the predated individuals, predation seemed biased towards breeding adults (55% predation in adults and 45% predation in juveniles). However, considering that most of the foals present in Arga during 2017 and 2018 were not sampled, as wolves consumed them too quickly and thoroughly to leave any remains, these values are most likely biased. Furthermore, we detected by genetics 14 different families in an area of 53.31 km2, which is almost half of the families identified in 2016 based on observational data by Ringhofer *et al.* (2017) in, approximately, the same area. Considering less than half of the individuals of the dataset were included in the genealogy, it is expected that the number of herds observed in Serra d'Arga by the Kyoto University is considerably higher than the number of families obtained in the genealogies.

Overall, we could not observe clear factors determining wolf predation risk on horse herds. Nevertheless, considering all the analyses above, it is possible to conclude males seem to be more predated than females. Males are extremely important since they are responsible for defending the herds against predators and other competitor males. If the male is not experienced, not only is incapable of protecting the herd from predators, due to absence of effective anti-predator behavior, but also less likely to reproduce successfully (Serôdio 1992). For this reason, owners should be advised to not remove the resident males from the herds and to not introduce unexperienced males (Serôdio 1992). Introduced females are also at disadvantage due to the adverse environmental conditions and lower food availability that might affect their reproductive success, however, the impact of an unexperienced female in a herd seems unlikely as damaging as the presence of an unexperienced male.

# 4. General Conclusions

This work was developed under a collaboration between CIBIO-InBIO and several institutions such as ACERG and Kyoto University, in order to assess socioeconomic conflicts related to wolf predation in *Garrano* horses in Alto Minho region and to study wolf-horse interactions in Serra d'Arga, respectively. This approach has made possible to understand in more depth the role of wolf predation on the endangered population of free-ranging *Garrano* horses. The study of wolf predation on free-ranging horses is only possible in the few regions worldwide where both species are sympatric. In this context, northwestern Portugal is revealed as an excellent case study which combined with the developed procedures based on non-invasive genetic analysis and GPS telemetry have created interesting research opportunities. In particular, our innovative approach focusing on the genetic identification of the consumed individual prey as well as the individual predator who consumed them based on scats allows the study of predation in a less invasive way and during longer periods, which has not been done before. Similarly to what has been reported in studies from northern Spain and Central Asia (Llaneza *et al.* 1996; Hovens and Tungalaktuja 2005; van Duyne *et al.* 2009; Llaneza and López-bao 2015; Lagos and Bárcena 2018), horses in Serra d'Arga are positively selected in relation to other livestock and wild ungulate species, but apparently it is having a strong population impact on horse populations. Despite the data supporting this premise, some topics need to be taken into consideration to produce new and reliable results, including i) annual wolf population census and extensive studies on wolf telemetry to allow an accurate quantification of predation rates; ii) regular census of the wild ungulate species and livestock species, including free-ranging horses, to accurately estimate prey selection by wolves; iii) adequate and updated data on wolf damages to quantify the socioeconomic impact of horse depredation; iv) proper sanitary studies on free-ranging horses to assess the compensatory effect of wolf predation; and v) the development of further studies on horse-wolf interactions in other areas with different horse abundances, wolf predation risk and habitat characteristics.

Based on the results provided by this study and other previous research on wolf-horse interactions, a proper management of the free-ranging horse herds in Alto Minho, and particularly in Serra d'Arga, should consider some effective measures to reduce horse depredation, such as:

i) regular health evaluations of *Garranos* ranging freely in the mountains should be mandatory, as a measure to reduce mortality and increase productivity (Gomes 1996; Álvares 2011);

ii) horse carcasses should be allowed to remain in mountain areas where wolves could scavenge them, contributing to a reduction of predation on healthy animals (Álvares 2011; Lagos and Bárcena 2015). Previous EU sanitary regulation (1774/2002) oblige owners to remove the carcasses of dead livestock, however, this law has been reviewed. The current EU sanitary regulation (1069/2009) now considers the possibility of leaving carcasses in open areas to feed endangered and protected wildlife in specified remote regions, which include the municipalities in our study area. This procedure should be encouraged and promoted by national authorities in order to benefit the horse owners and the wolf population.

iii) inform and teach horse owners regarding the correct management of *Garrano* herds related to the removal of individuals (Álvares 2011). Often, groups of males (bachelor groups) are removed from the mountains, which reduces the number of groups formed and causes females to roam alone when dispersing in search of a new group. Moreover, inexperienced males that had been living confined are occasionally introduced in these groups, having never contacted with wolves, thus, being unable of protecting the herd or maintaining its cohesion. Therefore, a correct management of the herds should help to prevent the disruption of its social structure and reduce wolf predation;

iv) horse herds should be effectively protected against the risk of wolf attacks, by testing innovative damage prevention measures. Although livestock guarding dogs (LGDs) are considered to be not suitable to protect free-ranging horses against wolves as they cannot accompany the herd (Woodroffe *et al.* 2005), these LGDs can be habituated as juveniles to confined horse females that then are released together with the free-ranging herds. However, as owners rarely interact with free-ranging horses and LGDs have special food requirements, these habituated dogs should be fed regularly in order to avoid abandoning the horse herd. Other alternative successful measure that has been explored for damage prevention in countries such as the United States is the use of donkeys, mules, alpacas or llamas (Walton and Feild 1989). These large herbivores are naturally aggressive towards canids, have strong anti-predator defenses and a strong herding instinct, being quite successful at chasing away the predator (Walton and Feild 1989; Wilbanks 1995). In Portugal, the introduction of mules in herds of free-ranging horses could be an effective measure against wolves since they are normally sterile and will remain with the herd without being aggressive towards the other individuals. In fact, the use of mules to protect *Garrano* herds from wolf attacks have already been used in Peneda-Gerês region by some horse owners (F. Álvares, *pers. com.*) Donkeys could be a problematic solution because they could crossbreed with *Garranos* and, besides, males are particularly aggressive towards all animals if not neutered before being introduced in a herd, and females will become aggressive if they have a foal (Wilbanks 1995).

v) ensure foal survival by assuring their confinement during the first months of age. Several studies report foals as particularly vulnerable to wolf predation during their first months of life (Gomes 1996; Sampaio 1998; Álvares 2011; Lagos 2013; Lagos and Bárcena 2018; this study). Some *Garrano* owners in Alto Minho already have the practice of bringing pregnant females to fenced pastures to give birth and to be kept there until foals are old enough to defend themselves from wolves (ACERG 2000). This practice – named “recria” – ensures the survival of a larger number of foals and the preservation of the *Garrano* breed. Thus, if “recria” becomes a common practice among the remaining horse owners, especially in areas with high wolf predation risk, the number of wolf attacks on horses would most likely be greatly reduced.

vi) an important measure to minimize the conflict between wolves and horse owners, would be changing the current economic compensation system for wolf damages on free-ranging horses. The current system is flawed and inefficient, failing to cover the costs of wolf predation on free-ranging horses and leaving owners without appropriate economic aid. By creating a compensation scheme similar to the one currently used in Swedish reindeer husbandry area, where the payments are issued according to the detection of reproduction in predators in order to offset any reindeer losses (Zabel and Uller 2008; Persson *et al.* 2015), would become possible to ensure that horse owners would obtain economic support to maintain their free-ranging husbandry practice, while protecting the Iberian wolf and minimizing illegal persecution.

The optimization or implementation of all these measures described above, would allow an expectable decrease in wolf predation on free-ranging horses, forcing wolves to find alternative prey, such as wild ungulates. In fact, the decreasing availability of livestock due to the implementation of efficient damage prevention measures could trigger a shift in the diet of wolves, which could translate in an increase of predation on wild boar, one of the most abundant available prey. If so, wolves would control wild boar's density in the area while maintaining a sustainable diet, certainly depending on a much lesser extent of human resources such as livestock. Nonetheless, the reintroduction of other wild ungulate species, such as red deer or fallow deer, would benefit both horses and wolf populations by promoting a shift in the wolf diet from domestic to wild prey.

Finally, it is important to point out that most of the bibliographic sources cited in this study regarding the *Garrano* population are written in Portuguese or Spanish and, consequently, do not allow an easy and wide access to information by the international scientific community. This study also compiled information collected from unpublished studies or datasets in Portuguese or Spanish, making them accessible for future research outside Iberian Peninsula. Overall, the obtained results and the new methodological procedures that emerged from this experimental study, allowed a better understanding on the patterns of wolf predation in free-ranging horses in northwestern Portugal and, at a wider context, provided new insights and opportunities to study predator-prey interactions as well as the human-carnivore conflict related to livestock depredation.

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# 6. Supplementary Material

**Table S1** – Studies reporting wolf predation on horses, listed by country, region and bibliographic reference (respective estimated F.O. is represented in the Figures 2 and 4).

| Country | Region | References |
|---|---|---|
| **Portugal** | Peneda-Gerês National Park | Álvares 1995<br>Petrucci-Fonseca 1990<br>Lançós 1998<br>Ferrão da Costa 2000<br>Vos 2000<br>Guerra 2004<br>Casimiro 2017 |
| | Gerês-Xurés International Park | Álvares *et al.* 2000 |
| | Vez/Soajo | Álvares 2011 |
| | Pitões das Junias | Álvares 2011 |
| | Larouco | Roque *et al.* 2001<br>Álvares 2011 |
| | Parque Natural do Alvão | Carreira and Petrucci-Fonseca 2000<br>Carreira 2010<br>Passinha 2018 |
| | Leiranco | Pimenta 1998 |
| | Arada | Quaresma 2002 |
| | Trancoso | Quaresma 2002 |
| **Spain** | Galicia | Lagos 2013<br>Llaneza and López-bao 2015<br>Lagos and Bárcena 2018 |
| | Basque Country | Echegaray *et al.* 2007<br>Echegaray and Vilà 2010 |
| | Northern Spain (West Galicia, Cantabrian Mountains, Douro Meseta) | Cuesta *et al.* 1991<br>Blanco *et al.* 1992 |
| | NW Asturias | Llaneza *et al.* 1996 |
| | SW Asturias | Llaneza *et al.* 1996 |
| | Coruña | Guitián *et al.* 1979 |
| **Italy** | Abruzzo | Fico *et al.* 1993 |
| | Northern Apennines | Ciucci *et al.* 1996<br>Meriggi *et al.* 199<br>Milanesi *et al.* 2011<br>Meriggi *et al.* 2015 |
| | Liguria | Imbert *et al.* 2016 |
| | Pollino National Park | Ciucci *et al.* 2018 |
| **Mongolia** | Hustai National Park | Hovens and Tungalaktuja 2005<br>van Duyne *et al.* 2009 |
| | Bogdkhan Mountain Strictly PA | Nakazawa *et al.* 2008 |
| **Inner Mongolia (China)** | Dalai Lake National Nature Reserve | Zhang *et al.* 2009<br>Huashan *et al.* 2014 |
| | western Daxing'anling Mountains | Wakabayashi *et al.* 2007 |
| | Xinbacrbuvou Banner | Honghai *et al.* 1998 |
| **Poland** | Białowieza Primeval Forest | Jędrzejewski *et al.* 2000 |
| | Carpathian Mountains | Gula 2008 |
| **Turkey** | Kars | Capitani *et al.* 2016 |
| **Nepal** | Bhijer and Dho Valley | Subba 2012 |
| | Annapurna-Manaslu Conservation Area | Chetri *et al.* 2017 |
| **Bhutan** | Wangchuck Centennial National Park (WCNP) | Jamtsho 2017 |
| **India** | Gya-Miru Wildlife Sanctuary (GMWS) | Namgail *et al.* 2007 |
| **Bulgaria** | Rhodope Mountains (West and East) | Genov *et al.* 2008 |
| **United States of America** | Idaho, Montana and Wyoming | Musiani *et al.* 2003 |
| | Montana | Haney *et al.* 2007 |
| **Canada** | Alberta | Musiani *et al.* 2003 |

**Table S2** – Citations (in Portuguese) regarding traditional husbandry practices of *Garrano* horses and their interaction with wolves in northwest Portugal from the following ethnographic studies: Fontes, 1977; Coutinho 1997; Dias 1948; Capela 2005; Sousa and Alves 1997.

| Book | Citation |
|---|---|
| **“Etnografia Transmontana. Volume II: O comunitarismo de Barroso”**<br><br>(Fontes, 1977) | “Cada lavrador tinha um cavalo, ou égua, ou mais. (…) Burros, havia poucos; em terras mais acidentadas abundavam mais.” (Page 101).<br><br>“No Maio, ia a vezeira para a serra, sem pastor.” (Page 102).<br><br>“Na Serra (as éguas) dormiam todas juntas em roda, como as vacas. Com esta diferença: a cabeça para dentro e o rabo para fora. (…) As crias mais novas iam para o meio. Estes animais defendem-se ao couce, por isso ficam com o rabo para fora. Os cavalos inteiros eram os vigias da noite. Não entravam na roda, mas andavam à volta do grupo, cheirando esta e aquela e vigiando o lobo.” (Page 102).<br><br>“Quando as éguas vêem o lobo, cheiram-no e este deita-se na terra fresca, espanando-se logo, cegando assim as éguas, para se poder depois atirar a elas, pela frente, pois por trás não consegue.” (Page 102).<br><br>“Estavam bravas, mordiam, estavam gordas, valentes, e se o laço as prendia, quantas vezes o dono ia de rasto, preso à corda, pelo monte fora.” (Page 102).<br><br>“Uma vez ou outra o lobo tinha sorte, matando uma; outras eram roubadas, tanto vacas como éguas.” (Page 102). |
| **“Mosaicos da Serra D’Arga”**<br><br>(Coutinho 1997) | “Os nevões são frequentes, acontecendo que há anos em que a Serra permanece coberta de geada durante alguns dias e as temperaturas são, nestas ocasiões, muito baixas, fazendo com que o lobo desça aos povoados à procura dos rebanhos, sobretudo de noite.” (Page 11).<br><br>“Daí a grande amplitude térmica nesta região, favorável a alguns tipos de fauna, como o coelho bravo, a perdiz, o javali, o lobo, a raposa, etc… e aos animais domésticos habituais.” (Page 11).<br><br>“Os pastores, em número de dois em Arga de Baixo, acompanham (o gado), podendo ser mulheres, ao contrário de Arga de Cima onde as mulheres, geralmente, não vão para o monte por causa do lobo.” (Page 49).<br><br>“Os animais, especialmente os de grande porte, como a vaca ou o cavalo, são levados para o monte ou soltam-nos pra o pasto perto das povoações e, por esse motivo, muito exposto ao público o que arrasta consigo algumas superstições (…)” (Page 50).<br><br>“Participavam, habitualmente, nas batidas à raposa ou aos lobos, quando essas eram organizadas.” (Page 54).<br><br>“Na Serra d’Arga há de tudo. O valentão do lobo, que desce ao povoado e destrói os rebanhos no pasto; a manhosa raposa, que assalta as capoeiras, o noctívago javali, que destrói as colheitas; a gineta, o gato bravo, o texugo, que entram por qualquer buraco para conquistar a presa.” (Page 57). |

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| **"Vilarinho da Furna. Uma aldeia comunitária"**<br><br>(Dias 1948) | "Os rebanhos vigiados têm o nome de *vezeiras*, porque os pastores são dados, à vez, por cada um dos donos do gado. Mas nem todos são *apastorados*, havendo também rebanhos ao *feirio*. Os rebanhos apastorados são, de longe, os mais importantes, pois ao feirio só ficam praticamente os cavalos e éguas *Garranos*, que pastam tanto na Serra do Gerês, como em vários tratos de terreno galego (…) Estes cavalos vivem em plena liberdade, semi-selvagens, a ponto de terem de ser laçados ou apanhados com artimanhas, pelos próprios donos, quando estes os querem levar à feira. Parte destes cavalos destina-se ao exército, pelas suas qualidades de animal de montanha." (Page 97/98).<br><br>"Na vezeira das vacas há um, ou dois pastores, conforme os currais onde pernoitam, pois existem lugares mais perigosos e difíceis de vigiar." (Page 100).<br><br>"Durante o dia fica sempre um pastor ou dois, a quem cabe preparar a ceia para os que chegam do lugar, pois a entrada ao serviço faz-se ao fim da tarde, de maneira a estarem de noite sempre o dobro dos pastores, para poderem defender os animais de qualquer perigo, sobretudo dos ataques dos lobos." (Page 101).<br><br>"Isto é um rebanho sem pastor, que vive entregue a si próprio durante grandes temporadas, ou todo ao ano. Cândido de Figueiredo, regista *feirio*, como termo do Gerês, significando gado vacum que anda em liberdade na serra à procura de pastos. Vemos, porém, que também serve para designar o gado cavalar em liberdade." (Page 120).<br><br>"As éguas têm as crias na serra, e aí crescem em inteira liberdade. É certo que por vezes algumas são presas dos lobos, embora isto não seja muito frequente, pois o gado cavalar em estado semi-selvagem sabe-se defender das feras. (Page 121). |
| **"As freguesias do concelho de Paredes de Coura nas memórias paroquiais de 1758"**<br><br>(Capela 2005) | "As espécies mais referidas e também as mais ameaçadoras são os *lobos*, os *javalis* (…) Em maior número e por isso também mais ameaçadores são os lobos, relativamente os quais as comunidades tiveram que instituir e promover sistemas defensivos. O memorialista de Parada do Monte (Melgaço) diz que sucede ver-se 6 e 7 juntos e ordinariamente 2 e 3, que "no tempo das neves andam de noite pelas portas dos principais lugares da freguesia e matam muitos cães e comem-nos"; em grandes matilhas ou em pequenos grupos são muito frequentes nestas serranias e fazem entradas nos lugares e freguesias." (Page 631)<br><br>"(…) organizaram mecanismos colectivos de defesa contra os lobos, as montarias e os fojos, e instituíram até prémios a quem trouxesse cabeça ou corpo do animal morto ou quiçá, vivo (…)." (Page 631).<br><br>"E descreve-os cuidadosamente: "E alocando os tais bichos até os meterem dentro de duas altas paredes que junta os dous braços e por fim deles um grande e alto buraco ou fojo colmado com um ténue mato e vai a saltar o lobo e não vencendo o salto dá com o corpo no fojo, aonde com tiros ou chuços o matam (…)." (Page 631). |
| **"Alto Minho: População e economia nos finais de setecentos"**<br><br>(Sousa and Alves 1997) | "Já a criação do gado cavalar era exclusivo dos lavradores, como meio de transporte pessoal, surgindo o concelho de Coura como aquele em que a sua criação era mais numerosa (…)." (Page 78).<br><br>"Tanto a serra do Gerez, como a d'Amarella, se cobrem de neve no Inverno, o que junto á sua elevação, as torna inuteis para uzo dos povos circunvizinhos, que só a ellas lanção seus gados no tempo de Verão, e são-lhes muito prejudiciaes os lobos que nellas se crião (…)" (Page 107).<br><br>"Esta elevada serra d'Arga occupa hum espaço de 5, para 6 legoas quadradas, e a parte superior he tão plana que ali tem tres fregueszias, com o sobrenome da mesma serra: produz muitos pastos, e criação de bestas; e ao mesmo tempo bastantes lobos, que pela contiguidade das serras, se communicão de humas a outras, sendo a principal origem delles na Peneda." (Page 118). |

**Table S3** – Average weights of each prey class detected in wolf diet, used to estimate Consumed Biomass.

| Prey class | | Mean Weight (kg) | References |
|---|---|---|---|
| Wild Ungulates | *Capreolus capreolus* | 24 | Pimenta (1998) |
| | *Sus scrofa* | 67 | Llaneza *et al.* (1996) |
| Domestic Ungulates | *Equus caballus* | 290 | Costa *et al.* (2018) |
| | *Capra hircus* | 30 | SPREGA, 2017 |
| | *Bos taurus* | 400 | SPREGA, 2017 |

**Table S4** – Estimated population size of the main species of domestic and wild ungulates represented on wolf diet in Serra d'Arga (based in information of numbers or abundances from official statistics and published data at the level of parishes included in the study area: see methods section for further details).

| Study Area | Prey Species | Year | Population Size | Source |
|---|---|---|---|---|
| Serra d'Arga ($53.31\ km^2$) | *Capreolus capreolus* | 2003 | 80 | Ferreira, 2003 |
| | *Sus scrofa* | 2017 | 533 | Terras de Sicó |
| | *Equus caballus* | 2017 / 2018 | 201 / 177 | Kyoto University |
| | *Capra hircus* | 2009 | 633 | INE, 2011 |
| | *Bos taurus* | 2009 | 160 | INE, 2011 |

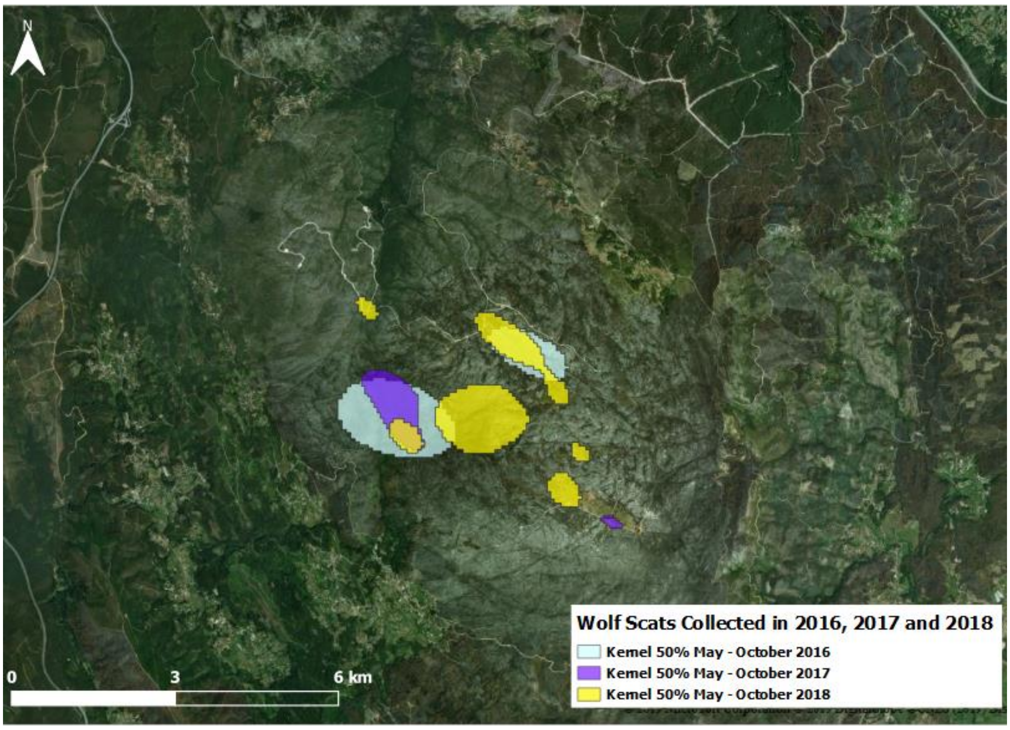


**Figure S1.** Kernel Density Estimation with 50% of the locations of wolf scats collected in Serra d'Arga during wolf breeding season (between may and october of 2016, 2017 and 2018).

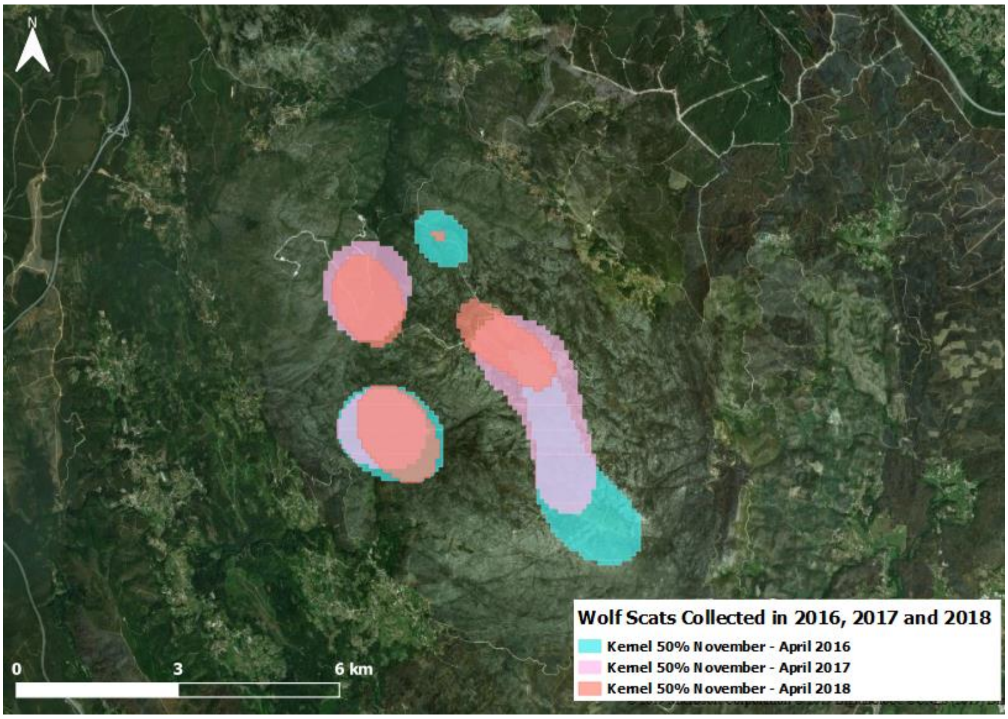


**Figure S2.** Kernel Density Estimation with 50% of the locations of wolf scats collected between in Serra d'Arga outside wolf breeding season (november and april of 2016, 2017 and 2018).

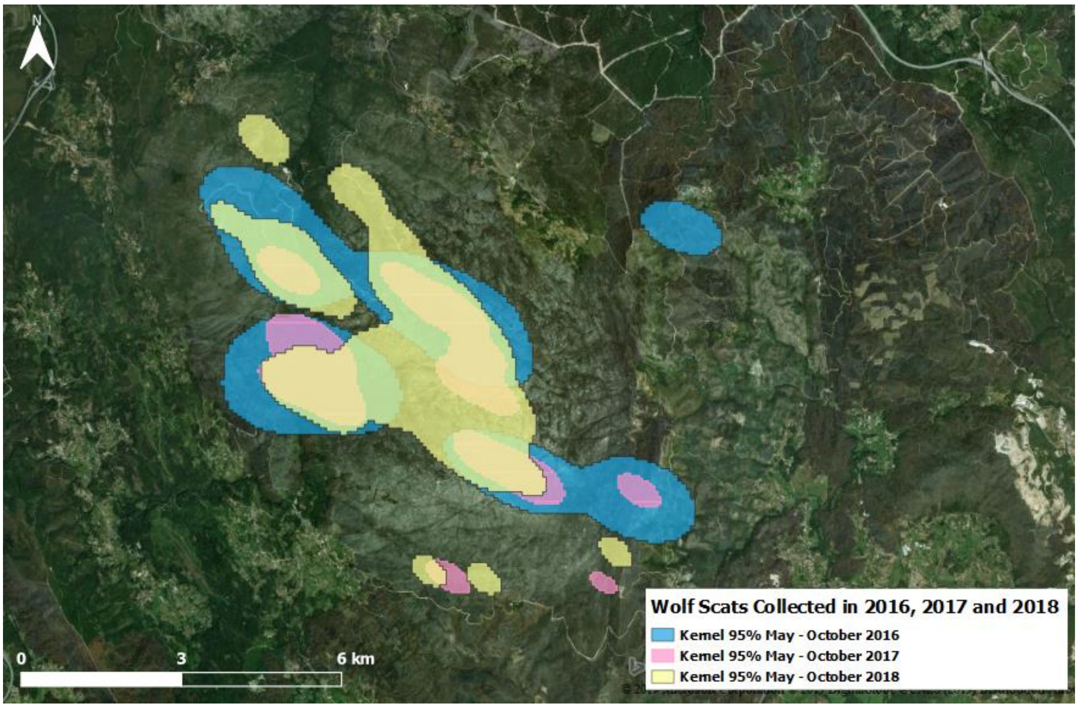


**Figure S3.** Kernel Density Estimation with 95% of the locations of wolf scats collected in Serra d'Arga during wolf breeding season (between may and october of 2016, 2017 and 2018).

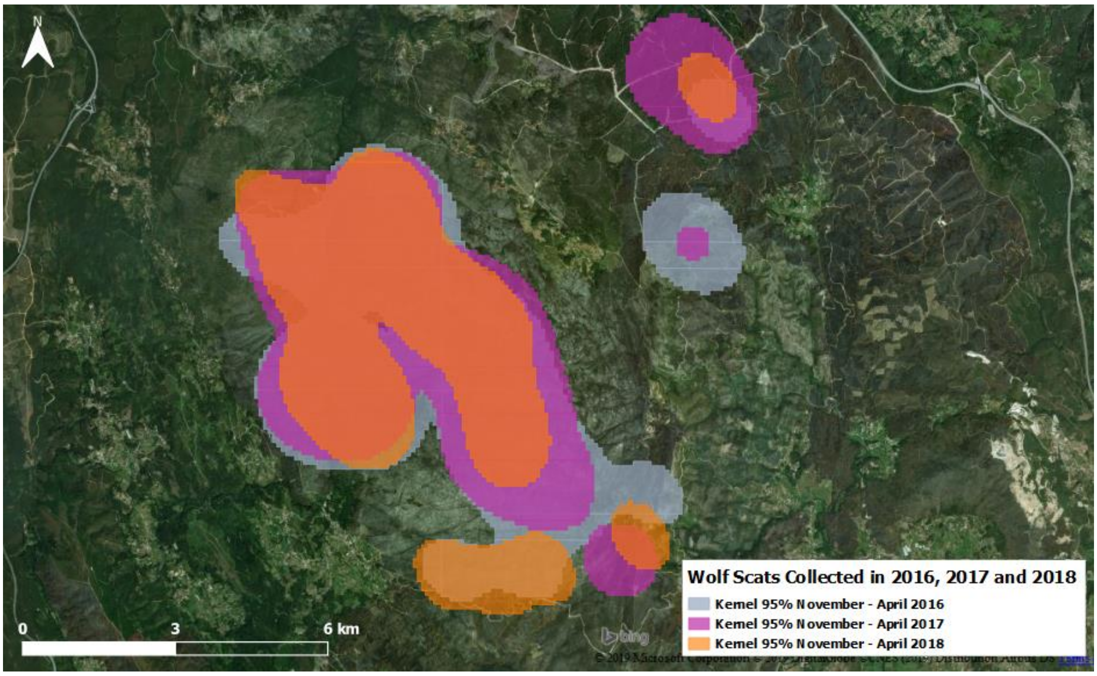


**Figure S4**. Kernel Density Estimation with 95% of the locations of wolf scats collected in Serra d'Arga outside wolf breeding season (between november and april of 2016, 2017 and 2018).