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## Technical Report of Action D1: Wolf activity monitoring and feeding ecology analysis post implementation of conservation actions

Lisbon, 2024

Coordinator:



Partners:



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## ACKNOWLEDGEMENTS

LIFE WolFlux Partners and collaborators, Instituto da Conservação da Natureza e Florestas (ICNF), in particular to the wildlife rangers who collected swabs at depredation sites, Associação para a Conservação do Habitat do Lobo Ibérico (ACHLI) and wolf monitoring teams (Universidade de Aveiro and CIBIO), and all local people that supported and provided valuable information.

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**Suggested citation:** Cadete, D.\*, Pinto, S.\*, Ferreira, E., Carvalho, J., Caroliny, A., Severino, P., Echegaray, J., Lino, A., Aliácar, S. 2024. Wolf activity monitoring and feeding ecology analysis post implementation of conservation actions. (p. 130 + Appendixes + Annex) [Technical Report of Action D1, LIFE17 NAT/PT/554 – WolFlux]. ZL Zoo Logical – Associação de Inovação para o Conhecimento, Divulgação e Conservação da Fauna.

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À memória do Vigilante Armando Dias (ICNF), cujo trabalho e dedicação à conservação da natureza, e em particular, ao lobo, não serão esquecidos.

This report honours the memory of Armando Dias, ICNF ranger, whose work and dedication to nature conservation, particularly to the Iberian wolf, will not be forgotten.

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## 1. Abstract

From 2020 to 2024, we continued implementing a large-scale, non-invasive integrated monitoring program to assess the demographic, genetic, and ecological status of the Iberian wolf population south of the Douro River and evaluate the effects of the LIFE WolFlux project conservation actions. The results confirm the success of the monitoring approach, although gathering data remains challenging, particularly in regions with extremely low wolf abundance. Law changes related to damage compensation could have affected forensic analysis sample size and wolf detection. Our findings indicate an increasingly precarious wolf status, with a decreased confirmed wolf range from 2019 to 2024. Also, confirmed reproduction events showed the same negative trend. Evidence suggests that connectivity levels varied with covered distance and location. It was higher in the central region (comprised by Leomil, Lapa and Trancoso packs), and lower in the western (Arada and Montemuro packs) and eastern areas (Almeida/border). Moreover, long distance extraterritorial movements were not detected during the project timeframe. However, the detection of first-generation migrants, even if low, suggests potential for connectivity among regions and possibly with wolf populations in Spain. Regarding feeding ecology, a slight increase in wild prey depredation was registered, yet the wolf population still shows a high dependence on human activity. Supported by fine-scale habitat modelling, we identify practical conservation tasks that should be implemented together with the projects and partners' broader conservation actions. We also recommend the continuity of the monitoring program to support this population's recovery actions closely.



# Introduction

## 2. Introduction

### 2.1 Framework

Action D1 overall goal was to establish a comparison with the reference situation, established in A.3, through a systematic survey, namely:

- Minimum population size;
- Breeding success;
- Mortality;
- Sex-ratios;
- Feeding ecology;
- *Rendez-vous* sites fidelity;
- Gene flow.

Nonetheless, given that the wolf population south of Douro is so precarious, to ensure that no data was discarded throughout the project timeline, an opportunistic survey took place immediately after the end of Action A3 (Figure 1).



Figure 1 - Monitoring Actions along the project's timeline.

### 2.2 Project Area

The study area is in central Portugal, encompassing 3 NUTS III areas: Aveiro, Viseu-Dão Lafões, and Beira e Serra da Estreña. The total area of approximately 9.000 km<sup>2</sup> includes 7 Natura 2000 network sites (Figure 2).

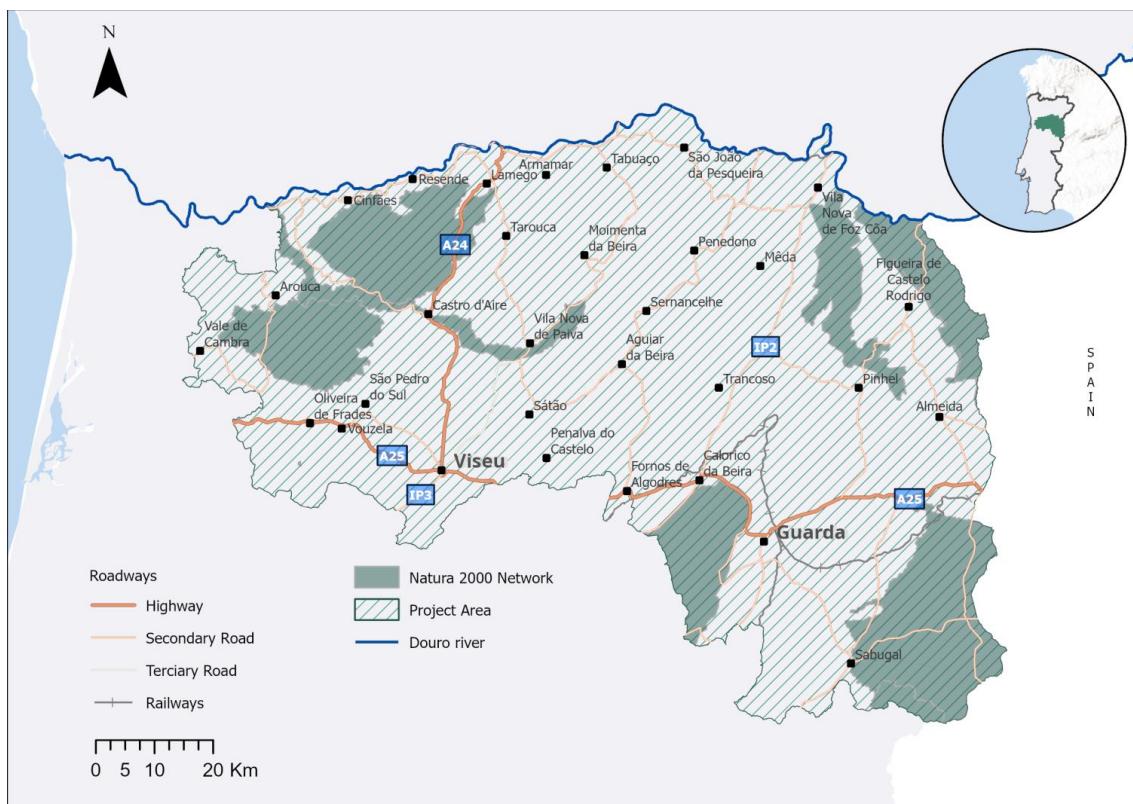


Figure 2 - Spatial overview of the project area. For higher resolution please see Appendix 1.

The area has diverse natural and socioeconomic features. Although the overall human population density is low at 51.59 inhabitants per square kilometre, it varies significantly among municipalities (standard deviation=43.01; maximum=192.1; minimum=11.3) (INE, 2017). The road network density is approximately 0.15 km/km<sup>2</sup> and includes three major highways: A25, A24, and IP2. The landscape varies from mountainous areas, such as Serra da Estrela, Serra da Arada, and Serra de Montemuro, to hilly landscapes in the central and southeast sections and plateau areas with deep river valleys along the border with Spain (Figure 3). The husbandry systems in the region reflect this diversity, with an ancient goat-herding community system in the Arada pack territory. At the same time, free-range cattle have replaced former sheep herds near the Spanish border.

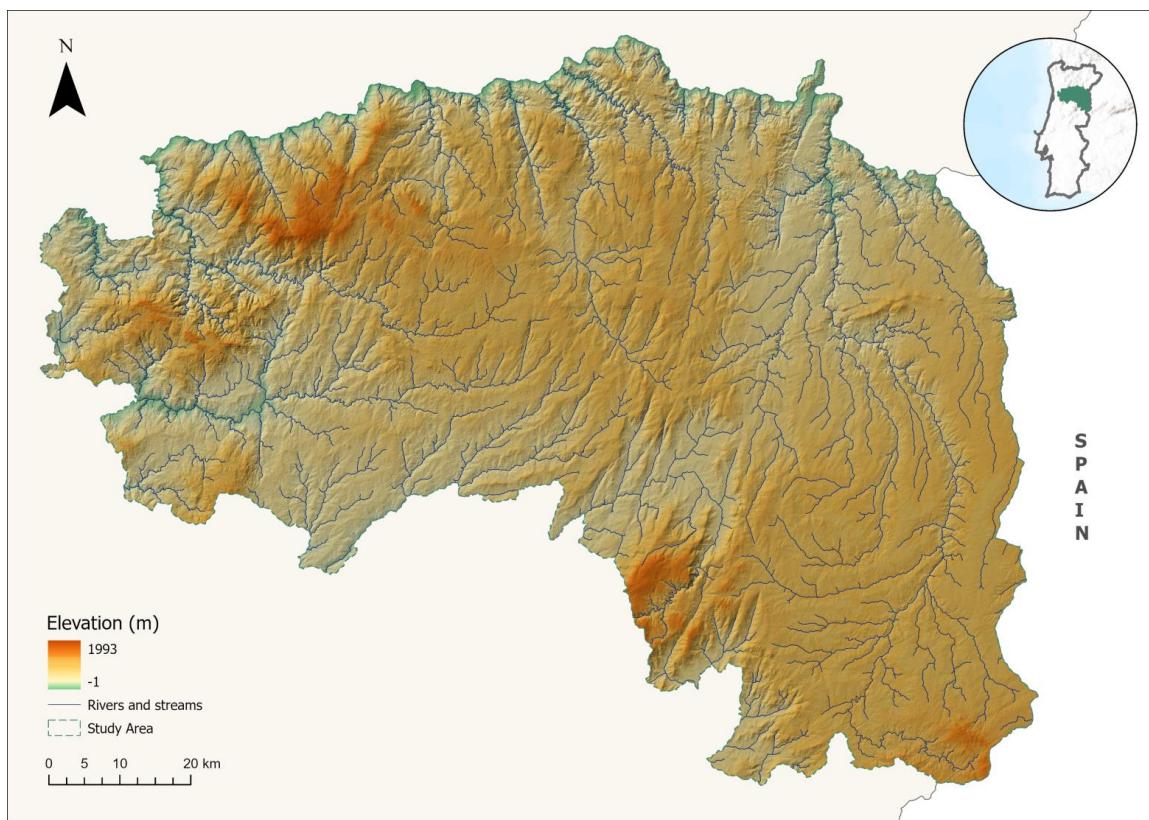


Figure 3 - Elevation, rivers, and streams of the project area. For higher resolution please see Appendix 2.



# Methods

### 3. Methods

#### 3.1 Setting up collaborations with monitoring partners

In the scope of the project, a protocol was established with institutions responsible for ongoing and long-term wolf monitoring initiatives, namely ACHLI (Associação para Conservação do Habitat do Lobo Ibérico) and each of its monitoring field teams – Universidade de Aveiro and CIBIO. Universidade de Aveiro is responsible for monitoring Arada and Montemuro packs, while CIBIO's work is focused on Leomil, Lapa and Trancoso. Within this framework, both teams shared data on wolf presence and breeding occurrence. Yearly wolf monitoring reports were analysed to extract data regarding connectivity, mortality, and population trend, which are presented in the following sections. Collaboration with ACHLI avoided the duplication of efforts and maximized the amount of information obtained and the efficiency in the allocation of LIFE WolFlux resources.

Moreover, a collaboration protocol was established with ARCA, the consultancy responsible for monitoring the Centre and East of the project area during the National Wolf Survey (NWS), promoted by Instituto de Conservação da Natureza e das Florestas (ICNF) in 2019, 2020 and 2021. On the scope of this collaboration both teams (ARCA and WolFlux) shared information and samples for genetic analysis. These results are presented together with the ones collected by the LIFE WolFlux. NWS results were requested to ICNF and are also presented in this report.

Finally, a collaboration with the autonomous government Junta de Castilla y León in Spain allowed the analysis of transboundary samples, to analyse the occurrence of genetic admixture between Portuguese and Spanish wolves south of the Douro. These data were integrated in section 5.4. *Individual genetic profiles*.

#### 3.2 Systematic Survey (2023-24)

Wolves have a long history of human persecution in Europe, so their habits are highly elusive. Consequently, their presence is difficult to detect, mainly when they occur at low densities and possibly do not systematically mark their territory against potential competitors. The current standard methods to monitor/survey wolf populations are indirect, relying mainly on the collection of biological samples for posterior molecular genetic analysis (Marucco et al., 2009, 2011; Serrinha et al., 2018; R. Torres, Hipólito, et

al., 2018). Hence, this action's primary field method consisted of surveys along mountain ridges, forests, and unpaved roads that offered *a priori* and a high probability of detecting wolf scat for posterior molecular analysis.

Most routes surveyed by the Scat Detection Dog Team for scat sampling during action D1 were the same as those previously travelled for action A3 (2019-2020). Minor changes during action D1 transect selection were related to logistical or terrain constraints, including significant habitat quality decreases or different infrastructure construction.

### 3.2.1 Surveys with Scat Detection Dog Team

A team, consisting of a wildlife biologist (the handler) and a trained Iberian wolf scat detection dog, was sent out to survey 5-10 km per 10x10 km UTM cells to find biological samples (Figure 4). This method has already been proven effective in Action A3. The search routes were chosen based on landscape features, the target species' etho-ecological preferences, and practical restrictions such as private land.

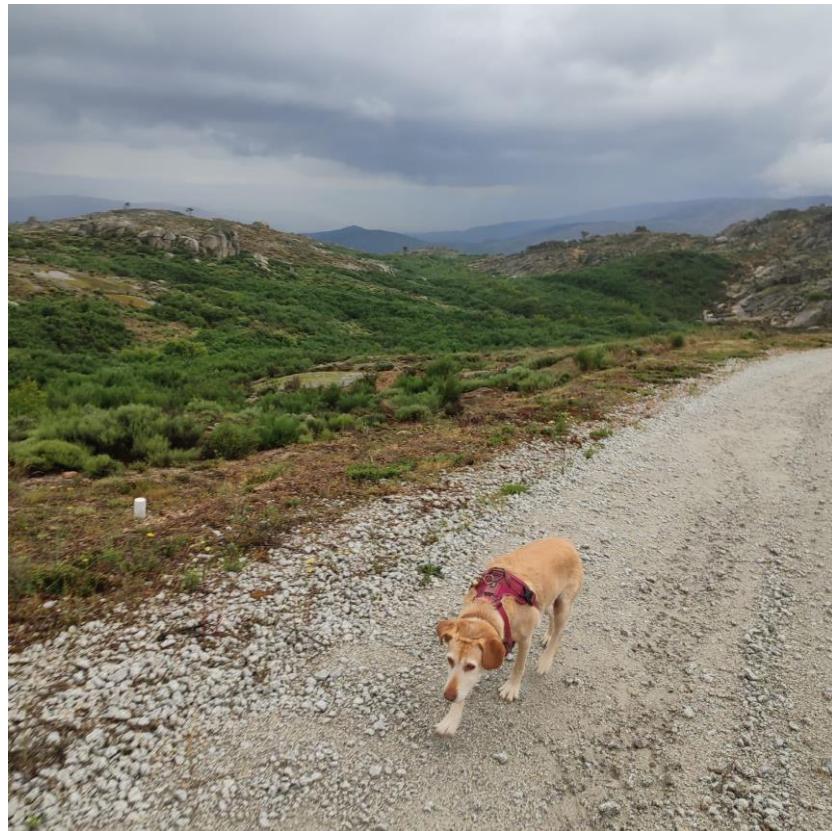


Figure 4 - Alice, the project's trained wolf scat detection dog searching for scat in a survey.

Biological samples, such as scats, were collected for species identification using molecular analysis when conditions allowed for DNA extraction. When the size of the scat permitted, an additional sample for subsequent molecular analysis was collected to

improve DNA extraction success rates. A small portion of each scat was also collected to analyze wolf feeding ecology across the project area. Each detected scat was geotagged and stored according to the lab's standard procedure.

Previous studies have shown that a well-trained scat detection dog team (SDDT) can locate aged and degraded samples. Some of these findings may not be suitable for molecular analysis, but they can help indicate recent target species presence, potential recolonization areas, or recent past local extinction areas. The application of SDDT implies several advantages when compared to the traditional "only human" surveying method: faster in situ primary "screening," higher canine cumulative linear covered distances, and higher sample detection ranges and rates when compared to human visual range and detection rates. Furthermore, the possibility of deploying "off-road" surveying actions is important in several field scenarios, such as small-density forest road areas, mountain/hilly areas, target species reported sights, and damage site inspections. The SDDT method was always deployed understanding its best practices, so it was not used over potential target species' resting sites and/or den/rendezvous sites. The SDDT application (non-invasive method) was planned and deployed to minimize impacts on target species and local wildlife. Concerning domestic dogs' physiology, this method is of better use over moderate to cold temperatures. Further, wolves' biological and etho-ecological features, such as significant movement range and scent-marking rates, make SDDT application as a large-scale "screening" method more suitable, efficient, and optimized probably from middle Autumn to early Spring. During this period, optimal atmospheric conditions are more common, and there is greater availability of samples over the terrain. However, it is a year-round application method that requires careful planning and adjustments to enhance its efficiency, such as selecting one-off or priority actions.

### 3.2.2 Kilometric Abundance Index

Before the widespread use of molecular analysis in species' assignment from biological samples, kilometric abundance index (KAI) was used in the Iberian Peninsula as the main method to determine wolf relative abundance. This index consists in counting the number of scat per surveyed distance length:

$$KAI = \frac{\text{Number of scat}}{\text{Transect surveyed lenght}}$$

The scat samples considered for the determination of the KAI were those that had not undergone genetic analysis, those that were tested but provided inconclusive results, and

those that were genetically confirmed as belonging to wolves. Thus, all scats that were genetically assigned to other species than wolf, were excluded from this analysis

### 3.2.3 Sampling Effort

From 6<sup>th</sup> February 2023 to 27<sup>th</sup> February 2024, 441,26 km was surveyed in 221 transects in a sub-set of 54 10x10km UTM cells (Figure 5 and Appendix 3). The transects initially conducted during Action A3 were repeated in Action D1.

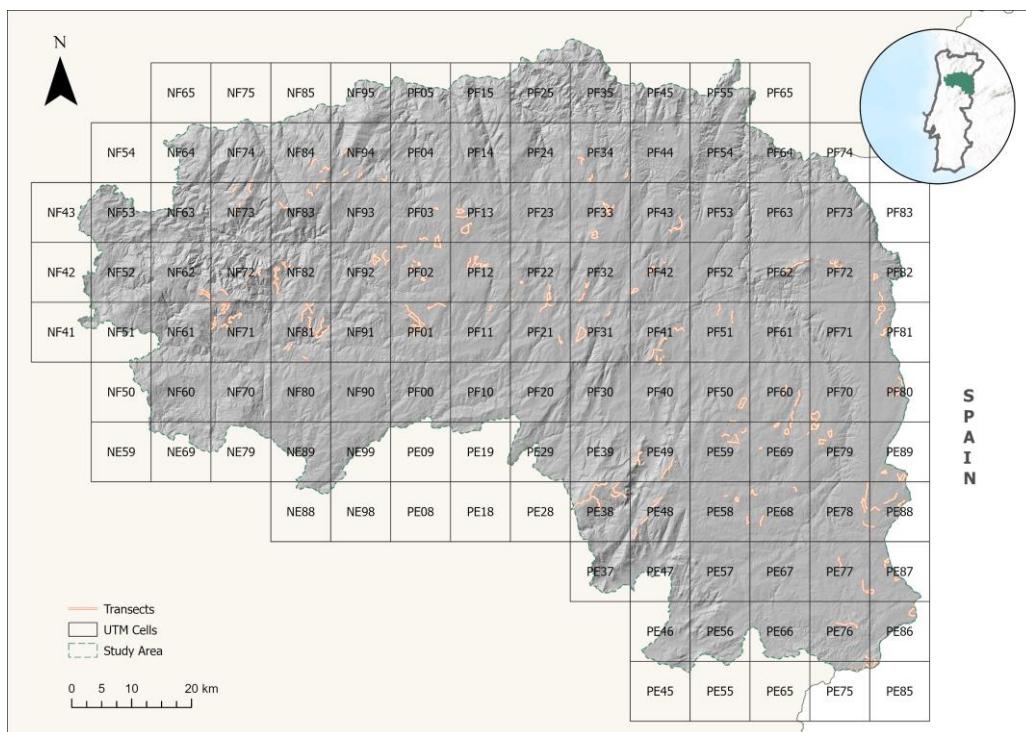


Figure 5 - Spatial overview of the Scat Detection Dog Teams survey transects of Action D1. For higher resolution, please see Appendix 4.

### 3.3 Opportunistic Survey (2020-2022)

An opportunistic survey was conducted to maximize the collection of wolf-related data in the project area and document it during the non-systematic sampling period. The methodology consisted of applying camera trapping and transect survey following a potential wolf-related event (e.g., livestock attack) or relevant information collected by the Rewilding Portugal team (e.g., wild prey content in a wolf-assigned scat, howls, tracks, etc.).

#### 3.3.1 Sampling Effort

Between January 15, 2020, and February 5, 2022, 152,57 km were surveyed along 63 transects distributed across 13 10x10 km UTM cells (Appendix 5). Between January 5, 2020, and August 24, 2023, 29 camera traps were deployed. 1057 night/traps were achieved along

14 UTM 10x10 km cells (Appendix 6). Figure 6 depicts the total effort deployed during this period. The majority of the effort was concentrated in the eastern region, as this area had less available information and fewer recorded individuals.

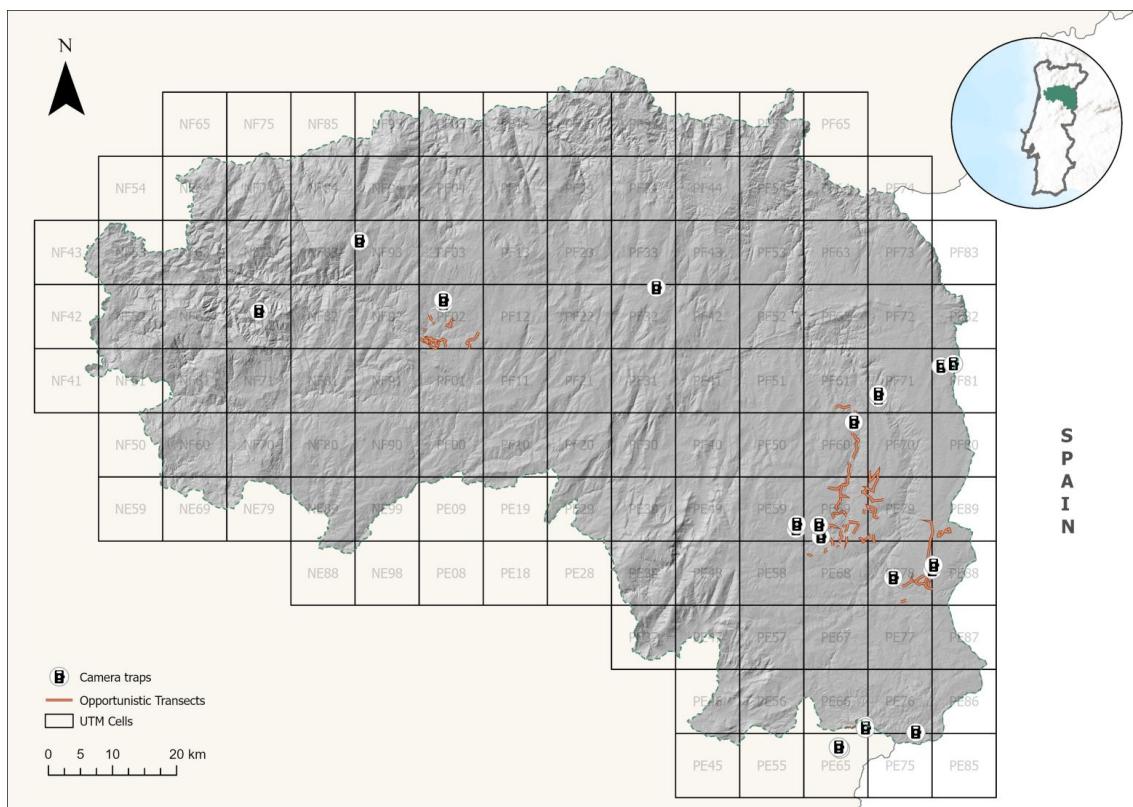


Figure 6 - Spatial overview of opportunistic transects and camera traps deployed between 2020 and 2022. For higher resolution please see Appendix 7.

### 3.4 Genetic Analysis of eDNA collected from Livestock Attacks

Following a LIFE WolFlux collaboration protocol with Instituto da Conservação da Natureza e Florestas (ICNF), ICNF Ranger teams working over the project's area were trained during an A3 theoretical and practical session to collect swab samples of saliva from wounded or killed animals found during livestock damage inspections. This non-invasive genetic sampling (NGS) method has proven to be very efficient for molecular data collecting and demographic/spatial information input in previous projects (Cadete et al., 2012, 2015; Palacios et al., 2017). Accordingly, during D1's opportunistic and systematic surveying, swab samples were collected from 38 livestock damage inspections, recorded geographically and stored for post molecular analysis.

### 3.5 Feeding Ecology Analysis

Wolf scats were dried in an oven at 60°C for over 3h for sterilisation. Then, the samples were washed through a 1-3mm mesh sieve (Kruuk & Parish, 1981) to separate the undigested parts, such as hair (Wagner et al., 2012). The identification of hairs followed a two-fold framework to obtain a reliable determination of the consumed food item (Sangiliano et al., 2016). First, we performed a macroscopic comparison of hairs with a reference collection of potential wolf prey, using parameters such as colour, shape, length and thickness (Karanth & Sunquist, 1995). Second, we carried out a microscopic analysis (100-400x), to observe the structure of cuticle, medulla and cortex of hair. These characteristics were then compared with a reference collection, including illustrated atlas (De Marinis & Asprea, 2006; Teerink, 1991; Valente et al., 2015). Twenty hairs were collected and identified for each sample (Lovari et al., 2015). For each category, we calculated the absolute and relative frequency of occurrence in the diet (Lucherini et al., 1995).

## 3.6 Genetic Analysis

### 3.6.1 Sample reception and storage

Since the end of action A3, between May 5, 2020 and February 28, 2024 – corresponding to the opportunistic and systematic sampling periods of action D1 – a total of 68 scat samples (and 21 replicates) and 192 swabs (corresponding to 38 livestock attacks) were received at the genetics laboratory of the Wildlife Research Unit (Department of Biology, University of Aveiro). Four additional samples, collected in the context of action A3 but not analysed in time for the action report, are also reported here (Appendix 8): (a) two scat samples (WFL169 and WFL172); (b) two hair samples (WLP01 and WLP02).

Scat samples were stored in 95% ethanol immediately after collection and kept at -20°C after arrival to the lab, until DNA isolation. Storage of non-invasive samples in a cool environment has a positive effect on sample conservation and thus in DNA isolation (Murphy et al., 2002). All information regarding sampling date and location was stored in a database and shared with the project team.

### 3.6.2 DNA isolation from scat and swabs

Both scat and swab samples are challenging in what concerns DNA isolation. In both cases, DNA from target species are expected in low quantity and quality and special measures

must be taken in order to avoid cross contamination among samples or environmental contamination with human or high DNA concentration samples (Taberlet et al., 1999). Besides the low quantity and quality of target species DNA, isolates from scats will also include DNA from preys, microbiome and parasites; and isolates from swabs will more likely have more DNA from livestock than from predator.

Sample manipulation was performed in a laboratory used only for DNA extraction from non-invasive samples. Throughout the procedure, disposable gloves, cap and mask were used, reducing the possibility of contamination by human DNA. Sterile and disposable consumables were used, as well as reagents and equipment exclusively for the treatment of non-invasive samples.

DNA isolation from scat samples was performed using QIAGEN® QiAamp DNAStool kit, following the manufacturer's protocol, with modifications. This commercial kit has been previously used by the project team and been proven to be very efficient for stool DNA extraction. In each extraction procedure a maximum of 10 samples were treated, including a negative control. DNA isolation from swab samples was performed using the InnuPREP Forensic Kit, according to the protocol recommended by the manufacturer. Whenever possible (and always for high priority livestock attacks) swabs from different attacks were handled separately. The use of negative controls during DNA extraction and amplification has ensured that there is no cross-contamination between samples and/or reagents. Subsequently, the samples were subjected to molecular marker analysis according to the procedures established for the present study.

### **3.6.3 Species identification using a fragment of the mitochondrial DNA control region**

For mitochondrial lineage determination, we amplified a DNA fragment corresponding to the d-loop of the mtDNA (mitochondrial DNA) chromosome control region. By default, for scats, a larger fragment of 442 base pair (bp) mitochondrial control region d-loop fragment was amplified using the Thr-L 15926 and DL-H 16340 primers (Vilà et al., 1999). This fragment is often used in the molecular distinction between dog and wolf (e.g. (Godinho et al., 2007, 2011; Vilà et al., 1999) and was also employed by our team (R. Torres, Fernandes, et al., 2018) and previous team (Roque et al., 2010), in the monitoring of wolf populations south of Rio Douro. In cases where amplification of the larger fragment wasn't possible, a more specific primer pair (dogDL1/dogDL3, Leonard et al. 2002) was used. For swabs, the first pair of primers (Thr-L 15926/DL-H 16340), which are not 100% specific for wolf and dog,

preferentially amplified DNA from the mammal preys (which was present in much higher quantity), so the second pair of primers (dogDL1/dogDL3, Leonard et al, 2002) was successfully used.

For the determination of mitochondrial lineages, generated sequences were compared with haplotypes previously described for wolf and dog (Vilà et al., 1997), using only a 261bp fragment (out of the 442 bp sequenced) corresponding to the fragment used by these authors. The PCR reactions were prepared in a separate room dedicated to DNA isolation and post-PCR procedures, which is equipped with an ultraviolet (UV) light PCR chamber. This light was switched on between camera use intervals, thus destroying any DNA molecules in the chamber before the procedure. All PCR reactions included a negative control to certify the absence of contamination between samples. After successful amplification, PCR products were purified and sequenced using the above-mentioned primers.

The determination of the origin of the samples was additionally checked by genotyping all samples using the microsatellite marker DBAR1. This marker has an allele frequency greater than 95% for the Iberian wolf (Godinho et al., 2011), thus being practically diagnostic in distinguishing between Iberian wolf and dog. This marker was amplified using the Qiagen Multiplex Kit™, following the manufacturer's instructions. The size of alleles was determined by automated sequencer fragment analysis as detailed in the section on determining individual profiles.

### **3.6.4 Molecular Determination of Sex**

Molecular determination of sex was performed based on the genotyping of the amelogenin gene. Amelogenin is a protein linked to the formation of enamel in mammals (Delgado et al., 2005) and in placental mammals presents two distinct forms associated with sex chromosomes (Iwase et al., 2007). In the case of dogs, there are currently specific primers for amplification of fragments associated with the amelogenin gene. This marker has already been successfully used for dog sexing (Steckler, 2010) and has been tested by our team on dog samples of known sex with satisfactory results.

### **3.6.5 Identification of individual profiles**

At first, for scats and swab samples, the microsatellite marker DBAR1 was separately amplified. In cases where amplification was successful and the results did not present ambiguities, eight additional markers were amplified in two amplification sets (AHT137,

AHTk171, INRA21; C04.140, C20.253, FH2001, FH2161, INU055). These markers were multiplex amplified using the Qiagen Multiplex Kit™, following the manufacturer's instructions.

As a standard procedure, a wider microsatellite marker panel, with 16 loci, was only amplified in cases of previously successful amplifications. These 16 microsatellite loci (AHT121, CPH09, CPH14, PEZ3, REN54P11, REN162C04; CXX279, FH2848, INU005, REN169D01, REN169O18; AHTk211, FH2054, INU055, INU030, REN247M23) are part of the panel recommended by the International Society of Animal Genetics (ISAG) and were amplified in three multiplex marker sets, after quantification of the total DNA present in the extracted samples. Amplification products were submitted to fragment analysis using capillary electrophoresis in an automated sequencer.

### 3.6.6 Genetic Data Analysis

Mitochondrial DNA sequences were aligned using the CLUSTALW algorithm (implemented in MEGA X software), and subsequently edited manually. The comparison between the haplotypes obtained in this work and the reference haplotypes (Vilà et al., 1997) was made using the same program and POPART (Leigh & Bryant, 2015). Identification of individual genetic profiles (genotypes) based on microsatellite loci, of genotype matches (recaptures), estimation of the probability of identity and assignment tests were performed in GenAIEx (Peakall & Smouse, 2006, 2012a, 2012b). Probability of identity was estimated using a conservative approach for small and closely related populations, by assuming populations of siblings (Waits et al., 2001). Assessment of genetic structure and posterior probability of assignment of individual genotypes to inferred genetic clusters was performed using STRUCTURE (Pritchard et al., 2000).



# Results

## 4. Results

### 4.1 Setting up collaborations with monitoring partners

#### 4.1.1 Monitoring Framework

The South of Douro wolf subpopulation is often referred to in technical literature as consisting of two wolf nuclei: Arada-Trancoso and the border region. However, due to task assignments to different research teams, the South of Douro was divided into three areas: West, Central, and East (bordering with Spain). It's important to note that this division is purely for organizational purposes to identify the projects conducted in each area. The entire subpopulation has been monitored since 1996, although varying methods, criteria, and efforts have been utilized over the years (Table 1).

*Table 1 - Wolf monitoring projects developed in South of Douro subpopulation.*

YEAR	WEST	CENTRAL	EAST
<b>1996</b>	(ICN, 1997)	(ICN, 1997)	(ICN, 1997)
<b>1997</b>	(ICN, 1997)	(ICN, 1997)	(ICN, 1997)
<b>1998</b>	(Grilo et al., 2002)	(Grilo et al., 2002)	-
<b>1999</b>	(Grilo et al., 2002)	(Grilo et al., 2002)	-
<b>2000</b>	(Bastos, 2001)	(Bastos, 2001)	-
<b>2001</b>	(Roque et al., 2005)	(Roque et al., 2005)	-
<b>2002</b>	(Pimenta et al., 2005)	(Pimenta et al., 2005)	(Pimenta et al., 2005)
<b>2003</b>	(Pimenta et al., 2005)	(Pimenta et al., 2005)	(Pimenta et al., 2005)
<b>2004</b>	(Álvares et al., 2005b) (Álvares et al., 2005a)	-	-
<b>2005</b>	(Roque & Petrucci-Fonseca, 2006)	(Roque & Petrucci-Fonseca, 2006)	-
<b>2006</b>	(Roque et al., 2011)	(Roque et al., 2011)	-
<b>2007</b>	(Roque et al., 2011)	(Roque et al., 2011)	-
<b>2008</b>	(Roque et al., 2011)	(Roque et al., 2011)	-
<b>2009</b>	(Roque et al., 2011)	(Roque et al., 2011)	-
<b>2010</b>	(Roque et al., 2011)	(Roque et al., 2011)	-
<b>2011</b>	(R. Torres et al., 2016)	(Roque et al., 2017)	(Cadete et al., 2012)
<b>2012</b>	(R. Torres et al., 2016)	(Roque et al., 2017)	-
<b>2013</b>	(R. Torres et al., 2016)	(Roque et al., 2017)	-
<b>2014</b>	(R. Torres et al., 2016)	(Roque et al., 2017)	(Cadete et al., 2015)
<b>2015</b>	(R. Torres et al., 2016)	(Roque et al., 2017)	-
<b>2016</b>	(R. Torres, Fernandes, et al., 2018)	(Roque et al., 2018)	(Palacios et al., 2017)

	(R. Torres, Hipólito, Lino, Barros, et al., 2021)		
<b>2017</b>	(R. Torres, Hipólito, et al., 2018) (R. Torres, Hipólito, Lino, Barros, et al., 2021)	(Serronha et al., 2018)	-
<b>2018</b>	(R. Torres, Hipólito, Lino, Barros, et al., 2021) (R. Torres et al., 2019)	(Serronha et al., 2019)	-
<b>2019</b>	(Cadete et al., 2021) (R. Torres et al., 2019) (R. Torres, Hipólito, Lino, Barros, et al., 2021) (Pimenta et al., 2023)	(Cadete et al., 2021) (Serronha et al., 2020) (Pimenta et al., 2023) (Pimenta et al., 2023)	(Cadete et al., 2021) (Pimenta et al., 2023)
<b>2020</b>	(R. Torres, Hipólito, Lino, Ferreira, et al., 2021) (R. Torres, Hipólito, Lino, Barros, et al., 2021) (Pimenta et al., 2023)	(Serronha et al., 2020) (Serronha et al., 2021) (Pimenta et al., 2023)	Unpublished data (LIFE WolFlux) (Pimenta et al., 2023)
<b>2021</b>	(R. Torres, Hipólito, Lino, Ferreira, et al., 2021) (R. Torres et al., 2023a) (Pimenta et al., 2023)	(Serronha et al., 2021) (Cardoso et al., 2022) (Pimenta et al., 2023) (Pimenta et al., 2023)	Unpublished data (LIFE WolFlux) (Pimenta et al., 2023)
<b>2022</b>	(R. Torres et al., 2023a) (R. Torres et al., 2023b)	(Cardoso et al., 2022) (Cardoso et al., 2024)	Unpublished data
<b>2023</b>	(R. Torres et al., 2023b)		Unpublished data
<b>2024</b>	Unpublished data	Unpublished data	Unpublished data

#### 4.1.2 Population Trend

Between 2019 and 2024, the monitoring projects and the new Portuguese National Wolf Survey (NWS) mentioned in Table 1, yielded significant data regarding population trend. In the scope of the NWS (Pimenta et al., 2023), five packs were confirmed in South of Douro population, and one was considered probable (Figure 7).

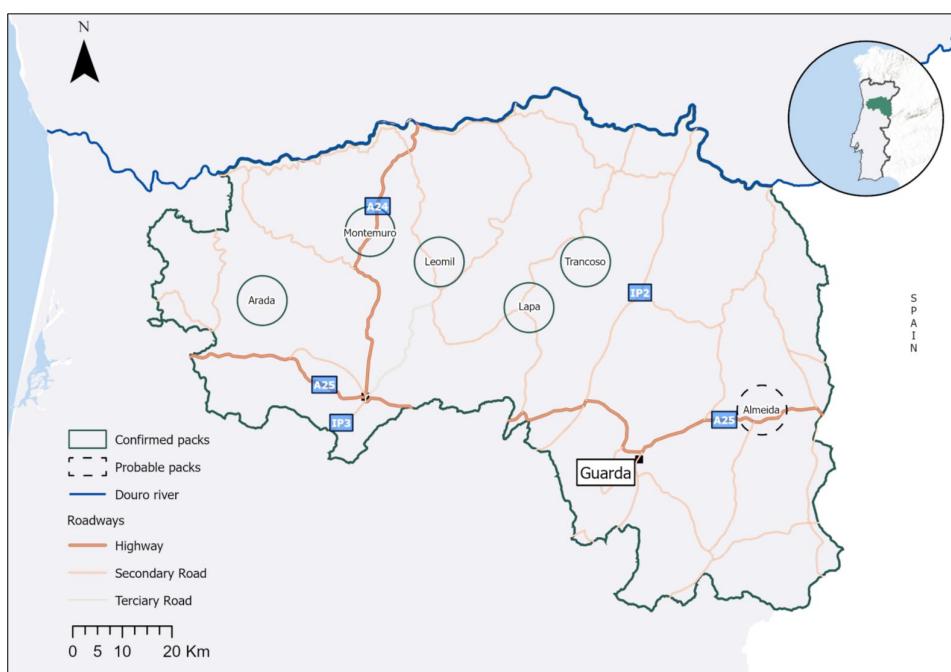


Figure 7 - Packs detected during the last national wolf survey. Adapted from Pimenta et al., 2023. For higher resolution please see Appendix 9.

The findings from the studies mentioned in Table 1 indicate a fluctuation in wolf pack confirmations over the five-year period analysed, with a notable peak in 2021 (Figure 8). Initially, there was a gradual increase in the number of confirmed packs from 2019 to 2021, reaching a maximum of six confirmations in the latter year. This trend is likely due to the increased effort during the National Wolf Survey between 2009 and 2021. After 2021, a slight decrease in confirmations was observed, accompanied by an increase of non-survey in Almeida pack. Despite these variations, confirmed packs remained the predominant category in all years.

The Leomil, Montemuro, and Arada packs exhibit the highest number of confirmations, followed by Lapa and Trancoso packs, which show a less stable situation, since they were not detected in two monitoring years (Figure 8). Almeida pack was never confirmed, which may be due to a more precarious situation, but also to less monitoring efforts in the area. It is noteworthy that wolf presence was detected in the Cinfães area, a previously confirmed pack area (Cadete et al., 2020); however, the criteria were insufficient to classify this territory as occupied by a wolf pack.

Pack	Pack Status					Years of confirmation
	2019	2020	2021	2022	2023	
Arada	C	C	C	C	C	5/5
Montemuro	C	C	C	C	C	5/5
Leomil	C	C	C	C	C	5/5
Lapa	ND	ND	C	C	C	3/5
Trancoso	C	C	C	ND	ND	3/5
Almeida	P	P	P	NS	NS	0/5
Total packs confirmed	4/6	4/6	5/6	4/6	4/6	

Packs  
■ Confirmed  
■ Not Detected  
■ Probable  
■ Non Surveyed

Figure 8 - Pack status as collected by other partners (ACHLI and NWS) during Life WolFlux project's time frame.

Regarding reproduction detection, Montemuro, Arada, and Leomil exhibit the highest number of confirmations, while Lapa and Almeida do not record such events (Figure 9). The Trancoso pack had one probable reproduction event.

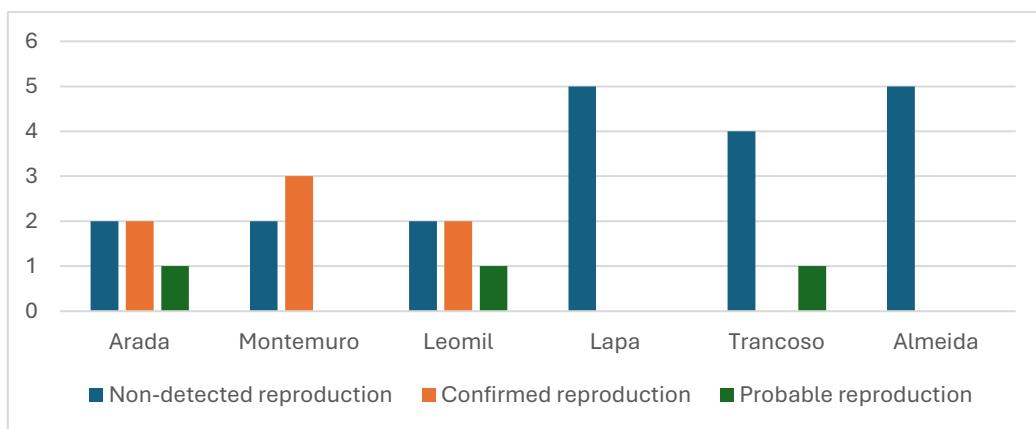


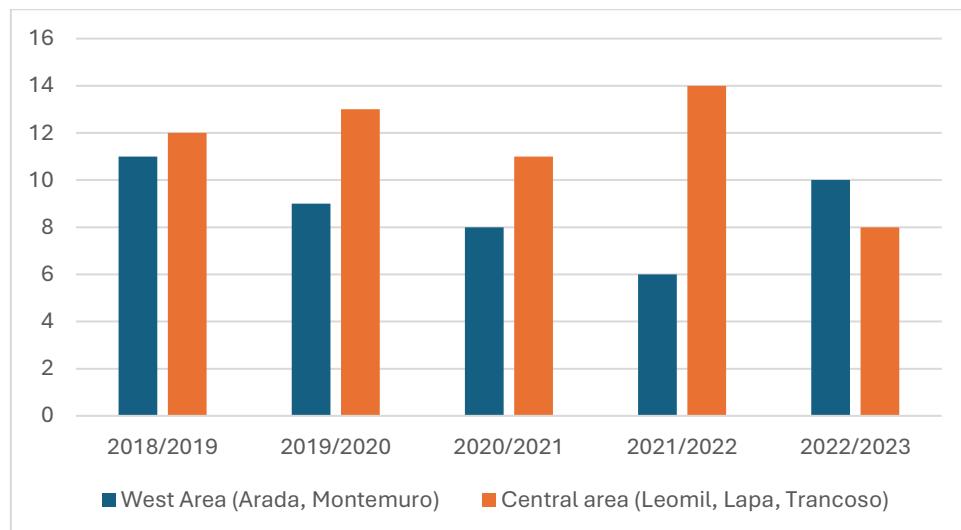
Figure 9 - Detected reproductive success in wolf packs between 2019 and 2023.

Figure 10 illustrates the minimum number of individuals recorded in two distinct areas—west (Arada, Montemuro) and central/east (Leomil, Lapa, Trancoso)—over five monitoring periods from 2018/2019 to 2022/2023.

In general, the central/eastern area consistently recorded higher minimum number of individuals than the western area. There was an initial count of 12 wolves in 2018/2019, which marginally increased in 2019/2020. A decline was observed in 2020/2021, followed by a more significant increase in 2021/2022. However, a significant decrease occurred the following year, and only 8 wolves were recorded.

In contrast, the western area exhibited a different trend, commencing with 11 wolves in 2018/2019, then experiencing a gradual decrease, reaching a minimum of 6 wolves in 2021/2022. Subsequently, a recovery occurred, resulting in a count of 10 wolves in 2022/2023.

These data indicate fluctuations in minimum wolf numbers in both areas, with the central/eastern area demonstrating a generally higher number of individuals, despite a recent decline. The western area, while lower overall, exhibits signs of slight recovery in the most recent year.



*Figure 10 - Minimum number of wolves detected in the projects' west Area (Arada, Montemuro) and central area (Leomil, Lapa, Trancoso) from 2018/2019 to 2022/2023, showing fluctuations in population numbers over time. Data collected by Universidade de Aveiro and CIBIO teams, owned and kindly shared by ACHLI. Please note that overall population size includes Eastern section (border region with Spain).*

### 4.1.3 Mortality

Two mortality events were recorded along the study area during the project time frame. The first occurred during the winter of 2019, and it involved a roadkill of a juvenile male in the western part of Leomil´s pack known home range. The other case was probably related to the same pack, which occurred during the intermediary period (December 2021) and was located in its eastern known home range. This animal was probably fatally injured after staying trapped in a snare. Due to its advanced decomposition state, it was impossible to determine its age and sex.

### 4.1.4 Connectivity

The most robust evidence of connectivity between packs is the detection of identical genetic profiles in the territories of two distinct packs. This indicates that the same individual was present in both territories, confirming an extraterritorial movement.

In the western region of the project area, only one extraterritorial movement was recorded during 11 years of monitoring and scat analysis (R. Torres et al., 2023b). The genotype Cinf2 was initially identified in the territory of Arada pack (February 2011) and was recaptured several months later in the currently non-confirmed Cinfães pack territory (R. Torres et al., 2023b). As evident in Figure 7, the territory allocated to Montemuro pack is intersected by the A24 highway. Although wolf genotypes have been captured in both the western and eastern sections of the road, only a single recapture of the female Mont12 has been recorded in both areas to date. This evidence strongly indicates the isolation of the western packs, which show minimal movement toward the eastern packs, and limited interaction even among them.

In the eastern region, however, several extraterritorial movements were observed. A male wolf (LSD59), initially detected in the Leomil pack area during winter 2014, has been frequently recorded in the Trancoso pack since the summer of the same year. In August 2019, the same individual was detected in the Lapa pack territory (Serronha et al., 2020). Between 2020 and 2021, two different extraterritorial movements were recorded. One female (LSD83) from the Leomil pack was subsequently detected in the Lapa pack, and another female from the Trancoso pack (LSD69) was recaptured in the Lapa pack territory (Serronha et al., 2021). Between 2021 and 2022, a male wolf from Lapa pack, identified as LSD86, was recorded in the Leomil pack area (Cardoso et al., 2022). Another significant recapture occurred in the Almeida pack region. In 2016, this pack was confirmed by detecting five distinct genotypes. One of the genotypes belonged to a male previously

detected in the Leomil pack area in the summer of 2011 (Pimenta et al., 2023). These extraterritorial movements demonstrate a substantial degree of connectivity among Leomil, Lapa, and Trancoso packs (with movements occurring in all directions), and also indicate potential connectivity with the border region of Spain.

## 4.2 Systematic (2023-2024) and Opportunistic (2020-2022) survey carried out by the LIFE WolFlux team

### 4.2.1 Kilometric Abundance Index

Scat samples assigned to domestic dog or other species, by genetic analysis, were excluded from the Kilometric Abundance Index (KAI). However, presumed wolf scat samples, including all those from which DNA isolation was unsuccessful or not analysed, were included in the index estimation. Out of the 54 10x10 km UTM cells surveyed, 30 did not yield any scat (resulting in a KAI of 0). In other areas, the KAI reached a high value of 3.96 scat/km, with an average KAI of 0.17 scat/km. Spatially, the KAI was higher in the western and eastern limits of the project area and lower or non-existent in its central area (Figure 11).

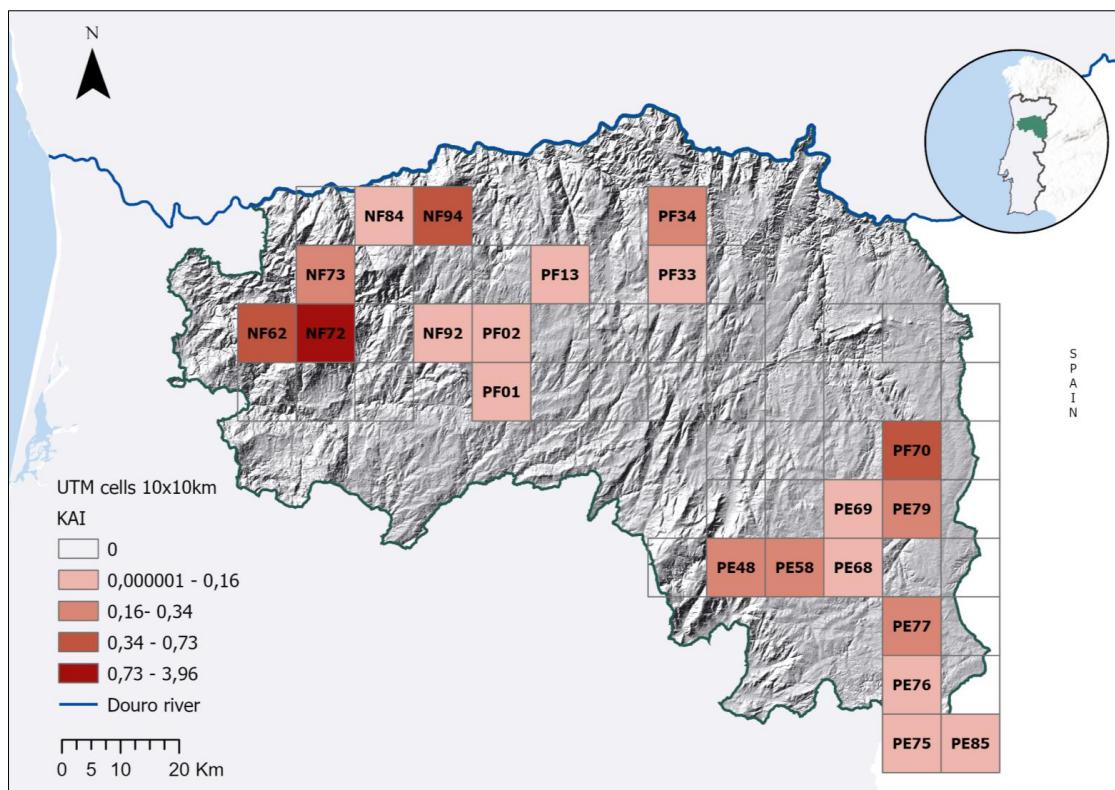


Figure 11 - Kilometric Abundance Index (KAI) obtained in the project area during Action D1 (2023-2024). For higher resolution please see Appendix 10.

In comparison to Action A3, the number of cells whose KAI was zero doubled (only 16 UTM cells in A3), and a significant reduction in KAI values is observed across the project area, particularly in the central region (Figure 12). The A3 KAI average value for the surveyed UTM cells was 1,57 scat/km, whereas in D1, this value was 0.11 scat/km

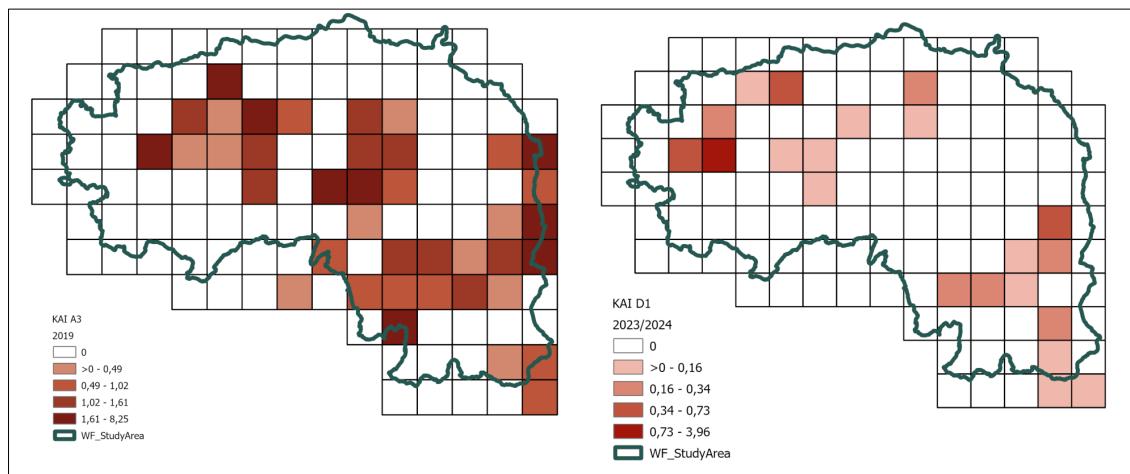


Figure 12 - Comparison between average KAI values/UTM cell between Action A3 and Action D1. For higher resolutions please see Appendix 11.

However, a significant part of the territories assigned to wolf packs in the scope of the NWS, had a positive KAI value, namely Arada, Leomil, Trancoso and Almeida. In the Montemuro and Lapa territories, no scat was detected during the transect surveys.

#### 4.2.2 Sample assignment

From the 72 scat samples that were received until the end of February 2024, all were submitted to DNA isolation. When replicates were available and the analysis of the first sample failed, a second or third replicate per sample was analysed. From the 72 samples selected for DNA isolation, 66 were successfully amplified, which represents an amplification success rate of 92%. From those, 64 samples were successfully sequenced (sequencing success rate of 97%). The overall success rate (amplification and sequencing) was 89%, which means that from the 72 analysed scat samples, 64 were successfully assigned to a carnivore mammal species. These sequences were compared to all deposited sequences on GenBank database, having been matched with haplotypes from: Iberian wolf (34 samples; 53%); domestic dog (27 samples; 42%) and red fox (3 samples; 5%) (Figure 13). The sequences identified with haplotypes from Iberian wolf and dog were corroborated by comparing them with a set of reference haplotypes for wolf (including Iberian wolf) and dog from Europe. Based on the generated DNA sequences, scat samples were assigned to one haplotype, whenever possible (Appendix 8).

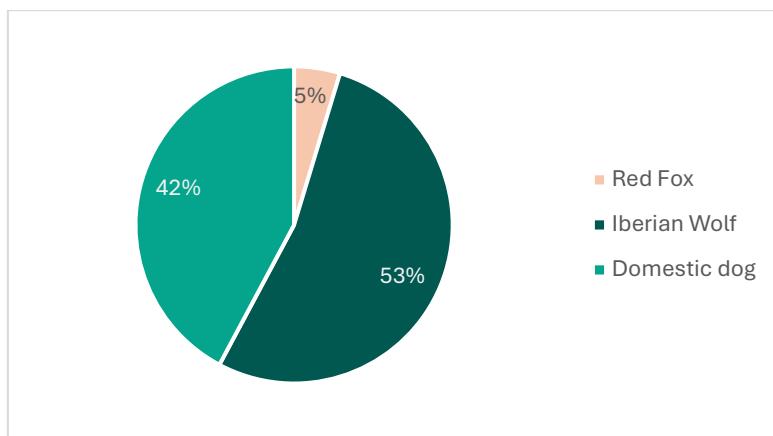


Figure 13 - Species assignment of scat samples.

DBAR genotyping of dog and Iberian wolf samples was consistent with species identification retrieved from mitochondrial haplotype, in all cases where results from both markers were available.

From the 192 swab samples (corresponding to 38 livestock attacks) that were received in the lab, 141 (corresponding to 30 attacks) were submitted to genetic analysis and 54 samples (38%), from 19 attacks (63%) were successfully amplified. Success rate varied between 0% and 100% in individual attacks, with an average of 2 to 3 successfully amplified swabs per individual attack, in the cases of attacks with successful amplification of predator DNA. From these 54 swab samples, all were successfully sequenced, generating a sequence of 186 nucleotides that allowed discrimination between dog and Iberian wolf.

The overall success rate (amplification and sequencing) was 38%, which means that from the 141 analysed swab samples, 54 were successfully identified as individuals of the genera *Canis*. These sequences were compared to all deposited sequences on GenBank database, having been matched with haplotypes from *Canis lupus signatus* ( $n = 11$ ) and *Canis lupus familiaris* ( $n = 43$ ). This result was corroborated by comparing these sequences with a set of reference haplotypes for dog and wolf (including Iberian wolf) for Europe. Collectively, these results allowed the detection of Iberian wolf and/or dog presence in 19 out of 30 livestock attacks (Appendix 8).

On average, for each attack, information on predator species was only possible to obtain from less than half of the swabs. In 19 of 30 attacks, at least one of the swabs provided some information about predator species feeding in the carcasses while in 11 of the attacks none of the swabs provided any information (Appendix 12).

Genotyping of the microsatellite marker DBAR1 was performed for all samples in which the mtDNA was successfully sequenced and identified as dog or wolf (n= 54). We were able to successfully amplify the same alleles consistently in the three replicates for only 14 samples (6 corresponding to wolves and 8 corresponding to dogs). Some samples presented inconclusive results (n = 10), since it was not possible to obtain a minimum of three replicates. For the remaining 40 samples, the amplification of DBAR1 marker failed systematically. The amplification success rate for DBAR1 was 26%.

During the opportunistic period, 11 UTM 10x10 Km cells (1100 Km<sup>2</sup>) were confirmed (Figure 14). The project's collaborative protocol with ICNF, in the scope of the NWS, was critical to improve species' detection over the project study area and proved to be beneficial for both undergoing surveys.

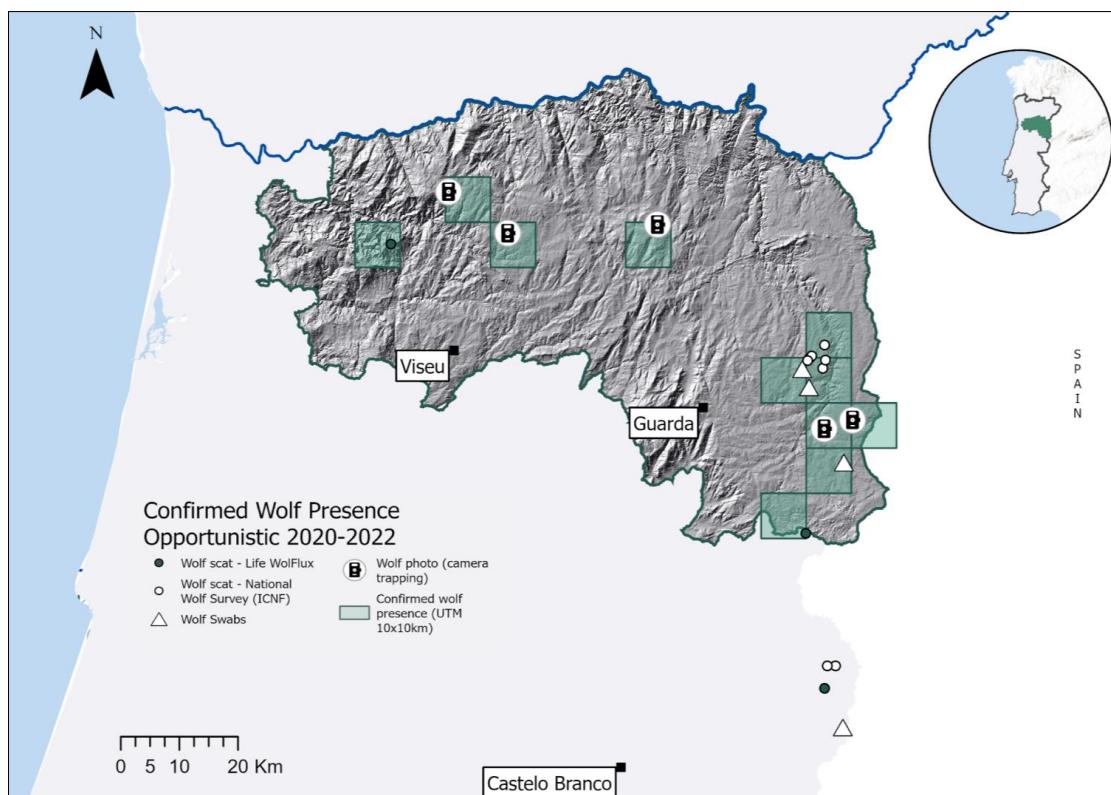


Figure 14 - Confirmed wolf presence during the opportunistic sampling period (2020-2022). Scat samples were collected by LIFE WolFlux and NWS (ICNF) surveying actions. LIFE WolFlux camera trapping and forensic analysis of canid attacks were very useful tools to confirm wolf presence during this period. For higher resolution please see Appendix 13.

Over the Central and Western sections, opportunistic camera trapping deployment and scat surveying allowed wolf presence confirmation along four more UTM 10x10 Km cells (Figure 15.)



Figure 15 - Wolf records obtained through camera trapping, in Trancoso pack territory, during the opportunistic sampling period.

In the scope of the project's Action D1 period (2023-24), it was possible to confirm wolf presence over 7 UTM 10X10 cells considering scat-only based surveying (Figure 16).

This result represents only two cells less than only-scat based on Action's A3 surveying results. As observed previously for Action A3 results, during D1, a substantial quantity of scat that remained unanalysed due to degradation suggests potential (unconfirmed) recent wolf presence or actual undetected wolf presence. This scenario may be the case for scat located over the border sites of confirmed wolf cells or in past confirmed presence areas obtained during D1 opportunistic sampling period and A3 timeframe (e.g. unanalysed and unsuccessfully analysed scat over the border with Spain – Easter Study Area Region)

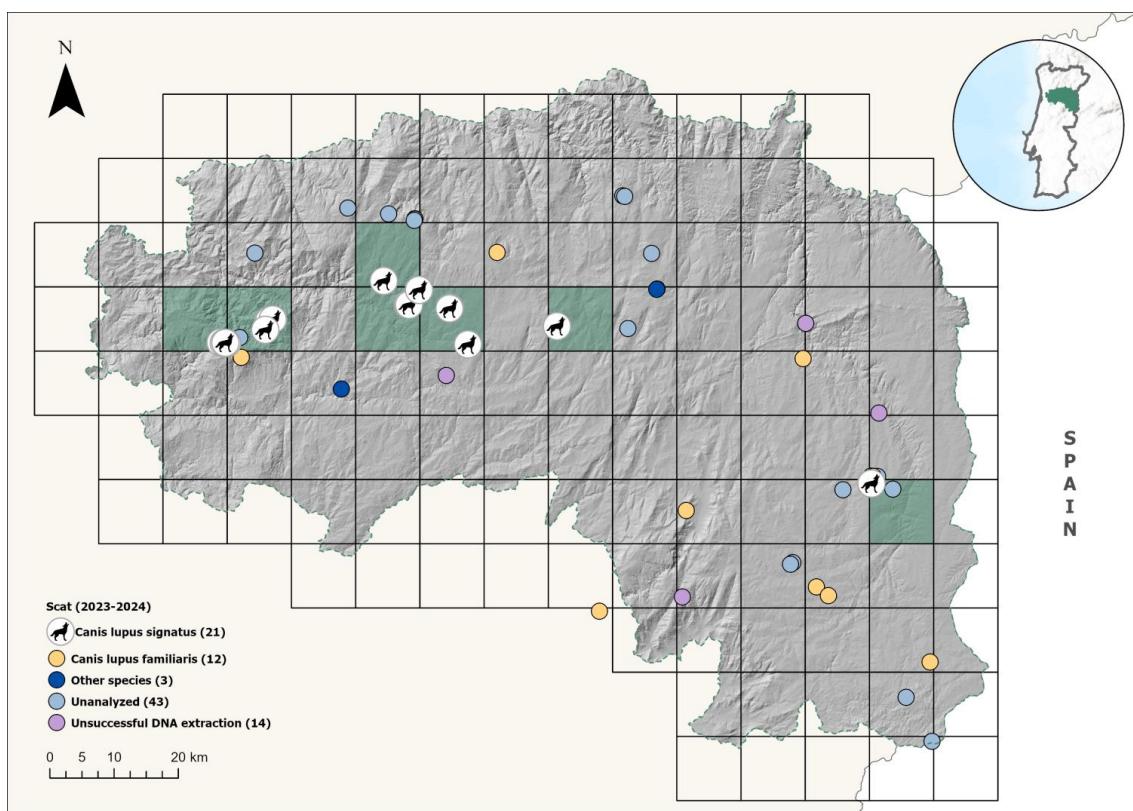
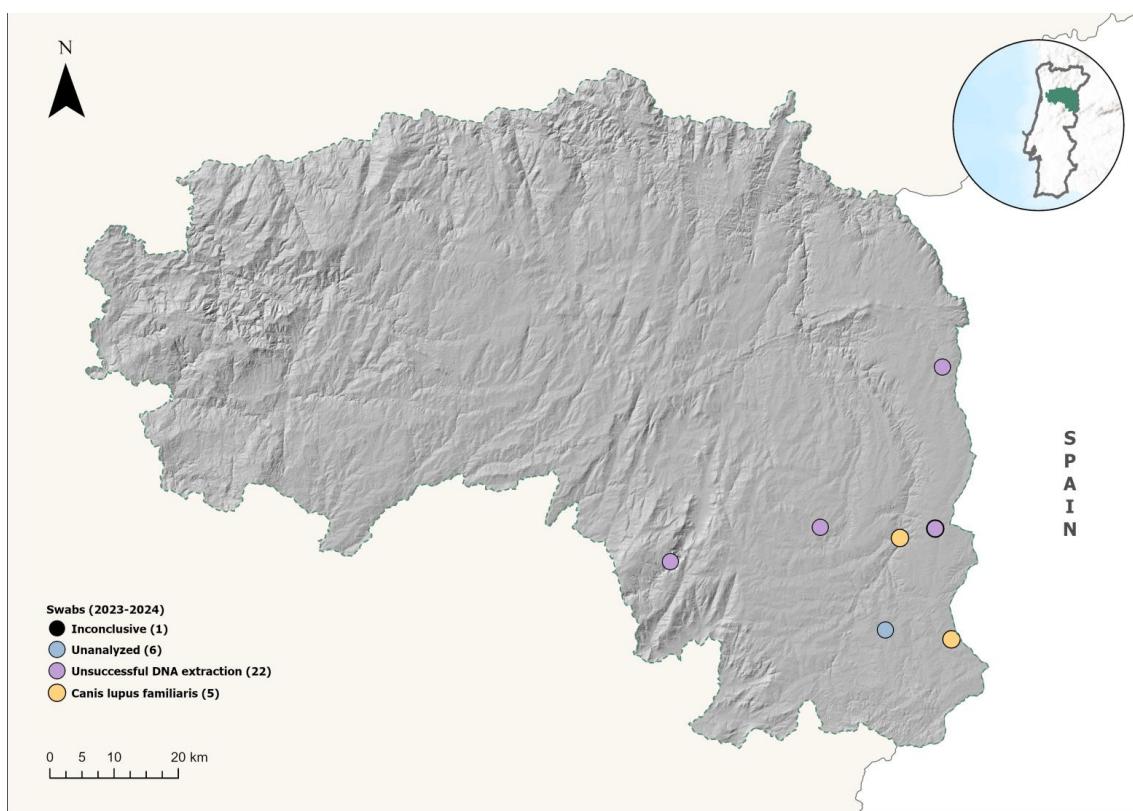


Figure 16 - Genetic assessment of scat collected between 2023 and 2024, in the scope of the project's Action D1. This representation includes scat that did not undergo genetic analysis, and green cells represent wolf presence confirmed by such NGS method. For higher resolution please see Appendix 14..

For Action A3's confirmed wolf range, swab sampling contributed crucially - allowing wolf presence confirmation over 11 UTM 10x10 Km<sup>2</sup>-, in which five were confirmed only by using this NGS method (please see Cadete et al. 2021). During that action, saliva samples were collected from 38 attacks to livestock, while during present Action D1 only 7 attacks were surveyed, producing no wolf presence confirmation results (Figure 17). This is primarily

attributable to the decision to collect saliva samples exclusively in the Eastern region of the project area.



*Figure 17 - Genetic assessment of swabs collected between 2023 and 2024, in the scope of project's Action D1. For higher resolution please see Appendix 15.*

#### 4.2.3 Individual Genetic Profiles

NGS allowed the successful genotyping of individual wolves in the project area's Western, Central, and Eastern sections. In addition to the 13 wolves (individual genetic profiles) reported in action A3, we retrieved 5 wolves from the scat and swab samples collected during action D1, and 2 more in the Castilla y León (Spain) region, in the scope of an agreement with Spanish authorities. Thus, a total of 22 wolves were documented throughout the project, 18 of which were recorded in the LIFE WolFlux area (Figure 18).

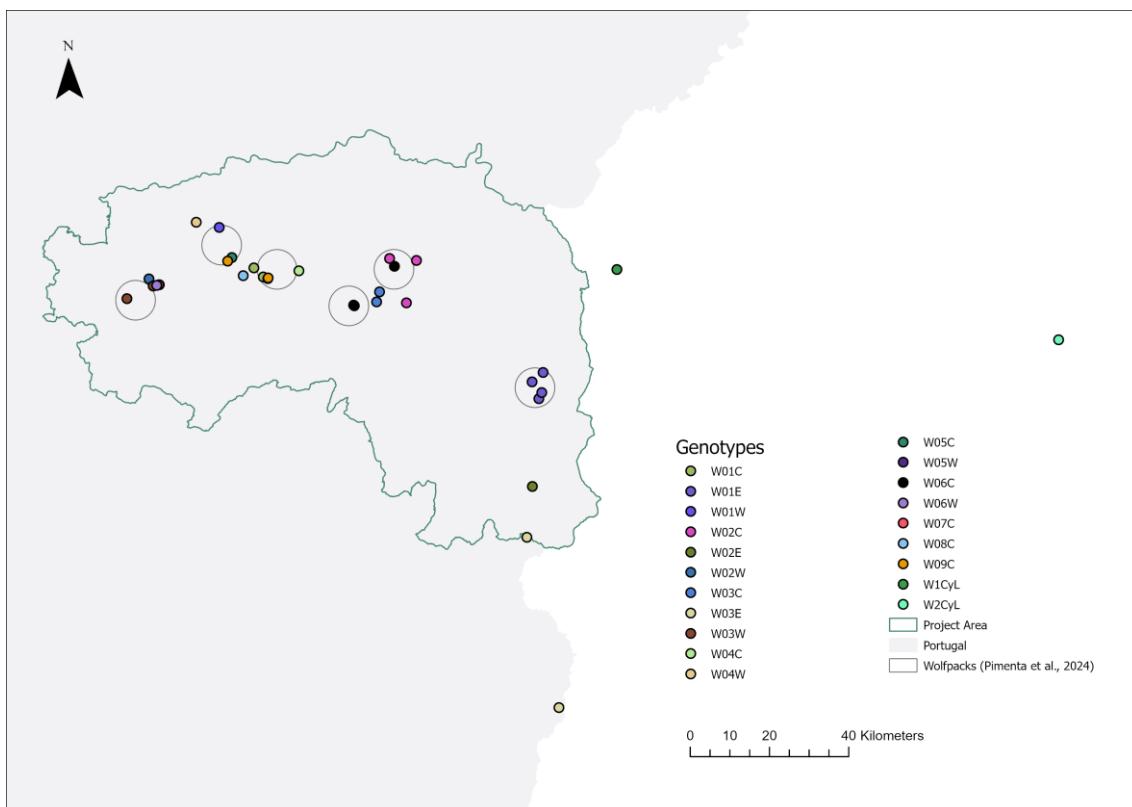
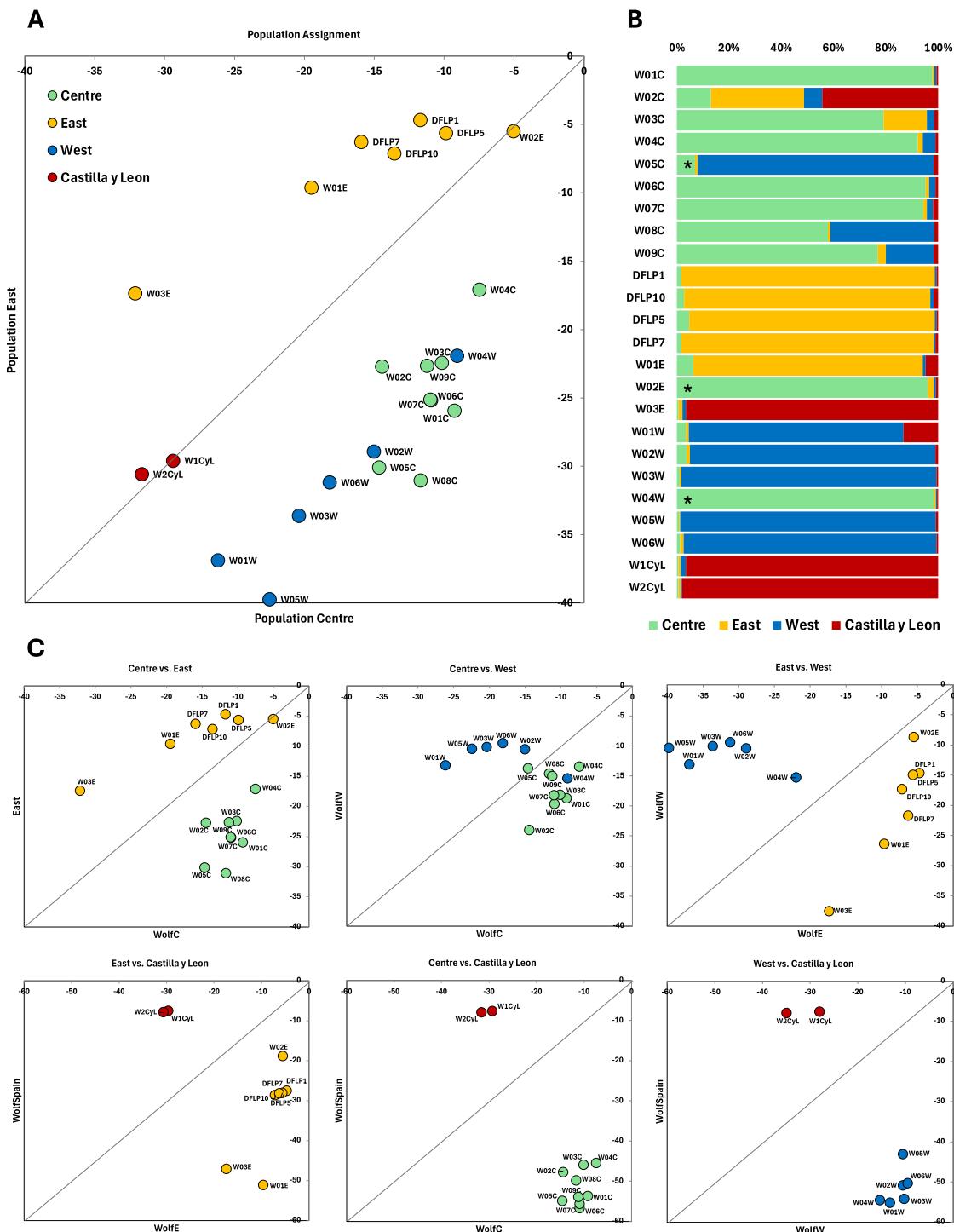


Figure 18 - Individual genotypes obtained by swabs and scat between 2019 and 2024 (A3 + D1). Empty larger circles indicate the location of detected wolf packs in the NWS (ICNF) – Pimenta et al. 2023. For higher resolution please see Appendix 16.

We used 24 microsatellite markers for genotyping the samples, but due to the overall low quality of the DNA, and to be able to generate data for a larger number of samples, we set a minimum threshold of 11 markers for generating an individual genetic profile, following the procedure described in the action A3 final report. New wolf genotypes were retrieved using an average of 20 markers (range: 17 to 23, two genotypes with 12 and 14 markers). Genotyping error – defined as the ratio of mismatched alleles over the total number of scored alleles (Pompanon et al., 2005) – was on average 2.1% for the Iberian wolf genotypes. The individual genetic profiles thus defined allowed the identification of 7 new individuals and 9 recaptures from previously sampled individuals (action A3) or newly reported individuals (action D1). These numbers must be considered as minimum numbers but not as population size estimates, because the number of genotyped samples and recaptures is still very low to allow accurate population size estimates.



**Figure 19 - Assignment of genotypes sampled throughout the project to sampling regions/inferred genetic clusters.** (A) Multi-population assignment test including all genotypes from the four sampling regions. (B) proportion of individual genotypes assigned to each of the four genetic clusters (closely matching the a priori division in Centre, East and West Portugal and Castilla y Leon). (C) Pairwise assignment tests between all pairs of regions. \* - genotypes assigned with high posterior probability (>90%) to a genetic cluster different from the region where they were sampled, in all methods (W05C, W04W and W02E).

The probability of identity – that is, the probability of two individuals show the same genotype in the population was conservatively estimated (considering a population of siblings and based on the 20 Iberian wolf genotypes identified during the project) as being  $7.8 \times 10^{-4}$  (considering 11 markers, minimum number of loci in a genotype) or  $5.2 \times 10^{-6}$  (considering 20 markers, average number of loci per genotype) for wolves. During action D1, four wolves (genotypes W01E, W03E, W03W and W09C) were sampled more than once. In the following analyses, only one sample was used per individual genotypes, so 18 recaptures (9 from baseline A3 monitoring and 9 from after the baseline monitoring) were removed from the dataset. On the other hand, four additional samples (DFLP1, DFLP 5, DFLP 7 and DFLP10), sampled in 2015 in the eastern area of the study area, where included as reference samples in the following analyses. As in the final report from action A3, Iberian wolf genotypes were compared (by means of ordination analysis and population assignment procedures) with dogs from Central Portugal in search for hybrids, previously reported to the area (R. T. Torres et al., 2017). No evidence of hybridization was found, as in the A3 final report, so we did not find relevant to provide further detail of these results in this report. In most cases, wolves from different regions were also unambiguously assigned to their putative populations with exception to three individuals that were assigned to genetic clusters/populations different than those where they were sampled: W04W (sampled in the West region but clearly assigned to Centre genetic cluster); W05C (sampled in the Centre region but clearly assigned to West genetic cluster); W02E (sampled in the East region but clearly assigned to Centre genetic cluster). These genotypes were clearly misassigned in both methods). A fourth genotyped (W03E) was assigned to a genetic cluster shared with genotypes from Castilla y Leon by the Bayesian clustering algorithm but that affinity was not retrieved by the other methods, despite W03E does differ substantially from the other genotypes from other regions. While these results do suggest a high degree of heterogeneity in the genotypes sampled in the East region – where Iberian wolf presence is more scattered and irregular – these results should be viewed carefully because of the scarcity of data (including genetic data), in particular in the East region. While these misassignments all refer to genotypes sampled during the A3 action, the clarification of these patterns was only possible after integrating additional data collected in action D1. The misassignment of genotypes to genetic clusters (gene pools) from regions other than the one where they were sampled are, nevertheless, evidence of migration and likely gene flow. As expected, wolf packs occurring in the Centre region have an important role in this exchange of individuals with West and East regions. Available data are scarce, and results should be viewed

carefully but our results also suggest that there might a role of Spanish populations, on the other side of the border, in the survival of Iberian wolf in the Eastern Portugal border area. Through a non-invasive approach, wolf tagging was conducted using individual genetic profiles to provide a Capture-Recapture analysis. Eight individual wolves were captured and recaptured throughout A3 plus D1 period. This analysis provided data both on short to medium scale connectivity and survival rates (Figure 20 and Figure 21). Extraterritorial incursion of individual wolf W02 between known ranges of Leomil, Lapa and Trancoso packs and a medium to large scale dispersal of wolf W03E from the southern limit of the study area (Malcata Natural Reserve) to Castelo Branco region, indicate habitat connectivity (landscape permeability). W03 CR analysis show a minimum survival of 4 years and landscape permeability over Arada mountain area. Regarding the eastern area, a probable solitary male wolf shows small scale movements since he was first NGS tagged in 2019 in the north of A25 highway, until last recapture in 2021 in the south of A25 (wolf W01E in Figure 21). It is likely that this is the same individual that was subsequently recaptured in an inferior passage on A25 in 2024 using camera trapping (Figure 22) – which indicates a minimum five-year survival in the eastern region. Apart from animal W09C CR analysis which suggests connectivity between Leomil and Montemuro Pack (Central and Western study area sections), no other CR data suggest medium to large scale connectivity between the three geographical sectors defined along the study area. Nonetheless, other data, such as migrants from the first generation indicate that a level of connectivity does exist in the project area.

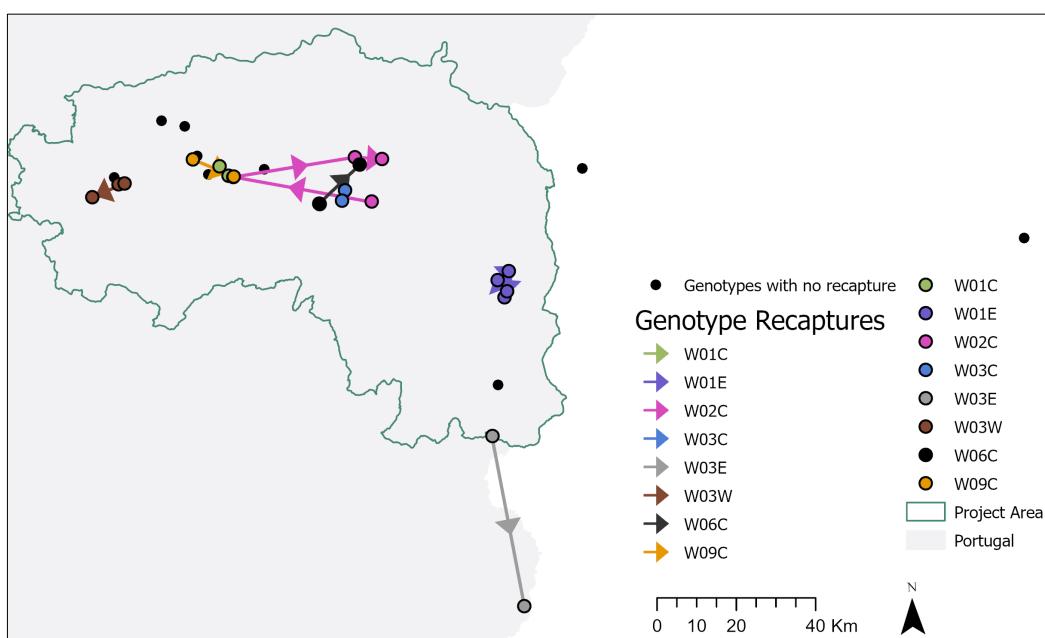


Figure 20 - Genotype Capture-Recapture (CR) obtained between 2019 and 2024 (A3 + D1). For higher resolution please see Appendix 17

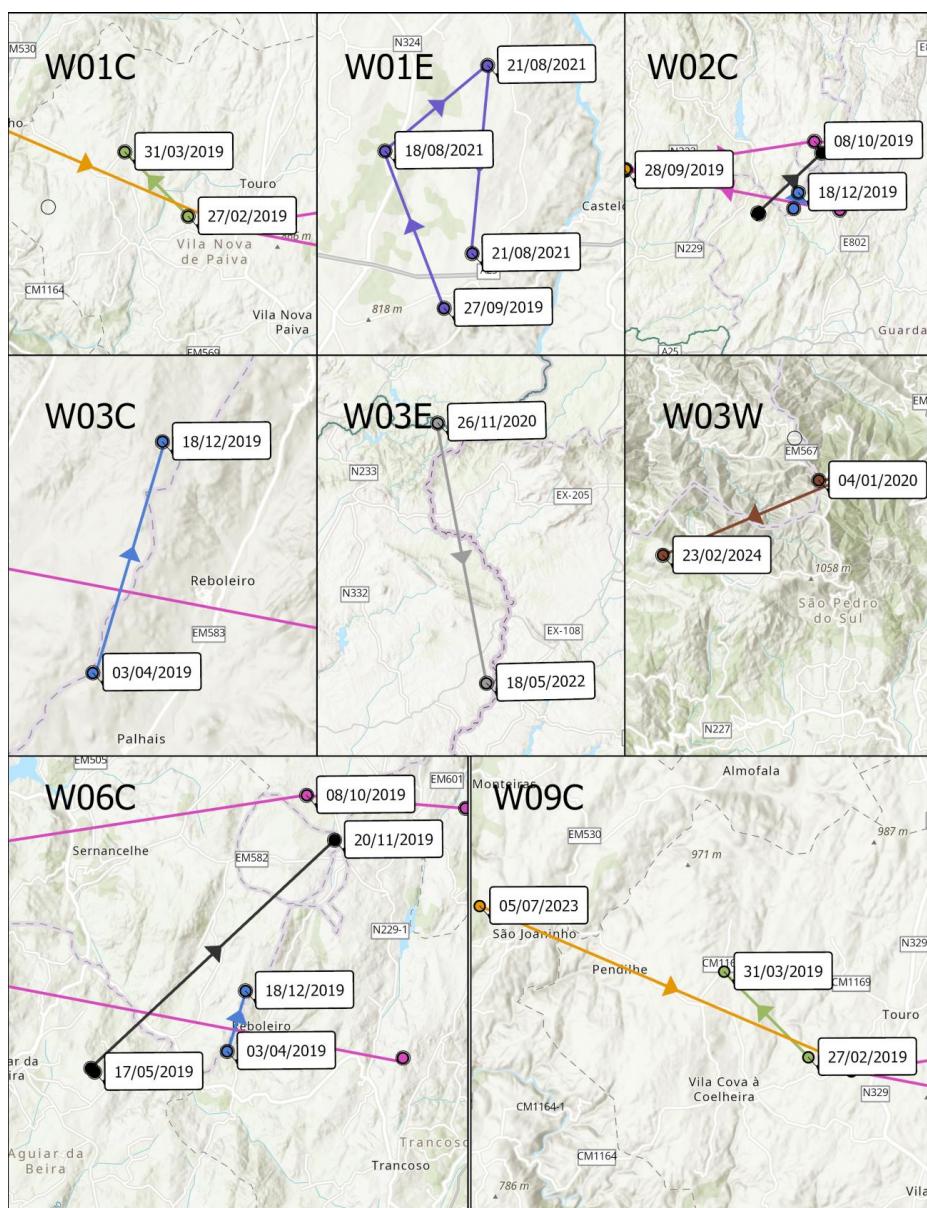


Figure 21 - Spatial overview of individual genetic profiles CR between 2019 and 2024 (A3 + D1). For higher resolution please see Appendix 18



Figure 22 - A winter 2024 photograph of probable male wolf WO1E in the same territory where he was first genotyped in 2019 and later recaptured three times throughout 2021. Photo kindly shared by ASCENDI (highway management company).

### 4.3 Attacks on Livestock

Between 2019 and 2023 there were 434 attacks declared to the authorities, of which 410 (94%) has been assessed as wolf attacks. The number of declared wolf damages has decreased over time (Figure 23). As stated in the report of action A.6 we believe this reduction is influenced by a change in the damage compensation system that entered into force in 2017 together with a reduction in wolf presence in certain areas, as shown in the wolf monitoring data presented in this report. The average of wolf attacks per year in the period 2012-2015, prior to the approval of the new damage compensation law, was 453, whereas the average number of wolf attacks during the period of execution of the LIFE WolFlux (2019-2023) is 82 attacks/year.

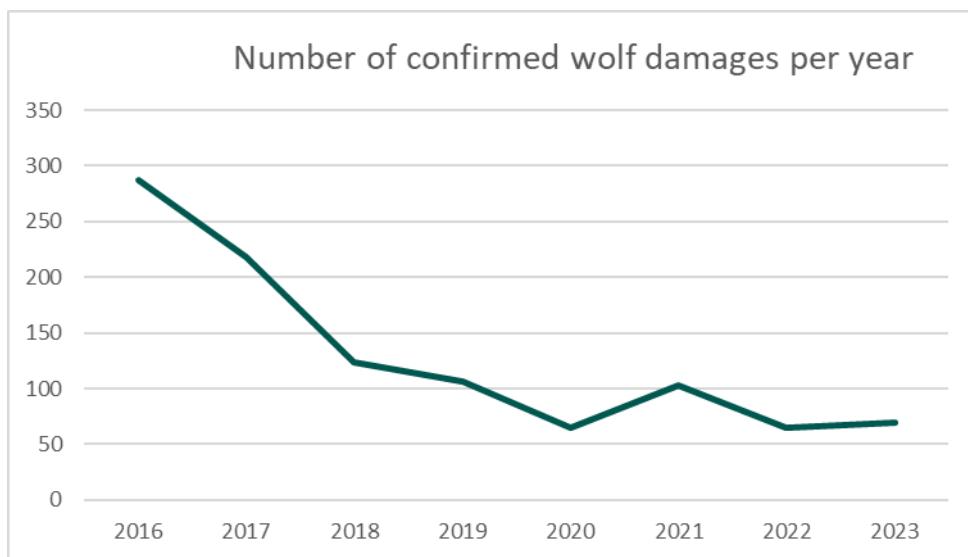


Figure 23 - Number of confirmed wolf attacks in the LIFE WolFlux area in the last 8 years.

Sheep are the most frequently affected livestock in wolf attacks (Figure 24), and the district of Guarda registers the highest number of attacks (Table 2), despite the majority of wolf packs being concentrated in the Viseu and Aveiro regions. This discrepancy is attributed to the parish of Reboleiro in Trancoso, which accounts for 58% of the attacks recorded in the Guarda district.

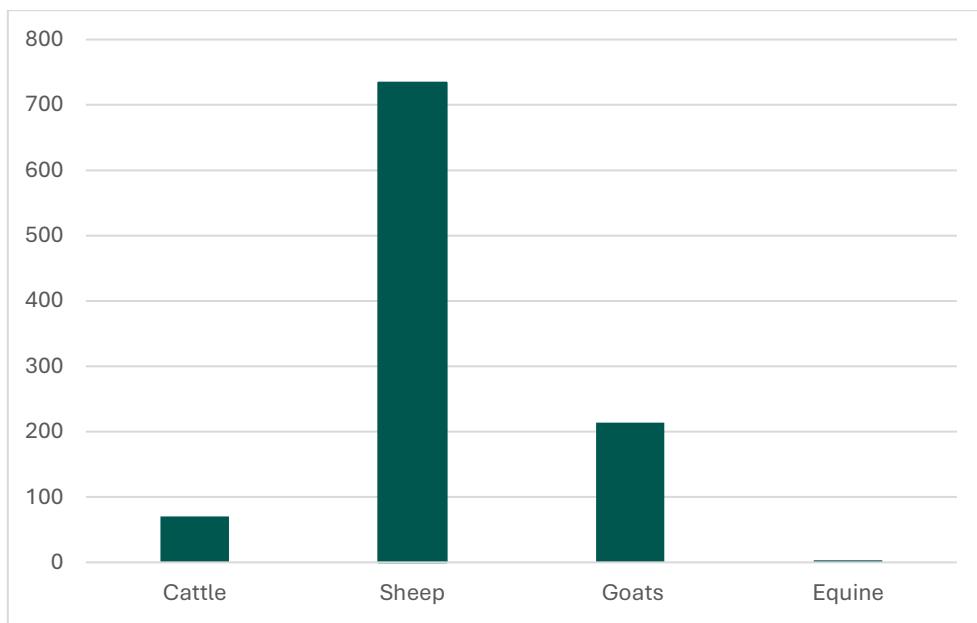


Figure 24 - Number of animals damaged (injured or killed) in declared wolf attacks in the LIFE WolFlux area between 2019 and 2023.

Table 2 - Number of wolf attacks declared per district, between 2019 and 2023.

District	Number of attacks
AVEIRO	44
CASTELO BRANCO	7
GUARDA	247
VISEU	112
	410

#### 4.4 Population Trend (2019-2024)

Figure 25 illustrates the area of confirmed wolf presence from 2019 to 2023/2024, utilising data from LIFE WolFlux and associated projects, including ACHLI wolf monitoring surveys (implemented by CIBIO and Aveiro University teams) and the National Wolf Survey (NWS). It is noteworthy that only data that included dates were incorporated. Consequently, the NWS confirmed a more extensive area of wolf presence than what is depicted in Figure 25, as it represents the total confirmed wolf presence between 2019 and 2021.

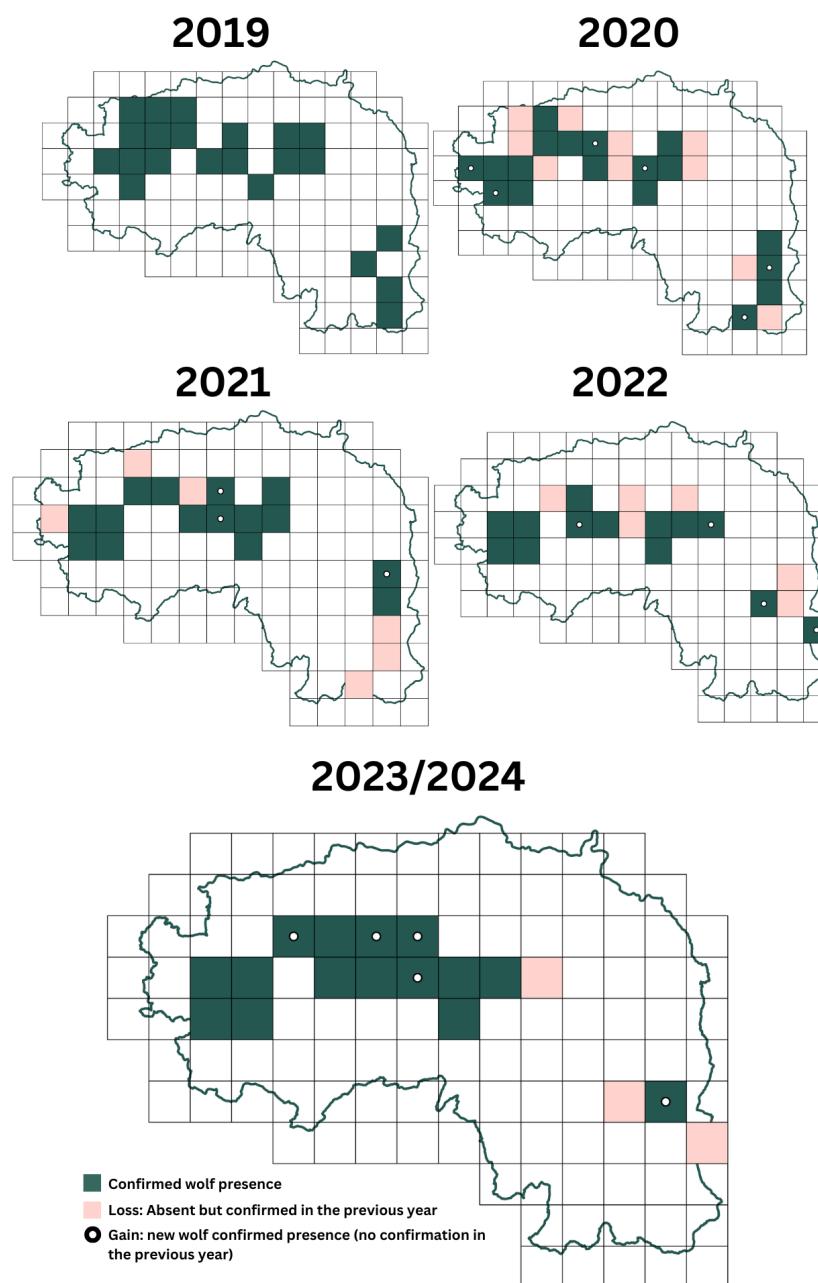


Figure 25 - Population trend during LIFE WolFlux project reveals spatial instability. Data provided by the LIFE WolFlux project and the protocol established with ACHLI. For higher resolution please see Appendix 19..

Losses (UTM cells with no wolf presence confirmation but that were confirmed in the previous year, depicted in pink) and gains (UTM cells with wolf presence confirmation but that were not confirmed in the previous year, depicted with a white dot) occur in all regions of the project area. As a result, there is no clear spatial pattern of wolf decline in the project area. Moreover, the evolution of wolf presence over the years shows significant instability.

Throughout the project's duration, a consistent downward trend in confirmed wolf presence has been observed within the study area. While 2019 marked the peak of confirmed wolf presence, a notable decline occurred in 2020. Subsequently, the confirmed presence has persistently remained at levels lower than those recorded in 2019. Despite extensive monitoring efforts in 2023/2024, it was only in the final phase of the project that the area of newly confirmed records exceeded the losses, indicating a slight increase in the confirmed wolf distribution (Figure 26).

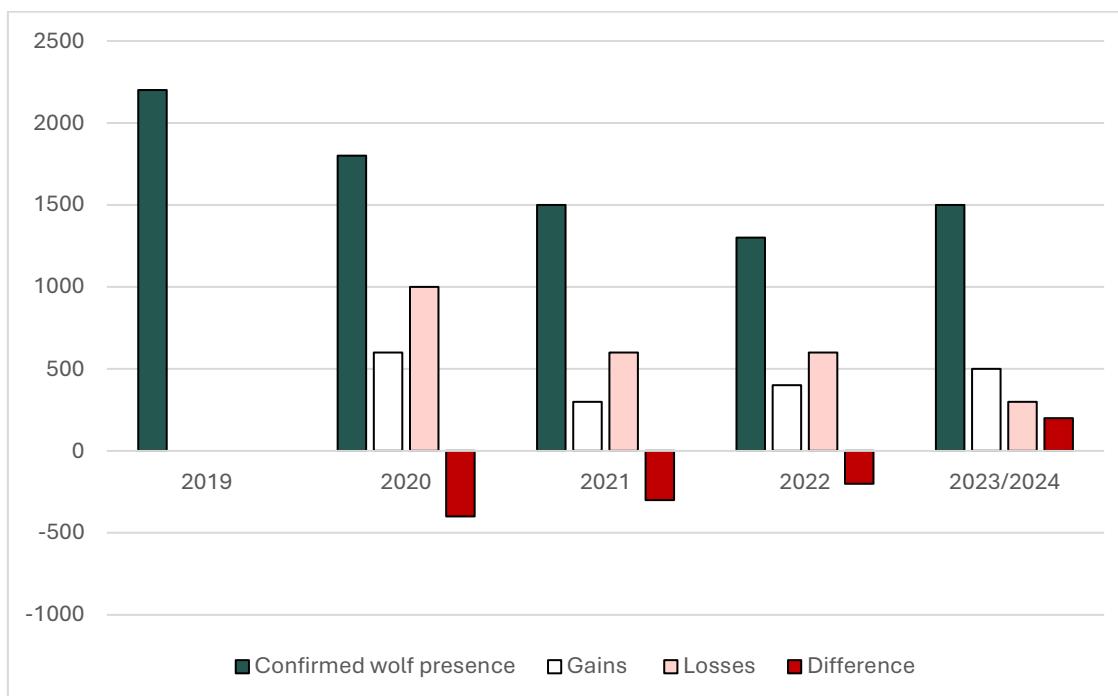


Figure 26 - Trend of confirmed wolf presence during the project period. Data provided by the LIFE WolFlux project and the protocol established with ACHLI.

The varying survey efforts across different years make direct comparisons challenging and potentially misleading. Despite these limitations, the data presented in Figure 25 and Figure 26 still provide valuable insights into wolf population trends and distribution patterns over time, which we believe are relevant enough to be considered for management and conservation decisions.

The direct comparison between 2019 (A3) and 2023/2024 (D1) (Figure 27) shows a similar lack of spatial pattern as seen in Figure 25. Wolf presence decreased in the project area's eastern, central, and western sections; however, gains were only detected in the western and central regions of the project area.

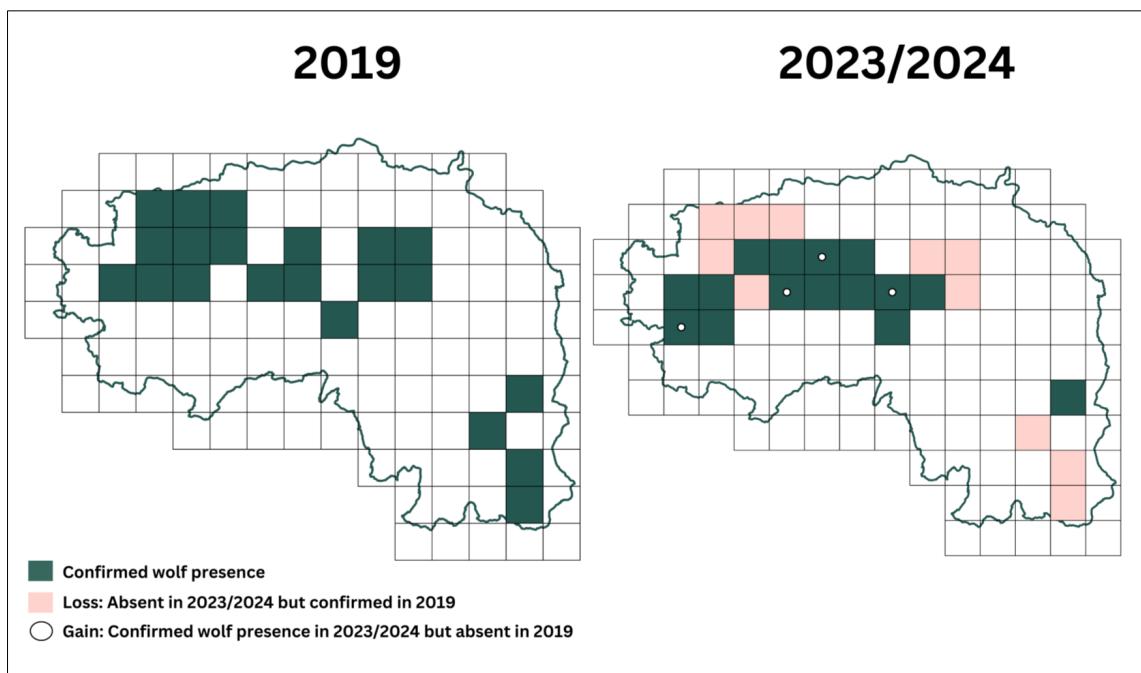


Figure 27 - Comparison of wolf presence in the project area in 2019 and 2023/2024. Data provided by the LIFE WolFlux project and the protocol established with ACHLI. For higher resolution please see Appendix 20.

The overall comparison between 2019 and 2023/2024 (Figure 28), shows that from the initial 2100 km<sup>2</sup> of wolf confirmed presence, 700 km<sup>2</sup> were not re-confirmed by the end of the project.

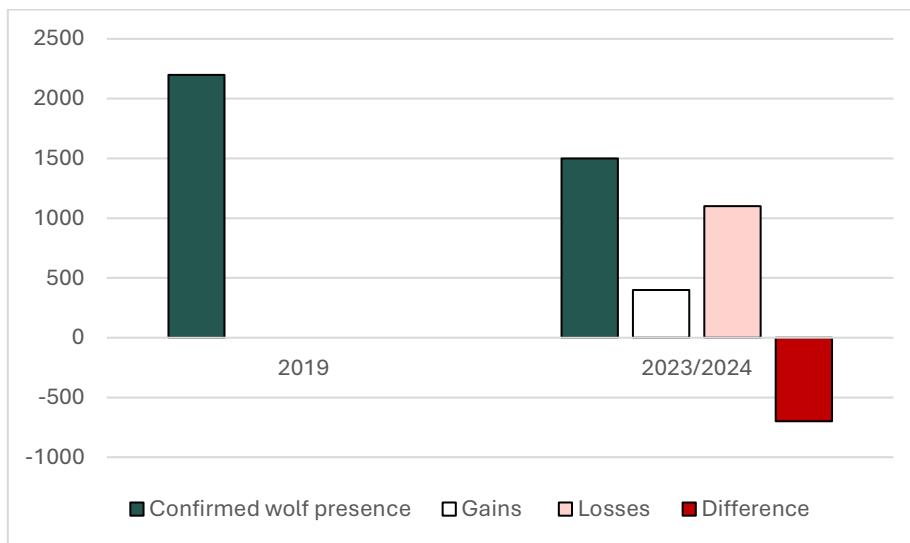


Figure 28- Comparison of confirmed wolf presence area between 2019 and 2023/2024.

## 4.5 Feeding Ecology

We analysed 36 genetically confirmed wolf scats in 2020 and 20 in 2024 (Figure 29). Wild boar remains at approximately the same frequency as in the first report (15-20%; Cadete et al., 2021). Neither lagomorphs nor mesocarnivores were recorded in the samples analysed,

contrary to what was previously reported. There was a higher percentage of goats and a greater representation of sheep (Fig 26). At the time of the first report, wild prey accounted for between 20 to 25 % of the wolf's diet in the study area. Now, that percentage ranges between 25 and 30 %. An important result should be highlighted: the appearance of roe deer in 10% ( $n = 2$ ) of the samples analysed, which matches the 10% increase in the representativeness of roe deer envisaged in the LIFE indicators (Action D2).

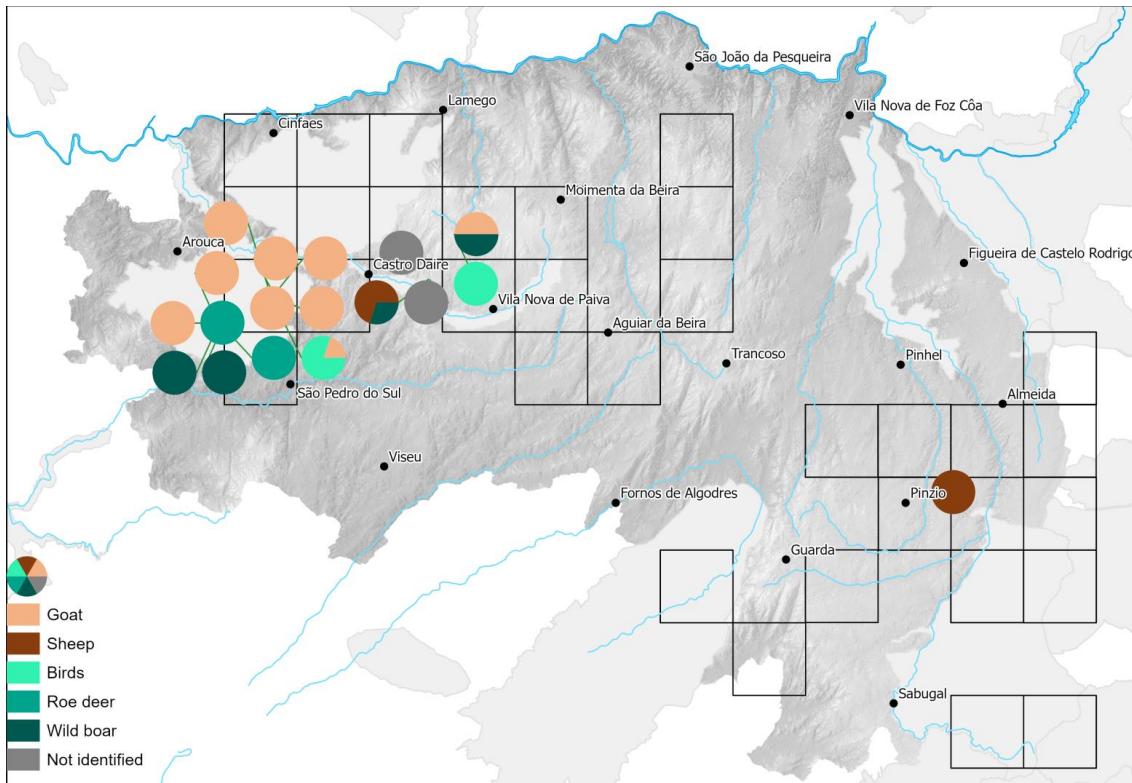


Figure 29 - Spatial overview of feeding ecology results. For higher resolution please see Appendix 21.

## 5. Ecological Modelling

Ecological niche modelling is an invaluable tool for understanding species distribution patterns, particularly for those inhabiting fragmented, human-modified landscapes, such as the Iberian wolf. Hence, in the scope of this project, a Species Distribution Model (SDM) was developed (Figure 30). The ability to predict habitat suitability for this species, which faces various conservation challenges, requires careful consideration of both environmental and anthropogenic factors. Previous research has underscored the importance of variables like topography, land cover, prey availability, and human

disturbance in defining suitable habitats for the wolf. The current distribution model introduces a new variable — distance to intensive livestock production units, particularly poultry and rabbit farms. Despite its potential significance, this variable has not been previously included in ecological niche models for the Iberian wolf. Intensive farming units can serve as crucial food resources, especially in regions south of the Douro River where wild prey may be limited. By integrating this variable, we aimed to generate a model that provides a more accurate representation of the wolf's ecological niche and better reflect the complex interactions between wolves and anthropogenic landscapes.

The model (Figure 30), developed using the MaxEnt algorithm, incorporates 155 confirmed wolf presence records collect during LIFE WolFlux project, and environmental variables such as altitude, distance to roads, livestock density, land cover, and human footprint. Model evaluation via the area under the receiver operating characteristic curve (AUC) yielded a value of 0.902, indicating strong predictive accuracy. Furthermore, an omission rate analysis demonstrated that at higher thresholds, the model adopts a more conservative stance, potentially underestimating wolf presence but reducing false positives.

Spatial analysis of model outputs highlights a complex pattern of habitat suitability, with optimal areas primarily concentrated in the western and central parts of the project. These areas, characterized by higher elevations and relative continuity, suggest a stable ecological niche that supports wolf presence. In contrast, the eastern region reveals fragmented, less suitable habitat, potentially limiting gene flow between western and eastern wolf populations. This spatial discontinuity could contribute to further population fragmentation, heightening conservation challenges for wolf populations south of the Douro River.

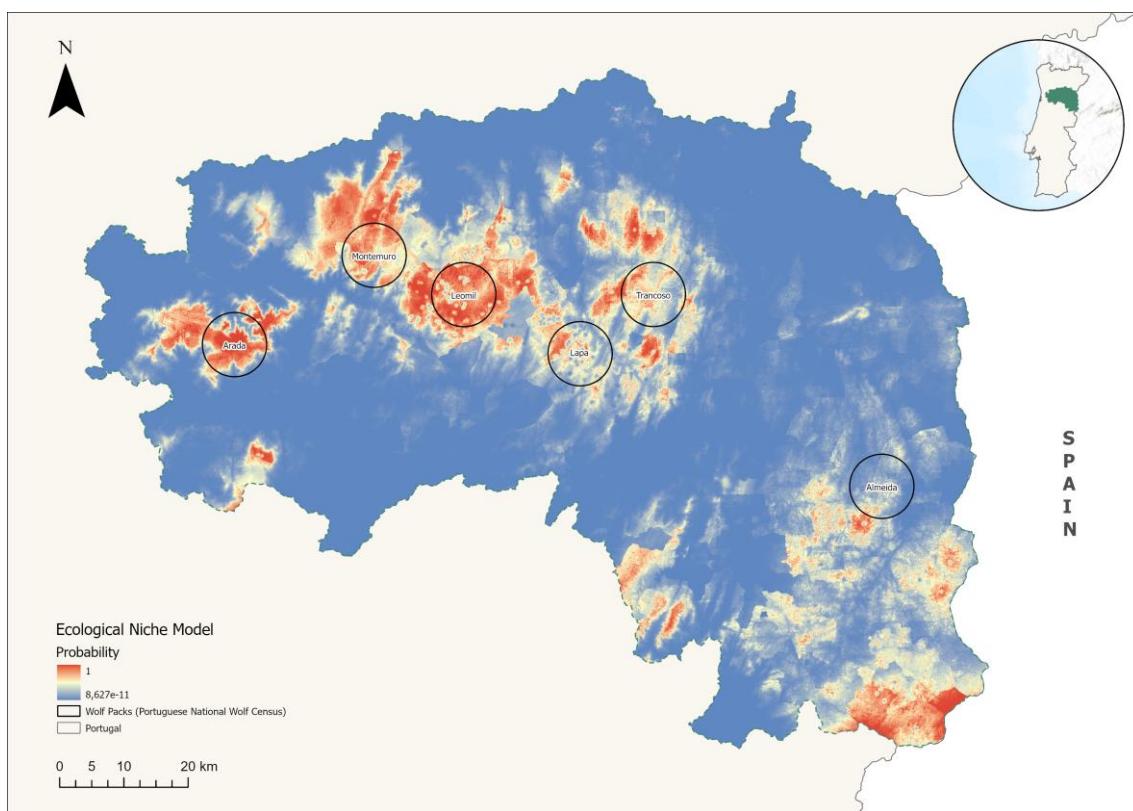


Figure 30 - Ecological Niche Model produced using MaxEnt, using all wolf data collected in the scope of LIFE WolFlux's project. For further information, please see Annex 1.

Variable analysis revealed that altitude was the most significant factor, with wolf presence probability peaking between 1,200 and 1,300 meters, suggesting a preference for reduced human presence at higher elevations. Distance to intensive farming emerged as a secondary but crucial variable, with higher wolf presence near these units, reflecting the species' reliance on anthropogenic food sources in prey-scarce areas. Additionally, increased distance from roads was associated with higher wolf presence, indicating the species' tendency to avoid human-impacted areas. Livestock density and land cover types, such as shrublands, also contributed to the model, while human footprint values inversely correlated with habitat suitability, highlighting the negative impact of human pressure on wolf distribution.

Another study (Pinto & Costa, 2023), has generated a cost surface model to identify areas where wolf movement has low cost (i.e., areas with more suitable habitat) and where it faces the greatest resistance. This model incorporated wolf presence data obtained in the scope of Action A3 (2019) and variables such as terrain ruggedness, distance to roads and railways, land cover, and burned areas to reflect landscape permeability.

Results indicate that only 15.7% of the area presents low-to-moderate movement costs, primarily concentrated in the western parts where natural habitats are less fragmented. The central region, however, poses a higher degree of connectivity challenges due to the prevalence of anthropogenic barriers, which can hinder gene flow between wolf populations.

The primary connectivity corridor, spanning 174.7 km, links key wolf territories but crosses critical points such as highways and secondary roads (Figure 31). However, it is important to point out that for this study's analysis, wolf confirmed presence data prior to Project's LIFE WolFlux start was not included, namely those located on the Northeastern limit of the study area (please see Cadete et al., 2012; Cadete et al., 2015). Such findings would generate another corridor connecting the Northern eastern region of the study area with Trancoso pack range and subsequently with Lapa and Leomil pack ranges.

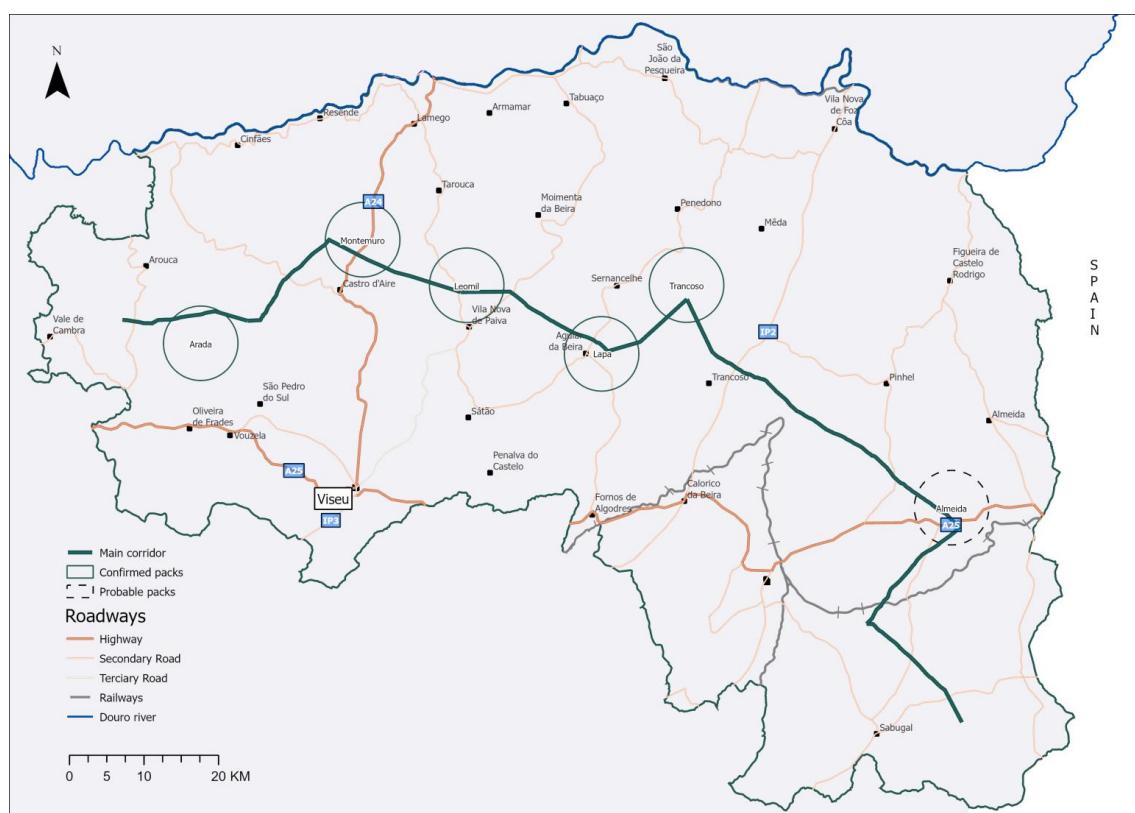


Figure 31 - Connectivity corridors of wolf movement, determined by a cost surface model that used A3 wolf presence data among other variables. Adapted from Pinto & Costa, 2023. For higher resolution please see Appendix 22.



# Discussion

## 6. Discussion

### 6.1 Methodological Considerations

A multi-source NGS program (forensic analysis, scat molecular analysis and camera trapping) was first implemented in the 2010/11 period to study a Portuguese Iberian wolf population nucleus in the Portuguese border region south of the Douro River (Cadete et al. 2012). The aim was to enhance the collection of wolf demographic and genetic data. Following projects in the same area deployed this same methodological approach (Cadete et al. 2015, Palacios et al. 2016). During project LIFE WolFlux project, this same multi-source approach was applied, now for the first time in Portugal in a much broader geographical scale. A new survey method was also added – The Scat Detection Dog Team with an improvement in scat sample size. Partnerships were established and managed with national governmental and non-governmental organizations to optimize data gathering and minimize survey overlapping. Although data collection over the entire south of Douro River wolf range still showed to be highly challenging and demanding due to target species low density and several other factors (please see Cadete et al. 2012), typical of humanized landscapes, this multi-source integrated monitoring methodological model proved to be efficient and long-term sustainable along the Portuguese south of Douro River wolf range.

The non-invasive integrated monitoring program proved to be the optimal strategy to improve demographic and molecular data collection among project's area, especially among its Eastern section where target species' abundance was known to be extremely low. We have shown that efforts to monitor the entire south of Douro River wolf subpopulation must imply a multiple data source system capable of performing species' demographic and genetic regular status assessment. Additionally, we have demonstrated the importance of establishing collaboration protocols with different organizations and smaller geographical scope projects, in a cooperative model, thus avoiding survey overlapping, increasing the data set and preventing target species' unnecessary disturbance. Demographic and genetic data on this wolf population still showed to be very difficult to obtain, as expected, due to its low abundance, biology, etho-ecological traits and practical constraints related with the landscape and its humanized condition.

The integration of molecular analysis has yielded valuable insights into population structure, connectivity, and gene flow. However, the consistent decline in population size

and the limited success in detecting new individuals indicate that more robust conservation measures are urgently required.

## 6.2 Comparison between Action A3 (2019) and D1 (2020-2022) opportunistic sampling

The LIFE WolFlux Project recognized that the Iberian wolf population south of the Douro River was highly vulnerable and faced severe threats, posing a significant risk of local extinction (Pimenta et al., 2005)

Addressing this conservation challenge was one of the primary objectives of the project, and the baseline monitoring has reinforced this concern (Cadete et al., 2021). The situation along the Spanish border was especially alarming when analysing the overall demographic species status over the study area.

Despite substantial efforts since the project's inception to collect samples and establish partnerships to enhance sample size, evidence of target species' presence in the eastern region remained sparse, indicating a very low number of individuals in that area. Much of the target species information from this region has stemmed from reported livestock attacks. Nevertheless, it is notable, at a project overall scale, that approximately 60% of all monitored attacks between April 2019 and November 2021 were attributed solely to domestic dogs (Lino et al., 2023).

Thus, during Action D1, we intensified efforts on scat and swab sampling in the area where the knowledge gap on the species was greater: the Eastern region South of River Douro. The stronger emphasis, after establishing the baseline scenario, on an area where Iberian wolf was rarer and its presence was more irregular resulted on a decrease on the percentage of detection of Iberian wolf (from 42% to 21%,

) in livestock attacks (though the actual number of analysed attacks did not differ substantially, from n=38 in A3 to n=30 in the following period). Nevertheless, focusing survey efforts on this region did not significantly impact the detection success of target species in collected and analysed scats. In fact, both total counts (37 to 34) and proportion (47% to 54%) of confirmed Iberian wolf scat were equivalent between both periods. The number of retrieved genotypes and newly sampled individuals was lower during the opportunistic monitoring and action D1 than during action A3 (

) though the number of recaptures was equivalent, namely from individuals originally sampled during action A3 (such as W01E and W03W). New data and new genotypes sampled after the baseline study, allowed us to clarify the position of same genotypes (e.g. W05C and W04W) that were more strongly assigned, this time, to a region other than the one they were sampled, as well as to identify additional mis-assigned individuals (e.g. W02E).

### **6.3 Comparison between Action A3 (2019) and D1 (2023-2024) systematic sampling**

Despite extensive conservation efforts, the South of Douro wolf population continues to be one of the most critically endangered wolf nuclei in the Iberian Peninsula (Silva et al., 2018). This population has faced numerous challenges and threats, including habitat loss and fragmentation, human-wildlife conflict, genetic isolation and low effective population size (Pimenta et al., 2023). The prolonged isolation from other wolf populations has resulted in a substantial decrease in genetic diversity, which is already evident in osteological abnormalities and increased susceptibility to virus infections, such as parvovirus, against which this wolf population lacks antibodies (Barroso et al., 2016)

The A3 methodology, which has demonstrated high efficacy, was implemented during the D1 systematic survey (2023/2024), and most transects were replicated. Despite comparable effort, the yielded results exhibit discrepancies. During Action A3 (February 2019 - 14th January 2020), out of 195 collected scat samples, 79 were not submitted for laboratory analysis due to insufficient quality, 4 were not successfully extracted, 30 were duplicates, 39 were attributed to dogs, 6 to other species, and 37 to wolves. In comparison, during the systematic sampling period of Action D1, 86 scat samples were collected, of which 20 were not analysed because were replicates, 12 were attributed to dogs, 3 were attributed to other species, 11 were not successfully extracted, and 21 were attributed to wolves, representing a decrease of 16 wolf scat compared to Action A3. Even though the possibility that during a screening survey program, stochastic and external factors could have negatively affected D1's Scat Detection Dog Team (SDDT) results (e.g. challenging survey weather and terrain conditions during) or that other more severe and/or permanent interferences may have occurred (e.g. habitat loss and/or quality decrease, increased domestic dog sympatry) may be considered, findings sustain an overall decreased wolf presence throughout the study area, specially over its Central and Eastern sections. The

kilometric abundance index (KAI) values exhibit a consistent decreasing trend: those obtained during D1 are substantially lower than those of A3. Spatially, a substantial disparity exists between the central, especially in the area attributed to Trancoso pack, and eastern regions, which was not observed during the A3 period.

Regarding wolf presence, during Action A3, the LIFE WolFlux project confirmed 18 UTM cells 10x10km, whereas in Action D1, only 7 cells were confirmed. Upon analysing the aggregate data from LIFE WolFlux and ACHLI, the same negative trend is observed: of the initial 21 confirmed UTM cells in 2019, only 15 were reconfirmed in 2023/2024. Despite this significant difference, 5 of the currently unconfirmed wolf presence UTM cells were previously confirmed solely by swabs collected during livestock attack inspections (Figure 32).

During the D1 systematic period, however, only 7 attacks were inspected, and none of the swabs were assigned to wolves. Therefore, the explanation for this substantial decrease may be attributed to the modifications imposed by the new legislation regarding damage inspection procedures by ICNF, to the sampling being concentrated in the Eastern region of the project area, and/or to an actual reduction in livestock depredation events.

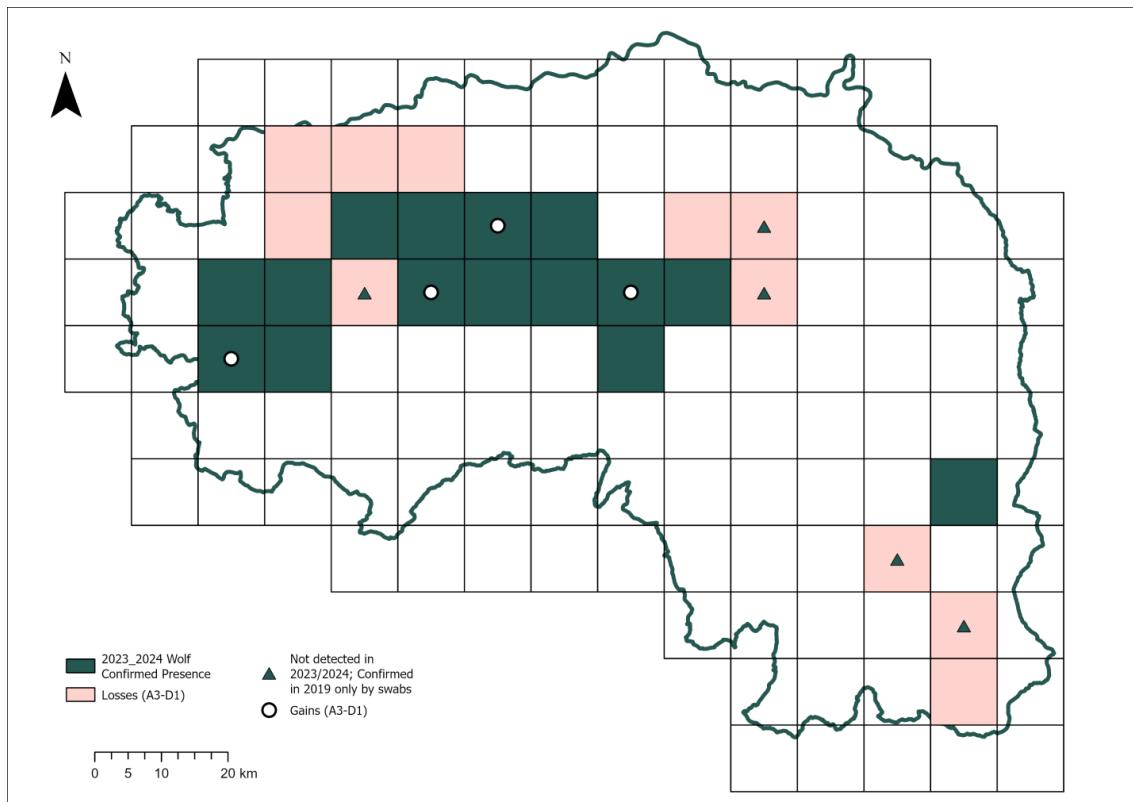


Figure 32 - Wolf confirmed presence during D1 Action (2023/2024). Data: LIFE WolFlux project and ACHLI protocol (CIBIO and Aveiro University teams). Losses are depicted in pink and represent those UTM cells which

were confirmed in Action A3, but not in D1; green triangles show the UTM cells that were confirmed solely by swab genetic assessment during A3. Gains are illustrated by white dots and represent those UTM cells which were not previously confirmed in Action A3. For higher resolution please see Appendix 24.

Such a limited amount of data, particularly to what refers to genetic data and Iberian wolf genotypes, necessarily limits the establishment of a baseline scenario and the comparison between the baseline and the end of the project. This could suggest that results can be biased due to a smaller sampling effort. However, such a limited amount of data is more a result of the low level of presence of Iberian wolf in the territory and the difficulties of handling non-invasive genetic sampling techniques. In fact, more than 530 samples (comprising scats and swabs from livestock attacks) were collected and analysed throughout the project, more than doubling the number of samples that were originally planned in the project. Also, the success rate on identifying Iberian wolf samples and retrieving genotypes was high, considering the nature and particularities of non-invasive genetic sampling. Such a low amount of information from such a large monitoring effort is an alarming result in itself.

## 6.4 Connectivity

Although species distribution modelling has indicated that the most suitable wolf habitat is located in the western area (which is indeed the least humanized region of the project area), there is limited evidence supporting high connectivity between Arada and Montemuro packs with extraterritorial movements, despite over two decades of research (Table 1). Natal habitat-biased dispersal has been attributed to Iberian wolves (Silva et al., 2018), which means that individuals tend to disperse to familiar landscapes. In this context, low connectivity among Arada and Montemuro is particularly unexpected because both regions share the same landscape features and visible barriers between the two mountains - the national road N225 and Paiva river - should have minimal impact. We posit that the principal distinctions between these two regions may be attributed to practices associated with animal husbandry systems, which in the Montemuro pack might reduce livestock accessibility to wolves. However, further investigation is necessary to determine if this is indeed the case, and if so, measures such as the reinforcement of wild prey reintroductions should be evaluated and strategically planned.

Conversely, genetic evidence from the Montemuro pack is also unexpected, as despite wolf presence being recorded on both sides of the highway (A24) that bisects the pack's territory,

only a single recapture has been documented thus far, according to ACHLI and Universidade de Aveiro findings (Table 1). Further investigation is necessary to determine the origin of individuals currently present in the Montemuro pack on the eastern region of the highway, given that this pack is crucial for ensuring genetic flow between the western packs and the central packs of the project area.

Leomil, Lapa and Trancoso pack territories lie in a more humanized landscape, as the orography of this central region exhibits less pronounced relief, which facilitates increased human activities. Nevertheless, wolf movements appear to be less restricted than in the western region, as evidenced by well-documented recaptures of individuals among the three packs.

Findings obtained during LIFE WolFlux project to assess connectivity of Iberian wolf population South of River Douro, based on the limited project's available data, we summarized some relevant indicators (Appendix 23) that could help us to characterize the baseline scenario as well as the project evolution. We searched for evidence of migration of individuals (such as mis-assigned individuals or long-distance migration) or gene flow (proportion of admixture from different genetic clusters, understood as different gene pools or reproductive units). Three individuals captured during the baseline study did confirm migration between regions, between west and centre regions (W05C, W04W) and from centre towards east region (W02E). We did not sample any mis-assigned individual after the baseline study, but one genotype sampled during the opportunistic monitoring (W03E) did show some affinity with two reference samples, from the Spanish side of the border (Castilla y Leon). While we should be carefully interpreting this result because of the limited amount of data, this assignment pattern suggests that Iberian wolf populations on the Spanish side of the border may have a role on the persistence of Iberian wolf on the East region South of River Douro.

Another evidence of flow of individuals between populations, would be the recapture of the same individual in two different regions. We did report 18 recaptures throughout the project but all within the same region where a genotype was first sampled. We were however able to report two long-distance migrations (more than 30km apart) in two cases: a north-south movement from individual W03E and an east-west movement of individual W02C. While recaptures of a same individual (genotype) in distant locations (30Km) are the most reliable evidence of the occurrence of long-distance movements, the assignment with very high probability of a genotype (W05C:90% ; W04W: 98%; W02E: 96%) to the gene pool (genetic

cluster) of a different population can also be considered as an evidence of recent migration, for these individuals are most likely first generation migrants.

Moreover, the connectivity analysis carried out using pairwise assignment tests showed a degree of genetic admixture between wolves of Central and Western packs and Central Packs are genetically closer to the wolves in the East (next to the border) than the wolves of the Western packs. These aligns with previous findings of wolves from Leomil dispersing to the border area (Palacios et al. 2017) and as previously pointed out by other authors (Pimenta et al., 2023), Leomil pack is of key importance for this populational nucleus survival, due to its productivity, stability and geographical location. It has been historically identified as a “source pack” of dispersal animals and for such reason special care for its home range and peripheral packs is required.

However, connectivity between central Portugal and the border regions of Spain requires improvement. The Ecological Niche Model indicates a need for ecological improvements between the central and eastern/border regions, namely in the municipalities of Pinhel and Figueira de Castelo Rodrigo, which would benefit connectivity and facilitate migration. It is highly recommended that conservation and recovery efforts are implemented across the border in articulation between the Portuguese and Spanish authorities, over Salamanca and Northern Extremadura regions.

Despite all effort for sampling Iberian wolf in the areas South of River Douro, that are closer to the border with Spain (East region), very few individuals were sampled from 2019 to 2024. These results are alarming for itself and reveal that the Iberian wolf has barely survived in the region after the establishment of a pack between 2012 and 2016 (Cadete et al., 2021; Palacios et al., 2017). This highlights the need for continuous conservation measures and addressing the causes that prevent the species to establish viable populations in the region.

Other long-term monitoring projects (ACHLI) and the LIFE WolFlux have not found evidence that supports significant connectivity between Arada and Montemuro packs.

## 6.5 Wolf Feeding Ecology

During the D1 systematic survey, a substantial quantity of wolf scat was located in proximity to intensive poultry and rabbit farms, indicating extensive utilization of these animals discarded remains (Figure 33). This intensive use is of particular concern given that the

future operations of these farms are uncertain and contingent upon uncontrolled factors such as entrepreneurial decisions and economic conditions. Furthermore, novel threats may arise from the consumption of intensive animal farming remains, such as avian influenza. To date, Portugal has the status of country free from avian influenza in caged birds (DGAV, 2023); however, some sporadic cases have indeed been reported (DGAV, 2024). It is established that the disease is contagious and can be transmitted to canines and felines. Infected mammals exhibit a diverse range of responses, from asymptomatic to lethal (DGAV, 2023). In this sense, special conservation and restoration efforts should be applied in packs where dependence on intensive livestock farms is detected, like in Leomil, Lapa and in less severe dependence, also Montemuro pack.



Figure 33 - Poultry remains from intensive farming, likely consumed by wolves or another wild carnivore.

Although the number of scats analysed in this report is lower ( $n= 20$ ), the concentration of droppings in the Arada pack allows for a comparative analysis between the two sampling periods (2020 and 2024). The results are encouraging. In 2020, out of seven scats analysed for this pack, it was only possible to detect the partial presence of one wild species in one scat, wild boar. In 2024, and considering the same spatial coverage, 12 scat were analysed, with two detecting the sole presence of roe deer and another two the sole presence of wild boar. This result shows that the presence of wild species in the wolf's diet has increased from 14 to 33 per cent. Unfortunately, the representativeness of the sampling considering the other packs did not allow us to extend the comparative analysis. It is important to stress that the present diet analysis was based on a very small sample size ( $n = 20$ ), which may lead, particularly, to an under-representation of wild prey in wolf's diet. The increase in wild boar abundance over the last decades, which can be found in functional densities along the LIFE WolFlux area ((Linck et al., 2023) – Action D.4), seems to be related to an increase in its frequency in wolves' diet (Lino et al. 2023 and this report).

## 6.6 Remarks about LIFE WolFlux actions and its influence in wolf monitoring results

The main threats identified to wolf conservation in the Sectorial Plan of Natura 2000 Network and the National Wolf Conservation Action are the lack of wild prey, conflicts with husbandry, negative attitudes and habitat destruction. These threats are preventing the population to thrive, creating social and ecological barriers that hamper connectivity between packs and the recolonization of new territories.

In light of this scenario, the project LIFE WolFlux was conceived to address these threats with the objective of contributing to the improvement of the conservation status and viability of the wolf subpopulation south of the Douro River, with the ultimate goal of enabling the wolf to thrive and fulfill its ecological role as an apex predator within the ecosystem.

The LIFE WolFlux project has met the foreseen targets in most of the actions and achieved some noteworthy outcomes, such us:

- Implementation of the most collaborative and extensive monitoring framework that was ever carried out south of the Douro River (Action D.1). Until now, different entities have studied specific packs, having a compartmentalized assessment of the whole

subpopulation. On the other hand, NWS was a large-scale collaborative study, but had less resources dedicated to genetic analysis for swab collection. LIFE WolFlux project has collected samples over the whole wolf range south of the Douro River, giving important insights on gene flow, dispersion and survival rates which can now be deepened and improved in future projects. The collaboration with other entities (ACHLI, ARCA, ICNF) has been fundamental to collect more information, avoid duplication of efforts and integrate the knowledge of other teams working locally.

- Extensive implementation of a combination of a set of damage preventive measures (108 guarding dogs and 51 wolf proof fences of different types) being displayed simultaneously over 5 years in the territories of all packs, predation hot spots and areas of recolonization (action C.2) supported by a team of veterinaries (action C.1). Efforts of previous LIFE and non-LIFE projects included a smaller number of measures, were concentrated in specific regions or packs or have used less variety of damage preventive measures.
- The first time that a civil surveillance team was actively searching for snares with 36 snares being found and removed (action C.3). Previous efforts had focused mainly on poison.
- The first Global Hunting Management Plan for roe deer specially designed as a tool to increase a wolf prey. This plan has allowed to influence more than 15.000 hectares of habitat Leomil pack, where the recovery of wild prey is key to reduce the dependence of the pack on leftovers of poultry and rabbit industries. Complementarily, more than 50 hectares of woodland, 40 hectares of pastures and almost 50 ponds have been created or restored over the whole WolFlux intervention area. The measures implemented have positively influenced roe deer which has duplicated or triplicated compared with densities prior to project implementation (action D.2).
- The LIFE WolFlux project has encompassed an economic dimension to promote socio-economic development through nature and has created incentives for farmers that coexist with wolf, adding value to their products (actions E.5 and E.6).
- The LIFE WolFlux has allowed to engage with a broad audience of different relevant stakeholders, produced a variety of communication materials (e.g. guide for damage prevention (actions E.1, E.2, E.3 and E.4) and, the most important of all, has had a body of technicians of Rewilding Portugal always present in the ground supporting and working side by side with livestock breeders, hunters, entrepreneurs and local authorities.

However, we have found challenges over the project that have hampered the implementation of actions in its full potential, which might have an influence on wolf monitoring results, such us:

- ICNF did not allow roe deer reinforcement within the areas of some packs, where the species' densities were extremely low (< 1 individual/ 100 ha), despite demonstrated social support to this initiative. Even though roe deer is increasing south of the Douro River and habitat restoration measures carried out in the scope of the LIFE WolFlux (action C.4) have had positive results, reinforcing roe deer would speed up the time for the species to reach functional densities, which still does not happen in the Central and Western regions. As vast literature shows, wild prey needs to be abundant enough to be incorporated into wolf's diet.
- Even though the surveillance team detected several snares and Rewilding Portugal made official complaints to the authorities (action C.3), only few cases were investigated and reached the Public Ministry and all of them were archived after some months. During action C.3 it was identified that there is a lack of resources and investigation dedicated to environmental crime, particularly when complaints are against an unknown subject, as is the case of snares. Moreover, on the scope of action C.3, various situations of risk of retaliation towards wolves were communicated to the authorities (GNR/SEPNA and ICNF), suggesting them the need to reinforce patrols in certain areas and periods of time but an answer was never received. These situations limited the impact of action C.3 which aimed to reduce the impunity towards poaching that persists nowadays.
- The implementation of damage preventive measures (action C.2) has succeeded, since farmers are highly satisfied with the livestock guarding dogs and fences, and many of them have experienced a reduction of damages (action D.3). However, one of the main problems identified by livestock breeders as a difficulty to live alongside wolves in the survey carried out in 2019 (action A.7) and repeated in 2022/23 (action D.4) was the damage compensation system. Livestock breeders considered compensation very slow and insufficient. Since 2019, the situation has deteriorated, with a decrease in the number of farmers reporting damages and a reduction in those receiving compensation payments, as addressed in action D.4. Considering that damage on livestock is one of the main motivations for retaliation against wolves, the LIFE WolFlux partnership has dedicated (and it is still dedicating) efforts to lobby in favor of a new damage



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# Conclusions

## 7. Conclusions and recommendations

Wolf survey and feeding ecology analysis results stress persistent challenges after the implementation of conservation measures within the LIFE WolFlux project. Despite substantial efforts, the wolf population south of the Douro River remains endangered. The acquired data reveals fluctuations in wolf range, with notable declines in confirmed packs and number of individuals in recent years, according to data obtained by LIFE WolFlux and ACHLI.

Two packs identified in studies prior to the project period were not detected (e.g. Almeida pack (Cadete et al., 2012, 2015; Palacios et al., 2017) and Cinfães pack (Pimenta et al., 2005, 2023)), which also suggests the high instability of occupation of these territories and a probable phenomenon of group extinction, even if possibly temporary (extinction/recolonization dynamics). Moreover, there are only two packs with regular breeding success south of the Douro River, based on the long-term monitoring carried out by ACHLI in collaboration with the University of Aveiro and BIOPOLIS-CIBIO. The presence of dispersing wolves in Castelo Branco is a positive prospect and thus early monitoring and conservation efforts will be needed to promote the establishment of individuals in the territory.

Regarding trophic ecology results, we note the positive fact of the increased use of a wild trophic resource (roe deer) in the western part of the study area (Freita and Arada mountains). However, in addition to the need of sample size increase to confirm this positive trend, the use of wild prey should be increased and extended to the entire study area. Special attention should be given to the plateau region of Nave and Lapa mountains, where dependence on animal carcasses (necrophagy) of anthropogenic origin is still very high, which is not sustainable from a conservation biology point of view.

Despite the difficulty in obtaining genetic information due to the extremely low abundance of the species, especially in the border region of the study area, the obtained data indicates good levels of connectivity between the groups in the centre of the study area (Leomil, Lapa and Trancoso). However, evidence of connectivity between the Arada and Montemuro packs is barely existent. While some evidence exists, suggesting connectivity between the packs in the West (Arada and Montemuro) and those in the Centre (Leomil, Lapa and Trancoso), the available data to substantiate this connection is insufficient.

The occurrence of genetic flow from Spanish nuclei is fundamental for the rescue of the wolf population south of the Douro, to avoid inbreeding depression. This study yielded data indicating potential connectivity among wolf packs inhabiting the plateau region encompassing Moimenta da Beira, Vila Nova de Paiva, Trancoso, and Sernancelhe (the central study area) and those in the Spanish border region along an east-west axis. Connectivity in the opposite direction, which is the one with the greatest biological value due to the need for migrants to arrive from Spanish nuclei, the evidence is very scarce and must be taken with great caution due to the small sample size.

In contrast to the trend observed for the rest of Europe where wolf populations have expanded in recent decades, wolves in Portugal are not only failing to expand but are experiencing a decline. Notably, the subpopulation south of the Douro River is at risk of functional extinction within the next few decades if urgent conservation efforts are not implemented to reverse the current situation. Initiatives funded by LIFE, such as the LIFE WolFlux project, have made substantial progress towards wolf conservation south of the Douro River by contributing to the mitigation of the primary threats to the species. However, these efforts are insufficient to facilitate the recovery of the wolf from its unfavorable conservation status and precarious situation south of the Douro River, a condition that has been recognized since the 1990s. Bolder conservation efforts need to be supported by the national authorities and more national resources need to be dedicated and coordinated to reverse the situation through the National Action Plan for Wolf Conservation (PACLobo). There is a critical need for an urgent and decisive shift in public policy strategy, transitioning from passive to active approaches and from conservation to proactive restoration and recovery initiatives.

Specifically, we share the following recommendations for future action and research, derived from the results and learnings acquired during the LIFE WolFlux project:

- More collaboration and coordination between relevant stakeholders such as ICNF, GNR/SEPNA, NGOs, livestock breeders, hunters, local communities, and general public.
- Modify the wolf damage compensation system through a participatory process involving relevant stakeholders to reach a social consensus. In the social surveys carried out in action A.7 and D.4 livestock breeders identified the improvement of the damage compensation system as one of the important factors to coexist with

wolves. Demands included quicker and fairer payments as well as being listened to by the authorities.

- Dedicating more funds of the Common Agricultural Policy and other financial instruments to prevent damage and offer technical support to livestock breeders on the implementation of damage preventive measures. These were two main conclusions for improving and expanding damage prevention that emerged from the transboundary round table seminar on damage prevention conducted with livestock breeders and authorities in Guarda in 2023.
- Increasing abundance and diversity of wild prey through active reinforcements and reintroduction with the support of local communities. Even if roe deer is expanding, densities are far from optimal in many areas. Apart from the wild boar, other potential wolf prey species are absent or in very low densities in the wolf range south of the Douro River. Results from LIFE WolFlux show how wild prey increases in wolf diet, as abundances are higher. This trend has also been observed in other European countries (eg. (Janeiro-Otero et al., 2020; Meriggi et al., 2011)
- Reduce wolf mortality resulting from targeted and non-targeted poaching, primarily through enhancement of prevention, detection, investigation, and punitive mechanisms for environmental crimes in general and those specifically perpetrated against this protected species. As registered in social surveys of actions A.7 and D.4, the use of snares is widespread, and poison is used in some areas. The impact of direct persecution by shooting is barely documented but was mentioned by several interviewees and local stakeholders over the years. Currently, the means to prevent, investigate and punish are insufficient, as documented in action C.3.
- Assessing the permeability of motorways like A24 and IP2 and improving connectivity where needed. The Niche Model and genetic capture-recaptures suggest the need to give more attention to improve the permeability of these infrastructures.
- Analysis of additional wolf genotypes from Salamanca and Extremadura to conduct a more robust analysis of gene flow with Portuguese wolves south of the Douro River.
- Preserve and restore wolf habitat and corridors from wildfires and other sources of disturbance. Throughout the duration of the project, various developments, particularly in the domains of clean energy and mining, were either approved or submitted for environmental impact assessment within the territories of the packs

and important areas of connectivity. A comprehensive, sensible, and coordinated land-use planning approach is essential to ensure a reduction in disturbance and ensure optimal landscape connectivity.

- Increasing efforts in creating nature-based and wolf-based economies that bring benefits to communities that live alongside wolves. Key actors interviewed both in 2029 and 2023 rarely point tourism as one of the advantages that the wolf can bring. Nonetheless, when initiatives like training guides, support nature business or add value to local products are implemented, as the LIFE WolFlux did on the scope of actions E.5 and E.6, are well received by local stakeholders.



# References

## 8. References

- Álvares, F., Quaresma, S., Grilo, C., & Costa, G. (2005a). *Plano de Monitorização do Lobo-ibérico na área do Projecto Eólico de Cinfães (Serra de Montemuro/Distritos de Viseu e Aveiro). Análise global dos resultados (2002-2005). Ano 3—Fase II* (p. 39 + Anexos).
- Álvares, F., Quaresma, S., Grilo, C., & Costa, G. (2005b). *Plano de Monitorização do lobo-ibérico nos Parques Eólicos da Serra da Freita (Freita I e Freita II): Análise da situação actual (Serras de Arada e Freira/Distritos de Viseu e Aveiro). Fase I. Relatório Anual.* ProSsistemas - Consultores de Engenharia.
- Bastos, T. (2001). *Estudo da ecologia de duas alcateias pertencentes à população lupina a sul do rio Douro. Relatório de estágio profissionalizante para a obtenção de licenciatura em Biologia Aplicada aos Recursos Animais.*, Faculdade de Ciências da Universidade de Lisboa.
- Cadete, D., Aliácar, S., Borges, C., & Simões, F. (2015). *Action A.2: Ex-ante detailed survey of wolf presence in Portuguese project areas. Evaluating the effectiveness of the scat detection dog team. Final Report (Ribeiro S. & Petrucci-Fonseca F. Coord). Project LIFE MedWolf (LIFE11NAT/IT/069)* (p. 81). Grupo Lobo/INIAV/FCUL.
- Cadete, D., Pinto, S., Aliácar, S., Mendes, M., Fernandes, J., Lino, A., Ferreira, E., Carvalho, J., Torres, R., & Fonseca, C. (2020). *Survey and feeding ecology of the Portuguese subpopulation of Iberian wolf south of the Douro river: Towards a non-invasive integrated monitoring program* (p. 73 + Annexes) [Technical Report of Action A3, LIFE17 NAT/PT/554 – WolFlux]. ZL Zoo Logical – Associação de Inovação para o Conhecimento, Divulgação e Conservação da Fauna.
- Cadete, D., Pinto, S., Aliácar, S., Mendes, M., Fernandes, J., Lino, A., Ferreira, E., Carvalho, J., Torres, R., & Fonseca, C. (2021). *Survey and feeding ecology of the Portuguese*

*subpopulation of Iberian wolf south of the Douro river: Towards a non-invasive integrated monitoring program. Technical Report of Action A3, LIFE17NAT/PT/554 – WolFlux* (p. 73 pp + Annexes). ZL Zoo Logical – Associação de Inovação para o Conhecimento, Divulgação e Conservação da Fauna.

Cadete, D., Pinto, S., Borges, C., Simões, F., & Petrucci-Fonseca, F. (2012). *O lobo na região fronteiriça portuguesa a sul do rio Douro: Desafios à monitorização e conservação*. III Congreso Ibérico do Lobo, Lugo.

Cardoso, J., Serrinha, A., Gil, P., Godinho, R., & Álvares, F. (2022). *Plano de Monitorização do Lobo a Sul do rio Douro – Zona Este: Relatório Ano X (julho 2021 – junho 2022)*. Relatório Técnico (p. 104 pp+ Anexos). Associação BIOPOLIS/CIBIO-InBIO.

Cardoso, J., Serrinha, A., Gil, P., Godinho, R., & Álvares, F. (2024). *Plano de Monitorização do Lobo a Sul do rio Douro – Zona Este: Relatório Ano XI (julho 2022 – junho 2023)*. Relatório Técnico (p. 106pp+ Anexos). Associação BIOPOLIS/CIBIO-InBIO.

De Marinis, A. M., & Asprea, A. (2006). How Did Domestication Change the Hair Morphology in Sheep and Goats? *Human Evolution*, 21(2), 139–149.  
<https://doi.org/10.1007/s11598-006-9010-0>

Delgado, S., Girondot, M., & Sire, J.-Y. (2005). Molecular evolution of amelogenin in mammals. *Journal of Molecular Evolution*, 60(1), 12–30.  
<https://doi.org/10.1007/s00239-003-0070-8>

DGAV. (2023). *Infecção por vírus da gripe aviária de alta patogenicidade em animais de companhia (mamíferos)*. Direcção Geral de Agricultura e Veterinária.  
[https://www.dgav.pt/wp-content/uploads/2023/11/GAAP\\_Animais\\_Companhia\\_v2.pdf](https://www.dgav.pt/wp-content/uploads/2023/11/GAAP_Animais_Companhia_v2.pdf)

DGAV. (2024). *Situação Epidemiológica da gripe aviária de alta patogenicidade em Portugal—Outubro de 2023 a Agosto de 2024 [Nota Técnica]*. Direcção Geral de

Agricultura e Veterinária. [https://www.dgav.pt/wp-](https://www.dgav.pt/wp-content/uploads/2024/08/NotaTecnica_GAAP_PT_2024.pdf)

[content/uploads/2024/08/NotaTecnica\\_GAAP\\_PT\\_2024.pdf](https://www.dgav.pt/wp-content/uploads/2024/08/NotaTecnica_GAAP_PT_2024.pdf)

Godinho, R., Llaneza, L., Blanco, J. C., Lopes, S., Álvares, F., García, E. J., Palacios, V.,

Cortés, Y., Talegón, J., & Ferrand, N. (2011). Genetic evidence for multiple events of hybridization between wolves and domestic dogs in the Iberian Peninsula. *Molecular Ecology*, 20(24), 5154–5166. <https://doi.org/10.1111/j.1365-294X.2011.05345.x>

Godinho, R., Lopes, S., & Ferrand, N. (2007). *Estudo da diversidade e estruturação genética das populações de lobo (Canis lupus) em Portugal. Relatório Final* (p. 56). CIBIO/UP.

Grilo, C., Lucas, P. M., Fernández-Gil, A., Seara, M., Costa, G., Roque, S., Rio-Maior, H., Nakamura, M., Álvares, F., Petrucci-Fonseca, F., & Revilla, E. (2019). Refuge as major habitat driver for wolf presence in human-modified landscapes. *Animal Conservation*, 22(1), 59–71. <https://doi.org/10.1111/acv.12435>

Grilo, C., Moço, G., Cândido, A., Alexandre, A., & Petrucci-Fonseca, F. (2002). *Bases para a definição de corredores ecológicos na conservação de uma população marginal e fragmentada: O caso da população lupina a sul do rio Douro—1ª Fase. Relatório técnico PRAXIS XXI* (p. 106). Centro de Biologia Ambiental.

ICN. (1997). *Conservação do lobo em Portugal. Projecto realizado ao abrigo do programa Life. Relatório Final.*

Iwase, M., Kaneko, S., Kim, H., Satta, Y., & Takahata, N. (2007). Evolutionary history of sex-linked mammalian amelogenin genes. *Cells, Tissues, Organs*, 186(1), 49–59. <https://doi.org/10.1159/000102680>

Janeiro-Otero, A., Newsome, T. M., Van Eeden, L. M., Ripple, W. J., & Dormann, C. F. (2020). Grey wolf (*Canis lupus*) predation on livestock in relation to prey availability.

- Biological Conservation*, 243, 108433.
- <https://doi.org/10.1016/j.biocon.2020.108433>
- Karanth, K. U., & Sunquist, M. E. (1995). Prey Selection by Tiger, Leopard and Dhole in Tropical Forests. *Journal of Animal Ecology*, 64(4), 439–450.  
<https://doi.org/10.2307/5647>
- Kruuk, H., & Parish, T. (1981). Feeding Specialization of the European Badger *Meles meles* in Scotland. *Journal of Animal Ecology*, 50(3), 773–788.  
<https://doi.org/10.2307/4136>
- Leigh, J. W., & Bryant, D. (2015). popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9), 1110–1116.  
<https://doi.org/10.1111/2041-210X.12410>
- Linck, P., Ares-Pereira, G., Rossa, M., Macedo, T., Torres, R., Fonseca, C., & Carvalho, J. (2023). *Monitoring the success of the release of wild ungulates and their situation at the end of the project. Technical Report of Monitoring Action D2, LIFE17 NAT/PT/554 – WOLFLUX. University of Aveiro, Aveiro. 28 pp.*
- Lino, S., Rossa, M., Fernandes, J. M., Barros, T., Lino, A., Hipólito, D., Ferreira, E., Aliácar, S. C., Cadete, D., Fonseca, C., Torres, R. T., Rosalino, L. M., & Carvalho, J. (2023). Dog in sheep's clothing: Livestock depredation by free-ranging dogs may pose new challenges to wolf conservation. *European Journal of Wildlife Research*, 69(6), 107.  
<https://doi.org/10.1007/s10344-023-01740-9>
- Lovari, S., Pokheral, C., Jnawali, S., Fusani, L., & Ferretti, F. (2015). Coexistence of the tiger and the common leopard in a prey-rich area: The role of prey partitioning—Lovari—2015—Journal of Zoology—Wiley Online Library. *Journal of Zoology*, 295(2).  
<https://zslpublications.onlinelibrary.wiley.com/doi/10.1111/jzo.12192>
- Lucherini, M., Lovari, S., & Crema, G. (1995). Habitat use and ranging behaviour of the red fox (*Vulpes vulpes*) in a Mediterranean rural area: Is shelter availability a key factor?

*Journal of Zoology*, 237(4), 577–591. <https://doi.org/10.1111/j.1469-7998.1995.tb05016.x>

Marucco, F., Avanzinelli, E., & Boitani, L. (2011). Non-invasive integrated sampling design to monitor the wolf population in Piemonte, Italian Alps. *Hystrix, the Italian Journal of Mammalogy*, 23(1), 5–13. <https://doi.org/10.4404/hystrix-23.1-4584>

Marucco, F., Pletscher, D. H., Boitani, L., Schwartz, M. K., Pilgrim, K. L., & Lebreton, J.-D. (2009). Wolf survival and population trend using non-invasive capture–recapture techniques in the Western Alps. *Journal of Applied Ecology*, 46(5), 1003–1010. <https://doi.org/10.1111/j.1365-2664.2009.01696.x>

Meriggi, A., Brangi, A., Schenone, L., Signorelli, D., & Milanesi, P. (2011). Changes of wolf (*Canis lupus*) diet in Italy in relation to the increase of wild ungulate abundance. *Ethology Ecology & Evolution*, 23(3), 195–210. <https://doi.org/10.1080/03949370.2011.577814>

Merow, C., Smith, M. J., & Silander Jr, J. A. (2013). A practical guide to MaxEnt for modeling species' distributions: What it does, and why inputs and settings matter. *Ecography*, 36(10), 1058–1069. <https://doi.org/10.1111/j.1600-0587.2013.07872.x>

Murphy, M. A., Waits, L. P., Kendall, K. C., Wasser, S. K., Higbee, J. A., & Bogden, R. (2002). An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. *Conservation Genetics*, 3(4), 435–440. <https://doi.org/10.1023/A:1020503330767>

Palacios, V., Garcia, E., Santos, R., Borges, C., & Simões, F. (2017). *Action D.3: Assessment of wolf presence in expansion areas in Portugal. Final Report (Ribeiro S. & Petrucci-Fonseca F. Coord).* Project LIFE MedWolf (LIFE11NAT/IT/069) (p. 61). Grupo Lobo/INIAV/FCUL.

Peakall, R., & Smouse, P. E. (2006). genalex 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6(1), 288–295.  
<https://doi.org/10.1111/j.1471-8286.2005.01155.x>

Peakall, R., & Smouse, P. E. (2012a). GenAIEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics*, 28(19), 2537. <https://doi.org/10.1093/bioinformatics/bts460>

Peakall, R., & Smouse, P. E. (2012b). *GenAIEx Tutorial 4: Advanced Frequency-Based Analysis. Software Tutorial.*

Phillips, S. J. (2017). A Brief Tutorial on Maxent.  
[https://biodiversityinformatics.amnh.org/open\\_source/maxent/Maxent\\_tutorial2017.pdf](https://biodiversityinformatics.amnh.org/open_source/maxent/Maxent_tutorial2017.pdf)

Pimenta, V., Barroso, I., Álvares, F., Barros, T., Borges, C., Cadete, D., Carneiro, C., Casimiro, J., Ferrão da Costa, G., Ferreira, E., Fonseca, C., García, E., Gil, P., Godinho, R., Hipólito, D., Llaneza, L., Marcos Perez, A., Martí-Domken, B., Monzón, A., ... Torres, R. (2023). *Situação populacional do Lobo em Portugal: Resultados do Censo Nacional de 2019/2021*. ICNF.

Pimenta, V., Barroso, I., Álvares, F., Correia, J., Costa, G., Moreira, L., Nascimento, J., & Petrucci-Fonseca, F. (2005). *Situação populacional do lobo em Portugal: Resultados do censo nacional 2002/2003*. Instituto de Conservação da Natureza e das Florestas.

Pinto, S., & Costa, D. (2023). *Análise da Conectividade Espacial da Subpopulação de lobo-ibérico (*Canis lupus signatus*) a Sul do Rio Douro. [Unpublished Manuscript]*. Universidade Nova de Lisboa, Análise e Visualização de Dados Espaciais.

Pompanon, F., Bonin, A., Bellemain, E., & Taberlet, P. (2005). Genotyping errors: Causes, consequences and solutions. *Nature Reviews Genetics*, 6(11), 847–859.  
<https://doi.org/10.1038/nrg1707>

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155(2), 945–959.  
<https://doi.org/10.1093/genetics/155.2.945>

Rio-Maior, H., Nakamura, M., Álvares, F., & Beja, P. (2019). Designing the landscape of coexistence: Integrating risk avoidance, habitat selection and functional connectivity to inform large carnivore conservation. *Biological Conservation*, 235, 178–188. Scopus. <https://doi.org/10.1016/j.biocon.2019.04.021>

Roque, S., Espírito-Santo, C., Grilo, C., Rio Maior, H., & Petrucci-Fonseca, F. (2005). A *população lupina a sul do rio Douro em Portugal: Análise temporal, atitudes públicas e aperfeiçoamento dos corredores ecológicos. Relatório final do Projecto POCTI/MGS/34733/2000 (Programa Sapiens). Relatório Final de Execução Técnica SAPIENS.* (p. 274). Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa.

Roque, S., Godinho, R., Cadete, D., Pinto, S., Pedro, A. S., Bernanrdo, J., Petrucci-Fonseca, F., & Álvares, F. (2011). *Plano de Monitorização do Lobo Ibérico nas áreas dos Projectos Eólicos das Serras de Montemuro, Freita, Arada e Leomil—Ano IV e Análise Integrativa dos Resultados (2006-2011)* (p. 193pp + Anexos). CIBIO-UP/Grupo Lobo.

Roque, S., Godinho, R., Cadete, D., Pinto, S., Petrucci-Fonseca, F., & Álvares, F. (2010). *Plano de Monitorização do Lobo Ibérico nas áreas dos Projectos Eólicos das Serras de Montemuro, Freita, Arada e Leomil – Ano III. Relatório Anual* (p. 141pp + Anexos). CIBIOUP/ Grupo Lobo.

Roque, S., Marti, B., Godinho, R., Petrucci-Fonseca, F., & Álvares, F. (2017). *Plano de Monitorização do Lobo a Sul do Rio Douro—Zona Este: Relatório Final ANO IV*, (p. 111pp + Anexos). CIBIO-UP/Grupo Lobo.

Roque, S., Marti, B., Godinho, R., Petrucci-Fonseca, F., & Álvares, F. (2018). *Plano de Monitorização do Lobo a Sul do Rio Douro—Zona Este: Relatório Final Ano V* (p. 103pp + Anexos). CIBIO-UP/Grupo Lobo.

Roque, S., Palmegiani, I., Petrucci-Fonseca, F., & Álvares, F. (2012). *O custo da necrofagia: Estratégias de uso do território por uma alcateia a sul do rio Douro em Portugal. III Congresso do Lobo Ibérico*, Lugo.

Roque, S., & Petrucci-Fonseca, F. (2006). *Factores que influenciam a população de lobo no Centro de Portugal: Implicações para uma estratégia de conservação—Ano 2006. Relatório de Progresso de Execução Material POCI*. Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa.

Sangiuliano, A., Lovari, S., & Ferretti, F. (2016). Dietary partitioning between European roe deer and European brown hare. *European Journal of Wildlife Research*, 62(5), 527–535. <https://doi.org/10.1007/s10344-016-1023-z>

Serronha, A., Gil, P., Godinho, R., & Álvares, F. (2019). *Plano de Monitorização do Lobo a Sul do Rio Douro – Zona Este: Relatório Final Ano VII* (p. 112 pp + Anexos). CIBIO/InBio.

Serronha, A., Gil, P., Godinho, R., & Álvares, F. (2020). *Plano de Monitorização do Lobo a Sul do Rio Douro – Zona Este: Relatório Final Ano VIII e análise global* (p. 123 pp. + Anexos). CIBIO/InBio.

Serronha, A., Gil, P., Godinho, R., & Álvares, F. (2021). *Plano de Monitorização do Lobo a Sul do Rio Douro – Zona Este: Relatório Ano IX (julho 2020 – junho 2021)* (p. 98 pp + Anexos). BIOPOLIS/CIBIO/InBio.

Serronha, A., Gil, P., Roque, S., Godinho, R., Petrucci-Fonseca, F., & Álvares, F. (2018). *Plano de Monitorização do Lobo a Sul do Rio Douro—Zona Este: Relatório Final Ano VI* (p. 111pp + Anexos). CIBIO/InBio.

Silva, P., López-Bao, J. V., Llaneza, L., Álvares, F., Lopes, S., Blanco, J. C., Cortés, Y., García, E., Palacios, V., Rio-Maior, H., Ferrand, N., & Godinho, R. (2018). Cryptic population

- structure reveals low dispersal in Iberian wolves. *Scientific Reports*, 8(1), 14108.
- <https://doi.org/10.1038/s41598-018-32369-3>
- Steckler, D. (2010). *Verifying parentage and gender of domestic dog conceptuses using microsatellites*. University of Pretoria.
- Taberlet, P., Waits, L. P., & Luikart, G. (1999). Noninvasive genetic sampling: Look before you leap. *Trends in Ecology & Evolution*, 14(8), 323–327. [https://doi.org/10.1016/s0169-5347\(99\)01637-7](https://doi.org/10.1016/s0169-5347(99)01637-7)
- Teerink, B. J. (1991). *Hair of West-European mammals: Atlas and identification key*. <https://www.semanticscholar.org/paper/Hair-of-West-European-mammals-%3A-atlas-and-key-Teerink/472babe24af6184eb8b682a30317b492e4dd7277>
- Torres, R., Carvalho, J., Ferreira, E., Barros, T., Vale, R., Oliveira, L., Figueiredo, A., Martins, R., & Fonseca, C. (2016). *Plano de Monitorização do lobo a sul do rio Douro—Zona oeste (PMLSD-O): Ano IV e análise integrativa dos resultados (2011-2015). Relatório Final* (p. 196 pp.). Aveiro, Departamento de Biologia, Universidade de Aveiro.
- Torres, R., Fernandes, T., Barros, T., Ferreira, E., Carvalho, J., & Fonseca, C. (2018). *Plano de monitorização do lobo a sul do rio Douro—Zona oeste (PMLSD-O): Fase II - ano I (2016/2017). Relatório Final* (p. 115 pp.). Aveiro, Departamento de Biologia, Universidade de Aveiro.
- Torres, R., Ferreira, E., Lino, A., Alves, C., Fonseca, C., & Hipólito, D. (2023a). *Plano de monitorização do lobo a sul do rio Douro – zona oeste (PMLSD-O): Fase III – ano II. Relatório Final* (p. 118). Departamento de Biologia, Universidade de Aveiro.
- Torres, R., Ferreira, E., Lino, A., Alves, C., Fonseca, C., & Hipólito, D. (2023b). *Plano de monitorização do lobo a sul do rio Douro – zona oeste (PMLSD-O): Fase III – ano III. Relatório Final* (p. 103). Departamento de Biologia, Universidade de Aveiro.
- Torres, R., Hipólito, D., Barros, T., Ferreira, E., Carvalho, J., & Fonseca, C. (2018). *Plano de monitorização do lobo a sul do rio Douro—Zona oeste (PMLSD-O): Fase II - ano II*

(2017/2018). *Relatório Final* (p. 112 pp.). Aveiro, Departamento de Biologia, Universidade de Aveiro.

Torres, R., Hipólito, D., Barros, T., Ferreira, E., & Fonseca, C. (2019). *Plano de monitorização do lobo a sul do rio Douro – zona oeste (PMLSD-O): Fase II – ano III (2018/2019). Relatório Final* (p. 108). Departamento de Biologia, Universidade de Aveiro.

Torres, R., Hipólito, D., Lino, A., Barros, T., Ferreira, E., & Fonseca, C. (2021). *Plano de monitorização do lobo a sul do rio Douro – zona oeste (PMLSD-O): Fase IV e análise integrativa dos resultados (2016/2020). Relatório Final.* (p. 144). Departamento de Biologia, Universidade de Aveiro,.

Torres, R., Hipólito, D., Lino, A., Ferreira, E., & Fonseca, C. (2021). *Plano de monitorização do lobo a sul do rio Douro – zona oeste (PMLSD-O): Fase III – ano I (junho 2020 – maio 2021). Relatório Final* (p. 119). Departamento de Biologia, Universidade de Aveiro.

Torres, R. T., Ferreira, E., Rocha, R. G., & Fonseca, C. (2017). Hybridization between wolf and domestic dog: First evidence from an endangered population in central Portugal. *Mammalian Biology*, 86, 70–74. <https://doi.org/10.1016/j.mambio.2017.05.001>

Valente, A., Rocha, R., Fonseca, C., Pedro Ferreira, J., & Lozano, E. (2015). *Atlas dos Pelos dos Mamíferos Terrestres Ibéricos*. Afrontamento.

Vilà, C., Amorim, I. R., Leonard, J. A., Posada, D., Castroviejo, J., Petrucci-Fonseca, F., Crandall, K. A., Ellegren, H., & Wayne, R. K. (1999). Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*. *Molecular Ecology*, 8(12), 2089–2103. <https://doi.org/10.1046/j.1365-294x.1999.00825.x>

Vilà, C., Savolainen, P., Maldonado, J. E., Amorim, I. R., Rice, J. E., Honeycutt, R. L., Crandall, K. A., Lundeberg, J., & Wayne, R. K. (1997). Multiple and ancient origins of the domestic dog. *Science (New York, N.Y.)*, 276(5319), 1687–1689. <https://doi.org/10.1126/science.276.5319.1687>

Wagner, C., Holzapfel, M., Kluth, G., Reinhardt, I., & Ansorge, H. (2012). Wolf (*Canis lupus*)

feeding habits during the first eight years of its occurrence in Germany. *Mammalian Biology*, 77(3), 196–203. <https://doi.org/10.1016/j.mambio.2011.12.004>

Waits, L. P., Luikart, G., & Taberlet, P. (2001). Estimating the probability of identity among

genotypes in natural populations: Cautions and guidelines. *Molecular Ecology*,

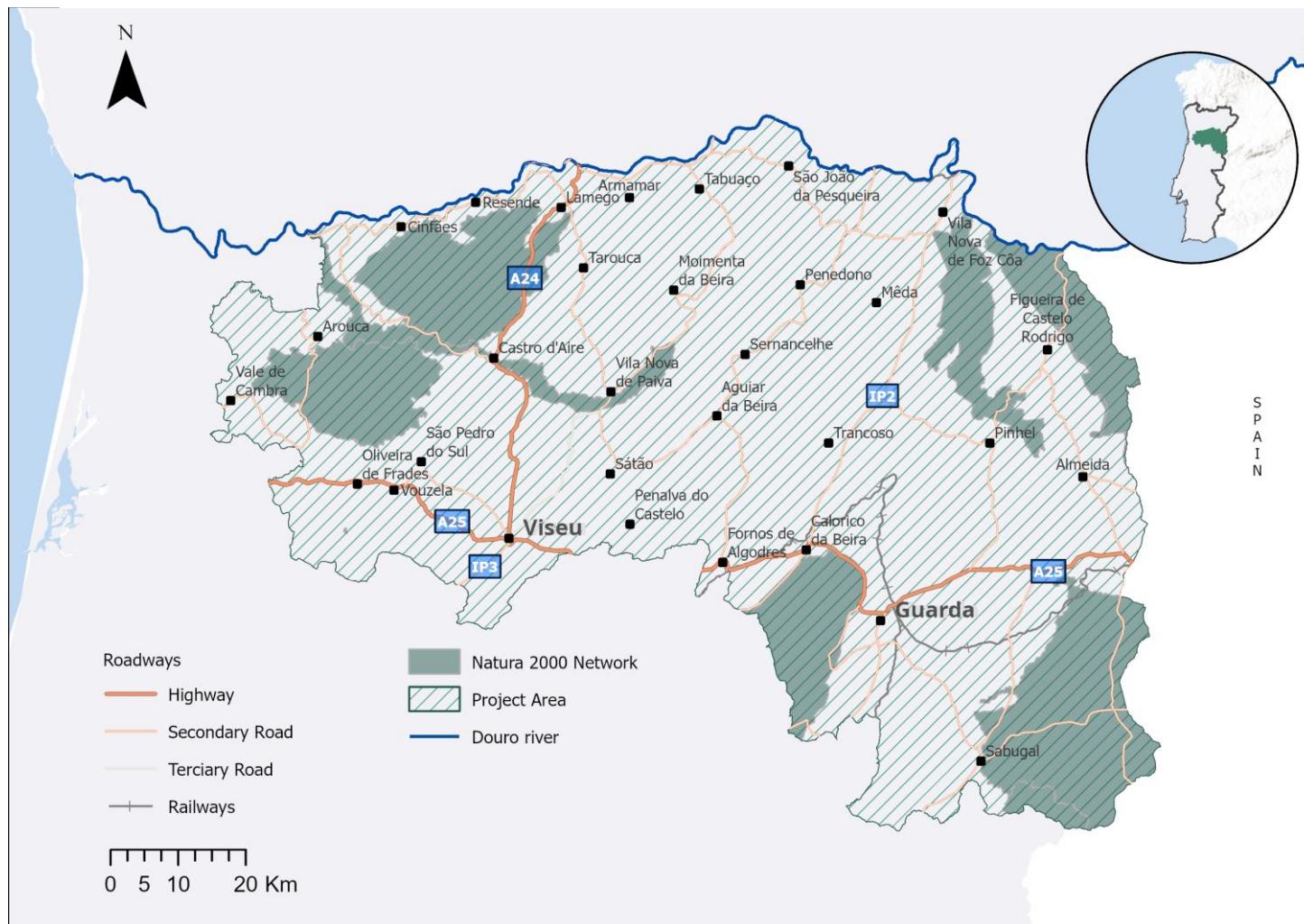
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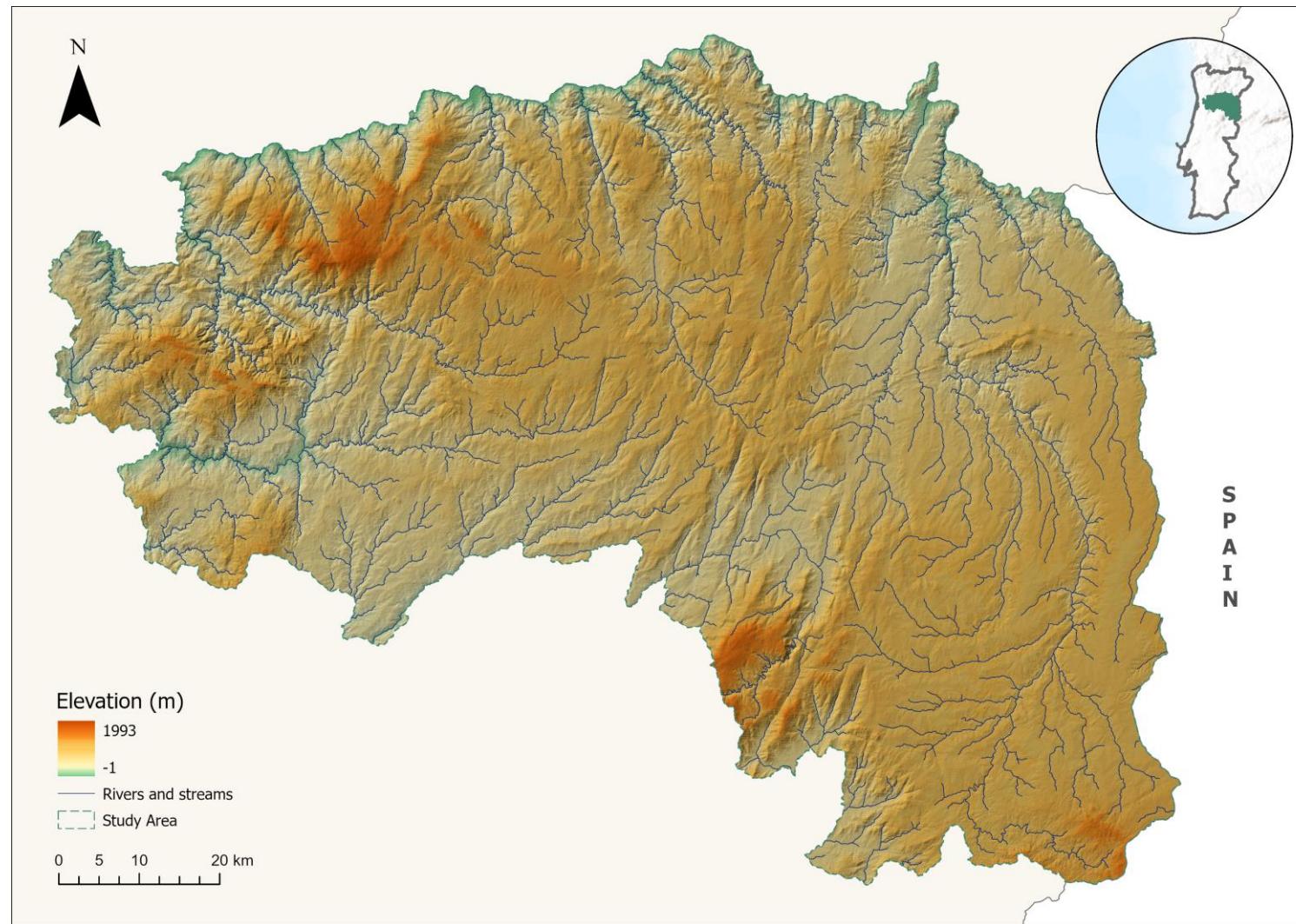


# Appendices

## 9. Appendixes

*Appendix 1 - Spatial overview of the project area.*



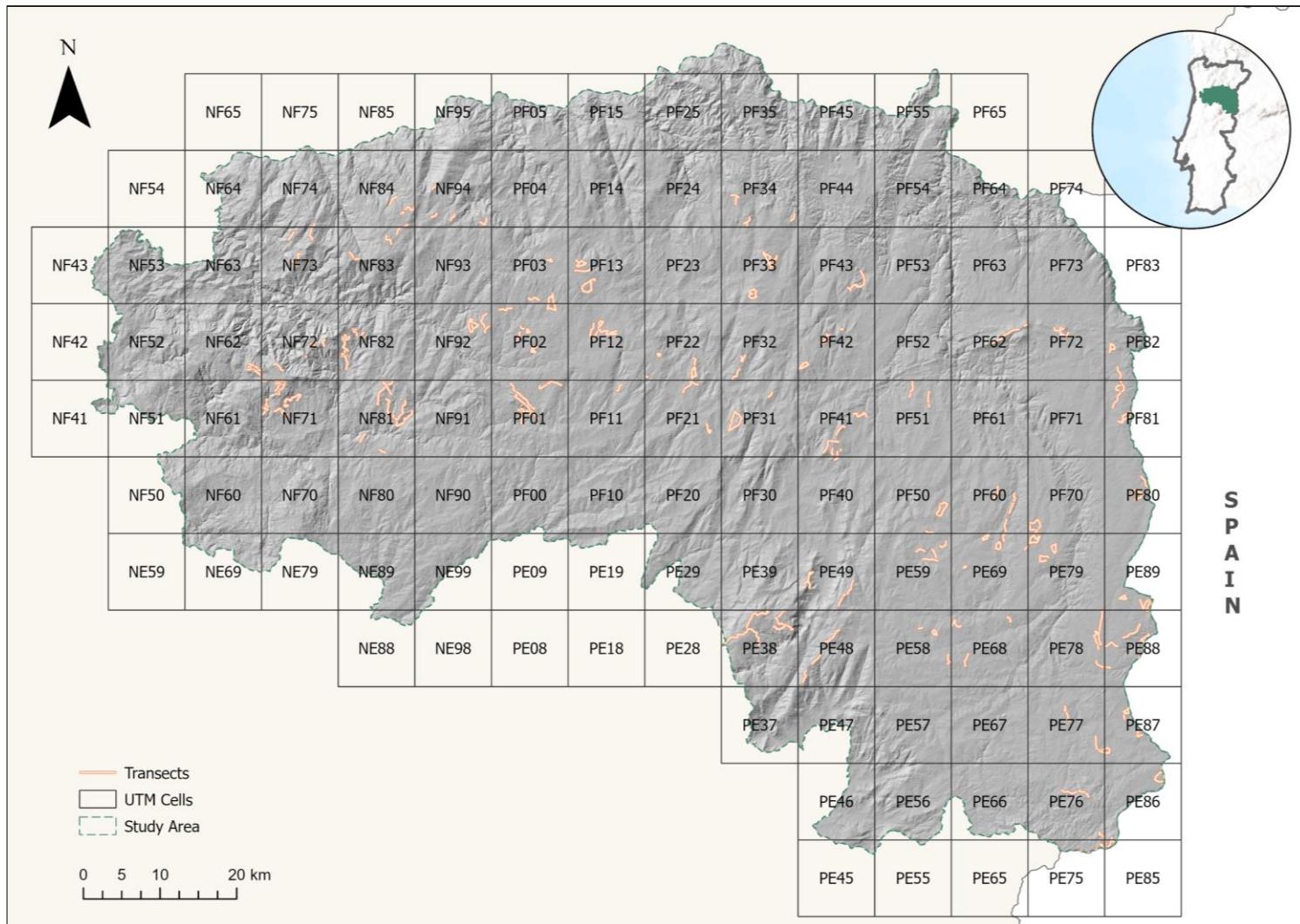
**Appendix 2 - Elevation, rivers and streams of the project area.**

**Appendix 3 - Number of transects and lenght (km) per 10x10 km UTM cell.**

<b>10X10 KM UTM CELL</b>	<b>TRANSECTS</b>	<b>TOTAL TRANSECT LENGTH (KM)</b>
NF62	5	6.76
NF71	13	20.87
NF72	6	5.15
NF73	4	5.72
NF74	1	0.47
NF81	7	22.18
NF82	12	13.62
NF83	4	5.84
NF84	4	5.07
NF92	3	11.54
NF94	5	5.34
PE38	5	20.23
PE48	3	6.57
PE49	4	8.27
PE58	4	5.59
PE59	4	7.09
PE68	3	5.56
PE69	5	8.67
PE75	5	6.26
PE76	8	12.73
PE77	5	5.75
PE78	4	10.25
PE79	5	11.35
PE85	4	3.53
PE86	3	8.28
PE87	5	8.06
PE88	6	10.56
PE89	3	9.14
PF01	7	20.47
PF02	8	11.85
PF03	3	6.61
PF11	1	1.21

<b>PF12</b>	3	11.50
<b>PF13</b>	4	14.90
<b>PF21</b>	3	5.94
<b>PF22</b>	4	7.85
<b>PF31</b>	2	8.42
<b>PF32</b>	2	4.35
<b>PF33</b>	3	12.27
<b>PF34</b>	3	5.42
<b>PF40</b>	1	0.77
<b>PF41</b>	7	9.82
<b>PF42</b>	4	7.32
<b>PF43</b>	1	5.40
<b>PF50</b>	3	6.43
<b>PF51</b>	4	4.58
<b>PF60</b>	4	6.90
<b>PF62</b>	2	5.24
<b>PF70</b>	2	5.21
<b>PF72</b>	1	5.46
<b>PF80</b>	3	5.74
<b>PF81</b>	4	11.95
<b>PF82</b>	2	5.17
<b>SUM</b>	221	441.26
<b>AVERAGE</b>	4	8.33

#### **Appendix 4 - Spatial overview of the Scat Detection Dog Teams survey transects of Action D1.**



***Appendix 5 - Effort of opportunistic transects.***

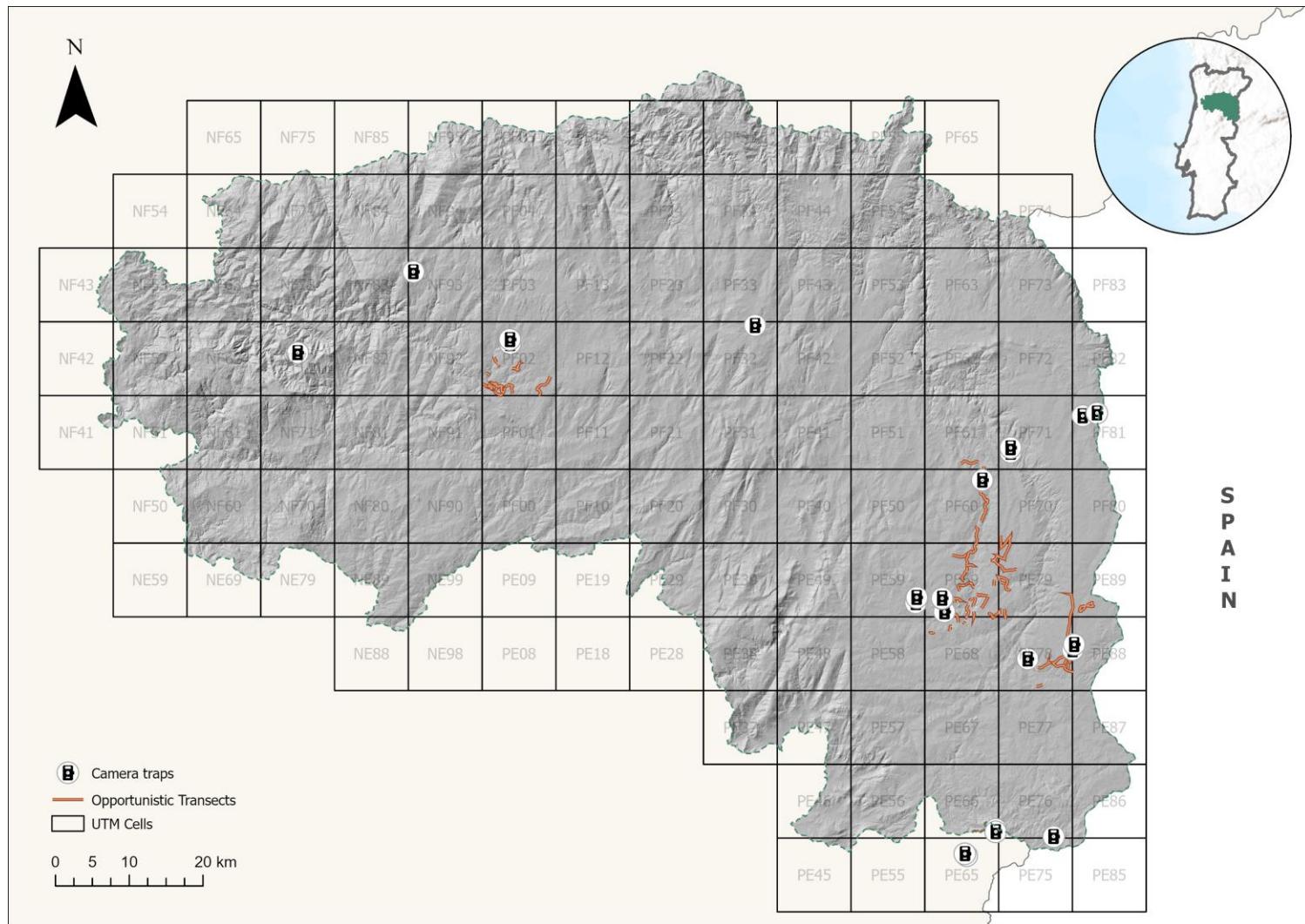
UTM 10X10 KM	TRANSECTS	LENGTH (KM)
<b>PE69</b>	20	41.82
<b>PE66</b>	1	5.33
<b>PE76</b>	1	0,2
<b>PE79</b>	6	17.62
<b>PF60</b>	2	11.41
<b>PF02</b>	8	21.88
<b>PF01</b>	2	0.59
<b>PF70</b>	1	2.88
<b>PE78</b>	7	34.36
<b>PE68</b>	8	6.72
<b>PE89</b>	3	5.14
<b>PF61</b>	2	3.03
<b>PE88</b>	2	1.78
<b>TOTAL</b>	63	152.57
<b>AVERAGE</b>	5	11.74

### Appendix 6 - Effort of opportunistic camera trapping.

#### UTM 10X10 KM    CAMERA TRAPS    NUMBER OF NIGHT/TRAPS

UTM 10X10 KM	CAMERA TRAPS	NUMBER OF NIGHT/TRAPS
PE65	2	27
PE76	1	16
PE66	2	52
PE78	1	30
PE88	2	36
PE69	3	16
PE59	3	81
PF60	2	112
PF71	2	37
PF81	2	164
NF72	1	11
PF02	4	205
PF32	2	232
NF93	2	38
<b>TOTAL</b>	<b>29</b>	<b>1057</b>
<b>AVERAGE</b>	<b>2</b>	<b>141</b>

**Appendix 7 - Spatial overview of opportunistic transects and camera traps deployed between 2020 and 2022.**



**Appendix 8 - Summary of the results of the 205 scat samples (A3 – 112; D1: 93) received in the genetic lab for analysis. In grey shading, samples that were not selected for DNA isolation, that were replicates of other samples or that consistently failed in amplification. \* - recapture. (1) – this sample was incorrectly assigned to a different genotype (W08C) in A3 report, but is a recapture of genotype W02C; (2) – this sample was incorrectly assigned to a different genotype (W09C) in A3 report, but is a recapture of genotype W6C.**

	Date of Collection	Extraction	Amplification	mtDNA	Assigned species	Assigned haplotype (Villá et al. 1997)	Genotype
<b>Action A3</b>							
WFL001	27/02/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01C
WFL002	27/02/2019	OK	OK	<i>Vulpes vulpes</i>	Red fox	-	-
WFL010	05/03/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D4	D01C
WFL013	29/03/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W02C
WFL016	29/03/2019	Not selected for DNA isolation					
WFL020	30/03/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D1	-
WFL021	30/03/2019	OK	Failed 3X				
WFL024	30/03/2019	OK	Failed 3X				
WFL026	30/03/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL028	30/03/2019	OK	OK	Failed			
WFL029	30/03/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL032	31/03/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01C*
WFL034	31/03/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL039	01/04/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6	-
WFL040	02/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL041	02/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL042	02/04/2019	Not selected for DNA isolation					
WFL043	02/04/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6	D02C
WFL044	03/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03C
WFL051	04/04/2019	Not selected for DNA isolation					
WFL053	04/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL055	27/04/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6	D03C
WFL056	27/04/2019	OK	Failed 3X				
WFL060	28/04/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6	D04C
WFL062	29/04/2019	OK	Failed 3X				
WFL063	30/04/2019	Not selected for DNA isolation					
WFL064	30/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01W
WFL065	30/04/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-

WFL076E	10/05/2019	OK	OK	<i>Vulpes vulpes</i>	Red fox	-	-
WFL079C	12/05/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D4	D05C
WFL080C	12/05/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D8	-
WFL081C	13/05/2019	OK	Failed 3X				
WFL082C	13/05/2019	OK	Failed 3X				
WFL084C	13/05/2019	OK	OK	<i>Vulpes vulpes</i>	Red fox	-	-
WFL085W	15/05/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W04W
WFL088C	15/05/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W05C
WFL089C	17/05/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL091C	17/05/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W06C
WFL093C	24/05/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W06C*
WFL094C	27/06/2019	OK	OK	<i>Vulpes vulpes</i>	Red fox	-	-
WFL095C	08/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D26	-
WFL096C	09/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D8	-
WFL097C	09/08/2019	OK	OK	Failed			
WFL098E	13/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	D01E
WFL099E	13/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D1	-
WFL100E	13/08/2019	OK	Failed 3X				
WFL101E	13/08/2019	OK	OK	<i>Vulpes vulpes</i>	Red fox	-	-
WFL102E	14/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL103E	14/08/2019	OK	Failed 3X				
WFL110E	19/08/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL111C	21/08/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W04C
WFL113C	23/08/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D19	-
WFL114C	22/08/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL115C	27/09/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL116C	27/09/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W07C
WFL117C	28/09/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W02C*
WFL118O	04/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL119C	08/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W02C*(1)
WFL120C	09/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL122O	23/10/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D3	-
WFL123O	23/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL124OA	25/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL124OB	25/10/2019	OK	replicate				
WFL125O	25/10/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	D01W
WFL126O	25/10/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-

WFL127O	25/10/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL128O	08/11/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL129O	08/11/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL130O	08/11/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W02W
WFL131C1	20/11/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL131C2	20/11/2019	OK	replicate				
WFL132C1	20/11/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W06C* (2)
WFL132C2	20/11/2019	OK	replicate				
WFL133C1	21/11/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D4	D06C
WFL133C2	21/11/2019	OK	replicate				
WFL134C	21/11/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D16	-
WFL135C	22/11/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D6	-
WFL137C1	24/11/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	D07C
WFL137C2	24/11/2019	OK	replicate				
WFL138C	22/11/2019	OK	OK	Failed			
WFL139C	26/11/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D6	-
WFL142O	06/12/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D6	-
WFL143O	06/12/2019	OK	OK	Failed			
WFL144O	06/12/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D22	-
WFL148C1	09/12/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D1	-
WFL148C2	09/12/2019	OK	replicate				
WFL150C1	11/12/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	D08C
WFL150C2	11/12/2019	OK	replicate				
WFL151C1	11/12/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D3	D09C
WFL151C2	11/12/2019	OK	replicate				
WFL152C	14/12/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL153C	14/12/2019	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL154O	04/01/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL155O	04/01/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03W
WFL158O	04/01/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL159C	05/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D3	-
WFL160C	05/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D26	-
WFL166C1	07/01/2020	OK	Failed 3X				
WFL166C2	07/01/2020	OK	Failed 3X				
WFL166C3	07/01/2020	OK	Failed 3X				
WFL169C1	09/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL169C2	09/01/2020	replicate (not selected for DNA isolation)					

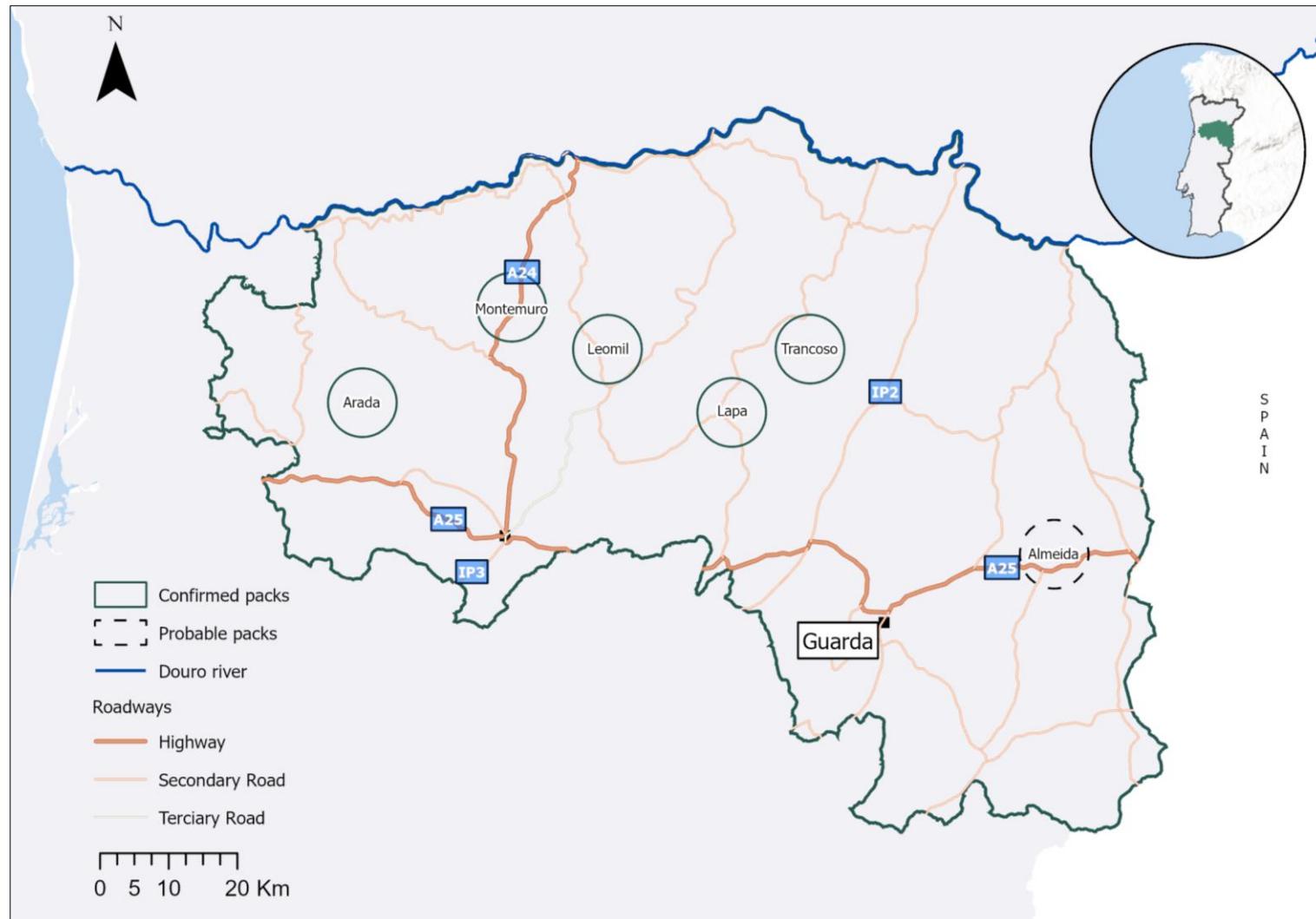
WFL172C	10/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	-
WFL173E1	11/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D6	D02E
WFL173E2	11/01/2020	OK	replicate				
WFL174E	12/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D4	-
WFL179E1	12/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	Close to D4	D03E
WFL179E2	12/01/2020	OK	replicate				
WFL181E1	13/01/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D26	D04E
WFL181E2	13/01/2020	OK	replicate				
WFL181E3	13/01/2020	OK	replicate				
WFL182E	13/01/2020	OK	Failed 3X				
WFL184E	13/01/2020	OK	OK	<i>Herpestes ichneumon</i>	Egyptian mongoose	-	-
WFL188E1	14/01/2020	OK	OK	<i>Canis lupus familiaris</i>			-
WFL188E2	14/01/2020	OK	replicate				
<b>Action D1</b>							
WFL189E	14/05/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	
WFL190E	17/09/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a	
WF1 CYL	05/02/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a	
WF2 CYL	11/02/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6a	
WF3 CYL	11/02/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6a	
WF4 CYL	28/02/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W3	W1CyL
WF5 CYL	05/03/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	D6a	
WF6 CYL	13/03/2020	OK	Failed 3x				
WF7 CYL	06/05/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W3	W2CyL
WFL191E	26/11/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03E
WFL_OSSO		OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D26a/D3/D15	
WFL192E	11/02/2020	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D3/D14	
SDED35	21/01/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
SDED36	21/01/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL195	26/11/2021	OK	OK	<i>Canis lupus familiaris</i>	Dog		
WFL196	02/09/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL201W	07/12/2022	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL202E	10/12/2022	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D15/D26a	
SDED31	05/07/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
SDED34	18/08/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01E*

SDED37	21/08/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01E*
SDED38	21/08/2021	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W01E*
SDED14	02/11/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
SDED4	08/07/2020	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFLP01	01/04/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D4	
WFLP02	13/05/2019	OK	OK	<i>Canis lupus familiaris</i>	Dog	D3	
WFLP03	25/03/2021	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D15/D26a	
WFL205Eb	09/05/2023	OK	OK	failed			
WFL205Ea	09/05/2023	OK	Failed 3x				
WFL206Ea	09/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a/D19/D21	
WFL206Eb	09/05/2023	replicate (not selected for DNA isolation)					
WFL207Ea	11/05/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL207Eb	11/05/2023	replicate (not selected for DNA isolation)					
WFL208Ea	11/05/2023	OK	Failed 3x				
WFL208Eb	11/05/2023	OK	OK	failed			
WFL213Ea	23/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a/D19/D21	
WFL213Eb	23/05/2023	replicate (not selected for DNA isolation)					
WFL214a	24/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a/D19/D21	
WFL214b	24/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a/D19/D21	
WFL214c	24/05/2023	replicate (not selected for DNA isolation)					
WFL215a	24/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a/D19/D21	
WFL215b	24/05/2023	replicate (not selected for DNA isolation)					
WFL215c	24/05/2023	replicate (not selected for DNA isolation)					
WFL218	28/05/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D6a	
WFL219Ca	29/05/2023	OK	Failed 3x				
WFL219Cb	29/05/2023	OK	Failed 3x				
WFL220E	09/05/2023	OK	Failed 3x				
WFL221E	21/06/2023	OK	Failed 3x				
WFL222a	26/04/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	D15	
WFL222b	26/04/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	D15	
WFL222c	26/04/2023	replicate (not selected for DNA isolation)					
WFL222d	26/04/2023	replicate (not selected for DNA isolation)					

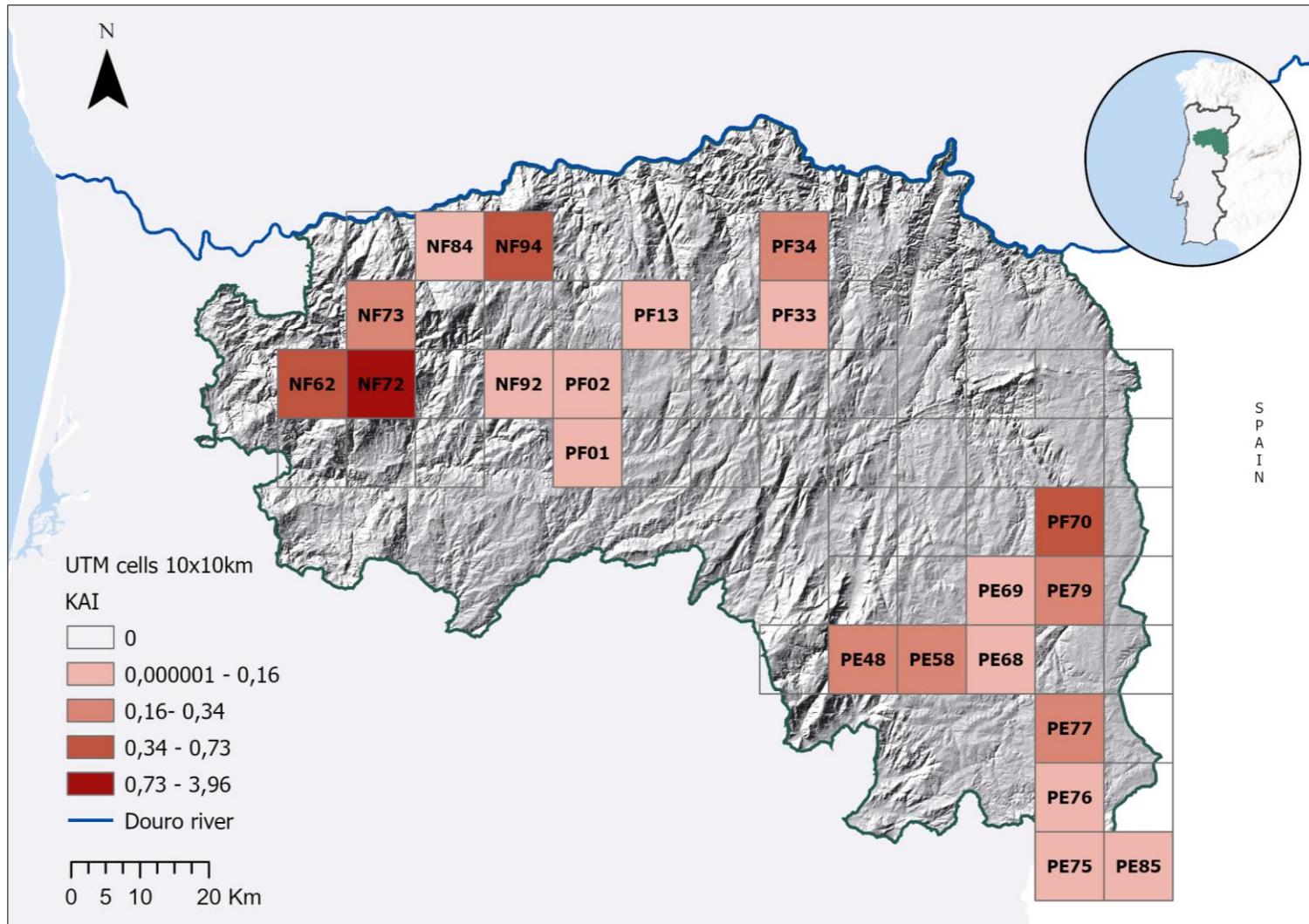
WFL222e	26/04/2023	replicate (not selected for DNA isolation)					
WFL230O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W05W
WFL231O	06/02/2024	OK	Failed 3x				
WFL232O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W06W
WFL233O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	
WFL236O	06/02/2024	OK	Failed 3x				
WFL237O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03W*
WfL238O	06/02/2024	OK	Failed 3x				
WFL240O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03W*
WFL243O	06/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL245O	22/02/2024	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D6	-
WFL246O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	
WFL247O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL248O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W03W*
WFL249O	23/02/2024	OK	Failed 3x				
WFL250O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL251O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL252O	23/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL253O	23/02/2024	OK	Failed 3x				
WFL254O	24/02/2024	OK	OK	<i>Vulpes vulpes</i>	Red fox		
WFL255C	25/02/2024	OK	Failed 3x				
WFL256C	25/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL257C	25/02/2024	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D4	-
WFL258C	28/02/2024	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL259CA	31/03/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D7a	-
WFL259CB	31/03/2023	replicate (not selected for DNA isolation)					
WFL260CA	28/03/2023	OK	OK	<i>Canis lupus familiaris</i>	Dog	close to D6a/W6/D6	
WFL260CB	28/03/2023	replicate (not selected for DNA isolation)					
WFL261C	29/03/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	Falhou
WFL262C	29/03/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W08C
WFL263CA	07/07/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL263CB	07/07/2023	replicate (not selected for DNA isolation)					
WFL264CA	07/07/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W09C
WFL264CB	07/07/2023	replicate (not selected for DNA isolation)					
WFL265CA	08/07/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	-
WFL265CB	08/07/2023	replicate (not selected for DNA isolation)					

WFL266O	05/07/2023	OK	OK	<i>Canis lupus signatus</i>	Iberian wolf	W1	W09C*
WFL267A	07/07/2023	OK	OK	<i>Vulpes vulpes</i>	Red fox		
WFL267B	07/07/2023	replicate (not selected for DNA isolation)					
WFL268A	07/07/2023	OK	OK	<i>Vulpes vulpes</i>	Red fox		
WFL268B	07/07/2023	replicate (not selected for DNA isolation)					

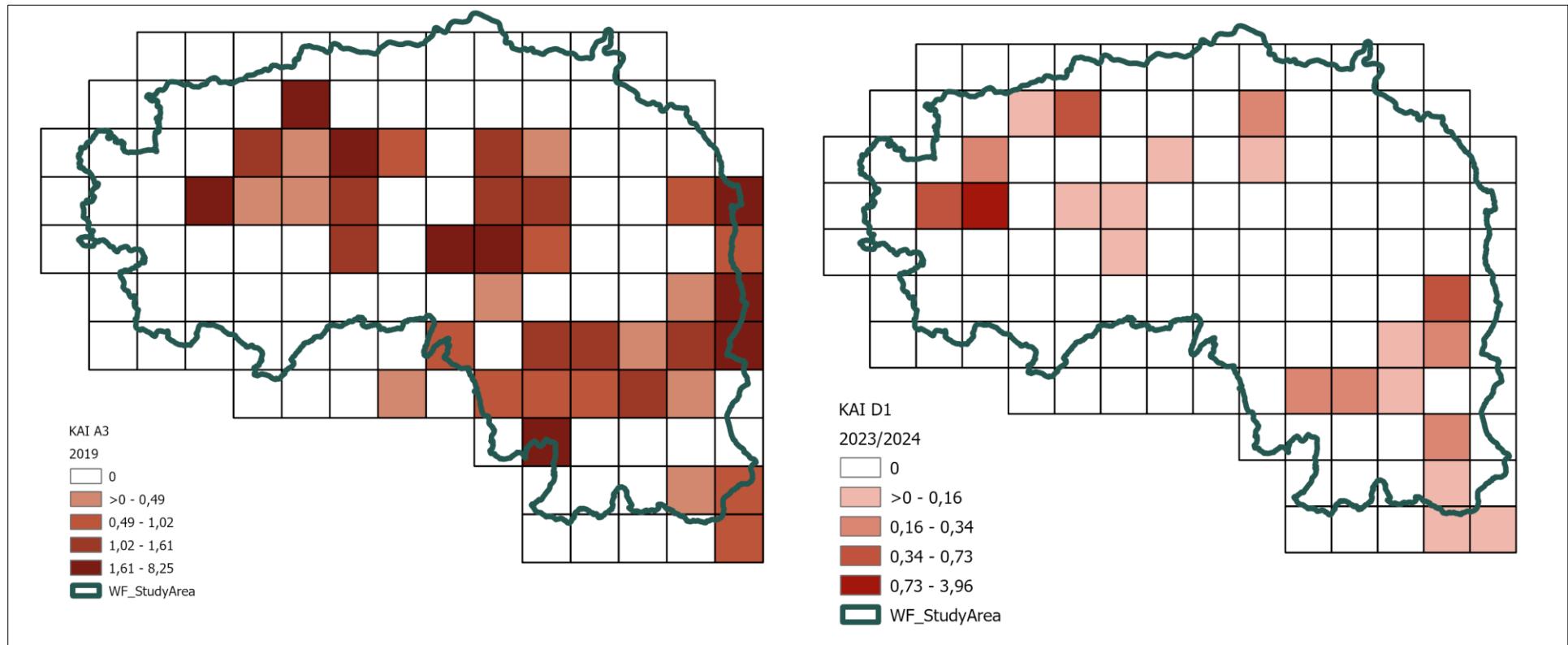
**Appendix 9 - Packs detected during the last national wolf survey. Adapted from Pimenta et al., 2023.**



**Appendix 10 - Kilometric Abundance Index (KAI) obtained in the project area during Action D1 (2023-2024).**



**Appendix 11 - Comparison between average KAI values/UTM cell between Action A3 and Action D1.**

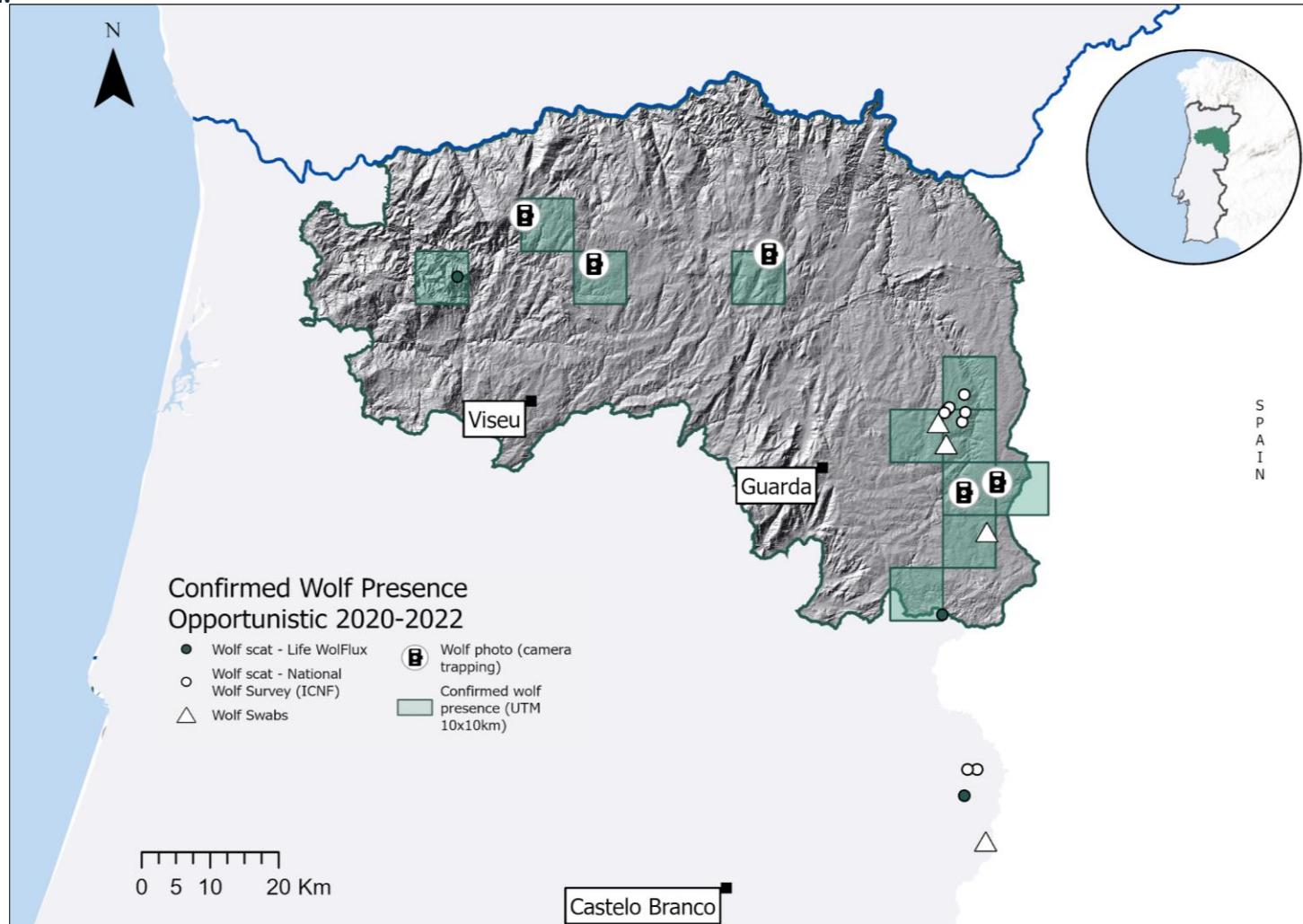


**Appendix 12 - Assignment of swabs obtained from livestock attacks to Iberian wolf and dog.**

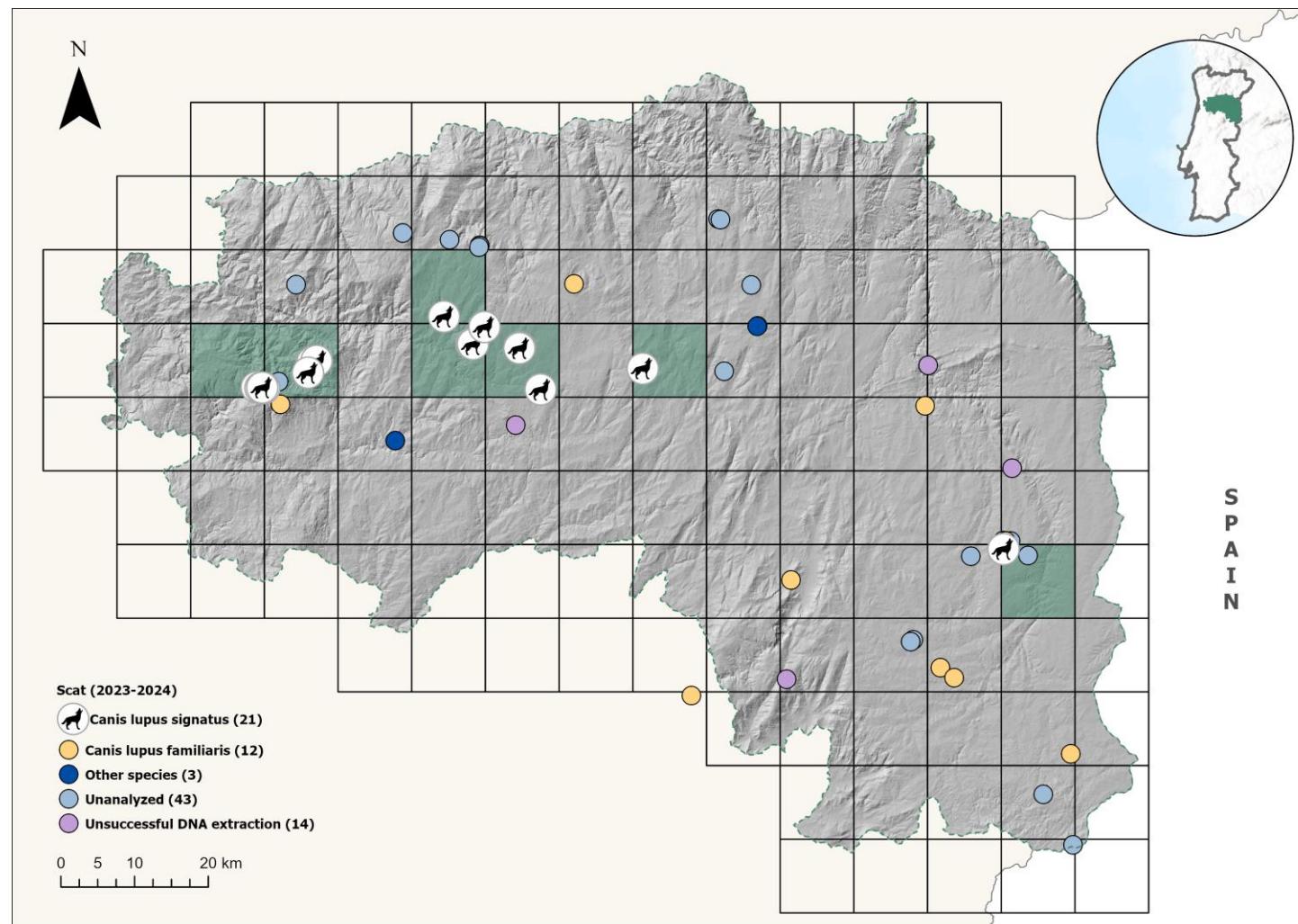
Livestock Attack	Locality	Swabs (totals)	Assigned Swabs		Genotype
			Dog	Wolf	
<b>Action A3</b>					
WFA01	Menoita/Guarda	6	6	0	D04E
WFA02	Meios/Guarda	6	1	0	
WFA03	Gosendinho	6	0	0	
WFA04	Cerdeira/Gralheira/Cinfães	6	0	3	
WFA05	Barreiro	6	1	2	
WFA06	Reboleiro	7	4	0	D10C
WFA07	Montes	6	0	0	
WFA08		6	1	0	
WFA09	Mizarela/Arouca	6	1	1	
WFA10	Regoufe	3	0	1	
WFA11	Sul	6	0	1	
WFA12	Sul	6	0	4	
WFA13	Reboleiro	3	2	0	
WFA14	Marmeiro	2	1	1	
WFA15	Monte Real	6	3	0	
WFA16		6	4	0	
WFA17	Vila Cortês da Serra	10	3	0	
WFA18	Rainho	4	4	0	
WFA19	Rodelas	1	0	0	
WFA20	Chãos	8	1	2	
WFA21	Rodelas	2	1	0	
WFA22	Castelo Mendo	6	0	5	W01E
WFA23	Reboleiro, Trancoso	6	3	0	
WFA24	Beselga, Penedono	6	4	0	
WFA25	Reboleiro	18	3	10	W03C
WFA26	Pala	6	6	0	
WFA27	Castanheira	6	0	0	
WFA28	Touro	6	3	0	D11C
WFA29	Reboleiro	6	2	0	
WFA30	Beselga, Penedono	6	2	0	
WFA31	Pereiro	4	0	0	
WFA32	Arrifana do Côa	7	0	0	
WFA33	Beselga, Penedono	6	3	0	
WFA34	Ribeira das Naus	6	0	0	
WFA35		6	0	1	

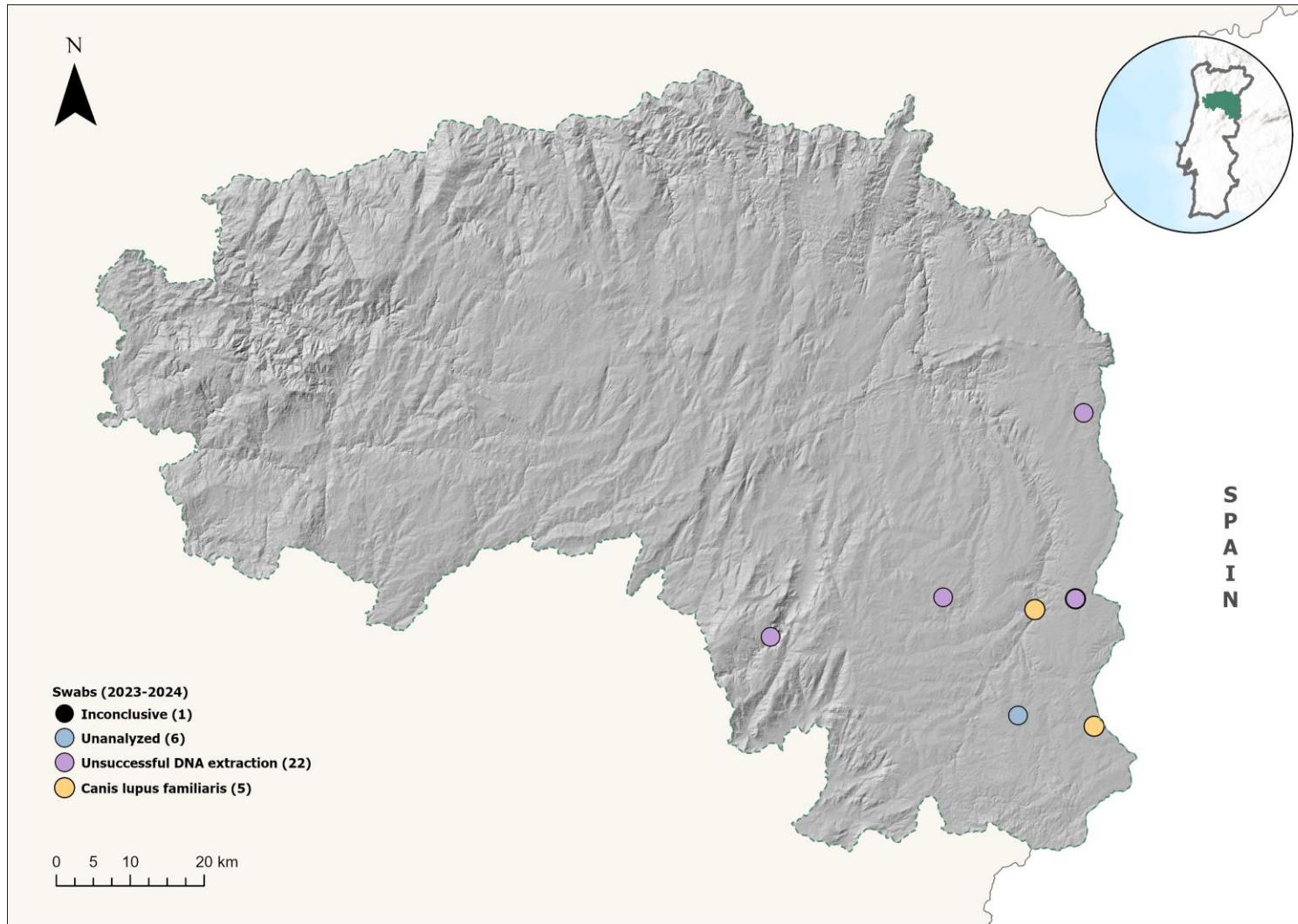
WFA36	Nave	6	0	5	W02E
WFA37	Reboleiro	6	4	0	D12C
WFA38	Aveloso	6	0	4	W02C
<b>Action D1</b>					
WFA39	Monte da Velha	6	1	3	
WFA40	Malhada Sorda	6	6	0	
WFA41	Amoreira	6	0	0	
WFA42	Vila Maior	6	not analysed		
WFA43	Freixo	2	0	1	
WFA44	Quinta da Boa Vista	6	not analysed		
WFA45	Reboleiro	6	not analysed		
WFA46	Reboleiro	4	not analysed		
WFA47	Rebolosa	6	0	1	
WFA48	Castelo Mendo	5	3	0	
WFA49	Gurgulhão	6	0	0	
WFA50	Almeida	4	not analysed		
WFA51	Atalaia-Safurdão	6	not analysed		
WFA52	Arrifana do Côa	6	0	0	
WFA53	Torre-do-Terrenho/ Sebadelhe-da-Serra	6	not analysed		
WFA54	Póvoa do Concelho	3	0	0	
WFA55	Póvoa do Concelho	4	1	0	
WFA56	Quinta da Carvalheira, Meda	6	5	0	
WFA57	Monfortinho	6	4	0	
WFA58	Monte Leal	6	not analysed		
WFA59	Mangide	6	3	0	
WFA60	Charco Fundo /Monfortinho	2	0	0	
WFA61	Almeida/Malpartida	5	3	0	
WFA62	Almeida/Malpartida	6	4	0	
WFA63	Mangide/Almeida	4	3	0	
WFA64	Mangide/Almeida	4	2	0	
WFA65	Nave-Longa/ Salvaterra do Extremo	8	0	6	W03E cf
WFA66	Alvendre (Guarda)	3	0	0	
WFA67	Freixo	4	0	1	
WFA68	Freineda	6	2	0	
WFA69	Aldeia da Dona	6	0	0	
WFA70	Castanheira	3	0	0	
WFA71	Castelo Branco	5	3	0	
WFA72	Malpartida	3	0	0	
WFA73	Jardo	3	2	0	
WFA74	Aldeia da Ponte	6	0	0	
WFA75	Aldeia da Ponte	1	1	0	
WFA76	Quintela da Lapa	6	0	0	

**Appendix 13 - Confirmed wolf presence during the opportunistic period (2020-2022). Scat samples were collected by LIFE Wolflux and NWS (ICNF) surveying actions. LIFE Wolflux camera trapping and forensic analysis of canid attacks were very useful tools to confirm wolf presence during this period.**

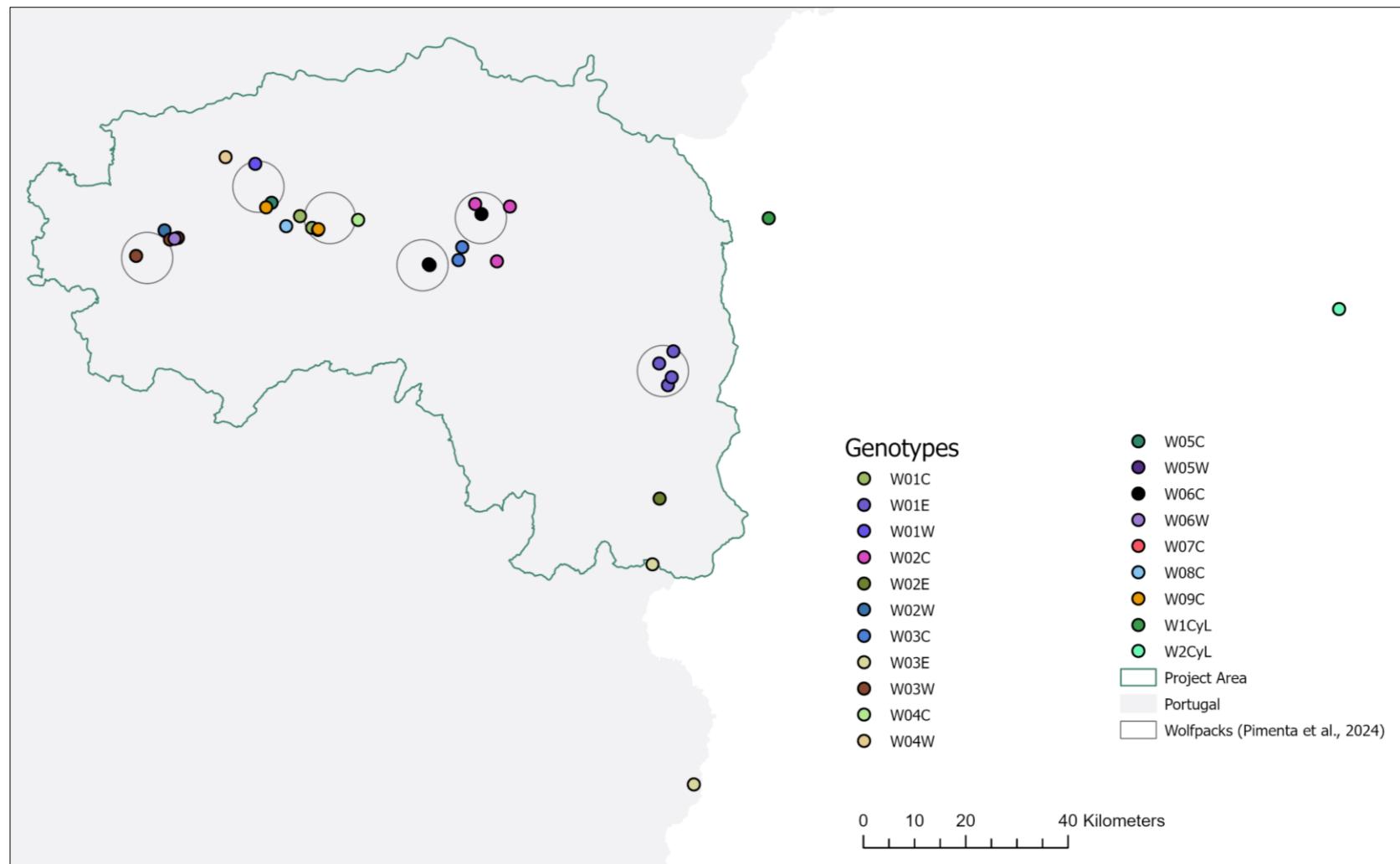


**Appendix 14 - Genetic assessment of scat collected between 2023 and 2024, in the scope of the project's Action D1. This representation includes scat that did not undergo genetic analysis, and green cells represent wolf presence confirmed by such NGS method.**

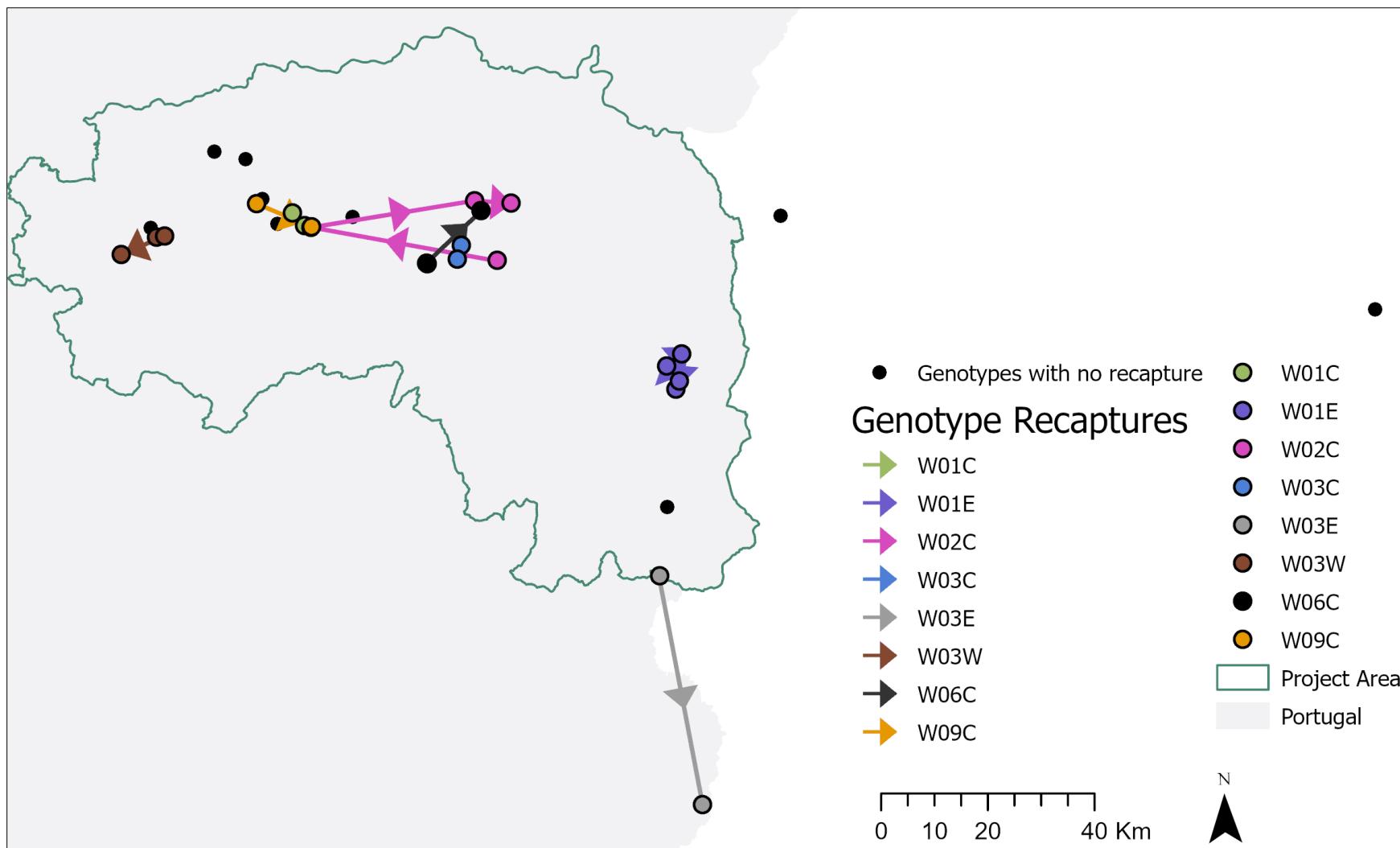


**Appendix 15 - Genetic assessment of swabs collected between 2023 and 2024, in the scope of project's Action D1.**

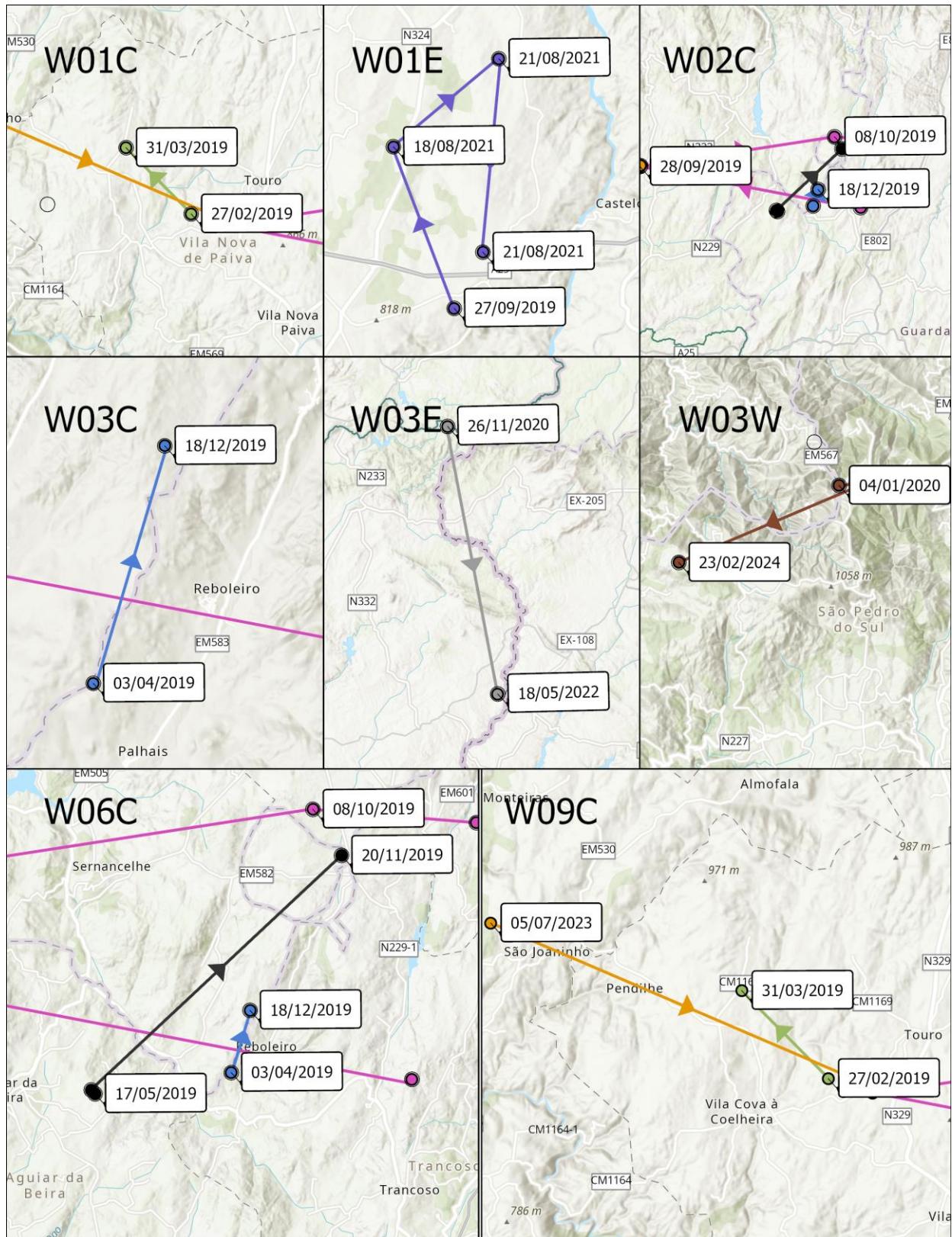
**Appendix 16 - Individual genotypes obtained by swabs and scat between 2019 and 2024 (A3 + D1). Empty larger circles indicate the location of detected wolf packs in the NWS (ICNF) – Pimenta et al. 2023**



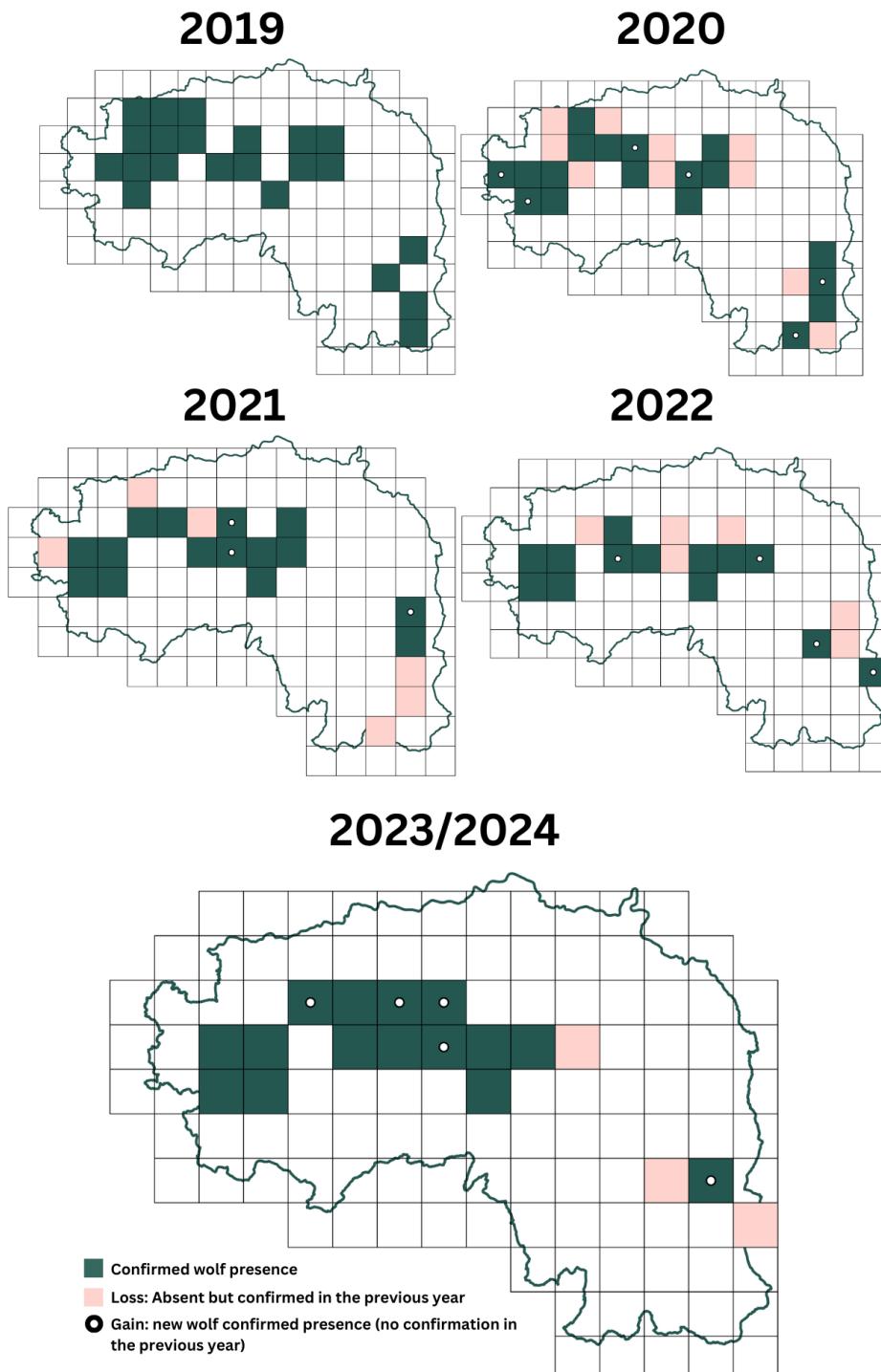
**Appendix 17 - Genotype Capture-Recapture (CR) obtained between 2019 and 2024 (A3 + D1).**



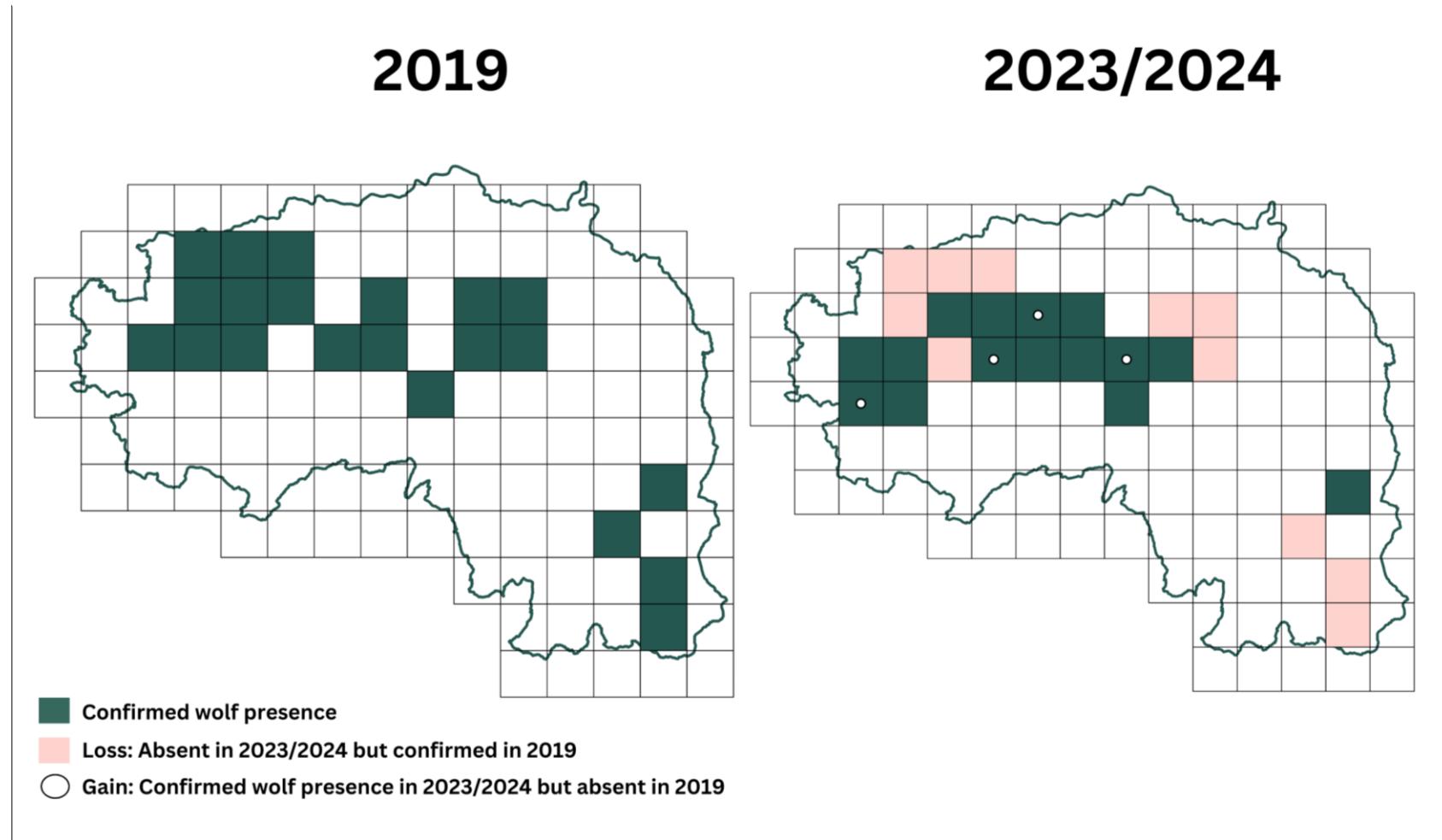
**Appendix 18 - Spatial overview of individual genetic profiles CR between 2019 and 2024 (A3 + D1).**



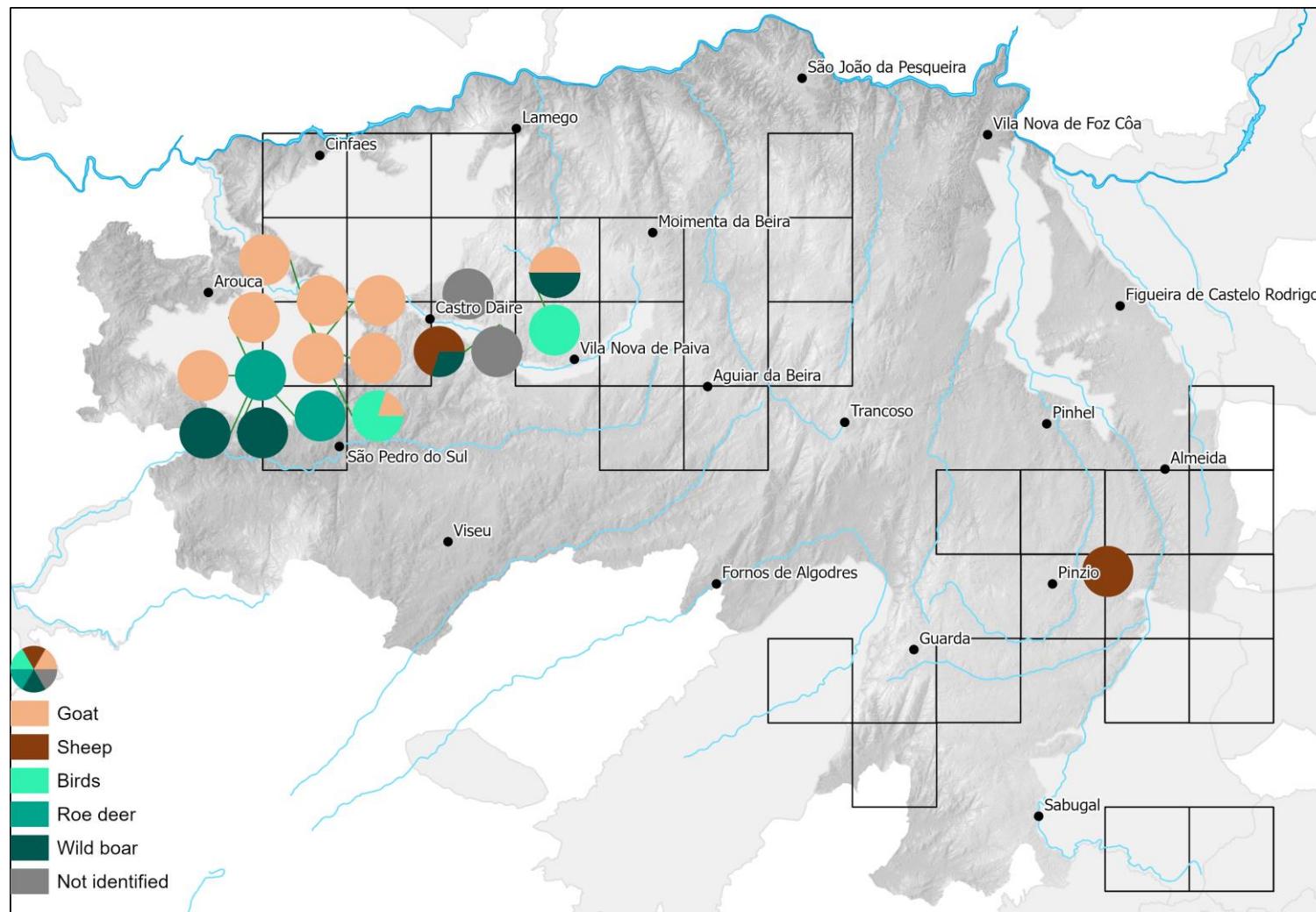
**Appendix 19 - Population trend during LIFE WolFlux project reveals spatial instability. Data provided by the LIFE WolFlux project and the protocol established with ACHLI.**



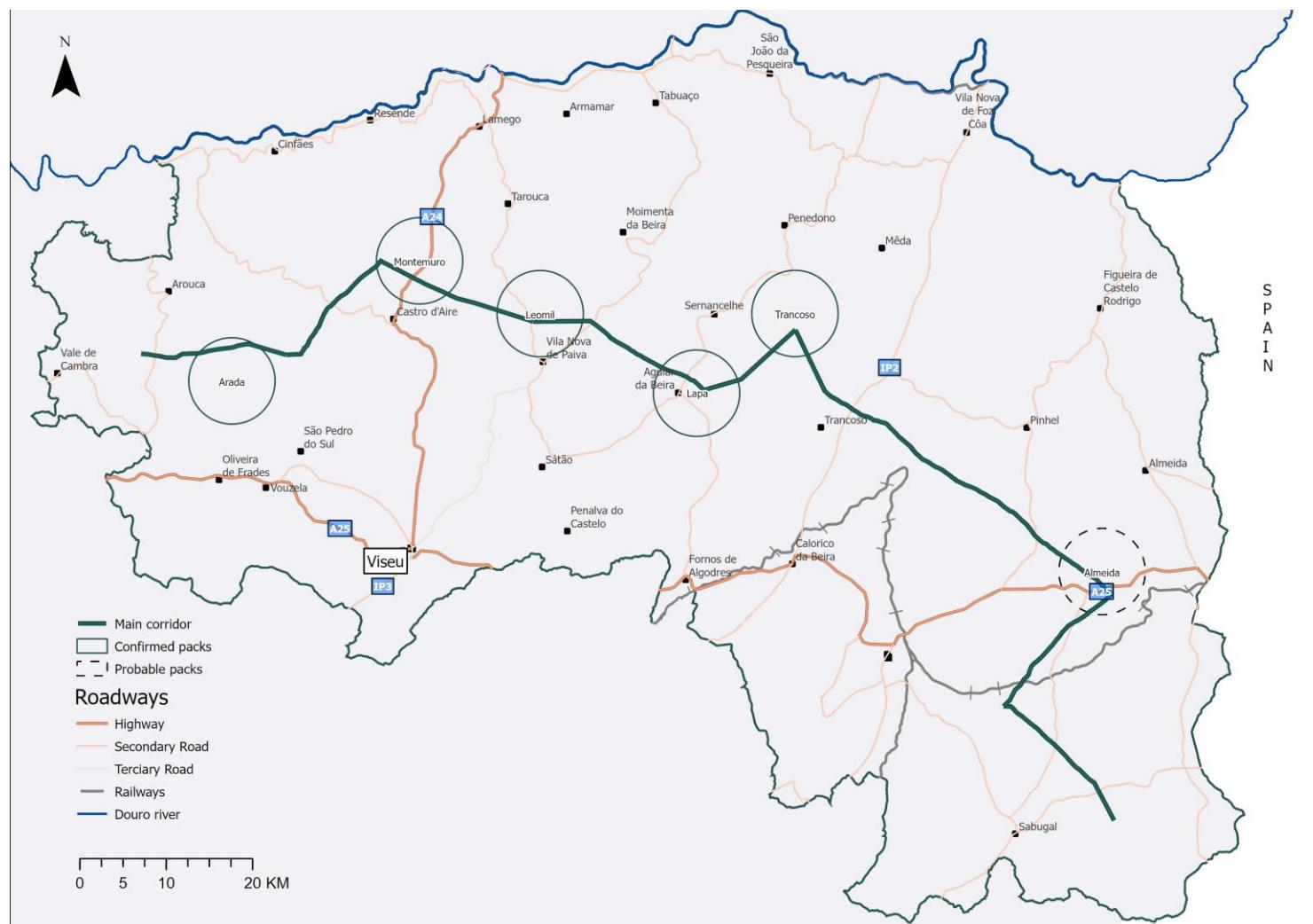
**Appendix 20 - Comparison of wolf presence in the project area in 2019 and 2023/2024. Data provided by the LIFE WolFlux project and the protocol established with ACHLI.**



### Appendix 21 - Spatial overview of feeding ecology results.



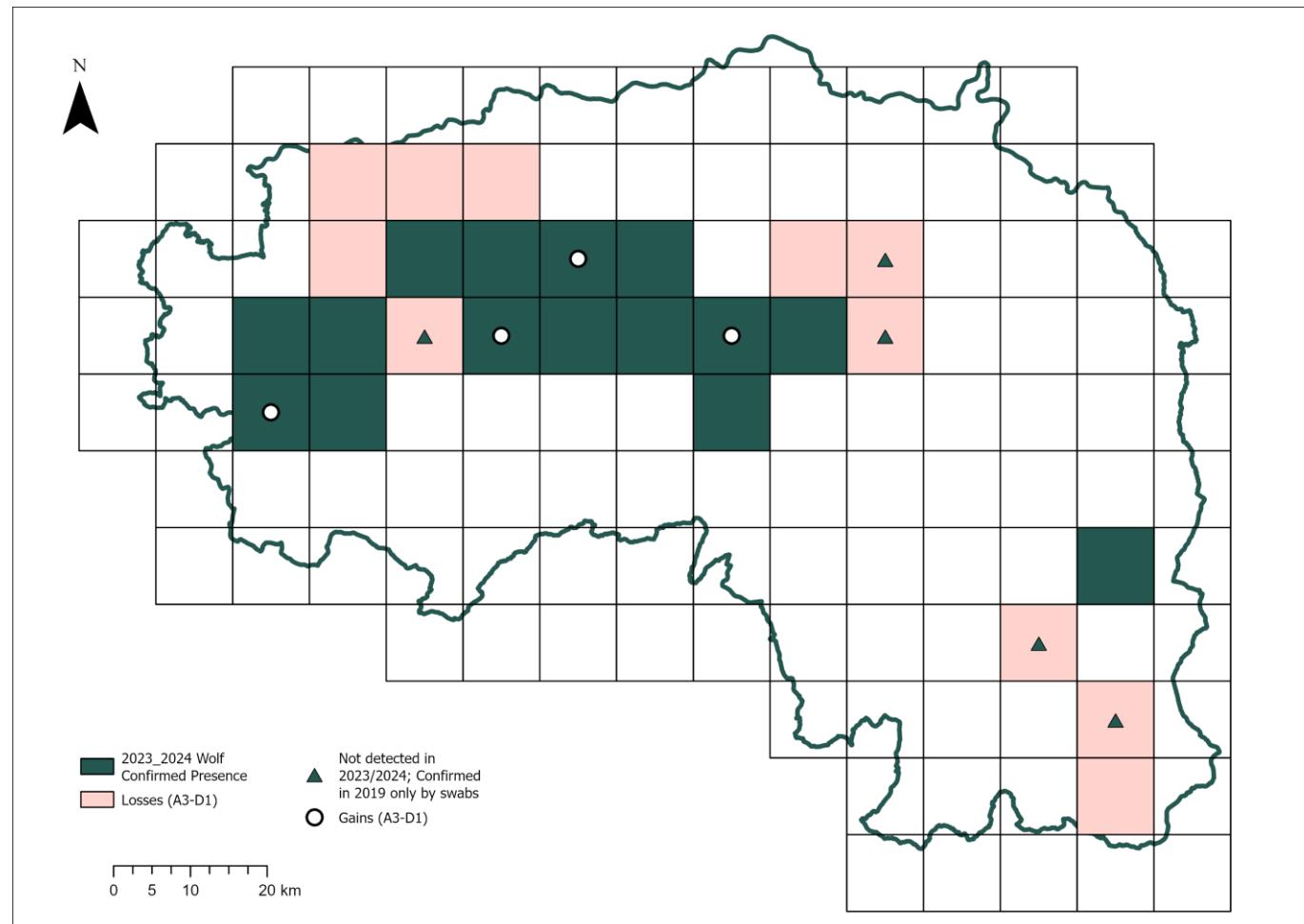
**Appendix 22 -Connectivity corridors of wolf movement, determined by a cost surface model that used A3 wolf presence data among other variables. Adapted from Pinto & Costa, 2023.**



**Appendix 23 - Project indicators summary and variation (%) from baseline (action A3) to end of project (action D1). Misassigned individuals: individual genotypes sampled in one population but assigned to the gene pool from other population, with very high probability (>90%). Cross-population recaptures: individuals sampled in one population and recaptured in a different population. Average proportion of admixture: average proportion of individual genotypes assigned to the gene pool of a different population than the population where they were sampled.**  
 \* - includes the two genotypes retrieved from samples collected in Castilla y Leon, Spain.

Indicators	Baseline (A3)	After Baseline	End of Project (A3 to D1)	Variation (%) from baseline to end of project
<b>Analysed Samples (% of analysed)</b>				
Scats analysed	93	72	165	77%
Scats identified (%)	78 (84%)	63 (88%)	141 (85%)	81%
Livestock attacks analysed	38	30	68	79%
Livestock attacks identified (%)	31 (82%)	19 (63%)	50 (74%)	61%
Swabs analysed (from livestock attacks)	225	141	366	63%
Swabs identified (%)	125 (56%)	54 (38%)	179 (49%)	43%
<b>Confirmed Iberian wolf samples (% of identified)</b>				
in scats (%)	37 (47%)	34 (54%)	71 (50%)	92%
in livestock attacks (%)	13 (42%)	4 (21%)	17 (34%)	31%
<b>Genotyped Iberian wolf samples</b>				
Samples (scats + swabs)	22	16*	38	72%
Different individuals	13	7*	20	53%
Recaptures	9	9	18	100%
<b>Connectivity</b>				
Misassigned individuals	3	0	3	0%
Cross-population recaptures	0	0	0	0%
Long-distance recaptures (more than 30Km)	1	1	2	100%
Average proportion of admixture	0.352	0.338	0.348	-1%

**Appendix 24 - Wolf confirmed presence during D1 Action (2023/2024). Data: LIFE WolFlux project and ACHLI protocol (CIBIO and Aveiro University teams). Losses are depicted in pink and represent those UTM cells which were confirmed in Action A3, but not in D1; green triangles show the UTM cells that were confirmed solely by swab genetic assessment during A3. Gains are illustrated by white dots and represent those UTM cells which were not previously confirmed in Action A3.**





# Annex

## **Annex 1**

# Iberian Wolf Distribution Model

## South of Douro River, Portugal

**2024**

## **Index**

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## 1. Introduction

Ecological niche modelling has become a pivotal tool for understanding the distribution patterns of species. It is particularly useful for those species inhabiting fragmented and human-modified landscapes. The Iberian wolf is one such species, and understanding its niche dynamics is critical for effective conservation. Previous studies on the ecological niche of the Iberian wolf have predominantly focused on variables such as topography, land cover, prey availability, and human disturbance (Grilo et al., 2019). These studies have demonstrated that the wolf's presence is closely associated with areas offering refuge from human activities, such as rugged terrains and dense forests, where wolves can avoid direct human encounters while accessing prey resources (Grilo et al., 2019).

Topographical variables, including altitude and slope, have consistently been highlighted as crucial determinants of habitat suitability for wolves. Higher altitudes and steep slopes provide natural refuges that limit human access and reduce the likelihood of human-wolf conflicts (Grilo et al., 2019). Vegetation cover, particularly the presence of dense vegetation, is another critical factor as it offers both protection from human activities and suitable habitats for prey species (Grilo et al., 2019). In contrast, open landscapes, particularly those fragmented by roads and human settlements, are typically avoided by wolves, underscoring the species' need for refuge (Grilo et al., 2019). Moreover, proximity to urban areas often correlates with higher mortality risks due to poaching and conflicts with livestock owners (Grilo et al., 2019). These findings highlight the importance of incorporating human disturbance factors into niche models to accurately predict wolf populations' distribution in human-dominated landscapes.

One of the novel aspects of the current distribution model is the inclusion of the variable which reflects distance to intensive livestock production units (poultry and rabbit farming). To the authors knowledge, this variable has not been previously used in previous models despite its potential significance. Intensive livestock farming units represent a critical trophic resource for wolves South of Douro river, particularly in areas where wild prey is scarce (Roque et al., 2012) and reliance on livestock may be higher.

The insights gained from this model are expected to be particularly valuable for future recommendations regarding habitat connectivity and the potential for gene flow between fragmented populations. Given that the project's primary objective was to analyse the connectivity of wolf groups south of the Douro River, understanding the ecological niche of the species in this context can provide significant insights into the challenges and

opportunities for conservation in this region. By integrating environmental variables and novel anthropogenic factors, this study seeks to enhance the predictive power of ecological niche models and contribute to more informed conservation strategies for the Iberian wolf.

## 2. Data and Methods

Six variables were selected and analysed to accurately model the distribution and habitat use. These variables were chosen based on their demonstrated relevance in previous studies and their potential influence on the species' ecological niche. Table\_1 summarises the key variables used in the analysis. Spatial rendering of the 6 environmental variables can be found between Annex 2 and Annex 7.

The "Distance to Intensive Farming" variable was developed using a multi-step process to identify and analyse potential intensive livestock production units across the study area. Initially, a shapefile containing building footprints for Europe was utilised as the primary dataset. From this dataset, buildings were selected based on specific size criteria that are typical of intensive farming units, namely its elongation. To ensure that these selected buildings were not located close to villages (and confused with non-intensive livestock shelters), only those structures more than 50 meters away from village perimeters were retained for further analysis. Even though modern Portuguese legislation demands that these units should be located even further away from villages, this distance was chosen due to old production units, built in previous eras.

Following this initial selection, a manual classification process was carried out. A total of 524 buildings were carefully examined using the ESRI "World Imagery" base map, allowing for precise identification and classification of buildings corresponding to intensive farming units. This process ensured that the variable accurately reflects the distribution of such units in relation to wolf presence, thereby contributing to a more nuanced understanding of how these anthropogenic factors influence the species' distribution and habitat use.

Table\_1 -- Variables used in the Iberian wolf Ecological Niche Model.

VARIABLE	DEFINITION	DATASET	PUBLISHER
<b>DISTANCE TO ROADS</b>	DISTANCE ACCUMULATION TO ROAD'S NETWORK	ROADS	OPEN STREET MAPS
<b>HUMAN FOOTPRINT</b>	HUMAN PRESSURE ON THE ENVIRONMENT	LAST OF THE WILD PROJECT, VERSION 3 (LWP-3): 2009 HUMAN FOOTPRINT, 2018 RELEASE	NASA SOCIOECONOMIC DATA AND APPLICATIONS CENTER (SEDAC)
<b>LIVESTOCK</b>	LIVESTOCK DENSITY	NATIONAL CENSUS OF AGRICULTURE	INSTITUTO NACIONAL DE ESTATÍSTICA
<b>LAND COVER</b>	DISTRIBUTION AND CLASSIFICATION OF LAND COVER	CARTA DE OCUPAÇÃO DO SOLO CONJUNTURAL DE 2023	DIRECÇÃO GERAL DO TERRITÓRIO
<b>ALTITUDE</b>	AVERAGE ALTITUDE (M)	EU_DEM_V11_E20N20	COPERNICUS
<b>DISTANCE TO INTENSIVE FARMING</b>	DISTANCE ACCUMULATION TO INTENSIVE FARMING	INDIVIDUAL BUILDING FOOTPRINTS FOR EU27 FROM THE HIERARCHICAL CONFLATION OF OSM, MICROSOFT BUILDINGS AND ESM R2020	EUROPEAN COMMISSION, JOINT RESEARCH CENTRE (JRC)

MaxEnt (Maximum Entropy) is a widely used tool for species distribution modeling due to its ability to provide accurate predictions even with limited presence data (Merow et al., 2013). The core principle of MaxEnt is to estimate the probability distribution of a species' occurrence by maximizing entropy, subject to environmental and anthropogenic constraints provided by the input variables (Phillips, 2017). Specifically, MaxEnt uses presence-only data, comparing the environmental conditions at known presence locations with those at randomly generated background locations (Phillips, 2017).

The model was developed using 155 presence records of the Iberian wolf (which comprises all the confirmed wolf presence within the project's period) along with a suite of environmental and anthropogenic covariates relevant to the species' distribution. The algorithm iteratively adjusts the model to maximize the difference between the environmental conditions at presence points and the conditions at the background points, thereby identifying the most suitable habitat for the species (Merow et al., 2013). Nonetheless, it is important to note that like in other machine learning methodologies, the data is divided into subsets: training, validation and testing. Therefore, of the initial 155 presence record points of the Iberian wolf, only 87 were used to train the model.

To avoid overfitting the model to the training data, MaxEnt includes a regularization feature that smooths the predicted distribution, ensuring that the model generalizes well to new

data (Phillips, 2017). The final model produced by MaxEnt represents the distribution with the highest entropy, meaning it provides the broadest possible prediction of habitat suitability while remaining consistent with the constraints imposed by the input variables.

### 3. Results

#### 3.1 Analysis of the model performance

The area under the receiver operating characteristic curve (AUC) is a common metric for evaluating machine learning models' performance. The obtained AUC value was 0.902 (Fig. 1Fig. 2), indicating excellent discriminatory power and suggesting that the model can reliably differentiate between presence and absence locations of the species

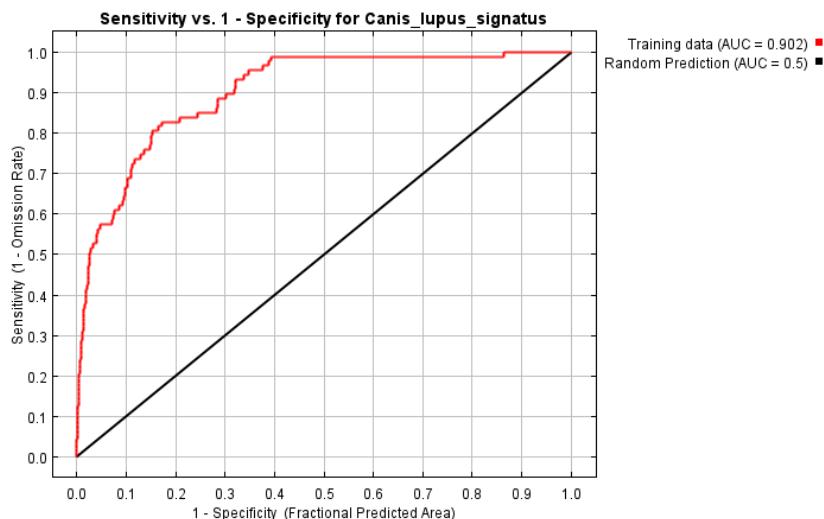
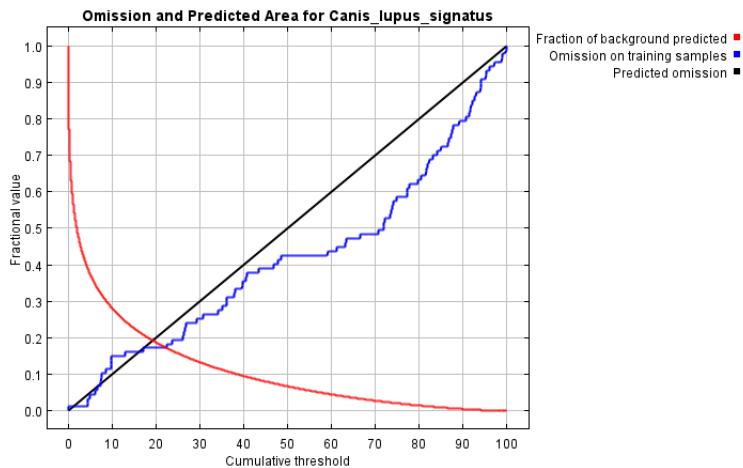


Fig. 1 - Receiver Operating Characteristic (ROC) curve for the distribution model. The curve compares the model's sensitivity (true positive rate) versus 1-specificity (false positive rate) across different threshold settings.

Fig. 2 depicts the relationship between the omission rate, predicted area, and cumulative threshold for the distribution model. The omission rate on training samples, shown by the blue line, represents the proportion of known species occurrences that the model fails to predict correctly. It indicates the percentage of actual presence locations that are omitted from the model's predictions.



*Fig. 2 - Relationship between omission rate, predicted area, and cumulative threshold for the model. The blue line shows the omission rate, the red line represents the fraction of the area predicted as suitable, and the black line indicates predicted omission.*

The cumulative threshold is a value used to determine which areas are predicted as suitable for the species. Lower thresholds result in more areas being classified as suitable, while higher thresholds make the model more selective, predicting fewer areas as suitable.

As the cumulative threshold increases, the omission rate (blue line) also rises. This behaviour is expected since the model becomes more conservative, identifying fewer areas as suitable for the species. At a specific threshold that balances sensitivity (the ability to correctly identify presence) and specificity (the ability to correctly identify absence), the model exhibits an omission rate of 17.2% (Table\_2). This means that the model fails to predict 17.2% of the known occurrences of the species, reflecting a conservative approach where the model tends to underestimate the species' presence (resulting in more false negatives) rather than overestimate it (avoiding false positives).

Table\_2 also shows that at lower cumulative thresholds, the omission rate remains low, indicating that the model successfully predicts most species occurrences. However, as the threshold increases, the predicted suitable area (red line) decreases, and the omission rate climbs, reinforcing the idea that the model becomes more restrictive in its predictions.

Table\_2 - Thresholds and corresponding omission rates.

Cumulative threshold	Cloglog threshold	Description	Fractional predicted area	Training omission rate
1.000	0.025	Fixed cumulative value 1	0.576	0.011
5.000	0.104	Fixed cumulative value 5	0.373	0.046
10.000	0.192	Fixed cumulative value 10	0.281	0.149
0.023	0.002	Minimum training presence	0.863	0.000
7.497	0.153	10 percentile training presence	0.320	0.092
22.412	0.387	Equal training sensitivity and specificity	0.172	0.172
22.546	0.388	Maximum training sensitivity plus specificity	0.172	0.172
4.273	0.090	Balance training omission, predicted area and threshold value	0.393	0.011
9.306	0.181	Equate entropy of thresholded and original distributions	0.291	0.115

### 3.2 Iberian Wolf Distribution Model

Based on the spatial analysis provided by the MaxEnt model, the results indicate a complex pattern of habitat suitability across the study region, with significant variability in the species' probability of occurrence. The areas where the probability of wolf presence is highest are primarily concentrated in the western and central parts of the project area, particularly around Montemuro, Leomil, and Arada packs (Fig. 3). These regions are characterized by a relatively continuous ecological niche, suggesting that they offer favorable conditions for the species. The model highlights that these areas have the essential resources and environmental conditions necessary for the species' persistence, such as higher elevations, which reduce human disturbances and offer better protection.

In contrast, the eastern part of the region, shows a distinct gap in habitat suitability, creating a significant discontinuity in the ecological niche. This gap may act as a barrier to movement and gene flow between the western and eastern wolf populations, potentially isolating these groups and leading to fragmented populations. The analysis also reveals that there are relatively few areas within the study region where the habitat is considered optimally suitable for the wolf. Most of the suitable habitats are limited to small, scattered patches, which further emphasizes the vulnerability of South of Douro River wolf population. This patchiness in habitat suitability is likely influenced by a combination of factors, including land cover, altitude, particularly slope, and distance from human infrastructure.

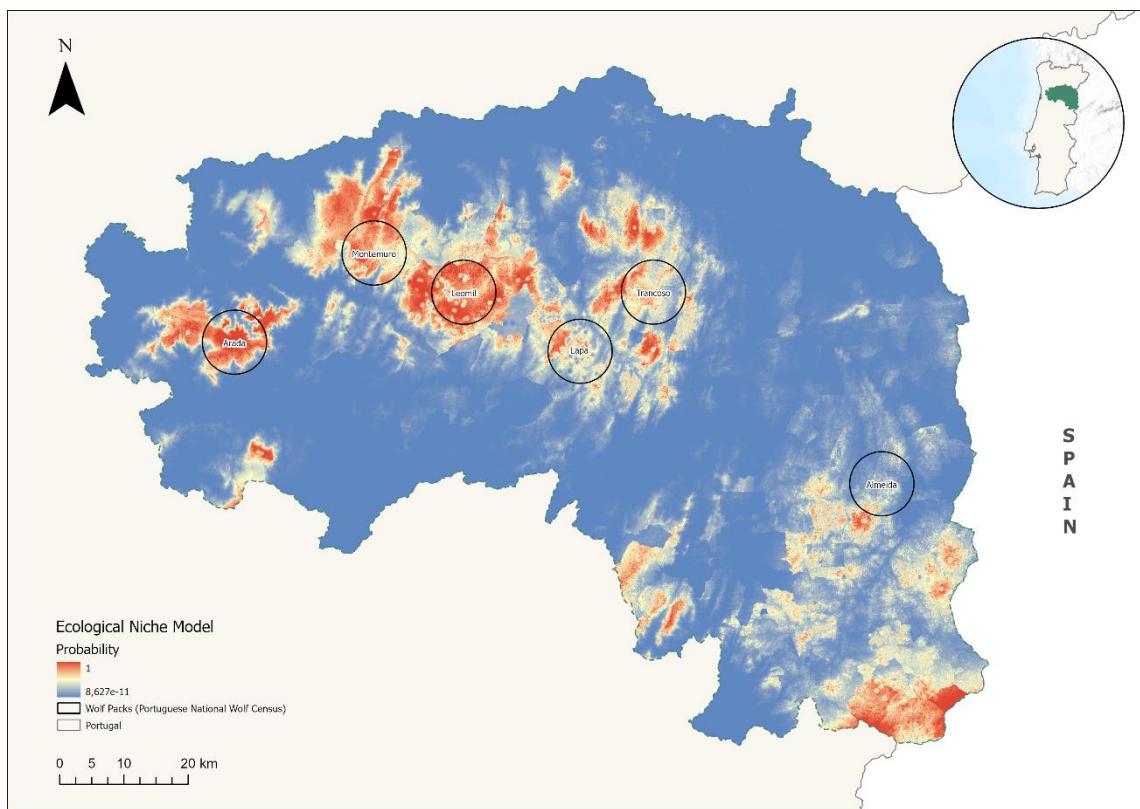


Fig. 3 - Iberian wolf ecological niche South of Douro river. The model was built using the totality of wolf records (genetic confirmation obtained from scat and swabs, and camera trapping), collected between 2019 and 2024 in the scope of Life WolFlux project. Source for pack location: (Pimenta et al., 2023)

Each of the six environmental variables contributed differently to the distribution model (Fig. 4).

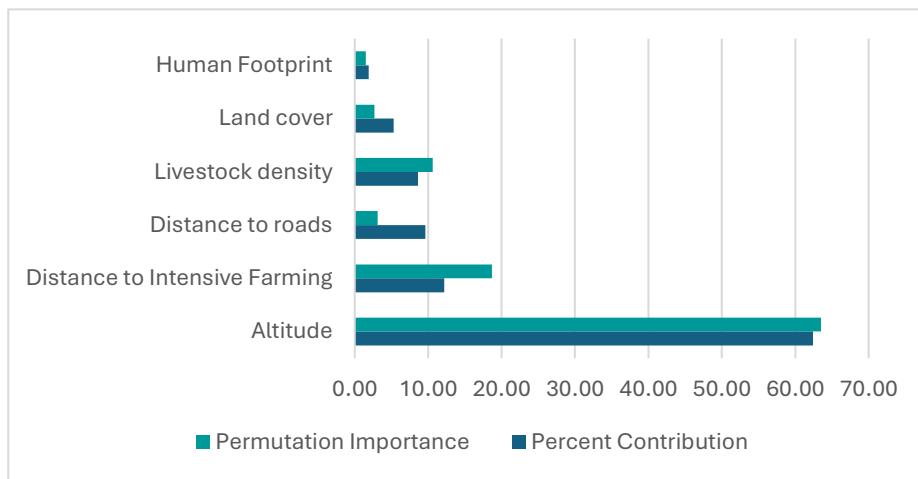
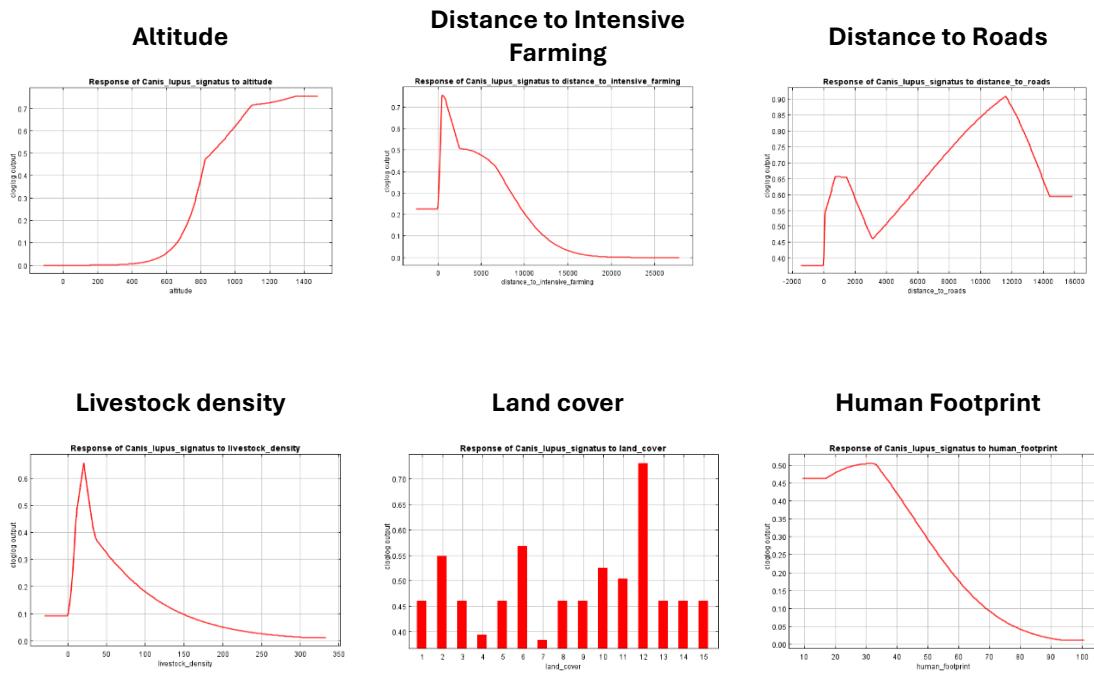


Fig. 4 - Estimates of relative contributions of the environmental variables to the MaxEnt model.



*Fig. 5 - Environmental variables response curves of the MaxEnt model. These curves show how the predicted probability of presence changes as each environmental variable varies, keeping all other environmental variables at their average sample value.*

## 4. Discussion

Altitude was the most significant variable in the model, contributing the most to predicting the presence of the Iberian wolf (Fig. 4). The response curve for this variable (Fig. 5) shows that the probability of wolf presence increases steadily with higher elevations. The probability is lowest at lower altitudes and begins to rise significantly around 500 meters, peaking between approximately 1,200 and 1,300 meters. This suggests that the species prefers higher altitudes, likely due to reduced human density and better availability of refuges.

Distance to intensive farming was the second most important variable for the model (Fig. 4). The probability of wolf presence is highest in areas close to intensive farming, with a sharp decline in probability as the distance increases (Fig. 5). This suggests that, as previously reported, these units are an important trophic resource for the species, especially in low livestock density (Roque et al., 2012).

Distance to roads was the third most important variable (Fig. 4). The probability of wolf presence increases as the distance to roads increases (Fig. 5). This reflects a clear tendency for wolves to avoid areas near roads, likely to minimize the risk of encounters with humans.

Livestock density was also an important variable for the model (Fig. 4). The response to the variable (Fig. 5) shows that the probability of wolf presence has a steep peak at a relatively low livestock density and a steady decrease as the livestock density increases. Even though wolf reliance on livestock has been extensively documented for the South of Douro population (Lino et al., 2023), these results suggest that high livestock densities might have a detrimental effect on wolf-suitable habitat. The same results were observed by Rio-Maior et al. (2019), who, utilizing other methodologies determined that wolves avoided areas with high density livestock.

Land cover and human footprint had a more modest contribution for the model (Fig. 4). Land cover response curve (Fig. 5) indicates that the Iberian wolf exhibits different responses to various land cover classes. Shrublands and spontaneous herbaceous vegetation are highly favourable, while others, such as waters or artificialized areas, are unsuitable.

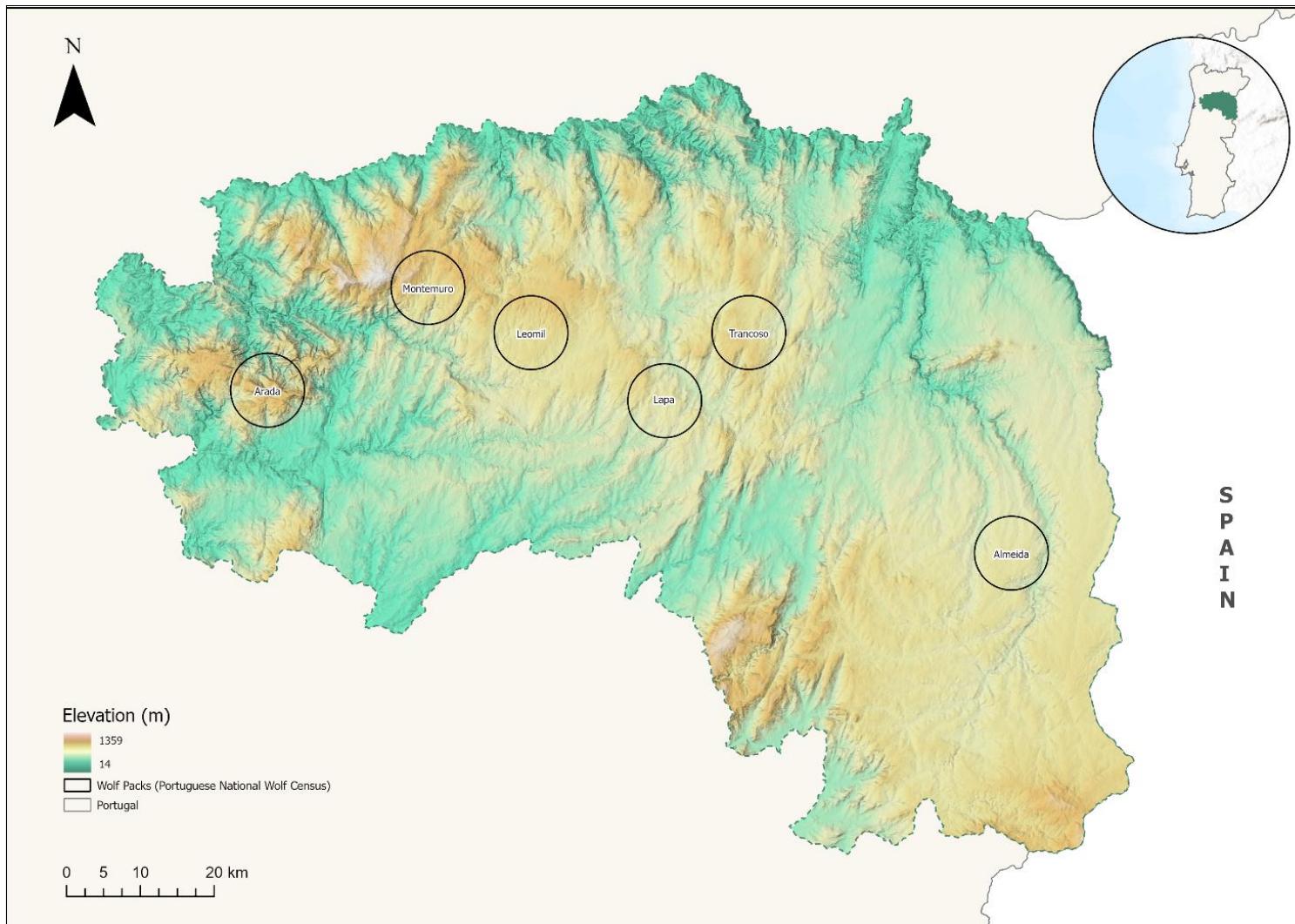
As expected, wolf presence probability is maximum when human footprint is low, but as the values of the variable increase, the probability decreases (Fig. 5)

## 5. References

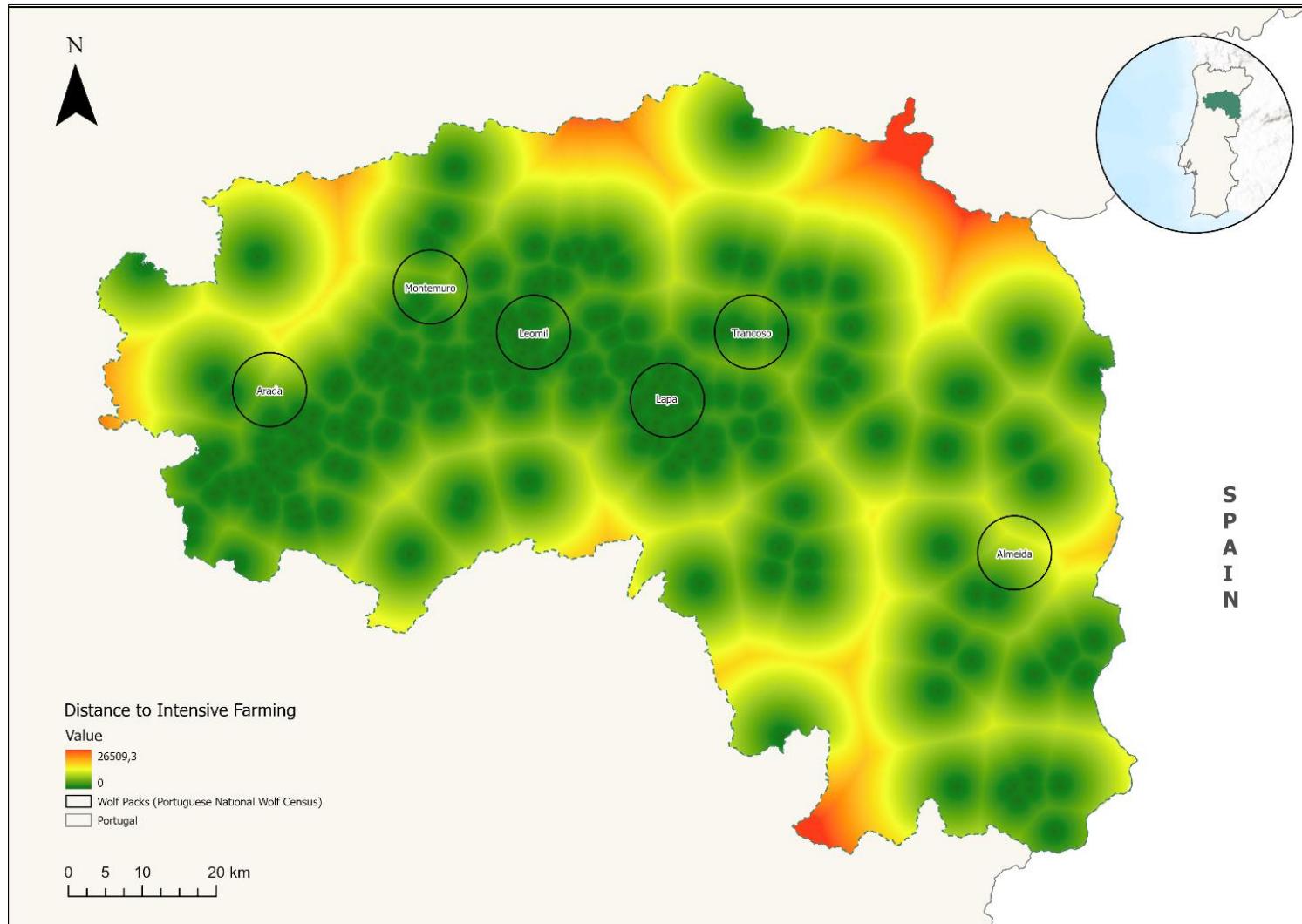
- Grilo, C., Lucas, P. M., Fernández-Gil, A., Seara, M., Costa, G., Roque, S., Rio-Maior, H., Nakamura, M., Álvares, F., Petrucci-Fonseca, F., & Revilla, E. (2019). Refuge as major habitat driver for wolf presence in human-modified landscapes. *Animal Conservation*, 22(1), 59–71. <https://doi.org/10.1111/acv.12435>
- Lino, S., Rossa, M., Fernandes, J. M., Barros, T., Lino, A., Hipólito, D., Ferreira, E., Aliácar, S. C., Cadete, D., Fonseca, C., Torres, R. T., Rosalino, L. M., & Carvalho, J. (2023). Dog in sheep's clothing: Livestock depredation by free-ranging dogs may pose new challenges to wolf conservation. *European Journal of Wildlife Research*, 69(6), 107. <https://doi.org/10.1007/s10344-023-01740-9>
- Merow, C., Smith, M. J., & Silander Jr, J. A. (2013). A practical guide to MaxEnt for modeling species' distributions: What it does, and why inputs and settings matter. *Ecography*, 36(10), 1058–1069. <https://doi.org/10.1111/j.1600-0587.2013.07872.x>
- Phillips, S. J. (2017). A Brief Tutorial on Maxent. [https://biodiversityinformatics.amnh.org/open\\_source/maxent/Maxent\\_tutorial2017.pdf](https://biodiversityinformatics.amnh.org/open_source/maxent/Maxent_tutorial2017.pdf)
- Pimenta, V., Barroso, I., Álvares, F., Barros, T., Borges, C., Cadete, D., Carneiro, C., Casimiro, J., Ferrão da Costa, G., Ferreira, E., Fonseca, C., García, E., Gil, P., Godinho, R., Hipólito, D., Llaneza, L., Marcos Perez, A., Martí-Domken, B., Monzón, A., ... Torres, R. (2023). Situação populacional do Lobo em Portugal: Resultados do Censo Nacional de 2019/2021. ICNF.
- Rio-Maior, H., Nakamura, M., Álvares, F., & Beja, P. (2019). Designing the landscape of coexistence: Integrating risk avoidance, habitat selection and functional connectivity to inform large carnivore conservation. *Biological Conservation*, 235, 178–188. <https://doi.org/10.1016/j.biocon.2019.04.021>
- Roque, S., Palmegiani, I., Petrucci-Fonseca, F., & Álvares, F. (2012). O custo da necrofagia: Estratégias de uso do território por uma alcateia a sul do rio Douro em Portugal. III Congresso do Lobo Ibérico, Lugo.

## 6. Annexes

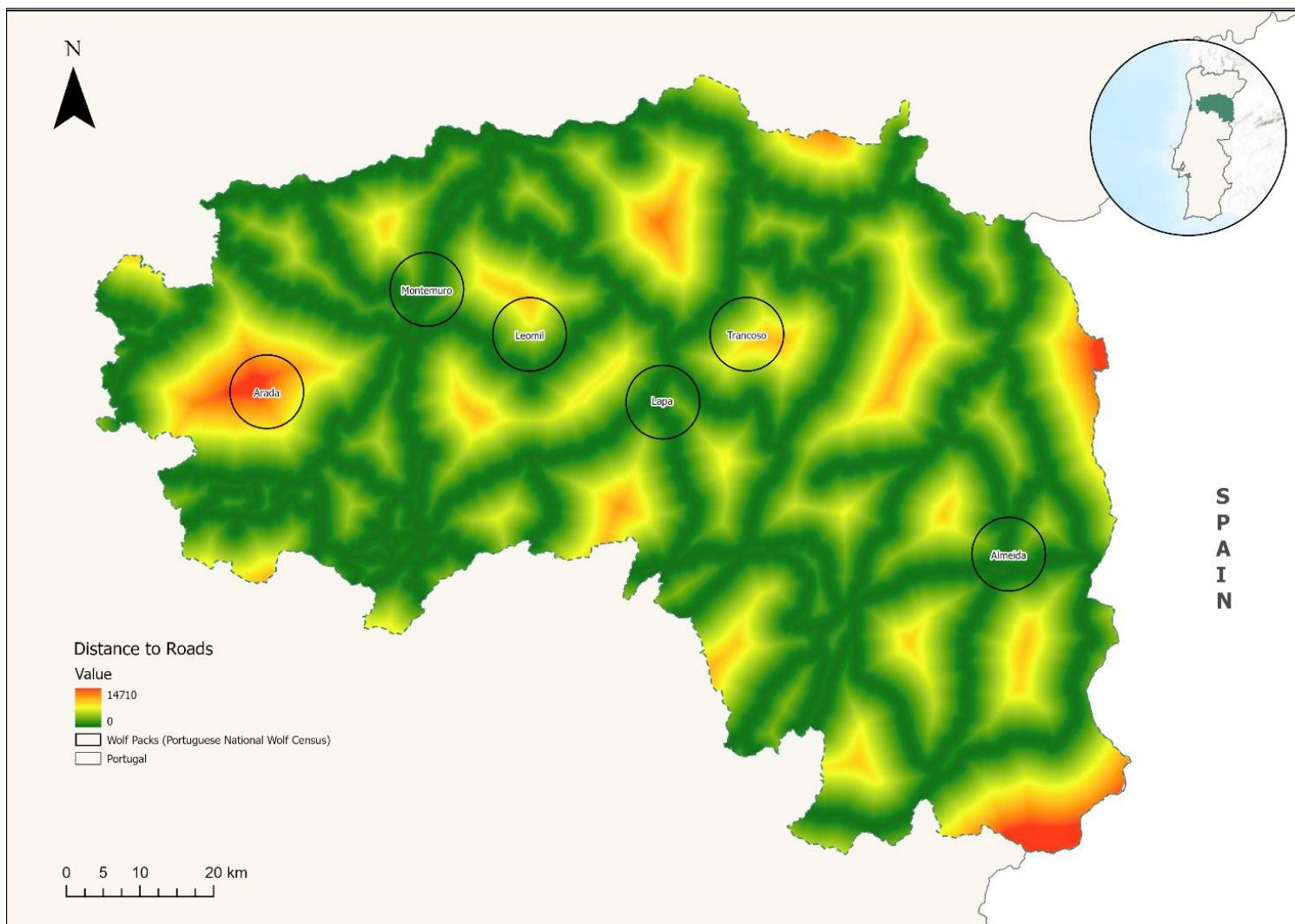
Annex 2 - Elevation of the study area.



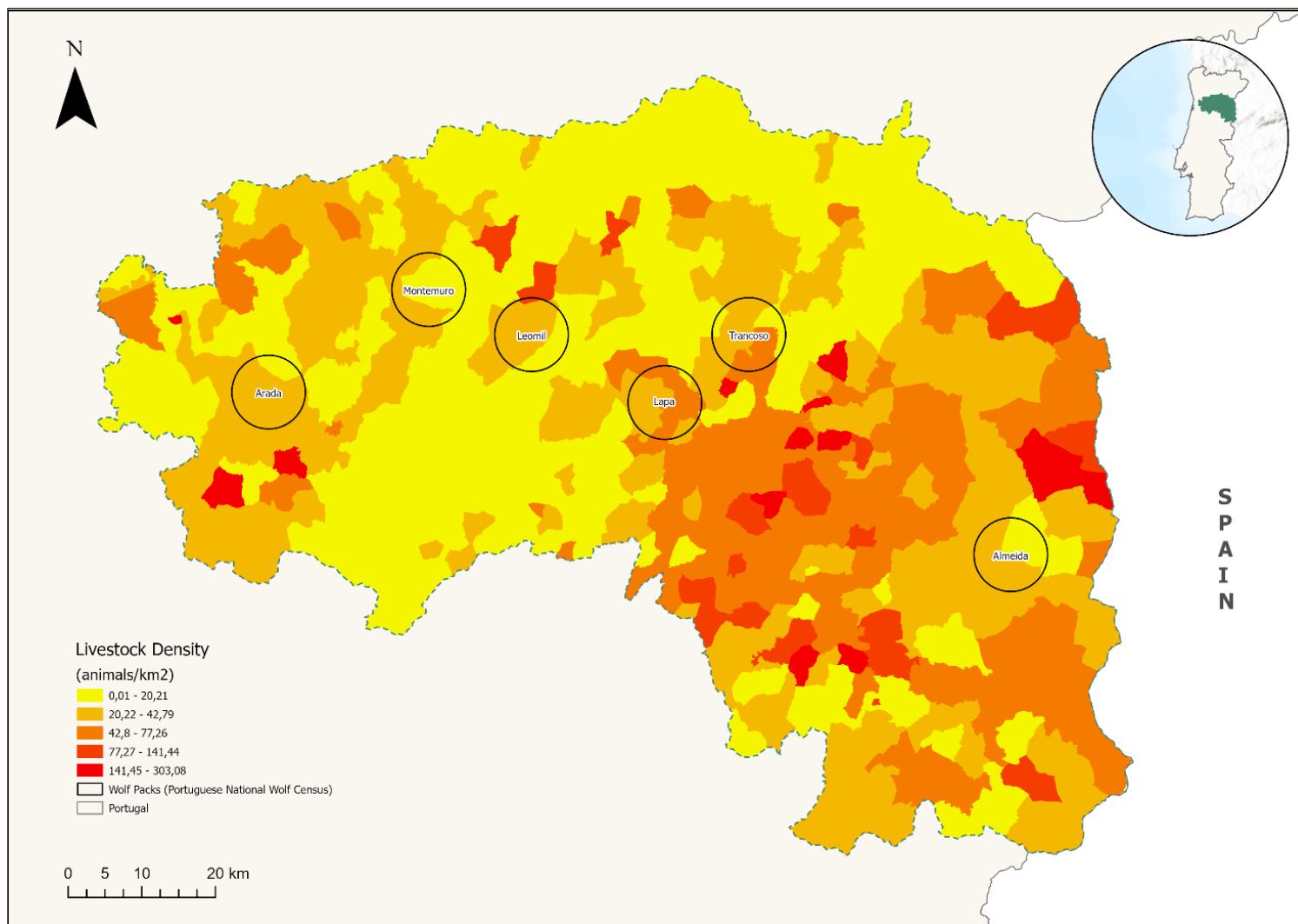
Annex 3 - Distance to intensive farming across the project area.



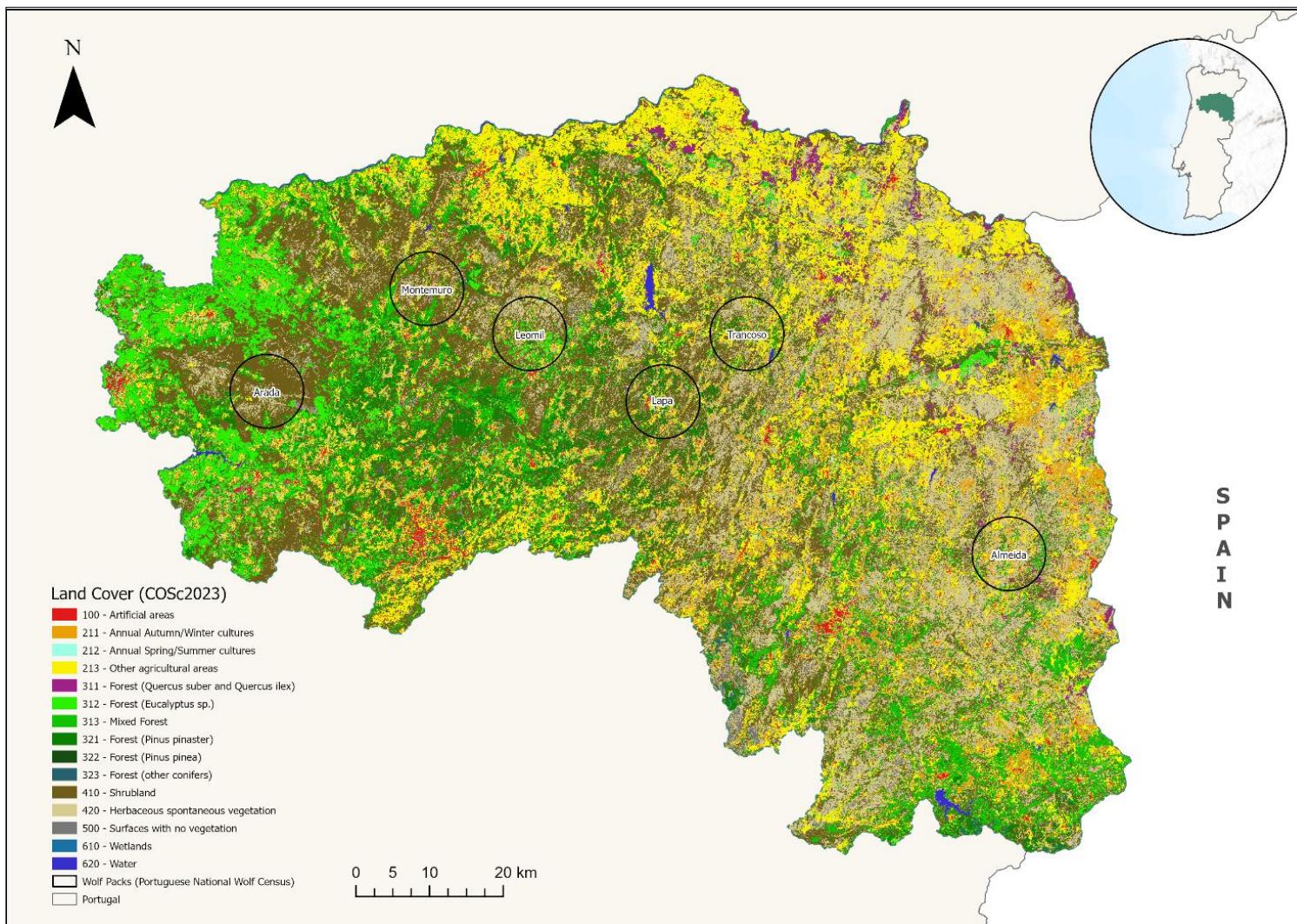
Annex 4 - Distance to roads across the study area.



Annex 5 - Livestock density across the project area.



Annex 6 - Land cover across the project area.



*Annex 7 - Human Footprint across the project area.*

