BEST PRACTICE ACTIONS FOR WOLF CONSERVATION IN MEDITERRANEAN-TYPE AREAS

Action D.3

Assessment of wolf presence in expansion areas in Portugal

Final Report

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1. Introduction

According to national wolf surveys carried out in 1994-1996 and 2002-2003, there are two different situations: while the population north of Douro River seems to be stabilized, even with an apparent and slight increase mainly in the border with Castilla y León (Spain), the Iberian wolf population south of Douro River still presents a high level of fragmentation and isolation. The MedWolf Project is focused on the most endangered Iberian wolf nucleus in Portugal, the south eastern portion of the wolf range south of Douro River, along the border with Spain. This area is of crucial importance for the consolidation of the south of the Douro river wolf population and the south-eastern expansion of the Portuguese wolf range, promoting its future connectivity with the Spanish wolf population.

In 2013 and 2014 several methodologies were used to assess the wolf status in the area: from traditional methods (sign surveys, howling sessions, transects) to more innovative ones (camera trapping, genetic analyses of scats, forensic analyses, and the use of a scat detection dog team). From two probable packs identified in the last national survey (2003-2004) the results obtained in the MedWolf indicated a 6-fold increase in the area of wolf presence, but confirmed the presence of only one pack, genetically identifying a minimum of 8 wolves, although no reproduction has been confirmed since 1995.

2. Goals

The goal was to update the wolf status in the intervention area in the last year of the project, and assess the population trend by undertaking a comparative analysis with the surveys conducted in previous years.

Specifically we sought to:

1. produce a reliable and updated wolf distribution map;
2. identify potential wolf packs;
3. estimate the minimum population size;
4. understand demographic patterns that might influence population trend;
5. detect the presence of different individuals from the ones identified in the previous surveys.

Non-invasive methods, including direct and indirect field techniques and genetic analysis will be implemented. During 2016 the sampling effort increased, mainly in the number of transects and kilometres covered to search for wolf signs and the number of howling stations, to increase the possibility of confirming reproduction, as well as the use of the scat detection dog team that was expanded to the entire project area in order to increase sampling collection and allow a better understanding of the dynamics of the wolf packs in the region.

3. Study area

The MedWolf study area (5,026 km^2) is located in the centre of Portugal, South of the Douro River, in the bordering region with Spain (Fig. 1). A detailed description of the study area can be seen in Cadete et al. (2015).

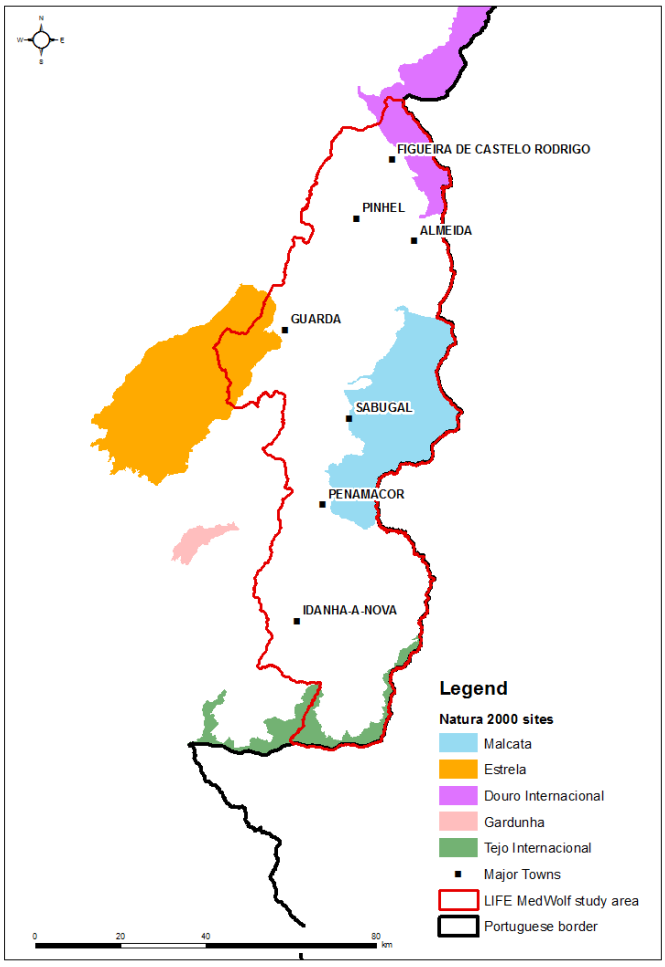


Figure 1. MedWolf study area in Portugal.

Farming and husbandry are the main economic activities. Livestock in the area includes semi-free range cattle, raised for meat production, and sheep and/or goat flocks. The wild prey community in the area includes wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*) only in the southern range.

4. Methods

4.1. Forensic analysis

The use of forensic genetic analysis allows to accurately detect wolf presence in kill sites, and previous surveys have shown its success to confirm wolf presence in the study area (Cadete et al. 2015).

During 2015 and 2016, attacks on livestock reported to ICNF were forensically investigated using molecular identification of the DNA present in hair or saliva samples collected from carcasses or close to kill sites. Swabs for saliva sampling were collected by ICNF wardens during visits to kill sites for damage assessments. Saliva samples were collected with cotton swabs from visible bite wounds and consumed body parts (muscle, skin and bone), and geo-referenced. Swabs were refrigerated until sent to the laboratory, and hair samples were kept in paper envelopes in dry places (Kelly et al. 2012).

A total of 147 visits to kill sites were carried out in 2015-2016, and 372 genetic samples were collected in 59% (n=87) of the cases, depending on the conditions of the carcass (e.g. level of consumption, decomposition). An average of 4.3 swabs/attack were collected.

4.2. Genetic analysis

The same molecular methods used in Action A.2 were used to analyse the biological samples received. The establishment of an improved sample collection protocol during A.2 proved to improve saliva derived samples genotyping and was also applied for Action D.3.

The samples analysed include scats collected during the wolf survey carried out in 2016 in the MedWolf study area, but also during the tests to compare the performances of the DT and the HT, both in the MedWolf area and in an area with high wolf density (Montesinho, see the scat detection dog tests' report). We will present results corresponding to all the scats analysed, distinguishing between both areas (MedWolf and Montesinho). We analysed scats collected

by both the DT and the HT. All scats detected by the DT were considered wolf scats *a priori*, before genetic analysis, since the dog is trained to mark only wolf scats. Scats detected by the HT were considered *a priori* wolf scats or *Canis* sp. scats, as discussed below, in section 4.3.2.1. Scats considered as *Canis* sp. by the HT are scats that could belong to wolf but, according to the field team's experience there is still a degree of uncertainty in the species assignment.

Considering the high success rate previously obtained in wolf identification from swab samples, and in order to gather as much information as possible, samples collected by ICNF wardens in the MedWolf area during wolf damages assessments in 2015, were analysed, in addition to all the swab samples collected during 2016. One hair sample collected by ICNF wardens during a damage assessment was also analysed. As before, the results regarding species assignment of the samples collected in damage sites were provided to ICNF and incorporated in the damage assessment process.

As previously, all samples assigned to wolf were tested for locus K alleles to investigate the presence of hybrids. Locus k is associated to hybrids wolf-dog signalling as in dogs the dominant black variant is determined by a three base pair deletion in the β-defensin gene CBD103 (Greco 2009). This was considered relevant, since the presence of hybrids showing a black unusual colour has been reported in Spain and Italy.

4.2.1. DNA extraction

4.2.1.1. Swab and hair samples

DNA was isolated using the Forensic DNA Extraction kit (AnalyticJena, Jena, Germany) according to manufacturer instructions.

4.2.1.2. Scat samples

Sample preparation and handling

Each scat was handled individually, in a dedicated room in order to prevent external contamination. From each sample, a portion of a visible transparent film (mucosal outer layer) at scat surface, was retrieved using a sterile scalp and moved to a sterile eppendorf tube that was immediately stored at -20 ºC until DNA extraction. Each DNA extraction was accomplished using a batch of 11 samples and one blank control (empty kit column) using the Innuprep stool kit (AnalytiKJena) according to manufacturer instructions. The starting

quantity of biological material was dependent of the faecal sample quality as scats were collected after being exposed to different and unknown atmospheric conditions for an undetermined amount of time. Quality was evaluated after visual analysis of the scats, according to the following criteria: high - fresh scat; medium; low – when there was a high percentage of dirt, prey hair or degradation of the organic material. DNA precipitation time, with ethanol at -20 ºC was increased for several hours (vs. 20 min) and DNA elution was performed in a final volume of 180 µL. All DNA samples were maintained frozen at -20 ºC until use.

Microsatellite PCR Amplification and genotyping

In order to avoid cross-contamination among DNA samples during extraction and PCR all reactions were prepared in a laminar flow hood using aerosol-resistant pipette tips and pre- and post-PCR experiments were carried out in separate rooms. In all cases, the occurrence of contamination events was monitored by including negative and positive controls in each PCR experiment. We used 16 canid nuclear microsatellite markers to amplify scat DNA in short amplicons: AHT121 (Holmes et al, 1993), C22.279, FH2001, FH2054, FH2010, , FH2611, FH4012 and FH3210 , REN247M23 **,** e PEZ06, PEZ08 , FH2361 , VWF.X , C38 , INU30 (Finnzymes, Inc.) All forward primers were fluorescently labelled (6-FAM, Hex, from Applied Biosystems). We conducted PCRs in eight multiplex groups using the Qiagen multiplex kit according to the manufacturers recommended protocols (including Q-solution). Products were electrophoresed on an ABI 310 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and alleles scored relative to an internal size standard, Genescan 350 Rox (Applied Biosystems), using GeneScan v3.7. Non-invasive genotypes were validated by replicated independent PCRs until each allele was observed at least twice. To summarise, the analysis proceeded as follows: (1) all DNA samples underwent PCR amplifications under standard conditions at all loci (if no product was detectable at one locus, the PCR was repeated up to 4 times), (2) PCR products and microsatellite profiles were checked for quality and rated. Samples producing incomplete genotypes were PCR re-amplified until three times from two DNA extractions or until each allele was observed at least twice.

As FH4012 and VWF.X loci may amplify fox specific microsatellite, we have been aware for the detection of peaks that corresponds to fox molecular weight microsatellite and those have been recorded.

4.2.2. Data analysis

Standard measures of genetic diversity were calculated in GenAlEx 6.5b using a reference set of 131 dogs and 92 Iberian wolf genotypes obtained from tissue samples. To determine genetic structuring and individual assignments based on the autosomal microsatellite data set, we used Structure version 2.3.3 running for 100,000 iterations (including 10,000 burn-in), with the 'admixture' model and assuming independent allele frequencies. GIMLET software was used for pooling identical genotypes among several genotypes. *ML-Relate* software was used to calculate maximum likelihood estimates of relatedness and relationship from codominant genetic data.

4.2.3. Molecular sexing

The sex of each sample was identified by amplifying a fragment from SRY, a Y-linked gene according to Olivier et al. (1999) and a marker associated to X chr according to Seddon et al. (2005), for female confirmation. Sexing PCR reactions included all positive controls for male and female.

4.2.4. Hybrid signal detection

To analyse the presence of putative wolf-dog hybrids in the MedWolf area the presence of the K locus mutated allele was screened, using the primer pair Locus SK described by Greco (2009). This locus can give a signal for hybrids wolf-dog determined by a three base pair deletion in the β-defensin gene CBD103.

PCR mix contained 5 µl of 2x Qiagen Multiplex PCR Master Mix, 1 µM of the pair of primers, 1 µl of DNA samples and 2 µl of water to bring to a final volume of 10 µl. Amplification conditions were 95ºC for 15 minutes, 32 cycles of 94ºC for 15 seconds, 55ºC for 15 seconds and 72ºC for 30 seconds and 72ºC for 10 minutes. PCR products were separated and fluorescently detected by capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Peaks were scored using GeneScan software.

4.2.5. Connectivity with western packs

Prepared in collaboration with: Raquel Godinho (CIBIO-InBIO)

To verify the existence of connectivity between the packs of the MedWolf area and the remaining Portuguese nucleus south of the Douro river, all available scat samples (n=57) collected in 2016 in the project area were provided to CIBIO-InBIO Laboratory (in the scope of a formal agreement established with this entity). The results were compared with the data gathered westward by CIBIO-InBIO during monitoring actions of three packs - Lapa, Leomil, Trancoso (identified in previous national census). This study is being developed, since October 2011, in an area that comprises 34 UTM 10 x 10 km cells, and includes part of 10 municipalities adjacent to the project area: Aguiar da Beira, Castro Daire, Mêda, Moimenta da Beira, Penedono, Sátão, Sernancelhe, Tarouca, Trancoso e Vila Nova de Paiva (Roque et al. 2017).

DNA from scats was extracted following Frantz et al. (2003) and Boom et al. (1990) protocols. To prevent potential contamination, all pre-PCR procedures were carried out in a dedicated laboratory used only for the manipulation of low quality DNA under sterile conditions and positive air pressure. Negative controls were included throughout the entire process to monitor for potential DNA contaminations. The quality of DNA extracted from scats was assessed based on the amplification of a 420bp fragment of the mtDNA hypervariable region one (HV1; Vilà et al. 1999) and four replicas of four microsatellites. Scat samples exhibiting wolf mtDNA and successfully genotyped for the four nuclear markers were subsequently used for the whole process.

Individual genotypes were determined using a set of 19 loci that were genotyped four times independently to minimize genotyping errors. The consensus genotypes over the four replicas were determined following rules at Godinho et al. (2015). The set of 19 loci is the same implemented by CIBIO at national wolf monitoring programs (e.g., Roque et al. 2017), and includes the subset of nine markers defined at the National Protocol for Genetic Analysis of Wolf and Dog Samples (ICNF, 2013). Additionally, a molecular sexing test was conducted for wolf samples following Seddon (2005). Multiple identical genotypes from non-invasive samples were identified using GIMLET (Valière 2002).

Since it was not possible for CIBIO-InBIO to analyse all the samples collected in 2016 in the MedWolf area (not all original samples were available for further analysis), the results of this analysis will only be considered in the definition of the wolf distribution (species

identification), and to identify recaptures of individuals originating from the westward packs (individual genotypes).

4.3. Livestock damages

Recorded livestock depredations can provide a first approximation of changes in large carnivore distribution and numbers. Information of reported wolf attacks registered and validated by the ICNF (Instituto da Conservação da Natureza e das Florestas) during the study period, from January to December 2016, was analysed. This information included the number of attacks, and number and type of killed, injured and disappeared animals.

A comparative analysis was also made with official data from previous years, 2012 to 2015, to understand the evolution of wolf caused damage in the study area. To identify areas with highest concentrations of wolf damage we used kernel density estimators (QGIS version 2.8.2, Heatmap, radius=8,000 m).

4.4. Field work

The same field methodologies used in 2013 and 2014 were developed in 2016: interviews, camera trapping, sign surveys, howling sessions, and the use of a scat detection dog team. Watching stations were not made in 2016 since they depend on the results obtained by other methodologies, and during 2016 no optimal conditions to perform them were found (e.g. positive results during howling stations, camera trapping, detection of rendezvous sites, or high concentrations of wolf scats).

In 2016, the field work was carried out by a different team composed by two field technicians (human team: HT) with wide experience in wolf surveys and the development of methodologies for wolf monitoring. The composition of the scat detection dog team (DT) was also different, with a new handler involved.

4.4.1. Interviews to local people

Interviews (n=20) were conducted throughout the study area to collect information about wolf sightings, reproduction and mortality. These were performed opportunistically, mainly to livestock breeders, shepherds and hunters. Additionally, relevant information gathered during the 377 interviews

conducted in 2016 to the general public, livestock owners, hunters, media workers, and environmental police officers, in the scope of Action D.5, was also considered. Relevant data regarding previous years was also considered to design the field work and analysed. When compared to previous surveys, the total number of interviews done in 2016 (n=397) was higher than in the 2014 (n=68), or 2013 (n=292) surveys (Cadete et al. 2014, 2015).

4.4.2. Camera trapping

Bushnell Trophy camera traps were set-up in locations where wolf signs were found or where information about wolf presence was obtained. The cameras were programmed to take 3 photos per event (trigger speed less than 1s), and were placed at a height of approximately 0.75 m above ground and tightly tied to trees (Fig. 2).

Figure 2. Camera trap set-up on a tree.

As roads were frequented by humans, we placed the traps off the roads and baited them with rotten liver to attract the animals. The cameras were checked and re-baited every week and the memory card was usually removed and replaced before it was fully exposed.

4.4.3. Sign survey

4.4.3.1. Transects

The HT conducted transects to detect signs of wolf presence in accordance with a selective sampling approach. Areas where there was a high probability of locating wolf signs were given priority; transects with forest trails, firebreaks and trails that at least partly crossed mountain passes, hill ranges or cross-trails were selected. We conducted transects of 2-4 km along 66 UTM 10X10 cells: 65 cells comprising the MedWolf study area (originally this area included 66 UTM 10X10 cells but one of them was not sampled because it is mostly comprised by private fenced farms and appropriate transects could not be defined) and one cell including part of Serra da Estrela which had not been sampled in previous surveys (Fig. 3). This new cell was sampled in 2016 due to the existence of recent information about possible wolf presence in the area. We repeated the same transects carried out in 2014 and added new transects defined by the new field team (51% were old, and 49% new transects).

Most of the cells were sampled (a minimum of two transects per cell) by the HT three times from January to October 2016, during the first, second and third trimesters (Fig. 4). Transects not covered by the HT in a trimester were performed by the DT, to ensure all the cells were sampled three times. The majority of transects performed by the HT were carried out by car, at a maximum speed of 20 km/h, stopping and sampling on foot every crossroads (Fig. 5). These type of transects has proven effective in monitoring wolf populations in the Iberian Peninsula. Field technicians collected scats and classified them as: wolf scat (based on size, shape, smell, and content the observer considers it as a wolf scat), or *Canis* sp. scat (doubtful species assignment, could be dog or wolf). In some cases, very old and decomposed scats were not collected, since they would not be successful in DNA analysis (as confirmed in previous surveys), and would also make it impossible for the HT to make a serious and well founded *a priori* species assignment. Each scat collected was kept in sterile plastic containers for posterior genetic analysis (Fig. 6).

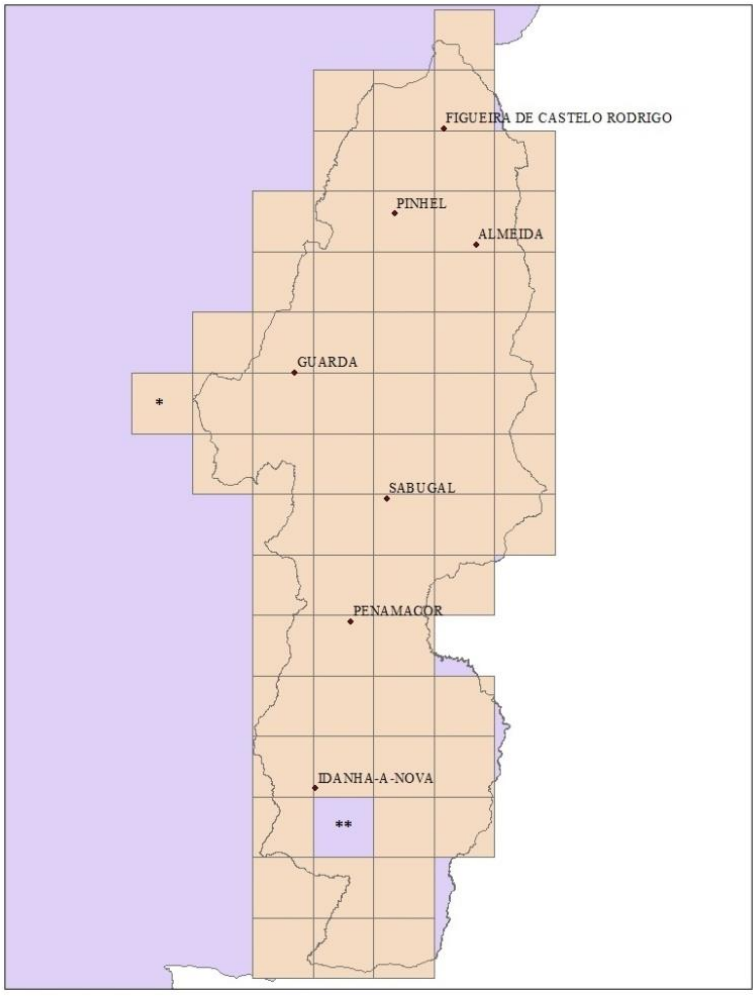
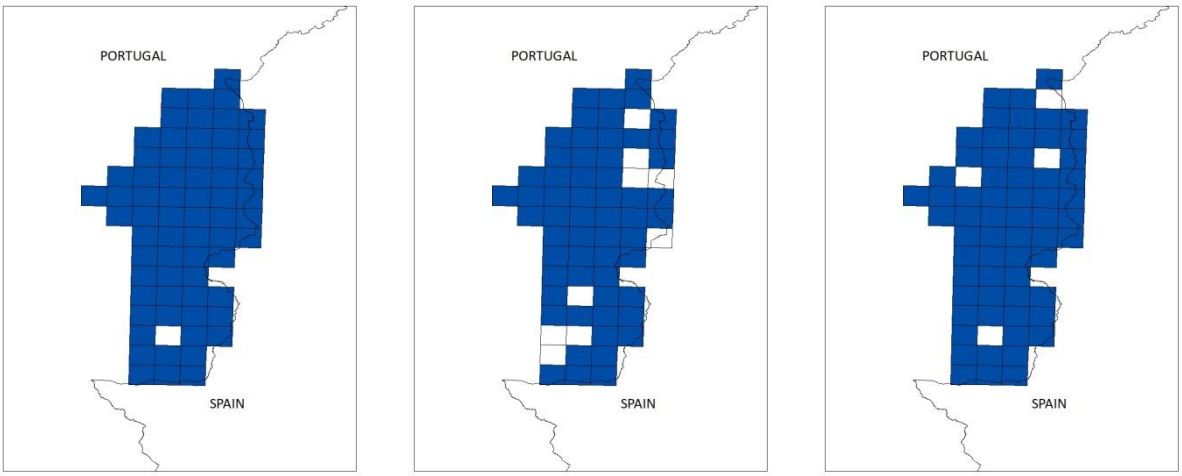


Figure 3. UTM cells sampled (10x10 km^2).
* UTM cell added to the original study area, comprising part of Serra da Estrela; ** UTM cell not sampled since it was not possible to define adequate transects for wolf survey.



1st trimester **2nd trimester** **3rd trimester**

Figure 4. UTM cells sampled by the HT during 2016.

Figure 5. Transects performed by car sampling every crossroads on foot.

Figure 6. Collecting a wolf scat for posterior genetic analysis.

In this analysis, we will consider wolf scats without genetic confirmation as the scats assigned by the field team as wolf scats, excluding *Canis* sp. scats. We assume a small error in the species assignment, since more than 90% of scats assigned to wolf by the same field technicians were genetically confirmed wolf scats in previous studies carried out in other areas of the Iberian Peninsula such as Picos de Europa.

To identify areas with highest concentrations of scats we used kernel density estimators (QGIS version 2.8.2, Heatmap, radius=8,000 m).

4.4.3.2. Scat detection dog

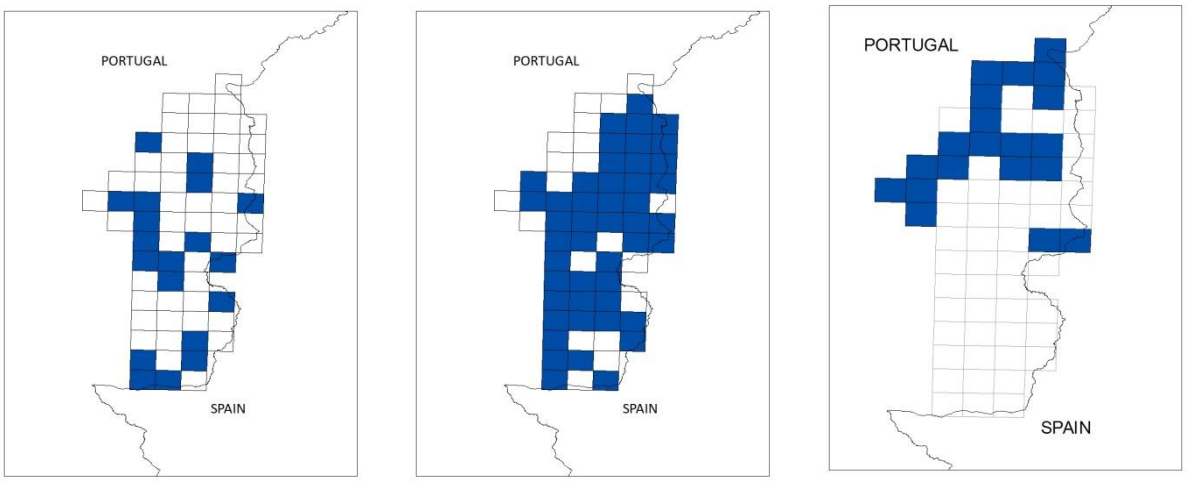
The use of detection dogs for wildlife monitoring and biodiversity conservation is increasing around the world, being especially useful to detect the presence of elusive and scarce species such as large carnivores. In Portugal, the use of these dogs in wolf surveys was initiated in the LIFE MedWolf Project (Cadete et al. 2015). An adult male dog, mixed-breed, named Zeus, was rescued from a shelter in Portugal and trained during May 2013 by the Conservation Canines program (University of Washington, Center for Conservation Biology). After which the scat detection dog team joined the wolf survey at the end of 2013, and continued to be used in 2014 within the estimated wolf range. In 2016 the training and use of the dog continued with a new handler designated by Conservation Canines. The new handler went through a 3 month training period of scat detection dog handling/surveying in the USA during 2014 (Fig. 7).

In 2016 the new scat detection dog team was used alongside the human team, covering all transects of the entire study area. In the beginning of the first trimester of 2016, the handler continued the dog training to correct any bad habits the dog may have acquired since the initial training. During this training period most of transects performed by the DT were accompanied by the HT. Since then, the DT performed alone all the remaining transects. The DT sampled, at least once, all the UTM cells comprising the MedWolf area, investing more effort and repeating transects in those where wolf presence was suspected by previous information (2 cell surveyed three times, 12 cells surveyed two times and 52 cells surveyed once) (Fig. 8).

The detection dog performed transects off leash. To optimize the dog's work, the handler allowed the dog to survey 10 m on each side of the road when needed and allowed the dog to

work into odours even if they were greater than 10 m off the road. Each scat detected and marked by the dog was collected and kept in sterile plastic containers for posterior genetic analysis. All the scats collected were considered wolf scats since the dog was trained to mark only wolf scats, discarding other scats, and thus even older and more decomposed scats were collected for analysis.

Figure 7. Scat detection dog team during 2016.



1st trimester **2nd trimester** **3rd trimester**

Figure 8. UTM cells sampled by the DT during 2016.

4.4.3.3. Wolf signs found using other methods

During 2016 scat detection dog tests were performed to analyse the efficiency of the scat detection dog when compared to human observers, being the results of these tests analysed in a specific report (Palacios et al. 2017). The wolf signs collected during the tests carried out in the MedWolf area, and other wolf signs collected opportunistically by the HT during the wolf surveys apart from those collected during the transects will be taken into account to estimate the wolf distribution range in the study area.

4.4.4. Howling sessions

Howling points consist in eliciting a wolf response via human imitation of howling. At every howling point, the respective researcher howled at 2-3 min intervals until either there was a response or three to four series had failed to elicit a reply. A single series consisted of 3 to 5 howls, each 5-8 seconds long, separated by a pause of 1-2 seconds, in accordance with Harrington and Mech (1982). Sessions started at sunset and lasted during the early night-time hours. Howling points were spread over the area at sites that offered good conditions for simulated howling and for receiving responses over the entire area and thought to be a possible breeding area according to the results of the abovementioned methods. Howling sessions took place between August and October as, in the NW of the Iberian Peninsula (V. Palacios, unpublished data), this is when pups usually remain at the rendezvous sites and the reply rate reaches a prolonged peak. When no response was obtained, we sought other information, such as sign concentrations, to assess possible pack presence and breeding. No attempts of simulated howling were made on rainy or windy nights as wolf response and howl audibility would have been reduced.

4.5. Mortality

Mortality data was gathered from interviews to shepherds, hunters and farmers and also from the official records available from the Dead Wolves Monitoring System (SMLM), managed by the ICNF. In the scope of this national and centralized System every wolf found dead in the Portuguese territory is collected by wardens from the ICNF being necropsied to assess the cause of death and determine physiological parameters, as well as collect biological samples

that will be available to the scientific community to pursue diverse types of research (e.g. genetic analysis).

5. Results

5.1. Genetic analysis

From March 2015 to December 2016, 510 non-invasive biological samples from the MedWolf study area were received: 137 scats (referring to the transects carried out during the survey and transects conducted during the scat detection dog tests in 2016), as well as 372 swabs and 1 hair sample (the swab and hair samples were collected by ICNF wardens during livestock damage assessments in 2015 and 2016). Additionally, 134 scat samples were collected in 2016 during the DT team tests in Montesinho, a higher wolf density area. In total, PCR amplification was successful for 245 swab samples (66%), 87 scats (32%) and one hair sample.

5.1.1. Analysis of scat samples

Scat samples collection in the field were searched using two methods: a Scat Detection Dog Team (DT), having a trained dog to detect wolf scats by scent, and a Human Team (HT) trained to search scats along predetermined transects. A total of 271 scat samples were analysed, 137 from the MedWolf area (transects and scat detection dog tests in low density areas) and 134 from Montesinho (scat detection dog tests in high wolf density areas). Considering only scats from the MedWolf area (137), we successfully got PCR amplification from 57 (42%, Table 1), increasing the 29% obtained from scats in 2013-2014. From Table 1 we have to note a strongly lower percentage value for samples with positive amplification (20.93%) for samples collected by the DT than by the HT (51.16%). This feature is associated to lower scat quality collected under the DT (e.g. older scats or mostly soil samples). This is supported by the fact that most of these samples were visually rated as having “low quality”. Samples classified as bad quality at the laboratory were not extracted. A possible explanation would be that while the scat detection dog is able to detect wolf scent in old/decomposed scats, the HT did not collect all these low quality scats, either because they did not detect them or discarded them if they were very old and decomposed.

Table 1. Summary of PCR amplification for all the scats collected during 2016 in the MedWolf and Montesinho areas.

Collector	Area	Nr.	Nr. positive PCR amplification	% positive PCR amplification
Dog Team[1]	MedWolf	43	9	20.93
	Montesinho	41	9	21.95
	Total	84	18	21.43
Human Team[1]	MedWolf	86	44	51.16
	Montesinho	23	6	26.09
	Total	109	50	45.87
Scats marked by DT and detected by HT	MedWolf	8	4	50
	Montesinho	70	15	21.43
	Total	78	19	24.36
Total	MedWolf	137	57	41.61
	Montesinho	134	30	22.39
	Total	271	87	32.10

[1] Wolf signs found exclusively by the DT or the HT.

All samples were analysed by PCR amplification of the 16 loci mentioned above but species assignment was performed using the 14 most amplified loci. Largely incomplete genotypes were removed from the analysis. Species assignment analysis using Structure software 2.3 was then performed using 42 genotypes presented graphically in Figure 9.

Comparing PCR amplification rates with 2013-2014 results from Action A.2 (36.4% in 2013 and 41% in 2014) it is of note an increase in the amplification rate of samples selected from the HT in 2016 (51.16%, Table 1). This is probably due to the age of the scats collected since in 2016 UTM cells were sampled every trimester, increasing the probability of finding more fresh scats. DNA amplification rates from the DT samples in the MedWolf were similar than those obtained in 2013-2014 values: 21% vs. 22%. This may be related with the dog sensitivity for wolf scats, independently of scat integrity and age, factors affecting DNA presence on scat samples.

In fact, most of the samples collected by the DT were considered to be of lower quality than the ones collected by the HT, according to the visual rating of the quality of the scats prior to genetic processing (Table 2). We obtained similar values of visual rating in 2016 and in 2014.

Table 2. Visual rating of the quality of the scats collected by the DT and the HT in 2016, and of the overall scats collected in 2014.

Visual Rating	DT	%	HT	%	DT & HT	%	TOTAL	%	TOTAL (2014)	% (2014)
High	4	5.19	11	10.78	5	6.67	20	7.87	10	7.04
Medium	25	32.47	47	46.08	30	40.00	102	40.16	62	43.66
Low	48	62.34	44	43.14	40	53.33	132	51.97	70	49.30
Total	77	100.00	102	100.00	75	100.00	254	100.00	142	100.00

Scats collected in 2016 and rated without sample are not included in the table: seven collected by the DT, seven collected by the HT, and three collected by both DT and HT.

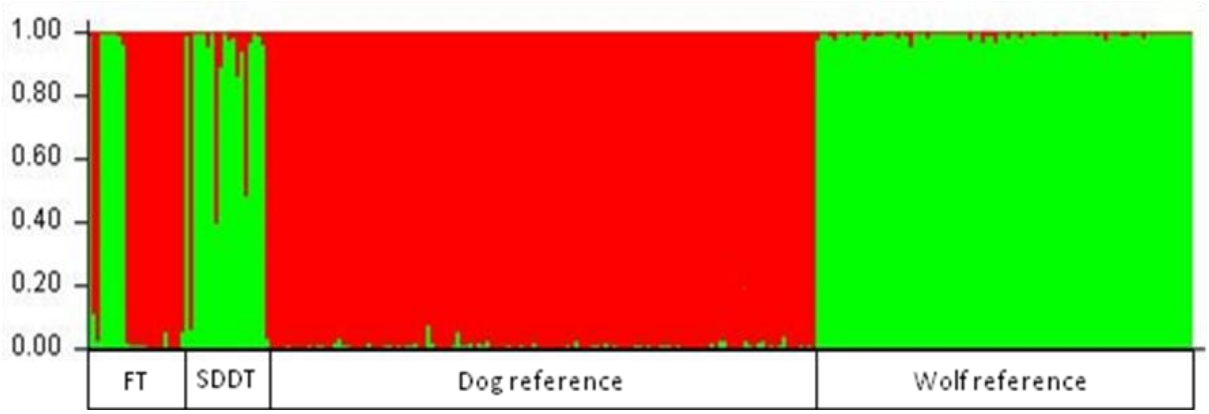


Figure 9. Probabilistic assignment to the genetic clusters inferred by the Bayesian analysis with K=2 of dogs and Iberian wolves reference genotypes (DT=dog team samples, HT=human Team samples).

Species assignment revealed a total of 26 scat samples affiliated to wolf and 17 samples affiliated on dog (Table 3). All of the scats marked by the DT which could be confirmed genetically were wolf scats, which can be considered an improvement in the dog's accuracy since in 2013-2014 when 34% of scats collected by the same dog were not wolf scats. On the other hand, 9 scats collected by the HT in the MedWolf area (24% of the scats collected by the HT which could be confirmed genetically) were wolf scats (six *a priori* considered wolf scats and three *Canis* sp. scats). In 2014, 30% of scats collected by the HT were wolf scats. However, none of the dog scats in 2016 were *a priori* classified as wolf scats by the HT: all were classified as *Canis* sp. scats (Table 3).

Table 3. Summary of species assignment based on molecular, dog selection and human evaluation of scat samples.

Area	Confirmed genetically		Marked by the DT as wolf scat	*A priori* HT assignment	
	Species	Nr.		Wolf	*Canis* sp.
MedWolf	Dog	16	0	0	16
	Wolf	12	4	6	3
Montesinho	Dog	1	0	0	1
	Wolf	14	12	9	1

5.1.2. Species assignment from swab and hair samples

We have analysed 246 samples (245 swabs and 1 hair) from livestock damages in the MedWolf area. From these, 104 (42%) samples showed only fox alleles and were not included in the species affiliation analysis. From the remaining 142 we were able to assign 103 (73%) genotypes to wolf and 39 (27%) genotypes to dog using Structure 2.3 software.

Wolf presence was not confirmed in 75.9% of the kill sites where swabs were collected. Considering the kill sites where species identification (fox, wolf, dog) was possible, we see that the wolf presence was confirmed in 38.9% (n=21), and the presence of dogs was confirmed in 16.7% (n=9). Only in one kill site the presence of both species was confirmed. We should be careful interpreting these results, since dogs and wolves eat carrion and the presence of both species in a carcass does not confirm who was the responsible of the attack. To ensure the species responsible for the killing is identified it is important: 1) to reduce the time between the attack and the inspection to minimize carcass consumption as carrion; and 2) to collect samples in bites related to the attack (presence of hematomas), which are distinct from bites related to consumption post-mortem (Llaneza et al. 2005a).

5.1.3. Wolf assigned sample sexing

Total wolf assigned samples were sexed and results are presented in Table 4 for swab and scat samples. From a total of 116 samples assigned to wolf in the MedWolf area (103 from damages and 13 from scats), 42 (36.2%) remained inconclusive (Inc). Considering only the 13 wolf assigned scat samples from Montesinho, 8 (61.5%) remained inconclusive.

Table 4. Sexing of wolf assigned samples: M=Male, F=Female and Inc=inconclusive. Zag,ZLIFE=swab samples; DLIFE16=scat samples; PLIFE2016=hair sample.

ID	Sexing	ID	Sexing	ID	Sexing	ID	Sexing
Zag1	M	Zag94	Inc	ZLIFE138	M	Zlife16-234	Inc
Zag2	M	Zag95	Inc	ZLIFE139	M	Zlife16-235	Inc
Zag3	M	Zag96	Inc	ZLIFE140	M	Zlife16-236	Inc
Zag4	M	ZLIFE9	M	ZLIFE141	M	Zlife16-237	Inc
Zag5	M	ZLIFE10	M	ZLIFE142	M	Zlife16-238	Inc
Zag6	M	ZLIFE11	M	ZLIFE143	M	DLIFE16-2	Inc
Zag7	M	ZLIFE1	M	ZLIFE144	M	DLIFE16-7	F
Zag8	M	ZLIFE2	M	ZLIFE145	M	DLIFE16-11	M
Zag9	M	ZLIFE3	M	ZLIFE146	M	DLIFE16-15	Inc
Zag10	M	ZLIFE4	M	ZLIFE177	Inc	DLIFE16-16	Inc
Zag40	M	ZLIFE14	Inc	ZLIFE178	Inc	DLIFE16-21	M
Zag41	M	ZLIFE15	Inc	ZLIFE179	Inc	DLIFE16-22	Inc
Zag42	M	ZLIFE16	Inc	Zlife180	Inc	DLIFE16-24	M
Zag43	M	ZLIFE17	Inc	ZLIFE187	Inc	DLIFE16-54Z	M
Zag46	M	ZLIFE18	Inc	ZLIFE188	Inc	DLIFE16-73Z	M
Zag47	M	ZLIFE19	M	ZLIFE189	Inc	DLIFE16-56T	M
Zag48	M	ZLIFE20	M	ZLIFE190	Inc	DLIFE16-57T	M
Zag49	M	ZLIFE21	M	ZLIFE191	Inc	DLIFE16-117T	M
Zag50	M	ZLIFE22	M	ZLIFE192	Inc	PLIFE2016	M
Zag51	M	ZLIFE23	M	ZLIFE193	Inc	ZLIFE5	M
Zag52	M	ZLIFE60	M	ZLIFE194	Inc	*DLIFE16-4T	M
Zag58	M	ZLIFE61	M	ZLIFE195	Inc	*DLIFE16-31T	Inc
Zag59	M	ZLIFE62	M	ZLIFE196	Inc	*DLIFE16-41T	Inc
Zag60	M	ZLIFE63	M	ZLIFE197	Inc	*DLIFE16-46T	M
Zag61	M	ZLIFE99	M	Zlife16-226	Inc	*DLIFE16-77T	M
Zag62	M	ZLIFE100	M	Zlife16-227	Inc	*DLIFE16-90T	Inc
Zag88	M	ZLIFE104	M	Zlife16-228	Inc	*DLIFE16-91T	M
Zag89	M	ZLIFE105	M	Zlife16-229	Inc	*DLIFE16-97T	Inc
Zag90	M	ZLIFE106	M	Zlife16-230	Inc	*DLIFE16-133T	Inc
Zag91	M	ZLIFE107	M	Zlife16-231	Inc	*DLIFE16-148T	F
Zag92	Inc	ZLIFE136	M	Zlife16-232	Inc	*DLIFE16-156T	Inc
Zag93	Inc	ZLIFE137	M	Zlife16-233	Inc	*DLIFE16-158T	Inc
*Samples collected in Montesinho.						*DLIFE16-160T	Inc

We can see that only males were identified through molecular sexing in samples derived from livestock damages. Concerning scats, molecular sexing on samples from the MedWolf area detected one female and seven males. These results may indicate a strong male-biased sex ratio in the study area.

All the 142 swab assigned samples were grouped by genotype matching algorithm from GeneAlex 5.0 producing 21 individual genotypes from wolf assigned samples and 6 individual genotypes from the 39 dog assigned samples.

5.1.4. Overall analysis for the period 2015-2016

Although we could not get complete genotyping for all 16 loci in most of the samples (90%), we have proceeded with standard genetic analysis, selecting the most amplified 14 microsatellite locus.

A total of 69 regrouped genotypes from 2015-2016: 21 wolf assigned genotypes and 6 dog assigned genotypes from livestock damages, 26 wolf assigned genotypes and 16 dog assigned genotypes for scats, were re-assigned and are shown in Figure 10. This reassignment confirms 47 samples assigned to wolf, 34 from the MedWolf area.

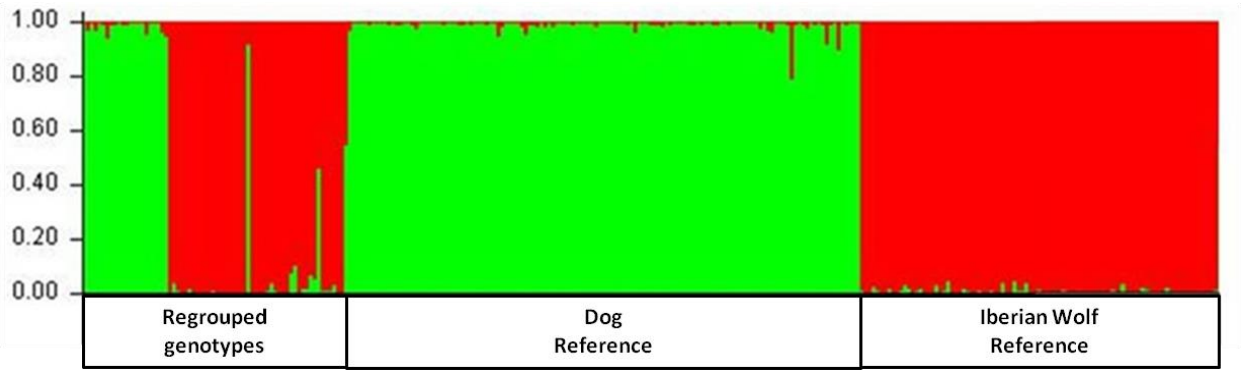


Figure 10. Probabilistic assignment to the genetic clusters inferred by the Bayesian analysis with K=2 of dogs and Iberian wolves reference genotypes.

After regrouping all MedWolf genotypes together (from livestock damages and from scats) taking in consideration sexing information and 10 microsatellites loci (most of them having a tetranucleotide repeat), the final number of individual genotypes accounts for 27 individuals in the MedWolf area, that is, there were identified six recaptures among scats and swab

samples. Two of the scat samples of 2016 (DLIFE16-11 and DLIFE16-21), represent the same individual found in one livestock attack from 2015 (Zag1-zag5) (Table 5).

Table 5. 2015-2016 genotype regrouping samples.

Genotype ID	Sample ID	Sample ID	Sample ID	Molecular sexing	Year
2	Zag40,41,42,43,46	Zlife14,15,16,17,18		M	2015
3	Zag47-Zag52			M	2015
4	Zag58-Zag62			M	2015
5	Zag88-Zag91			M	2015
6	Zag92-Zag96			Inc	2015
7	ZLIFE1-ZLIFE4	Zlife60,61,62,63		M	2015
8	ZLIFE5			M	2015
9	zlife9,10,11			M	2015
10	Zlife_19,20,21,22,23			M	2015
13	Zag6-Zag10			M	2015
18	DLIFE16-11	DLIFE16-21	Zag1-Zag5	M	2015-2016
11	ZLIFE99-100			M	2016
12	Zlife177-180			Inc	2016
14	Zlife192-Zlife197			Inc	2016
15	Zlife16 226-238			Inc	2016
16	DLIFE16-2			Inc	2016
17	DLIFE16-7			F	2016
1	Zlife104-Zlife107	Zlife187-Zlife191	Zlife136-Zlife146	M	2016
19	DLIFE16-15			Inc	2016
20	DLIFE16-16			Inc	2016
21	DLIFE16-22			Inc	2016
22	DLIFE16-24			M	2016
28	DLIFE16-57T			M	2016
33	DLIFE16-117T			M	2016
38	PLife2016			M	2016
39	DLIFE16-54z			M	2016
40	DLIFE16-73z			M	2016

In summary, in the MedWolf area we identified 27 different genotypes: 10 from swabs collected in 2015; 1 identified in 2015 (one swab) and in 2016 (two scats); 16 identified in 2016 (five from swabs, one from a hair sample, and 10 from scats). One out of 11 genotypes

identified in 2015 was recaptured in 2016. In 2013, five out of 20 genotypes identified were recaptured in 2014. But 2015 samples derive only from swabs collected at kill sites, while for the other years scats collected throughout the study area were also included.

Molecular sexing of 2015-2016 samples reveals a strong male-biased ratio (male:female ratio=18:1), when comparing to the results obtained in 2013-2014 (male:female ratio=2.9:1.0, Cadete et al. 2015). If only data from 2016 are considered the ratio decreases to 9:1, despite continuing abnormally high for wolf populations (sex ratio being usually closer to 1:1, e.g. Mech 1970). If only results from scat samples are considered (since these samples were collected systematically throughout the study area, and not opportunistically as the swabs) the ratio decreases to 6:1, which is still very high but closer to what is expected in wild wolf populations, and closer to the 4:1 ratio registered in the Montesinho scats.

There is the probability that the samples that remained inconclusive for sexing for scat and swab samples may retain some female individuals. Nevertheless, those samples were repeated for 3-4 times and never amplified for sex fragments although all the PCR reactions included a positive reaction for male and female that always amplified.

However, caution is required when interpreting the results obtained since DNA quality has limited the chance of genotyping of all loci. Although we consider a final number of 27 individuals recognized in 2015 and 2016 in the project study area, this number has chances to be overestimated, a very common conclusion when using molecular methods for individuals accounting (Creel et al. 2003). Considering sex ratio bias, we would like to note that molecular sexing protocol identifies clearly two bands corresponding to chrY and chrX amplification for unequivocally male identification.

5.1.5. Relatedness

Samples DLIFE16-54z and DLIFE73z were removed from relatedness analysis due to very incomplete genotypes. Maximum likelihood estimates of relatedness and relationship from 25 individual genotypes using M-L Related software (Kalinowski et al. 2006) were calculated and are presented as a triangular matrix in Table 6.

Table 6. Triangular matrix showing the relationship between each pair of individuals that has the highest likelihood among four relationships: U=Unrelated; HS(red)=Half Sibs; FS(blue)=Full Sibs; PO(green)=Parent/Offspring.

	13	8	14	21	38	1	10	2	4	9	7	11	15	16	18	22	28	3	5	6	12	19	20	33	17
13	-																								
8	U	-																							
14	U	HS	-																						
21	U	HS	U	-																					
38	U	U	U	PO	-																				
1	U	U	U	HS	U	-																			
10	U	PO	U	U	U	U	-																		
2	U	U	U	U	U	U	HS	-																	
4	U	HS	U	U	U	U	U	U	-																
9	U	U	U	FS	PO	U	U	U	U	-															
7	U	PO	U	U	U	U	U	U	FS	HS	-														
11	U	U	U	U	FS	U	U	U	PO	HS	PO	-													
15	U	FS	U	U	U	U	U	U	U	U	PO	PO	-												
16	U	FS	U	FS	HS	U	U	U	U	FS	PO	HS	FS	-											
18	U	U	U	U	FS	U	U	U	U	PO	FS	U	HS	HS	-										
22	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	-									
28	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	-								
3	U	U	U	HS	U	U	FS	HS	FS	U	HS	U	U	HS	U	U	U	-							
5	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	-						
6	U	PO	U	U	U	U	U	U	HS	U	FS	U	U	HS	U	U	HS	U	U	-					
12	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	FS	U	HS	U	U	-				
19	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	-			
20	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	-		
33	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	HS	-	
17	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	-

The relatedness matrix presented in Table 6 reveals that individuals 4, 7, 11, 15, 16 are more related with each other while the individuals 17 and 19 are mostly no related with the other individuals. These data may indicate the existence of two family groups in the period of 2015-2016.

5.1.6. Overall analysis for the period 2013-2016

Individual genotypes previously found in 2013 and 2014 amounted to 50 individual genotypes, where 20 showed recaptures and 30 appeared to be isolated individuals. Further genotyping and regrouping end up with 42 wolf genotypes. In 2015 and 2016, 27 individual genotypes were detected with six recaptures (Table 7). To compare genotypes and search genotype matching between samples from 2015-2016 and 2013-2014, all the samples, except DLIFE16-53 and DLIFE16-74 due to incomplete genotypes, were analysed together in a unique file containing 67 genotypes.

As molecular genotyping of non-invasive samples is generally associated to dropout and/or false alleles, to identify individual genotypes and genotype matching, with higher accuracy we have selected 6 loci having mostly tetranucleotide repetition motifs as allele peaks are more stable and less prone to deviation. Using loci FH4012, C38, Ren 437, FH2361, VWFX and PEZ06, Psibs was estimated as $0.7x10^{-4}$ giving high confidence that any two matching samples originated from the same individual and were not derived from full-siblings who happened to be identical at the same loci. Genotypes from different samples mismatching at three or fewer loci were re-examined for possible genotyping errors and in some cases additional genotyping was undertaken to resolve any ambiguities. After regrouping of all the 67 genotypes, we were able to identify only one recapture between samples from 2013-2014 and 2015-2016 (bold in Table 7).

Matching genotypes were then given a consensus ID (number followed by "D") and the composite genotype was used in subsequent analyses. Genotypes from 1D to 48D correspond to 2013-2014 samples, and from 49D to 86D correspond to 2015-2016 samples. All samples analysed to estimate global relatedness are described in Table 7.

Table 7. Genotypes from the MedWolf area used for relatedness estimation.

Regrouped genotypes	Sample ID
SLIFE14_6	1D
SLIFE14_334_335_325_326_327_176_177_180_181_135-140_158-163_226-233_325-327	2D
SLIFE14_9_SLIFE118_SLIFE119	3D
SLIFE14_10	4D
SLIFE14_90_91_92_93	5D
SLIFE14_14	6D
SLIFE14_246_247_250_364_367	7D
SLIFE14_339_341_342	8D
SLIFE14_254_255_256	9D
SLIFE14_320_309	10D
SLIFE14_184_370_371_372	16D
SLIFE14_188_189	13D
SLIFE14_190_192	14D
SLIFE14_251_252_253	15D
SLIFE14_331	19D
SLIFE14_348_349_351	20D
SLIFE14_116-127_SLIFE_44-50_SLIFE14_336-338	22D
SLIFE14_61 _69_ 70-71_ 107-114_12-13	23D
SLIFE14_379-381_Plife14_11_PLIFE14_12_SLIFE14_300_282_DLIFE14_89tz	25D
SLIFE_18	26D
SLIFE20_21	27D
SLIFE23	28D
SLIFE68	29D
SLIFE75_76	30D
SLIFE78_82_73_80	31D
SLIFE94	32D
SLIFE97	33D
SLIFE120	34D
SLIFE121	35D
SLIFE125	36D
SLIFE175_177_163	37D
SLIFE179	38D
BATOCAS	39D

Regrouped genotypes	Sample ID
DLIFE14_8z	40D
DLIFE14_23za=DLIFE74	41D
DLIFE14_25z	42D
DLIFE14_56	43D
DLIFE14_59	44D
DLIFE14_61B	45D
DLIFE14_73z	46D
DLIFE_54	47D
DLIFE_72_79	48D
ZLIFE_104-107_187-191_136-146	49D
Zag_40_41_42_43_46	50D
Zag_47-52_ZLIFE14_15_16_17_18_DLIFE14_70z	51D
Zag58_Zag62	52D
Zag88_Zag91	53D
Zag92_Zag96	54D
ZLIFE1_ZLIFE4=ZLIFE60_61_62_63	55D
ZLIFE5	56D
ZLIFE9_10_11	57D
ZLIFE_19_20_21_22_23	58D
ZLIFE99_100	59D
ZLIFE177_180	60D
Zag6_Zag10	61D
ZLIFE192_ZLIFE197	62D
ZLIFE16 226_238	63D
DLIFE16_2	64D
DLIFE16_7	65D
DLIFE16_11=DLIFE16_21=Zag1_Zag5	66D
DLIFE16_15	67D
DLIFE16_16	68D
DLIFE16_22	69D
DLIFE16_24	70D
DLIFE16_57T	76D
DLIFE16_117T	81D
PLIFE2016	86D

The mean expected heterozygosity estimated was He=0.682 (±0.029) that is similar to previous values found in the previous report and in other reports concerning Iberian wolf (0.617±0.031, Godinho et al. 2011). The inbreeding coefficient shows a positive value (Fis=0.163) indicating an excess of homozygous individuals that could also be explained by the low number of individuals and the high isolation of this population nucleus and errors associated to non-invasive genetics such as dropout alleles.

Relatedness estimation among the remaining 67 individual genotypes is presented in Table 8.

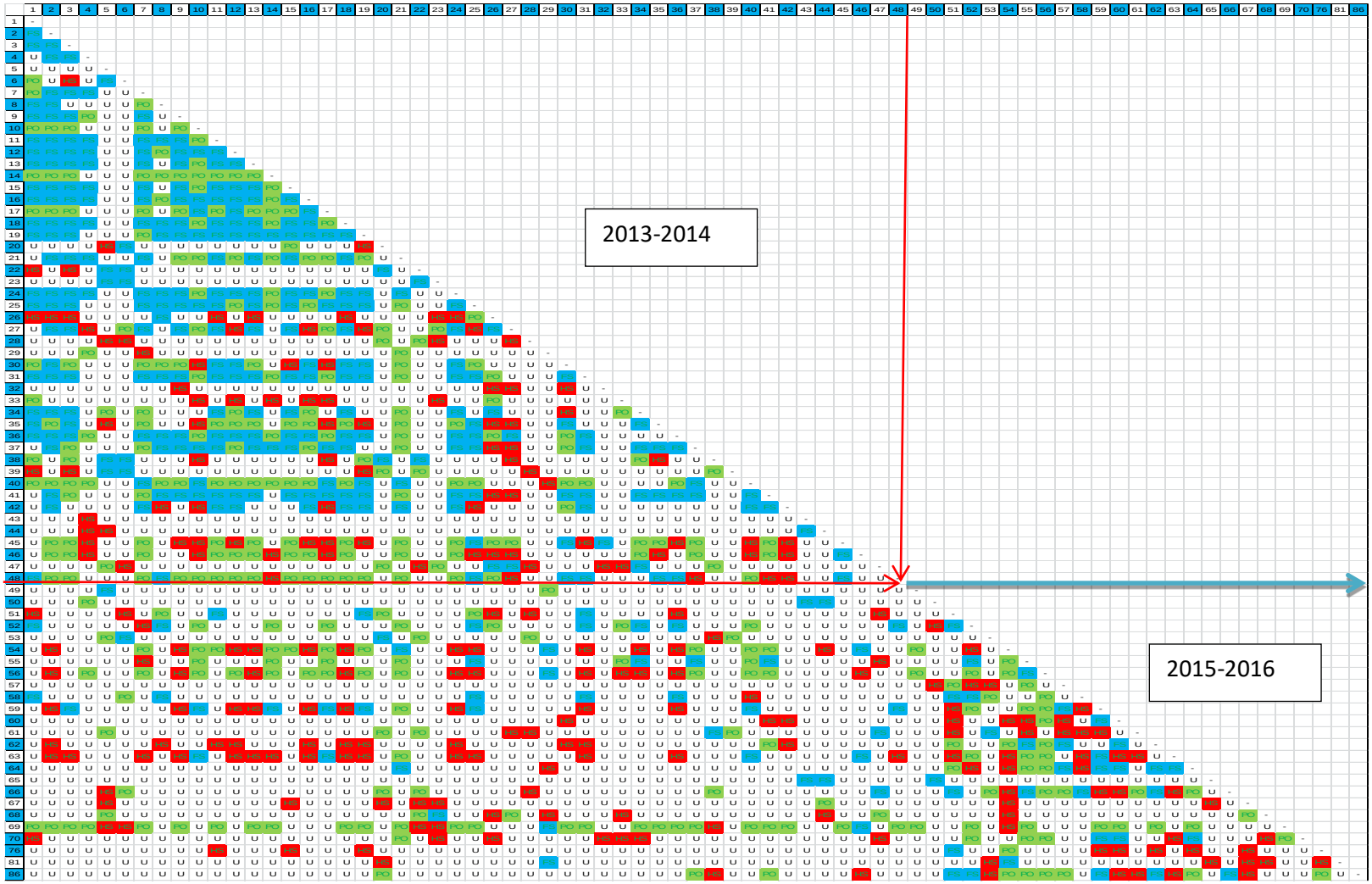
We should note that Table 8 represents the probability of relationship with a 95% confidence using genotypes from six loci producing high quality data (low allele dropout or false alleles).

Globally, using 6 loci for individual identification, relatedness estimation provided 67 genotypes from MedWolf area being 42 from the period of 2013-2014 and 25 for the period of 2015-2016 (one being recaptured from 2013).

The intersection of related individuals in the two periods under analysis shows that individual genotypes from 1D to 48D (2013-2014) marked by red arrows are more related than with individuals genotype from 49D to 86D (from 2015-2016) marked by a blue arrow in table 8.

In fact relationship between 2013-2014 and 2015-2016 individuals is rarefied and a new group more related seems to appear in 2016. Mortality may be reason of loss of continuity and unrelated genotypes should correspond to lone wolves or dispersers. Also Echegaray and Vilà (2010) reported that most genotypes within the Basque Country were observed only once. Marucco et al. (2009) discussed that problems of recapture with wolves arise during scat sampling due to differing individual behaviours leads to different probabilities of finding their scats. They stated that young wolves were more likely to be sampled off or away from human trails than at marking sites. Also Echegaray and Vilà (2010) stated that young and solitary wolves being subordinates do not intensively mark the territory. Therefore, a sampling design based on collection of scats along wolf tracks increases the probability of characterizing each individual, especially young and dispersing wolves. These are possible causes for low recaptures in the MedWolf area.

Table 8. Triangular matrix showing the relationship between each pair of individuals that has the highest likelihood among four relationships: U(White)=Unrelated; HS(red)=Half Sibs; FS(blue)=Full Sibs; PO(green)=Parent/Offspring. Individual genotypes from 1D to 48D (red arrows) refer to 2013-2015 and individual genotypes from 49D to 86D (blue arrow zone) refer to 2015-2016).



5.1.7. Hybrid signal detection

All samples 2013-2016 assigned to wolf were amplified for K locus. However in all samples assigned to wolf, no insertions were detected at K locus meaning that no hybrids for black colour were detected.

5.1.8. Connectivity with western packs

The results confirmed the dispersion of two males from a westward pack (Leomil), one after summer 2011 and the other after winter 2014, dates when their presence had been last confirmed in the original pack (Raquel Godinho, pers. com.). Both males seem to have established in different areas, the first dispersant closer to the original pack, in the Almeida pack region (ca. 70 kms from the Leomil pack), and the second dispersant further south in the region of the Sabugal/Malcata pack (ca. 90 kms from Leomil). The sampling years also provide indication of a minimum longevity for both wolves, if we consider a mean dispersal age of 2 years, ranging from 4-7 years, which is considerable for wild wolves in an area where human caused mortality is expected to be high. These results confirm the connectivity between the packs of the study area and those located west (included in a more stable nucleus of the Portuguese population South of the Douro river), and the successful dispersal of wolves resulting in the establishment of packs in the MedWolf area.

5.2. Livestock damages

During the period 2012-2016, 449 wolf attacks on livestock occurred in the MedWolf area, affecting a total of 1,213 heads (Table 9). Livestock damages reached a maximum peak (n=149) in 2014, being 2016 the year with less wolf attacks registered (n=65). Almeida was the county most affected (64% of wolf attacks), and no livestock damages were registered in Penamacor or Idanha-a-Nova. Wolf attacks affected mainly cattle and small ruminants (50% cattle; 34% small ruminants; 9% donkeys; 7% ostriches) (Fig. 11).

Table 9. Wolf attacks registered in the study area from 2012 to 2016.

County	2012	2013	2014	2015	2016	Total
Fig. Castelo Rodrigo	0	1	7	0	0	8
Pinhel	0	10	11	27	12	60
Almeida	46	48	116	44	34	288
Guarda	8	13	3	5	10	39
Sabugal	13	12	12	8	9	54
Penamacor	0	0	0	0	0	0
Idanha-a-Nova	0	0	0	0	0	0
Total	67	84	149	84	65	449

Although the majority of wolf attacks were on cattle, most of the animals killed were small ruminants (883 small ruminants, 310 cattle, 46 ostriches, and 44 donkeys; 69%, 24%, 4%, and 3% respectively) (Fig. 12).

Taking into account all the wolf attacks occurred during 2012-2016, a total of 1,283 head of livestock were killed, 1.5 ostriches/attack, 1.1 donkeys/attack, 5.8 sheep/attack, and 1.4 cattle/attack.

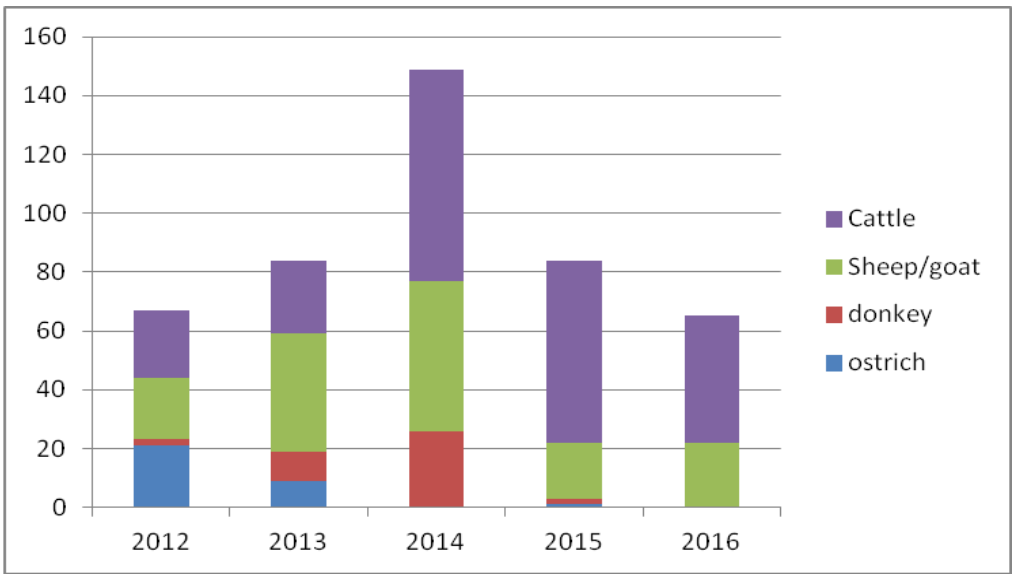


Figure 11. Number of wolf attacks registered in the study area per species from 2012 to 2016.

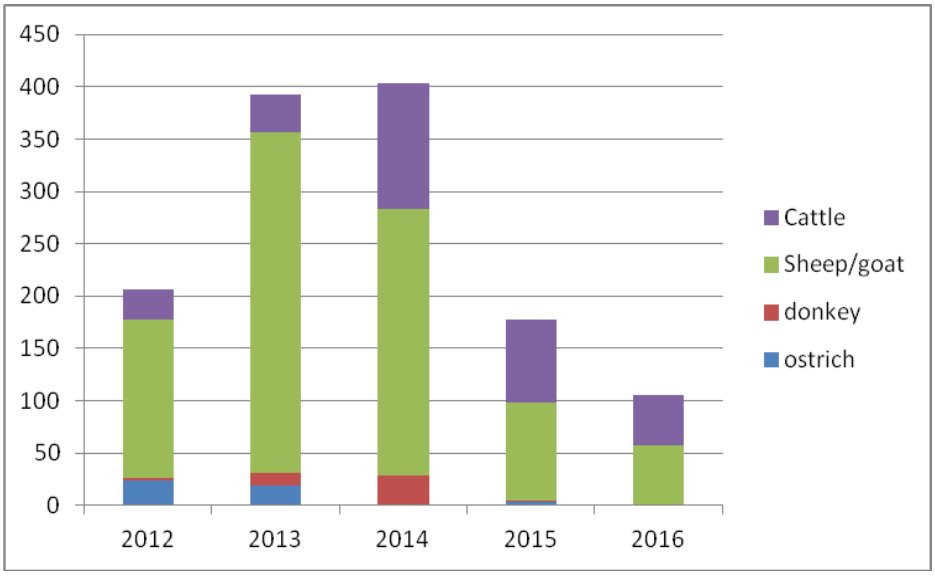


Figure 12. Number of livestock heads killed in wolf attacks in the study area from 2012 to 2016.

In 2016, 65 wolf attacks were registered in the MedWolf area, 34 in Almeida, 10 in Guarda, 12 in Pinhel, and 9 in Sabugal (Fig. 13).

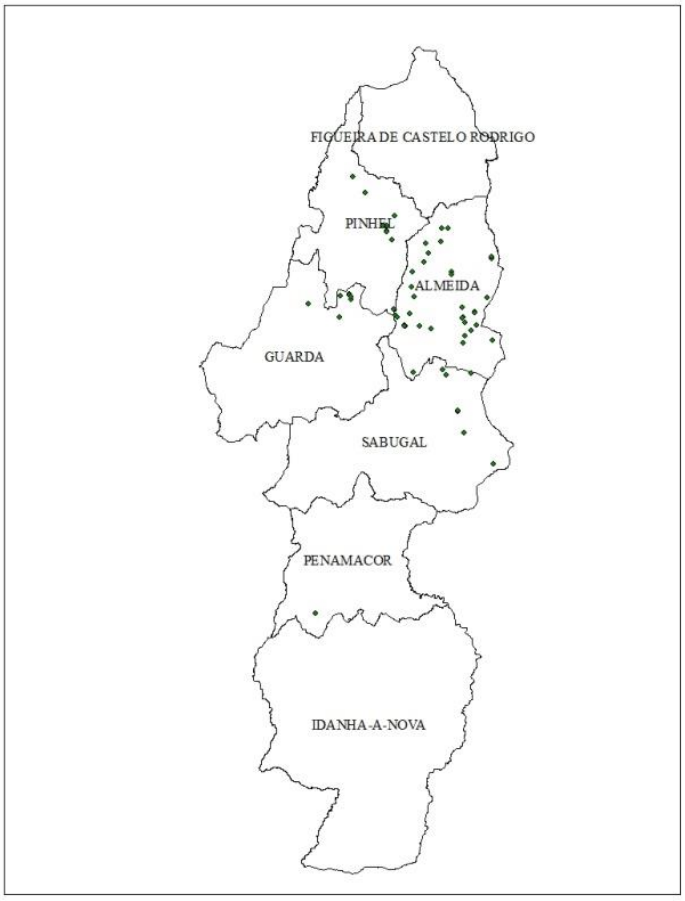


Figure 13. Location of the livestock damages registered in the MedWolf area during 2016.

Damages focused on cattle and small ruminants (44 cattle and 21 small ruminants, 68% and 32% respectively). The total number of livestock heads affected was 156: 98 small ruminants (58 killed and 40 disappeared), and 58 cattle (47 killed and 11 disappeared).

5.3. Camera trapping

A total of 29 camera traps were set-up in 11 UTM 10x10 cells for a total of 1,039 trap-nights (Fig. 14). It represented a decrease of 64% and 58% in the number of trap-nights, when compared to the 2014 and 2013 surveys, respectively, but to only less 3 UTM cells surveyed, when compared to the 2014 survey (Cadete et al. 2014, 2015). This is due by the fact that in 2016 greater emphasis was put on sign surveys since scats' KAI is fundamental to confirm the presence of reproductive packs (see 5.4.), which reduced the availability to maintain the previous trap-night effort. Considering the low wolf density in the study area, it may have influenced the probability of obtaining wolf records compared to previous years, when 25 photos were obtained in 6 events in 2013, and 15 photos in 7 events in 2014.

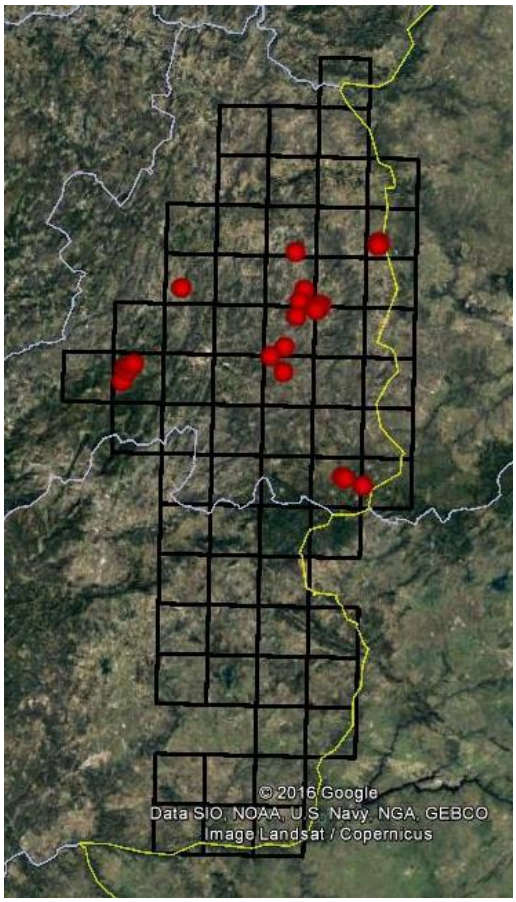


Figure 14. Location of the camera traps installed during 2016.

The cameras registered fox (*Vulpes vulpes*), domestic dog (*Canis lupus familiaris*), wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), badger (*Meles meles*), egyptian mongoose (*Herpestes ichneumon*), domestic goat (*Capra hircus*), cattle (*Bos taurus*), and stone marten (*Martes foina*) (Table 10, Fig. 15).

Table 10. Camera traps installed during 2016 and results obtained (t-n= trap-nights).

Code	UTM	t-n	Start date	End date	Species
M1	PF70	7	18-feb	25-feb	
M2	PE38	5	20-feb	25-feb	fox, dog
M3	PE38	5	20-feb	25-feb	fox, dog
M4	PE38	3	08-mar	09-mar	fox, dog
M5	PE38	34	08-mar	11-abr	dog, badger, stone marten
M6	PE38	34	08-mar	11-abr	fox, dog, badger
M7	PF70	60	08-mar	28-jun	fox, dog, wild boar, cattle
M8	PF70	3	09-mar	20-mar	
M9	PE38	21	11-mar	01-abr	fox, dog, stone marten
M10	PF81	17	12-mar	29-mar	
M11	PF81	8	12-mar	20-mar	wild boar
M12	PE79	29	13-mar	22-may	
M13	PF81	27	15-mar	28-jun	dog, cattle
M14	PF81	22	20-mar	10-jun	fox, dog
M15	PE38	13	29-mar	11-abr	fox, dog
M16	PE76	59	31-mar	28-jun	fox, dog, wild boar
M17	PE76	83	31-mar	28-jun	fox, dog, wild boar, cattle
M18	PF70	37	22-may	28-jun	fox, dog, badger, cattle, stone marten
M19	PE86	11	31-may	11-jun	fox, dog, goat
M20	PF60	53	22-jul	07-oct	fox, dog, wild boar, roe deer, badger, stone marten
M21	PE79	53	22-jul	07-oct	fox, dog, wild boar, badger
M22	PE69	19	23-jul	11-ago	fox, dog
M23	PE68	2	25-jul	27-jul	fox
M24	PF60	44	24-ago	07-oct	fox, wild boar, badger, stone marten
M25	PE69	44	24-ago	07-oct	fox, wild boar
M26	PF61	29	30-ago	21-sep	fox, dog, wild boar, Egyptian mongoose
M27	PE68	33	04-sep	07-oct	fox, dog, wild boar, stone marten
M28	PF40	8	08-oct	16-oct	fox, dog, wild boar, roe deer, domestic goat
M29	PE38	7	09-oct	16-oct	dog, roe deer

Figure 15. Pictures of the different species recorded by the camera traps in the MedWolf area during 2016.

Foxes and dogs were recorded in almost all cameras (74%) and throughout the year, confirming the presence of these canids within the wolf range along the year. The presence of dogs was associated to livestock only in 24% of the camera sites. It is also worth mentioning the records of wild prey in 42% of the cameras, with the wild boar being more frequent than the roe deer (11 and 3 cameras, respectively) as expected, since wild boar exists in higher densities throughout the study area. No wolves were captured in the camera traps. However, in July, pictures of a single wolf were recorded in Sabugal by the wind farm monitoring team (BIOTA - Estudos e Divulgação em Ambiente, Lda., courtesy of Barbara Monteiro, Fig. 16).

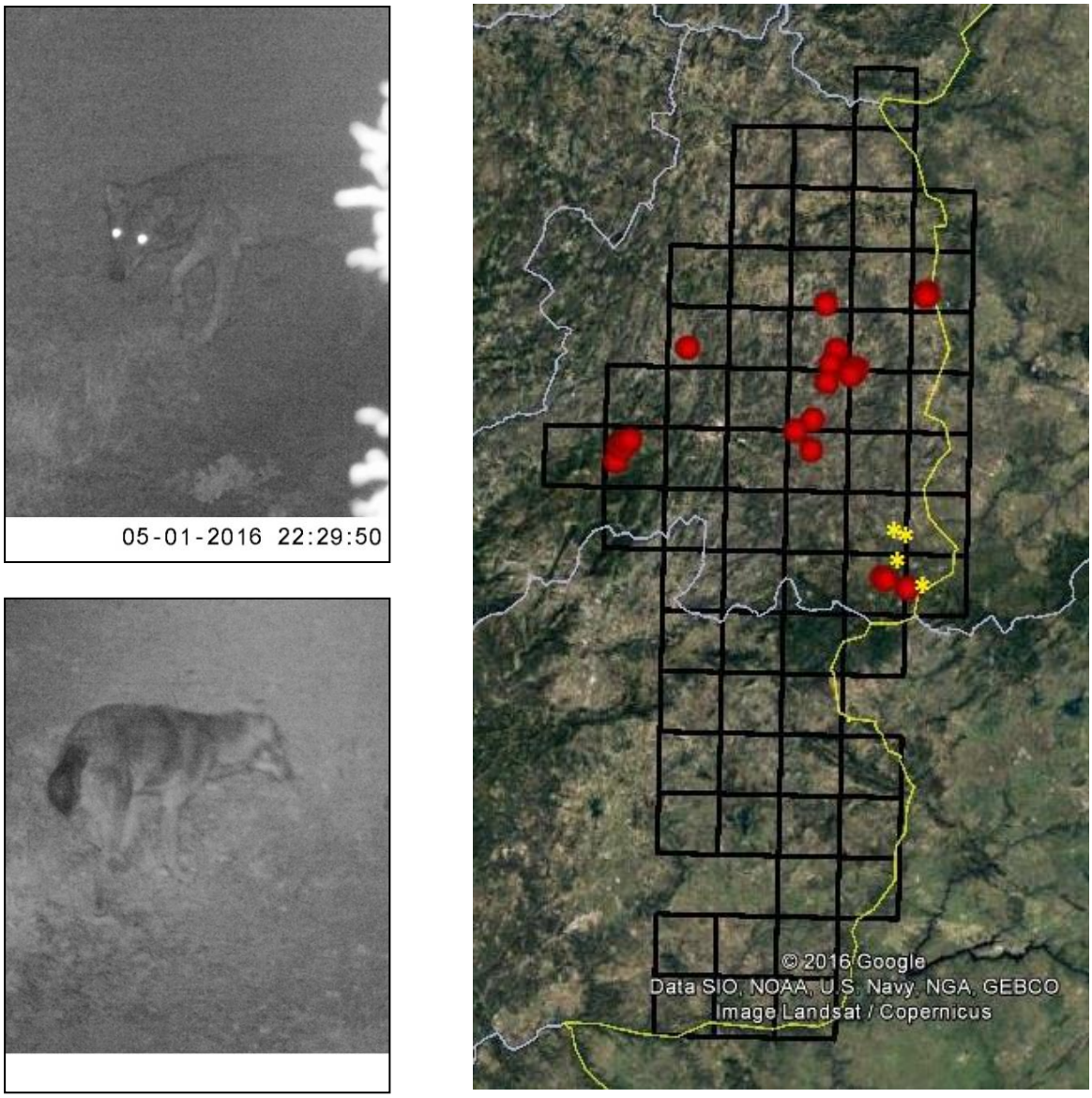


Figure 16. Wolf pictures obtained in Sabugal (courtesy of BIOTA - Estudos e Divulgação em Ambiente, Lda.). Location of the cameras where the pictures were taken (*) compared to the location of the cameras installed in this study (red circles).

These pictures were registered in four different camera traps. Three cameras located in Soito, recorded one wolf each, and another wolf was photographed 8 km away, in Serra das Mesas (Fig. 16). Nevertheless, it is not possible to determine if the pictures belong to the same or different individuals, since no distinguishing features were observed.

5.4. Sign survey

In 2016 we carried out 555 transects, 452 performed by the human team and 103 by the scat detection dog team, increasing 3-fold the number of transects carried out with respect to Cadete et al. (2015) (Table 11).

Table 11. Comparative sampling efforts developed in 2014 and 2016.

	2014*	**2016**
Human team (HT)		
UTM cells sampled	65	66
Transects done	130	452
km covered	310.8	1,426.9
Transects/UTM cell	2	6.8
km/UTM cell	4.8	21.6
Scat detection dog team (DT)		
UTM cells sampled	17	66
Transects done	98.5**	103
km covered	197	314.16

* Data from Cadete et al. (2015); ** In 2014 the scat detection dog team surveyed 98.5 sites, sampling 2 km per site (Cadete et al. 2015).

During the first trimester of 2016, 66 UTM cells were sampled by the HT and 16 by the DT[1], with wolf scats[2] (n=12) being found in 4 (Table 12, Fig. 17).

[1] These include only cells exclusively sampled by the DT; transects carried out by the HT and the DT together, during the training period of the dog, where considered as HT transects, since the DT had not major intervention on these transects.

[2] In this section we consider wolf scat every scat assigned to wolf by the human team (excluding *Canis* sp. assigned scats) or marked by the scat detection dog as a wolf scat, without genetic confirmation (see the Methods section and the results of genetic analyses below).

Table 12. Sampling effort and wolf scats found during the first trimester of 2016.

1st Trimester	**Nr. transects**	**Nr. km**	**Nr. UTM cells**	**Nr. scats**
Human team	151	469.84	66	7
Dog team	17	49.65	16	5
Cell with wolf presence	**Nr. scats**	**Nr. transects**	**Nr. km**	**KAI**
Human team transects				
29T PE38	3	2	8.96	0.33
29T PE76	1	4	13.26	0.08
29T PE79	1	4	13.45	0.07
29T PF81	2	3	13.18	0.15
Dog team transects				
29T PE76	5	1	3.75	1.33

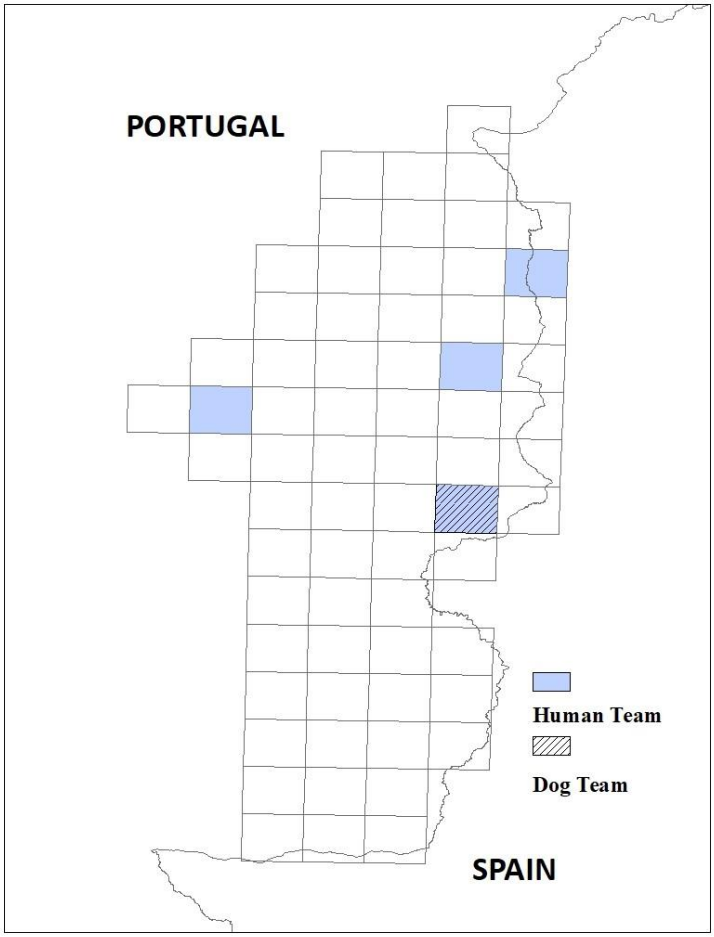


Figure 17. UTM cells with estimated wolf presence according to transects

carried out during the first trimester of 2016.

During the second trimester 58 UTM cells were sampled by the HT and 47 by the DT, and wolf scats (n=19) were found in 11 (Table 13, Fig. 18).

Table 13. Sampling effort and wolf scats found during the second trimester of 2016.

2nd Trimester	**Nr. transects**	**Nr. km**	**Nr. UTM cells**	**Nr. scats**
Human team	107	349.52	58	3
Dog team	68	212.56	47	16
Cell with wolf presence	**Nr. scats**	**Nr. transects**	**Nr. km**	**KAI**
Human team transects				
29T PF40	1	6	21.73	0.05
29T PE76	2	2	7.45	0.27
Dog team transects				
29T PE38	1	2	5.57	0.18
29T PE47	3	2	6.01	0.50
29T PE58	1	1	1.96	0.51
29T PE69	1	3	6.79	0.15
29T PE76	3	3	8.31	0.36
29T PE79	2	2	5.1	0.39
29T PE86	1	2	7.62	0.13
29T PE89	2	2	7.17	0.28
29T PF70	1	3	13.25	0.08
29T PF81	1	3	12.92	0.08

During the third trimester 63 UTM cells were sampled by the HT and 16 by the DT, and wolf scats (n=9) were found in 6 (Table 14, Fig. 19). In none of the cells, the KAI of scats, together with the length of transects conducted, suggested the existence of a reproductive pack (probability of wolf reproduction >0.60 for KAI values of 0.65 scats/km sampling 15.6±8.5 km/site, Llaneza et al. 2014).

In total, human and dog teams found wolf scats in 13 UTM cells (20% of the cells comprising the MedWolf area, Fig. 20). Other 35 scats collected during the transects and assigned by the human team to *Canis* sp., were collected for genetic analyses.

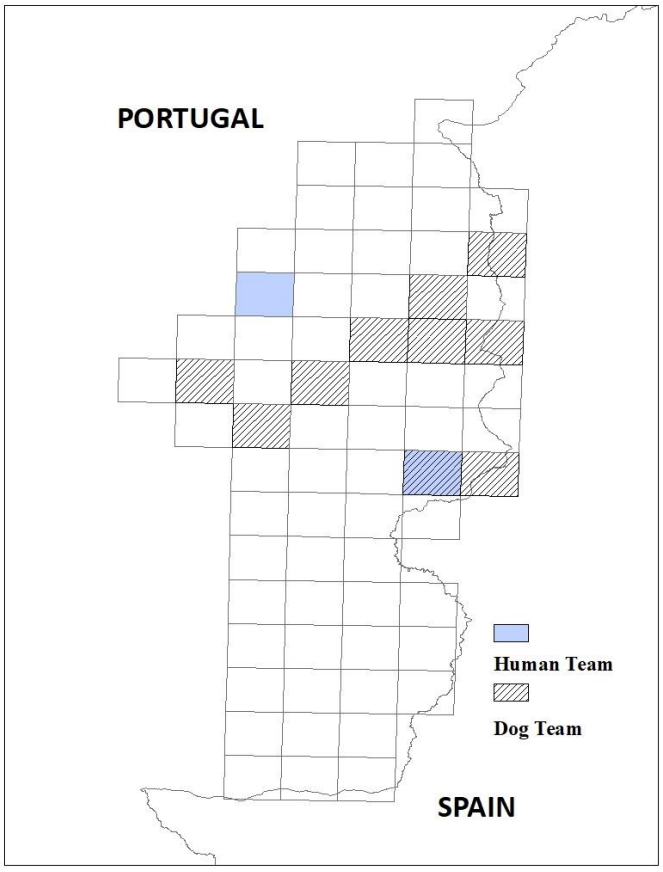


Figure 18. UTM cells with estimated wolf presence according to transects carried out during the second trimester of 2016.

Table 14. Sampling effort and wolf scats found during the third trimester of 2016.

3rd Trimester	**Nr. transects**	**Nr. km**	**Nr. UTM cells**	**Nr. scats**
Human team	194	607.54	63	3
Dog team	18	54.02	16	6
Cell with wolf presence	**Nr. scats**	**Nr. transects**	**Nr. km**	**KAI**
Human team transects				
29T PE69	1	9	26.13	0.04
29T PE75	1	2	11.16	0.09
29T PE76	1	6	13.19	0.08
Dog team transects				
29T PE37	1	1	4.70	0.21
29T PE38	1	1	5.35	0.19
29T PF40	4	1	2.07	1.93

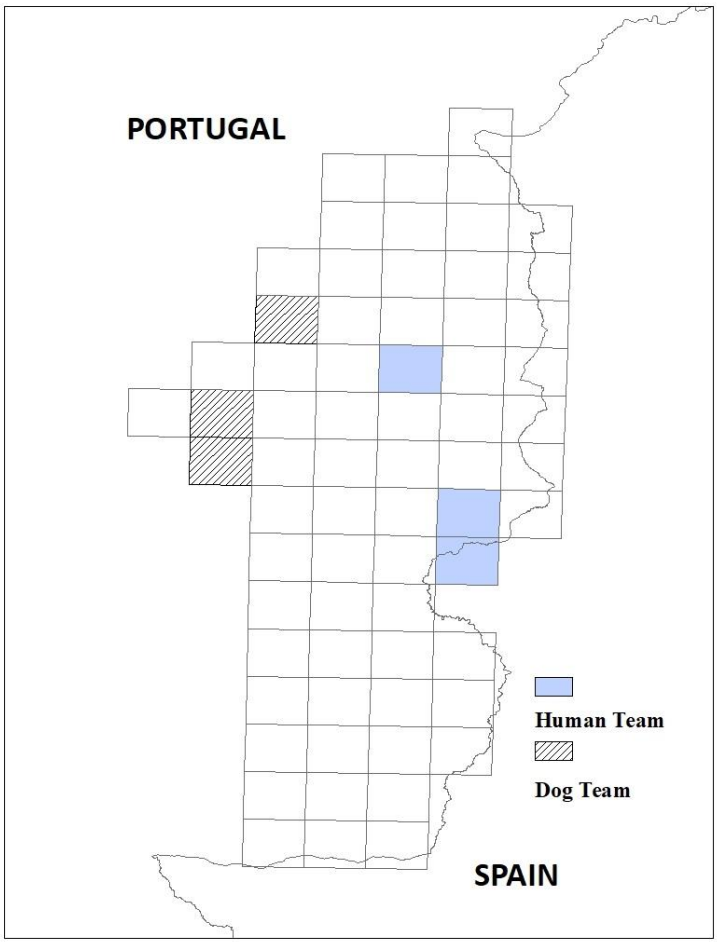


Figure 19. UTM cells with estimated wolf presence according to transects carried out during the third trimester of 2016.

Besides the 40 scats a priori considered wolf scats by the HT and the DT found during the transects, other 36 scats assigned to wolf were collected in 2016, four opportunistically collected and 32 during the scat detection dog tests carried out in the MedWolf area, increasing the wolf presence in three UTM cells: 29T PE48 (two scats), 29T PE77 (one scat), and 29T PF60 (three scats). Considering all the field work carried out in 2016, we estimated wolf presence in 16 UTM cells (24% of the UTM cells comprising the study area, Fig. 21).

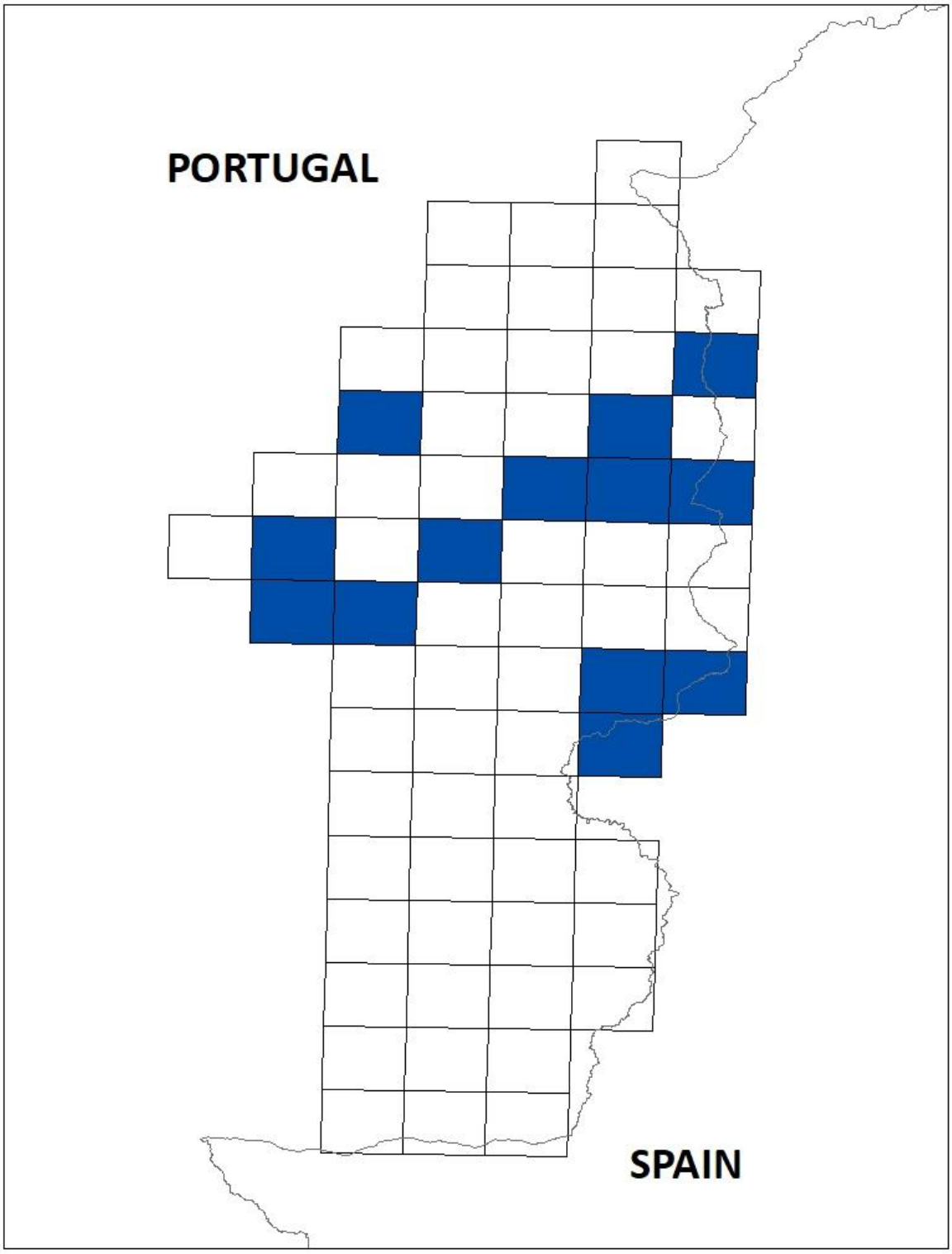


Figure 20. Estimated wolf presence in the MedWolf area according to transects carried out in 2016.

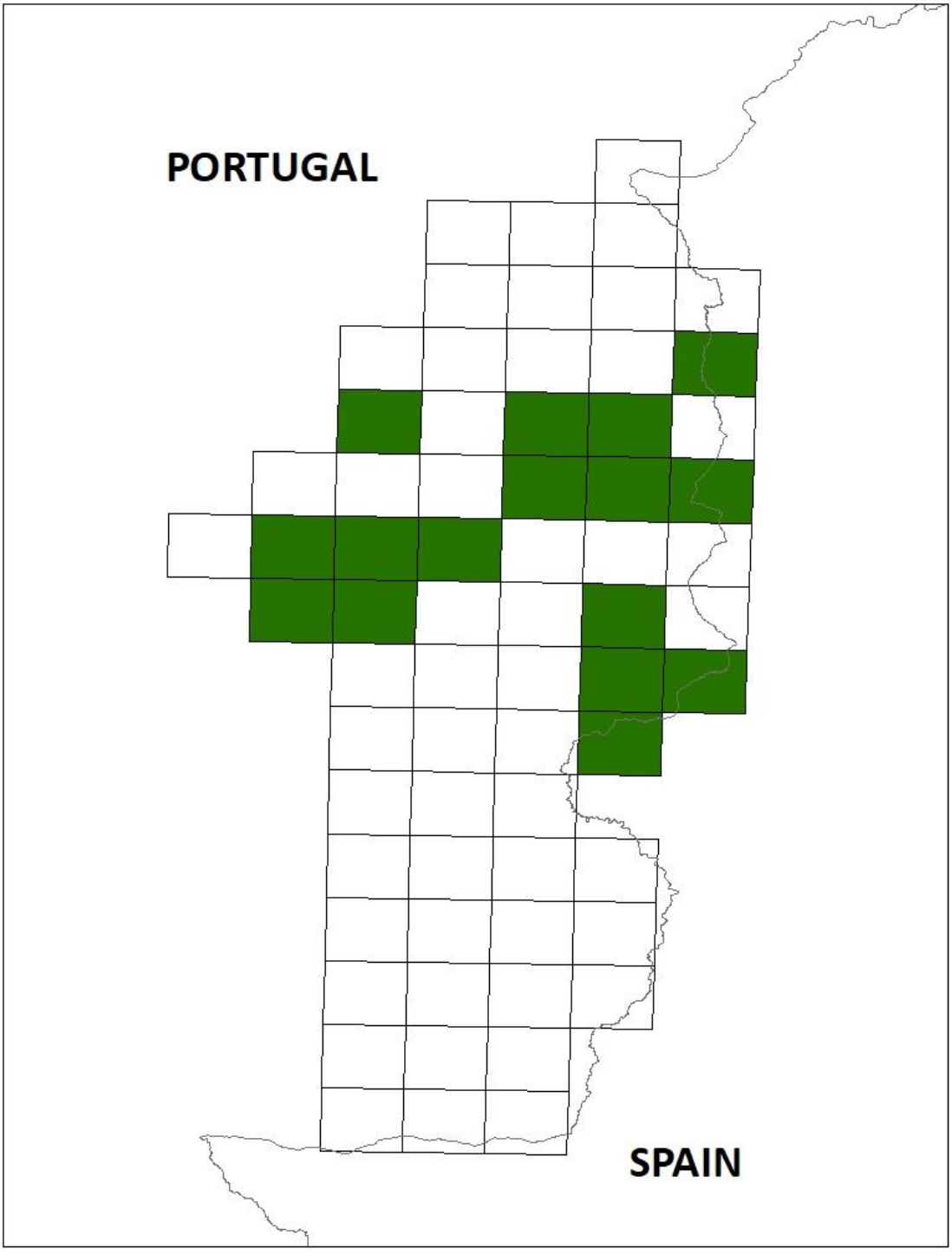


Figure 21. Estimated wolf presence in the MedWolf area according to the field work carried out in 2016.

5.5. Howling sessions

A total of 50 howling points were conducted from August 24th to October 17th, sampling 11 UTM 10x10 cells (Table 15, Fig. 22). All howling points were conducted with optimal wind conditions. Dogs responded in all cases except in the trials carried out in Serra da Malcata. No wolf response was obtained, despite the increased effort (compared to the 24 and 2 howling points done in the 2013 and 2014 surveys, respectively). The absence of response may be due to the fact that no sites with high concentration of scats, suggesting the existence of a breeding area, were found (areas where the probability of success is higher, Llaneza et al. 2005b), despite the increased effort in sign survey, as previously described.

Table 15. Howling points carried out in the MedWolf area during 2016.

Date	Cell	Nr. of howling points
24/08/2016	PE69	2
24/08/2016	PF60	2
28/08/2016	PE76	1
28/08/2016	PE86	5
07/09/2016	PE65	2
07/09/2016	PE66	3
07/09/2016	PE75	1
07/09/2016	PE76	1
10/09/2016	PE68	4
10/09/2016	PE69	2
10/09/2016	PE78	3
11/09/2016	PF60	1
13/09/2016	PE75	5
09/10/2016	PE38	5
10/10/2016	PE65	7
10/10/2016	PE66	2
10/10/2016	PE75	1
10/10/2016	PE76	1
17/10/2016	PF70	2

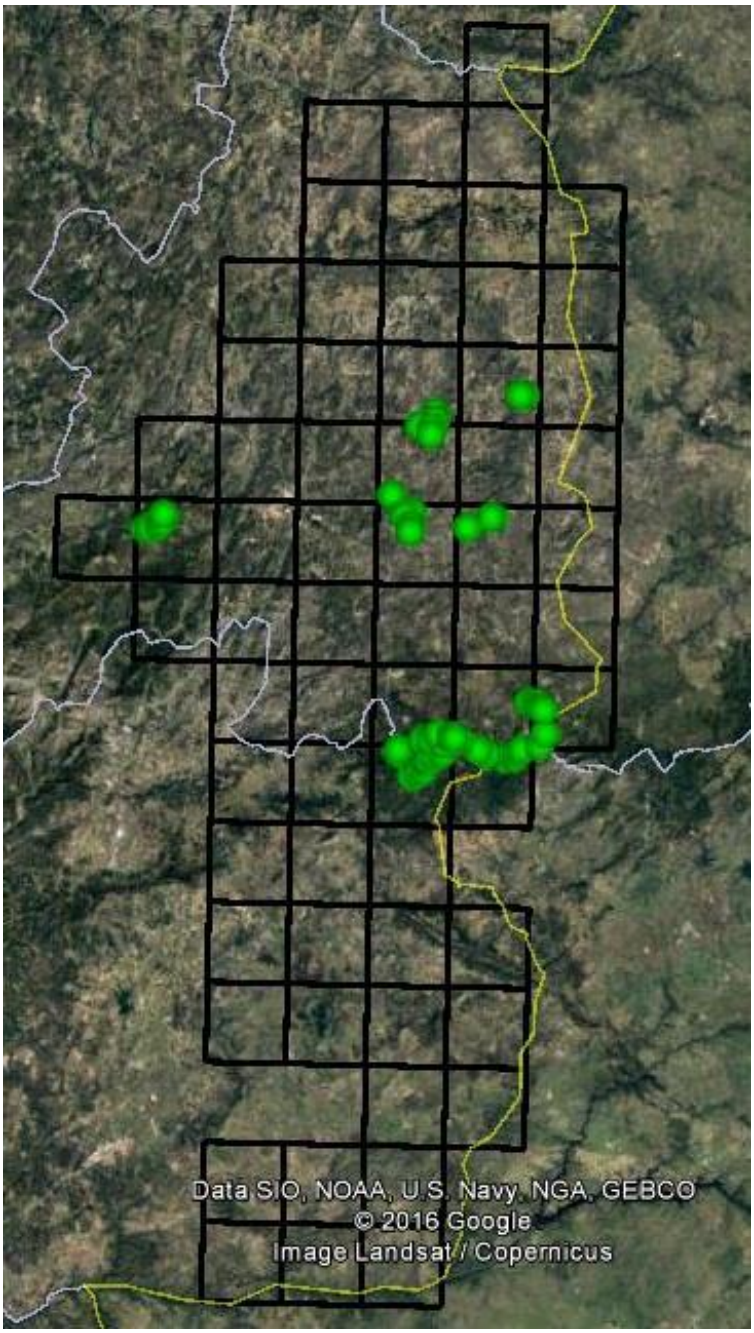


Figure 22. Location of the howling points conducted in the MedWolf area during 2016.

5.6. Reproduction

No wolf positive results were obtained during howling and watching stations. Nevertheless, unconfirmed information of a sighting of a wolf pup and pups howling in Almeida in 2014, was obtained from a farmer.

5.7. Mortality

No confirmed record of wolf mortality was obtained during field work or from the official records. We only obtained unconfirmed information about a possible wolf illegally killed (shot) in 2015 in Trinta (Guarda), Serra da Estrela.

6. Discussion and conclusions

6.1. Genetic analysis

A considerably high number of non-invasive samples, 1,516, were received and analysed during A.2 and D.3 Actions of the MedWolf project, comprising a period of 4 years, from 2013 to 2016.

DNA amplification from DNA of non-invasive samples poses technical problems related mainly with the presence and quality of DNA in the samples. From this study, non-invasive samples showing higher percentage of PCR amplification were those from swabs from livestock damages. Generally, when DNA was present, PCR amplification was readily suitable.

For scats, DNA presence and quality were two main problems. The percentages of amplification varied depending on if it were collected by the HT or the DT. As dogs find the scats by smell and humans by sight, the scat detection dog detects older and more decomposed scats (bad samples for PCR amplification) that field technicians are less prone to find. That could be the reason of a better amplification rate for scats found by the HT. However, although showing lower PCR amplification rates, the specificity of the DT for wolf derived samples was higher.

All samples were amplified tested for 16 microsatellite samples. This is a high number of loci for the analysis of non-invasive environmental samples where DNA integrity is mostly affected. Our conclusion is that a subset of loci should be selected for non-invasive DNA based monitoring, attending to some loci characteristics such as null alleles, dropout alleles, allele bias. We have given preference to tetranucleotide repeats for genotype identification of individuals. These considerations follow those found by Perez et al. (2006) or Mondol et al. (2009) for individual identification of leopards in a landscape.

Regrouping all samples using six microsatellite loci enabled detection of 42 individual genotypes in 2013-2014 and 27 individual genotypes in 2015-2016. This strategy was undertaken to minimize overestimation of the number of individuals due to genotyping errors as allelic dropout or false allele amplification.

Concerning relatedness, data pointed to the probable existence of 2 groups in the study area. Also some genotypes mostly unrelated were detected. Those individuals may correspond to lone wolves or dispersers. By geo-referencing the genotypes, and integrating with data arising from other wolf monitoring methods, it was possible to obtain a more complete picture of the status and trend of the wolf nucleus in the MedWolf intervention area.

A total of 137 scats from the MedWolf area were analysed in 2016: 98 collected during the wolf survey, and 39 during the scat detection dog tests. Species assignment was possible for 28 scats (22%), 12 wolf scats and 16 dog scats. All of the *a priori* considered wolf scats that could be genetically confirmed were effectively wolf scats, and none of the genetically confirmed dog scats were *a priori* classified by the HT as wolf scats or were marked by the DT as a wolf scat. These results confirm the high success of both teams involved in the 2016 wolf survey in the MedWolf area, and support their future use with expected high levels of accuracy.

Regrouping all the samples from 2015-2016, using six microsatellite loci enable us to find, 11 individual genotypes from scats (all of them in 2016, since no scats were collected in 2015). Additionally, of the 372 swabs and 1 hair sample collected during livestock damage assessments in 2015 and 2016, it was possible to find 16 individual genotypes (10 in 2015 and 6 in 2016) different from those found via scat analysis.

Special care should be taken also when considering the male dominated sex ratio, since it could be influenced by the sampling and sexing molecular methods and the type of samples used. Nevertheless, Sastre et al. (2008), targeting both Y and X chromosomes, obtaining a very high sexing rate, found only males in a sample of scats collected in the Pyrenees Mountains (Spain) (37 males and zero females, out of 39 scat samples). Blanco et al (1990), based on the number of wolves found dead (where sexing was possible), found a 6:1 sex ratio outside the usual wolf range in Spain, which is similar to the one obtained from scat sexing for the entire MedWolf area in 2016. It has been proposed that the higher presence of males may result from intrinsic population regulation, and may be common in recent re-colonization areas, possibly since males may disperse longer distances (Mech 1970, Mech 1987). Data of

2016, from three packs located west of the study area (included in a more stable nucleus of the Portuguese population South of the Douro river), obtained a sex ratio of 1.25:1, based on molecular sexing of scats (Roque et al. 2017). The number of different genotypes identified in this area of low wolf density can be explained by isolate dispersers from neighbour areas of higher densities.

Finally, since 2013, and considering a total of 298 wolf assigned samples, no signal of hybridization was found in the MedWolf area, despite being a more peripheral, isolated, and recently expanded nucleus, where a higher probability of hybridization would be expected (Godinho et al. 2011). Despite the presence of dogs throughout the study area, the existence of a male dominated population, as suggested by the sexing results, may have decreased the hybridization risk, as the dynamics of hybridization seems to result from crosses between male dogs with female wolves (Godinho et al. 2011).

6.2. Wolf distribution

According to the results of the genetic analyses of samples (swabs and scats) collected in 2016, wolf presence was confirmed in 11 UTM 10x10 cells. The results of the genetic analysis performed by CIBIO-InBIO (Raquel Godinho, pers. comm.) allowed to confirm the presence of the species in one more UTM 10x10 cell. Thus, the distribution in the MedWolf area comprises 12 UTM 10x10 cells, increasing by 10 cells the distribution estimated in 2002, but reducing by one cell the range estimated in 2014 (Fig. 23). Wolf presence includes Pinhel, Almeida, Sabugal, and Guarda counties. We found no evidences of wolf presence in Figueira de Castelo-Rodrigo, Penamacor, and Idanha-a-Nova counties. It is important to emphasize that the sampling effort was bigger in the surveys carried during the MedWolf project, namely in 2016.

A genetically confirmed wolf distribution map by 4x4 UTM cells was also produced (Annex I).

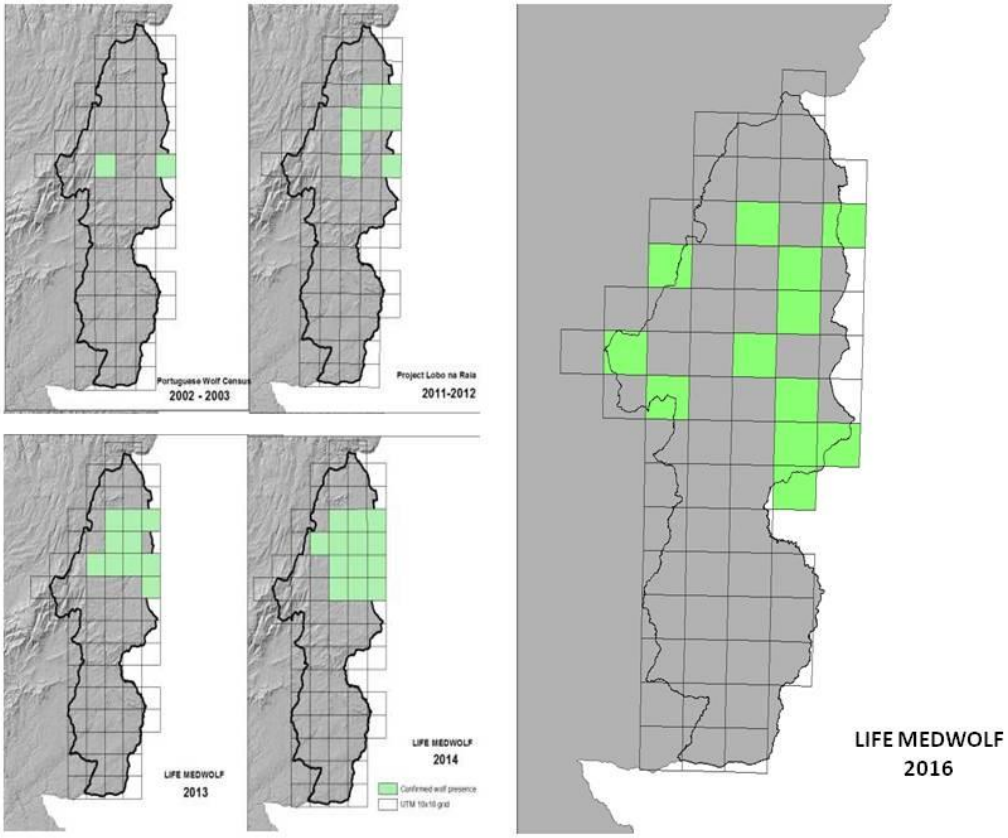


Figure 23. Evolution of the wolf range in the MedWolf area from 2002 to 2016 (including only genetically confirmed samples). Considering UTM 10x10, cells with presence confirmed by camera trapping coincide with those confirmed by genetically confirmed samples.

6.3. Wolf population

According to the genetic analyses, 27 individuals were identified in the MedWolf area in 2015-2016 (11 in 2015, and 17 in 2016, being one individual identified in both years). We found a very low recapture rate during the period 2013-2016 (see genetic analyses section) which could be due to an extremely high renewal rate. However, these results differ from those obtained in the contiguous area south of Douro river, where eight out of nine individuals genetically identified in 2016 where recaptures from previous years (Roque et al. 2017). As pointed out in a study carried out in Picos de Europa, Spain, a low recapture rate could be underestimated due to a low sample size, and these results should be considered preliminary results (García et al. 2013). For this reason, to avoid errors, for population estimates in the study area we only consider the population identified in 2016: 17 different genotypes (11 from scats, 5 from swabs and one from a hair sample). We obtained in 2016 a minimum

density of 1.42 wolves/100 km^2 (considering 12 UTM 10x10 cells with wolf presence genetically confirmed), 1.06 wolves/100 km^2 (considering 16 UTM 10x10 cells with wolf presence based on *a priori* species assignment of scats by the HT), and 0.26 wolves/100 km^2 for the overall study area (66 UTM 10x10 cells). These results yield a wolf density similar than that obtained in the Portuguese adjacent area south of the Douro River comprising three wolf packs (scat genetic analysis confirmed 1.5 wolves/100 km^2, Roque et al. 2017), but higher than the density estimated in the Spanish distribution range south of the Douro river (recolonizing area) in 2001 (15 packs/19,500 km^{2,}, and 0.52-0.77 wolves/100 km^2, according to Llaneza and Blanco 2005[3]).

Since we do not have data from radio-collared wolves or enough data obtained from genetic recaptures (only one case for the period 2015-2016), it is impossible to estimate home range sizes of individuals or packs, and to identify pack territories. However, as an alternative approach, based on the concentrations of scats found during the surveys and livestock damages records (Fig. 24), we can identify by means of kernel density distribution estimators six zones with wolf presence that could, potentially, contain wolf packs:

1. Almeida (Côa and Cabras riversides);
2. Sabugal/Malcata;
3. Border with Spain (F. de Castelo Rodrigo/Almeida);
4. Border with Spain (Almeida/Sabugal);
5. Serra da Estrela/South West Guarda;
6. North Guarda/South West Pinhel.

Most of the individuals genetically identified (15 out of 17) were located inside these six zones. The other two were found outside these areas, in Pinhel and Sabugal municipalities.

[3] Llaneza and Blanco (2005) estimated the wolf population multiplying the number of packs by 6.7-10 individuals, based on mean pack size data.

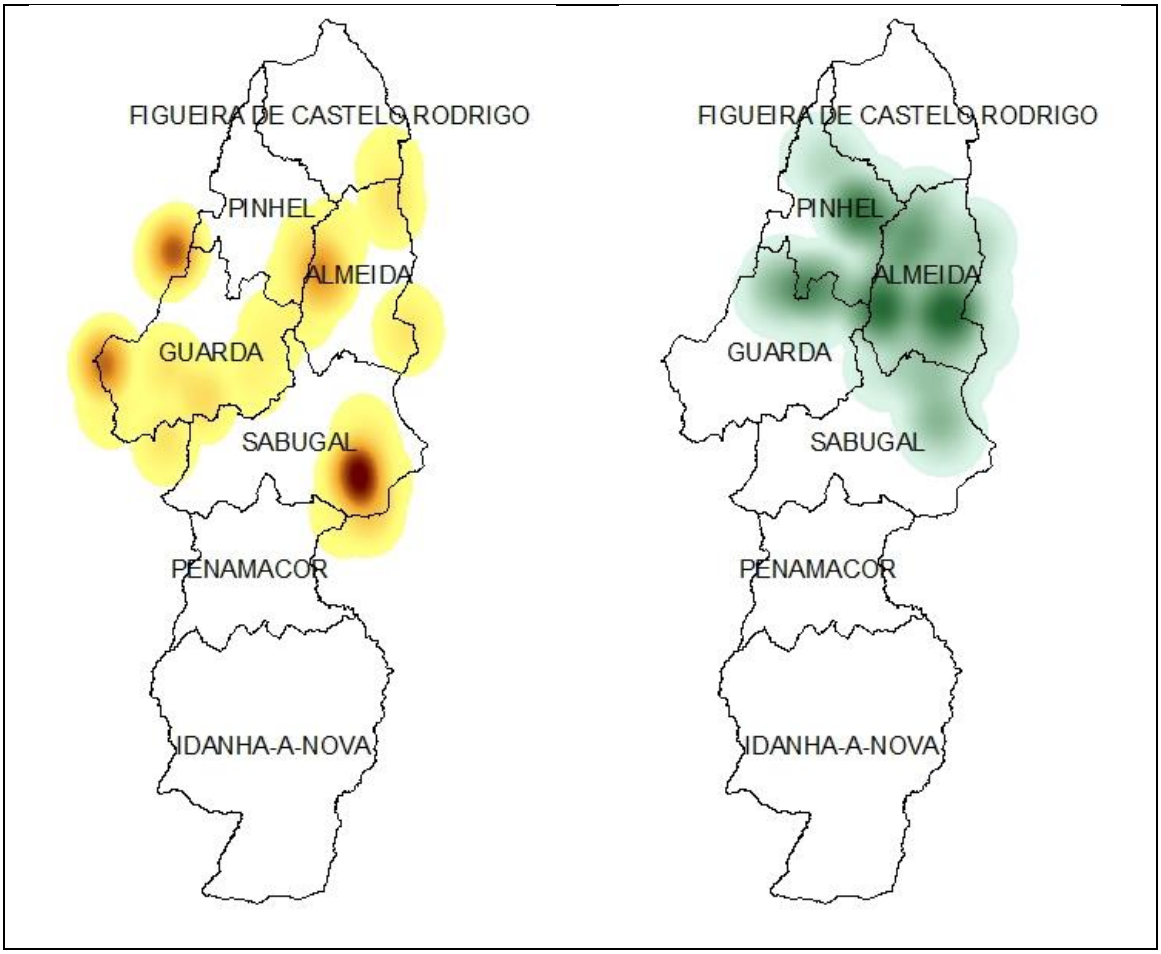


Figure 24. Highest concentrations of scats found during surveys in 2016 (left) and wolf damages recorded during 2015-2016, in the MedWolf area.

Almeida (Côa and Cabras riversides)

The existence of a pack of wolves in this area was considered probable in 1995, 2002-2003, and confirmed in 2011, 2013, and 2014 (ICN 1997, Pimenta et al. 2005, Roque et al. 2011, Cadete et al. 2015). In 2014, the minimum calculated pack size was 7 individuals, 6 males and at least one female. The existence of a pack of wolves in this area is considered confirmed due to information obtained in previous years altogether with the amount of livestock damages and the presence of five different individuals genetically confirmed in 2016 (four males and one unknown sex). Reproduction was not confirmed in 2016.

Sabugal/Malcata

In this area, the existence of a pack of wolves was considered probable in 1995, and during the national census carried out in 2002-2003 the area was considered as of probable wolf

presence area (ICN 1997, Pimenta et al. 2005). In 2016 a relatively high concentration of wolf scats was found and three different individuals were genetically identified (two males and other of unknown sex). The existence of a pack of wolves in Sabugal/Malcata in 2016 is thus considered probable.

Border with Spain (F. de Castelo Rodrigo/Almeida)

In this area several wolf scats were found and two individuals were identified by genetic analyses (unknown sex). The existence of a nearby pack of wolves in Vitigudino (Spain) confirmed in 2013-2014 (Sáenz de Buruaga et al. 2015) suggests that this area could be part of the territory of the Spanish pack.

Border with Spain (Almeida/Sabugal)

In this area the probable existence of a pack was reported in 2002-2003, 2013, and 2014 (Pimenta et al. 2005, Cadete et al. 2015). In 2016 the presence of the species was detected (two different individuals identified genetically, one male and one unknown sex) and several livestock damages were recorded in the area. Although it was not possible to confirm the existence of a pack different from Almeida or Sabugal, wolves are present in the area.

Serra da Estrela/South West Guarda

The presence of wolves in this area has been confirmed in 2016 by means of wolf scats, genetically confirming the presence of two different individuals (one female and one male). We have reliable information about one wolf killed in 2015. Further research is needed to see if a pack of wolves will establish its territory in the area.

North Guarda/South West Pinhel

In this area one individual (male) was detected by means of genetic analysis of wolf scats. In 2002-2003 this area was considered an area of probable wolf presence (Pimenta et al. 2005).

6.4. Wolf population trend

The results obtained in 2016 confirm the establishment and consolidation of the wolf presence in half of the MedWolf area (see Distribution Range section, Fig. 23, Table 16). The two wolf packs estimated in 1995 were present in all the later surveys, despite changes in location, except in the most recent carried out in 2016, where the presence of one more pack was considered probable (Fig. 25). Wolf presence was now confirmed in all the areas previously considered as probable, suggesting a slow recovery of the wolf population as reported by Torres and Fonseca (2016). This slow recovery may indicate the instability of wolf packs in the area. In fact, there is only one report of reproduction in 1995 (Pimenta et al. 2005), and information about possible reproduction in the Almeida's pack in 2014 (not confirmed information). Of major interest is the connection found between the packs of the study area and the packs located west, which can be relevant source packs to maintain the MedWolf nucleus and thus deserve special attention.

The observed trend differs from the stably expanding trend reported for the Spanish wolf population south of Douro river, where the population, extinct in 1988, recolonized the area reaching 15 wolf packs in 2000-2001 (Llaneza and Blanco 2005), and from other European populations (Chapron et al. 2014). The high dependence on livestock to survive may influence the population trend, with high levels of livestock damages being a major limiting factor to wolf expansion (Llaneza and Blanco 2001), due to the high levels of human caused mortality (Boitani 2000, Llaneza and Blanco 2001, Boitani 2003, Torres and Fonseca 2016).

However, our results yield promising expectations for the future. The presence of wolves in Serra da Estrela (and surrounding areas) and the existence of a probable pack in Sabugal/Malcata reveal an expanding range that may foster a positive trend in the future. In these areas we detected the presence of wolves but low levels of damages to livestock (see 6.3.). The reason for this lack of livestock damages records is not known, since it can result from a real absence of damages, or to the fact that damages are not being claimed, or even to low densities of livestock or higher wild prey densities. If the presence of wolves in these areas consolidates in the future, maybe this wolf nucleus will have the chance to recover in areas with abundant wild prey and low levels of conflict due to livestock damages.

Table 16. Wolf trend in the MedWolf intervention area since 1995.

	1997[1]	**2002-2003**[2]	**2013**[3]	**2014**[3]	**2016**[4]
Range					
Nr. UTM 10x10 cells	13[5]	2[5]	10	13	12
Packs/wolf presence areas					
Almeida (Côa and Cabras riversides)	Probable pack	Probable pack	Confirmed pack[6]	Confirmed pack	Confirmed pack
Sabugal/Malcata	Probable pack	Probable presence			Probable pack
Border with Spain (F. de Castelo Rodrigo/Almeida)		Probable presence			Probable pack[7]
Border with Spain (Almeida/Sabugal)		Probable pack	Probable pack	Probable pack	Confirmed presence
Serra da Estrela/South West Guarda					Confirmed presence
North Guarda/South West Pinhel		Probable presence			Confirmed presence

[1] ICN (1997); [2] Pimenta et al. (2005); [3] Cadete et al. (2015); [4] this study; [5] presence not confirmed genetically; [6] the authors estimate the presence of 1-2 packs (one confirmed and other possible) in this area; [7] this area is considered as part of a Spanish pack's territory.

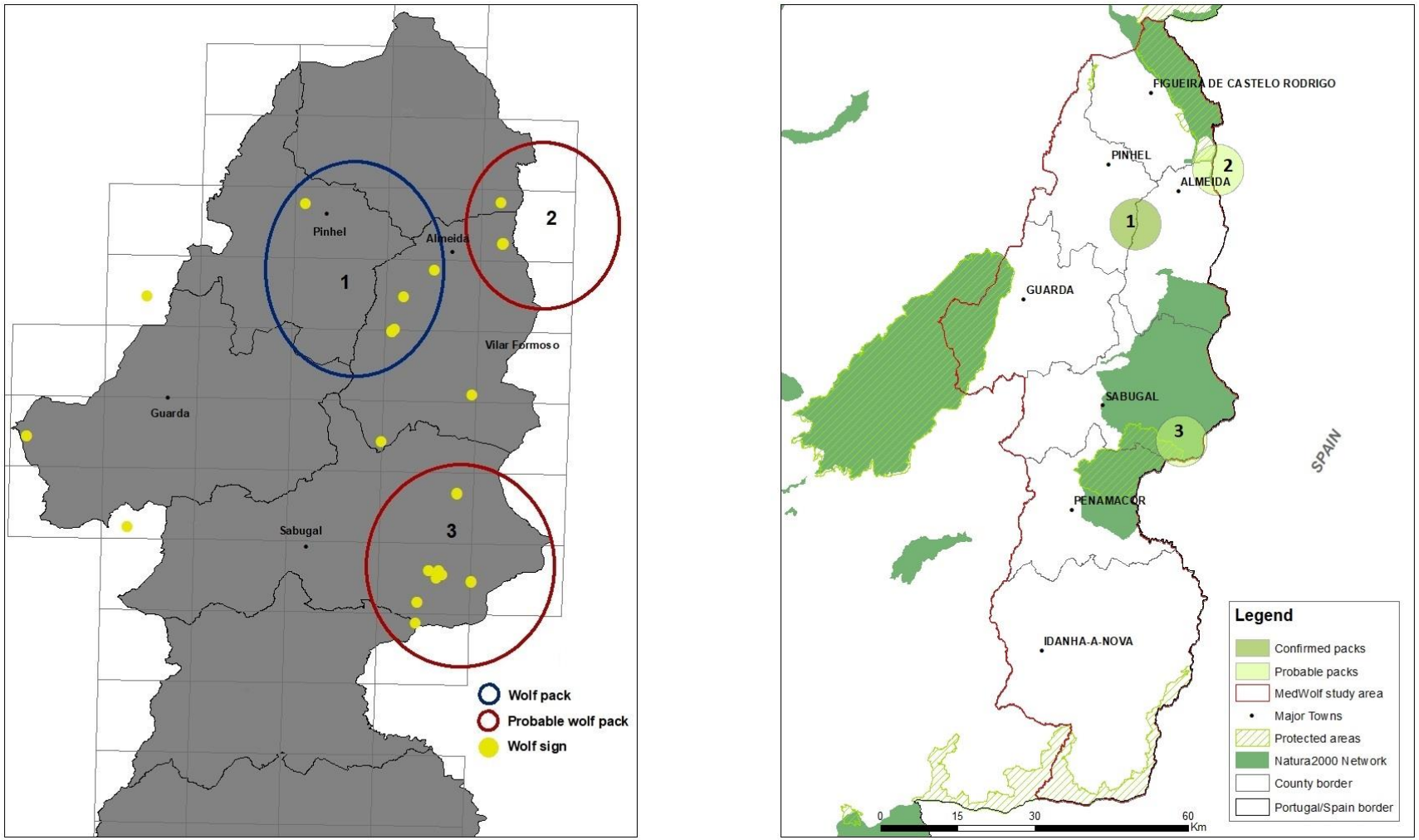


Figure 25. Maps with location of wolf packs identified in the MEDWOLF area in 2016: 1) Almeida (Côa and Cabras riversides); 2) Border with Spain (F. de Castelo Rodrigo/Almeida); 3) Sabugal/Malcata; with indication of wolf records (scat or swab genetically confirmed) (left), and of protected areas (right).

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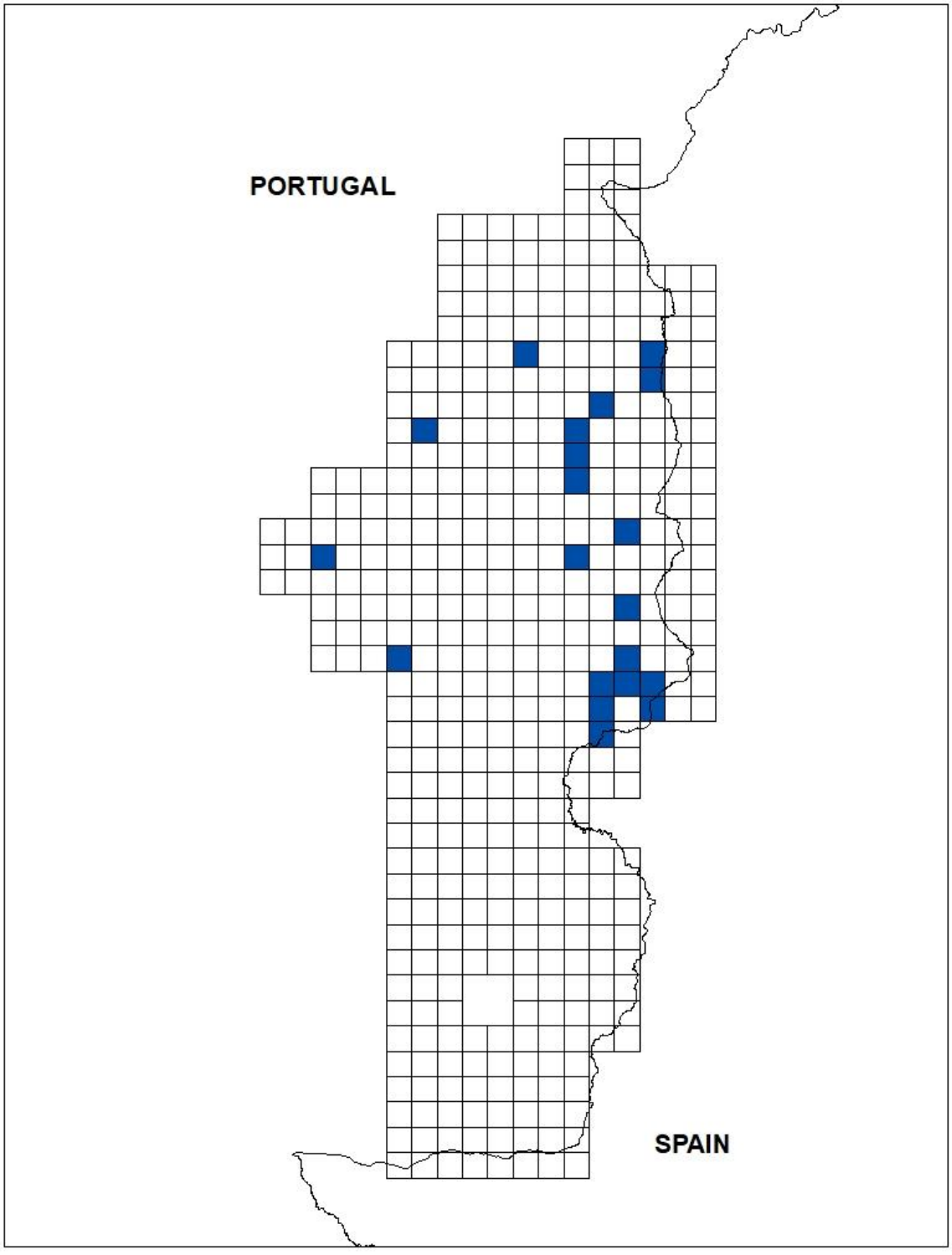
Annex I



Genetically confirmed wolf distribution in the MedWolf area in 2016, based on 4x4UTM cells. Cells with presence confirmed only by camera trapping (n=2) are also included.