

Surveillance of prion diseases in Portuguese Cervids “CWD risk assessment in Portugal project- results”

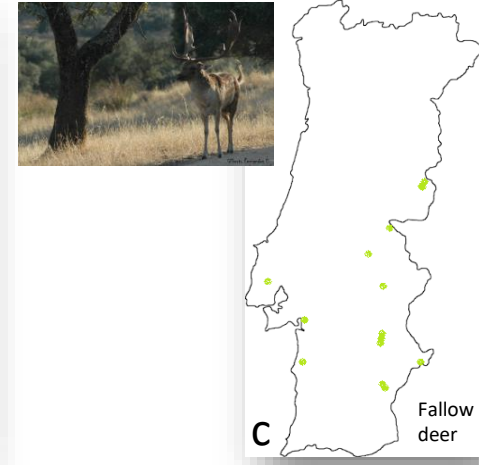
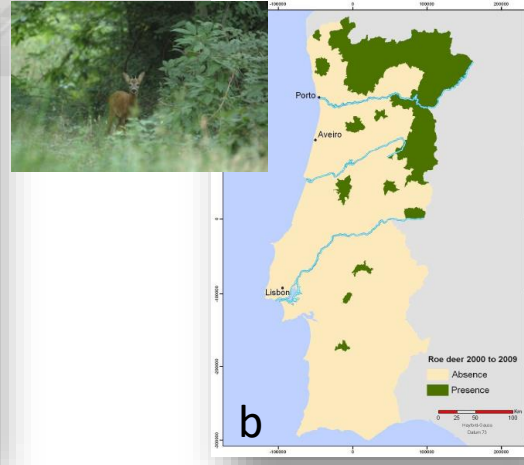
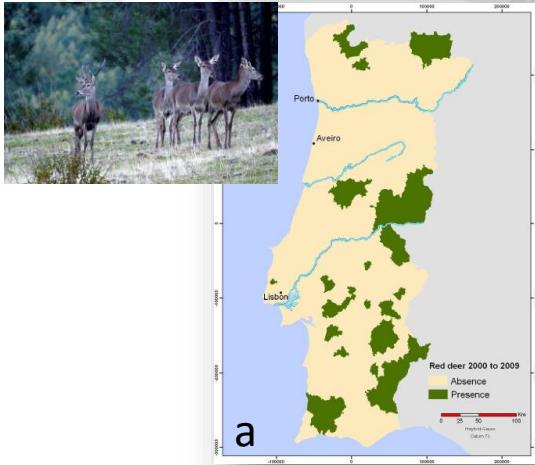
Leonor Orge; Juan Maria Torres, Jorge C. Pereira, Nuno Gonçalves-Anjo, Estela Bastos, Ana C. Matos, Adelina Gama, Anabela Alves, Alexandra Esteves, Luís Figueira, Carla Lima, Filipe Silva¹, Fernanda Seixas, Isabel Pires, João Silva, Madalena Vieira-Pinto, Maria L. Pinto, Paula Mendonça, Paulo Carvalho, Paula Tavares, Roberto Sargo and Maria A. Pires

TSE EURL-NRL meeting
Istituto Superiore di Sanità, Rome,
2-3 October 2023

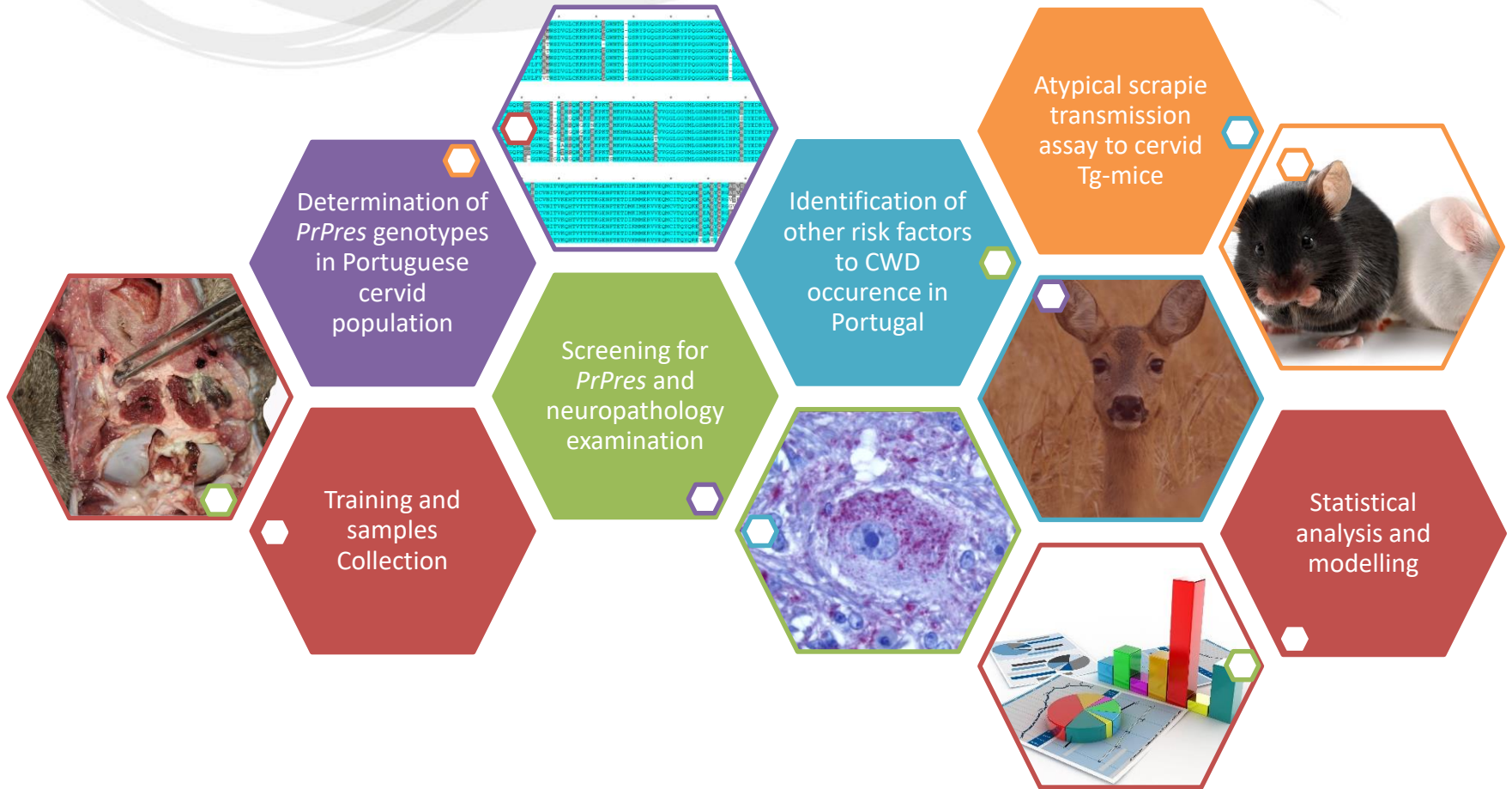
- PTDC/CVT-CVT/29947/2017, was financed by FCT (Budget: 239.897,23€)
- Started in October 2018, and it was extended until September 2022 in consequence of the COVID pandemic situation.
- Institutions:
 - UTAD (Coordinator)
 - INIAV (TSE diagnosis)
 - IPCB (Samples collection)
 and formal collaboration of INIA-Madrid (Senior Research Juan Maria Torres)



Aims

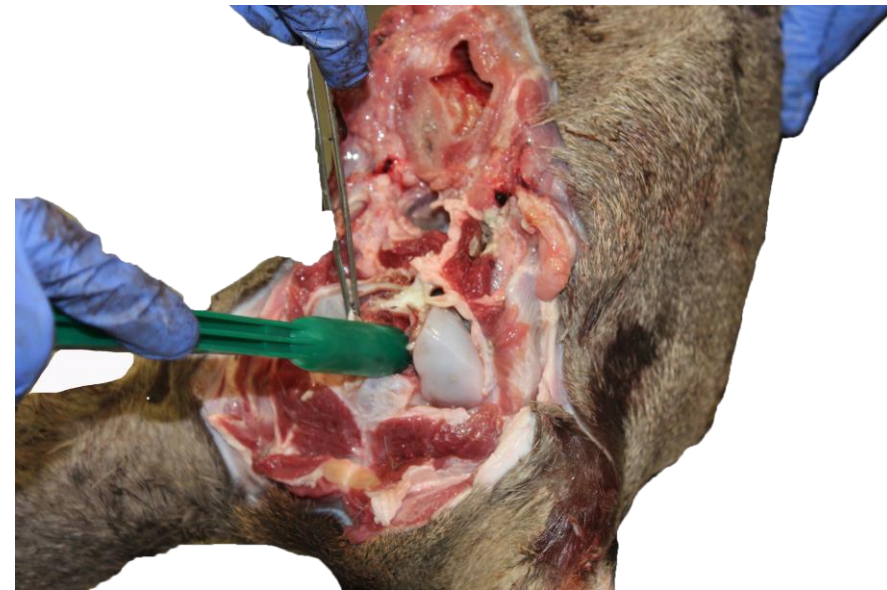


- To identify prion diseases in cervids;
- To determine the *prnp* genetic variability in the cervids population (genetic susceptibility / resistance to CWD);
- To evaluate the risk of exposure of the cervids population in Portugal to prions (CWD, BSE, Classical and Atypical scrapie);
- To increase CWD awareness among stakeholders.



Task 1: Training and sample collection

- to standardize the sampling and sample identification (brainstem, cerebellum and lymph node for PrPres screening and neuropathology; muscle for genotyping)



Task 1: Training and sample collection

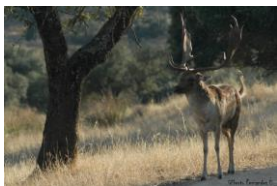
560 cervids (hunting; tuberculosis surveillance programme; found dead)



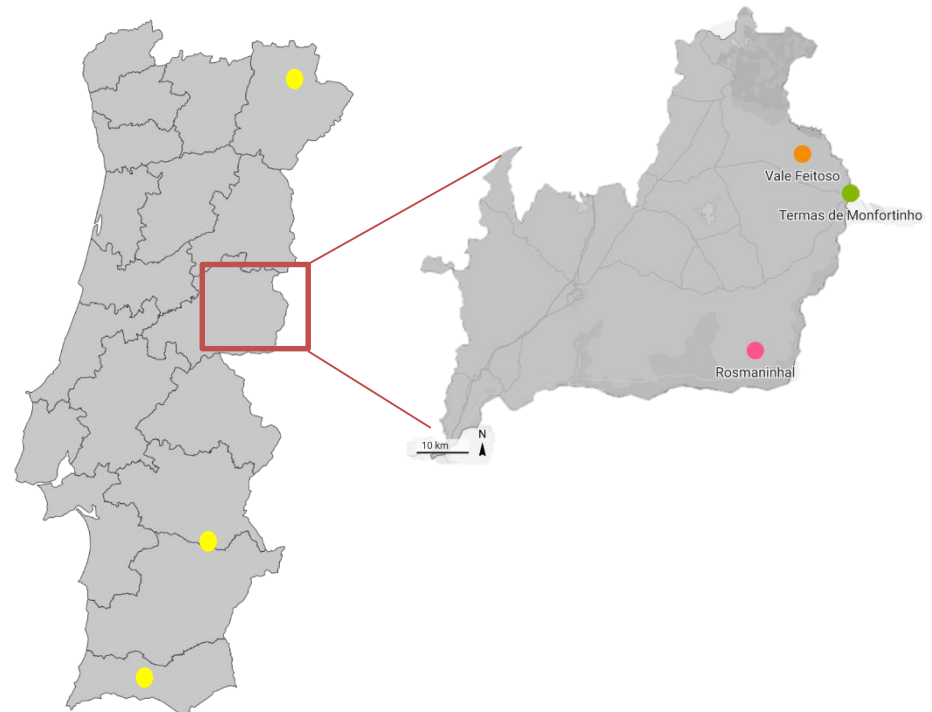
229 *Cervus elaphus*
(red deer)



258 *Dama dama*
(fallow deer)

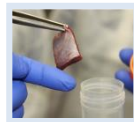
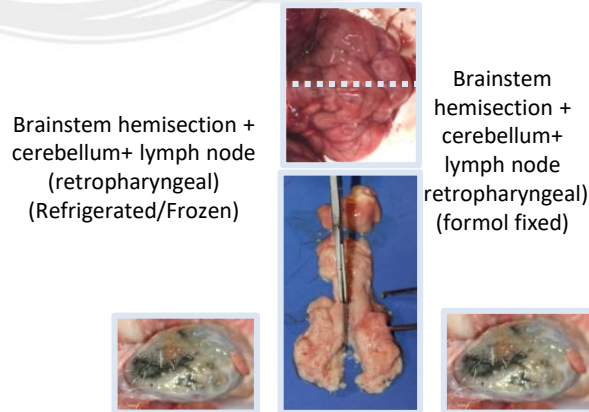


73 *Capreolus capreolus*
(roe deer)

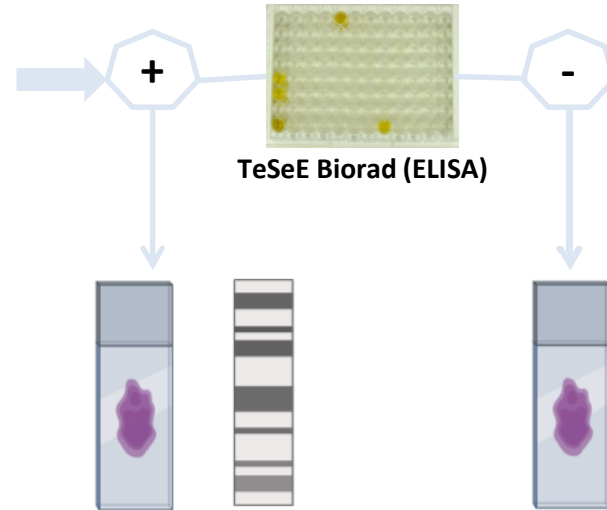


Task 1: Training and sample collection

Task 2: Screening for *PrPres* (lymph nodes and brainstem) and neuropathological examination



Masseter muscle (frozen)



Confirmatory
(histopathology/
immunohistochemistry/
Western Immunoblot)

Neuropathology

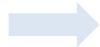
Task 3: Determination of *prnp* genotypes in Portuguese cervid population



(Picture created in biorender.com for PrionwastingRisk dissemination flyer)

Task 2: Screening for PrPres (lymph nodes and brainstem) and neuropathological examination

TeSeE[®] SAP ELISA from Bio-Rad (INIAV)



310-390 mg



210-250 mg
(cortex)



All PrPres negative

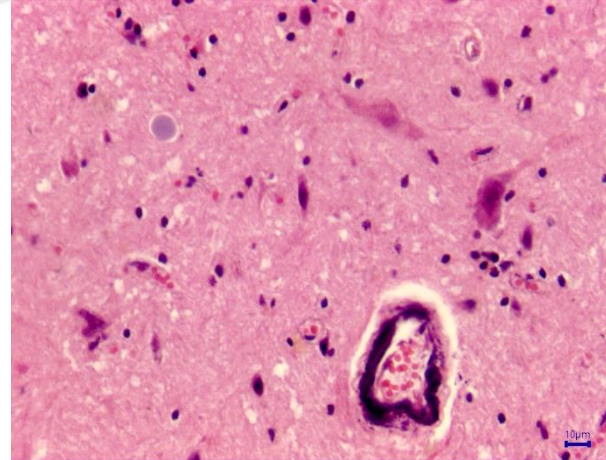


- 62% no lesions observed
- 6.4% discrete inflammation (0.3% lymphoplasmocytic menigitis associated with presence of parasitic forms)
- 0.3% choroid plexus papilloma
- 11.8% common age-related brain changes (intraneuronal lipofuscin; polyglucosan bodies and vascular mineralization)
- 19.3% unsuitable (autolysis and freezing artifacts)

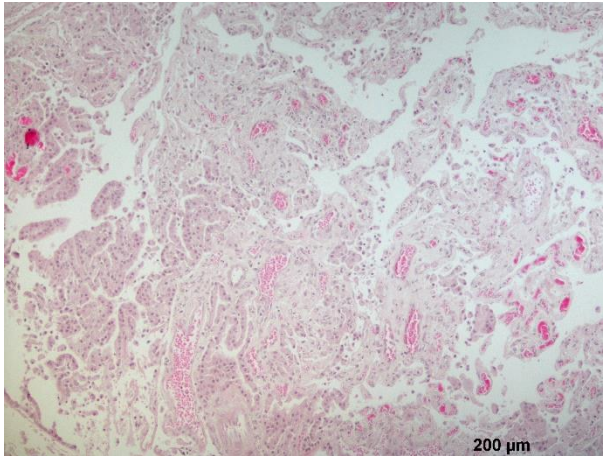
Task 2: Neuropathological examination



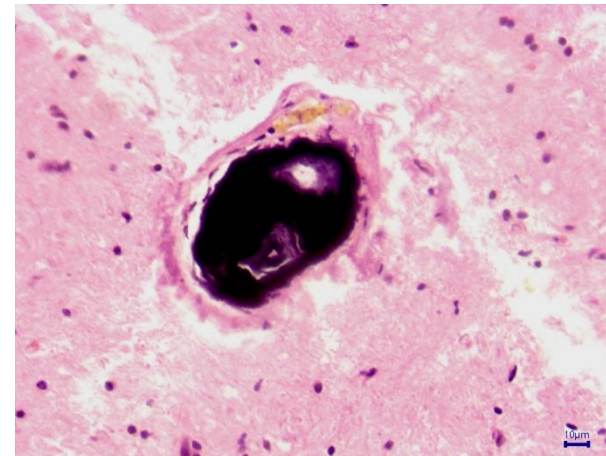
Red deer (#IPC192) (HE)



Fallow deer (#INI16) (HE)



Red deer (#IPC193) (HE)



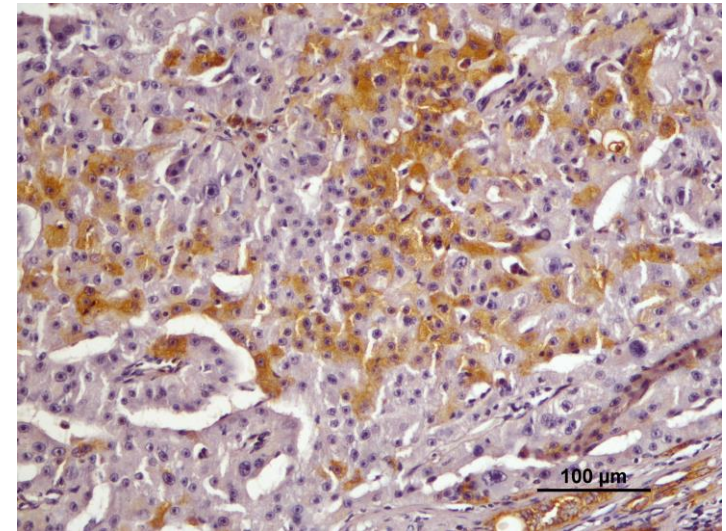
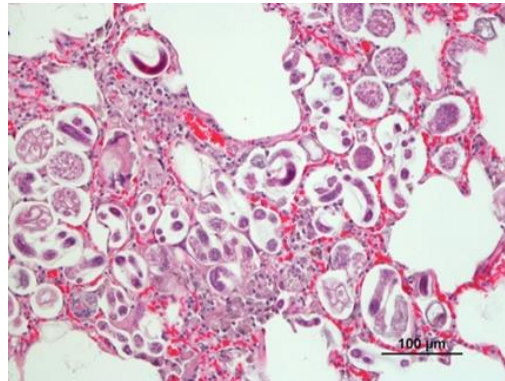
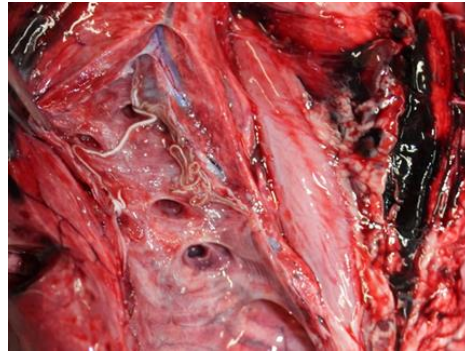
Fallow deer (#INI16) (HE)

Task 2: other lesions observed

Most cases of *Capreolus capreolus* were from necropsies

Other lesions were diagnosed:

- parasites
- traumatic lesions
- renal carcinoma.



Task 3: Determination of *prnp* genotypes in Portuguese cervid population

Genomic DNA Extraction

- ✓ Frozen Muscle and Lymphoid tissues
- ✓ NZY Tissue gDNA Isolation kit (NZYTech, Lda. - Genes and Enzymes)



PCR Amplification

- ✓ Full exon 3 of *PRNP* gene (771 bp)
- ✓ Primers F223-ACACCCTCTTTATTTTGCAG and R224-AGAAGATAATGAAAACAGGAAG)



Sanger Sequencing and Analysis

- ✓ Eurofins Genomics GmbH in Germany.
- ✓ SnapGene Viewer v. 5.1.5
- ✓ Unipro UGENE v. 40.0
- ✓ Jalview 2.11.1.4

Red deer Samples Analysis

Synonymous or
silent variation



408 [gcT/gcC]
codon A136A - Alanine



Non-Synonymous or
Non-silent variation



292 [Acc/Gcc]
codon T98A - Threonine to Alanine
676 [Cag/Gag]
codon Q226E - Glutamine to Glut. acid

Haplotypes

- ❖ T98-Q226 (TQ)
- ❖ T98-E226 (TE)
- ❖ A98-Q226 (AQ)

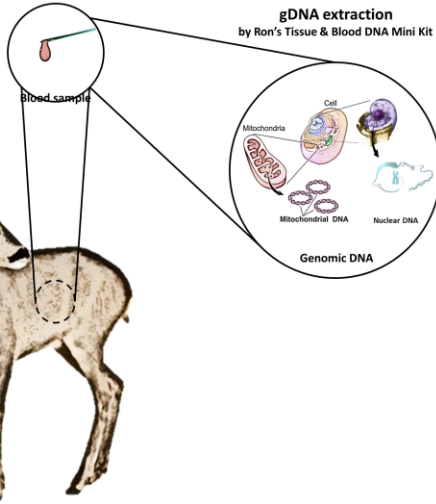
Task 3: Determination of *prnp* genotypes in Portuguese cervid population

Fallow Deer Samples Analysis



- ❖ no intra species variation
- ❖ haplotype TE with N138 of fallow deer was observed in all samples

Task 3: Determination of *prnp* genotypes in Portuguese cervid population



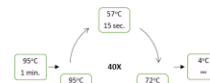
PCR amplification

mtDNA
D-loop hypervariable region I (HVI) – 436 bp

Forward primer:
L-PRO (5'-CGTCAGTCTCACCATCAACCCCAAGC-3')

Reverse primer:
H-16493 (5'-TGAGATGGCCCTGAAGAAAGACC-3')

+ 10 µL MyTaq™ HS Red Mix (2x)
+ 7 µL H₂O
+ 100 ng gDNA
+ 1 µL Primer L-PRO (100 ng/µL)
+ 1 µL Primer H-16493 (100 ng/µL)

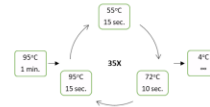


nDNA
Prnp gene – 830 bp

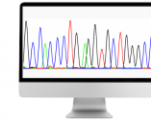
Forward primer:
223 (5'-ACACCCCTCTTATTGGCAG-3')

Reverse primer:
224 (5'-AGAAGATAATGAAAACAGGAAG-3')

+ 10 µL MyTaq™ HS Red Mix (2x)
+ 7 µL H₂O
+ 100 ng gDNA
+ 1 µL Primer 223 (100 ng/µL)
+ 1 µL Primer 224 (100 ng/µL)



Sequencing by **euoifins** Genomics



Bioinformatics analyses

SnapGene®
v.5.3.1

Unipro **UGENE**

NEW

**Non-Synonymous or
Non-silent variation**

**(c.254G>A)
Codon G85E**

Haplotype

T98-P168-Q226 (TPQ)

Task 4: Identification of other risk factors

4.1 Imports of cervids and hunting tourism from countries with CWD

- Data requested from the Competent Authorities on:
 - import of cervids (Direção Geral de Alimentação e Veterinária, DGAV);
 - number of non-resident licenses in Portugal for the 2018/2019 and 2019/2020 hunting seasons (Instituto de Conservação Natureza e Florestas, ICNF).
- Online surveys carried out and distributed to hunting managers and hunters to assess the risk associated with hunters coming to Portugal from countries with documented cases of CWD





Instituto Nacional de
Investigação Agrária e
Veterinária, I.P.



Task 4: Identification of other risk factors

4.1 Imports of cervids



Mod. CI-005/2 (11.2022)

Task 4: Identification of other risk factors

4.1 Hunting tourism from countries with documented cases of CWD

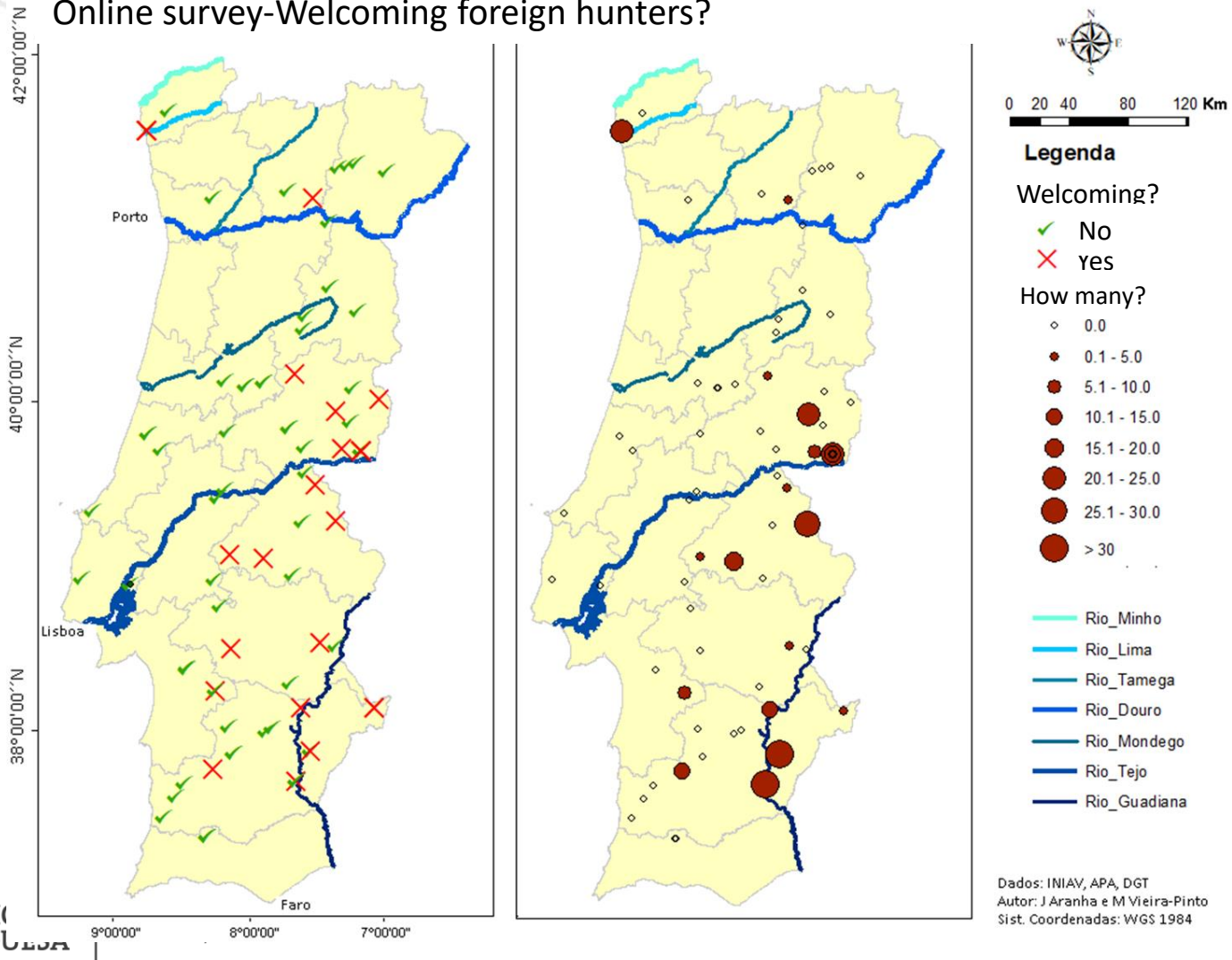
Table 1. Non-resident hunting licenses in Portugal (ICNF)

Country	USA	Finland	Norway	Sweden
Hunting Season 2018/2019 (N=2067)	12 (0,6%)	33 (1,6%)	29 (1,4%)	47 (2,3%)
Hunting Season 2019/2020 (N=1892)	13 (0,7%)	15 (0,8%)	5 (0,3%)	49 (2,6%)
Total (N=3959)	25 (0,6%)	48 (1,2%)	34 (0,9%)	96 (2,4%)

Task 4: Identification of other risk factors

4.1 Hunting tourism from countries with documented cases of CWD

Online survey- Welcoming foreign hunters?



Task 4: Identification of other risk factors

4.1 Hunting tourism from countries with documented cases of CWD

Table 2. Foreign hunters according to hunting managers (online surveys)

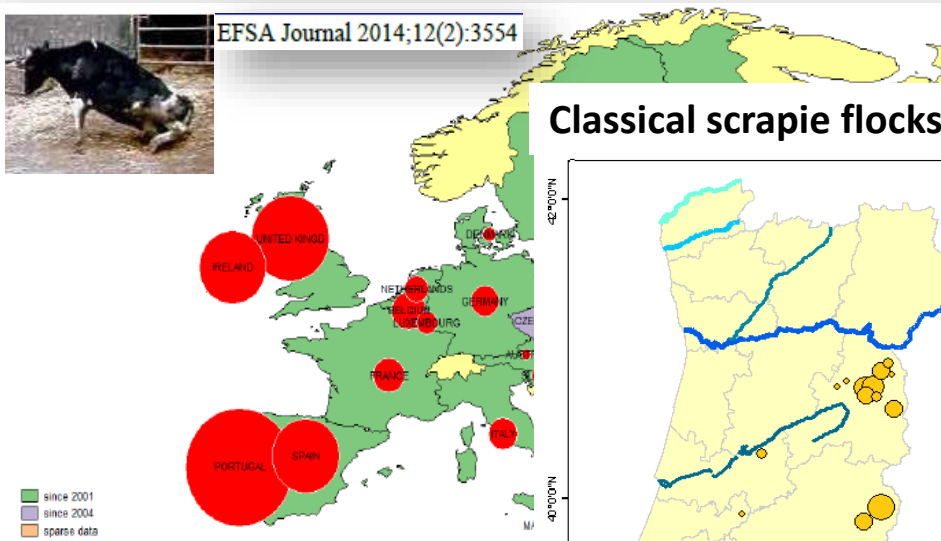
Hunting Season	Presence of foreign hunters	Presence of foreign hunters (countries CWD)
2018/2019 (N=92)	27(29,3%)	5(18,51%)
2019/2020 (N=92)	21(22,80%)	3(14,28%)
Total (N=184)	48(26,08%)	8(16,67%)

Task 4: Identification of other risk factors

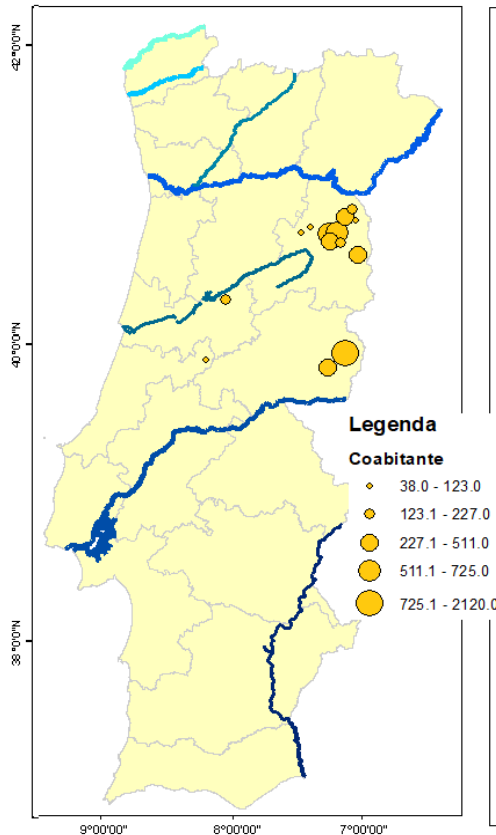
4.2 Georeferencing system to co-localize the BSE and scrapie affected farms and the geographical distribution of cervid population



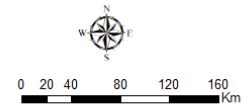
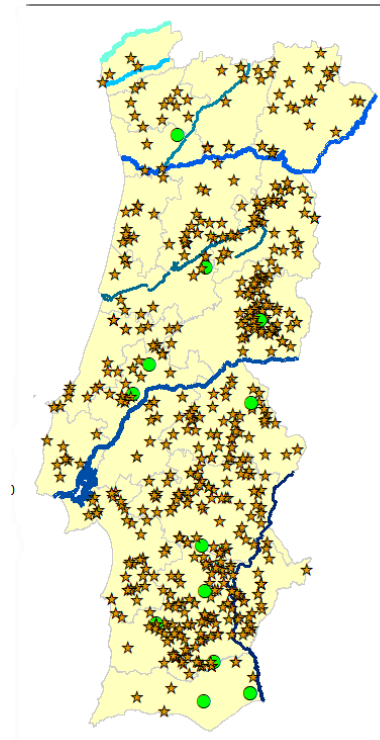
EFSA Journal 2014;12(2):3554



Classical scrapie flocks



Atypical scrapie flocks



Species

● Goat

★ Sheep

— Rio_Lima

— Rio_Tamega

— Rio_Douro

— Rio_Mondego

— Rio_Tejo

— Rio_Guadiana

Dados: INIAV, DGT
Autores: José Aranha, Madalena Vieira-Pinto
Data: 2022
Sistema de coordenadas: WGS 1894

Task 4: Identification of other risk factors

4.2 Georeferencing system to co-localize the BSE and scrapie affected farms and the geographical distribution of cervid population


 utad

 Laboratory of Fluvial and
Terrestrial Ecology (LEFT)

João Alexandre Cabral, José Aranha



cibio

U.PORTO

Nuno Mouta, Joana Vicente



cesam

 universidade de aveiro
centre for environmental
and marine studies

João Carvalho, Rita Tinoco Torres



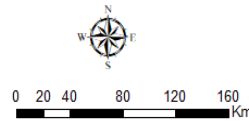
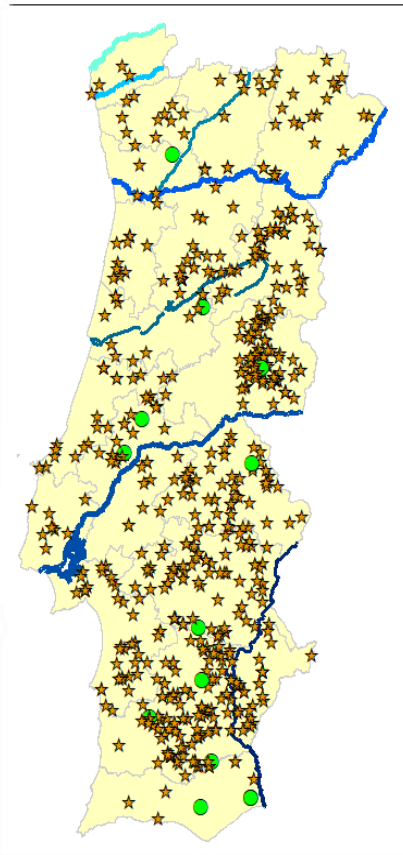
Renata Carvalho

A multidisciplinary modelling approach to analyse the risk of Classical Scrapie spill-over from small ruminants to wild cervids

(submitted to Web Ecology journal)

Task 5: Atypical scrapie transmission assay to cervid Transgenic-mice models

Atypical scrapie flocks



Species

- Goat
- ★ Sheep
- Rio_Lima
- Rio_Tamega
- Rio_Douro
- Rio_Mondego
- Rio_Tejo
- Rio_Guadiana



- 226Q-DePrPTg146
 - 226E-ElkPrPTg152
- (around 2x the level expression in deer brain)

Dados: INIAV, DGT
Autores: José Aranha, Madalena Vieira-Pinto
Data: 2022
Sistema de coordenadas: WGS 1894

Mod. CI-005/2 (11.2022)

Task 5: Atypical scrapie transmission assay to cervid Transgenic-mice models



Table 1. Inoculation of 226Q-DePrPTg146 and 226E-ElkPrPTg152 mouse lines with Chronic Wasting Disease

Chronic Wasting Disease isolate	Inoculum code	226Q-DePrPTg146		226E-ElkPrPTg152	
		MST±SD ^a (n/n ₀) ^b	PrP ^{res} signature	MST±SD (n/n ₀)	PrP ^{res} signature
CWD elk # 3	1140	244±22 (5/5)	21K	163±20 (6/6)	21K
CWD White-tailed deer # 1	1137	273±18 (7/7)	21K	185±23 (7/7)	21K

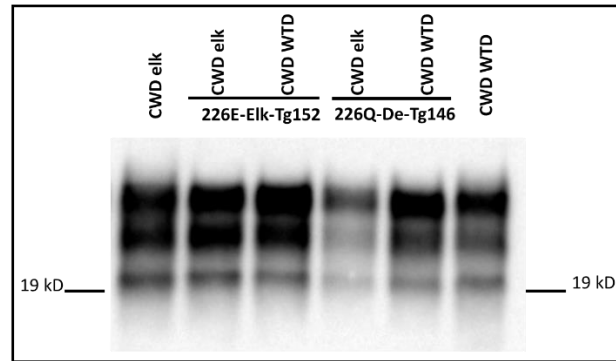


Figure 1. Electrophoretic profiles of PrP^{res} as detected by Sha31 mAb in brain extracts from elk or deer transgenic mouse lines mice infected with the prion agents indicated in the top. CWD from elk or white-tailed deer (WTD) were included as controls at the final left and right lanes respectively.

Task 5: Atypical scrapie transmission assay to cervid Transgenic-mice models



Table 2. Inoculation of 226Q-DePrPTg146 and 226E-ElkPrPTg152 mouse lines with atypical scrapie

Atypical scrapie isolate	Inoculum code	226Q-DePrPTg146		226E-ElkPrPTg152	
		MST±SD ^a (n/n ₀) ^b	PrP ^{res} signature	MST±SD (n/n ₀)	PrP ^{res} signature
Sh-Sc Atypical (07-20) [no WM labelling]	1394	>650; (7341; WB pending;)	NA ^c	>650; (7388; WB pending)	NA
Sh-Sc Atypical (12-33794) [no WM labelling]	1395	>650; (7380; WB pending;)	NA	>590; (7451)	NA
Sh-Sc Atypical (10-20882) [WM labelling]	1396	>650; (7367; WB pending)	NA	>590; (7430)	NA
Goat-Sc Atypical (10-36867)	1196	>650; (7392; WB pending)	NA	>650; (7408; WB pending)	NA
Goat-Sc Atypical (12-09106) Cerebellum	1199	>650; (7393; WB pending;)	NA	>650; (7410; WB pending)	NA

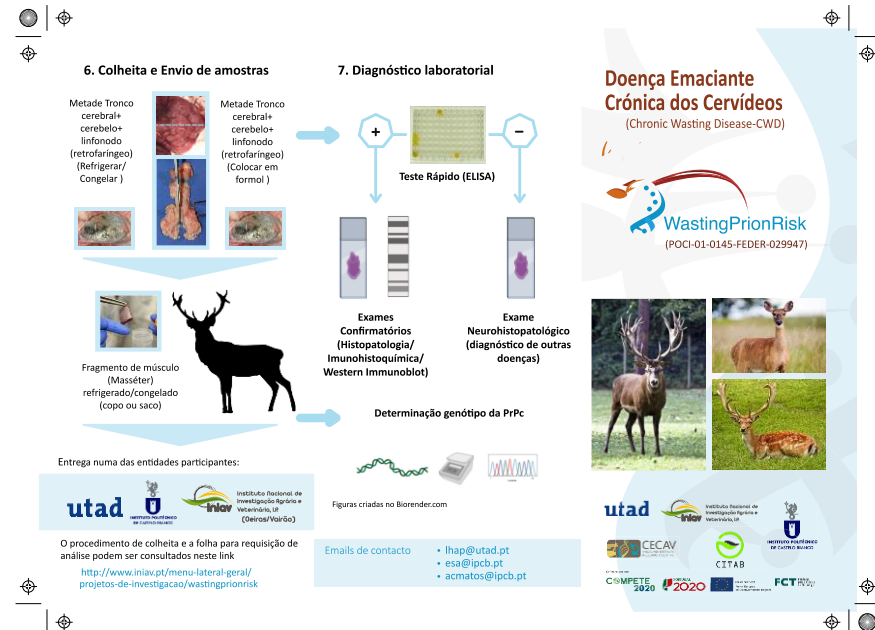
^aMean survival times in days post-inoculation ± standard deviation.

^bAttack rate where “n” is the number of PrP^{res} positive animals by WB and “n₀” is the total number of inoculated animals.

^cNA: Not available.

Task 7: Dissemination of results and establishment of a collaborative work platform and a database for TSEs in cervids

- Site of the Project
 - <https://wastingprionrisk.pt/>
- Communications of the results to DGAV



Task 7: Dissemination of results and establishment of a collaborative work platform and a database for TSEs in cervids



Review

Neuropathology of Animal Prion Diseases

Leonor Orge ^{1,2,*}, Carla Lima ³, Carla Machado ², Paula Tavares ³, Paula Mendonça ², Paulo João Silva ², Maria de Lurdes Pinto ¹, Estela Bastos ⁴, Jorge Cláudio Pereira ¹, Nuno Gonçalves Adelina Gama ¹, Alexandra Esteves ¹, Anabela Alves ¹, Ana Cristina Matos ⁵, Fernanda Seixas Isabel Pires ¹, Luis Figueira ⁶, Madalena Vieira-Pinto ¹, Roberto Sargo ¹ and Maria dos Anjos Pires ^{1*}



Review

Scrapie at Abattoir: Monitoring, Control, and Differential Diagnosis of Wasting Conditions during Meat Inspection

Alexandra Esteves ^{1*}, Madalena Vieira-Pinto ¹, Hélder Quintas ², Leonor Orge ^{1,3}, Adelina Gama ¹, Anabela Alves ¹, Fernanda Seixas ¹, Isabel Pires ¹, Maria de Lurdes Pinto ¹, Ana Paula Mendonça ³, Carla Lima ⁴, Carla Neves Machado ³, João Carlos Silva ³, Paula Tavares ⁴, Filipe Silva ¹, Estela Bastos ^{5,6}, Jorge Pereira ¹, Nuno Gonçalves-Anjo ⁶, Paulo Carvalho ³, Roberto Sargo ¹, Ana Matos ^{7,8}, Luís Figueira ⁸ and Maria dos Anjos Pires ^{1*}

PRION
<https://doi.org/10.1080/19336896.2023.2191540>

RESEARCH PAPER

Estimating sequence diversity of prion protein gene (*PRNP*) in Portuguese populations of two cervid species: red deer and fallow deer

Jorge C. Pereira ^a, Nuno Gonçalves-Anjo ^b, Leonor Orge ^{a,c}, Maria A. Pires ^a, Sara Rocha ^b, Luís Figueira ^d, Ana C. Matos ^d, João Silva ^e, Paula Mendonça ^e, Paulo Carvalho ^e, Paula Tavares ^e, Carla Lima ^e, Anabela Alves ^a, Alexandra Esteves ^a, Maria L. Pinto ^a, Isabel Pires ^a, Adelina Gama ^a, Roberto Sargo ^a, Filipe Silva ^a, Fernanda Seixas ^a, Madalena Vieira-Pinto ^a, and Estela Bastos ^b



Chapter

TSE Monitoring in Wildlife Epidemiology, Transmission, Diagnosis, Genetics and Control

Carla Neves Machado, Leonor Orge, Isabel Pires, Adelina Gama, Alexandra Esteves, Ana Paula Mendonça, Ana Matos, Anabela Alves, Carla Lima, Estela Bastos, Fernanda Seixas, Filipe Silva, João Carlos Silva, Luis Figueira, Madalena Vieira-Pinto, Maria De Lurdes Pinto, Nuno Gonçalves-Anjo, Paula Tavares, Paulo Carvalho, Roberto Sargo and Maria Dos Anjos Pires

frontiers
in Bioengineering and Biotechnology

EDITORIAL
published: 20 January 2021
doi: 10.3389/fbioe.2020.638513

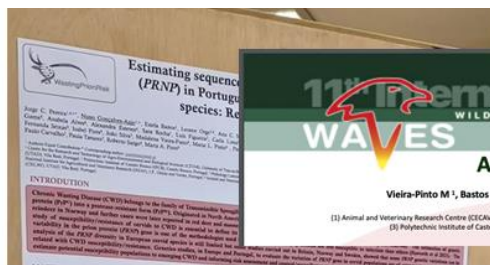


OPEN ACCESS Check for updates

Editorial: The Design of Molecular Tools in Relation to Prions and Their Biosafety

Maria Lurdes Pinto ^{1*}, Leonor Orge ², Maria dos Anjos Pires ³ and Jesús R. Requena ⁴

Task 7: Dissemination of results and establishment of a collaborative work platform and a database for TSEs in cervids



Chronic wasting disease risk assessment in Portugal - setting up a project

Orge L^{1,2}, Machado C¹, Silva J¹, Mendonça P¹, Carvalho P¹, Tavares P¹, Lima C¹, Alves A², Bastos E³, Esteves A², Figueira L⁴, Matos AC⁴, Pinto ML², Pires I², Gama A², Sargo R², Silva F², Seixas F², Vieira-Pinto M², Pires MA²

Chronic Wasting Disease – Characterization of prnp gene in Portuguese cervids

Genetic approach to the sequence of the prnp gene in Portuguese cervids species

Nuno Gonçalves-Anjo¹, Estela Bastos¹, Sara Rocha¹, Jorge C. Pereira¹, Luis Figueira², Ana C. Matos², Carla Machado³, João Silva³, Paula Mendonça⁴, Paulo Carvalho⁴, Paulo Tavares⁴, Carla Lima⁴, Anabela Almeida⁴, Alexandre Esteves⁴, Maria J. Pinto⁴, Isabel Bicho⁴, Adalina Gama⁴

Molecular Genetics Approaches

Current state of Chron

Gonçalves-Anjo, N¹; Pereira, JC¹; Bastos, E^{1*}

¹ Centre of the Research and Technology of Agro-Environmental and Biological Science

² Polytechnic Institute of Castelo Branco (IPC), Castelo Branco, Portugal

³ Animal and Veterinary Research Centre (CECAV), UTAD, Vila Real, Portugal

⁴ Pathology Laboratory, UEISPSA, National Institute for Agricultural and Veterinary I

* ebastos@utad.pt

ESVP/ECVP Poster Abstracts Exotic - Wild Life - Poultry - Fish

RENAL CARCINOMA IN ROE DEER (CAPREOLUS CAPREOLUS):
MOLECULAR AND HISTOPATHOLOGICAL STUDY OF TWO CASES

M.A. Pires¹, A.P. Silva¹, A.L. Gonçalves¹, R. Sargo¹, L. Lourenço¹, A. Radar-Chavrovitch¹, M.L. Pinto¹, F. Seixas¹, A. Gama^{1*} and E. Bastos^{1*}



Human and Animal Prion Diseases in Portugal Who is the model?

Nuno Gonçalves-Anjo¹; Jorge Pereira¹; Adalina Gama²; Leonor Orge^{2,3}; Maria dos Anjos Pires^{2,3}; Estela Bastos^{1*}

¹ CITAB (Centre for the Research and Technology of Agro-Environmental and Biological Sciences), UTAD, Vila Real, Portugal
² Animal and Veterinary I
³ Pathology Laboratory, U

ESVP/ECVP Poster Abstracts Exotic - Wild Life - Poultry - Fish

PARASITIC PNEUMONIA IN ROE DEER (CAPREOLUS CAPREOLUS):
FIRST MOLECULAR IDENTIFICATION OF DICTYOCAULUS CAPREOLUS
IN PORTUGAL

A. Gama¹, M. Castro¹, I. Pires¹, F. Seixas¹, M.L. Pinto¹, M.A. Pires¹, T. Coutinho¹, A.P. Lopes¹ and E. Bastos^{1*}

Task 7: Dissemination of results and establishment of a collaborative work platform and a database for TSEs in cervids

Abordagens evolutivas no gene *PRNP* e nas doenças priónicas

Estudo do p53 em carcinoma renal em *Capreolus capreolus*

Relatório de Estágio

Licenciatura em Genética e Biotecnologia



Ana Luísa Barreiro Gonçalves

68607

Relatório de Estágio

Licenciatura em Genética e Biotecnologia



Abordagens moleculares aplicadas à identificação de parasitas pulmonares em Cervídeos

Universidade de Trás-os-Montes e Alto Douro

Genética e Biotecnologia



Miguel António Pinho de Castro al6868

Orientadora: Estela Maria Bastos Martins de Almeida

Coorientadora: Adelina Maria Gaspar Gama

Doença Emaciante Crónica: Caracterização do Gene *prnp* nos

Cervídeos Portugueses

Análise da diversidade e estrutura genética de uma população de corço usando DNA mitocondrial

Relatório Final de Estágio

Licenciatura em Genética e Biotecnologia

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Task 7: Dissemination of results and establishment of a collaborative work platform and a database for TSEs in cervids



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Weaknesses



Representativeness of the cervid sample size to detect rare prion diseases?



Limited number of found dead cervids tested



Limited number of responses to the inquiries unabled the risk analysis;



Delay in Atypical scrapie transmission assay to cervid Transgenic-mice models;



Delay in publishing some results;

Conclusions

- ✓ Prion diseases in cervids still not diagnosed in Portugal;
- ✓ There is *prnp* genetic susceptibility of Portuguese cervids to prion diseases;
- ✓ New *prnp* polymorphism identified in roe deer (G85E);
- ✓ Potential risk of CWD introduction by hunting tourism;
- ✓ Recommendation for more complete records on the geographical area of hunting activity from non-residents in Portugal (better analysis of the risk associated with the entry and spread of infectious diseases);
- ✓ Model-based spatial analysis applicable to other diseases and different wild species



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