



EIAV epidemiologic Report in Portuguese feral Garranos populations

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Dissertação de Mestrado em Contaminação e Toxicologia Ambientais.

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Santos Silva Carvalho

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To my nephews and Family

“Anybody who has been seriously engaged in scientific work of any kind, realizes that over the entrance to the gates of the temple of science are written the words: ‘You must have faith.’”

Max Planck

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Abstract

The Garranos are an endangered horse breed currently with approximately 2000 animals distributed in five major feral populations located in Minho and Trás-os-Montes regions of the North of Portugal. Of the five populations two are located in the Viana do Castelo district, Arcos de Valdevez (AVV) and Viana do Castelo (VC); two at the Braga district, Terras do Bouro (TB) and Vieira do Minho (VM); and finally, one located at Vila Real district, Montalegre (MONT).

The Associação de Criadores de Equinos de Raça Garrana (ACERG) initiatives started in 1995. ACERG activities begin with a Census of feral small horses that resemble the description of Garranos reported by Ruy de Andrade (D'Andrade, 1938) in the article entitled 'Garranos' of Bolletim Pecuário in 1938. Later, in 1997 ACERG started the Garrano Studbook based on the previous data collected during 1995/97. In 2009 the compilation of all information in a digital database allowed a more easy access to field data of all Garranos populations collected during the last 15 year of ACERG action. In this thesis we first characterize the Garranos populations by making use of the information compiled in the digital database of Garranos.

The disease Equine Infectious anaemia (EIA) was first described in the 19th century (Lignee, 1843), but its etiologic agent, the Equine Infectious anaemia virus (EIAV), was only identified in the 20th century, first as a "retravirus" (Charman et al., 1976) and later as a lentivirus (Gonda et al., 1978; Parekh et al., 1980).

In the 20th century several epidemiological studies revealed a worldwide distribution of EIAV (Johnson, 1976), and the infection process was associated with blood feeding insects (Hawkins et al., 1973; Hawkins et al., 1976; Issel & Foil, 1984), figuring EIAV in the OIE List of notifiable diseases for it's socio-economical and animal health importance.

OIE disease control policies dictates that all the equines transferred between different countries should present an EIAV seronegative test, and that seropositive animals should be quarantined or euthanized. Due to the in force OIE control policies, several EIAV tests are performed every year in Portugal. To date there are no OIE reports of EIAV in Portugal.

A recent EIAV epidemiological study performed in the aim of the PhD thesis of Fidalgo-Carvalho (Fidalgo-Carvalho, 2008) revealed the presence of the EIAV infection in Portugal by using EIAV tests different from the OIE official tests. Fidalgo-Carvalho performed the EIAV epidemiological study in only two of the five Garranos populations. More specifically in two feral Garrano populations AVV and VC. In this project we aimed to perform an EIAV epidemiological study in the five populations of garranos: VC, AVV, VM, TB, and MONT.

Thus, in the first part of this thesis a detailed characterization of the Garranos populations was performed and in the second part an EIAV seroprevalence study was conducted in the characterized populations.

This study confirmed previous results from Fidalgo-Carvalho and demonstrated the presence of endemic EIAV infections in all the five Garranos populations studied.

Resumo

Os Garranos são uma raça de cavalo atualmente ameaçada, com cerca de 2.000 animais, distribuídos em cinco grandes populações localizadas na região norte de Portugal. Dos cinco núcleos, dois estão localizados no distrito de Viana do Castelo, Arcos de Valdevez (AVV) e Viana do Castelo (VC); dois no distrito de Braga, Terras do Bouro (TB) e Vieira do Minho (VM) e, finalmente, um localizado no distrito de Vila Real, Montalegre (MONT). Em 1995 iniciaram-se as iniciativas da Associação de Criadores de Raça Garrana de Equinos (ACERG). Nessa altura a ACERG iniciou um censo dos Garranos selvagens que correspondiam às características descritas por Ruy de Andrade 19 (D'Andrade, 1938). ACERG começou a livro da raça Garrana, em 1997, com base nos dados recolhidos entre 1995-1997. Em 2009, a compilação de todas as informações em um banco de dados digital permitiu um acesso mais fácil para o campo de dados de todas as populações Garranos recolhidos durante os 15 anos acção da ACERG.

A doença anemia infecciosa equina (EIA) foi descrita pela primeira vez no século 19 (Lignee, 1843), mas seu agente etiológico, o vírus da anemia infecciosa equina (EIAV), foi identificado apenas como "retravirus (Charman" et al. 1976) e mais tarde como um lentivírus (Gonda et al., 1978; Parekh et al., 1980) no século 20.

Nessa época vários estudos epidemiológicos revelaram uma distribuição mundial do vírus da EIA (Johnson, 1976), e associaram o processo de infecção a insectos hematófagos (Hawkins et al., 1973, Hawkins et al., 1976; Issel & Folha, 1984), constando na lista de doenças notificação obrigatória da OIE devido a importância sócio-econômica e para a saúde animal.

As políticas de controle OIE determinam que todas as transferências de equinos entre os diferentes países devem ser seronegativas para EIAV, e que os animais seropositivos devem ser colocados em quarentena ou sacrificados. Devido a estas políticas é obrigatória a elaboração de relatórios sobre o real estado de da doença EIAV em diferentes países.

Recentemente um relatório epidemiológico por Fidalgo Carvalho Fidalgo-Carvalho, (2008) revelou a presença da infecção EIAV em Portugal, mais especificamente em duas populações selvagens Garrano (AVV e VC). Neste projeto, procura-se realizar um estudo epidemiológico em cinco das populações ferais dos garranos, VC, AVV, VM, TB e Mont.

Na primeira parte deste trabalho foi feita uma caracterização detalhada das populações de Garranos e, na segunda parte um estudo de soroprevalência EIAV foi nas populações caracterizadas.

Este estudo revelou, mais uma vez a presença de infecções endêmicas EIAV em populações aparentemente saudáveis de Garranos.

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After one year of research, I would like to show my gratitude to everyone that made this thesis possible.

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To Associação de Craidores de Equinos de Raça Garrana (ACERG), I am thankful for the support in the collection of blood samples and the contact with the breeders. I am also indebted to ACERG for the availability of their database, in which all the population work was set upon. Within ACERG, I cannot forget Dr José Vieira Leite, Eng. Conceição Silva and Eng. Joao Paulo Ribeiro, for arrangement of accommodations and ideal work conditions during my stay in Vieira do Minho.

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In the end, I realize this is more from you all than me. I merely made the most of the strong foundation you provided, which supported me every step of the way and prevented me from failing. To all, the biggest "Thank you" anyone could ever say.

Abbreviations list

ACERG Associação de Criadores de Equinos de Raça Garrana

AGID Agar gel immunodifusion

AIDS Acquired Immunodeficiency Syndrome

AVV Arcos de Valdevez

BIV Bovine immunodeficiency virus

CA Capsid

dUTPase Deoxyuridine 5'-triphosphate nucleotidohydrolase

EIAV Equine Infectious Anemia Virus

ELISA Enzyme Linked Immunosorbent Assay

ERV Endogenous Retroviruses

FIV Feline immunodeficiency virus

HIV Human immunodeficiency virus

ICTV International Committee on the Taxonomy of Viruses

IN Integrase

Mont Montalegre

MA Matrix

NC Nucleocapsid

OIE Organization for International Epizooties or world organization for
animal health.

PR Protease

RT Reverse transcripase

RT-PCR Reverse transcriptase-polymerase chain reaction

SIV Simian immunodeficiency virus

TB Terras de Bouro

VC Viana do Castelo

VLPs Virus like particles

VM Vieira do Minho

WB Western blotting

Chapter I: Characterization of five feral populations of Garranos

1: General introduction: The Garrano: The Portuguese Celtic Horse and ACERG Activity

Together with the Puro Sangue Lusitano (PSL) and the Sorraia, the Garranos are one of the three autochthonous horse breeds of Portugal.

In the 18th century and beginning of 19th Century (Lima, 1867)) the distribution region of Garrano it was an extensive region from the North to the southeast of Portugal, Minho and Trás os Montes to Alentejo. But, with the industrialization of agriculture in the late 19th and during the 20th century, we assisted to a decreased economical interest in strong tow horses like the garrano. This fact, lead to a significant declining of the garrano distribution and rescue of these animals into feral populations on the mountain tops of Northern Portugal (Gouveia, 1999; Portas et al., 2001). Currently the Garrano inhabits the North region of Portugal distributed in five major nuclei located at Arcos de Valdevez, Viana do Castelo, Vieira do Minho, Terras de Bouro and Montalegre. The last three populations are located in the national natural Park, Penêda Geres

This horse breed was first characterized by Ruy D'Andrade. This author described the Garrano morphological standard like a small horses with a dark brown or bay coat and abundant mane and tail. The head is small with concave or straight profile, and white marks are an exception. They present lively eyes and small mobile ears, long and muscled neck, flat withers and straight and short back with a sloping group and set low tail. The chest is wide and deep with a well round abdomen and straight shoulders. Finally the legs are strong and solid with short cannons and broad joints and well shaped feet of tough hooves (D'Andrade, 1938).

The current ACERG breed morphological standard is very similar to the described by Ruy d'Andrade. Garranos can be described like small horses with a maximum height of 1.35 m measured at withers, with a dark brown coat, abundant mane and tail, a small head with concave or straight profile and preferably with no white marks. The chest is wide and deep with a well round abdomen, straight shoulders, strong and solid legs and hooves.

Very few characterization studies of the garrano populations have been described. The only available report refers to 2000 living specimens which fewer than 1000 are breeding mares and only 30 are breeding stallions (Portas & Leite, 2000). These numbers are a result of wolf predation and the little attention given to foals due to the low economic value of this breed.

Moreover, genetic studies showed a high level of heterozygosity in Garranos populations. The reported levels of heterozygosity were very similar to those from non-endangered breeds (Luis et al., 2006) (Morais et al., 2005). But, according to the Food and Agriculture Organization of the United Nations (FAO), the reported Garrano effective population numbers place this breed in the list of breeds classified as endangered (Galal & Hammond, 1996). The heterozygosity levels can be explained by the recent habits of crossbreeding this breed with other breeds like heavy horses breeds, for improvement of meat production (Gomes & Oom, 2000).

Celtic horse breeds are also considered historical patrimonial due to the relation with the Celtic occupation in Europe during 12th to 1st centuries BC (Index 1). Furthermore these breeds importance extends to the American continent. Recent studies have supported the historical reports that American horses descend from Iberian horses, including Celtic Horses, took by Cristovão Colombo and other explores during the XVth century A.D. (Luis et al., 2006).

In 1993 the Associação de Criadores de Equinos de Raça Garrana (ACERG) was created in Portugal, and started a recuperation program of this breed. The establishment of the zoothechnical and genealogic books where all the living specimens were registered was the first attempt to prevent the extinction of the breed and remove non primitive characteristics introduced by crossbreeding of Garrano for meat production.

In the last two decades the Garranos Association (ACERG) made a strong effort to prevent the extinction of this breed by the implementation of breeding and protection programs (Gouveia, 1999; (PRODER), 2009) appealing for the attention of the breeders to these animals through economic compensations. Moreover, the organization and integration of the Garrano horse breed in equestrian sports competition brought a new economic and cultural interest to the breed and stimulated the habit of stabling the best specimens for sportive and reproductive purposes.

During the first five years of ACERG registration program (Gouveia, 1999) all the animals that presented the morphological standard characteristics of Garranos (D'Andrade, 1938) were identified, brand marked with a 'G' on the left thigh and an identification number on the shoulder. The animals were registered and blood collected to create a DNA bank for future genetic parentage testing. The first registered horses formed the Zoothechnical Book of Garranos.

From there on all the Garrano foals were registered and brand marked in the presence of the certificated Garrano dams. Brand marks consist in a letter that designates the year of birth followed by a sequential number. Garrano names should begin by the letter of the year. The letters of the year are sequential letter that are established by

Fundação Alter Real (FAR) for all the Portuguese breeds. The letter A was first attributed to animals born in 1982. For example foals that have born in the year of 2010 have names that begin with the letter F. The Garrano marks (letter and numbers) are made on the shoulder and registered at the genealogic birth book. Parentage confirmed by genetic testing.

In this first party of the master project five of the major populations of Garranos, VC, AVV, VM, TB and MONT were characterized through data available in the ACERG ruralbit progen's database.

At the present only Garranos that are descendent from animals can be registered in the adult stud book. Exceptionally, in the last 5 years a small number of animals that presented high standard morphological characteristics entered directly into the adult book, without passing through the genealogic book. At the present no direct entry into the adult book is permitted without passing through the genealogic book.

For foals registration in the birth book is mandatory to fill in the ACERG registration sheet. This registration sheet contains parental information, month of birth, gender, breeder (owner) and location.

Foals are subjected to parental genetic test in order to confirm the testimonies of the breeder, which are usually conducted at the time of entrance in the adult stud book.

To enter into the adult stud book (aged more than 3 years old) animals should be surveyed by an ACERG technician and approved as studs. For that, a detailed description of the Garrano should be performed. The Garrano detailed description ('resenho') should be in accordance to the morphological breed standards, like height measured at the withers less than 1.35 cm and the absence of white marks. An evaluation and classification of the Garranos is also performed at the time of adult entry into stud book. After that, animal records are updated annually for last survey, owner's change or transaction, location, as well as reproductive performances and contest performances.

In 2009 the compilation of all the Garrano information in a digital database, the ACERG ruralbit progen's database. This database allowed a more easy access to field data of all Garranos populations which keeps being updated monthly based on the breeder reports and ACERG technician's information.

2: Methods

2.1: Data collection

a: Environmental data

Impact of environmental conditions in the dynamic and state of populations is well known. Humidity and temperature are known to be very important in the transmission of EIAV, because EIAV transmission vectors, horse and deer flies, are highly sensitive to these conditions. The presence of EIAV vectors has been previously reported in two of the studied populations, VC and AVV (Fidalgo-Carvalho, 2008). Thus environmental data regarding annual precipitation and mean temperature was collected from the Instituto de Metereologia Portugal to verify the existence of conditions for presence of vectors in all studied Garrano populations.

b: Population's data

In order to extrapolate the EIAV prevalence rates of the sampled animals to the real population, the estimation of the real population size was needed. A detailed characterization of the actual state of the studied populations is described, such as population size, gender, age distribution and mortality and birth dynamics. For that all the Information relative to animals belonging to the five populations, VC, AVV, VM, TB and MONT was analyzed by consulting the ACERG ruralbit progen's database.

Information considering the size of populations was obtained through count of registered animals in ACERG online database (genpro.ruralbit.com) (a), and compared to data collected by inquiry near the ACERG technicians and main breeders (b).

Only adults registered animals were considered to calculate the population size of Garranos. All juveniles were neglected due to the overestimation of their real number in the database. As there is no control of the juveniles until they enter in the adult stud book.

Garrano data of AVV, TB, VM and VC populations was collected until February 1st of 2010. MONT data was collected on 24 August 2010. Animals reported as missing animals by the breeders were considered as dead. Based on ACERG experience breeders usually report missing animals to avoid revealing the animal's cause of dead. All the few dead reports are strictly related to wolf predation as there is an economical compensation in these specific situations. Garranos owners receive an economic compensation, trough the Proder program, per each registered and surveyed Garrano.

Data collected consisted in the registration number, gender, year of birth, genealogic (birth) book registration, genealogy and paternity test confirmation, last breeding year, location and last survey date information along with height at the withers.

c: Inquiry

A direct inquiry concerning the total approximate number of animals in each population was conducted to the main breeders and to ACERG's technicians responsible for animal's surveys and marking. The inquiry results were used as comparative measures to test the database liability.

The inquiry was made in an informal way, by direct questioning and in randomly selected areas of the populations and periods of time, however only main breeders were questioned.

The question only concerned the approximate total number of the adult population, excluding all juveniles born in the last three years. Approximate number of male and females could not be obtained through this questioner.

A secondary inquiry was conducted to the ACERG technicians for acknowledgment of living conditions of the animals. A report with all stabled and semi-stabled registered animals was kindly yield by an ACERG technician. No distinguish was made between full stabled and semi-stabled animal. All stabled animals integrate the livestock group.

2.2: Data analysis

All data collected from ACERG database was analyzed with SPSS(SPSSinc., Mar 11 2009). Animals were firstly spliced by population and separately analyzed. ACERG data based on individual information was then converted into population information by count of total registered animals, males, females, deaths and births.

Active population was obtained based on breeding data and birth entries, regardless of the parentage genetic confirmation test. Thus, total active dams are equal to the birth entries of foals into the genealogic book in one year, in each population. Calculation of the total adults registered into the ACERG books

Only Garranos registered into the zootechnical book and into the adult stud book were accounted to calculate the total size of Garrano populations.

Garranos from the zootechnical book that presently aged more than 30 years old in 2010 were excluded for the calculation of the population size.

Animals reported as livestock were excluded from the real population and treated separately.

Frequencies of total livestock animals among the inside and outside of the studied populations were calculated to verify the assumption of the mainly feral state of the studied populations..

2.3 Characterization of the 'population structure

a: Gender distribution

Females and males were recounted to estimated populations followed by the count of the reproductive active animals.

Frequency rates in total population and active population as well as female/male ratios were calculated to enable comparison between populations.

b: Age Distribution

Age by population was distributed in a poisson probability curve to enable the comparison between populations of older/younger and homogeneous/heterogeneous populations.

2.4: Population dynamics

Mortality and birth rates were calculated for total population and within genders and age classes. Frequencies of adults lost in the last year, juveniles lost after three years and new entries in the adult book were also calculated for better characterization of the real population dynamic.

3- RESULTS

3.1: Environment conditions.

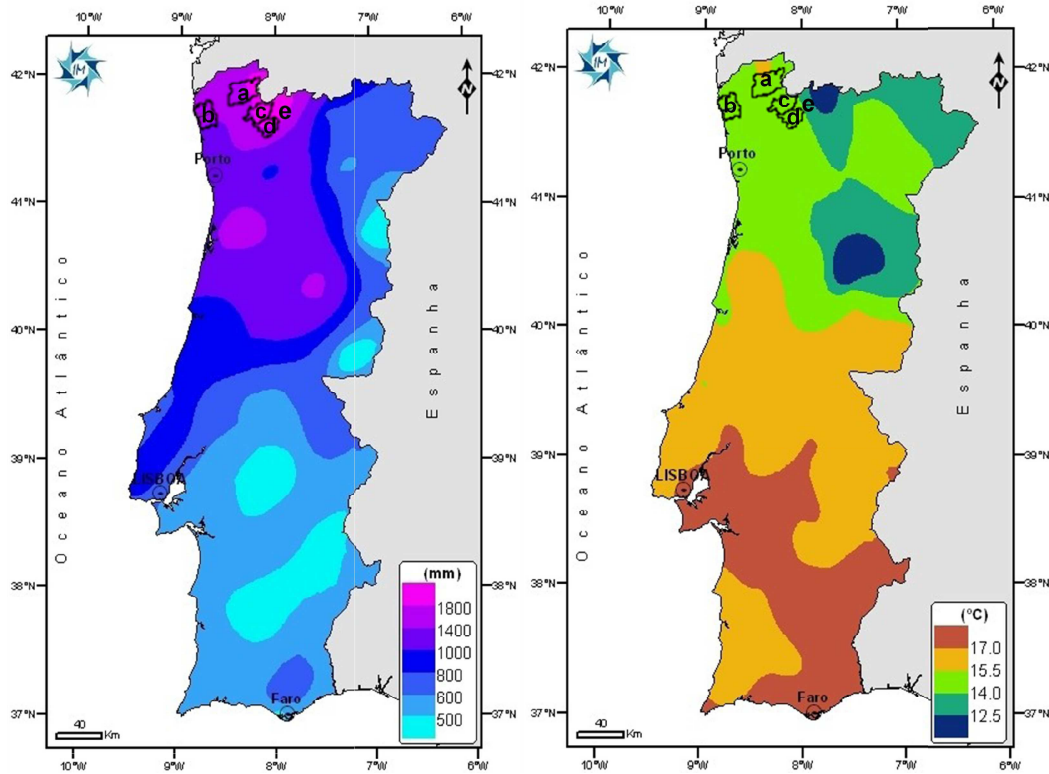


Figure 2: Maps for total precipitation (mm^3) and mean temperatures ($^{\circ}\text{C}$) for 2009 in Portugal. The four studied regions highlighted: a-Arcos de Valdevez, b-Viana do Castelo, c-Terras de Bouro, d-Vieira do Minho and e- Montalegre

The total precipitations for 2009 was similar in the studied populations, Vieira do Minho, Arcos de Valdevez and Terras de Bouro, with more the 1800 mm^3 in this year. Viana do Castelo shows a little lower total precipitation with a value between 1400 and 1800 mm^3 in 2009.

Mean temperatures in 2009 were equal to all studied populations except Montalegre and were between 12.5 and 15.5 C° , with slight lower mean in Montalgre.

3.2: Estimation of the population size of Garranos

For the estimation of the real population size two models were tested and compared to the inquiry results, a) the total number of registered animals into the adult books and b) the total number of registered animals into the stud book, c) the number of animals surveyed at least once by an ACERG technician in the last three years.

Inquiry responses were obtained at different levels, breeders and ACERG technicians. In case of highly incoherent responses preference was given to ACERG technicians responses. Results were organized in table 1. Results were then used to test

the liability of the ACERG database and for selection of the best estimation model of the real population size.

Data presented in table 1 show that the inquiry results to ACERG breeders and technicians is very similar. However, the numbers obtained by inquiring is very different from the total number of adult entries into the ACERG adult books.

Population size	Breeder 1	Breeder 2	ACERG Tech	Adult entries
Arcos de Valdevez	>400	>400	around 450	1073
Terras de Bouro	-	-	[150,200]	381
Vieira do Minho	[300,400]	around 300	[250,300]	527
Viana do Castelo	[100,150]	-	arround 100	141
Montalegre	-	-	-	530

Table 1 – Comparison of the total population size numbers obtained by inquiring Garrano breeders and technicians and the numbers obtained by the sum of total adult entries. Information regarding Montalegre population was not collected.

a) Total number of animals registered into the adult books

The total number of animals registered in the adults books consist in the sum of the number of adults present into the zootechnical book and the number of adults present into the adult stud book, the genealogic book. Results show in table 1 a clear overestimation of the population size based on the number of adult entries into ACERG books and the numbers obtained by the inquiry of ACERG breeders and technicians.

b) Total number of animals registered into the stud book

Population	Total registered adults	ACERG results	Inquiry	Stud registered	Book
Arcos de Valdevez	1073	450		353	
Terras de Bouro	381	[150,200]		99	
Vieira do Minho	527	[250,300]		201	
Viana do Castelo	141	100		53	
Montalegre	530	-		125	

Table 2. Comparison between the different methods used to estimate the total population size of Garrano populations. Inquiry not conducted in Montalegre region

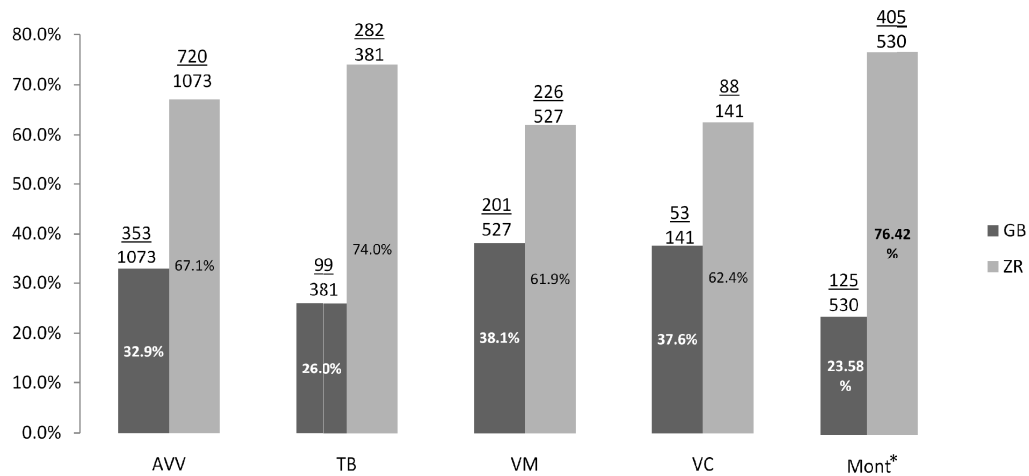


Figure 1 - Percentage of garranos present in the adult book that entered through the genealogic book (GB, the stud book) versus the direct entries in the Zootechnical book (ZR). AVV –Arcos de Valdevez, TB – Terras de Bouro, VM – Vieira do Minho, VC – Viana do Castelo, Mont – Montalegre

c) Total number of animals surveyed by ACERG in the last three years.

Data from the interval of days between surveys for all studied females was collected from ACERG database. The mean interval of days between surveys was then calculated with SPSS (table 3). All animals last surveyed for more than this mean value were considered lost of track.

	N	Minimum	Maximum	Mean	Std. Deviation	Variance	Approx. Years
interval between surveys	1140	262	4011	902.24	598.428	358115.684	3

Table 3 - Mean of days between surveys for all registered studied females through the last 15 years of ACERG activity. The mean of day's interval between surveys is 902 which approximates to 3 years.

This model establishes that the real population is constituted by all animals that had been surveyed at least in 2007, three years ago.

Table 4 shows the comparison between the estimation of the population size obtained by calculating all the adult entries into the ACERG books (adult entries), the adults surveyed in the last 3 years by ACERG actions and the inquiring results to ACERG technicians. Data demonstrated that the population size obtained by the number of surveyed animals since 2007 is very similar to the results obtained by inquiry to ACERG technicians.

Population size	Adult entries	Adults Surveyed	ACERG inquiring results
Arcos de Valdevez	1073	441	450
Terras do Bouro	381	167	[150,200]
Vieira do Minho	527	272	[250,300]
Viana do Castelo	141	95	100

Table 4 – Real population size estimated by adult entries, adults surveyed in the last 3 years, and the inquiring results based on ACERG technicians.

3.3: Mode of leaving: Feral versus livestock state

The original region of Garranos Arcos de Valdevez (AVV), Vieira do Minho (VM), Terras de Bouro (TB), Viana do Castelo (VC)) and Montalegre (Mont) has a total of 2741 registered horses (Fig 3).

Of these 2741 animals only 152 animals are in livestock conditions, which corresponds to 5.3% of the total population on the natural Garrano region. All the other Garranos, 2589 animals corresponding to 94.7% of these populations are maintained in a

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feral state at the mountain tops of these regions. Most of the livestock animals, 58,6%, which corresponds to 89 animals, are found on the expansion region , outside of the four populations studied (Fig 4).

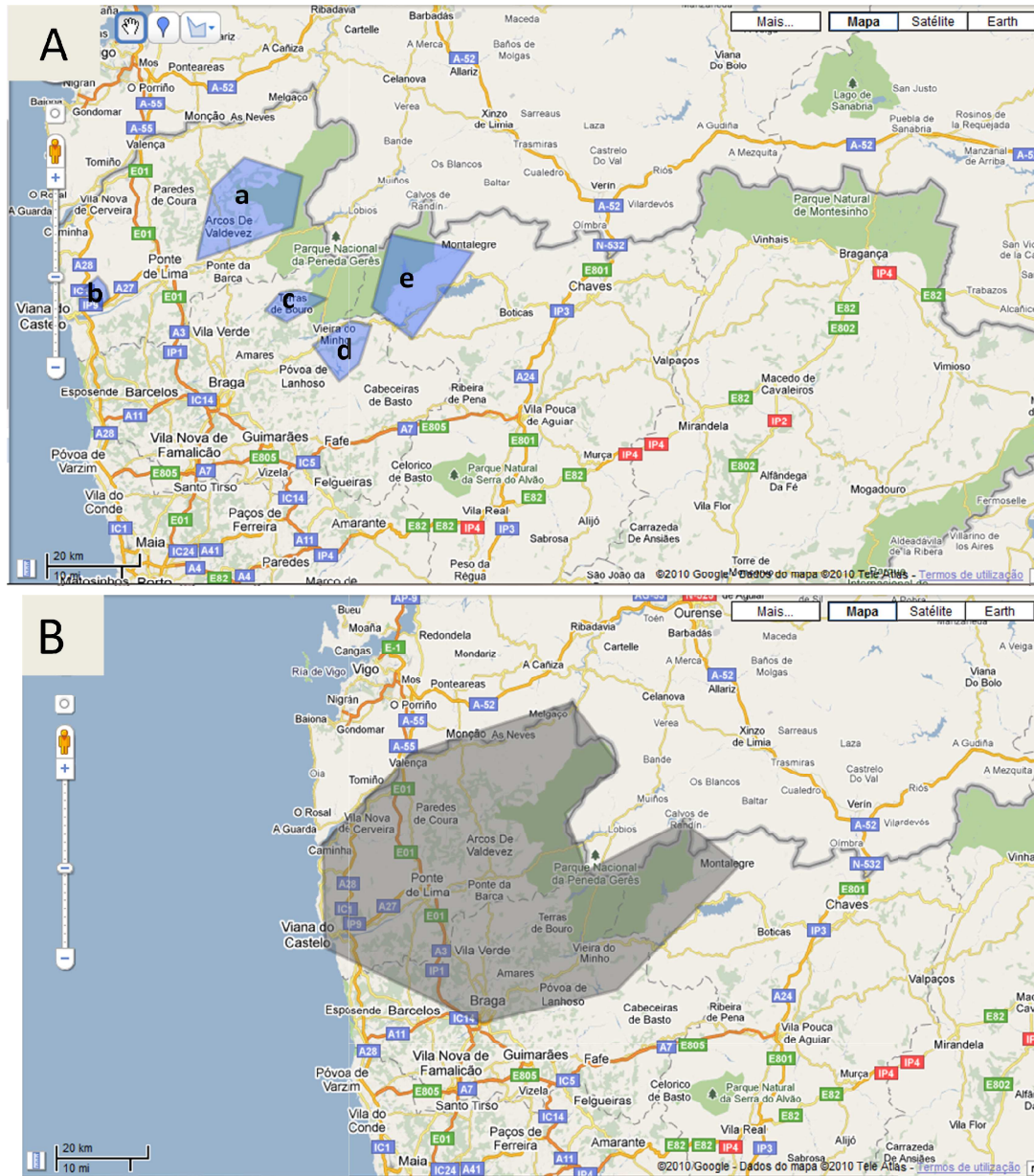


Figure 3: Original (A) and Expansion (B) region of Garranos from the five original populations. a – Arcos de Valdevez, b – Viana do Castelo, c- Terras de Bouro, d- Vieira do Minho and e – Montalegre.

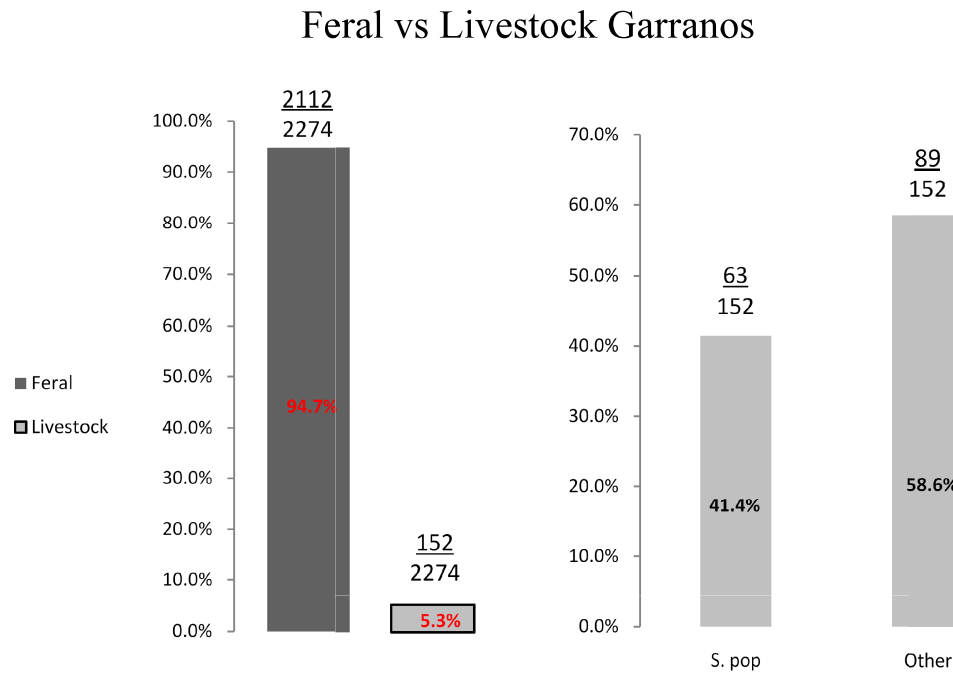


Figure 4 – A: Distributions of the Garranos accordingly livestock versus feral mode state of living. B: Comparison between the number of livestock and feral garranos at the original (natural) and the expansion region.

4: Characterization of Livestock Animals

The five populations studied have low percentages of livestock animals. In Viana do Castelo and Montalegre there are no reports of Garranos living in livestock conditions. In Terras do Bouro only 3% (5 animals) of the animals are maintained stabled. Arcos the Valdevez (AVV) and Vieira do Minho (VM) have less than 10% of stabled animals, with 8,6% (38 animals) for AVV and 7.4% (20 animals) for VM respectively. Outside this populations 10,8% of the animals are in livestock conditions.

The distribution by gender of livestocked animals showed that in Arcos de Valdevez (AVV), Vieira do Minho (VM) 84.2% (32 animals) and 70% (14 animals) were mares. But, in Terras the Bouro 60% of livestocked animals are stallions (3 out of 5 livestocked animals).

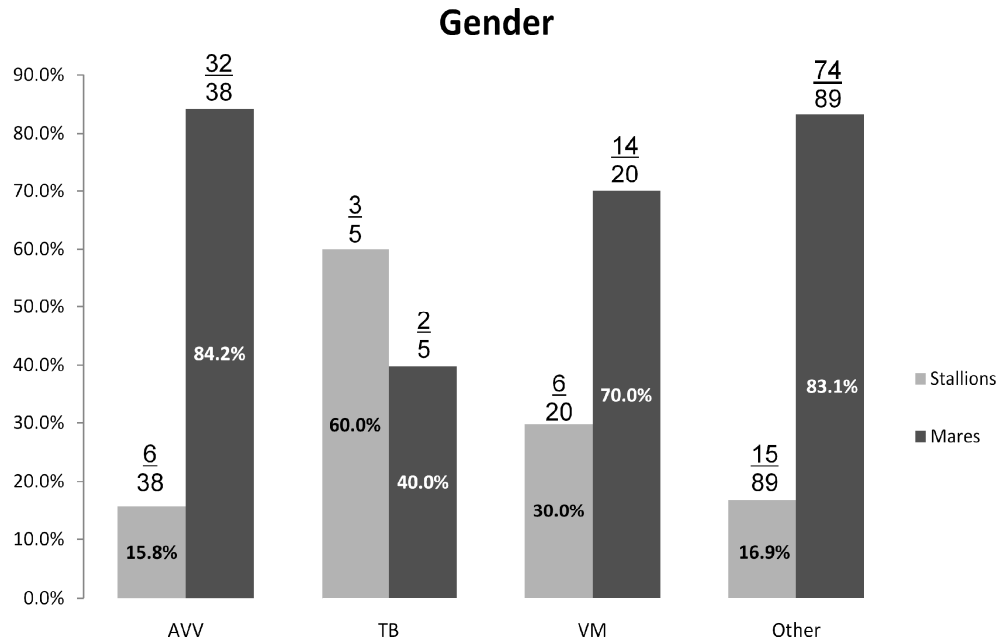


Figure 5 - Distribution by Gender of livestock animals from the four studied populations. VV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont – montalegre and other - all other registered animals not included in populations studied.

5: Calculation of population size of Garranos at the feral state

Considering population size, and after the adjustments previously described in Methods, it's observed that Arcos de Valdevez is the biggest population with a total of 411 animals, followed by Vieira do Minho, Montalegre and Terras de Bouro with 272, 217 and 167 checked animals respectively. Viana do Castelo is the smallest population with a total of 95 animals.

However considering the percentages of surveyed animal in the last three years for the total of registered animals, it is observed that Viana do Castelo is actually the better represented by ACERG database, with 67% of the registered animals being checked in the last three years. The other three populations had less than half of the registered animals surveyed, Vieira do Minho 47,8%, Terras do Bouro 43,8% and Arcos de Valdevez 41,1%.

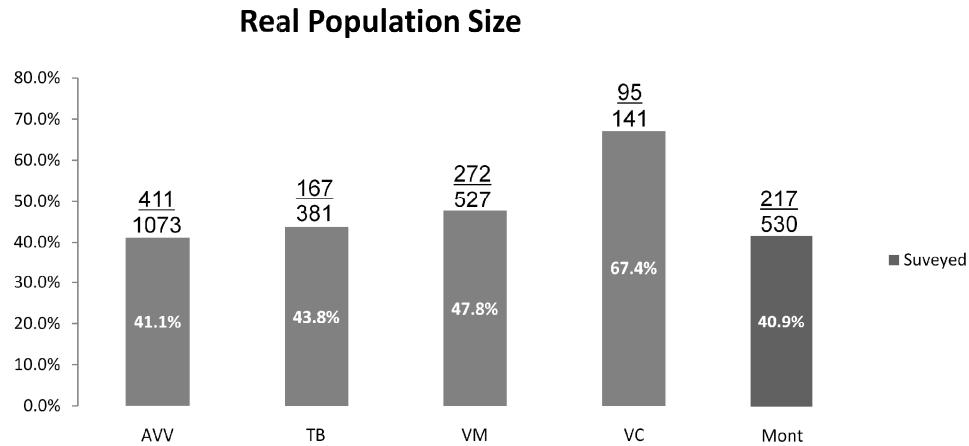


Figure 6 - Distribution of Surveyed animals from the five major populations AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont – montalegre.

6: Characterization of feral populations in 2009.

a: Gender

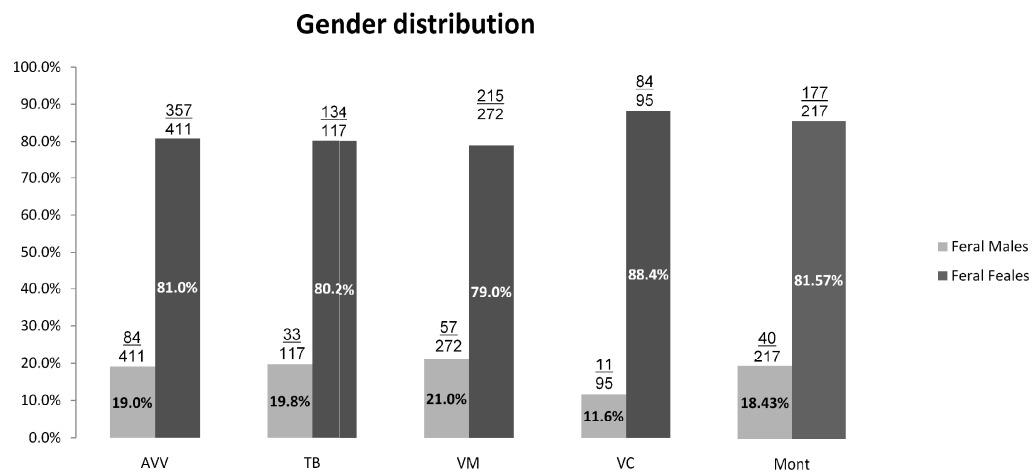


Figure 7 - Gender Distribution of animals belonging to the four studied population AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont - montalegre

The distribution by gender reveals similar results for every population with almost 80% of females. Viana do Castelo is the populations with higher percentage of females, 88,4%(84 out of 95 animals) . On the other hand Vieira do Minho is the population with highest percentage of males, 21% (57 out of 272 animals).Montalegre,

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Arcos de Valdevez and Terras de Bouro are not far from this values with 18.43% (40 out of 217), 19.0% (84 out of 411) and 19.8% (33 out of 117).

Nucleus	Surveyed	Active population	Feral Males	Active Males	Feral Females	Active Females
Arcos de Valdevez	441	272	84	47	357	225
Terras de Bouro	167	117	33	23	134	94
Veira do Minho	272	138	57	33	215	105
Viana do Castelo	95	83	11	7	84	76
Montalegre	230	164	40	26	177	138

Table 5 – Count of total feral and active population, males and females for the four Populations.

b: Active population

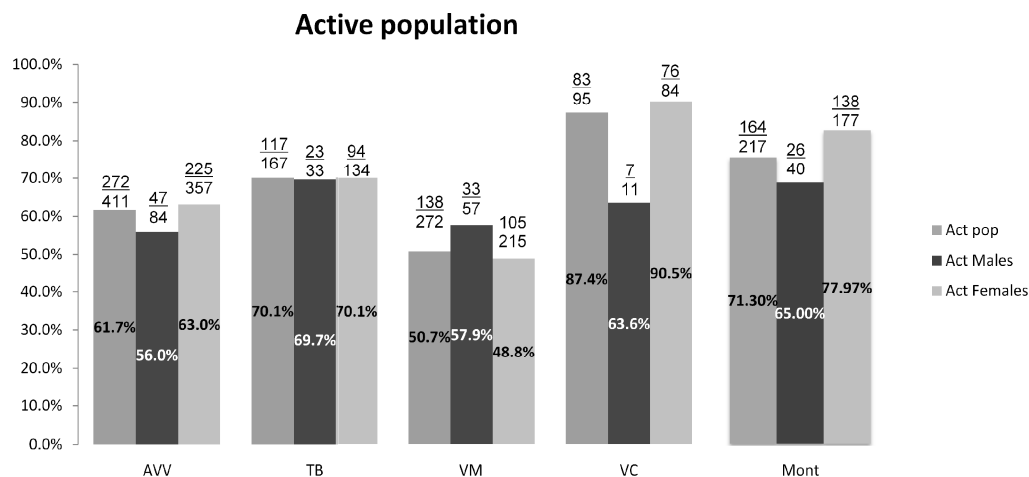


Figure 8 - Frequency of active animals of the studied nucleus: AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont - montalegre

All nucleus show high percentages of active population, and coherently distributed between gender. Vieira do Minho has a slightly lowest frequency of active population, with 54.8%, comparing to the 61.7%, 70.1%, 71.3% and 87.4% of Arcos de Valdevez, Terrasde Bouro, Montalegre and Viana do Castelo respectively.

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Considering active males and females frequencies Viana do Castelo has an unusual high frequency of active females (90,5%), specially when compared with frequency of active males (63.6%). In all other population this values are not discrepant. In this matter Terras do Bouro is particularly coherent with less than 5% difference between Active males (69.7%) and females (70.1%) frequencies. All other populations, except Vieira do Minho, present a slight higher frequency of active females,

c: Age Classes

Calculation of mean ages in the populations shows that Montalegre and Arcos de Valdevez are the older population with 14.28 (std deviation of 4.782) and 14.26 years (Std deviation of 4.795) and Vieira do Minho and Viana do Castelo as the youngest ones with 12.44 years (std. dev. 5.034) and 12.14 (std dev. 4.701) respectively. Terras do Bouro is in the middle with 13.35 years (std. Dev. 4.701).

Nucleus		[3-6[	[6-9[	[9-12[	[12-15[	[15-18[	[18-21	[21-24[	>24	Total
Arcos de Valdevez		12	61	106	85	102	46	20	9	441
Terras do Bouro		11	29	38	37	23	20	6	3	167
Vieira do Minho		25	54	63	58	27	23	17	5	272
Viana do Castelo		9	25	20	15	14	11	1	0	95
Montalegre		16	27	26	47	47	28	0	26	217

Table 6 - Counting of surveyed adult animals (>3) by age classes and by population. Age classes are presented in 3 year intervals until 24. Animals over 24 are grouped in a single class.



Figure 9 - Distribution accordingly to age classes of the adult surveyed animals from the studied populations. AVV – Arcos de Valdevez, TB- Terras de Bouro, VM - Vieira do Minho, VC- Viana do Castelo, Mont – Montalegre. Age classes of 3 years intervals.

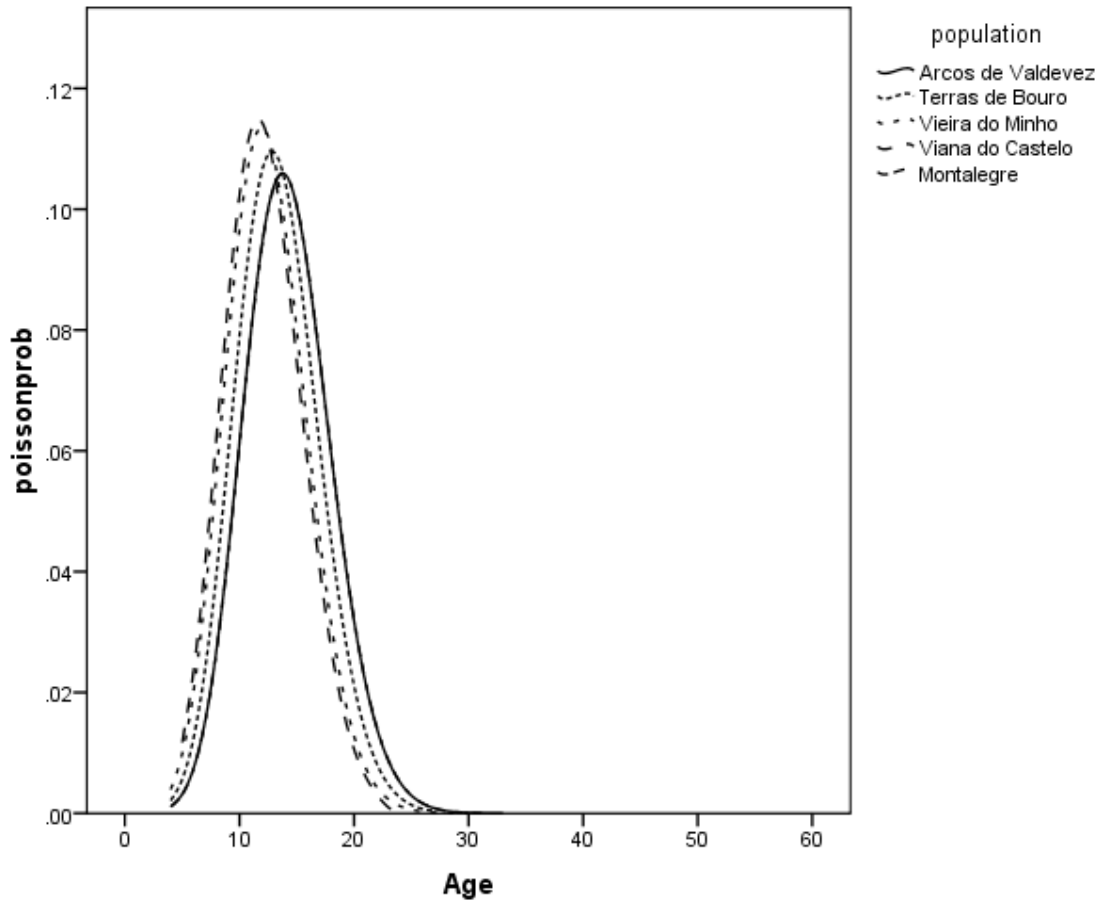


Figure 10 - Poisson distribution of animals by age.

The distribution by age classes among different populations shows that all populations are mainly constituted by animals between 6 and 15 years. Only Viana do Castelo shows a maximum frequency for the class between 6 and 9 years old, all the other populations have this maximum point for the class of 12 to 15 years old. Arcos de Valdevez also shows a second maximum point for the class 15 to 18 years after a slightly decrease for the 12 to 15 years class in the middle. The Poisson distribution of the age by population clearly indicate that Montalegre and Arcos de Valdevez are the oldest population with two overlapped curves, opposing to Viana do Castelo and Vieira do Minho which show very similar distribution curves. Terras do Bouro is somehow in the middle like predicted from the mean age.

Poisson distribution curves of animals by age do not show any difference between gender in any of the studied populations as female and male curves are perfectly overlapped in all four studied populations (fig 11).

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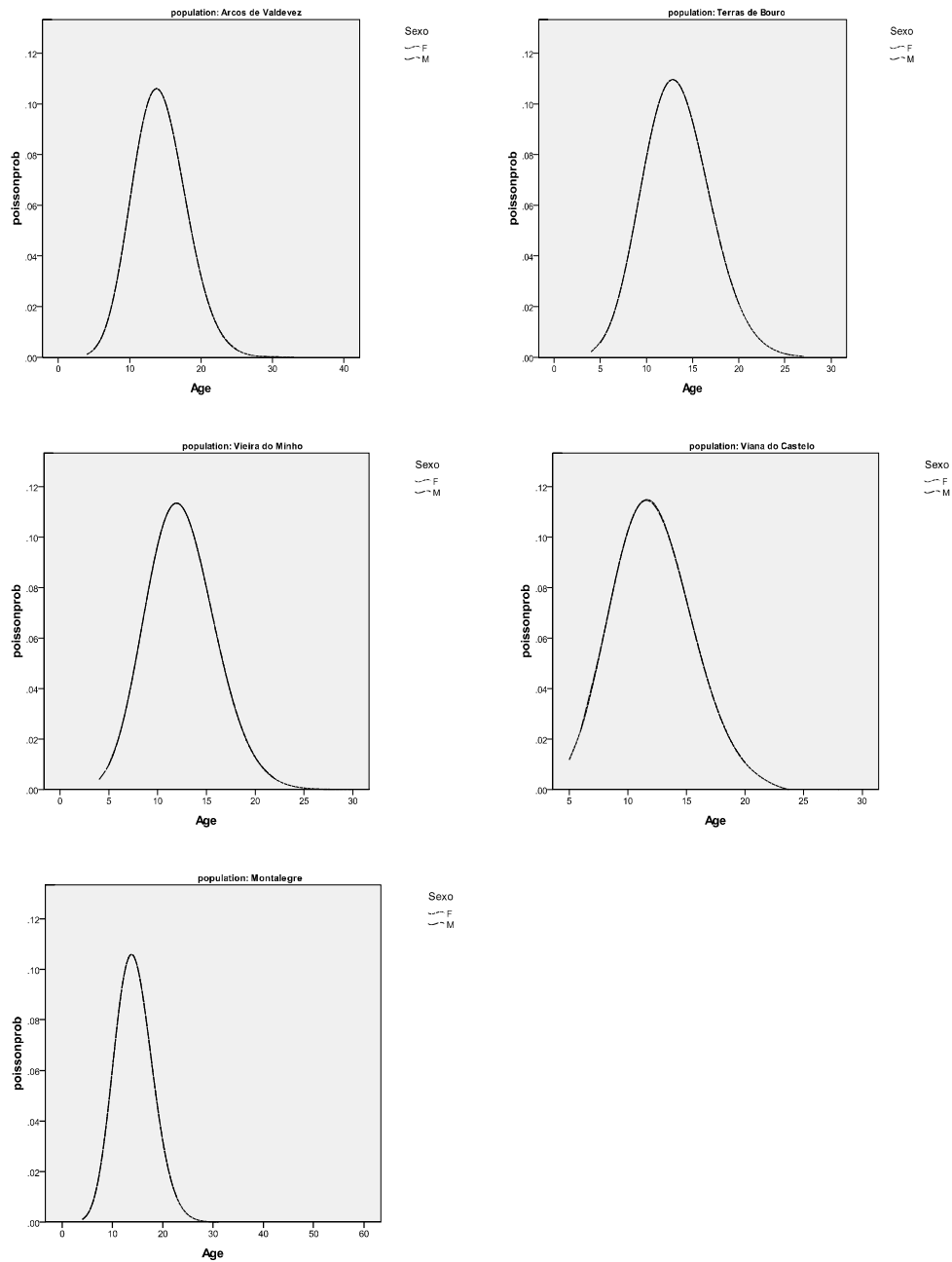


Figure 11 - Age and gender distribution of the different Garrano populations (Arcos de Valdevez , Terras de Bouro, Vieira do Minho, Viana do Castelo and Montalegre (mont) M-Males, F- Females.

7: Population dynamics

la: Adult entries in 2009

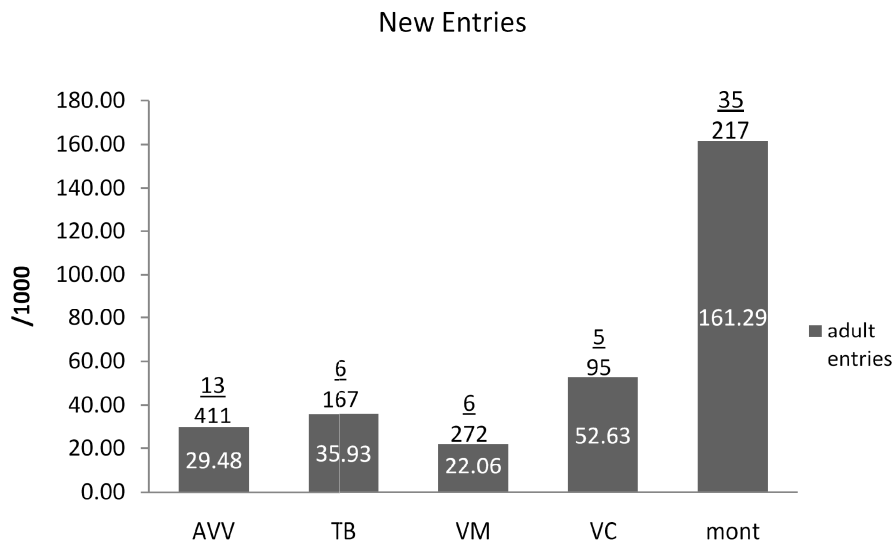


Figure 12 - frequency of juveniles that reach adult age (3 years) in 2009 (adult entries) (B). AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont - montalegre

Respecting the number of young juveniles that achieved to adult in 2009, Montalegre shows the best results with 161.29 animals per each thousand animals in the population. On the other side Vieira do Minho has the worst value with only 22.06 animal per every thousand reaching the adult state. In the middle are Viana do Castelo, Arcos de Valdevez, and Terras de Bouro with 52.63, 29.48 and 35.93 per thousand animals.

b: Mortality and Birth rates

Population		Checked	Mort	Birth	Males Mort	Males Birth	Females Mort	Females Birth
Arcos de Valdevez	de	441	12	225	4	88	8	137
Terras de Bouro	de	167	5	94	0	36	5	58
Vieira do Minho	do	252	4	105	1	38	3	67
Viana do Castelo	do	95	1	84	0	35	1	49

Montalegre 217 2 138 0 67 2 71

Table 7 - Counting of checked animals, deaths, births in 2009 by population. Checked - animals surveyed and considered the actual population, Mort – animals reported dead in 2009, Birth - animals born in 2009, Males Mort - male animals reported dead in 2009, Males Birth – male animals born in 2009, Females Mort – females animals reported dead in 2009, Females Birth - females born in 2009.

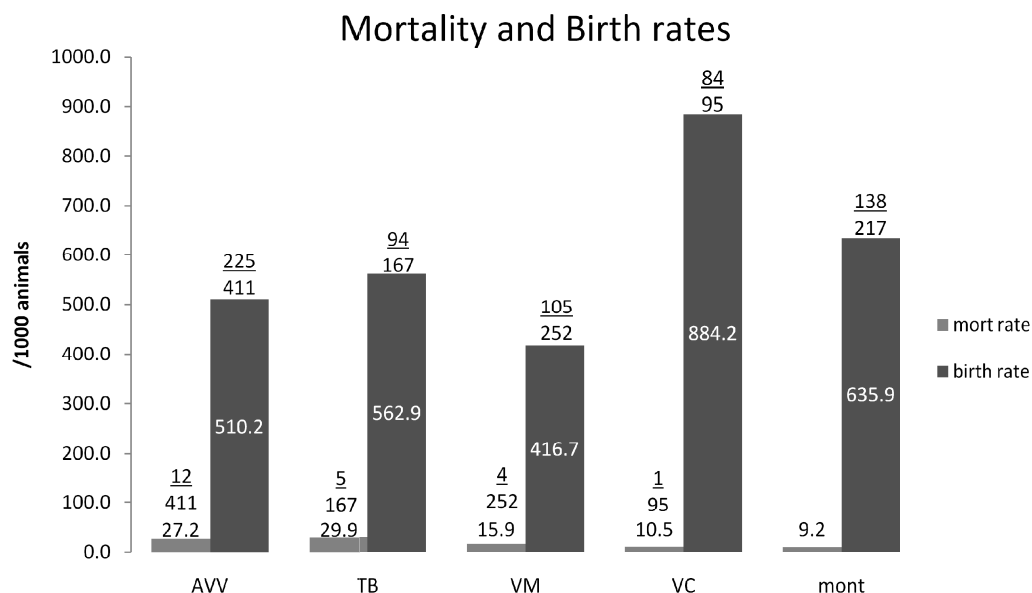


Figure 13 - Mortality and birth rates in the four studied populations: AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont - montalegre

Mortality and birth rates of 2009 are highly disproportional. The highest birth rates, Viana do Castelo and Montalegre ,884.2 and 635.9 animals per each thousand, contrasts with the lowest mortality rates of 10.5 and 9.5 animals per thousand. On the other hand Vieira do Minho, with the lowest birth rate of 416.7 per thousand, has a mortality no higher than 15.9 animals per thousand. Arcos and terras do Bouro and montalegre present the higher mortality rates with 27.2 and 29.9 , and birth rates of 510.2 and 562.9 per each thousand animals respectively.

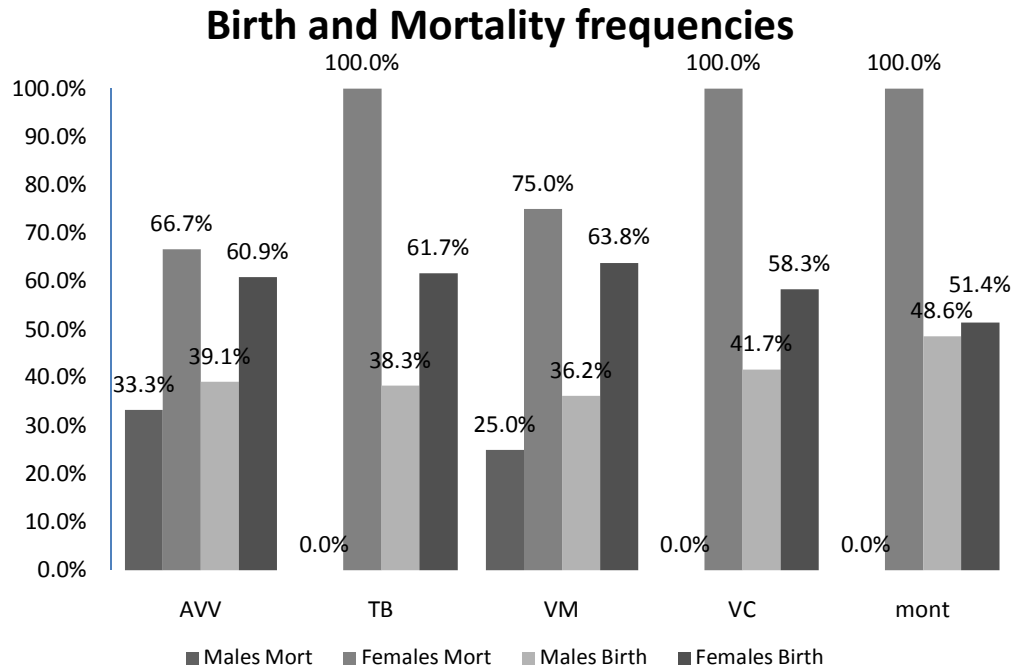


Fig 14 - Mortality and birth ratios distribution accordingly gender and Garrano nucleus. AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo, mont – montalegre.

When mortalities and births are distributed by gender, it's observed that in all populations, females mortality frequency is considerably higher than males. Actually in Vieira do Minho and Terras do bouro, in 2009, there were no males mortalities.

Considering births frequencies, the values are not so unbalanced. Vieira do Minho has the biggest difference in this chapter with more than 2/3 (63.8%) of females births, while Montalegre is the most balanced one with 51.4% of female foals

c: Missing rates

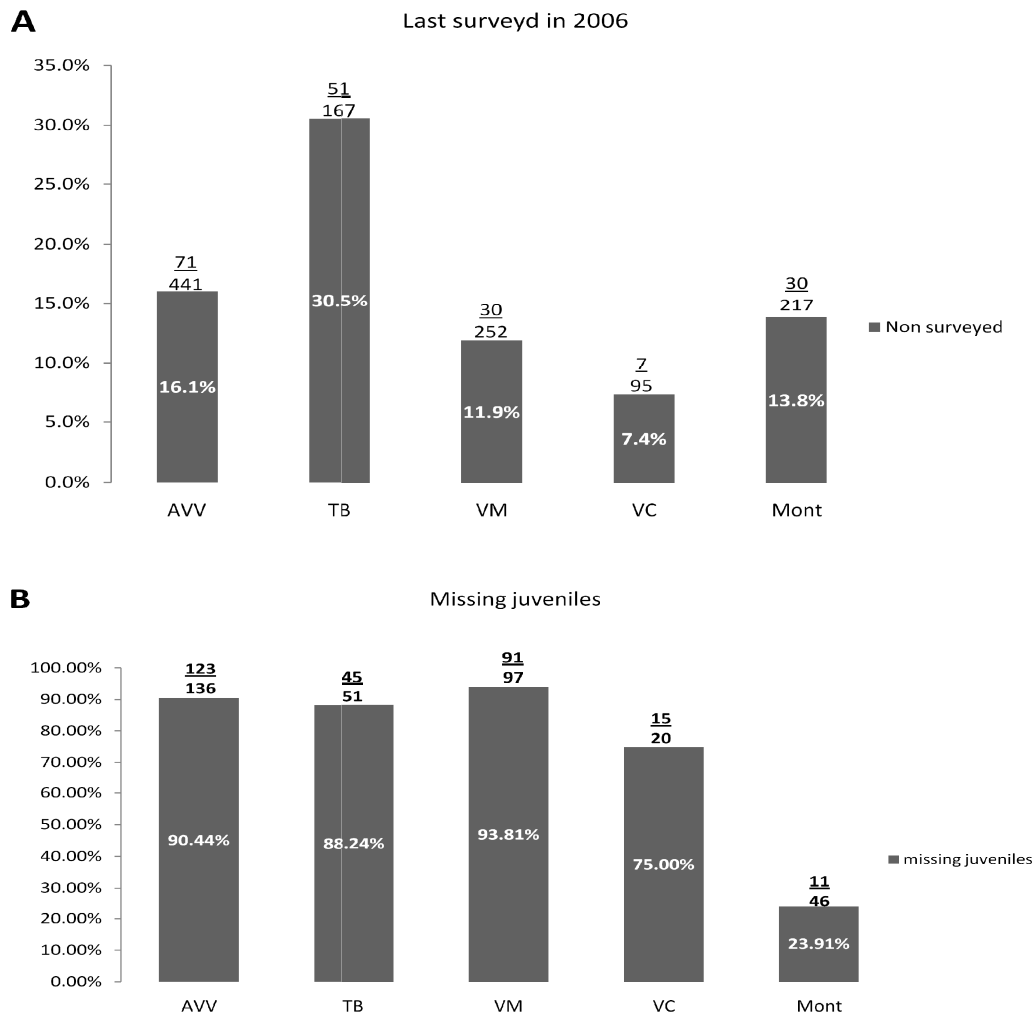


Fig 15 - Frequency of lost animals in 2009 (last surveyed 4 years ago) (A), and frequency of juveniles born in 2006 that didn't made till adults (B). AVV.- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC- Viana do Castelo , Mont - Montalegre

Considering the animals that were last surveyed in 2006 and as so given as lost in 2009 Terras de Bouro showed the worst results with 51 animals lost, corresponding to 30.5% of the total population. On the other side Viana do Castelo has the best values with 7 lost animals (7.4%). Beside that all the other three populations had similar frequencies between 11.9 for Terras de Bouro and 16.1% for Arcos.

Taking into account the high birth rates and low new adults rates, a juvenile missing rate was calculated for the foals born in 2006. For the four of the five populations less the 25% of the juveniles born in 2006 reached adult age in 2009. Viana do Castelo presents the worst results with 93.81% of the juveniles being either dead or lost during

juvenile state. Arcos and Terras de Bouro do not show much better results with missing rates of 90.44 and 88.24%. Only Viana is a little better, but still only a quarter (25%) of the animals can be surveyed after the first three years of live. In this chapter Montalegre presents great values, completely opposing to the other 4 populations, with only 23.9% juveniles lost and the correspondent 76.1% reaching the adult stage and the adult book.

4: Discussion

4.1: ACERG Intervention

The garranos breed is currently endangered with little more than 2000 animals registered in their original distribution region (Portas & Leite, 2000) .

In the end of seventies and during the eighties, after the Salazar's strong legislations concerning community fields, Garrano populations were restricted to five major populations located in Arcos de Valdevez, Vieira do Minho, Terras de Bouro, and Viana do Castelo (FIG.3A). During this time the low economical interest of these breed lead to the abandon and exposure of young foals to wolf predation (Portas et al., 1998). Even more the abandon of agriculture practices, which highly valued the strength of these breed, lead to crossbreeding for meat production purposes (Morais et al., 2005). The degradation of the Garranos breed state continued until 1990 and almost ended in the extinction of these breed. However, since the appearance of the ACERG in 1995, the number of garranos has increased as well as their distribution area (Fig3). The establishment of the breed registration studbook and the financial support to the breeders lead to an increase in the distribution area to almost all north region of Portugal (Fig3B). Even if most of animals still leave in complete feral conditions (Fig 4A), and as so are highly exposed to wolf predation, the increase interest in the foals and the year control of the leaving animals it's starting to reveal good results in the increase of population size, as well as attracting the interest of new breeders, as it is proved by the highest percentage of stabled animals outside the original distribution zone (Fig 4B). In the original region the interest in these breed seem to be increasing with a considerable number of livestock animals in populations like Arcos de Valdevez (8,6%) and Vieira do Minho (7,4%) (fig5). The percentage of mares (84,2% and 70,0%) (fig 5) also indicates that the stabled animals might be mainly for reproductive purposes and as so leading to a recovery in the breed conservation status.

Beside these direct results, the recent creation of an online database with all the information collected in the last 15 years facilitates population studies as these one to evaluate the current state and development of the breed.

4.2: Population's size in 2009

The total number of live registered animals is considerably different between populations. From all the populations studied Arcos de Valdevez was the one with a bigger size, with 1073 live registered specimens, opposing to Viana do Castelo with few then 150 live registered animals. Vieira do Minho and Terras de Bouro have total population effectives of 527 and 381 animals respectively. However these values have

little meaning as they do not reflect the real population size. Direct inquiry to the breeders and to ACERG personal showed that these numbers were highly overestimated.

As so for a better characterization of the populations an estimation model was developed based on the mean number of days between surveys in all populations. This method (check methods) considers as lost every animal that hasn't been surveyed for more than three years, and as so, out of the population. This model seems to estimate quite well the population size when compared to the breeder's inquiry.

This model show that Arcos de Valdevez is actually the more overestimated population with only 41% of the animals registered being considered alive. However is important to notice that the big size of this population makes the control and monitorization by ACERG much harder. This population size effect is observed in Viana do Castelo, which total number of registered animals is 147 animals, almost 1/10th of Arcos the Valdevez, and has almost 70% of its animals surveyed in the last three years (fig 6). As so considering the number of animals to control Terras do bouro seems to be poorly characterized by the database, as shows a lower percentage of surveyed animals than Vieira do Minho (43.8 and 47.8 respectively), despite the last one presents a much high number animals to control, 527 against 381 (fig6). This effective monitorization program might explain the better genetically conservation status of Vieira do Minho population showed by Morais et al 2005.

4.3: Population structure

The five populations present a clear disequilibrium between sexes, with a much higher number of females than males. Viana do Castelo almost reaches the 8 times ratio female/male (83/11), however, once again the small size of the population may influence and intensify this difference. All the other populations have ratios around 4 (compare in Tab 5) which seems more plausible as there are no big differences in birth rates between genders (fig 8). Beside the percentage of active populations is quite similar between genders and as so sexual selective pressures should be similar. Considering age distribution, most animals are between the 6 and 15 years (adults) (fig 9), and with a long reproductive life ahead, which may lead to a faster recovery of the breed. Fact also supported by the mean age of the populations. With mean ages between 12 and 14 years old, only Vieira do Minho and Montealegre are significantly older than ($p \leq 0.001$). Vieira do Minho and Viana do Castelo. Terras do Bouro does not show significant differences in age for these two group swith a mean of 13.35 years.

4.4: Population dynamics

According to the reported births and mortality and respective rates the populations should be in a excellent progression and conservation state. All populations show birth rates above the 400birth/1000animals, with Viana actually reaching the 800births/1000animals (Fig 13). This high number of birth reports is a result of the activity and economic incentives from ACERG. However after reporting the births it seems to lack some follow up on the foals by the breeders, which rarely report their deaths or disappearances, fact proved by the small number of foals registered in the birth book that reach the adult book (fig 15B). In fact this follow up only seems to be happening in Montalegre where at least 3 in every 4 born animals in 2009 reached the adult stage (fig.15B). Beside this population all the other show opposing results. Even in Viana do Castelo small population, only 25% of the foals born in 2006 entered in the adult book in the present year, leaving the other 75% with no report of current status. By these study all these animals are considered lost of track and not integrating part of the population. All the other three populations have even worst percentages of lost juveniles with values close to the 90% (Fig 15B).

As so to evaluate the real dynamic of the population in this scenario, it is better to compare the mortality rates with new entries on the adult book and exclude all foals, as their current numbers are too hard to estimate accurately.

The mortality rates are also highly underestimated; they seem to resume the deaths of adult animals caused by wolf predation or accidents.

According to these reports the highest mortality rate is observed in Terras de Bouro with 29,9 deaths per 1000 animals (fig 13), which is closer to the rate of new entries, 35,93 for this population (fig 13), than the birth rate itself. This is observed in all populations, where there seem to be a slight difference between the new entries and mortality rates the real population dynamics. Comparing these two rates all populations show a slight increase in population size in 2009 as new entries always surpasses the mortality rates (fig 12 and 13). This observation is coherent with the information passed by ACERG personal that populations are slowly increasing in population size since the beginning of their activity.

On the other hand the estimation model used in this study shows that a considerable percentage of animals were lost of track in 2009 (fig 15A). Considering these percentages, and the dynamic of the population of new entries and mortalities, the size of surveyed population seems to be decreasing, but this might only be a result of the selection breeding program developed by the ACERG that intends to recover the old specific characters of this breed. In the future, with the decrease of animals directly

registered in the studbook, a new estimation model should be developed to better determine the animals lost of track.

Chapter II:EIAV Epidemiology: Seroprevalence in Garranos Portuguese populations in 2009

1General Introduction:

1.1: Equine Infectious Anaemia

Equine Infectious anaemia was first described in the 19th century (Lignee, 1843), and was the first nonplant virus filterable agent to be discovered (Valle & Carre, 1904). Despite its early discovery only in 1967 became possible the in vitro replication of the EIA virus (Watanabe, 1966; Kobayashi & Kono, 1967; Kobayashi & Kono, 1967; Kono & Kobayashi, 1967; Kono & Yokomizo, 1968), which lead to further characterization, and its identification as a “retravirus” (Charman et al., 1976) and later as a lentivirus (Gonda et al., 1978; Parekh et al., 1980).

Epidemiological studies revealed a worldwide distribution of EIA virus (Johnson, 1976), and the infection process was soon associated with blood feeding insects (Hawkins et al., 1973; Hawkins et al., 1976; Issel & Foil, 1984).

The discovery of a human lentiviral disease, Acquired Immunodeficiency Syndrome (Chiu et al., 1985), brought a new interest to retrovirus and opened the space for EIAV to be used as an Animal model for AIDS studies (Cohen, 2004; Leroux et al., 2004). Beside, recent studies also demonstrate promising utility of EIAV in gene therapy procedures (Bienemann et al., 2003; Waddington et al., 2003).

Despite two decades of intensive research activity and more than 150 years of knowledge about Equine infectious anaemia, there is no available vaccine to prevent EIAV infection, exception made to one developed in china, which efficiency is still under evaluation (Cohen, 2004).

1.2: EIAV as a Retrovirus

Retroviruses were first described in the 70's, when the discovery of RNA dependent DNA polymerase (Reverse Transcriptases) showed that RNA genomes may be copied into DNA (Baltimore, 1970). These viruses have encapsulated two single mRNA strains, which through tRNA-primed reverse transcription originate a DNA double strand “provirus” (M.Coffin et al., 1997), (Weiss, 1996). The double strand DNA provirus will be integrated in the host cell genome, preferentially in euchromatin (Weiss, 1996).

Expression of provirus endow with mRNA for translation into viral proteins, and full length transcripts leads to genomic RNA for packing into new virions (Weiss, 1996).

Even if provirus transcription is normally controlled through normal cellular transcription regulators, some complex retroviruses (HIV, EIAV) require other regulatory proteins as tat or env for RNA transcription and transport of viral transcripts from nucleus to cytoplasm, respectively. In fact, because of their efficiency inserting genetic information into the host cell genome, retroviruses are commonly used as vectors in genetic manipulation methods.

The appearance of retrovirus is not yet fully understood; however it is pointed that retroviruses might have evolved from the positive-strand RNA virus, as they both present a tRNA-like 3' end (Weiner & Maizels, 1987; Wang et al., 1993).

According to the most recent taxonomy classification of retroviruses by the International Committee on the Taxonomy of Viruses (ICTV), this family can be split into two sub family: Orthoretrovirinae with six different genera; Alpharetroviruses, Betaretroviruses, Deltaretroviruses, Gammaretroviruses, Epsilonretroviruses, and Lentiviruses; and spumaretrovirinae with a single genera Spumavirus (Gifford & Tristem, 2003), (Hull, 2001). EIAV is included in the Lentivirus genera, as well as, Human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2), simian immunodeficiency virus (SIV); feline immunodeficiency virus (FIV); bovine Immunodeficiency Virus (BIV), caprine arthritis/encephalitis virus (CAEV), and Maedi-visna virus. Unlike the previous classification based on presence (complex) or absence (simple) of accessory genes, this new taxonomic system is supported in the biochemical and genetic properties of retroviruses.

1.3: Retrovirus and Lentivirus Characterization

All retrovirus encode three main polyproteins; gag, pol, env. The pol gene in the gag-pol fusion encodes the viral enzymes protease, reverse transcriptase, dUTPase and integrase. The processing of gag polyprotein by the protease originates the matrix, capsid, nucleocapsid and p9 proteins. Finally the env encodes the outer envelope glycoproteins gp90 and gp45 (Weiss et al., 1996).

Beside these main structural components, lentivirus encodes several accessory genes with regulatory or auxiliary functions (Weiss, 1996). From all, EIAV is the least complex one, with only three accessory genes (tat, rev and S₂), in contrast to the six accessory genes of other Lentiviruses.

Understanding the function and interaction of these accessory genes may be the key to develop new antiviral components, for which EIAV is commonly used as an animal experimental model for AIDS therapeutic and vaccination studies.

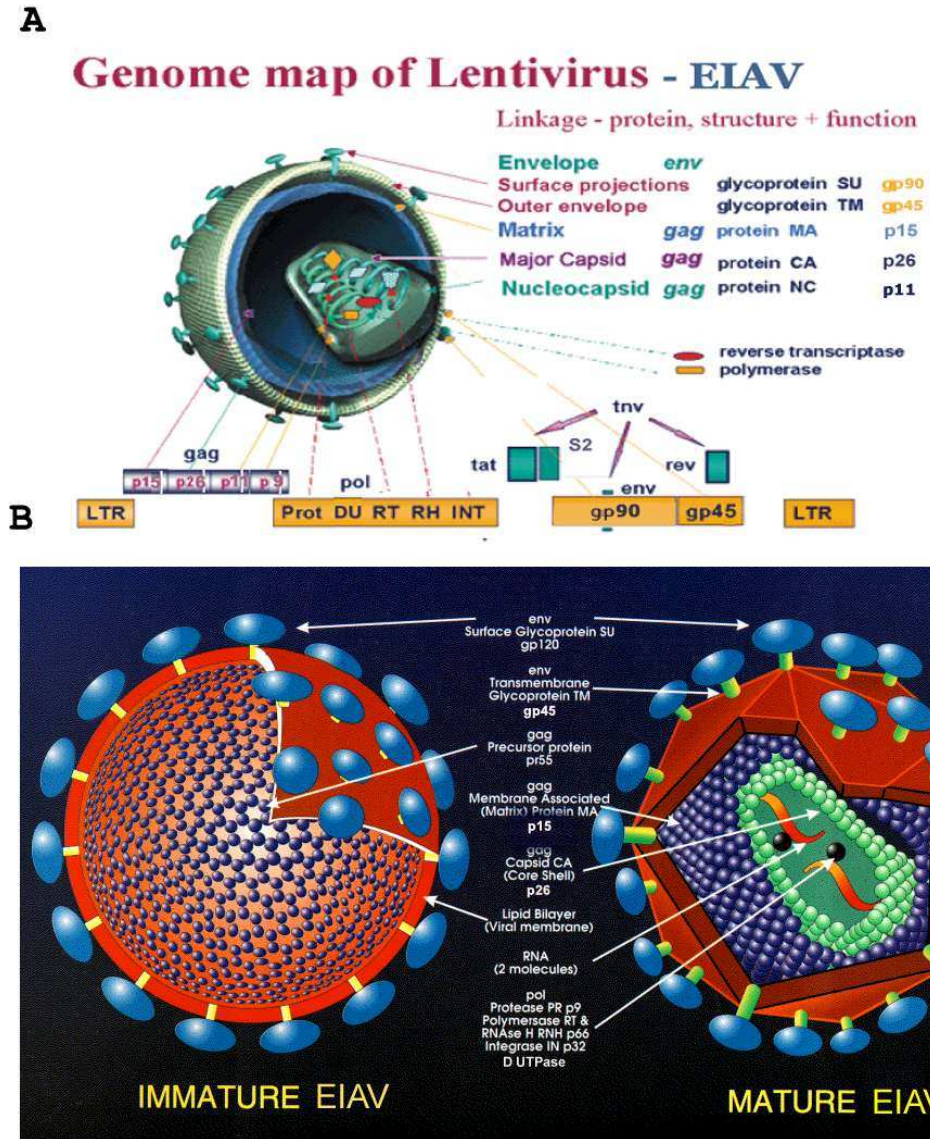


Fig 18 - A: EIAV genome encodes for structural (gag), enzymatic (pol), and accessory viral proteins (rev, at and S2). Enzymatic proteins encoded in pol, Protease (PR), Reverse transcription (RT), Integrase (IN) and dUTPAase (DU) is synthesised as a gagpol precursor. The Gag polyprotein is cleaved by the protease to produce, Matrix (p15), capsid (p26), nucleocapsid (p11) and L domain (YPXL) containing protein (p9). At the cell surface EIAV possess the surface glycoprotein (Gp90) that is anchored to the surface via the transmembrane (TM) and fusion domain of the glycoprotein Gp45. Rev accessory gene is essential for nucleocytoplasm transport of non splicing viral mRNA, Tat is involved for viral transactivation and S2 is a viral protein with unknown functions.

B: Virus release from cellular membranes marks the beginning of viral maturation and involves proteolytical events necessary for structural viral rearrangements culminating in

an infectious viral particle. During this process a hexagonal viral core is formed by capsid proteins that contain the nucleocapsid protein in its lumen, which supports the two copies of viral RNA. The matrix protein is in close contact with the lipid bilayer. Retroviral particles also contain a wide range of cellular components, such as the lipid bilayer and cellular proteins (ubiquitin, Major Histocompatibility Complex II, chaperones, and others) (Figure A and Figure B were exported from Fidalgo-Carvalho, 2008, after adaption from Harvard AIDS Institute Library of Images and www.uti.it/infectionline/HIV_ciclo.htm).

1.4: Life Cycle

Despite high variability among retrovirus, Lentiviruses share, as previously referred, several physical and genomic properties, as well as, a replication cycle.

This process can be divided in four stages: a) entrance; during which occurs cell binding, fusion and penetration; b) propagation; consisting in reverse transcription, integration and transcription of provirus, protein production and viral mRNA particles assembly; c) budding; throughout packaging and egress of new virion particles from cellular membrane, and d) post-budding maturation; for reorganization of new virion interior architecture (Tang et al., 1999).

The host cell response to viral infection and the cellular processes involved in viral replication are only now, starting to be revealed. In example, several studies with retrovirus and with (+)RNA virus show high similarity of processes in the entrance and propagation stages of the life cycle (Ahlgren et al., 2003),.

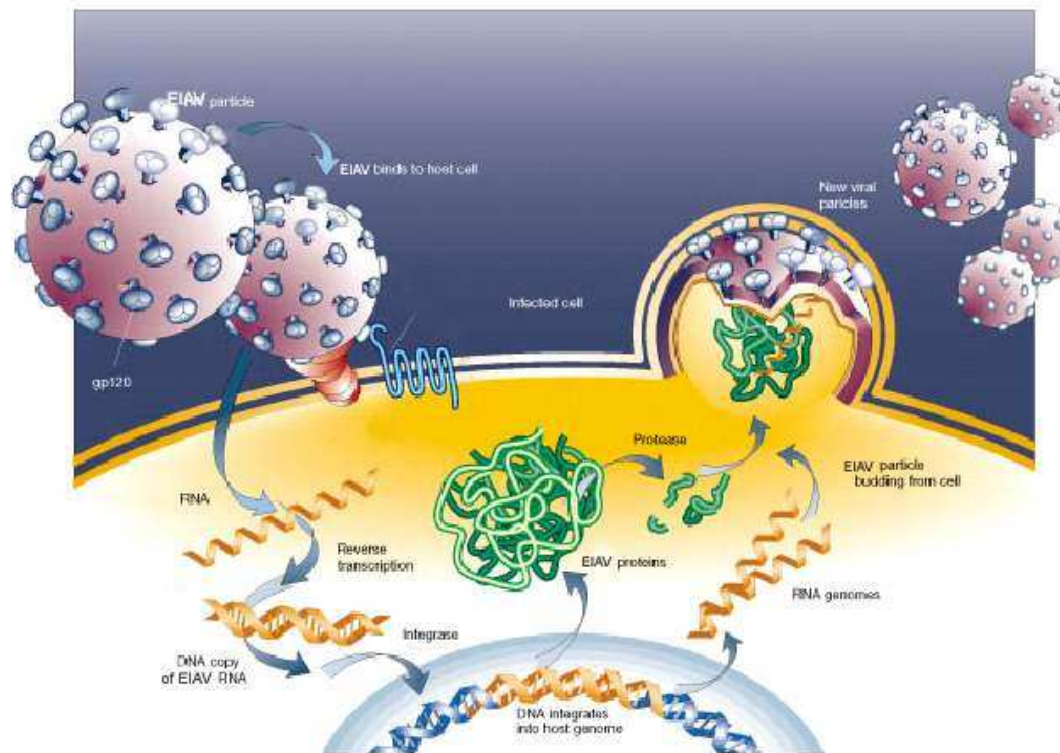


Fig 19 - EIAV is a macrophage-tropic lentivirus, viral entry is thought to be receptor mediated, although an EIAV receptor remains unknown. After entry the virus is uncoated, viral RNA is retrotranscribed to DNA and the pre-integration complex is formed. This complex comprises viral and cellular proteins and is responsible for the nuclear translocation of proviral DNA and consequently for its integration into the host genome. After integration viral transcription and protein synthesis is initiated, assembly of viral proteins and RNA occurs and budding of viral particles by cellular membrane extrusion release new viral particles. After viral maturation these viral particles are able to infect new target cells and the viral cycle is completed (Figure 1.2 was extracted from Fidalgo-Carvalho, 2008 after adaption from Weiss, RA. 2001. Nature 410:963).

1.5: Lentivirus Transmission

Retrovirus can be transmitted vertically, prenatally, perinatally and post-natally; horizontally as cell free virus or infected cells; and genetically as integrated DNA provirus during cellular division of infected cells.

Even if these are the most common transmission processes of Lentiviruses, there might be other transmission courses specific of some viruses. FIV, SIV and Leukaemia Virus in cats are frequently transmitted through bites and scratches, and EIAV is associated to transmission through blood feeding biting flies.

This is the main natural transmission pathway of EIAV between animals, as vertical transmission is only significant if the breeding mares are in the acute infection period, approximately 2 months after exposure (Kemen & Coggins, 1972). During chronic stage of the infection only 11% of the offspring are infected and asymptomatic carrier mares (5-6 months after exposure till animal's death) do not transmit the EIAV to their breeds (Kemen & Coggins, 1972).

In the end it was this long asymptomatic carrier stage that gave Lentiviruses their name, as from the Latin Lenti means slow.

1.6: EIAV Vaccine

Genomic and antigenic variation are globally recognised among scientific community as characteristic of lentivirus infections and widely accepted as the main obstacle to development of efficient vaccines (Craig & Montelaro; Craig et al., 2007). This variation is related to increases virus replication episodes during stress of immune suppression of unapparent animals (Craig et al., 2009), however in EIAV case over 90% of the infected animals progress from short chronic stage into a new unapparent stage by immunologic control of virus replication (Craig et al., 2009). This makes EIAV a unique animal model for research and development in other Lentivirus prophylactic vaccines.

Still despite all the efforts to the development of an efficient vaccine for EIAV recent studies have shown that 13% variation Env from the most efficient current vaccine decreases by 50% in disease protection (Craig et al., 2006; Craig et al., 2009). As so a definitive vaccine would finally enable a efficient prophylactic program to EIAV disease and uncover the path to the development of vaccines to other Lentivirus such as HIV.

1.7: EIAV: global distributed disease

Even if nowadays EIA is considered a rare horse disease in Europe, EIAV is considered worldwide distributed with several epidemiologic reports in Europe in the late 70's (Steck, 1967), (Feiling et al., 1974), (Toma, 1974), (Papadopoulos, 1975), (tabl...). How the disease was controlled was never correctly explained and doubts about the official diagnostic assay used in the borders are frequent (Fidalgo-Carvalho, 2008).

Portugal was supposed to have escaped all of epidemics with no reported cases of disease until recently. In 2008, Fidalgo-Carvalho, in a follow up study of Garranos from the north of Portugal revealed seroprevalences of almost 40% (Fidalgo-Carvalho, 2008).

The long unapparent state of EIAV, during which animal are carriers without any sign of infection, makes harder to diagnose the disease in borders and transactions and prevent the spread of the virus.

1.8: EIAV Control Plan

Currently EIAV figures in the OIE List B of notifiable diseases as is considered of socio-economical and/or public health importance within countries and that are significant in the international trade of animals and animal products.

OIE control policies dictate that seropositive animals should be quarantined or eliminated to prevent spread of disease. These policies have sacrificed thousands of animals' worldwide and limited further research in EIAV, especially in the isolation and characterization of primary isolates.

EIAV epidemiologic report in Portuguese feral Garranos populations

Country	Epidemiologic report	Reference
Bulgaria	EIA in Bulgaria	(Dimitrov et al., 1975)
Czechoslovakia	EIA in Czechoslovakia	(Celer & Zakopal, 1975)
England	EIAV case report in Newmarket	(Rossdale et al., 1975)
Europe	Diagnosis and prophylaxis of EIAV	(Toma, 1974),
Germany	Outbreak of EIAV in Kassel	(Feiling et al., 1974)
Germany	Epidemic situation of EIAV in Horses	(Primus & Zettl, 1975)
Greece	Epidemiological situation of EIA in greece	(Papadopoulos, 1975)
Ireland	EIA in Ireland: 2005 outbreak	(Cullinane et al., 2007)
Ireland	Real-time quatitive RT-PCR and PCR assay for a novel European field isolate of EIAV based on sequence determination of gag gene	(Quinlivan et al., 2007)
Italy	Epidemiological situation of EIA in Italy	(Codazza, 1975)
Netherlands	EIAV seroprevalence in the Netherlands	(de Boer et al., 1979)
Yugoslavia	Epidemiological situation of EIA in Yugoslavia	(Petrovic et al., 1975)

Table 8 – Epidemic reports of EIAV in the Europe in the last 50 years. Table was extracted from Fidalgo-Carvalho, 2008.

1.9: EiaV diagnostic methods

Agar gel immunodiffusion test (AGID)

Over the past 30 years the control of EIAV infections of horses has been based primarily on the identification and elimination of seropositive horses identified predominantly by a standardized AGID assay done in centralized reference laboratories. AGID, also known as the Coggins test, is the prescribed test for the International horse trade (Coggins et al., 1972), (Pearson & Knowles, 1984). Precipitating antibody is rapidly produced as a result of EIA infection and can be detected by the AGID test. Specific reactions are indicated by precipitin lines between the EIA antigen and the positive standard serum.

Horses in the first 2-3 weeks after infection will usually give negative serological reactions. In rare cases the post infection time prior to the appearance of detectable antibody may extend up to 60 days. EIAV transmission from AGID negative horses has been reported (McConnell & Katada, 1981).

Enzyme linked immunosorbent assay (ELISA).

There are three ELISAs that are approved by the United States Department of Agriculture for the diagnosis of EIA and are available internationally; a competitive ELISA and two non competitive ELISAs. The competitive ELISA and one non-competitive ELISA detect antibody produced against p26 core protein antigen. The other non competitive ELISA incorporates both p26 core protein and gp45 (viral transmembrane protein) antigens (Burki & Rossmanith, 1990), (Matsushita et al., 1989), (Pearson & Knowles, 1984), (Shane et al., 1984).

1.10: EIAV Presence in Portugal

Until very recently there were no reported cases of EIAV infection in Portugal. Only in 2008 Fidalgo-Crvalho presented the first report of this disease in two Garrano populations in Portugal recurring to more sensitive assays than the one officially accepted.

In here it is intended to support the prevalence of EIAV in Portugal recurring to more sensitive ELISA assays and to western blotting, questioning once again the validity of the Coggins test.

2.Methods

2.1: Sampling

Blood samples were collected from forty six (46) feral garranos belonging to the four major populations from September 2009 to January 2010. From those 11 animals were from Arcos de Valdevez (2,5%), 5 from Terras do bouro(3,0%), 19 Vieira do Minho

(7,0%) and 11 from Viana do Castelo(11.6%). For all populations Gender(fig...) and age classes (fig...) were represented. 13 blood samples collected in 2008 from animals belonging to Montalegre were also analyzed, after confirmation of the live status of the animals in the database, corresponding to 6.0% of the total population (Fig20).

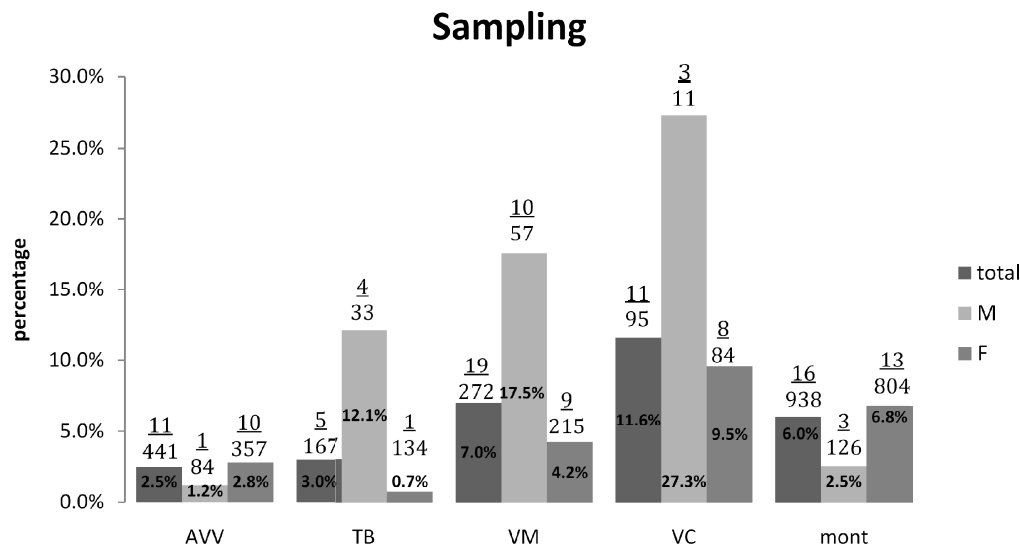


Fig 20 - Sample representation of total population, and gender within population. AVV- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC, Viana do Castelo, Other – all other surveyed animals outside the four populations studied.

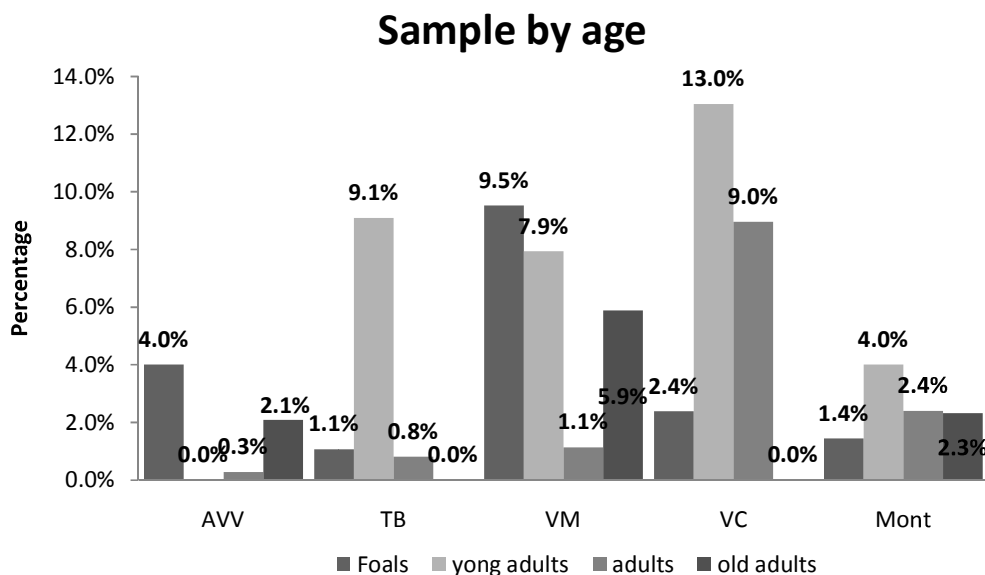


Fig 21 - Sample representativity for age classes within populations. Foals - 1 to 3 years old, young adults – 3 to 7 years, Adults – 7 to 20 and old adults > than 20 years old. . AVV- Arcos de Valdevez, TB- Terras de Bouro, VM- Vieira do Minho, VC, Viana do Castelo, Mont - Montalegre.

2.2: Sample collection

Whole blood (15ml) was collected from jugular venipuncture from apparently healthy Garranos, with the help of sterilized needles and vacuum tubes containing EDTA. Plasmas were obtained from whole blood by centrifugation at 1800 rpm for 15 minutes at 4°C and plasma stored at -20°C until use. All samples were collected from September 2009 to January 2010. Serologic assays were conducted from April to July 2010.

2.3: Serologic Assays

a: Elisa ConA

Antibody status against EIAV gp90 glycoprotein was tested by a concanavalin A ELISA, as described (Hammond et al., 1999). The use of Concanavalin A in envelope glycoprotein based ELISA was previously described by Robinson et al. in 1990 due to its ability to bind the lentiviral glycoprotein surface envelopes. In summary, Immulon II (Dynatech Laboratories, USA) 96 well plates were coated for 1 hour at room temperature (RT) with 50 µg/ml Concanavalin A (Vector Laboratories, USA). Plates were then adsorbed for 1 hour at RT with 1 µg/well of disrupted EIAVpv purified viral particles in 1%/Triton X-100. Plates were washed 5 times with a 0.025% tween20 1X PBS solution and incubated for 1 hour with blocking solution (5% of inactivated horse serum and 5% of non fat milk and 0.25% tween20 in PBS) at RT. Next, the Garranos plasma was incubated at an initial concentration of 1:50 and continued two fold dilutions until 1:6400. Plates were again washed 5 times with 0.025% tween20 1X PBS and incubated for 1 hour with an anti-horse peroxidase conjugated secondary antibody (IgG-HRP diluted in 1:35000 in blocking solution) at RT. After the secondary antibody plates were washed 5 times with 0.025% tween20 1X PBS, and 200 µl of TM blue added at RT for 20 minutes with gentle shaking. The reaction was then stopped with 1 M of H₂SO₄, and the Absorbances read at 450nm. A positive reaction was defined as one in which the A_{450nm} in the test well was greater than two times the absorbance of the negative control plasma. Horses with plasma reactivity consistently between 2 and 1.5 fold higher than the control were considered 'suspicious'.

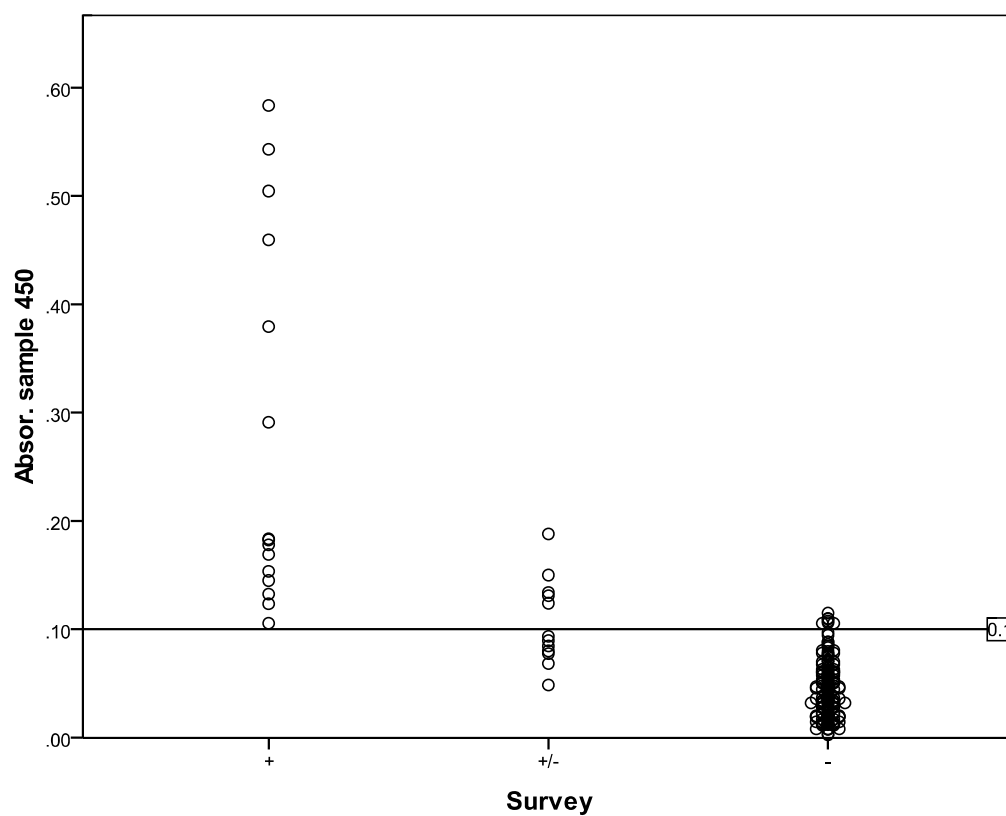
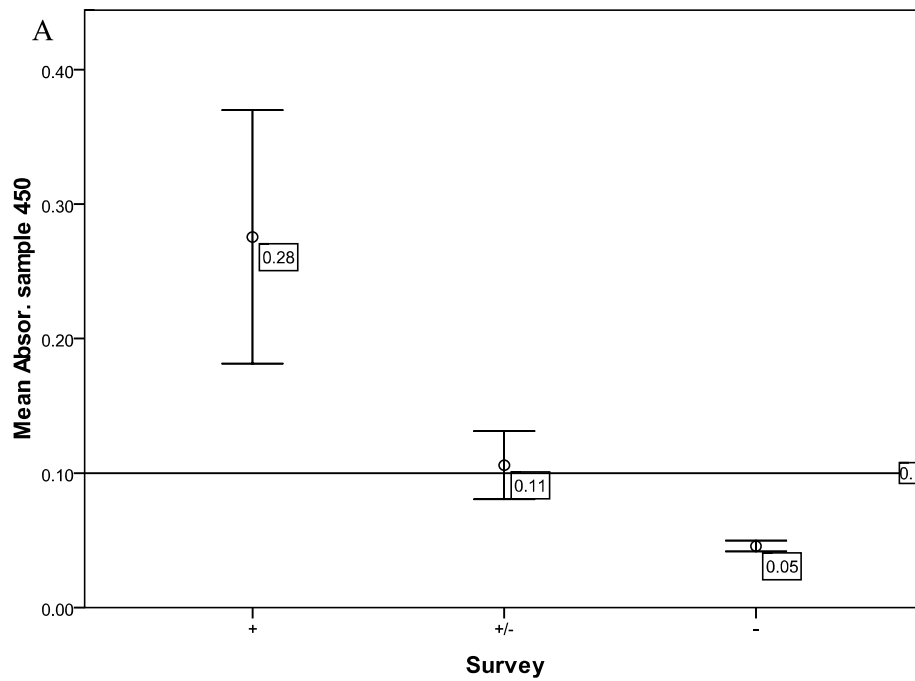
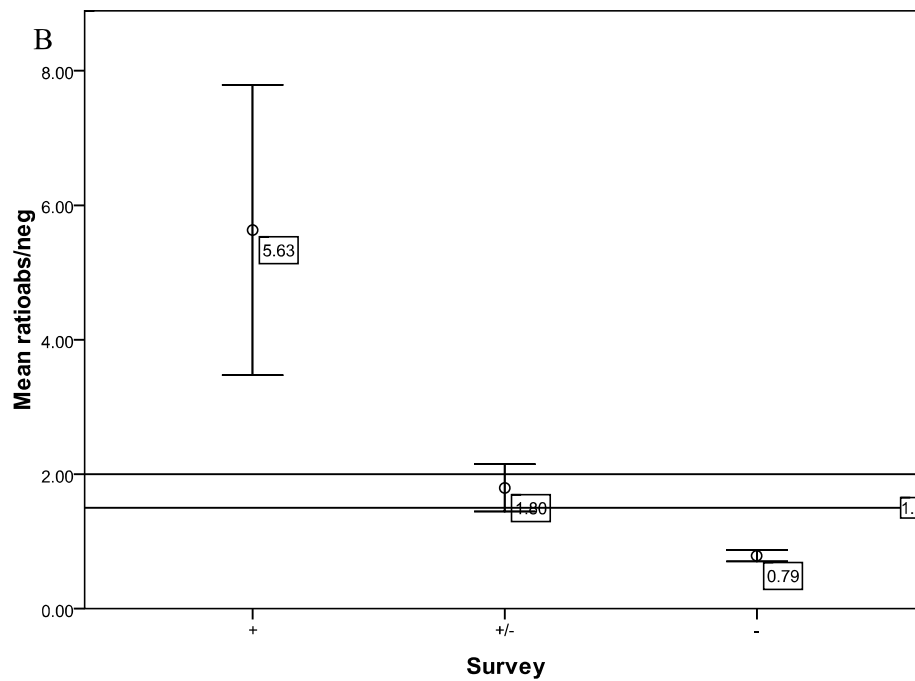


Fig 22 - Boxplot distribution of the absorbance obtained from Elisa Con-A assay on animals serum samples. All animals were tested in triplicates.



Error Bars: 95% CI



Error Bars: 95% CI

Fig 23 –Samples Absorbance (A) and Samples absorbance/negative control absorbance ratio (B) means by survey group with 95% intervals.

b: P26 standard Elisa

Positive and suspicious samples were confirmed for the capsid protein p26 by standard elisa. Immulon II (Dynatech Laboratories, USA) 96 well plates were adsorbed with 0.1 µg/well of purified p26 in 1X PBS solution at RT for 1 hour. Washing, blocking, secondary antibody and substrate were exactly the same as the Elisa ConA assay.

c: Western Blotting

Positive and suspicious samples were analyzed by Western blotting to visualize plasma antibodies against reduced purified p26 capsid protein and EIAVpv depending on the assay.

p26 assay

Plasma samples were incubated with western blot strips prepared with p26. .40 micrograms of p26 gradient purified viral particles were loaded and run in a 12% NuPAGE Bis-Tris Gel (Invitrogen lab.). After electrophoresis matrix protein was transferred to a nitrocellulose membrane and blocked 5% of inactivated horse serum and 5% of non fat milk and 0.25% tween20 in PBS. Several strips were then cut from the blocked membrane, and each strip incubated with a 1:50 dilution of heat inactivated plasma for 2 hours at RT. Strips were washed 3 times for 8 minutes in 0.025% Tween PBS and incubated with an anti-equine secondary antibody (1:35 000 dilution) for 1 hour. After 3 washes, 10 minutes/each, signal was detected with West Pico substrate.

EIAVpv assay

IN this case plasma sample we incubated with western blot strips prepared with EIAV pv viral proteins. 10 micrograms of EIAVpv gradient purified viral particles were loaded and run in a 12% NuPAGE Bis-Tris Gel (Invitrogen lab.). After electrophoresis matrix protein was transferred to a nitrocellulose membrane and blocked 5% of inactivated horse serum and 5% of non fat milk and 0.25% tween20 in PBS. Several strips were then cut from the blocked membrane, and each strip incubated with a 1:50 dilution of heat inactivated plasma for 2 hours at RT. Strips were washed 3 times for 8 minutes in 0.025% Tween PBS and incubated with an anti-equine secondary antibody (1:35 000 dilution) for 1 hour. After 3 washes, 10 minutes/each, signal was detected with West Pico substrate.

3: RESULTS

After calculation of prevalence frequencies of EIAV in populations Viana do Castelo showed the highest values with 18% (2/11) of the sampled animals infected and 9% (1/11) more suspected to be infected. Terras do bouro does not show any infection on

any of the 5 sampled animals. Montalegre shows 6.3 (2/16) of infected animals plus other 6.3% (1/16) suspicious of infection. Arcos de Valdevez also presents an equal percentage of infected and suspicious animals, 8% (1/11).

Infection frequencies by age classes reveals higher infection rates in younger animals, oals and young adults with 12% and 7.1% against 4.8% in adults.

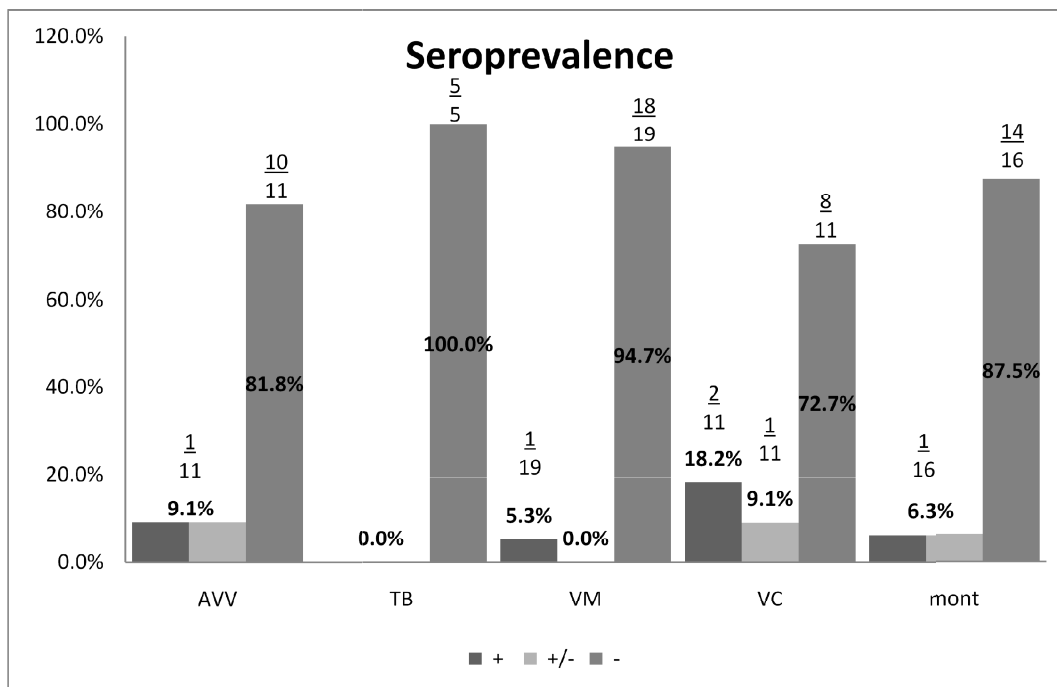


Fig 24 – Infection frequencies of sampled animals by population. Arcos de Valdevez (AVV), Terras de Bouro (TB), Vieira dom Minho (VM), Viana do Castelo(VC). (+) – seropositive, (+/-) – suspicious of seropositive, (-) – seronegative;.

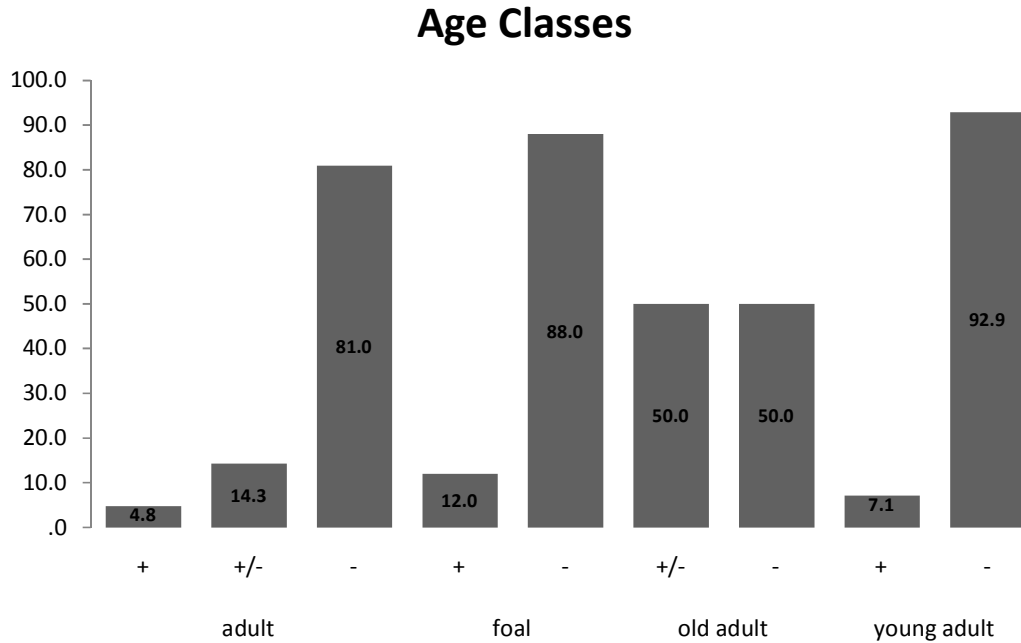


Fig 25 – Infection rates by age classes in sampled animals. Foals - 1 to 3 years old, young adults – 3 to 7 years, Adults – 7 to 20 and old adults > than 20 years old. (+) – seropositive, (+/-) – suspicious of seropositive, (-) – seronegative.

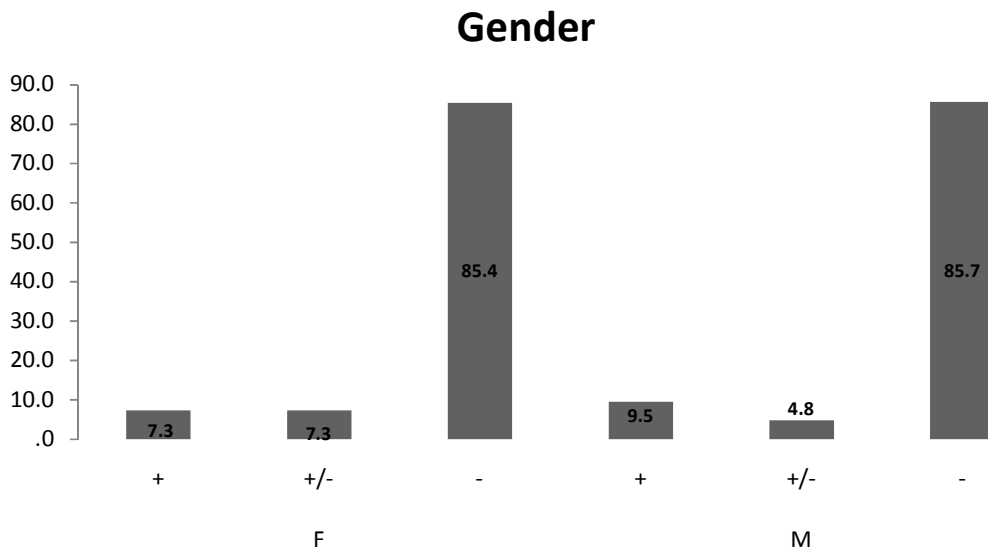


Fig 26- Infection rates between genders in all sampled animals. Foals - 1 to 3 years old, young adults – 3 to 7 years, Adults – 7 to 20 and old adults > than 20 years old. (+) – seropositive, (+/-) – suspicious of seropositive, (-) – seronegative

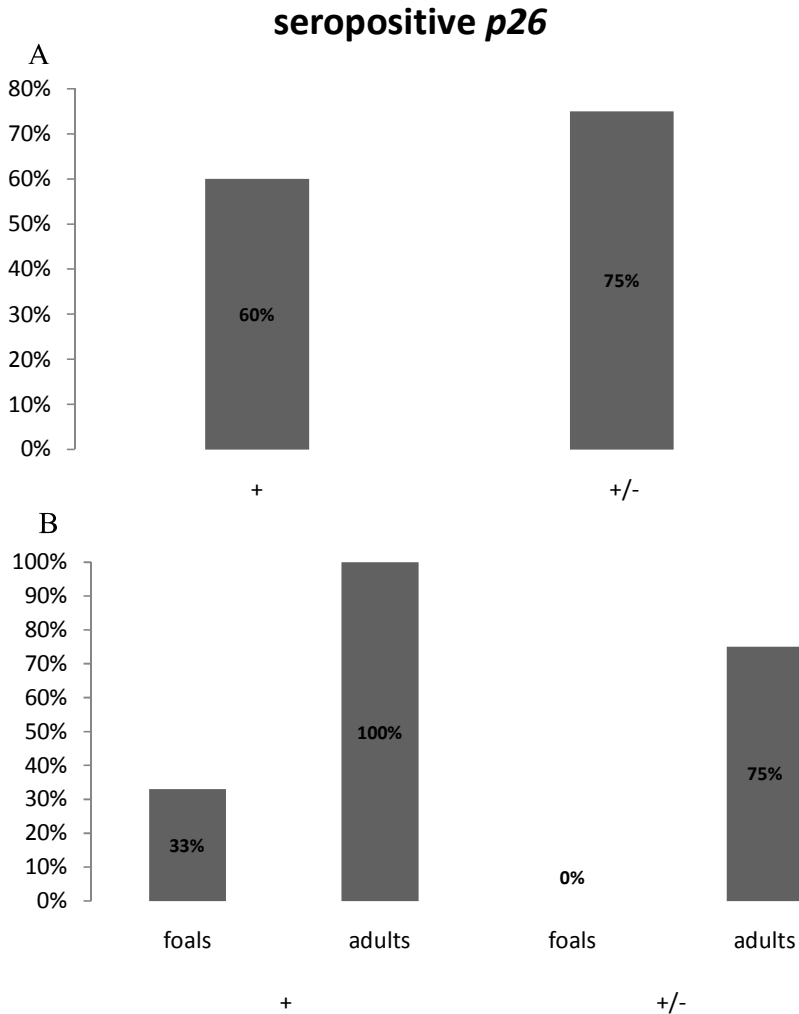


Fig 27 – Frequency of seropositive animals for Con-A confirmed with p26 (A). Con-A animals confirmed with p26 by age (B).

Confirmation with elisa for the capsid protein p26 showed that 3/5(60%) of the positive samples for EIAVpv by Elisa Con-A are also positive for p26, and that the 2 animals with negative response to the capsid protein are foals. The suspicious animals are all adults and from the 4 tested for p26 3 (75%) gave a positive response confirming the suspected infection.

Western blot showed positive bands for three of the 5 positive samples in Con-A EIAVpv elisa. Bands under the 6000 daltons are considered unspecific binding due to presence in negative control. A Viana do Castelo foal (e834) presented two bands on with approximately 41 Kda, probably a Gag cleavage intermediate product (p45) and other with approximately 15 kda, matrix protein (p15) . Both bands are present in the two positive

controls and absent in the negative, however p15 is intense in early infected positive control.

A negative sample in con-A EIAVpv elisa (p11) was also tested but results are not clear due to high contrast reactivity.

Other two samples gave slight positive bands with 41Kda, a Arcos de Valdevez foal (e982) and a Montalegre female (a453).

Two animals, E834 (foal from VC) and 1947(female VC) presented a band for purified p26 western blot, confirming the positive result in p26 Elisa. The band is absence in the negative control and in p11 sample which consistently gave negative results in Con-A and p26 Elisa. Positive control presented the band observed in two positives samples.

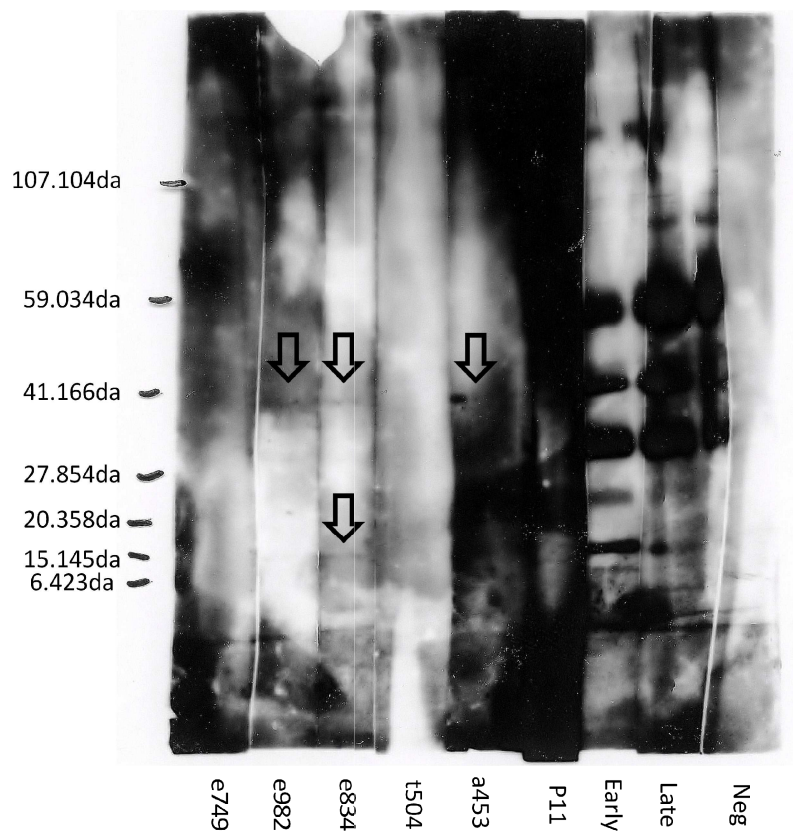


Fig 28 - Western blot for EIAVpv confirmation for positive samples in EIAVpv con-A Elisa and p26 Elisa. Negative control presented bands below 6500 daltons probably due to unspecific binding of the secondary antibody. Early – recently infected animal serum for positive control, Late – Old infected animal serum for positive control.

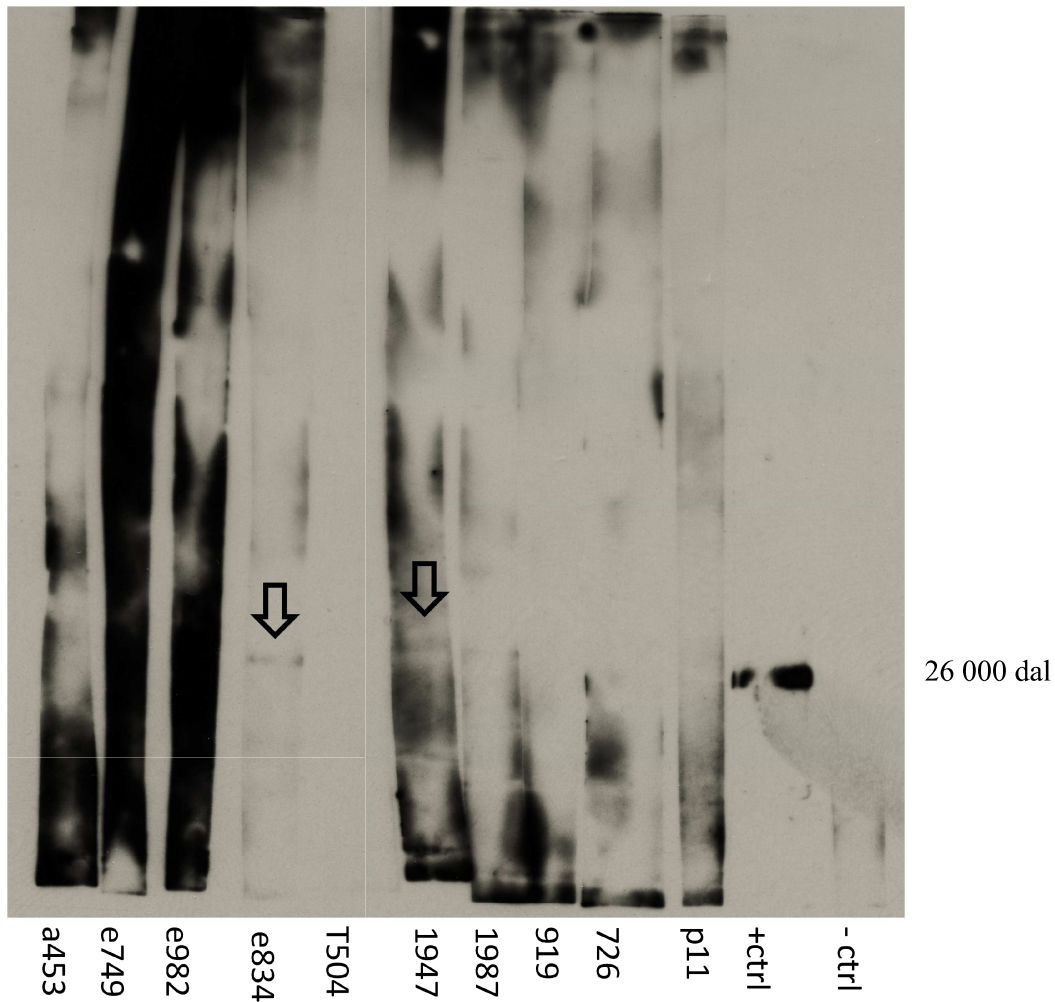


Fig 29 - Western blott of purified p26 for confirmation of positive samples by p26 and EIAVpv elisa.

4: Discussion

Until very recently there was not a single report of EIAV in Portugal despite the number of epidemic cases in Europe and the strong horse industry and equine tradition. Only in 2008 Fidalgo-Carvalho described, for the first time, the prevalence of EIAV in garranos horse in North of Portugal, recurring to a more sensitive serologic assay than the official AGID test (see introduction). The results confirm these report and three of the four sampled populations gave positive results to the serosurvey of EIAV. Only terras do bouro hadn't infected sampled animals (fig24), but is important to notice that this is also the worst sampled population with no more than 5 sampled animals (3% of the total population) and poor representation of the population structure with 4 males and 1 female. Previous reports have shown that males are less susceptible to disease than females (Fidalgo-CAarvalho, 2008), even if the current study does not show such

difference. In fact from all sampled animals males had a higher frequency of infection than females, 9.5% against 7.3% (fig26). Yet if we consider the suspicious animals confirmed positives with p26 there is not much different between genders (fig26). It's important to notice that most of the infected animals are foals (Fig25) and differences were reported in adult animals (Fidalgo-Carvalho, 2008). The higher missing rates foal and juveniles previously described (Fig15) might randomly eliminate infected animals and decrease the infection rates.

Beside Terras de Bouro all other sampled populations have infected animals.

Another population that is not very well represented by the sample, Arcos de Valdevez, shows 8% of infected animals and 8% suspicious of infection and confirmed positive with p26. Even if the total sample resumes 11 animals, correspondent to 2.5% of the total population, the distribution between genders and age classes is much better represented in this case.

Vieira do Minho and Viana do Castelo sample is close or over the 10% of the total population and also show infected animals. Viana do Castelo has a strong a total 2 infected animals, a foal and a young adult, and 1 adult animal (6%) of suspicious of infection by EIAVpv and confirmed with capsid protein p26 (Fig27)

In general adult animals have a lower response to EIAVpv viral proteins than to the capsid protein (p26). All suspicious adults for EIAVpv gave strong positives for purified p26 elisa. The foals, however, have the opposite response with good reactivity EIAVpv con-A Elisa and low reactivity to p26. This agrees with the stages of immune response descript to EIAV and other lentivirus (Fraser et al., 2001). In a first stage of infection unspecific antibodies against the exterior proteins present in complete viral proteins of EIAVpv are produced while specific neutralization antibodies against inside proteins like p26 and other Gag derived proteins only appear in late stages of infection.

Western blott results are quite inconclusive and incoherent with elisa. Only slight bands can be seen, and all Gag derived cleaved products. E834 a Viana do Castel foal gave positive bands to p26 capsid protein, p15 matrix protein and p45 gag intermediate cleavage product. This last one was also present in E982 a Arcos de Valdevez foal (fig28). Beside these two samples never presented any band for env viral proteins gp90 or gp120. However it's important to notice that p15 was indeed more intense in the early infected positive control (fig28)

Of all adults tested by western blot only a Viana do Castelo female was positive for p26 capsid protein (fig29), while a Montalegre female gave a positive band for p45 gag intermediate product.

These results might be related to reactivity issues observed during all procedure derived from the difference between the Portuguese strain to which the Garranos were exposed and the EIAVpv strain.

Chapter III: Conclusions

In the last 15 years ACERG has put an effort to conserve and protect the Garrano breed with a monitorization and conservation programme that intend to increase the value of the breed and recover the specific phenotypic characteristics of this breed which has been crossbreed during the last years for meat production purposes.

Despite the creation of a breed studbook for control of the animals, the number of specimens lost of track in 2009 is still considerable high, especially in Terras de Bouro. In fact, this population seems to be the one where the recovering programme has been less efficient with the higher percentage of lost animals in 2009 and mortality rates. However these results only concern to 2009, in order to fully evaluate the efficiency of ACERG program is important to make a retrospective study based on the online database.

All populations do not present much difference in structure despite big differences in size. Viana do Castelo is the only exception with a much higher ratio of females per male, which is probably explained by the extreme small size, less the 100 animals.

On the other side Viana do Castelo has lower mortalities rates due to the absence of wolf predation in this population, one of the main reasons for animal deaths report.

It is concluded that most of the animals come out of the populations due to anthropogenic actions and are not reported as dead by their breeders leading to high animals lost percentages. The fate of these animals is undetermined even by the ACERG and only with the improvement of the monitorization program and sensibilization of breeders can the cause of these disappearances be discovered.

Despite the official inexistence of EIAV in Portugal a total of 8 animals out of the 62 sampled animals ($\approx 13\%$) were seropositive to EIAVpv or capsid protein. Two of these animals were confirmed by western blot with clear bands for p26 capsid protein or p15 matrix protein or p45.

Unfortunately the sampling of Terras do Bouro and Arcos de Valdevez was not close to the 10% of the total population studied. In these case Terras do Bouro is actually very poorly represented with only one female, a young adult, and 4 males, two adults and two foals. With these sampling no extrapolation or conclusions to the total population can be taken, and assuming inexistence of the virus in Terras de Bouro is highly risky. In fact as this population is surrounded by Arcos de Valdevez and Vieira do Minho, and previous studies have shown the existence of vector flies in this region, the existence of infected

animals is more probable. Future prevalence studies should be conducted with better sampling to confirm this suspect.

Vieira do Minho and Viana do Castelo sampling almost reached the 10%, a common accepted minimum representation, and 2009 prevalence can be extrapolated to the total population. Viana do Castelo showed high prevalence rates with 27% infected animals. In such a small population it is important to refer that the three infected sampled animals were not related in anyway, either by breeder, location or reproduction.

This population also showed an higher infection rate for male then females which disagrees with previous conclusions that females are more susceptible than males (Fidalgo-Carvalho, 2008). Once again more studies shall be conducted to see if this is a sampling effect or if in fact the differences in infection rates between gender in adult animals previously reported is due to differences in susceptibility or not.

Prevalence rates don't seem to have any effect in mortality rates, and even if this effect might be disguised by anthropogenic action, any of the animals showed any sign of the disease during blood samples collection. This, once again, opens the question of how serious is the disease to the animals by itself and the control measures of OIE.

Beside like previously reported seroprevalence results are highly dependent on the assay (Motta, 2007). In this study EIAVpV viral proteins showed to give better reactivity in foals, that were recently infected, and capsid protein p26 had better reactivity with older animals that might be in a later stage of infection, possible already in a long unapparent state. Official coggins tests was not conducted but a previous follow up study show that all animals in Portuguese populations were negative for AGID test (Fidalgo-Carvalho, 2008). A future coggins AGID test might be conducted in these samples to confirm the suspect and once again question the sensibility of the official test used in the animals transactions.

As final remarks this study reveals once again the presence of endemic EIAV infections in feral populations, confirming a previous report in 2008 (Fidalgo-Carvalho, 2008). However, all infected animals did not presented any sign of disease questioning again the real impact of the disease in feral populations and the control measures in the borders that lead to thousands of sacrificed animals every year. Beside the differences shown in the different assays also leaves some suspicious about the efficiency of serologic assays compared to new available RT-PCR methods with given proves to be much more sensitive and efficient (Fidalgo-Carvalho, 2008).

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Annex I: The History and Origins of the Celtic horse.

The Wild Horse

Ancient wild horse populations were common in open plains of Europe, Asia and North America during the Pleistocene (Clutton-Brock & Clutton-Brock, 1999). However by the end of ice age, and throughout the Mesolithic period the range of wild horses was highly reduced (Clutton-Brock & Clutton-Brock, 1999). The climatic and vegetation changes in North America through this transition, lead to a decline in the body size that ended in extinction of wild horses around 10 500 BC (Guthrie, 2003). In Eurasia, still, two species survived until modern days: The Tarpan *Equus ferus ferus* and The Przewalski's horse *Equus ferus przewalskii* (Groves, 1986).

Nowadays, the Przewalski's horse is the only living wild horse in the world, thanks to a small number of specimens that were captured in Mongolian Steppes in the early 20th century and several repopulation and protection programs in Europe and North America (Clutton-Brock & Clutton-Brock, 1999).

In contrast, the Tarpan was commonly described in the 19th and 20th century as small animal with a mouse-dun coat and light underbelly, short frizzle mane, sooty to black limbs from the knees and hocks down and dark hair tail (Olsen, 2006), and went extinct in 1918/19 in Poland when the last specimen was killed during a failed capture attempt (Olsen, 2006).

Even if The Tarpam wild horse is considered extinct, reports of Tarpam stallions steeling domestic females are common, though, many may only be feral horses (Olsen, 2006).

Actually, many ancient domestic breeds were abandoned by man and formed groups that adopted a feral life style. Even though these animals shall not be considered wild horse species as they had been under man's domestication for a very long time in the past.

The Celtic horse in the Celtic cultures

According to historic and archaeological evidences the Celts took over Europe through the Hallstatt and La Tène cultures, from the 12th to 1st BC centuries (Lorrio & Zapatero, 2005). Their origins seem to be linked to culture divergence of Indo-Europeans from central Europe. By the 3rd century BC the Celts had conquered territory and controlled most of European continent from Iberian peninsula to Turkey, and as far north as Ireland and Scotland (Lorrio, 2007).

Even though Celtic presence in Iberian Peninsula is not questioned, how it occurred and the effects in the pre-Celtic cultures were a matter of controversy during the last century (Lorrio & Zapatero, 2005).

Nowadays there are still different theories about this process, but all differ in the possibility of an invasion by The Celts of central Europe (Gimpera, 1943), (Almagro, 1935; Almagro, 1939; Almagro, 1947-48), or the adaptation and evolution of indo-European Iberians into Celtic costumes, by contact with this cultures (Schulten (Schulten, 1914). However these theories are based only on ancient literacy, linguistics, archaeological sources, and studies of genetic similarity, evolution and divergence of the different recent breeds of Celtic horse and other animals directly related to the Celts can help to clarify this matter.

In fact a recent study of the origins of Iberic Horses based on mtDNA concluded that this maternal lineage was mainly of North African Horses (Royo et al., 2005). Still it's also proposed that a male-mediated introgression during Celts occupation might have happened, which could be proved by the use of Y-chromosome markers (Royo et al., 2005).

Future studies similar to this might help to understand the path of the Celts in the Iberian Peninsula, as well as their influence and contributions for the way we see it today.

Celtic Horse Breeds in the Europe

The Celtic Horse remains most of its ancestral primitive morphology; their height is below average (1.2-1.4 m till the shoulder), usually with a dark pigmented coat: black, brown or bays (Canon et al., 2000).

In current days there are different breeds of celtic horses in Iberian Peninsula: the Garrano o Facó/Caballo Galego, Asturcón, Pottoka, Jaca Navarra, Losino, and Menorquina and Mellorquina however very little differences can be found between them (Canon et al., 2000). This is probably a result of the more than two thousand years of reproductive isolation; conclusion sustained by the higher genetic distance between Atlantic and Mediteranic (Menorquina and Mellorquina) breeds then intra-Atlantic Breeds (Canon et al., 2000).

In the last two decades iberian celtic horse breeds have been subject of several monitorization and genetic studies (Dunner et al., 1998), (Canon et al., 2000; Morais et al., 2005; Royo et al., 2005; Royo et al., 2007), specially the Spanish breed Asturcón because of huge decreasing of specimen numbers during the civil war and of the similarity with celtic horses descriptions from ancient roman literature (Alvarez, 1995).

In current days almost every breeds of northern Iberian Celtic Horses are near to extinction and are currently protected by several conservation programmes in Portugal and Spain (Royo et al., 2005). Once again the asturcón, because of its historical and economic interest, was the first breed to call the attention of conservationists. In 1981 started the studbook of this breed and in 1987 appeared the ACPRA to control and protect this breed. Since then several studies have been conducted to evaluate the conservations status of this breed and to improve the action methods of ACPRA (Dunner et al., 1998), (Royo et al., 2007).